

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 March 2008 (27.03.2008)

PCT

(10) International Publication Number
WO 2008/034881 A1

(51) International Patent Classification:
C07K 14/62 (2006.01) **C12N 15/17** (2006.01)
A61K 38/28 (2006.01)

Per [DK/DK]; Gammel Mejerivej 7, DK-3050 Humle-
bæk (DK). **HAVELUND, Svend** [DK/DK]; Kurvej 24,
DK-2880 Bagsværd (DK).

(21) International Application Number:
PCT/EP2007/059990

(74) Common Representative: **NOVO NORDISK A/S**; Cor-
porate Patents, Novo Allé, DK-2880 Bagsværd (DK).

(22) International Filing Date:
20 September 2007 (20.09.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
06121113.2 22 September 2006 (22.09.2006) EP

(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.

(71) Applicant (*for all designated States except US*): **NOVO
NORDISK A/S** [DK/DK]; Novo Allé, DK-2880 Bagsværd
(DK).

(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).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **NIELSEN, Peter
Kresten** [DK/DK]; Granholmen 16, DK-2840 Holte
(DK). **HUBALEK, Frantisek** [CZ/DK]; Classens-
gade 19 A, 2. tv., DK-2100 Copenhagen Ø (DK).
LAUTRUP-LARSEN, Inger [DK/DK]; Kongsvænget
34, DK-2830 Virum (DK). **LUDVIGSEN, Svend**
[DK/DK]; Baunedalen 13, DK-3540 Lyngby (DK). **RI-
BEL-MADSEN, Ulla** [DK/DK]; Birkely 1, DK-2830
Virum (DK). **BALSCHMIDT, Per** [DK/DK]; Morten-
strupvej 42, DK-2970 Hørsholm (DK). **NØRGAARD,**

Published:

- with international search report
- with sequence listing part of description published sepa-
rately in electronic form and available upon request from
the International Bureau

(54) Title: PROTEASE RESISTANT INSULIN ANALOGUES

(57) Abstract: The present invention relates to novel insulin analogues exhibiting resistance towards protease, wherein at least two amino acids are substituted and/or deleted relative to the parent insulin molecule. A method for the preparation of such insulin analogues, insulin preparations containing the insulin analogues of the invention and a method of treating diabetes mellitus using these insulin analogues is also provided.

WO 2008/034881 A1

PROTEASE RESISTANT INSULIN ANALOGUES.**FIELD OF THE INVENTION**

The present invention relates to novel insulin analogues exhibiting resistance to-
wards protease, a method for the preparation of such insulin analogues, insulin preparations
5 containing the insulin analogues of the invention and a method of treating diabetes mellitus
using these insulin analogues.

BACKGROUND OF THE INVENTION

The oral route is by far the most widely used route for drug administration and is in
10 general very well accepted by patients, especially for chronic therapies. Administration of
therapeutic peptides or proteins is however often limited to parenteral routes rather than the
preferred oral administration due to several barriers such as enzymatic degradation in the
gastrointestinal (GI) tract, drug efflux pumps, insufficient and variable absorption from the
intestinal mucosa, as well as first pass metabolism in the liver. Human insulin is degraded by
15 various digestive enzymes found in the stomach (pepsin), in the intestinal lumen (chymotryp-
sin, trypsin, elastase, carboxypeptidases, etc.) and in mucosal surfaces of the GI tract (amin-
opeptidases, carboxypeptidases, enteropeptidases, dipeptidyl peptidases, endopeptidases,
etc.).

This is unfortunate because many peptides and many proteins have proven clinically
20 effective and could have more widespread use if easy to administer and acceptable to recipi-
ents.

Diabetes mellitus is a metabolic disorder in which the ability to utilize glucose is
partly or completely lost. About 5% of all people suffer from diabetes and the disorder ap-
proaches epidemic proportions. Since the introduction of insulin in the 1920's, continuous
25 efforts have been made to improve the treatment of diabetes mellitus. Since people suffering
from diabetes are subject to chronic treatment over several decades, there is a major need
for safe, convenient and life quality improving insulin formulations.

In the treatment of diabetes mellitus, many varieties of insulin formulations have been
suggested and used, such as regular insulin, isophane insulin (designated NPH), insulin zinc
30 suspensions (such as Semilente[®], Lente[®], and Ultralente[®]), and biphasic isophane insulin.
Some of the commercial available insulin formulations are characterized by a fast onset of ac-
tion and other formulations have a relatively slow onset but show a more or less prolonged ac-
tion. Fast-acting insulin formulations are usually solutions of insulin, while retarded acting insulin

formulations can be suspensions containing insulin in crystalline and/or amorphous form precipitated by addition of zinc salts alone or by addition of protamine or by a combination of both.

Human insulin consists of two polypeptide chains, the A and B chains which contain 21 and 30 amino acid residues, respectively. The A and B chains are interconnected by two disulphide bridges. Insulin from most other species is similar, but may contain amino acid substitutions in some positions. Within the last decade a number of human insulin analogues have been developed. They are designed for particular profiles of action, i.e. fast acting or prolonged action. Commercially available products comprising such insulin analogues include Levemir®, NovoRapid®, Humalog®, Apidra® and Lantus®.

Normally, insulin formulations are administered by subcutaneous injection.

However, administration by the oral route would be advantageous due to patient compliance, safety and convenience.

Oral administration of protein drugs such as insulin often results in very low bioavailability due to enzymatic and absorption barriers. The general approach for peptide and protein delivery is parenteral administration which is invasive and inconvenient. Therefore non-invasive routes like oral delivery of protein based pharmaceuticals are increasingly investigated. Recent formulation designs for oral protein/peptide delivery include co-formulations with protease inhibitors, permeation enhancers, polymer-based delivery systems and insulin conjugates. The latter includes hexyl-insulin-monoconjugate-2 (HIM2) (Nobex Cooperation and GSK), a human insulin analog with a PEG 7-hexyl group attached to B29. In for example US 7,030,082, US 6,867,183 and US 6,770,625 oral HIM2 has been reported to have increased proteolytic stability and bioavailability compared to insulin.

SUMMARY OF THE INVENTION

In one embodiment of the invention an insulin analogue with enhanced proteolytic stability and retained biological insulin activity is provided.

In a further embodiment of the invention an insulin analogue is provided wherein at least two amino acids are substituted and/or deleted relative to the parent insulin molecule.

In yet a further embodiment of the invention an insulin analogue is provided wherein at least two hydrophobic amino acids have been substituted with hydrophilic amino acids relative to the parent insulin, wherein the substitutions are within or in close proximity to two or more protease cleavage sites of the parent insulin and wherein such insulin analogue optionally further comprises one or more additional mutations.

It is also an object of the invention to provide insulin analogues according to the invention wherein the A-chain of the insulin analogue comprises at least one mutation and the B-chain of the insulin analogue comprises at least one mutation relative to the parent insulin.

The present invention is also related to nucleic acid sequences which code for the prepropeptide of the claimed insulin analogues. In a further embodiment the present invention is related to vectors containing such nucleic acid sequences and host cells containing such nucleic acid sequences or vectors.

A process for obtaining an insulin analogue according to the invention and use thereof as a pharmaceutical is also provided.

DESCRIPTION OF THE DRAWINGS

Fig. 1: Proteolytic stability of human insulin (HI) and insulin analogues towards chymotrypsin measured as percentage of intact insulin (analogue) at 37 °C.

Fig. 2: Proteolytic stability of human insulin (HI) and insulin analogues towards pepsin measured as percentage of intact insulin (analogue) at 25 °C.

DESCRIPTION OF THE INVENTION

An insulin analogue according to the invention is an insulin molecule having two or more mutations of the A and/or B amino acid chains relative to the parent insulin molecule.

It has been found that by substituting two or more hydrophobic amino acids within or in close proximity to two or more protease sites on an insulin with hydrophilic amino acids, an insulin analogue is obtained which is proteolytically stable compared to the parent insulin.

The following is a non-limiting list of embodiments, which is further described elsewhere herein:

Embodiment 1. An insulin analogue wherein at least two hydrophobic amino acids have been substituted with hydrophilic amino acids relative to the parent insulin, wherein the substitutions are within or in close proximity to two or more protease cleavage sites of the parent insulin and wherein such insulin analogue optionally further comprises one or more additional mutations.

Embodiment 2. An insulin analogue according to embodiment 1 wherein increased solubility relative to the parent insulin is obtained.

Embodiment 3. An insulin analogue according to any one of the embodiments 1-2 wherein the A-chain of the insulin analogue comprises at least one mutation and the B-chain of the insulin analogue comprises at least one mutation relative to the parent insulin.

Embodiment 4. An insulin analogue according to any one of the embodiments 1-3 wherein the insulin analogue further comprises at least one amino acid substitution in a pro-tease site of a first modified insulin analogue, wherein said at least one amino acid substitution is such that at least one hydrophobic amino acid has been substituted with at least one hydrophilic amino acid.

Embodiment 5. An insulin analogue according to any of the embodiments 1-4 wherein

- the amino acid in position A12 is Glu or Asp and/or the amino acid in position A13 is His, Asn, Glu or Asp and/or the amino acid in position A14 is Asn, Gln, Glu, Arg, Asp, Gly or His and/or the amino acid in position A15 is Glu or Asp; and
- the amino acid in position B24 is His and or the amino acid in position B25 is His and/or the amino acid in position B26 is His, Gly, Asp or Thr and/or the amino acid in position B27 is His, Glu, Lys, Gly or Arg and/or the amino acid in position B28 is His, Gly or Asp; and

which optionally further comprises one or more additional mutations.

Embodiment 6. An insulin analogue according to any of the embodiments 1-5 wherein the amino acid in position A14 is Glu, Asp or His, the amino acid in position B25 is His and which optionally further comprises one or more additional mutations.

Embodiment 7. An insulin analogue according to any of the embodiments 1-6 wherein the amino acid in position A14 is Glu, Asp or His, the amino acid in position B25 is His and the amino acid in position B30 is deleted.

Embodiment 8. An insulin analogue according to any of the embodiments 1-6 wherein the amino acid in position A14 is Glu, Asp or His and the amino acid in position B25 is His.

Embodiment 9. An insulin analogue according to any of the embodiments 1-6 wherein the one or more additional mutations is selected from a group consisting of: A(-3)Gly, A(-2)Gly, A(-1)Pro, A(0)Pro, A8His, A18Gln, A18Gln, A21Gln, A21Gly, B(-3)Gly, B(-2)Gly, B(-1)Pro, B(0)Pro, B1Glu, B1Gln, B1Glu, B1Gln, B3Gln, B10Pro, B14Thr, B16Glu, B17Ser, B26Asp, DesB26, DesB27, B27Glu, B27Glu, B28Asp, desB28, desB29, desB30, B31Leu, B32Glu.

Embodiment 10. An insulin analogue according to any of the embodiments 1-6 or 9 wherein the additional mutation is desB30.

Embodiment 11. An insulin analogue according to any the embodiments 1-10 wherein A14 is Glu.

5 Embodiment 12. An insulin analogue according to any of the embodiments 1-11 which shows increased stability towards one or more protease enzymes relative to the parent protein.

10 Embodiment 13. An insulin analogue according to any of the embodiments 1-12 which shows increased stability towards two or more protease enzymes relative to the parent protein.

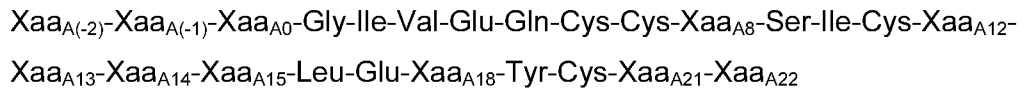
Embodiment 14. An insulin analogue according to any of the embodiments 1-13 wherein the parent insulin is selected from a group consisting of:

- human insulin;
- 15 • an insulin analogue of human insulin wherein the amino acid residue in position B28 is Pro, Asp, Lys, Leu, Val or Ala and the amino acid residue in position B29 is Lys or Pro and optionally the amino acid residue in position B30 is deleted;
- des(B26-B30) human insulin, des(B27-B30) human insulin, des(B28-B30) human insulin, des(B29-B30) human insulin, des(B27) human insulin or des(B30) human insulin;
- 20 • an insulin analogue of human insulin wherein the amino acid residue in position B3 is Lys and the amino acid residue in position B29 is Glu or Asp;
- an insulin analogue of human insulin wherein the amino acid residue in position A21 is Gly and wherein the insulin analogue is further extended in the C-terminal with two Arg residues;
- 25 • an insulin derivative wherein the amino acid residue in position B30 is substituted with a threonine methyl ester; and
- an insulin derivative wherein to the N ϵ position of lysine in the position B29 of des(B30) human insulin a tetradecanoyl chain is attached.

30 Embodiment 15. An insulin analogue according to any of the embodiments 1-6, 9 or 11-14 wherein the one or more additional mutations are selected to enhance chemical stability of insulin.

Embodiment 16. An insulin analogue according to embodiment 15 wherein the one or more additional mutations are selected from a group consisting of: A18Gln, A21Gln, A21GLy and B3Gln.

Embodiment 17. An insulin analogue according to any of the embodiments 1-4 or 12-14 comprising an A-chain amino acid sequence of formula 1:

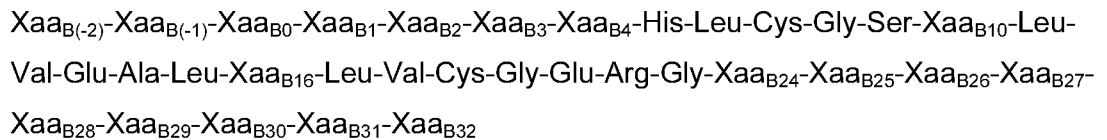


5

Formula (1) (SEQ ID No:1)

and a B-chain amino acid sequence of formula 2:

10



Formula (2) (SEQ ID No:2)

15

wherein

$\text{Xaa}_{A(-2)}$ is absent or Gly;

$\text{Xaa}_{A(-1)}$ is absent or Pro;

Xaa_{A0} is absent or Pro;

Xaa_{A8} is independently selected from Thr and His;

20

Xaa_{A12} is independently selected from Ser, Asp and Glu;

Xaa_{A13} is independently selected from Leu, Thr, Asn, Asp, Gln, His, Lys, Gly, Arg, Pro, Ser and Glu;

Xaa_{A14} is independently selected from Tyr, Thr, Asn, Asp, Gln, His, Lys, Gly, Arg, Pro, Ser and Glu;

25

Xaa_{A15} is independently selected from Gln, Asp and Glu;

Xaa_{A18} is independently selected from Asn, Lys and Gln;

Xaa_{A21} is independently selected from Asn and Gln;

Xaa_{A22} is absent or Lys;

$\text{Xaa}_{B(-2)}$ is absent or Gly;

30

$\text{Xaa}_{B(-1)}$ is absent or Pro;

Xaa_{B0} is absent or Pro;

Xaa_{B1} is absent or independently selected from Phe and Glu;

Xaa_{B2} is absent or Val;

Xaa_{B3} is absent or independently selected from Asn and Gln;

35

Xaa_{B4} is independently selected from Gln and Glu;

Xaa_{B10} is independently selected from His, Asp, Pro and Glu;

Xaa_{B16} is independently selected from Tyr, Asp, Gln, His, Arg, and Glu;

Xaa_{B24} is independently selected from Phe and His;

Xaa_{B25} is independently selected from Phe and His;

5 Xaa_{B26} is absent or independently selected from Tyr, His, Thr, Gly and Asp;

Xaa_{B27} is absent or independently selected from Thr, Asn, Asp, Gln, His, Lys, Gly, Arg, Pro, Ser and Glu;

Xaa_{B28} is absent or independently selected from Pro, His, Gly and Asp;

Xaa_{B29} is absent or independently selected from Lys and Gln;

10 Xaa_{B30} is absent or Thr;

Xaa_{B31} is absent or Leu;

Xaa_{B32} is absent or Glu;

the C-terminal may optionally be derivatized as an amide;

15 wherein the A-chain amino acid sequence and the B-chain amino acid sequence are connected by disulphide bridges between the cysteines in position 7 of the A-chain and the cysteine in position 7 of the B-chain, and between the cysteine in position 20 of the A-chain and the cysteine in position 19 of the B-chain and wherein the cysteines in position 6 and 11 of the A-chain are connected by a disulphide bridge;

20 wherein optionally the N-terminal A-chain amino acid sequence is connected to the C-terminal B-chain amino acid sequence by an amino acid sequence comprising 3-7 amino acids to form a single chain insulin molecule, wherein optionally the N-terminal of the B-chain is extended with 1-10 amino acids;

wherein if Xaa_{A8} is Thr and Xaa_{A12} is Ser and Xaa_{A13} is Leu and Xaa_{A14} is Tyr then

25 Xaa_{A15} is Glu or Asp; and

wherein if Xaa_{B24} is Phe and Xaa_{B25} is Phe and Xaa_{B26} is Tyr and Xaa_{B27} is Thr and Xaa_{B28} is Pro then Xaa_{B29} Gln.

