Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2658736 C 2014/08/12

(11)(21) 2 658 736

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2007/08/29
(87) Date publication PCT/PCT Publication Date: 2008/03/06
(45) Date de délivrance/Issue Date: 2014/08/12
(85) Entrée phase nationale/National Entry: 2009/01/21
(86) N° demande PCT/PCT Application No.: EP 2007/007539
(87) N° publication PCT/PCT Publication No.: 2008/025527
(30) Priorité/Priority: 2006/08/31 (EP06018171.6)

(51) Cl.Int./Int.Cl. *C07K 14/65* (2006.01), *C12N 15/12* (2006.01), *C12N 15/62* (2006.01), *A61K 47/48* (2006.01), *C12N 9/52* (2006.01)

(72) Inventeurs/Inventors:
FISCHER, STEPHAN, DE;
HESSE, FRIEDERIKE, DE;
KNOETGEN, HENDRIK, DE;
LANG, KURT, DE;
REGULA, JOERG THOMAS, DE;
SCHANTZ, CHRISTIAN, DE;
SCHAUBMAR, ANDREAS, DE

(73) Propriétaire/Owner:
F. HOFFMAN-LA ROCHE AG, CH

(74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : PROCEDE DE PRODUCTION DU FACTEUR DE CROISSANCE 1 ANALOGUE A L'INSULINE
(54) Title: METHOD FOR THE PRODUCTION OF INSULIN-LIKE GROWTH FACTOR-I

(57) Abrégé/Abstract:
Method for the production of IGF-I, characterized by cultivating a prokaryotic host cell comprising an expression vector containing a nucleic acid encoding a fusion protein comprising said IGF-I N-terminally linked to the C-terminus of a propeptide, whereby said propeptide ends C-terminally with amino acids -Y-Pro, wherein Y is selected from the group consisting of Pro, Pro-Ala, Pro-Gly, Pro-Thr, Ala-Pro, Gly-Pro, Thr-Pro, Arg-Pro, or Pro-Arg-Pro, recovering and cleaving said fusion protein with IgA protease, and recovering said IGF-I. IGF-I is useful for the treatment of neurodegenerative disorders like Alzheimer's Disease.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 March 2008 (06.03.2008)

PCT

(10) International Publication Number
WO 2008/025527 A1

(51) International Patent Classification:
C07K 14/65 (2006.01) *C12N 9/52* (2006.01)
C12N 15/62 (2006.01) *A61K 47/48* (2006.01)
C12N 15/12 (2006.01)

(21) International Application Number:
PCT/EP2007/007539

(22) International Filing Date: 29 August 2007 (29.08.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
06018171.6 31 August 2006 (31.08.2006) EP

(71) Applicant *(for all designated States except US)*: **F. HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacherstrasse 124, CH-4070 Basel (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **FISCHER, Stephan** [DE/DE]; Jakobifeldweg 11, 82398 Polling (DE). **HESSE, Friederike** [DE/DE]; Ganghoferstrasse 23, 80339 München (DE). **KNOETGEN, Hendrik** [DE/DE]; Barbarastrasse 33, 82377 Penzberg (DE). **LANG, Kurt** [DE/DE]; Langoner Strasse 10, 82377 Penzberg (DE). **REGULA, Joerg, Thomas** [DE/DE]; Lierstrasse 15, 80639 Muenchen (DE). **SCHANTZ, Christian** [DE/DE]; Im Wiesfeld 11, 82377 Penzberg (DE). **SCHAUBMAR, Andreas** [DE/DE]; St. Klara Strasse 41, 82377 Penzberg (DE).

(74) Agent: **SCHREINER, Siegfried**; Roche Diagnostics GmbH, Patent Department (TR-E)), Postfach 11 52, 82372 Penzberg (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report*

(54) Title: METHOD FOR THE PRODUCTION OF INSULIN-LIKE GROWTH FACTOR-I

(57) Abstract: Method for the production of IGF-I, characterized by cultivating a prokaryotic host cell comprising an expression vector containing a nucleic acid encoding a fusion protein comprising said IGF-I N-terminally linked to the C-terminus of a propeptide, whereby said propeptide ends C-terminally with amino acids -Y-Pro, wherein Y is selected from the group consisting of Pro, Pro-Ala, Pro-Gly, Pro-Thr, Ala-Pro, Gly-Pro, Thr-Pro, Arg-Pro, or Pro-Arg-Pro, recovering and cleaving said fusion protein with IgA protease, and recovering said IGF-I. IGF-I is useful for the treatment of neurodegenerative disorders like Alzheimer's Disease.

