HU000030373T2

(19) **HU**

(11) Lajstromszám: **E 030 373**

(13) **T2**

MAGYARORSZÁG
Szellemi Tulajdon Nemzeti Hivatala

EURÓPAI SZABADALOM
SZÖVEGÉNEK FORDÍTÁSA

(21) Magyar ügyszám: **E 10 753706**
(22) A bejelentés napja: **2010. 03. 18.**

(96) Az európai bejelentés bejelentési száma:
EP 20100753706
(97) Az európai bejelentés közzétételi adatai:
EP 2408800 A2 **2010. 09. 23.**
(97) Az európai szabadalom megadásának meghirdetési adatai:
EP 2408800 B1 **2016. 05. 25.**

(51) Int. Cl.: ***C07K 1/14*** *(2006.01)*
C07K 1/10 *(2006.01)*
C07K 1/18 *(2006.01)*

(86) A nemzetközi (PCT) bejelentési szám:
PCT/KR 10/001674
(87) A nemzetközi közzétételi szám:
WO 10107256

(30) Elsőbbségi adatok:
20090023953 **2009. 03. 20.** **KR**

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(54) **Eljárás fiziológiásan aktív polipeptid helyspecifikus konjugátumának előállítására**

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 408 800 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
25.05.2016 Bulletin 2016/21

(21) Application number: **10753706.0**

(22) Date of filing: **18.03.2010**

(51) Int Cl.:
C07K 1/14 *(2006.01)* ***C07K 1/10*** *(2006.01)*
C07K 1/18 *(2006.01)*

(86) International application number:
PCT/KR2010/001674

(87) International publication number:
WO 2010/107256 (23.09.2010 Gazette 2010/38)

(54) **METHOD FOR PREPARING A SITE-SPECIFIC CONJUGATE OF A PHYSIOLOGICALLY ACTIVE POLYPEPTIDE**

VERFAHREN FÜR DIE ZUBEREITUNG EINES ORTSSPEZIFISCHEN KONJUGATS EINES PHYSIOLOGISCH AKTIVEN POLYPEPTIDS

PROCÉDÉ DE PRÉPARATION D'UN CONJUGUÉ SITE-SPÉCIFIÉ D'UN POLYPEPTIDE PHYSIOLOGIQUEMENT ACTIF

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

(30) Priority: **20.03.2009 KR 20090023953**

(43) Date of publication of application:
25.01.2012 Bulletin 2012/04

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EP 2 408 800 B1

Description

FIELD OF THE INVENTION

[0001] The present invention relates to a method for preparing a site-specific physiologically active polypeptide conjugate, more particularly to a method for preparing said conjugate in a high yield by linking a physiologically active polypeptide with a non-peptidyl polymer.

BACKGROUND OF THE INVENTION

[0002] Peptides are easily denatured due to their low stability, degraded by *in-vivo* proteases, and excreted through the kidney due to their relatively small size. Accordingly, in order to maintain a specific concentration in the blood of a peptide drug in its active form, it is necessary to administer the peptide drug frequently to a patient. However, peptide drugs are usually administered in the form of injectable preparations, and such frequent administration cause severe discomfort for patients. To solve such problem, there have been developed a number of methods, e.g., a method for transferring the peptide drug through oropharyngeal or nasopharyngeal inhalation by increasing the permeation of the peptide drug through the biological membranes, a method for modifying a specific amino acid sequence which is sensitive to proteases (e.g., GLP-1 amino acid sequence for preventing loss of the titers by a dipeptidyl peptidase) in order to stabilize the peptide by inhibiting the degradation by the enzyme, and a method for chemically adding a non-peptidyl polymer with a high solubility, such as polyethylene glycol (PEG), on the surface of the peptide.

[0003] PEG, which has been used as one of non-peptidyl polymers, non-specifically binds to a specific site or multiple sites of a target peptide to attain the effect of increasing the molecular weight of the peptide, the resulting PEG-peptide resists the loss through the kidney and enzymatic hydrolysis, without causing any side-effects. For example, International Pat. Publication No. WO 2006/076471 describes sustaining the physiological activity of a B-type natriuretic peptide (BNP) used as congestive heart failure therapeutic agent by binding PEG thereto, and U.S. Pat. No. 6,924,264 describes increasing the in-vivo residence time of an exendin-4 drug by way of binding PEG to the lysine residue thereof.