Embodiment 18. An insulin analogue according to any of the embodiments 1-4 or 12-14 comprising an A-chain amino acid sequence of formula 3:

30 Gly-Ile-Val-Glu-Gln-Cys-Cys-Xaa_{A8}-Ser-Ile-Cys-Xaa_{A12}-Xaa_{A13}-Xaa_{A14}-Xaa_{A15}-Leu-Glu-Xaa_{A18}-Tyr-Cys-Xaa_{A21}

Formula (3) (SEQ ID No:3)

35 and a B-chain amino acid sequence of formula 4:

Xaa_{B1}-Val-Xaa_{B3}-Xaa_{B4}-His-Leu-Cys-Gly-Ser-Xaa_{B10}-Leu-Val-Glu-Ala-Leu-Xaa_{B16}-
 Leu-Val-Cys-Gly-Glu-Arg-Gly-Xaa_{B24}-His-Xaa_{B26}-Xaa_{B27}-Xaa_{B28}-Xaa_{B29}-Xaa_{B30}

Formula (4) (SEQ ID No:4)

5

wherein

Xaa_{A8} is independently selected from Thr and His;

Xaa_{A12} is independently selected from Ser, Asp and Glu;

Xaa_{A13} is independently selected from Leu, Thr, Asn, Asp, Gln, His, Lys, Gly, Arg,

10 Pro, Ser and Glu;

Xaa_{A14} is independently selected from Thr, Asn, Asp, Gln, His, Lys, Gly, Arg, Pro,
 Ser and Glu;

Xaa_{A15} is independently selected from Gln, Asp and Glu;

Xaa_{A18} is independently selected from Asn, Lys and Gln;

15 Xaa_{A21} is independently selected from Asn, and Gln;

Xaa_{B1} is independently selected from Phe and Glu;

Xaa_{B3} is independently selected from Asn and Gln;

Xaa_{B4} is independently selected from Gln and Glu;

Xaa_{B10} is independently selected from His, Asp, Pro and Glu;

20 Xaa_{B16} is independently selected from Tyr, Asp, Gln, His, Arg, and Glu;

Xaa_{B24} is independently selected from Phe and His;

Xaa_{B26} is absent or independently selected from Tyr, His, Thr, Gly and Asp;

Xaa_{B27} is absent or independently selected from Thr, Asn, Asp, Gln, His, Lys, Gly,
 Arg, Pro, Ser and Glu;

25 Xaa_{B28} is absent or independently selected from Pro, His, Gly and Asp;

Xaa_{B29} is absent or independently selected from Lys and Gln;

Xaa_{B30} is absent or Thr;

the C-terminal may optionally be derivatized as an amide;

wherein the A-chain amino acid sequence and the B-chain amino acid sequence are
 30 connected by disulphide bridges between the cysteines in position 7 of the A-chain and the
 cysteine in position 7 of the B-chain, and between the cysteine in position 20 of the A-chain
 and the cysteine in position 19 of the B-chain and wherein the cysteines in position 6 and 11
 of the A-chain are connected by a disulphide bridge.

Embodiment 19. An insulin analogue according to embodiment 18,

35

wherein

Xaa_{A8} is independently selected from Thr and His;

Xaa_{A12} is independently selected from Ser and Glu;

Xaa_{A13} is independently selected from Leu, Thr, Asn, Asp, Gln, His, Lys, Gly, Arg, Pro, Ser and Glu;

5 Xaa_{A14} is independently selected from Asp, His, and Glu;

Xaa_{A15} is independently selected from Gln and Glu;

Xaa_{A18} is independently selected from Asn, Lys and Gln;

Xaa_{A21} is independently selected from Asn, and Gln;

Xaa_{B1} is independently selected from Phe and Glu;

10 Xaa_{B3} is independently selected from Asn and Gln;

Xaa_{B4} is independently selected from Gln and Glu;

Xaa_{B10} is independently selected from His, Asp, Pro and Glu;

Xaa_{B16} is independently selected from Tyr, Asp, Gln, His, Arg, and Glu;

Xaa_{B24} is independently selected from Phe and His;

15 Xaa_{B26} is independently selected from Tyr, Thr, Gly and Asp;

Xaa_{B27} is independently selected from Thr, Asn, Asp, Gln, His, Lys, Gly, Arg, and Glu;

Xaa_{B28} is independently selected from Pro, Gly and Asp;

Xaa_{B29} is independently selected from Lys and Gln;

20 Xaa_{B30} is absent or Thr;

the C-terminal may optionally be derivatized as an amide;

wherein the A-chain amino acid sequence and the B-chain amino acid sequence are connected by disulphide bridges between the cysteines in position 7 of the A-chain and the cysteine in position 7 of the B-chain, and between the cysteine in position 20 of the A-chain and the cysteine in position 19 of the B-chain and wherein the cysteines in position 6 and 11 of the A-chain are connected by a disulphide bridge.

Embodiment 20. An insulin analogue according to any of the embodiments 1-16 wherein the C-terminal B-chain is connected to the N-terminal A-chain with 3-15 amino acids or 3-7 amino acids, to form a single chain insulin molecule, wherein optionally the N-terminal of the B-chain is extended with 1-10 amino acids.

Embodiment 21. A pharmaceutical composition comprising a biologically active amount of the insulin analogue according to any of the embodiments 1-20 and a pharmaceutically acceptable carrier.

Embodiment 22. A pharmaceutical composition comprising two or more insulin analogues according to any of the embodiments 1-20 wherein each analogue is defined by having at least one mutation, which is absent in at least one of the other variants.

Embodiment 23. A pharmaceutical composition according to any of the embodiments 11-20 which further comprises a pharmaceutical acceptable carrier and/or excipient, and optionally an adjuvant.

Embodiment 24. A method for the treatment of diabetes mellitus in a subject comprising administering to a subject an insulin analogue according to any of the embodiments 1-20 or a pharmaceutical composition according to any of the embodiments 21-23.

Embodiment 25. A method of reducing the blood glucose level in mammals by administering to a patient in need of such treatment a therapeutically active dose of an insulin analogue according to any of the embodiments 1-20 or a pharmaceutical composition according to any of the embodiments 21-23.

Embodiment 26. Method according to embodiment 24 or 25 being an oral administration.

Embodiment 27. Method according to embodiment 24 or 25 being parenteral administration.

Embodiment 28. Method according to embodiment 24 or 25 being intratracheal administration.

Embodiment 29. An insulin analogue according to any of the embodiments 1-20 for use as a pharmaceutical in the treatment or prevention of hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, obesity, syndrome X and dyslipidemia.

Embodiment 30. An insulin analogue according to any of the embodiments 1-20 for use as a pharmaceutical in delaying or preventing disease progression in type 2 diabetes.

Embodiment 31. An insulin analogue according to any of the embodiments 1-20 for use as a pharmaceutical in decreasing food intake, decreasing β -cell apoptosis, increasing β -cell function and β -cell mass, and/or for restoring glucose sensitivity to β -cells.

Embodiment 32. A nucleic acid sequence encoding an insulin analogue according to any of the embodiments 1-20, a derivative thereof, a partial sequence thereof, a degenerated sequence thereof or a sequence which hybridises thereto under stringent conditions.

Embodiment 33. A nucleic acid sequence encoding a precursor of an insulin analogue according to any of the embodiments 1-20, a derivative thereof, a partial sequence thereof, a degenerated sequence thereof or a sequence which hybridises thereto under stringent conditions.

Embodiment 34. An expression vector comprising a nucleic acid sequence according to embodiment 32 or 33.

Embodiment 35. A host cell comprising an expression vector according to embodiment 34.

5 Embodiment 36. A method of producing an insulin analogue comprising the step of cultivating the host cell of embodiment 35.

Embodiment 37. A method of preparing an insulin analogue according to any of the embodiments 1-20 wherein the substitution of amino acids is carried out by site-directed mutagenesis.

10 Embodiment 38. A process for preparing a pharmaceutical composition according to any of the embodiments 21-23 comprising mixing an insulin analogue according to any of the embodiments 1-20 with pharmaceutically acceptable substances and/or excipients.

Embodiment 39. A pharmaceutical composition obtainable by the process according to embodiment 38.

15

Insulin is a polypeptide hormone secreted by β -cells of the pancreas. Insulin consists of two polypeptide chains, A and B, which are linked by two inter-chain disulphide bridges. Furthermore, the A-chain features one intra-chain disulphide bridge.

20 The hormone is synthesized as a single-chain precursor proinsulin (preproinsulin) consisting of a prepeptide of 24 amino acid followed by proinsulin containing 86 amino acids in the configuration: prepeptide-B-Arg Arg-C-Lys Arg-A, in which C is a connecting peptide of 31 amino acids. Arg-Arg and Lys-Arg are cleavage sites for cleavage of the connecting peptide from the A and B chains.

25 The insulin analogues according to the invention may comprise further mutations. A mutation in an insulin molecule may be in the form of a substitution, a deletion or an addition of an amino acid residue in the A and/or B chain of the naturally occurring insulin molecule.

30 With "desB30" or "B(1-29)" is meant natural insulin B chain lacking the B30 amino acid residue and "A(1-21)" means the natural insulin A chain. The mini C-peptide and its amino acid sequence are indicated in the three letter amino acid code.

35 Herein terms like A1, A2, A3 etc. indicates the position 1, 2 and 3, respectively, in the A chain of insulin (counted from the N-terminal end). Similarly, terms like B1, B2, B3 etc. indicates the position 1, 2 and 3, respectively, in the B chain of insulin (counted from the N-terminal end). Using the one letter codes for amino acids, terms like A21A, A21G and A21Q designates that the amino acid in the A21 position is A, G and Q, respectively. Using the

three letter codes for amino acids, the corresponding expressions are A21Ala, A21Gly and A21Gln, respectively.

Herein the terms A(0) or B(0) indicate the positions N-terminally neighbouring A1 or B1, respectively. The terms A(-1) or B(-1) indicate the positions of the first amino acids N-terminally to A(0) or B(0), respectively. Thus A(-2) and B(-2) indicate positions N-terminally to A(-1) and B(-1), respectively, A(-3) and B(-3) indicate positions N-terminally to A(-2) and B(-2), respectively, and so forth.

The term connecting peptide covers a peptide chain which can connect the C-terminal amino acid residue of the B-chain with the N-terminal amino acid residue of the A-chain in insulin.

The term pro-peptide means a polypeptide sequence whose function is to allow the expressed polypeptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. exportation of the polypeptide across the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The pro-peptide may be the yeast α -factor pro-peptide, vide US 4,546,082 and 4,870,008. Alternatively, the pro-peptide may be a synthetic pro-peptide, which is to say a pro-peptide not found in nature. Suitable synthetic pro-peptides are those disclosed in US 5,395,922; 5,795,746; 5,162,498 and WO 98/32867. The pro-peptide will preferably contain an endopeptidase processing site at the C-terminal end, such as a Lys-Arg sequence or any functional analogue thereof.

The term "diabetes" includes type 1 diabetes, type 2 diabetes and other states that cause hyperglycaemia.

The term "treatment" of a disease includes treatment, prevention or alleviation of the disease.

In one embodiment of the invention the insulin analogue is particularly suitable for oral administration.

Hydrophobic amino acids are herein to be understood as the naturally occurring amino acids tryptophan (Trp, W), phenylalanine (Phe, F), valine (Val, V), isoleucine (Ile, I), leucine (Leu, L) and tyrosine (Tyr, Y) (with the three-letter and the one-letter abbreviation in brackets).

Hydrophilic amino acids are herein to be understood as natural amino acids that are not hydrophobic amino acids according to the definition above. In one embodiment hydro-

philic acids according to the invention are selected from the group consisting of: Glutamic acid (Glu, E), aspartic acid (Asp, D), histidine (His, H), glutamine (Gln, Q), asparagine (Asn, N), serine (Ser, S), threonine (Thr, T), proline (Pro, P), glycine (Gly, G), lysine (Lys, K) and arginine (Arg, R). In a further embodiment hydrophilic amino acids according to the invention are selected from the group consisting of: Glutamic acid (Glu, E), aspartic acid (Asp, D), histidine (His, H), glutamine (Gln, Q), asparagine (Asn, N), lysine (Lys, K) and arginine (Arg, R).

“An insulin” according to the invention is herein to be understood as human insulin, an insulin analogue or an insulin derivative.

The term “parent insulin” as used herein is intended to mean an insulin before any mutations according to the invention have been applied thereto. Non-limiting examples of parent insulins are e.g. a wild-type insulin such as human insulin or porcine insulin, an analogue of human insulin or a derivative of human insulin or an insulin analogue such as human insulin or an insulin analogue which has been PEGylated or acylated.

In one embodiment a parent insulin according to the invention is selected from the group consisting of:

- Human insulin,
- an insulin analogue of human insulin wherein the amino acid residue in position B28 of is Pro, Asp, Lys, Leu, Val, or Ala and the amino acid residue in position B29 is Lys or Pro and optionally the amino acid residue in position B30 is deleted,
- an insulin analogue which is des(B28-B30) human insulin, des(B27) human insulin or des(B30) human insulin,
- an insulin analogue of human insulin wherein the amino acid residue in position B3 is Lys and the amino acid residue in position B29 is Glu or Asp,
- an insulin analogue of human insulin wherein the amino acid residue in position A21 is Gly and wherein the insulin analogue is further extended in the C-terminal with two arginine residues,
- an insulin derivative wherein the amino acid residue in position B30 is substituted with a threonine methyl ester, and
- an insulin derivative wherein to the N ϵ position of lysine in the position B29 of des(B30) human insulin a tetradecanoyl chain is attached.