WO 2008/025527 A1

Method for the production of insulin-like growth factor-I

This invention relates to a method for the production of insulin-like growth factor-I (IGF-I), pharmaceutical compositions, and methods of use.

Background of the Invention

Human insulin-like growth factor I (IGF-I) is a circulating hormone structurally related to insulin. IGF-I is traditionally considered the major mediator of the actions of growth hormone on peripheral tissues. IGF-I consists of 70 amino acids and is also named somatomedin C and defined by SwissProt No. P01343. Use, activity and production are mentioned in, e.g., le Bouc, Y., et al., FEBS Lett. 196 (1986) 108-112; de Pagter-Holthuizen, P., et al., FEBS Lett. 195 (1986) 179-184; Sandberg Nordqvist, A.C., et al., Brain Res. Mol. Brain Res. 12 (1992) 275-277; Steenbergh, P.H., et al., Biochem. Biophys. Res. Commun. 175 (1991) 507-514; Tanner, J.M., et al., Acta Endocrinol. (Copenh.) 84 (1977) 681-696; Uthne, K., et al., J. Clin. Endocrinol. Metab. 39 (1974) 548-554; EP 0 123 228; EP 0 128 733; US 5,861,373; US 5,714,460; EP 0 597 033; WO 02/32449; WO 93/02695.

The regulation of IGF-I function is quite complex. In the circulation, only 0.2 % of IGF-I exists in the free form whereas the majority is bound to IGF-binding proteins (IGFBP's), which have very high affinities to IGF's and modulate IGF-I function. The factor can be locally liberated by mechanisms releasing IGF-I such as proteolysis of IGFBPs by proteases.

IGF-I plays a paracrine role in the developing and mature brain (Werther, G.A., et al., Mol. Endocrinol. 4 (1990) 773-778). In vitro studies indicate that IGF-I is a potent non-selective trophic agent for several types of neurons in the CNS (Knusel, B., et al., J. Neurosci. 10(1990) 558-570; Svrzic, D., and Schubert, D., Biochem. Biophys. Res. Commun. 172 (1990) 54-60), including dopaminergic neurons (Knusel, B., et al., J. Neurosci. 10(1990) 558-570) and oligodendrocytes (McMorris, F.A., and Dubois-Dalcq, M., J. Neurosci. Res. 21 (1988) 199-209; McMorris, F.A., et al., Proc. Natl. Acad. Sci. USA 83 (1986) 822-826; Mozell, R.L., and McMorris, F.A., J. Neurosci. Res. 30 (1991) 382-390)). US 5,093,317 mentions that the survival of cholinergic neuronal cells is enhanced by administration of IGF-I. It is further known that IGF-I stimulates peripheral nerve regeneration (Kanje, M., et al., Brain Res. 486 (1989) 396-398) and enhance ornithine decarboxylase activity US 5,093,317). US 5,861,373 and WO 93/02695 mention a method of treating

injuries to or diseases of the central nervous system that predominantly affects glia and/or non-cholinergic neuronal cells by increasing the active concentration(s) of IGF-I and/or analogues thereof in the central nervous system of the patient. WO 02/32449 is directed to methods for reducing or preventing ischemic damage in the central nervous system of a mammal by administering to the nasal cavity of the mammal a pharmaceutical composition comprising a therapeutically effective amount of IGF-I or biologically active thereof. IGF-I is absorbed through the nasal cavity and transported into the central nervous system of the mammal in an amount effective to reduce or prevent ischemic damage associated with an ischemic event. EP 0874641 claims the use of an IGF-I or an IGF-II for the manufacture of a medicament for treating or preventing neuronal damage in the central nervous system, due to AIDS-related dementia, Alzheimer's disease (AD), Parkinson's Disease, Pick's Disease, Huntington's Disease, hepatic encephalopathy, cortical-basal ganglionic syndromes, progressive dementia, familial dementia with spastic parapavresis, progressive supranuclear palsy, multiple sclerosis, cerebral sclerosis of Schilder or acute necrotizing hemorrhagic encephalomyelitis, wherein the medicament is in a form for parenteral administration of an effective amount of said IGF outside the blood-brain barrier or blood- spinal cord barrier.