[0004] These methods prolong the *in-vivo* residence time of a peptide drug by increasing the molecular weight of PEG, but as the molecular weight increases, the titer of the peptide drug becomes significantly reduced. In addition, the non-specific binding of PEG may shield the active domain of a physiologically active polypeptide to significantly lower the activity of the polypeptide.

[0005] Therefore, there is a need to develop an improved method for preparing a conjugate of a physiologically active polypeptide and a non-peptidyl polymer, in which the polymer is linked to the peptide in a site-specific manner that does not affect the polypeptide's activity.

[0006] The present inventors have completed the invention by confirming that a physiologically active polypeptide conjugate having a non-peptidyl polymer site-specifically linked can be prepared in a high yield by adjusting the pH and the alcohol content of the reaction medium.

SUMMARY OF THE INVENTION

[0007] Accordingly, it is an object of the present invention to provide a high-yield method for preparing a physiologically active polypeptide conjugate in which a non-peptidyl polymer site-specifically binds to a physiologically active polypeptide.

[0008] In accordance with one aspect of the present invention, there is provided a method for preparing a site-specific physiologically active polypeptide conjugate, comprising the steps of: i) subjecting to a reaction of a physiologically active polypeptide and a non-peptidyl polymer in a reaction medium which contains a specific amount of an alcohol and has a specific pH to enable the non-peptidyl polymer to bind to a target site of the physiologically active polypeptide; and ii) isolating and purifying the physiologically active polypeptide conjugate from the reaction mixture of step (i) by ion exchange chromatography using an alcohol, wherein process conditions and reactants are as set out in Claim 1.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, which respectively show:

Fig. 1: a purification profile of positional isomers of DA-exendin-4-PEG using SOURCE S column;
Fig. 2: a purification profile of positional isomers of CA-exendin-4-PEG using SOURCE S column;
Fig. 3: a graph showing Lys27-pegylated isomers obtained when CA-exendin-4 is pegylated at varying pH;
Fig. 4: a graph showing Lys27-pegylated isomers obtained when CA-exendin-4 is pegylated at varying pH and in 45% EtOH.