In one embodiment a parent insulin according to the invention is selected from the group consisting of:

human insulin;
 DesB30 human insulin;
 AspB28 human insulin;
 AspB28,DesB30 human insulin;
 5 LysB3,GluB29 human insulin;
 LysB28,ProB29 human insulin;
 GlyA21, ArgB31, ArgB32 human insulin; and
 DesB30, ArgB31, ArgB32 human insulin.

10 The term “insulin analogue” as used herein means a modified insulin wherein one or more amino acid residues of the insulin have been substituted by other amino acid residues and/or wherein one or more amino acid residues have been deleted from the insulin and/or wherein one or more amino acid residues have been added to the insulin.

In one embodiment an insulin analogue comprises less than 8 modifications (substitutions, deletions, additions) relative to the parent insulin. In one embodiment an insulin analogue comprises less than 7 modifications (substitutions, deletions, additions) relative to the parent insulin. In one embodiment an insulin analogue comprises less than 6 modifications (substitutions, deletions, additions) relative to the parent insulin. In another embodiment an insulin analogue comprises less than 5 modifications (substitutions, deletions, additions) relative to the parent insulin. In another embodiment an insulin analogue comprises less than 4 modifications (substitutions, deletions, additions) relative to the parent insulin. In another embodiment an insulin analogue comprises less than 3 modifications (substitutions, deletions, additions) relative to the parent insulin. In another embodiment an insulin analogue comprises less than 2 modifications (substitutions, deletions, additions) relative to the parent insulin.
 25 insulin.

The term “insulin derivative” as used herein means a chemically modified parent insulin or an analogue thereof, wherein at least one substituent is not present in the parent protein or an analogue thereof, i.e. a parent protein which has been covalently modified. Typical modifications are amides, carbohydrates, alkyl groups, acyl groups, esters, PEGylations and the like. Examples of derivatives of human insulin according to the invention are threonine methyl ester-B30 human insulin, N ϵ -B29-tetradecanoyl des(B30) human insulin, N ϵ -B29-tetradecanoyl GlnB3 des(B30) human insulin, N ϵ -B29-tridecanoyl human insulin, N ϵ -B29-tetradecanoyl human insulin, N ϵ -B29-decanoyl human insulin and N ϵ -B29-dodecanoyl human insulin.
 35 man insulin.

The term "human insulin" as used herein means the human hormone whose structure and properties are well-known. Human insulin has two polypeptide chains that are connected by disulphide bridges between cysteine residues, namely the A-chain and the B-chain. The A-chain is a 21 amino acid peptide and the B-chain is a 30 amino acid peptide, the two chains being connected by three disulphide bridges: one between the cysteines in position 6 and 11 of the A-chain, the second between the cysteine in position 7 of the A-chain and the cysteine in position 7 of the B-chain, and the third between the cysteine in position 20 of the A-chain and the cysteine in position 19 of the B-chain.

Mutations in the insulin molecule is denoted stating the chain (A or B), the position, and the three letter code for the amino acid substituting the native amino acid. By "desB30" is meant a natural insulin B chain or an analogue thereof lacking the B30 amino acid. Thus, A21Gly, B28Asp, desB30 human insulin is an analogue of human insulin where position 21 in the A chain is mutated to glycine, position 28 in the B chain is mutated to aspartic acid, and position 30 in the B chain is deleted.

A "protease" or a "protease enzyme" is a digestive enzyme which degrades proteins and peptides and which is found in various tissues of the human body such as e.g. the stomach (pepsin), the intestinal lumen (chymotrypsin, trypsin, elastase, carboxypeptidases, etc.) or mucosal surfaces of the GI tract (aminopeptidases, carboxypeptidases, enteropeptidases, dipeptidyl peptidases, endopeptidases, etc.), the liver (Insulin degrading enzyme, cathepsin D etc), and in other tissues.

A proteolytically stable insulin analogue is herein to be understood as an insulin analogue, which is subjected to slower degradation by one or more proteases relative to human insulin. In one embodiment a proteolytically stable insulin analogue according to the invention is subjected to slower degradation by one or more proteases relative to the parent insulin. In a further embodiment of the invention an insulin analogue according to the invention is stabilized against degradation by one or more enzymes selected from the group consisting of: pepsin (such as e.g. the isoforms pepsin A, pepsin B, pepsin C and/or pepsin F), chymotrypsin (such as e.g. the isoforms chymotrypsin A, chymotrypsin B and/or chymotrypsin C), trypsin, Insulin-Degrading Enzyme (IDE), elastase (such as e.g. the isoforms pancreatic elastase I and/or II), carboxypeptidase (e.g. the isoforms carboxypeptidase A, car-

boxypeptidase A2 and/or carboxypeptidase B), aminopeptidase, cathepsin D and other enzymes present in intestinal extracts derived from rat, pig or human.

In one embodiment an insulin analogue according to the invention is stabilized against degradation by one or more enzymes selected from the group consisting of: chymotrypsin, trypsin, Insulin-Degrading Enzyme (IDE), elastase, carboxypeptidases, aminopeptidases and cathepsin D. In a further embodiment an insulin analogue according to the invention is stabilized against degradation by one or more enzymes selected from the group consisting of: chymotrypsin, carboxypeptidases and IDE. In a yet further embodiment an insulin analogue according to the invention is stabilized against degradation by one or more enzymes selected from: chymotrypsin and carboxypeptidases.

$T_{1/2}$ may be determined as described in the Examples as a measure of the proteolytical stability of an insulin analogue according to the invention towards protease enzymes such as chymotrypsin, pepsin and/or carboxypeptidase A. In one embodiment of the invention $T_{1/2}$ is increased relative to human insulin. In a further embodiment $T_{1/2}$ is increased relative to the parent insulin. In a yet further embodiment $T_{1/2}$ is increased at least 2-fold relative to the parent insulin. In a yet further embodiment $T_{1/2}$ is increased at least 3-fold relative to the parent insulin. In a yet further embodiment $T_{1/2}$ is increased at least 4-fold relative to the parent insulin. In a yet further embodiment $T_{1/2}$ is increased at least 5-fold relative to the parent insulin. In a yet further embodiment $T_{1/2}$ is increased at least 10-fold relative to the parent insulin.

Protease cleavage sites (herein also mentioned as protease sites) are to be understood as amino acid residues that are recognized by proteases and/or amino acid residues whose peptide bond is cleaved by proteases. Protease cleavage sites may be determined by determining cleavage "hot-spots" by HPLC, MS or LC-MS analyses and/or by prediction based on enzyme specificity of the protease enzyme for which the protease cleavage site is to be determined. A skilled person in the art will know how to determine protease cleavage sites for example based on enzyme specificities as for example described in Handbook of Proteolytical Enzymes, 2nd ed., Barrett, A.J., Rawlings, N.D., Woessner, J.F. editors, Elsevier Academic Press 2004. For example chymotrypsin is predicted to cleave peptide bonds C-terminal to aromatic residues (Trp, Tyr, Phe or Leu), that are not followed by Pro. Similarly, trypsin is predicted to cleave peptide bonds C-terminal to basic residues Lys or Arg, that are not followed by Pro, elastase is predicted to cleave residues C-terminal to Ala, Val, Gly or Ser and carboxypeptidase A will remove any C-terminal amino acid, but not Arg, Lys or Pro.

Insulin-degrading enzyme (IDE) is predicted to cleave the following positions of human insulin B9-10, B10-11, B13-14, B14-15, B24-25, B25-26, A13-14 and A14-15.

The term substituting (an) amino acid "within or in close proximity" to a protease cleavage site is herein used to indicate the substitution of an amino acid within or in close proximity to a position of the parent insulin which has been determined to be a protease cleavage site. In one embodiment two or more hydrophobic amino acids within or in close proximity to two or more protease sites on an insulin are substituted, wherein said hydrophobic amino acids are substituted with hydrophilic amino acids. In a further embodiment two or more hydrophobic amino acids within two or more protease sites on an insulin are substituted with hydrophilic amino acids. In a yet further embodiment two or more hydrophobic amino acids situated next to two or more protease sites on an insulin are substituted with hydrophilic amino acids. In a still further embodiment two or more hydrophobic amino acids situated two amino acids away from two or more protease sites on an insulin are substituted with hydrophilic amino acids. In a yet further embodiment two or more hydrophobic amino acids situated three amino acids away from two or more protease sites on an insulin are substituted with hydrophilic amino acids. In a still further embodiment two or more hydrophobic amino acids situated up to four amino acids away from two or more protease sites on an insulin are substituted with hydrophilic amino acids. In a yet further embodiment two or more hydrophobic amino acids situated one, two or three amino acids away from or within two or more protease sites on an insulin are substituted with hydrophilic amino acids. In a still further embodiment two or more hydrophobic amino acids situated one or two amino acids away from or within two or more protease sites on an insulin are substituted with hydrophilic amino acids. In a yet further embodiment two or more hydrophobic amino acids situated next to or within two or more protease sites on an insulin are substituted with hydrophilic amino acids.

An insulin analogue according to the invention may have a net charge which is different than the net charge of the parent insulin. In one embodiment the net charge of an insulin analogue according to the invention is more positive than the net charge of the parent insulin. In one embodiment the net charge of an insulin analogue according to the invention is more negative than the net charge of the parent insulin. In one embodiment the average positive net charge of an insulin analogue according to the invention is between 0.5 and 5 as measured in an aqueous solution. In one embodiment the average positive net charge of an insulin analogue according to the invention is between 1 and 5. In one embodiment the aver-

age positive net charge of an insulin analogue according to the invention is between 1 and 4. In one embodiment the average positive net charge of an insulin analogue according to the invention is between 1 and 3. In one embodiment the average positive net charge of an insulin analogue according to the invention is between 2 and 3. In one embodiment the average negative net charge of an insulin analogue according to the invention is between -0.5 and -5 as measured in an aqueous solution. In one embodiment the average negative net charge of an insulin analogue according to the invention is between -1 and -5. In one embodiment the average negative net charge of an insulin analogue according to the invention is between -1 and -4. In one embodiment the average negative net charge of an insulin analogue according to the invention is between -1 and -3. In one embodiment the average negative net charge of an insulin analogue according to the invention is between -2 and -3.

In one embodiment an insulin analogue according to the invention may have increased solubility relative to human insulin. In a further embodiment an insulin analogue according to the invention has increased solubility relative to human insulin at pH 3-9. In a yet further embodiment an insulin analogue according to the invention has increased solubility relative to human insulin at pH 4-8.5. In a still further embodiment an insulin analogue according to the invention has increased solubility relative to human insulin at pH 4-8. In a yet further embodiment an insulin analogue according to the invention has increased solubility relative to human insulin at pH 4.5-8. In a further embodiment an insulin analogue according to the invention has increased solubility relative to human insulin at pH 5-8. In a yet further embodiment an insulin analogue according to the invention has increased solubility relative to human insulin at pH 5.5-8. In a further embodiment an insulin analogue according to the invention has increased solubility relative to human insulin at pH 6-8.

In one embodiment an insulin analogue according to the invention has increased solubility relative to human insulin at pH 2-4.

In one embodiment an insulin analogue according to the invention may have increased solubility relative to the parent insulin. In a further embodiment an insulin analogue according to the invention has increased solubility relative to the parent insulin at pH 3-9. In a yet further embodiment an insulin analogue according to the invention has increased solubility relative to parent insulin at pH 4-8.5. In a still further embodiment an insulin analogue according to the invention has increased solubility relative to parent insulin at pH 4-8. In a yet further embodiment an insulin analogue according to the invention has increased solubility

relative to parent insulin at pH 4.5-8. In a still further embodiment an insulin analogue according to the invention has increased solubility relative to parent insulin at pH 5-8. In a yet further embodiment an insulin analogue according to the invention has increased solubility relative to parent insulin at pH 5.5-8. In a further embodiment an insulin analogue according to the invention has increased solubility relative to parent insulin at pH 6-8.

In one embodiment an insulin analogue according to the invention has increased solubility relative to parent insulin at pH 2-4.

By "increased solubility at a given pH" is meant that a larger concentration of an insulin analogue of the invention dissolves in an aqueous or buffer solution at the pH of the solution relative to the insulin it is compared to, i.e. human insulin or the parent insulin. Methods for determining whether the insulin contained in a solution is dissolved are known in the art.

In one embodiment, the solution may be subjected to centrifugation for 20 minutes at 30,000 g and then the insulin concentration in the supernatant may be determined by RP-HPLC. If this concentration is equal within experimental error to the insulin concentration originally used to make the composition, then the insulin is fully soluble in the composition of the invention.

In another embodiment, the solubility of the insulin in a composition of the invention can simply be determined by examining by eye the container in which the composition is contained. The insulin is soluble if the solution is clear to the eye and no particulate matter is either suspended or precipitated on the sides/bottom of the container.

An insulin analogue according to the invention may have increased potency and/or bioavailability relative to the parent insulin when compared upon measurement.

Standard assays for measuring insulin potency or bioavailability are known to the person skilled in the art and include inter alia (1) insulin radioreceptorassays, in which the relative potency of an insulin is defined as the ratio of insulin to insulin analogue required to displace 50% of ^{125}I -insulin specifically bound to insulin receptors present on cell membranes, e.g. a rat liver plasma membrane fraction; (2) lipogenesis assays, performed e.g. with rat adipocytes, in which relative insulin potency is defined as the ratio of insulin to insulin analogue required to achieve 50% of the maximum conversion of $[3\text{-}^3\text{H}]$ glucose into organic-extractable material (i.e. lipids); (3) glucose oxidation assays in isolated fat cells in which the relative potency of the insulin analogue is defined as the ratio of insulin to insulin analogue to achieve 50% of the maximum conversion of glucose-1- $[^{14}\text{C}]$ into $[^{14}\text{CO}_2]$; (4) insulin radioimmunoassays which can determine the immunogenicity of insulin analogues by measuring the

effectiveness by which insulin or an insulin analogue competes with ¹²⁵I-insulin in binding to specific anti-insulin antibodies; and (5) other assays which measure the binding of insulin or an insulin analogue to antibodies in animal blood plasma samples, such as ELISA assays possessing specific insulin antibodies.

5

Insulin analogues according to the invention may optionally be analyzed for further protease sites which may be subject to further substitutions of one or more hydrophobic amino acids with hydrophilic amino acids. An insulin analogue according to the invention may be an insulin analogue which has at least two hydrophilic acids in protease sites compared to the parent insulin, the first modified insulin, and which has further at least one amino acid substitution in a new protease site of the first modified insulin wherein at least one hydrophobic amino acid has been substituted with at least one hydrophilic amino acid.

10

In one embodiment an insulin analogue is provided wherein the C terminal B chain is connected to the N terminal A chain with 3-15 amino acids. Such an insulin analogue is herein denoted "single-chain insulin" or "SCI" and has the general structure B-C-A wherein B is the human B insulin chain or an analogue or derivative thereof, A is the human insulin A chain or an analogue or derivative and C is the connecting peptide chain of 3-15 amino acid residues normally connecting B30 with A1. If the B chain is a desB30 chain the connecting peptide will connect B29 with A1. The single-chain insulin will contain correctly positioned disulphide bridges (three) as in human insulin that is between CysA7 and CysB7 and between CysA20 and CysB19 and an internal disulfide bridge between CysA6 and CysA11. In one embodiment an insulin analogue is provided wherein the C terminal B chain is connected to the N terminal A chain with 3-7 amino acids.