Reduction of brain and serum levels of free IGF-I has been related to the pathogenesis of sporadic and familial forms of AD. Furthermore, IGF-I protects neurons against Aβ-induced neurotoxicity (Niikura, T., et al., J. Neurosci. 21 (2001) 1902-1910; Dore, S., et al., Proc. Natl. Acad. Sci. USA 94 (1997) 4772-4777; Dore, S., et al., Ann. NY Acad. Sci. 890 (1999) 356-364). Recently, it was shown that peripherally administered IGF-I is capable of reducing brain Aβ levels in rats and mice (Carro, E., et al., Nat. Med. 8 (2002) 1390-1397). Furthermore, the study demonstrated that in a transgenic AD mouse model prolonged IGF-I treatment significantly reduced brain amyloid plaque load. These data strongly support the idea that IGF-I is able to reduce brain Aβ levels and plaque-associated brain dementia by clearing Aβ from the brain.

The recognition site of the IgA Protease is described as Yaa-Pro.!.Xaa-Pro. Yaa stands for Pro (or rarely for Pro in combination with Ala, Gly or Thr: Pro-Ala, Pro-Gly, or Pro-Thr. Xaa stands for Thr, Ser or Ala (Pohlner, J. et al., Bio/Technology 10 (1992) 799-804; Pohlner, J. et al., Nature 325 (1987) 458-462 and US 5,427,927). Naturally cleavage sites have been identified by Wood, S.G. and Burton, J., Infect Immun. 59 (1991) 1818-1822. Synthetic peptide substrates for the immunoglobulin

A1 protease from Neisseria gonorrhoeae (type 2) are the autoproteolytic sites Lys-Pro-Ala-Pro.!.Ser-Pro, Val-Ala-Pro-Pro.!.Ser-Pro, Pro-Arg-Pro-Pro.!.Ala-Pro, Pro-Arg-Pro-Pro.!.Ser-Pro, Pro-Arg-Pro-Pro.!.Thr-Pro and the IgA1 Cleavage Sites Pro-Pro-Thr-Pro.!.Ser-Pro and Ser-Thr-Pro-Pro.!.Thr-Pro.

WO 2006/066891 discloses conjugates consisting of an insulin-like growth factor-1 (IGF-I) and one or two poly(ethylene glycol) group(s), characterized in that said IGF-I has an amino acid alteration at up to three amino acid positions 27, 37, 65, 68 of the wild-type IGF-I amino acid sequence so that one or two of said amino acids is/are lysine and amino acid 27 is a polar amino acid but not lysine, is conjugated via the primary amino group(s) of said lysine(s) and said poly(ethylene glycol) group(s) have an overall molecular weight of from 20 to 100 kDa. Such conjugates are useful for the treatment of neurodegenerative disorders like Alzheimer's Disease.

WO2006/074390 refers to IGF-I variants and fusion proteins comprising IGF-I variants and certain fusion components. WO 2006/074390 refers to certain IGF-1 variants.

Methods for the recombinant production of IGF-I via a fusion protein are known, e.g., from EP0155655 and US 5,158,875. However microheterogenity of recombinantly produced IGGF-I is often found (Forsberg, G. et. al., Biochem. J. 271 (1990) 357-363).

Summary of the Invention

The invention provides a method for the recombinant production of IGF-I without N-terminal attached methionine in prokaryotes with high purity and yield.
The invention comprises a method for the production of IGF-I, characterized by

a) cultivating a prokaryotic host cell comprising an expression vector containing a nucleic acid encoding a fusion protein comprising said IGF-I N-terminally linked to the C-terminus of a propeptide,
b) whereby said propeptide ends C-terminally with amino acids -Y-Pro, wherein Y is selected from the group consisting of Pro, Pro-Ala, Pro-Gly, Pro-Thr, Ala-Pro, Gly-Pro, Thr-Pro, Arg-Pro, or Pro-Arg-Pro,
c) recovering and cleaving said fusion protein with IgA protease, and
d) recovering said IGF-I.