Fig. 5: a graph showing Lys27-pegylated isomers obtained when CA-exendin-4 is pegylated at pH 7.5 and in a varying amount (%) of ethanol;
Fig. 6: a graph showing Lys27-pegylated isomers obtained when CA-exendin-4 is pegylated at pH 7.5 and in a varying amount (%) of isopropanol;
Fig. 7: an SDS-PAGE analysis of CA-exendin-PEG-immunoglobulin Fc;
Fig. 8: an SDS-PAGE analysis of CA-exendin-PEG Lys12 and Lys27;
Fig. 9: an analysis profile of Lys12-pegylated isomers of CA-exendin-4 by peptide mapping;
Fig. 10: an analysis profile of Lys27-pegylated isomers of CA-exendin-4 by peptide mapping;
Fig. 11: a purification profile of positional isomers of oxyntomodulin-PEG using SOURCE S column;
Fig. 12: a purification profile of positional isomers of imidazo-acetyl oxyntomodulin-PEG using SOURCE S column;
Fig. 13: an analysis profile of Lys30-pegylated isomers of oxyntomodulin by peptide mapping;
Fig. 14: a purification profile of a conjugate of imidazo-acetyl oxyntomodulin-PEG and immunoglobulin Fc using SOURCE Q column;
Fig. 15: an SDS-PAGE analysis of a conjugate of imidazo-acetyl oxyntomodulin-PEG and immunoglobulin Fc;
Fig. 16: a purification profile of positional isomers of oxyntomodulin analogue-PEG using SOURCE S column;
Fig. 17: an analysis profile of Lys27-pegylated isomers of oxyntomodulin analogue by peptide mapping;
Fig. 18: a purification profile of positional isomers of imidazo-acetyl oxyntomodulin analogue-PEG using SOURCE S column;
Fig. 19: an analysis profile of Lys27-pegylated isomers of imidazo-acetyl oxyntomodulin analogue by peptide mapping;
Fig. 20: a purification profile of a conjugate of imidazo-acetyl oxyntomodulin anlaog-PEG and immunoglobulin Fc using SOURCE Q column;
Fig. 21: an SDS-PAGE analysis of a conjugate of imidazo-acetyl oxyntomodulin anlaog-PEG and immunoglobulin Fc;
Fig. 22: a purification profile of positional isomers of GLP-1-PEG using SOURCE S column;
Fig. 23: an analysis profile of Lys34-pegylated isomers of GLP-1 by peptide mapping;
Fig. 24: a purification profile of positional isomers of imidazo-acetyl GLP-1-PEG using SOURCE S column;
Fig. 25: an analysis profile of Lys34-pegylated isomers of imidazo-acetyl GLP-1 by peptide mapping;
Fig. 26: a purification profile of a conjugate of imidazo-acetyl GLP-1-PEG and immunoglobulin Fc using SOURCE Phe column;
Fig. 27: an SDS-PAGE analysis of a conjugate of imidazo-acetyl GLP-1-PEG and immunoglobulin Fc;
Fig. 28: a purification profile of positional isomers of GLP-2-PEG using SOURCE S column;
Fig. 29: an analysis profile of Lys30-pegylated isomers of GLP-2 by peptide mapping;
Fig. 30: a purification profile of positional isomers of imidazo-acetyl GLP-2-PEG using SOURCE S column;
Fig. 31: a purification profile of a conjugate of imidazo-acetyl GLP-2-PEG and immunoglobulin Fc using SOURCE Phe column;
Fig. 32: an SDS-PAGE analysis of a conjugate of imidazo-acetyl GLP-2-PEG and immunoglobulin Fc;
Fig. 33: a purification profile of positional isomers of human insulin-PEG (B1F) using SOURCE S column;
Fig. 34: a purification profile of positional isomers of human insulin-PEG (A1G) using SOURCE S column;
Fig. 35: a purification profile of positional isomers of human insulin-PEG (B29K) using SOURCE S column; and
Fig. 36: an analysis profile of A1G-, B1F-, or B29K-pegylated isomers of human insulin by peptide mapping.

DETAILED DESCRIPTION OF THE INVENTION

[0010] Hereinafter, the present invention is described in detail.

[0011] The present invention provides a method for preparing a site-specific physiologically active polypeptide conjugate, comprising the steps of:

i) treating a physiologically active polypeptide with a non-peptidyl polymer in a reaction medium which contains a specific amount of an alcohol and has a specific pH to enable the non-peptidyl polymer bind to a target site of the physiologically active polypeptide; and
ii) isolating and purifying the physiologically active polypeptide conjugate from the reaction mixture of step (i) by ion exchange chromatography using an alcohol, wherein process conditions and reactants are as set out in claim 1.

[0012] A physiologically active polypeptide conjugate herein refers to a substance in which a physiologically active polypeptide and a terminus of a non-peptidyl polymer are covalently linked to each other, and the present invention is characterized by linking a polypeptide with a polymer under a specific condition and isolating the resulting polypeptide conjugate having the polymer linked on a target site thereof.

[0013] The term "physiologically active polypeptide or peptide", as used herein, refers to a peptide which can exhibit