15

20

25

The present invention is also related to nucleic acid sequences which code for the claimed insulin analogues. In a further embodiment the present invention is related to vectors containing such nucleic acid sequences and host cells containing such nucleic acid sequences or vectors.

30

In still a further embodiment, the invention relates to a process for producing an insulin analogue comprising (i) culturing a host cell comprising a nucleic acid sequence encoding an insulin precursor; (ii) isolating the insulin precursor from the culture medium and (iii) converting the insulin precursor into an insulin analogue of the invention by in vitro enzymatic conversion.

In still a further embodiment, the invention relates to a process for producing an insulin analogue comprising (i) culturing a host cell comprising a nucleic acid sequence encoding an insulin precursor; (ii) isolating the insulin precursor from the culture medium and (iii) converting the insulin precursor into an insulin analogue of the invention.

5 In one embodiment of the present invention the host cell is a yeast host cell and in a further embodiment the yeast host cell is selected from the genus *Saccharomyces*. In a further embodiment the yeast host cell is selected from the species *Saccharomyces cerevisiae*.

10 By "insulin analogue" as used herein is meant a polypeptide derived from the primary structure of a naturally occurring insulin, for example that of human insulin, by deleting and/or substituting at least one amino acid residue occurring in the naturally occurring insulin and/or by adding at least one amino acid residue. The added and/or substituted amino acid residues can either be codable amino acid residues or other naturally occurring amino acid residues.

15 The insulin analogues according to the present invention may be human insulin or an analogue thereof mutated in one or more positions. In one embodiment the insulin analogues are designed for enhanced stability towards proteases based on the identified protease cleavage sites.

20 In one embodiment at least one cleavage site subjected to mutation is in a position selected from the group consisting of: B2-3, B6-7, B9-10, B10-11, B13-14, B14-15, B16-17, B22-23, B24-25, B25-26, A13-14, A14-15 and A19-20 of human insulin.

In one embodiment at least one cleavage site subjected to mutation is in a position selected from the group consisting of: B2-3, B6-7, B16-17, B22-23, B24-25 and/or A19-20 of human insulin.

25 In one embodiment at least one cleavage site subjected to mutation is in position B22-23.

In one embodiment at least one cleavage site subjected to mutation is in a position selected from the group consisting of: B9-10, B10-11, B13-14, B14-15, B24-25, B25-26, A13-14, A14-15.

30 In one embodiment cleavage sites subjected to mutation are in two or more positions selected from the group consisting of: B2-3, B6-7, B9-10, B10-11, B13-14, B14-15, B16-17, B22-23, B24-25, B25-26, A13-14, A14-15 and A19-20 of human insulin.

In one embodiment cleavage sites subjected to mutation are in two or more positions selected from the group consisting of: B2-3, B6-7, B16-17, B22-23, B24-25 and/or A19-20 of human insulin.

35

In one embodiment cleavage sites subjected to mutation are in two or more positions selected from the group consisting of: B9-10, B10-11, B13-14, B14-15, B24-25, B25-26, A13-14, A14-15.

5 In one embodiment an insulin analogue according to the invention is obtained wherein the A-chain of the insulin analogue comprises at least one mutation and the B-chain of the insulin analogue comprises at least one mutation relative to the parent insulin, wherein the at least one mutation in the A-chain is in one or more cleavage sites selected from the group consisting of: A13-14, A14-15 and A19-20 and the at least one mutation in the B-chain
10 is in one or more cleavage sites selected from the group consisting of: B2-3, B6-7, B9-10, B10-11, B13-14, B14-15, B16-17, B22-23, B24-25 and B25-26.

In one embodiment an insulin analogue according to the invention is obtained wherein the A-chain of the insulin analogue comprises one mutation and the B-chain of the
15 insulin analogue comprises at least one mutation relative to the parent insulin, wherein the one mutation in the A-chain is in the cleavage site A19-20 and the at least one mutation in the B-chain is in one or more cleavage sites selected from the group consisting of: B2-3, B6-7, B16-17, B22-23 and B24-25.

20 In one embodiment an insulin analogue according to the invention is obtained wherein the A-chain of the insulin analogue comprises at least one mutation and the B-chain of the insulin analogue comprises at least one mutation relative to the parent insulin, wherein the at least one mutation in the A-chain is in one or more cleavage sites selected from the group consisting of: A13-14 and A14-15 and the at least one mutation in the B-chain is in one
25 or more cleavage sites selected from the group consisting of: B9-10, B10-11, B13-14, B14-15, B24-25 and B25-26.

Amino acid residues suitable for substitution are selected with the purpose of removing the cleavage sites. In one embodiment amino acids are selected with the additional
30 purpose of increasing the solubility. In a further embodiment the increased solubility is in the pH range 3-9. In a yet further embodiment the increased solubility is in the pH range 4-8. The insulin or insulin analogue may be substituted in one or more positions by any natural amino acid or any natural amino acid may be added to the parent insulin or parent insulin analogue. For the sake of convenience, here follows the names of codable, natural amino acids with
35 the usual three letter codes & one letter codes in parenthesis: Glycine (Gly & G), proline (Pro

& P), alanine (Ala & A), valine (Val & V), leucine (Leu & L), isoleucine (Ile & I), methionine (Met & M), cysteine (Cys & C), phenylalanine (Phe & F), tyrosine (Tyr & Y), tryptophan (Trp & W), histidine (His & H), lysine (Lys & K), arginine (Arg & R), glutamine (Gln & Q), asparagine (Asn & N), glutamic acid (Glu & E), aspartic acid (Asp & D), serine (Ser & S) and threonine (Thr & T). If, due to typing errors, there are deviations from the commonly used codes, the commonly used codes apply. The amino acids present in the insulins of this invention are, preferably, amino acids which can be coded for by a nucleic acid. In one embodiment insulin or an insulin analogue is substituted by Gly, Glu, Asp, His, Gln, Asn, Ser, Thr, Lys, Arg and/or Pro and/or Gly, Glu, Asp, His, Gln, Asn, Ser, Thr, Lys, Arg and/or Pro is added to insulin or an insulin analogue. In one embodiment insulin or an insulin analogue is substituted by Glu, Asp, His, Gln, Asn, Lys and/or Arg and/or Glu, Asp, His, Gln, Asn, Lys and/or Arg is added to insulin or an insulin analogue.

The insulin analogues may be such wherein position 25 of the B chain may be modified from the natural Phe residue to His in combination with substitution of the natural Tyr at position A14 to Glu, Asp or His. One or more additional mutations may include desB30 and the insulin analogues could be further modified by N-terminal extension or C-terminal extension of the A-chain and/or the B-chain such as e.g. extension of the N-terminal of the A- and/or B-chain of the insulin analogue with GGP, GGPP, GP or GPP. Further examples include but are not limited to additional mutations wherein one or two Pro residues may be added to positions A0 and/or B0, Leu may be added to position B31 and/or Glu may be added to position B32. One or more additional mutations may be selected from position A8 modified to His, position A21 to Gly, position B1 to Glu or Gln, position B16 to Glu, position B26 to Asp, position B27 to Glu and/or position B28 to Asp.

In one embodiment an insulin analogue according to the invention is selected from the group consisting of:

- A14E, B25H, desB30 human insulin;
- A14H, B25H, desB30 human insulin;
- A14E, B1E, B25H, desB30 human insulin;
- A14E, B16E, B25H, desB30 human insulin;
- A14E, B25H, B28D, desB30 human insulin;
- A14E, B25H, B27E, desB30 human insulin;
- A14E, B1E, B25H, B27E, desB30 human insulin;
- A14E, B1E, B16E, B25H, B27E, desB30 human insulin;
- A8H, A14E, B25H, desB30 human insulin;

- A8H, A14E, B25H, B27E, desB30 human insulin;
 A8H, A14E, B1E, B25H, desB30 human insulin;
 A8H, A14E, B1E, B25H, B27E, desB30 human insulin;
 A8H, A14E, B1E, B16E, B25H, B27E, desB30 human insulin;
 5 A8H, A14E, B16E, B25H, desB30 human insulin;
 A14E, B25H, B26D, desB30 human insulin;
 A14E, B1E, B27E, desB30 human insulin;
 A14E, B27E, desB30 human insulin;
 A14E, B28D, desB30 human insulin;
 10 A14D, B25H, desB30 human insulin;
 A(-1)P, A(0)P, A14E, B25H, desB30 human insulin;
 A14E, B(-1)P, B(0)P, B25H, desB30 human insulin;
 A(-1)P, A(0)P, A14E, B(-1)P, B(0)P, B25H, desB30 human insulin;
 A14E, B25H, B30T, B31L, B32E human insulin;
 15 A14E, B25H human insulin;
 A14E, B16H, B25H, desB30 human insulin;
 A14E, B10P, B25H, desB30 human insulin;
 A14E, B10E, B25H, desB30 human insulin;
 A14E, B4E, B25H, desB30 human insulin;
 20 A14H, B16H, B25H, desB30 human insulin;
 A14H, B10E, B25H, desB30 human insulin;
 A14E, B25H, desB27, desB28, desB29, desB30 human insulin;
 A13H, A14E, B10E, B25H, desB30 human insulin;
 A13H, A14E, B25H, desB30 human insulin;
 25 A14E, A18Q, B3Q, B25H, desB30 human insulin;
 A14E, B24H, B25H, desB30 human insulin;
 A8H, A14E, B10D, B25H, B26G, desB27, desB28, desB29, desB30 human insulin;
 A8H, A14E, B10D, B25H-amide, desB26, desB27, desB28, desB29, desB30 human
 insulin;
 30 A14E, B25H, desB26, desB27, desB28, desB29, desB30 human insulin;
 A14E, B25H, B26G, desB27, desB28, desB29, desB30 human insulin;
 A14E, B25H-amide, desB27, desB28, desB29, desB30 human insulin
 A14E, B25H, B26G, B27G, B28G, desB30 human insulin;
 A14E, B26G, desB27, desB28, desB29, desB30 human insulin;
 35 A14E, B25H, desB26, desB27, desB28, desB29, desB30 human insulin;

- A14E, A18Q, A21Q, B3Q, B25H, desB30 human insulin;
 A14E, A18Q, A21Q, B3Q, B25H, B27E, desB30 human insulin;
 A14E, A18Q, B3Q, B25H, desB30 human insulin;
 A13H, A14E, B1E, B25H, desB30 human insulin;
 5 A13N, A14E, B25H, desB30 human insulin;
 A13N, A14E, B1E, B25H, desB30 human insulin;
 A(-2)G, A(-1)P, A(0)P, A14E, B25H, desB30 human insulin;
 A14E, B(-2)G, B(-1)P, B(0)P, B25H, desB30 human insulin;
 A(-2)G, A(-1)P, A(0)P, A14E, B(-2)G, B(-1)P, B(0)P, B25H, desB30 human insulin;
 10 A14E, B27R, B28D, B29K, desB30 human insulin;
 A14E, B25H, B27R, B28D, B29K, desB30 human insulin;
 A14E, B25H, B26T, B27R, B28D, B29K, desB30 human insulin;
 A14E, A18K, B25H, desB27, desB28, desB29, desB30 human insulin;
 A14E, A22K, B25H, desB27, desB28, desB29, desB30 human insulin;
 15 A14E, A22K, B25H, desB30 human insulin;
 A14E, desB1, desB2, desB3, B25H-amide, desB26, desB27, desB28, desB29,
 desB30 human insulin;
 A14E, desB1, desB2, desB3, B25H, desB27, desB28, desB29, desB30 human insu-
 lin;
 20 A14E, desB1, desB2, desB3, B16H, B25H, desB27, desB28, desB29, desB30 hu-
 man insulin;
 A14E, B25H, B27R, desB30 human insulin;
 A14E, B25H, B27H, desB30 human insulin;
 A14E, B25H, B27R, desB28-B30 human insulin;
 25 A14E, B25H, B27H, desB28-B30 human insulin;
 A14E, B25H, B27E, desB28-B30 human insulin;
 A14E, B25H, B27K, desB28-B30 human insulin;
 A14E, B27K, desB28-B30 human insulin;
 A14E, A18Q, B3Q, B25H, desB30 human insulin;
 30 A13E, A14E, B25H, desB30 human insulin;
 A12E, A14E, B25H, desB30 human insulin;
 A15E, A14E, B25H, desB30 human insulin;
 A13E, B25H, desB30 human insulin;
 A12E, B25H, desB30 human insulin;
 35 A15E, B25H, desB30 human insulin;

- A14E, B25H, desB27, desB30 human insulin;
A14E, B25H, B26D, B27E, desB30 human insulin;
EEAEAEAPK-B(1-29)-B25H-AAK-A(1-21)-A14E human insulin;
EEAEPK-B(1-29)-B25H-DGK-A(1-21)-A14E human insulin;
5 B(1-29)-B25H-AAK-A(1-21)-A14E human insulin;
B(1-29)-B1E, B25H-AAK-A(1-21)-A14E human insulin;
B(1-29)-B25H, B27E-AAK-A(1-21)-A8H, A14E human insulin;
B(1-29)-B1E, B25H, B27E-AAK-A(1-21)-A8H, A14E human insulin;
EEAEAEAPK-B(1-29)-B16E, B25H-AAK-A(1-21)-A8H, A14E human insulin;
10 B(1-29)-B25H, B29Q-TGLGGGQ-A(1-21)-A14E human insulin
B(1-29)-B16E, B25H, B29Q-TGLGGGQ-A(1-21)-A14E human insulin
B(1-29)-B25H, B29Q-TGLGGGQ-A(1-21)-A8H, A14E human insulin
A14E, B25H, B27R, desB30 human insulin;
A14E, B25H, B27N, desB30 human insulin;
15 A14E, B25H, B27D, desB30 human insulin;
A14E, B25H, B27Q, desB30 human insulin;
A14E, B25H, B27E, desB30 human insulin;
A14E, B25H, B27G, desB30 human insulin;
A14E, B25H, B27H, desB30 human insulin;
20 A14E, B25H, B27K, desB30 human insulin;
A14E, B25H, B27P, desB30 human insulin;
A14E, B25H, B27S, desB30 human insulin;
A14E, B25H, B27T, desB30 human insulin;
A13R, A14E, B25H, desB30 human insulin;
25 A13N, A14E, B25H, desB30 human insulin;
A13D, A14E, B25H, desB30 human insulin;
A13Q, A14E, B25H, desB30 human insulin;
A13E, A14E, B25H, desB30 human insulin;
A13G, A14E, B25H, desB30 human insulin;
30 A13H, A14E, B25H, desB30 human insulin;
A13K, A14E, B25H, desB30 human insulin;
A13P, A14E, B25H, desB30 human insulin;
A13S, A14E, B25H, desB30 human insulin;
A13T, A14E, B25H, desB30 human insulin;
35 A14E, B16R, B25H, desB30 human insulin;

A14E, B16D, B25H, desB30 human insulin;
 A14E, B16Q, B25H, desB30 human insulin;
 A14E, B16E, B25H, desB30 human insulin;
 A14E, B16H, B25H, desB30 human insulin;
 5 A14R, B25H, desB30 human insulin;
 A14N, B25H, desB30 human insulin;
 A14D, B25H, desB30 human insulin;
 A14Q, B25H, desB30 human insulin;
 A14E, B25H, desB30 human insulin;
 10 A14G, B25H, desB30 human insulin;
 A14H, B25H, desB30 human insulin;
 A8H, B10D, B25H human insulin; and
 A8H, A14E, B10E, B25H, desB30 human insulin.