The recovered IGF-I comprises no methionine residue attached at the N-terminus.

A preferred embodiment of the invention is a propeptide selected from the group consisting of peptides shown in SEQ ID NO: 2-5.

A further embodiment of the invention is a fusion protein comprising said IGF-I N-terminally linked to the C-terminus of a propeptide, characterized in that said propeptide ends C-terminally with amino acids -Y-Pro, wherein Y is selected from the group consisting of Pro, Pro-Ala, Pro-Gly, Pro-Thr, Ala-Pro, Gly-Pro, Thr-Pro, Arg-Pro, or Pro-Arg-Pro. Due to the –Y-Pro sequence the propeptide can be separated by IgA protease treatment from said IGF-I .

Preferably the fusion protein according to the invention is characterized by the formula Met-X_1-His_n-X_2-Y-Pro-[IGF-I], wherein

- Met denotes methionine,
- X_1 is a bond, serine or asparagine ,
- His is histidine,
- n is a number from 0 to 6,
- X_2 is a linker peptide, selected from the group of peptides SEQ ID NO: 6-10,
- Pro is proline, and
- Y is selected from the group consisting of Pro, Pro-Ala, Pro-Gly, Pro-Thr, Ala-Pro, Gly-Pro, Thr-Pro, Arg-Pro, or Pro-Arg-Pro.

Preferably the propeptide is shown by the formula Met-X_1-His_n-X_2-Y-Pro-, wherein

- Met denotes methionine
- X_1 is a bond, serine or asparagine
- His is histidine,
- n is a number from 0 to 6,
- X_2 is a linker peptide, selected from the group, consisting of peptides SEQ ID NO: 6-10,
- Pro is proline, and
- Y is selected from the group consisting of Pro, Pro-Ala, Pro-Gly, Pro-Thr, Ala-Pro, Gly-Pro, Thr-Pro, Arg-Pro, or Pro-Arg-Pro.

The propeptide is C-terminally linked to the N-terminus (glycine) of IGF-I. The propeptide preferably has a length of up to 30 amino acids. Preferaby X1 is a bond. Preferably n is 0 or 6. Preferably X2 is peptide SEQ ID NO:7. Preferably Y is Pro-Arg-Pro.

The invention further comprises pharmaceutical compositions containing an IGF-I according to the invention, preferably together with a pharmaceutically acceptable carrier.

The invention further comprises methods for the production of pharmaceutical compositions containing an IGF-I according to the invention.

The invention further comprises the use of an IGF-I according to the invention for the preparation of a medicament for the treatment of AD.

The invention further comprises methods for the treatment of AD, characterized in that a pharmaceutically effective amount of amino-reactive IGF-I is administered to a patient in need of such treatment, preferably in one to two applications per week.

Detailed Description of the Invention

It was surprisingly found that IgA protease, preferably IgA protease from Neisseria gonorrhoae, is capable of cleaving the amino acid sequence Y-Pro.!.Gly-Pro. Y is selected from the group consisting of Pro, Pro-Ala, Pro-Gly, Pro-Thr, Ala-Pro, Gly-Pro, Thr-Pro, Arg-Pro, or Pro-Arg-Pro. Preferably useful as cleavage site is Pro-Pro.!.Gly-Pro or Pro-Arg-Pro-Pro.!.Gly-Pro (SEQ ID NO:11) (.!. : cleavage position). The IgA protease cleavage site for the process according to the present invention has the amino acid consensus sequence Y-Pro.!.Gly-Pro, whereby Gly-Pro are the first two amino acids of IGF-I. Y preferably represents an amino acid sequence which ends with the amino acid(s) Pro, Pro-Ala, Arg-Pro or Pro-Arg-Pro. Such Y amino acid sequences, especially Pro-Arg-Pro can be prolonged by a further Ala or Pro-Ala group, as e.g. in Ala-Pro-Arg-Pro (SEQ ID NO:12) or Pro-Ala-Pro-Arg-Pro (SEQ ID NO:13). Particularly preferred are the cleavage amino acid sequences Pro-Arg-Pro-Pro.!.Gly-Pro (SEQ ID NO:11), Pro-Ala-Pro.!.Gly-Pro (SEQ ID NO:14), Pro-Pro-.!.Gly-Pro(SEQ ID NO:15), Ala-Pro-Arg-Pro-Pro.!.Gly-Pro (SEQ ID NO:16) or Pro-Ala-Pro-Arg-Pro-Pro.!.Gly-Pro (SEQ ID NO:17).