a physiological activity *in-vivo,* e.g., may be selected from a group consisting of insulinotropic peptide, blood factor, digestive hormone, adrenocorticotropic hormone, thyroid hormone, intestinal hormone, cytokine, enzyme, growth factor, neuropeptide, hypophyseotropic hormone, hypophysiotropic hormone, anti-obesity peptide, anti-viral peptide, and non-native peptide derivatives retaining physiologically active property, but not limited thereto. More particularly, the physiologically active polypeptide or peptide is selected from the group consisting of erythropoietin, GM-CSF (granulocyte macrophage-colony stimulating factor), amylin, glucagon, insulin, somatostatin, PYY (peptide YY), NPY (neuropeptide Y), GLP-1, GLP-2, exendin-4, oxyntomodulin, ghrelin, angiotensin, bradykinin, calcitonin, corticotropin, eledoisin, gastrin, leptin, oxytocin, vasopressin, LH (luteinizing hormone), prolactin, FSH (follicle stimulating hormone), PTH (parathyroid hormone), secretin, sermorelin, hGH (human growth hormone), growth hormone-releasing peptide, G-CSFs (granulocyte colony stimulating factor), interferons, interleukins, prolactin-releasing peptide, orexin, thyroid-releasing peptide, cholecystokinin, gastrin-inhibiting peptide, calmodulin, gastrin-releasing peptide, motilin, vasoactive intestinal peptide, ANP(atrial natriuretic peptide), BNP (barin natriuretic peptide), CNP (C-type natriuretic peptide), neurokinin A, neuromedin, renin, endothelin, sarafotoxin peptide, carsomorphin peptide, dermorphin, dynorphin, endorphin, enkepalin, T cell factor, tumor necrosis factor, tumor necrosis factor receptor, urokinase receptor, tumor inhibitory factor, collagenase inhibitor, thymopoietin, thymulin, thymopentin, tymosin, thymic humoral factor, adrenomodullin, allatostatin, amyloid beta-protein fragment, antimicrobial peptide, antioxidant peptide, bombesin, osteocalcin, CART peptide, E-selectin, ICAM-1, VCAM-1, leucokine, kringle-5, laminin, inhibin, galanin, fibronectin, pancreastatin, and fuzeon. In addition, the physiologically active polypeptide includes a precursor, a derivative, a fragment, or a variant thereof.

[0014] Active polypeptides are exendin, insulin, GLP-1, GLP-2, oxyntomodulin, ghrelin, angiotensin, bradykinin, calcitonin, or a derivative thereof. The derivative thereof may be prepared, e.g., by chemical substitution (e.g., alpha-methylation or alpha-hydroxylation), deletion (e.g., deamination or carbon deletion) or modification (e.g., N-methylation) of any groups on an amino acid residue, and particularly the preparation of the exendin derivative is described in detail in Korean Patent Application No. 2008-69234.

[0015] Meanwhile, the term "non-peptidyl polymer", as used herein, refers to a biocompatible polymer including two or more repeating units linked to each other by a covalent bond excluding a peptide bond.

[0016] The non-peptidyl polymer which can be used in the present invention may be selected form the group consisting of polyethylene glycol, polypropylene glycol, copolymers of ethylene glycol and propylene glycol, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, dextran, polyvinyl ethyl ether, biodegradable polymers such as PLA (poly(lactic acid)) and PLGA (polylactic-glycolic acid), lipid polymers, chitins, hyaluronic acid, and combinations thereof, and claimed is polyethylene glycol. A derivative thereof, which is known in the art or easily prepared based on the skill of the art, could be envisaged. The non-peptidyl polymer which can be used in the present invention functions to increase the molecular weight of a physiologically active polypeptide to prevent the loss of the conjugate through the kidney. Any non-peptidyl polymer, as long as it is resistant to *in-vivo* protease, can be used without any limitation. The molecular weight of the non-peptidyl polymer may be in the range of 0.5 to 100 kDa, preferably of 0.5 to 20 kDa, and the suitable molar ratio of the physiologically active polypeptide and the non-peptidyl polymer may be chosen in the range of from 1:1 to 1:50.

[0017] The non-peptidyl polymer used in the present invention has a reactive group at one end or at both ends. In case of the non-peptidyl polymer having a reactive group at both ends, it can bind to a physiologically active carrier and a protein drug which assist functioning as a long acting formulation.

[0018] The reactive group at one end or both ends of the non-peptidyl polymer is preferably selected from the group consisting of a reactive aldehyde group, a propionaldehyde group, a butyraldehyde group, a maleimide group and a succinimide group. Examples of the succinimide group include succinimidyl propionate, succinimidyl butanoate, hydroxy succinimidyl, succinimidyl carboxymethyl, or succinimidyl carbonate. In particular, when the non-peptidyl polymer has a reactive aldehyde group or a reactive succinimidyl group at one end, it is effective in linking at both ends with a physiologically active polypeptide and an immunoglobulin with minimal non-specific reactions. The aldehyde reactive group selectively binds to an N-terminus at a low pH, and can bind to a lysine residue to form an amine bond at a high pH, such as pH 9.0. In addition, the succinimidyl reactive group can form a stable amide bond with an amino terminus or lysine residue at pH 7.0~9.0.