15 Insulin is a polypeptide hormone secreted by β -cells of the pancreas and consists of two polypeptide chains, A and B, which are linked by two inter-chain disulphide bridges. Furthermore, the A-chain features one intra-chain disulphide bridge.

The hormone is synthesized as a single-chain precursor proinsulin (preproinsulin) consisting of a prepeptide of 24 amino acid followed by proinsulin containing 86 amino acids
 20 in the configuration: prepeptide-B-Arg Arg-C-Lys Arg-A, in which C is a connecting peptide of 31 amino acids. Arg-Arg and Lys-Arg are cleavage sites for cleavage of the connecting peptide from the A and B chains.

Three major methods have been used for the production of human insulin in micro-
 25 organisms. Two involve *Escherichia coli*, with either the expression of a large fusion protein in the cytoplasm (Frank et al. (1981) in Peptides: Proceedings of the 7th American Peptide Chemistry Symposium (Rich & Gross, eds.), Pierce Chemical Co., Rockford, Ill. pp 729-739), or use of a signal peptide to enable secretion into the periplasmic space (Chan et al. (1981) PNAS 78:5401-5404). A third method utilizes *Saccharomyces cerevisiae* to secrete an insu-
 30 lin precursor into the medium (Thim et al. (1986) PNAS 83:6766-6770). The prior art discloses a number of insulin precursors which are expressed in either *E. coli* or *Saccharomyces cerevisiae*, vide U.S. Pat. No. 5,962,267, WO 95/16708, EP 0055945, EP 0163529, EP 0347845 and EP 0741188.

The insulin analogues are produced by expressing a DNA sequence encoding the insulin analogue in question in a suitable host cell by well known technique as disclosed in e.g. US patent No. 6500645. The insulin analogue is either expressed directly or as a precursor molecule which has an N-terminal extension on the B-chain. This N-terminal extension may have the function of increasing the yield of the directly expressed product and may be of up to 15 amino acid residues long. The N-terminal extension is to be cleaved off in vitro after isolation from the culture broth and will therefore have a cleavage site next to B1. N-terminal extensions of the type suitable in the present invention are disclosed in U.S. Patent No. 5,395,922, and European Patent No. 765,395A.

The isolated insulin analogue can be acylated in the desired position by well known acylation methods and examples of such insulin analogues are described e.g. in the European patent applications having the publication Nos. EP 214826, EP 375437 and EP 383472.

The nucleic acid sequence coding for the respective insulin analogue polypeptide may be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beaucage et al. (1981) Tetrahedron Letters 22:1859-1869, or the method described by Matthes et al. (1984) EMBO Journal 3:801-805. According to the phosphoramidite method, oligonucleotides are synthesized, for example, in an automatic DNA synthesizer, purified, duplexed and ligated to form the synthetic DNA construct. A currently preferred way of preparing the DNA construct is by polymerase chain reaction (PCR).

The nucleic acid sequences may also be of mixed genomic, cDNA, and synthetic origin. For example, a genomic or cDNA sequence encoding a leader peptide may be joined to a genomic or cDNA sequence encoding the A and B chains, after which the DNA sequence may be modified at a site by inserting synthetic oligonucleotides encoding the desired amino acid sequence for homologous recombination in accordance with well-known procedures or preferably generating the desired sequence by PCR using suitable oligonucleotides.

The recombinant vector capable of replicating in the selected microorganism or host cell and which carries a nucleic acid sequence encoding the insulin analogue polypeptide in question may be an autonomously replicating vector, *i.e.*, a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extra-chromosomal element, a mini-chromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a

single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used. The vector may be linear or closed circular plasmids and will preferably contain an element(s) that permits stable integration of the vector into the host cell's genome or autonomous repli-

5 cation of the vector in the cell independent of the genome.

In one embodiment, the recombinant expression vector is capable of replicating in yeast. Examples of sequences which enable the vector to replicate in yeast are the yeast plasmid 2 μ m replication genes REP 1-3 and origin of replication.

The vectors may contain one or more selectable markers which permit easy selec-

10 tion of transformed cells. A selectable marker is a gene the product of which provides for bio-
cide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Selectable markers for use in a filamentous fun-

15 gal host cell include *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *pyrG* (orotidine-5'-phosphate decarboxylase) and *trpC* (anthranilate synthase. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. A well suited se-

lectable marker for yeast is the *Schizosaccharomyces pombe* TPI gene (Russell (1985) Gene 40:125-130).

20 In the vector, the nucleic acid sequence is operably connected to a suitable pro-
moter sequence. The promoter may be any nucleic acid sequence which shows transcrip-
tional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extra-cellular or intra-cellular polypeptides either ho-
mologous or heterologous to the host cell.

25 Examples of suitable promoters for directing the transcription in a bacterial host cell are the promoters obtained from the *E. coli lac* operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus licheniformis* alpha-
amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacil-*
lus amyloliquefaciens alpha-amylase gene (*amyQ*), and *Bacillus licheniformis* penicillinase

30 gene (*penP*). Examples of suitable promoters for directing the transcription in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus oryzae* TKA amy-
lase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, and *Aspergillus niger* acid stable alpha-amylase. In a yeast host, useful promoters are the *Sac-*
charomyces cerevisiae Ma1, TPI, ADH or PGK promoters.

The nucleic acid construct will also typically be operably connected to a suitable terminator. In yeast a suitable terminator is the TPI terminator (Alber et al. (1982) J. Mol. Appl. Genet. 1:419-434).

The procedures used to ligate the individual nucleic acid sequences contained in the expression vector such as DNA coding for the desired insulin analogue polypeptide, the promoter and the terminator, respectively, and to insert them into a suitable vector containing the information necessary for replication in the selected host, are well known to persons skilled in the art. It will be understood that the vector may be constructed either by first preparing a DNA construct containing the entire DNA sequence encoding the insulin analogue polypeptides of the invention, and subsequently inserting this fragment into a suitable expression vector, or by sequentially inserting DNA fragments containing genetic information for the individual elements (such as the signal, pro-peptide, connecting peptide, A and B chains) followed by ligation.

The vector comprising such nucleic acid sequence is introduced into the host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The host cell may be a unicellular microorganism, e.g., a prokaryote, or a non-unicellular microorganism, e.g., a eukaryote. Useful unicellular cells are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, *Streptomyces* cell, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp. Eukaryote cells may be mammalian, insect, plant, or fungal cells. In a preferred embodiment, the host cell is a yeast cell. The yeast organism used in the process of the invention may be any suitable yeast organism which, on cultivation, produces large amounts of the single chain insulin of the invention. Examples of suitable yeast organisms are strains selected from the yeast species *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe*, *Saccharomyces uvarum*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida* sp., *Candida utilis*, *Candida cacaoi*, *Geotrichum* sp., and *Geotrichum fermentans*.

The transformation of the yeast cells may for instance be effected by protoplast formation followed by transformation in a manner known *per se*. The medium used to cultivate the cells may be any conventional medium suitable for growing yeast organisms. The secreted insulin analogue polypeptide, a significant proportion of which will be present in the medium in correctly processed form, may be recovered from the medium by conventional procedures including separating the yeast cells from the medium by centrifugation, filtration

or catching the insulin precursor by an ion exchange matrix or by a reverse phase absorption matrix, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

5

PHARMACEUTICAL COMPOSITIONS

Another object of the present invention is to provide a pharmaceutical formulation comprising an insulin analogue according to the present invention which is present in a concentration from 0.1 mg/ml to 500 mg/ml, and wherein said formulation has a pH from 2.0 to 10.0. The formulation may further comprise protease inhibitor(s), a buffer system, preservative(s), tonicity agent(s), chelating agent(s), stabilizers and surfactants. In one embodiment of the invention the pharmaceutical formulation is an aqueous formulation, i.e. formulation comprising water. Such formulation is typically a solution or a suspension. In a further embodiment of the invention the pharmaceutical formulation is an aqueous solution. The term
10 “aqueous formulation” is defined as a formulation comprising at least 50 %w/w water. Likewise, the term “aqueous solution” is defined as a solution comprising at least 50 %w/w water, and the term “aqueous suspension” is defined as a suspension comprising at least 50 %w/w water.
15

In another embodiment the pharmaceutical formulation is a freeze-dried formulation, where to the physician or the patient adds solvents and/or diluents prior to use.
20

In another embodiment the pharmaceutical formulation is a dried formulation (e.g. freeze-dried or spray-dried) ready for use without any prior dissolution.

In a further embodiment the invention relates to a pharmaceutical formulation comprising an aqueous solution of an insulin analogue of the present invention, and a buffer, wherein said insulin analogue is present in a concentration from 0.1 mg/ml or above, and wherein said formulation has a pH from about 2.0 to about 10.0.
25

Formulations intended for oral use may be prepared according to any known method, and such formulations may contain one or more agents selected from the group consisting of sweetening agents, flavouring agents, colouring agents, and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets may contain the active ingredient in a mixture with non-toxic pharmaceutically-acceptable excipients which are suitable for the manufacture of tablets. These excipients may be for example, inert diluents, such as mannitol, maltodextrin, kaolin, calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example corn
30

starch; binding agents, for example, starch, gelatine, polymers or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration or release of the therapeutically active polypeptide.

5

The orally administerable formulations of the present invention may be prepared and administered according to methods well known in pharmaceutical chemistry, see Remington's Pharmaceutical Sciences, 17th ed. (A. Osol ed., 1985).

10

In one embodiment of the invention, the pharmaceutical compositions of the present invention may be administered by means of solid dosage forms such as tablets and capsules. The tablets may be prepared by wet granulation, by dry granulation, by direct compression or melt granulation.

15

Tablets for this invention may be prepared utilizing conventional tableting techniques. A general method of manufacture involves blending of an insulin analogue, a water-soluble diluent, hydrophilic binder and optionally a portion of a disintegrant. This blend is then granulated with an aqueous solution of the hydrophilic binder or an aqueous solution of the hydrophilic binder and surfactant and milled, if necessary. The granules are dried and reduced to a suitable size. Any other ingredients, such as lubricants, (e.g. magnesium stearate) and additional disintegrants, are added to the granules and mixed. This mixture is then compressed into a suitable size and shape using conventional tableting machines such as a rotary tablet press. The tablets may be film coated by techniques well known in the art.

20

25

Formulations for oral use may also be presented as hard or soft gelatine capsules where the active ingredient is mixed with an inert solid diluent, for example, such as mannitol, maltodextrin, calcium carbonate, sodium carbonate, lactose, kaolin, calcium phosphate or sodium phosphate, or a soft gelatine capsule wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin, or olive oil.

30

35

Capsules for this invention may be prepared utilizing conventional methods. A general method of manufacture involves blending a therapeutically active peptide, alginate, a water-soluble diluent, a hydrophilic binder, and optionally a portion of a disintegrant. This blend is then granulated with an aqueous solution of the hydrophilic binder or an aqueous solution of the hydrophilic binder and surfactant in water, and milled, if necessary. The granules are dried and

reduced to a suitable size. Any other ingredients, such as a lubricant, are added to the granules and mixed. The resulting mixture is then filled into a suitable size hard-shell gelatin capsule using conventional capsule-filling machines.

5 In a further embodiment of the invention the buffer is selected from the group consisting of sodium acetate, sodium carbonate, citrate, glycylglycine, histidine, glycine, lysine, arginine, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium phosphate, and tris(hydroxymethyl)-aminomethan, bicine, tricine, malic acid, succinate, maleic acid, fumaric acid, tartaric acid, aspartic acid or mixtures thereof. Each one of these
10 specific buffers constitutes an alternative embodiment of the invention.

 In a further embodiment of the invention the formulation further comprises a pharmaceutically acceptable preservative. The preservative is present in an amount sufficient to obtain a preserving effect. The amount of preservative in a pharmaceutical
15 formulation is the well-known to the skilled person and may be determined from e.g. literature in the field and/or the known amount(s) of preservative in e.g. commercial products. Each one of these specific preservatives constitutes an alternative embodiment of the invention. The use of a preservative in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*,
20 19th edition, 1995.

 In a further embodiment of the invention the formulation further comprises a chelating agent. The use of a chelating agent in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice*
25 *of Pharmacy*, 19th edition, 1995.

 In a further embodiment of the invention the formulation further comprises a stabilizer. The term "stabiliser" as used herein refers to chemicals added to polypeptide containing pharmaceutical formulations in order to stabilize the peptide, i.e. to increase the shelf life
30 and/or in-use time of such formulations. The use of a stabilizer in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

 In a further embodiment of the invention the formulation further comprises a surfactant. The term "surfactant" as used herein refers to any molecules or ions that are comprised
35

of a water-soluble (hydrophilic) part, the head, and a fat-soluble (lipophilic) segment. Surfactants accumulate preferably at interfaces, which the hydrophilic part is orientated towards the water (hydrophilic phase) and the lipophilic part towards the oil- or hydrophobic phase (*i.e.* glass, air, oil etc.). The concentration at which surfactants begin to form micelles is known as the critical micelle concentration or CMC. Furthermore, surfactants lower the surface tension of a liquid. Surfactants are also known as amphipathic compounds. The term "Detergent" is a synonym used for surfactants in general. The use of a surfactant in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

In a further embodiment of the invention the formulation further comprises protease inhibitors.

It is possible that other ingredients may be present in the insulin analogue pharmaceutical formulation of the present invention. Such additional ingredients may include wetting agents, emulsifiers, antioxidants, bulking agents, tonicity modifiers, chelating agents, metal ions, oleaginous vehicles, proteins (e.g., human serum albumin, gelatine or proteins) and a zwitterion (e.g., an amino acid such as betaine, taurine, arginine, glycine, lysine and histidine). Such additional ingredients, of course, should not adversely affect the overall stability of the pharmaceutical formulation of the present invention.

Pharmaceutical compositions containing an insulin analogue according to the present invention may be administered to a patient in need of such treatment at several sites, for example, at topical sites, for example, skin and mucosal sites, at sites which bypass absorption, for example, administration in an artery, in a vein, in the heart, and at sites which involve absorption, for example, administration in the skin, under the skin, in a muscle or in the abdomen.

Administration of pharmaceutical compositions according to the invention may be through several routes of administration, for example, lingual, sublingual, buccal, in the mouth, oral, in the stomach and intestine, nasal, pulmonary, for example, through the bronchioles and alveoli or a combination thereof, epidermal, dermal, transdermal, vaginal, rectal, ocular, for examples through the conjunctiva, uretal, and parenteral to patients in need of such a treatment.

Compositions of the current invention may be administered in several dosage forms, for example, as solutions, suspensions, emulsions, microemulsions, multiple emulsion,

foams, salves, pastes, plasters, ointments, tablets, coated tablets, rinses, capsules, for example, hard gelatine capsules and soft gelatine capsules, suppositories, rectal capsules, drops, gels, sprays, powder, aerosols, inhalants, eye drops, ophthalmic ointments, ophthalmic rinses, vaginal pessaries, vaginal rings, vaginal ointments, injection solution, in situ transforming solutions, for example in situ gelling, in situ setting, in situ precipitating, in situ crystallization, infusion solution, and implants.

Compositions of the invention may further be compounded in, or attached to, for example through covalent, hydrophobic and electrostatic interactions, a drug carrier, drug delivery system and advanced drug delivery system in order to further enhance stability of the insulin analogue compound, increase bioavailability, increase solubility, decrease adverse effects, achieve chronotherapy well known to those skilled in the art, and increase patient compliance or any combination thereof.