In accordance with the present invention the term "IgA protease" includes proteases which specifically cleave IgA and which are described, for example, in Kornfeld, S.J. and Plaut, A.G., Rev. Infekt. Dis. 3 (1981) 521-534 as e.g. IgA1 protease from Neisseria gonorrhoea (type 2). Recombinant IgA proteases such as those described in DE-A 36 22 221; Koomey, J.M., et al. Proc. Natl. Acad. Sci. USA 79 (1982) 7881-7885; Bricker, J., et al., Proc. Natl. Acad. Sci. USA 80 (1983) 2681- 2685; Pohlner, J., Nature 325 (1987) 458- 462; and Halter, R., et al., EMBO J. 3 (1984) 1595-1601 are also just as suitable. Preferably said IgA protease is IgA protease from Neisseria gonorrhoae. Preferably said IgA1 protease from Neisseria gonorrhoea (type 2) has the sequence SEQ ID NO:21.

IGF-I according to the invention refers to a human protein consisting of 70 amino acids which is also named somatomedin C and defined by SwissProt No. P01343. Use, activity and production are mentioned in, e.g., le Bouc, Y., et al., FEBS Lett. 196 (1986) 108-112; de Pagter-Holthuizen, P., et al., FEBS Lett. 195 (1986) 179-184; Sandberg Nordqvist, A.C., et al., Brain Res. Mol. Brain Res. 12 (1992) 275-277; Steenbergh, P.H., et al., Biochem. Biophys. Res. Commun. 175 (1991) 507-514; Tanner, J.M., et al., Acta Endocrinol. (Copenh.) 84 (1977) 681-696; Uthne, K., et al., J. Clin. Endocrinol. Metab. 39 (1974) 548-554; EP 0 123 228; EP 0 128 733; US 5,861,373; US 5,714,460; EP 0 597 033; WO 02/32449; WO 93/02695.

IGF-I according to the invention comprises an IGF-I selected from the group consisting of IGF-I, C-terminal truncated IGF-I (deletion of 3-6 amino acids), R36A (substitution of arginine at position 36 by alanine), R37A. Preferably said IGF-I is C-terminally linked to human Fc from IgG, preferably from IgG1 or IgG4.

C-terminal truncated IGF-I (deletion of 3-6 amino acids) an IGF-I of SEQ ID NO.1, in which at the C-terminus 3-6 amino acids are deleted.

R36A denotes an IGF-I of SEQ ID NO. 1, in which at amino acid position 36 arginine is substituted by alanine.

R37A denotes an IGF-I of SEQ ID NO. 1, in which at amino acid position 37 arginine is substituted by alanine.

The gene coding for a the fusion protein is preferably placed under the control of suitable (preferably inducible) expression signals so that fusion proteins can be produced according to the requirements. Suitable prokaryotic or eukaryotic (plant

as well as animal) cells can be used as host cells for the production of protein fusions; cell- free systems are, however, also possible.

A preferred embodiment of the process according to the present invention is characterized in that a host cell is transformed with a recombinant DNA or a recombinant vector, in which the DNA or the vector contains at least one copy of a gene which codes for a fusion protein according to the invention and the transformed cell is cultured in a suitable medium, the gene coding for the fusion protein is made to express in the transformed cell, the fusion protein is cleaved with IgA protease and IGF-I is isolated.