[0019] Further, the reactive groups at both ends of the non-peptidyl polymer may be the same or different. When a polyethylene glycol having a reactive hydroxyl group at both ends thereof is used as the non-peptidyl polymer, the hydroxyl group may be activated into various reactive groups by known chemical reactions, or a commercially available polyethylene glycol having a modified reactive group may be used.

[0020] The step (i) of the present invention is to prepare a physiologically active polypeptide conjugate by site-specifically linking a non-peptidyl polymer with a physiologically active polypeptide in a suitable reaction medium.

[0021] The term "site-specific" or "site-specifically", as used herein, refers to the linking a non-peptidyl polymer onto a specific target amino acid site of a physiologically active polypeptide, preferably an amine of the lysine residue or N-terminus. The site-specific linking or bond may prevent the formation of incidental conjugates in which the non-peptidyl polymer is linked to a physiologically important amino acid residue. For example, when a non-peptidyl polymer binds to

the N-terminus of exendin-4, *in-vitro* activity of the exendin-4 became reduced, but when a non-peptidyl polymer binds to a lysine residue, *in-vitro* activity was maintained. In particular, when a non-peptidyl polymer binds to 27th lysine residue rather than 12th lysine residue, much higher *in-vitro* activity was observed (*See* Example 10 and Table 2).

[0022] The present inventors have found that the presence of an alcohol in a reaction medium and the pH of the reaction medium in step (i) are critical factors for a site-specific bond of a non-peptidyl polymer and a physiologically active polypeptide. Accordingly, in the step (i) of the present invention, a non-peptidyl polymer becomes linked to a specific site of a physiologically active polypeptide, using a reaction medium containing a specific content of an alcohol and having a specific pH.

[0023] In a specific embodiment of the present invention, the ratio of a specific positional isomer of the polypeptide conjugate can be varied based on the pH of the reaction medium, or based on the concentration (%) of an alcohol at same pH. It is therefore possible to link a non-peptidyl polymer to a desired amino acid of a physiologically active polypeptide in a site-specific manner.

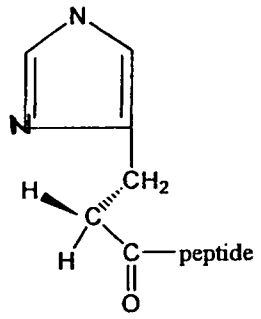
[0024] That is, the step (i) of the present invention is carried out at a specific pH to enable the non-peptidyl polymer to bind to a desired (or target) site, i.e., that dose not affect the polypeptide's activity. The pH range may depend on the types of physiologically active polypeptide. For instance, in case of an insulinotropic peptide (e.g., exendin-4), an isomer in which the polymer is linked to the 12th lysine was highly observed at low pH of the reaction medium, whereas an isomer in which the polymer is linked to the 27th lysine which does not affect the insulinotropic activity was highly observed at high pH of the reaction medium (*See* Example 3 and Fig. 3). Accordingly, the step (i) is preferably carried out at pH 7.5 to 9.0 so that the non-peptidyl polymer is selectively coupled to the 27th lysine.

[0025] Further, the step (i) of the present invention is carried out in a reaction medium containing an alcohol so that a non-peptidyl polymer can bind to a site that does not affect the polypeptide's activity. Examples of the alcohol include primary, secondary, and tertiary alcohol, preferably an alcohol having a carbon number of one to ten, more preferably ethanol or isopropanol. In case of an insulinotropic peptide (e.g., exendin-4), an alcohol may be present in the reaction medium, in an amount ranging, by volume, from 25% to 90%, preferably from 35% to 60%, based on the total volume of the reaction medium, in order to enable a non-peptidyl polymer to bind to the 27th lysine that does not affect the insulinotropic activity.