Compositions of the current invention are useful in the formulation of solids, semisolids, powder and solutions for pulmonary administration of insulin analogue, using, for example a metered dose inhaler, dry powder inhaler and a nebulizer, all being devices well known to those skilled in the art.

Compositions of the current invention may be useful in the formulation of controlled, sustained, protracting, retarded, and slow release drug delivery systems. More specifically, but not limited to, compositions may be useful in formulation of parenteral controlled release and sustained release systems (both systems leading to a many-fold reduction in number of administrations), well known to those skilled in the art. Even more preferably, are controlled release and sustained release systems administered subcutaneous. Without limiting the scope of the invention, examples of useful controlled release system and compositions are hydrogels, oleaginous gels, liquid crystals, polymeric micelles, microspheres, nanoparticles.

Methods to produce controlled release systems useful for compositions of the current invention include, but are not limited to, crystallization, condensation, co-crystallization, precipitation, co-precipitation, emulsification, dispersion, high pressure homogenisation, encapsulation, spray drying, microencapsulating, coacervation, phase separation, solvent evaporation to produce microspheres, extrusion and supercritical fluid processes. General reference is made to Handbook of Pharmaceutical Controlled Release (Wise, D.L., ed. Marcel Dekker, New York, 2000) and Drug and the Pharmaceutical Sciences vol. 99: Protein Formulation and Delivery (MacNally, E.J., ed. Marcel Dekker, New York, 2000).

Parenteral administration may be performed by subcutaneous, intramuscular, intraperitoneal or intravenous injection by means of a syringe, optionally a pen-like syringe.

Alternatively, parenteral administration can be performed by means of an infusion pump. A

further option is a composition which may be a solution or suspension for the administration of the insulin analogue compound in the form of a nasal or pulmonal spray. As a still further option, the pharmaceutical compositions containing the insulin analogue compound of the invention can also be adapted to transdermal administration, *e.g.* by needle-free injection or
5 from a patch, optionally an iontophoretic patch, or transmucosal, *e.g.* buccal, administration.

The insulin analogue according to the invention can be administered via the pulmonary route in a vehicle, as a solution, suspension or dry powder using any of known types of devices suitable for pulmonary drug delivery. Examples of these comprise of, but are not limited to, the three general types of aerosol-generating for pulmonary drug delivery, and may
10 include jet or ultrasonic nebulizers, metered-dose inhalers, or dry powder inhalers (Cf. Yu J, Chien YW. Pulmonary drug delivery: Physiologic and mechanistic aspects. Crit Rev Ther Drug Carr Sys 14(4) (1997) 395-453).

In a further embodiment, the formulation could be aerosolized by any known aerosolisation technology, such as nebulisation, to achieve a MMAD of aerosol particles less than
15 10 μm , more preferably between 1-5 μm , and most preferably between 1-3 μm . The preferred particle size is based on the most effective size for delivery of drug to the deep lung, where protein is optimally absorbed (cf. Edwards DA, Ben-Jebria A, Langer A, Recent advances in pulmonary drug delivery using large, porous inhaled particles. J Appl Physiol 84(2) (1998) 379-385).

Deep lung deposition of the pulmonal formulations comprising the insulin analogue compound may optional be further optimized by using modifications of the inhalation techniques, for example, but not limited to: slow inhalation flow (*eg.* 30 L/min), breath holding and timing of actuation.

25 The term "stabilized formulation" refers to a formulation with increased physical stability, increased chemical stability or increased physical and chemical stability.

The term "physical stability" of the protein formulation as used herein refers to the tendency of the protein to form biologically inactive and/or insoluble aggregates of the protein
30 as a result of exposure of the protein to thermo-mechanical stresses and/or interaction with interfaces and surfaces that are destabilizing, such as hydrophobic surfaces and interfaces. Physical stability of the aqueous protein formulations is evaluated by means of visual inspection and/or turbidity measurements after exposing the formulation filled in suitable containers (*e.g.* cartridges or vials) to mechanical/physical stress (*e.g.* agitation) at different temperatures for various time periods. Visual inspection of the formulations is performed in a sharp
35

focused light with a dark background. The turbidity of the formulation is characterized by a visual score ranking the degree of turbidity for instance on a scale from 0 to 3 (a formulation showing no turbidity corresponds to a visual score 0, and a formulation showing visual turbidity in daylight corresponds to visual score 3). A formulation is classified physical unstable with respect to protein aggregation, when it shows visual turbidity in daylight. Alternatively, the turbidity of the formulation can be evaluated by simple turbidity measurements well-known to the skilled person. Physical stability of the aqueous protein formulations can also be evaluated by using a spectroscopic agent or probe of the conformational status of the protein. The probe is preferably a small molecule that preferentially binds to a non-native con-
former of the protein. One example of a small molecular spectroscopic probe of protein structure is Thioflavin T. Thioflavin T is a fluorescent dye that has been widely used for the detection of amyloid fibrils. In the presence of fibrils, and perhaps other protein configurations as well, Thioflavin T gives rise to a new excitation maximum at about 450 nm and enhanced emission at about 482 nm when bound to a fibril protein form. Unbound Thioflavin T is essentially non-fluorescent at the wavelengths.

Other small molecules can be used as probes of the changes in protein structure from native to non-native states. For instance the "hydrophobic patch" probes that bind preferentially to exposed hydrophobic patches of a protein. The hydrophobic patches are generally buried within the tertiary structure of a protein in its native state, but become exposed as a protein begins to unfold or denature. Examples of these small molecular, spectroscopic probes are aromatic, hydrophobic dyes, such as anthracene, acridine, phenanthroline or the like. Other spectroscopic probes are metal-amino acid complexes, such as cobalt metal complexes of hydrophobic amino acids, such as phenylalanine, leucine, isoleucine, methionine, and valine, or the like.

The term "chemical stability" of the protein formulation as used herein refers to chemical covalent changes in the protein structure leading to formation of chemical degradation products with potential less biological potency and/or potential increased immunogenic properties compared to the native protein structure. Various chemical degradation products can be formed depending on the type and nature of the native protein and the environment to which the protein is exposed. Elimination of chemical degradation can most probably not be completely avoided and increasing amounts of chemical degradation products is often seen during storage and use of the protein formulation as well-known by the person skilled in the art. Most proteins are prone to deamidation, a process in which the side chain amide group in glutaminy or asparaginy residues is hydrolysed to form a free carboxylic acid. Other degradation pathways involves formation of high molecular weight transformation products

where two or more protein molecules are covalently bound to each other through transamination and/or disulfide interactions leading to formation of covalently bound dimer, oligomer and polymer degradation products (*Stability of Protein Pharmaceuticals*, Ahern, T.J. & Manning M.C., Plenum Press, New York 1992). Oxidation (of for instance methionine residues) can be mentioned as another variant of chemical degradation. The chemical stability of the protein formulation can be evaluated by measuring the amount of the chemical degradation products at various time-points after exposure to different environmental conditions (the formation of degradation products can often be accelerated by for instance increasing temperature). The amount of each individual degradation product is often determined by separation of the degradation products depending on molecule size and/or charge using various chromatography techniques (e.g. SEC-HPLC and/or RP-HPLC).

Hence, as outlined above, a "stabilized formulation" refers to a formulation with increased physical stability, increased chemical stability or increased physical and chemical stability. In general, a formulation must be stable during use and storage (in compliance with recommended use and storage conditions) until the expiration date is reached.

In one embodiment of the invention the pharmaceutical formulation comprising the insulin analogue compound is stable for more than 6 weeks of usage and for more than 3 years of storage.

In another embodiment of the invention the pharmaceutical formulation comprising the insulin analogue compound is stable for more than 4 weeks of usage and for more than 3 years of storage.

In a further embodiment of the invention the pharmaceutical formulation comprising the insulin analogue compound is stable for more than 4 weeks of usage and for more than two years of storage.

In an even further embodiment of the invention the pharmaceutical formulation comprising the insulin analogue compound is stable for more than 2 weeks of usage and for more than two years of storage.

Aqueous suspensions may contain the active compounds in admixture with excipients suitable for the manufacture of aqueous suspensions.

Oily suspensions may be formulated by suspending the active ingredient in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such

as a liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set forth above, and flavouring agents may be added to provide a palatable oral preparation. These formulations may be preserved by the addition of an anti-oxidant such as ascorbic acid.

5

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active compound in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional
10 excipients, for example, sweetening, flavouring, and colouring agents may also be present.

15

The pharmaceutical formulations comprising a compound for use according to the present invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil, for example, olive oil or arachis oil, or a mineral oil, for example a liquid paraffin, or
a mixture thereof. Suitable emulsifying agents may be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol anhydrides, for example sorbitan monooleate, and condensation products of said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening
20 and flavouring agents.

20

Syrups and elixirs may be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain a demulcent, preservative and flavouring and colouring agent.

25

30

In a further embodiment of the invention, the formulation further comprises a permeation enhancer. Bile salts and fatty acids are most often considered to increase the oral permeability of the lipid bi-layer membranes of the epithelial cell lining of the GI tract. In general, permeation enhancers increase paracellular and transcellular transport of macromolecules by reversible altering the membrane integrity. The bile salt is selected from the group consisting of cholate, deoxycholate, taurocholate, glycocholate, taurodeoxycholate, ursodeoxycholate, tauroursodeoxycholate, and chenodeoxycholate. The fatty acids is selected from the group of short, medium and long chain fatty acids, such as caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid etc. Others enhancers could be surfac-

tants such as monoglycerides, polyoxyethylene esters, sorbitan surfactants (nonionic) and sulphates (anionic).

In a further embodiment of the invention, the formulation further comprises a muco-adhesive polymer. An intimate contact of the drug delivery system to the mucosa of the gastrointestinal tract can be obtained by use of such a mucoadhesive polymer. An intimate contact of the dosage form to the membrane seems advantageous as an enzymatic degradation of the therapeutically active polypeptide on the way between the delivery system and the absorption membrane can be avoided. Moreover, a step concentration gradient on the absorption membrane representing the driving force for passive drug uptake can be provided.

In a further embodiment of the invention, the formulation further comprises an inhibitor of a proteolytic enzyme(s) to further circumvent the enzymatic barrier and achieving the delivery of the therapeutically active polypeptide such as aminopeptidase inhibitor, amastatin, bestatin, boroleucine and puromycin. Examples of protease inhibitors are sodium glycolate, camostat mesilate, bacitracin, soybean trypsin inhibitor and aprotinin.

Entrapment and encapsulation is a technique used in drug delivery systems for therapeutically active polypeptides to optimize delivery properties including protection against enzymatic degradation. Entrapment or encapsulation could be in the form of polymeric drug delivery systems such as hydrogels and nanocapsules/microspheres, and lipid drug delivery systems such as liposomes and micro emulsions.

Formulations of the current invention may be administered in several dosage forms, for example, as solutions, suspensions, micro- and nano suspension, emulsions, microemulsions, multiple emulsion, foams, salves, pastes, ointments, tablets, coated tablets, effervescent tablets, sublingual tablets, buccal tablets, capsules, for example, hard gelatine capsules and soft gelatine capsules, powder, granules, in situ transforming solutions, for example in situ gelling, in situ setting, in situ precipitating, in situ crystallization, stomach floating formulation such as floating suspension, floating tablet or the like.

In another embodiment, the present invention relates to an insulin analogue according to the invention for use as a medicament.

In one embodiment, an insulin analogue according to the invention is used for the preparation of a medicament for the treatment or prevention of hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, obesity, hypertension, syndrome X, dyslipidemia, cognitive disorders, atherosclerosis, myocardial infarction, stroke, coronary heart disease and other cardiovascular disorders, inflammatory bowel syndrome, dyspepsia and gastric ulcers.

In another embodiment, an insulin analogue according to the invention is used as a medicament for delaying or preventing disease progression in type 2 diabetes.

In another embodiment, an insulin analogue according to the invention is used as a medicament for decreasing food intake, decreasing β -cell apoptosis, increasing β -cell function and β -cell mass, and/or for restoring glucose sensitivity to β -cells.

In one embodiment of the invention, the derivative according to the invention is for use as a medicament for the treatment or prevention of hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, obesity, hypertension, syndrome X, dyslipidemia, cognitive disorders, atherosclerosis, myocardial infarction, coronary heart disease and other cardiovascular disorders, stroke, inflammatory bowel syndrome, dyspepsia and gastric ulcers or for delaying or preventing disease progression in type 2 diabetes or for decreasing food intake, decreasing β -cell apoptosis, increasing β -cell function and β -cell mass, and/or for restoring glucose sensitivity to β -cells, is provided.

In a further embodiment of the invention, a method for the treatment or prevention of hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, obesity, hypertension, syndrome X, dyslipidemia, cognitive disorders, atherosclerosis, myocardial infarction, coronary heart disease and other cardiovascular disorders, stroke, inflammatory bowel syndrome, dyspepsia and gastric ulcers or for delaying or preventing disease progression in type 2 diabetes or for decreasing food intake, decreasing β -cell apoptosis, increasing β -cell function and β -cell mass, and/or for restoring glucose sensitivity to β -cells, the method comprising administering to a patient in need of such treatment an effective amount for such treatment of an insulin analogue according to the invention, is provided.

The treatment with an insulin analogue according to the present invention may also be combined with a second or more pharmacologically active substances, e.g. selected from antidiabetic agents, antiobesity agents, appetite regulating agents, antihypertensive agents, agents for the treatment and/or prevention of complications resulting from or associated with diabetes and agents for the treatment and/or prevention of complications and disorders resulting from or associated with obesity. Examples of these pharmacologically active substances are: GLP-1 and GLP-1 derivatives and analogues, GLP-2 and GLP-2 derivatives and analogues, Exendin-4 and Exendin-4 derivatives and analogues, amylin and amylin derivatives and analogues, sulphonylureas, biguanides, meglitinides, glucosidase inhibitors, glucagon antagonists, DPP-IV (dipeptidyl peptidase-IV) inhibitors, inhibitors of hepatic enzymes involved in stimulation of gluconeogenesis and/or glycogenolysis, glucose uptake modulators, compounds modifying the lipid metabolism such as antihyperlipidemic agents as HMG CoA inhibitors (statins), compounds lowering food intake, RXR agonists and agents acting on

the ATP-dependent potassium channel of the β -cells; Cholestyramine, colestipol, clofibrate, gemfibrozil, lovastatin, pravastatin, simvastatin, probucol, dextrothyroxine, neteglinide, repaglinide; β -blockers such as alprenolol, atenolol, timolol, pindolol, propranolol and metoprolol, ACE (angiotensin converting enzyme) inhibitors such as benazepril, captopril, enalapril, fosinopril, lisinopril, alatriopril, quinapril and ramipril, calcium channel blockers such as nifedipine, felodipine, nifedipine, isradipine, nimodipine, diltiazem and verapamil, and α -blockers such as doxazosin, urapidil, prazosin and terazosin; CART (cocaine amphetamine regulated transcript) agonists, NPY (neuropeptide Y) antagonists, MC4 (melanocortin 4) agonists, orexin antagonists, TNF (tumor necrosis factor) agonists, CRF (corticotropin releasing factor) agonists, CRF BP (corticotropin releasing factor binding protein) antagonists, urocortin agonists, β 3 agonists, MSH (melanocyte-stimulating hormone) agonists, MCH (melanocyte-concentrating hormone) antagonists, CCK (cholecystokinin) agonists, serotonin re-uptake inhibitors, serotonin and noradrenaline re-uptake inhibitors, mixed serotonin and noradrenergic compounds, 5HT (serotonin) agonists, bombesin agonists, galanin antagonists, growth hormone, growth hormone releasing compounds, TRH (thyrotropin releasing hormone) agonists, UCP 2 or 3 (uncoupling protein 2 or 3) modulators, leptin agonists, DA agonists (bromocriptin, doprexin), lipase/amylase inhibitors, RXR (retinoid X receptor) modulators, TR β agonists; histamine H3 antagonists, gastrin and gastrin analogues and derivatives.