The expression of the fusion protein according to the invention can, for example, be improved at the DNA level by fusion with fragments of lysine-free beta-galactosidase gene, i.e., Y contains a part of a lysine-free beta-galactosidase protein. Other alternatives for increasing the expression of the fusion protein are known to the expert. The purification and separation of the expression product can be facilitated by fusion with other polypeptides, in particular, with polypeptides or proteins that are highly charged (e.g. poly(Lys, Arg)) or which can bind to particular substances with high affinity (e.g. streptavidin) (see e.g. EP-A 0 089 626, EP-A 0 306 610). Especially preferred linker peptides are peptides SEQ ID NO: 6-10, preferably N-terminally preceded by SHHHHHH (SEQ ID NO:18, NHHHHHH (SEQ ID NO:19) or HHHHHH (SEQ ID NO:20).

The present invention also provides a (recombinant) nucleic acid which codes for a fusion protein according to the present invention and in which an IgA protease cleavage site is incorporated in the junction region between the propeptide and IGF-I .

A recombinant DNA according to the present invention can be obtained in a manner known to one skilled in the area of molecular biology. For this a vector which contains a DNA sequence coding for the amino acid sequence of IGF-I is usually cleaved with restriction endonuclease(s) in the region of the 5' end of this gene and religated with oligonucleotides which contain the desired sequence.

In addition, the invention also provides a recombinant vector which contains at least one copy of a recombinant DNA according to the present invention. Vectors which are suitable as a basis for protein expression in prokaryotic organisms are known to the expert. This vector is preferably one which allows a high expression of

the recombinant DNA according to the present invention. The recombinant DNA on the vector is preferably under the control of an inducible expression signal (e.g. . lambda., tac, lac or trp promoter).

The vector according to the present invention can be present extrachromosomally (e.g. plasmid) as well as integrated in the genome of the host organism (e.g. bacteriophage lambda). The vector according to the present invention is preferably a plasmid. Vectors which are suitable in each case for gene expression in a particular host organism are known to one skilled in the area of molecular biology. It can be a eukaryotic vector, but preferably a prokaryotic vector. Examples of suitable vectors for the expression of the DNA according to the present invention in prokaryotes are, for instance, commercially available pUC and pUR vectors.

The invention also provides a cell, preferably a prokaryotic cell, particularly preferably an E. coli cell, which is transformed with the recombinant DNA according to the present invention or/and with a recombinant vector according to the present invention.

When the fusion protein is expressed in prokaryotes, sparingly soluble aggregates (refractile bodies, inclusion bodies) are formed which are inactive. Therefore the fusion protein must be transformed into its active form. Using procedures which are familiar to those skilled in the art (cf. e.g. EP-A 0 219 874, EP A 0 114 506, WO 84/03711) first a solubilization is carried out by addition of denaturing agents which is followed by renaturation and, if desired, further purification steps.

The conditions required for the treatment of an IGF-I fusion protein to be cleaved with IgA proteases are not critical. In this process it is, however, preferred that the ratio by weight of IGF-I fusion protein to IgA protease is 1:1 to 100:1. The reaction preferably takes place in a buffered aqueous solution of pH 6.5 to 8.5. The buffer concentration is preferably in the range between 50 and 500 mmol/l if desired, with addition of 0-100 mmol/l sodium chloride. The cleavage is preferably carried out at room temperature for at least 60 min up to 5 days, preferably between 24 – 72 h.

After solubilization, renaturation and cleavage with IgA protease the cleavage product obtained in this way is preferably purified by means of hydrophobic interaction chromatography, ion exchange chromatography and/or fractionation by size. The IGF-I produced in this way is free of methionine in position -1.

Pharmaceutical Formulations

IGF-I's can be administered as a mixture, or different species separated by e. g. hydrophobic interaction chromatography, ion exchange chromatography or size exclusion chromatography. The compounds of the present invention can be formulated according to methods for the preparation of pharmaceutical compositions, which methods are known to the person skilled in the art. For the production of such compositions, an IGF-I according to the invention is combined in a mixture with a pharmaceutically acceptable carrier, preferably by dialysis or diafiltration against an aqueous solution containing the desired ingredients of the pharmaceutical compositions. Such acceptable carriers are described, for example, in Remington's Pharmaceutical Sciences, 18th edition, 1990, Mack Publishing Company, edited by Oslo et al. (e.g. pp. 1435-1712). Typical compositions contain an effective amount of the substance according to the invention, for example from about 0.1 to 100 mg/ml, together with a suitable amount of a carrier. The compositions may be administered parenterally. The IGF-I according to the invention is administered preferably via intraperitoneal, subcutaneous, intravenous, or intranasal application.