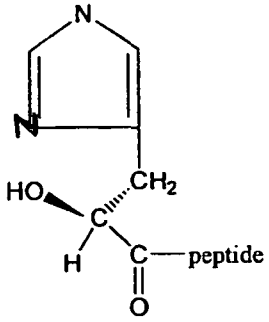
[0026] When a physiologically active polypeptide is exendin-4 or a derivative thereof, the pH employed in step (i) may be 7.0 to 10.0 in order to enhance the binding ratio of a non-peptidyl polymer to Lys27. In other embodiment of the present invention, when a physiologically active polypeptide is calcitonin, the pH employed in step (i) may be 4.0 to 6.0 in order to enhance the binding ratio of a non-peptidyl polymer to N-terminus. When a physiologically active polypeptide is oxyntomodulin or a derivative thereof, the pH employed in step (i) may be 7.0 to 10.0 in order to enhance the binding ratio of a non-peptidyl polymer to Lys27 or Lys30. When a physiologically active polypeptide is human insulin or a derivative thereof, the pH employed in step (i) may be 4.0 to 6.0 in order to enhance the binding ratio of a non-peptidyl polymer to Phel (1st phenylalanine) N-terminus in B chain. When a physiologically active polypeptide is human insulin or a derivative thereof, the pH employed in step (i) may be 7.0 to 10.0 in order to enhance the binding ratio of a non-peptidyl polymer to Glyl (1st glycine) N-terminus in A chain, or to Lys29 (29th lysine) in B chain. When a physiologically active polypeptide is GLP-1 or a derivative thereof, the pH employed in step (i) may be 7.0 to 10.0 in order to enhance the binding ratio of a non-peptidyl polymer to Lys34. When a physiologically active polypeptide is GLP-2 or a derivative thereof, the pH employed in step (i) may be 7.0 to 10.0 in order to enhance the binding ratio of a non-peptidyl polymer to Lys30.

[0027] Accordingly, the preferred site-specific physiologically active polypeptide conjugate of the present invention is an exendin-4 conjugate in which PEG is linked to Lys27, a calcitonin conjugate in which PEG is linked to N-terminus, an oxyntomodulin conjugate or an analogue thereof in which PEG is linked to Lys27 or Lys30, a human insulin conjugate or an analogue thereof in which PEG is linked to Phel N-terminus in B chain, Glyl N-terminus in A chain, or to Lys29 in B chain, or a GLP-1 or GLP-2 conjugate in which PEG is linked to Lys34 or Lys30.

[0028] In a preferred aspect of the present invention, a peptide derivative (or analogue) may be used to facilitate a site-specific bond between a non-peptidyl polymer and a physiologically active polypeptide. The derivative is a peptide having any of other non-target amino acid sites deleted or protected in order to prevent undesired linking. For example, in case of a insulinotropic peptide such as an exendin, various exendin derivatives may be used, such as Des-amino-histidyl (DA)-exendin-4 of formula (I), Beta-hydroxy-imidazopropionyl(HY)-exendin-4 of formula (II), Imidazo-acetyl(CA)-exendin-4 of formula (III), and Dimethyl-histidyl(DM)-exendin-4 of formula (IV), which are prepared using the methods where an alpha amine group of N-terminal amino acid, histidine is deleted, the N-terminal amine group is substituted with hydroxyl group, the N-terminal alpha amine group of histidine is modified with two methyl groups, or an alpha carbon of N-terminal histidine and an amine group bound thereto are deleted to leave an imidazoacetyl group, but not limited thereto. The structures of such exemplary exendin derivatives and methods of preparation thereof are described in Korean Unexamined Patent Publication No. 2009-0008151, which is included within the scope of the present invention by reference to the formulas on page 11 thereof.



Formula I



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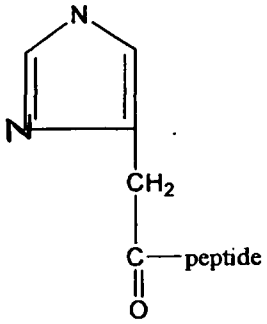
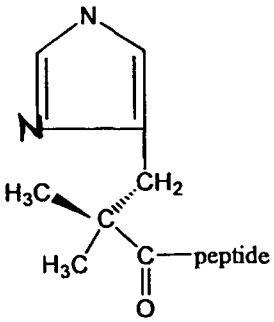


Formula III



Formula IV

[0029] Similarly, oxyntomodulin, GLP-1 and GLP-2 having a histidine as 1st amino acid are also used as a derivative having any structure selected from Formulas (I) to (IV).