It should be understood that any suitable combination of the derivatives according to the invention with one or more of the above-mentioned compounds and optionally one or more further pharmacologically active substances are considered to be within the scope of the present invention.

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference in their entirety and to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein (to the maximum extent permitted by law).

All headings and sub-headings are used herein for convenience only and should not be construed as limiting the invention in any way.

The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should

be construed as indicating any non-claimed element as essential to the practice of the invention.

The citation and incorporation of patent documents herein is done for convenience only and does not reflect any view of the validity, patentability, and/or enforceability of such patent documents.

This invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law.

EXAMPLES

EXAMPLE 1

Comparison of proteolytic stability (Half-life) of B25H, A14E, A14E_B25H and human insulin towards pepsin, chymotrypsin, and carboxypeptidase A

Proteolytic stability of human insulin and insulin analogues (0.06 mM, 10 μ L) towards chymotrypsin or pepsin (0.34-3.4 μ g) was measured after incubation in 100mM NH_4HCO_3 , pH 8.1 or 10 mM HCl, pH 2.0, respectively and 25°C at a final volume of 100 μ L. At various times (0, 5, 15, 30 and 60 min) samples were quenched with an equal volume of 0.5%TFA or 0.1 M TrisHCl, pH 8.0 (final pH 7.7) and transferred to 5°C. Human insulin and insulin analogues were immediately analyzed by RP-HPLC at 214 nm and the area under the peak corresponding to intact protein was determined.

Stability of human insulin and insulin analogues (0.06 mM, 13.9 μ g) towards carboxypeptidase A (6.8 μ L of working solution, 20mg/ml, 53 units/mg, Sigma C-9268) was measured after incubation in 5 mM NaP, 140 mM NaCl, 70 ppm Tween20, pH 7.4 and 37°C at a final volume of 400 μ L. At various times (5, 15, 30, 60 min) samples were quenched with an equal volume of 0.2%TFA and transferred to 5°C. Reference samples (0 min) were prepared without adding enzymes. Human insulin and analogues were immediately analyzed by RP-HPLC in a neutral buffer system at 214 nm and the area under the peak corresponding to intact protein was determined. Identical retention times were observed for human insulin and desB30. Half-lives ($T_{1/2}$) were obtained from the curves and the fold increase/decrease compared to human insulin was calculated (Stability relative fold). Similar half-lives were determined for human insulin, $T_{1/2}$ = 6.9 min, and desB30 human insulin, $T_{1/2}$ = 6.7.

Mutation Site(s) in Human Insulin	T _{1/2} [Min] (Fold) Pepsin	T _{1/2} (Min)/Fold Chymotrypsin	T _{1/2} (Min)/Fold Carboxypeptidase A
B25H	17.8 (16.2)	25.7 (5.1)	25.7 (2.1)
None	1.1 (1.0)	5.0 (1.0)	6.9 (1.0)
A14E, DESB30	3.7 (3.4)	33.2 (6.6)	15.7 (2.3)
None	1.1 (1.0)	5.0 (1.0)	6.9 (1.0)
A14E, B25H, DESB30	42.4 (38.5)	62.4 (12.5)	61.9 (9.0)
None	1.1 (1.0)	5.0 (1.0)	6.9 (1.0)

EXAMPLE 2

Identification of protease cleavage sites within insulin by MS analysis and enzyme specificity

Identification of protease cleavage sites were carried out by time course analysis of limited proteolysis of insulin and insulin analogues by various enzymes (pepsin, chymotrypsin, carboxypeptidase A) by LC-MS. Generated peptide fragments were identified by MS or MS/MS and quantified at 214 nm in order to identify minor and major cleavage sites (hot-spots). The following hot-spots for pepsin (B24-25, B25-26, B26-27) and chymotrypsin (B16-17, B24-25, B25-26, B26-27, A14-15 and A19-20) within human insulin were identified. Interestingly, the hot-spots overlap with insulin-degrading enzyme (IDE), see below, and cathepsin D (Hanne Refsgaard, Novo Nordisk A/S, personal communication).

Cleavage sites within the insulin sequence was also predicted based on enzyme specificity of chymotrypsin, trypsin and insulin-degrading enzyme (IDE) as shown below in example 3. The predicted cleavage sites for trypsin (basic residues, C-term. to Lys, Arg), elastase (aliphatic residues, C-term. to Ala, Val, Gly, Ser), carboxypeptidase A (broad specificity, but not Arg, Lys & Pro), pepsin A (aromatic residues, N-term. to Trp, Tyr, Phe, and Leu and various other residues), and chymotrypsin (aromatic residues, C-term. to Trp, Tyr, Phe, and Leu, but not Xxx-Pro) are described in several hand-books of biochemistry. Cleavage sites for IDE within insulin has recently been published: B9-10, B10-11, B13-14, B14-15, B24-25, B25-26, A13-14, A14-15 (Duckworth et al., 2006) and the following substitutions have been shown to inhibit IDE degradation of insulin: B10D and B4E_B16Q_B17F (Bennett et al., 2003).

EXAMPLE 3**Repeated cycles of hot-spot identification within A14E, B25H, desB30 human insulin**

5 Major hot-spots for chymotrypsin cleavage (B16-17, B24-25, A19-20) and minor cleavage sites (B1-2, B6-7), were identified in A14E, B25H, desB30 human insulin according to the method described in example 2. Insulin analogues were designed based on the identified cleavage sites and predicted cleavage sites from the enzyme specificity. Amino acid residues for substitution were selected in order to remove the cleavage sites and to increase
10 the solubility.

Chymotrypsin, analogue design based on hot-spots identified by LC-MS of digests of A14E, B25H, desB30 human insulin:

15 A14X1 B25H desB30X12 HI B1X2 B6X3 B16X6 B24X8 A19X11

X1= E/D/H/Q/N/S/T/M/P

X2= E/D/H/Q/N/S/T/M/P/native aa

X3= E/D/H/Q/N/S/T/M/P/native aa

20 X6= E/D/H/Q/N/S/T/M/P/native aa

X8= E/D/H/Q/N/S/T/M/P/native aa

X11= E/D/H/Q/N/S/T/M/P/native aa

X12= desB30/native aa

25 Note: substitution of B24 and/or A19 in human insulin is likely to significantly reduce receptor affinity.

30 A14X1 B25P desB30X12 HI B1X2 B6X3 B16X6 B24X8 A19X11

X1= E/D/H/Q/N/S/T/M/P

X2= E/D/H/Q/N/S/T/M/P/native aa

X3= E/D/H/Q/N/S/T/M/P/native aa

35 X6= E/D/H/Q/N/S/T/M/P/native aa

X8= E/D/H/Q/N/S/T/M/P/native aa

X11= E/D/H/Q/N/S/T/M/P/native aa

X12= desB30/native aa

- 5 Note: substitution of B25 with Pro is likely to significantly reduce chymotrypsin cleavage at B24-25.

10

Chymotrypsin, design based on identified and predicted hot-spots:

A14X1 B25H desB30X12 HI B1X2 B6X3 B11X4 B15X5 B16X6 B17X7 B24X8 A13X9
A16X10 A19X11

15

X1= E/D/H/Q/N/S/T/M/P

X2= E/D/H/Q/N/S/T/M/P/native aa

X3= E/D/H/Q/N/S/T/M/P/native aa

X4= E/D/H/Q/N/S/T/M/P/native aa

20 X5= E/D/H/Q/N/S/T/M/P/native aa

X6= E/D/H/Q/N/S/T/M/P/native aa

X7= E/D/H/Q/N/S/T/M/P/native aa

X8= E/D/H/Q/N/S/T/M/P/native aa

X9= E/D/H/Q/N/S/T/M/P/native aa

25 X10= E/D/H/Q/N/S/T/M/P/native aa

X11= E/D/H/Q/N/S/T/M/P/native aa

X12= desB30/native aa

- 30 Note: substitution of B11, B15, B24, A13, A16, and/or A19 in human insulin is likely to significantly reduce receptor affinity.

Chymotrypsin + trypsin, design based on identified and predicted hot-spots:

35

A14X1 B25H desB30X12 HI B1X2 B6X3 B11X4 B15X5 B16X6 B17X7 B24X8 A13X9
A16X10 A19X11 B22X13

X1= E/D/H/Q/N/S/T/M/P

- 5 X2= E/D/H/Q/N/S/T/M/P/native aa
X3= E/D/H/Q/N/S/T/M/P/native aa
X4= E/D/H/Q/N/S/T/M/P/native aa
X5= E/D/H/Q/N/S/T/M/P/native aa
X6= E/D/H/Q/N/S/T/M/P/native aa
10 X7= E/D/H/Q/N/S/T/M/P/native aa
X8= E/D/H/Q/N/S/T/M/P/native aa
X9= E/D/H/Q/N/S/T/M/P/native aa
X10= E/D/H/Q/N/S/T/M/P/native aa
X11= E/D/H/Q/N/S/T/M/P/native aa
15 X12= desB30/native aa
X13= E/D/H/Q/N/S/T/M/P/native aa

Chymotrypsin + trypsin + insulin-degrading enzyme (IDE), design based on identified and predicted hot-spots:

20

A14X1 B25H desB30X12 HI B1X2 B6X3 B11X4 B15X5 B16X6 B17X7 B24X8 A13X9
A16X10 A19X11 B22X13 B4X14 B9X15 B10X16 B13X17 B14X18

X1= E/D/H/Q/N/S/T/M/P

- 25 X2= E/D/H/Q/N/S/T/M/P/native aa
X3= E/D/H/Q/N/S/T/M/P/native aa
X4= E/D/H/Q/N/S/T/M/P/native aa
X5= E/D/H/Q/N/S/T/M/P/native aa
X6= E/D/H/Q/N/S/T/M/P/native aa
30 X7= E/D/H/Q/N/S/T/M/P/native aa
X8= E/D/H/Q/N/S/T/M/P/native aa
X9= E/D/H/Q/N/S/T/M/P/native aa
X10= E/D/H/Q/N/S/T/M/P/native aa
X11= E/D/H/Q/N/S/T/M/P/native aa
35 X12= desB30/native aa

X13= E/D/H/Q/N/S/T/M/P/native aa

X14= E/D/H/Q/N/S/T/M/P/native aa

X15= E/D/H/Q/N/S/T/M/P/native aa

X16= E/D/H/Q/N/S/T/M/P/native aa

5 X17= E/D/H/Q/N/S/T/M/P/native aa

X18= E/D/H/Q/N/S/T/M/P/native aa

EXAMPLE 4

Proteolytic stability (Half-life) of insulin analogues towards chymotrypsin

10

Insulin analogues were tested in stability assays and analogues exhibiting enhanced resistance to proteolytic digestion were identified by increased half-lives according to the methods described in example 1. The results demonstrate the potential of the further improved insulin analogues for increased bioavailability due to enhanced resistance to proteolytical degradation and improved solubility.

15

The stability of the following insulin analogues towards chymotrypsin have been tested relatively to human insulin:

20

Insulin analogues	Chymotrypsin digestion [Fold stability relative to Human Insulin]
A14E, B25H, desB27, desB28, desB29, desB30 human insulin	43.8
A8H, A14E, B25H, B27E, desB30 human insulin	38.9
EEAEAEAPK-B(1-29)-B25H-AAK-A(1-21)-A14E human insulin	33.2
A8H, A14E, B1E, B25H, B27E, desB30 human insulin	22.8
A14E, B25H, B28D, desB30 human insulin	17.4
A14E, B25H, B26D, desB30 human insulin	16.6
A14E, B25H, B27E, desB30 human insulin	14.1
A8H, A14E, B25H, desB30 human insulin	13.3
A8H,A14E,B10E,B25H,desB30 human insulin	12.6
A14E, B25H, desB30 human insulin	12,5

Insulin analogues	Chymotrypsin digestion [Fold stability relative to Human Insulin]
A14E, B1E, B25H, B27E, desB30 human insulin	11.9
A8H, A14E, B1E, B25H, desB30 human insulin	10.2
A8H, A14E, B1E, B16E, B25H, B27E, desB30 human insulin	8.3
A14D, B25H, desB30 human insulin	7.9
A14E, B27E, desB30 human insulin	7.7
A14E, B28D, desB30 human insulin	7.5
A8H, B10D, B25H human insulin	7.4
A14E, B1E, B16E, B25H, B27E, desB30 human insulin	5.5
A14E, B1E, B25H, desB30 human insulin	4.8
A14H, B25H, desB30 human insulin	4.5
A14E, B25H human insulin	4.1
A14E, B1E, B27E, desB30 human insulin	3.9
A8H, A14E, B16E, B25H, desB30 human insulin	2.3
A14E, B16E, B25H, desB30 human insulin	2.1
human insulin (reference)	1

EXAMPLE 5**Proteolytic stability of insulin analogues towards extracts from rat intestine (duodenum lumen)**

- 5 Insulin analogues were tested for their stability towards proteolytic degradation in intestinal extracts. The extracts were prepared by washing the luminal content of fasted animals. Insulin analogues were incubated with diluted extracts at 37°C at pH=7.4 and after 20h incubation the concentration of the intact compound was determined by RP-HPLC. The relative amounts of intact insulin analogues and intact human insulin were compared. Approximately
- 10 5-10 fold difference in the stability between human insulin and the analogue A14E B25H desB30 human insulin was found.

CLAIMS

1. An insulin analogue wherein at least two hydrophobic amino acids have been substituted with hydrophilic amino acids relative to the parent insulin, wherein the substitutions are within or in close proximity to two or more protease cleavage sites of the parent insulin and wherein such insulin analogue optionally further comprises one or more additional mutations.

2. An insulin analogue according to claim 1 wherein the A-chain of the insulin analogue comprises at least one mutation and the B-chain of the insulin analogue comprises at least one mutation relative to the parent insulin.

3. An insulin analogue according to anyone of claims 1 or 2 which shows increased stability towards one or more protease enzymes relative to the parent protein.