The pharmaceutical formulations according to the invention can be prepared according to known methods in the art. Usually, solutions of IGF-I are dialyzed or diafiltrated against the buffer intended to be used in the pharmaceutical composition and the desired final protein concentration is adjusted by concentration or dilution.

The scope of the claims should not be limited by the preferred embodiments set forth herein, but should be given the broadest interpretation consistent with the description as a whole. Names of the amino acids are abbreviated using either the one letter code (e.g. R) or the three letter code (e.g. Arg). R36A means an IGF-I mutant in which amino acid arginine36 is replaced by alanine.

Sequence Listing

SEQ ID NO: 1 amino acid sequence of human IGF-I (amino acids 49-118 from SwissProt P01343).

SEQ ID NO: 2 amino acid sequence of a preferred propeptide

SEQ ID NO: 3	amino acid sequence of a preferred propeptide
SEQ ID NO: 4	amino acid sequence of a preferred propeptide
SEQ ID NO: 5	amino acid sequence of a preferred propeptide
SEQ ID NO: 6-10	linker
SEQ ID NO: 11-17	cleavage sequences
SEQ ID NO: 18-20	others
SEQ ID NO: 21	amino acid sequence of an IgA1 protease from Neisseria gonorrhoea (type 2)

Examples

Example 1

The expression vector and the E. coli strain useful are described in EP 0 972 838. From an E. coli clone, expressing fusion protein are grown on selective agar plate, one inoculating loop is transferred to (100 ml) selective medium and cultivated for 13 h at 37°C to an optical density (578 nm) of 2–4 . This culture is stored on ice for the next 6 hours prior to the automated inoculation of the main culture which is performed at 37°C. The expression of IGF-I mutant is initiated at an optical density (578 nm) of 50 with the addition of 1.0 mM IPTG. The overall fermentation lasts up to 16 hours. The amount of protein is determined densitometrically by comparing the volumetric intensity of the protein band of the product with the band of an IGF standard on a SDS-PAGE gel. The culture broth is harvested by centrifugation.

To obtain purified inclusion body (IB) material, the harvested biomass out of standard fermentation is treated with the following procedure: 0.3 g/100 g bio dry weight Lysozyme and 5 U/1 g bio dry weight Benzonase are incubated for 20 min and homogenized. 30 U/1 g bio dry weight Benzonase is added and incubated for 60 min. at 37 °C. 0.5 L Brij-buffer / liter is added and incubated for 30 min. at RT. After centrifugation the pellet is resuspended in 300 ml Tris-EDTA-Puffer/100 g bio wet weight (purified IB wet weight), incubated for 30 min. at RT and centrifugated. 1g IBs/liter are solubilized at room temperature in 6.8 M guanidine-HCl, 0.1 M TrisHCl, 0.1 M DTT, pH 8.5 overnight. The turbid solution is dialyzed at 4°C against 6.8 M guanidine-HCl, 0.1 M TrisHCl, pH 8.0. After dialysis insoluble components were removed by centrifugation. Folding is performed by 50-fold dilution of the pro-IGF-I solution into 0.8 M arginine, 0.1 M TrisHCl, 0.1 M guanidine-HCl, 1 mM GSH, 1 mM GSSH, pH 8.5 at room temperature. After two