[0030] In a particular embodiment of the present invention, the present inventors have investigated the effects of the pH and the content of an alcohol (%) of the reaction medium on the site-specific binding aspect of the non-peptidyl polymer, and confirmed that the ratio of the insulinotropic peptide conjugates having the polymer linked to 12th lysine/27th lysine is varied depending on the change of pH and amount of ethanol or isopropanol, in particular. In particular, a more preferable isomer having the non-peptidyl polymer bound to the 27th lysine residue was largely obtained, when 35% to 55%, preferably about 45% of ethanol or isopropanol is used at pH 7.5.

[0031] After enhancing the ratio of the desired non-peptidyl polymer-physiologically active polypeptide conjugate by adjusting into a specific pH and an alcohol content in step (i), the desired conjugate can be isolated and purified by ion

exchange chromatography using an alcohol in step (ii).

[0032] The alcohol used in step (ii) is present in a purification solution, and its specific examples are same as defined in step (i). However, the alcohol of step (i) was employed for the purpose of increasing the polypeptide's reactivity and site-specificity by modification of secondary or tertiary structure thereof, whereas the alcohol of step (ii) was employed for the purpose of facilitating the high-throughput isolation and purification of the site-specific physiologically active polypeptide conjugate by reduction of non-specific bond between ion-exchange column and the conjugate. The isolation and purification may be carried out by using various methods known to a person skilled in the art, preferably by ion exchange chromatography, more preferably by high pressure ion exchange chromatography.

[0033] Meanwhile, in order to facilitate the isolation and purification of positional isomer, the ion exchange chromatography may be carried out at a specific pH. The pH was suitably adjusted to increase the conjugate's site-specificity in step (i), whereas it was re-adjusted to attach the conjugate to and to detach it from the ion exchange column in a purification solution containing an alcohol in step (ii). The suitable pH employed in step (ii) may be in the range from about 2.0 to about 6.0.

[0034] Furthermore, the physiologically active polypeptide of the present invention may further be linked with a physiologically active carrier. In this case, the non-peptidyl polymer should be a non-peptidyl polymer with both ends in order to bind with the physiologically active carrier. That is, a physiologically active carrier covalently binds to an end of the non-peptidyl polymer which is not covalently linked with the physiologically active polypeptide, and thus it can be prepared a conjugate in which both ends of non-peptidyl polymer are linked with the physiologically active polypeptide and the physiologically active carrier.

[0035] As described above, the physiologically active polypeptide conjugate prepared in step (ii) may further bind with a physiologically active carrier, and the resulting polypeptide-polymer-carrier conjugate shows completely different activities compared to the polypeptide-polymer conjugate, i.e., the outstanding physiological activities such as excellent prolonged duration of pharmacological effects of a physiologically active polypeptide, targeting to a specific site such as a lesion to be treated, or induction of necrosis.

[0036] The term "physiologically active carrier", as used herein, refers to a physiologically active substance showing additional activities distinct to the native polypeptide's physiological activity, which can sustain the polypeptide's physiological activities such as the pharmacological effects, or induce targeting to a specific site or necrosis, by binding to a non-peptidyl polypeptide together with a physiologically active polypeptide.

[0037] The physiologically active carrier used in the present invention includes a substance having afore-mentioned activities without any limitation, e.g., albumin, immunoglobulin Fc region, transferrin, aptamer, toxin, collagen, dextran, polysaccharides, fatty acids, fibrinogen, and the like. Preferably, the physiologically active carrier may be selected from an albumin, an immunoglobulin Fc region, and a transferrrin, more preferably an immunoglobulin Fc region.