4. An insulin analogue according to anyone of claims 1-3 comprising an A-chain amino acid sequence of formula 1:

Xaa_{A(-2)}-Xaa_{A(-1)}-Xaa_{A0}-Gly-Ile-Val-Glu-Gln-Cys-Cys-Xaa_{A8}-Ser-Ile-Cys-Xaa_{A12}-
Xaa_{A13}-Xaa_{A14}-Xaa_{A15}-Leu-Glu-Xaa_{A18}-Tyr-Cys-Xaa_{A21}-Xaa_{A22}

Formula (1) (SEQ ID No:1)

and a B-chain amino acid sequence of formula 2:

Xaa_{B(-2)}-Xaa_{B(-1)}-Xaa_{B0}-Xaa_{B1}-Xaa_{B2}-Xaa_{B3}-Xaa_{B4}-His-Leu-Cys-Gly-Ser-Xaa_{B10}-Leu-
Val-Glu-Ala-Leu-Xaa_{B16}-Leu-Val-Cys-Gly-Glu-Arg-Gly-Xaa_{B24}-Xaa_{B25}-Xaa_{B26}-Xaa_{B27}-
Xaa_{B28}-Xaa_{B29}-Xaa_{B30}-Xaa_{B31}-Xaa_{B32}

Formula (2) (SEQ ID No:2)

wherein

Xaa_{A(-2)} is absent or Gly;

Xaa_{A(-1)} is absent or Pro;

Xaa_{A0} is absent or Pro;

Xaa_{A8} is independently selected from Thr and His;

Xaa_{A12} is independently selected from Ser, Asp and Glu;

Xaa_{A13} is independently selected from Leu, Thr, Asn, Asp, Gln, His, Lys, Gly, Arg,

Pro, Ser and Glu;

Xaa_{A14} is independently selected from Tyr, Thr, Asn, Asp, Gln, His, Lys, Gly, Arg, Pro, Ser and Glu;

Xaa_{A15} is independently selected from Gln, Asp and Glu;

Xaa_{A18} is independently selected from Asn, Lys and Gln;

5 Xaa_{A21} is independently selected from Asn and Gln;

Xaa_{A22} is absent or Lys;

Xaa_{B(-2)} is absent or Gly;

Xaa_{B(-1)} is absent or Pro;

Xaa_{B0} is absent or Pro;

10 Xaa_{B1} is absent or independently selected from Phe and Glu;

Xaa_{B2} is absent or Val;

Xaa_{B3} is absent or independently selected from Asn and Gln;

Xaa_{B4} is independently selected from Gln and Glu;

Xaa_{B10} is independently selected from His, Asp, Pro and Glu;

15 Xaa_{B16} is independently selected from Tyr, Asp, Gln, His, Arg, and Glu;

Xaa_{B24} is independently selected from Phe and His;

Xaa_{B25} is independently selected from Phe and His;

Xaa_{B26} is absent or independently selected from Tyr, His, Thr, Gly and Asp;

Xaa_{B27} is absent or independently selected from Thr, Asn, Asp, Gln, His, Lys, Gly,

20 Arg, Pro, Ser and Glu;

Xaa_{B28} is absent or independently selected from Pro, His, Gly and Asp;

Xaa_{B29} is absent or independently selected from Lys and Gln;

Xaa_{B30} is absent or Thr;

Xaa_{B31} is absent or Leu;

25 Xaa_{B32} is absent or Glu;

the C-terminal may optionally be derivatized as an amide;

wherein the A-chain amino acid sequence and the B-chain amino acid sequence are connected by disulphide bridges between the cysteines in position 7 of the A-chain and the
30 cysteine in position 7 of the B-chain, and between the cysteine in position 20 of the A-chain and the cysteine in position 19 of the B-chain and wherein the cysteines in position 6 and 11 of the A-chain are connected by a disulphide bridge;

wherein optionally the N-terminal A-chain amino acid sequence is connected to the C-terminal B-chain amino acid sequence by an amino acid sequence comprising 3-7 amino

acids to form a single chain insulin molecule, wherein optionally the N-terminal of the B-chain is extended with 1-10 amino acids;

wherein if Xaa_{A8} is Thr and Xaa_{A12} is Ser and Xaa_{A13} is Leu and Xaa_{A14} is Tyr then Xaa_{A15} is Glu or Asp; and

5 wherein if Xaa_{B24} is Phe and Xaa_{B25} is Phe and Xaa_{B26} is Tyr and Xaa_{B27} is Thr and Xaa_{B28} is Pro then Xaa_{B29} Gln.

5. A pharmaceutical composition comprising a biologically active amount of the insulin analogue according to any one of claims 1-4 and a pharmaceutically acceptable carrier.

10

6. A method for the treatment, prevention or alleviation of hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, obesity, syndrome X or dyslipidemia in a subject comprising administering to a subject an insulin analogue according to any one of claims 1-4 or a pharmaceutical composition according to any one of claim 5.

15

7. Method according to claim 6 being an oral administration.

8. An insulin analogue according to anyone of claims 1-4 for use as a pharmaceutical in the treatment or prevention of hyperglycemia, type 2 diabetes, impaired glucose tolerance, type
20 1 diabetes, obesity, syndrome X and dyslipidemia.

9. An insulin analogue according to anyone of claims 1-4 for use as a pharmaceutical in delaying or preventing disease progression in type 2 diabetes.

25

10. An insulin analogue according to anyone of claims 1-4 for use as a pharmaceutical in decreasing food intake, decreasing β -cell apoptosis, increasing β -cell function and β -cell mass, and/or for restoring glucose sensitivity to β -cells.

30

11. A nucleic acid sequence encoding an insulin analogue according to any one of claims 1-4.

12. A process for preparing a pharmaceutical composition according to claim 5 comprising mixing an insulin analogue according to any one of claims 1-4 with pharmaceutically accept-

able substances and/or excipients.

Proteolytic stability of insulin analogs towards chymotrypsin

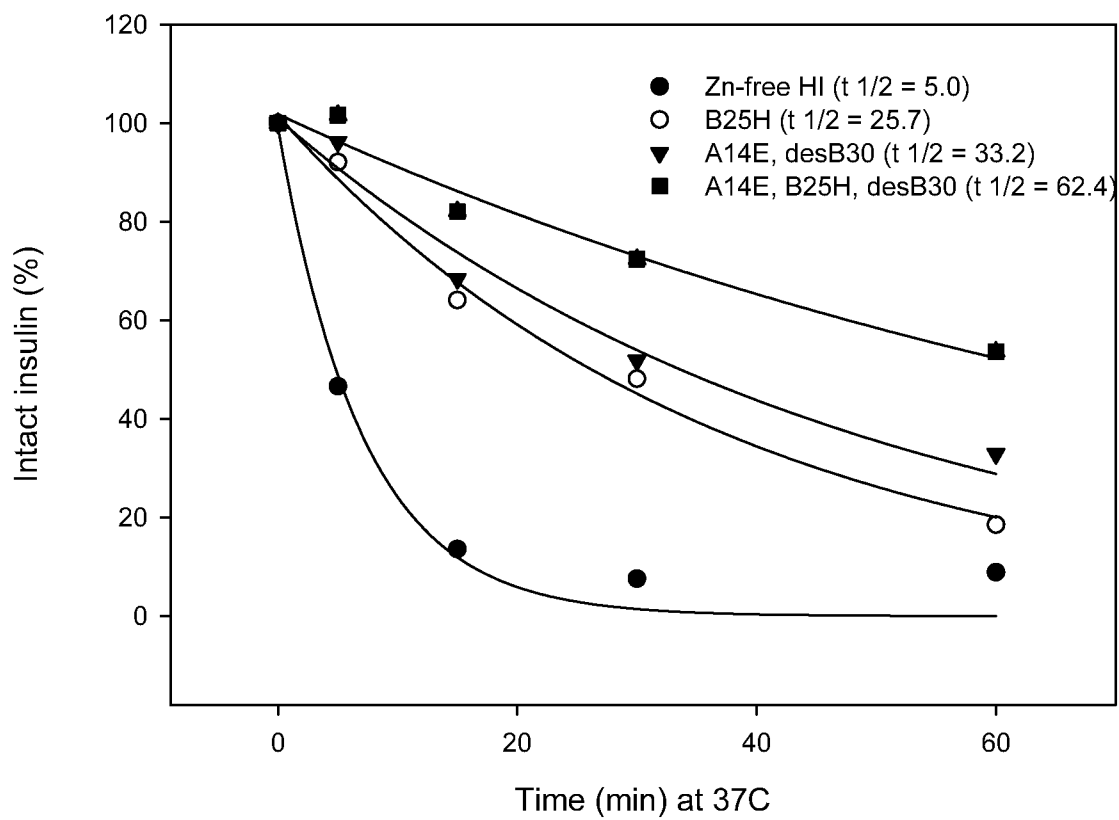


Fig. 1/2

Proteolytic stability of HI and insulin analogs towards pepsin

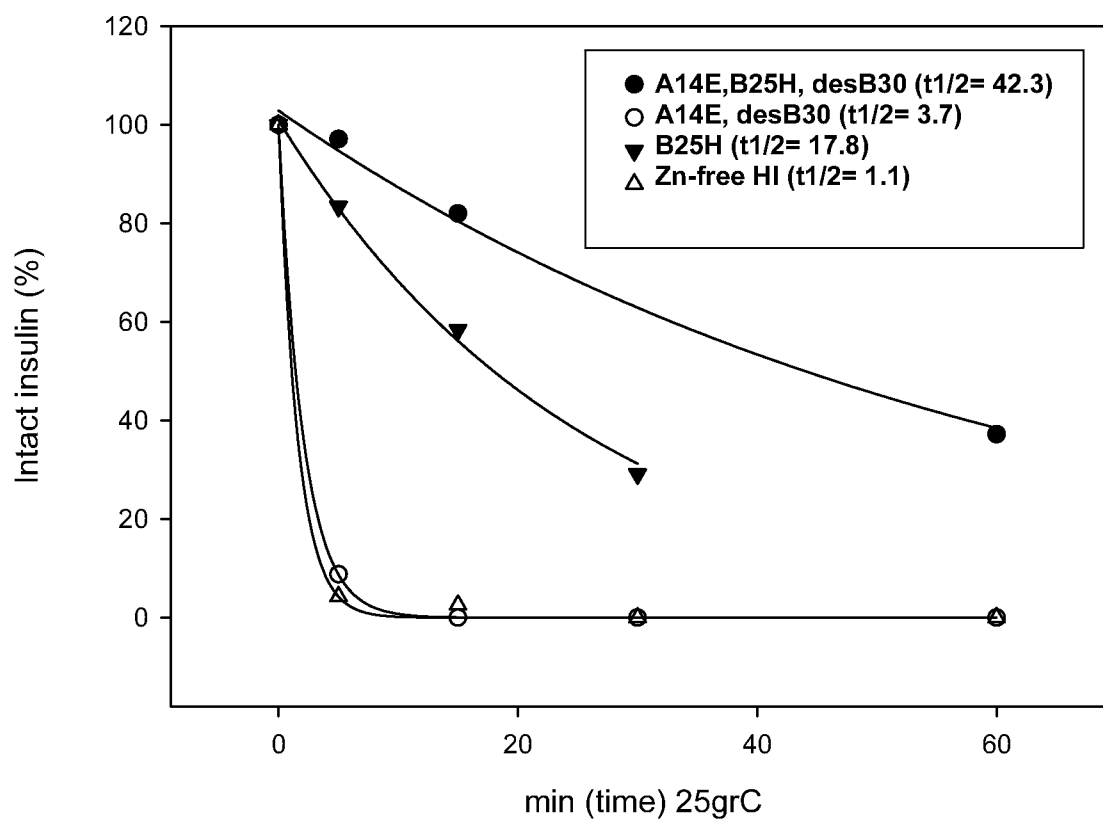


Fig. 2/2

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2007/059990

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/62 A61K38/28 C12N15/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SCHILLING R J ET AL: "DEGRADATION OF INSULIN BY TRYPSIN AND ALPHA CHYMOTRYPSIN" PHARMACEUTICAL RESEARCH (NEW YORK), vol. 8, no. 6, 1991, pages 721-727, XP002462223 ISSN: 0724-8741 the whole document</p>	1-12
A	<p>BENNETT R G ET AL: "Insulin inhibition of the proteasome is dependent on degradation of insulin by insulin-degrading enzyme." JOURNAL OF ENDOCRINOLOGY, vol. 177, no. 3, June 2003 (2003-06), pages 399-405, XP002462224 ISSN: 0022-0795 cited in the application the whole document</p>	1-12
-/--		



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

13 December 2007

Date of mailing of the international search report

02/01/2008

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Kools, Patrick

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2007/059990

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 214 826 A (NOVO INDUSTRI AS [DK] NOVONORDISK AS [DK]) 18 March 1987 (1987-03-18) claims 1-13 -----	1-12
A	WO 98/01473 A (NOVONORDISK AS [DK]; EGEL MITANI MICHU [DK]; BRANDT JAKOB [DK]; VAD KN) 15 January 1998 (1998-01-15) the whole document -----	1-12
A	EP 0 544 466 A (LILLY CO ELI [US]) 2 June 1993 (1993-06-02) the whole document -----	1-12
P, A	WO 2007/081824 A (UNIV CASE WESTERN RESERVE [US]; WEISS MICHAEL [US]) 19 July 2007 (2007-07-19) the whole document -----	1-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2007/059990

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 6 and 7 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 1-12 all partially
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 6 and 7 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.2

Claims Nos.: 1-12 all partially

The present claims 1-4 relate to an extremely large number of possible insulin analogues. Support and disclosure in the sense of Article 6 and 5 PCT is to be found however for only a very small proportion of the analogues claimed, see examples 1 and 4. The non-compliance with the substantive provisions is to such an extent, that the search was performed taking into consideration the non-compliance in determining the extent of the search of claims 1-4 (PCT Guidelines 9.19 and 9.23).

The search of claims 1-4 was restricted to those claimed insulin analogues which appear to be supported and disclosed in examples 1 and 4.

The use and method claims depending on the subject-matter of claims 1-4 were also restricted to the subject-matter searched for said product claims.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2)PCT declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2007/059990

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0214826	A	18-03-1987	AR 241801 A1	30-12-1992
			AT 113061 T	15-11-1994
			AU 593274 B2	08-02-1990
			AU 6206686 A	05-03-1987
			CA 1306212 C	11-08-1992
			CN 86106574 A	03-08-1988
			CS 8606310 A2	12-09-1990
			DE 3650101 D1	24-11-1994
			DE 3650101 T2	23-02-1995
			DE 10075008 I1	18-05-2000
			ES 2001624 A6	01-06-1988
			FI 863512 A	01-03-1987
			GR 862233 A1	31-12-1986
			HU 42526 A2	28-07-1987
			IE 66138 B1	13-12-1995
			IL 79887 A	21-11-1991
			JP 2662390 B2	08-10-1997
			JP 62053999 A	09-03-1987
			LU 90484 A9	21-02-2000
			NL 990042 I1	01-02-2000
			NO 863474 A	02-03-1987
			NZ 217406 A	29-05-1989
			PH 25772 A	18-10-1991
			US 5618913 A	08-04-1997
			YU 4188 A1	30-06-1990
			YU 148486 A1	30-04-1991
WO 9801473	A	15-01-1998	AU 3255397 A	02-02-1998
EP 0544466	A	02-06-1993	AU 2857692 A	27-05-1993
			BR 9204542 A	01-06-1993
			CA 2083360 A1	27-05-1993
			CN 1072723 A	02-06-1993
			CZ 9203424 A3	16-06-1993
			FI 925343 A	27-05-1993
			HU 63457 A2	30-08-1993
			JP 3304442 B2	22-07-2002
			JP 5286998 A	02-11-1993
			MX 9206724 A1	01-05-1993
			NO 924525 A	27-05-1993
			NZ 245170 A	26-07-1994
			US 5491216 A	13-02-1996
			ZA 9208916 A	18-05-1994
WO 2007081824	A	19-07-2007	NONE	