hours the solution is supplemented with 2 M sodium chloride, filtered and applied at a flow rate of 10 ml/min to a HIC column (Butyl Sepharose™ 4 Fast Flow; GE, Amersham Biosciences), which is equilibrated at room temperature with buffer containing 2 M NaCl, 0.8 M arginine, 0.1 M TrisHCl, 0.1 M guanidine-HCl, pH 8.5. The column is washed with equilibration buffer till baseline is achieved and then eluted with ten column volumes of a linear gradient starting with equilibration buffer and ending with buffer containing 0.1 M TrisHCl, 5% ethylene glycol, pH 8.5. Eluted fractions are analyzed by reversed phase high performance chromatography (rpHPLC). Fractions that contain protein with correctly formed SS-bridges were pooled. The reaction mix is supplemented with IgA1 protease from Neisseria gonorrhoea (type 2) (w/w ratio 1:50) and incubated over night at room temperature (see Figure 2). The reaction mix is diluted 1:2 with 50 mM acetic acid pH 4.5 and then applied to a cation IEC column (MacroCap™ SP support; GE, Amersham Biosciences, Uppsala, Sweden), equilibrated with 50 mM acetic acid or applied to a SEC Superdex™ 200 (General Electric). The column is washed till baseline is reached and then eluted with 20 column volumes of a linear gradient starting with 50 mM acetic acid and ending with 50 mM acetic acid supplemented with 1 M sodium chloride. Eluted fractions were analyzed by SDS-PAGE. Fractions containing a single band with IGF-I molecular size are pooled as IGF-I. Identity of IGF-I is verified by analytical size exclusion chromatography (SEC) with static light scattering detection, MS analysis of tryptic digests, MS analysis of Asp-N digests and analytical cation IEC or SEC.

CLAIMS:

1. A method for the production of IGF-I, comprising

   a) cultivating a prokaryotic host cell comprising an expression vector containing a nucleic acid encoding a fusion protein comprising said IGF-I N-terminally linked to the C-terminus of a propeptide, wherein Gly-Pro are the first two amino acids of IGF-I,

   b) whereby said propeptide ends C-terminally with amino acids -Y-Pro, wherein Y is selected from the group consisting of Pro, Pro-Ala, Arg-Pro, and Pro-Arg-Pro,

   c) recovering and cleaving said fusion protein with IgA protease wherein said IgA protease has the sequence SEQ ID NO:21, and

   d) recovering said IGF-I.

2. The method of claim 1, wherein said IGF-I is selected from the group of IGF-I (SEQ ID NO:1), an IGF-I of SEQ ID NO.1, in which at the C-terminus 3-6 amino acids are deleted, IGF-I of SEQ ID NO. 1, in which at amino acid position 36 arginine is substituted by alanine, IGF-I of SEQ ID NO. 1, in which at amino acid position 37 arginine is substituted by alanine.

3. The method of claim 1 or claim 2, wherein said IGF-I is C-terminally linked to human Fc from IgG.

4. The method of any one of claims 1 to 3, wherein the propeptide is shown by the formula

   $Met\text{-}X_1\text{-}His_n\text{-}X_2\text{-}Y\text{-}Pro\text{-}$,

   wherein

   - Met denotes methionine,
   - X_1 is a bond, serine or asparagine,
   - His is histidine,
   - n is a number from 0 to 6,

- X_2 is a linker peptide, selected from the group consisting of peptides SEQ ID NO: 6-10,
- Pro is proline, and
- Y is selected from the group consisting of Pro, Pro-Ala, Arg-Pro, and Pro-Arg-Pro.

5. A fusion protein comprising IGF-I linked to the C-terminus of a propeptide, wherein Gly-Pro are the first two amino acids of IGF-I, wherein said propeptide ends C-terminally with amino acids -Y-Pro, and wherein Y is selected from the group consisting of Pro, Pro-Ala, Arg-Pro, and Pro-Arg-Pro.

6. The fusion protein of claim 5, wherein said propeptide has a length of up to 30 amino acids.

7. The fusion protein of claim 5 or claim 6, characterized by the formula

$$Met\text{-}X_1\text{-}His_n\text{-}X_2\text{-}Y\text{-}Pro\text{-}[IGF\text{-}I\ ],$$

wherein

- Met denotes methionine,
- X_1 is a bond, serine or asparagine,
- His is histidine,
- n is a number from 0 to 6,
- X_2 is a linker peptide, selected from the group consisting of peptides SEQ ID NO: 6-10,
- Pro is proline, and
- Y is selected from the group consisting of Pro, Pro-Ala, Arg-Pro, and Pro-Arg-Pro.