[0038] The immunoglobulin Fc region of the present invention refers to the heavy chain constant region 2 (CH2) and the heavy chain constant region 3 (CH3) of an immunoglobulin, excluding the variable regions of the heavy and light chains, the heavy chain constant region 1 (CH1) and the light chain constant region 1 (CL1). It may further include a hinge region at the heavy chain constant region. Also, the immunoglobulin Fc region of the present invention may be a extended form containing a part or all of the Fc region including the heavy chain constant region 1 (CH1) and/or the light chain constant region 1 (CL1), except for the variable regions of the heavy and light chains, as long as it has a physiological function substantially similar to or better than the native immunoglobulin Fc, and may include immunoglobulin Fc regions modified by phosphorylation, sulfation, acrylation, glycosylation, methylation, farnesylation, acetylation, amidation, and the like. The range of immunoglobulin Fc, method for preparation thereof, and method for covalently linking an immunoglobulin Fc to a non-peptidyl polymer-physiologically active polypeptide conjugate are disclosed in Korean Patent Nos. 775343, 725314, 725315, and 824505.

[0039] In accordance with the method of the present invention, a desired polypeptide conjugate having an excellent physiological activity can be prepared in a high yield by site-specifically linking a non-peptidyl polymer to a specific amino acid of a physiologically active polypeptide, while minimizing the formation of additional conjugates.

[0040] The following Examples are intended to further illustrate the present invention.

Example 1: Preparation and isolation of pegylated DA-exendin-4 (Lys27) conjugate

<1-1> Preparation of pegylated DA-exendin-4 (Lys27) conjugate

[0041] In order to prepare a pegylated peptide conjugate by covalently linking Lys in the peptide with the PEG, desamino-histidyl-exendin-4 (DA-exendin-4, AP, U.S.) and 3.4K PropionALD(2) PEG (PEG having two propionaldehyde groups, IDB Inc., Korea) was subjected to a reaction at 4°C for 12 hours at a molar ratio of 1:30, with a peptide concentration of 3 mg/mL. At this time, 100 mM HEPES buffer (pH 7.5) containing 45% isopropanol was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto.

<1-2> Isolation of positional isomer

[0042] A mono-pegylated peptide was primarily purified from the reaction mixture of Example <1-1> by using an SOURCE Q ion exchange chromatography (XK 16 mL, GE healthcare, Korea) under the following condition, and positional isomers were isolated by using an SOURCE S ion exchange chromatography (XK 16 mL, GE healthcare, Korea) under the following condition. In this process, ethanol was used to facilitate the isolation of isomers, by including it in a purification solution. The pegylated sites were confirmed from eluted peaks by peptide mapping method.

Column: SOURCE Q
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Tris, pH 8.5) and B (A+0.5M NaCl); Gradient A 0→40%, 80 min

Column: SOURCE S
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM citric acid, pH 3.0 + 45% ethanol) and B (A+45% ethanol+0.5M KCl); Gradient A 0→100%, 45 min

[0043] From the analysis of the purification profile of positional isomers, it was found that a peak for Lys12-pegylated DA-exendin-4 was eluted earlier, and then a Lys27-pegylated peak was eluted in the last portion (Fig. 1).

Example 2: Preparation and isolation of pegylated CA-exendin-4 (Lys27) conjugate

[0044] The procedure of Example 1 was repeated except for using imidazo-acetyl-exendin-4 (CA-exendin-4, Bachem, U.S.) instead of DA-exendin-4 in Example 1 to obtain the pegylated CA-exendin-4 (Lys27) conjugate. From the analysis of the purification profile of positional isomers, it was found that a peak for Lys12-pegylated CA-exendin-4 was eluted earlier, and then a Lys27-pegylated peak was eluted in the last portion (Fig. 2).

Example 3: Change in ratio of pegylated CA-exendin-4 (Lys27) conjugate according to pH of reaction medium

[0045] To investigate the change in ratio of a polypeptide pegylated at a specific site by pH, CA-exendin-4 and 3.4K PropionALD(2) PEG were subjected to pegylation by reacting the peptide and the PEG at 4°C for 12 hours at a molar ratio of 1:30, with a peptide concentration of 3 mg/mL. At this time, 100 mM citric acid (pH 3.0), 100 mM NaOAc (pH 4.5), 100 mM Na-P (pH 7.5), 100 mM Na-P (pH 8.5), 100 mM HEPES (pH 8.0), and 100 mM Na-Borate (pH 9.2) buffers were used as a reaction medium, respectively, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto. Each reaction mixture was purified by the method described in Example 1, followed by analyzing the ratio of the Lys27-pegylated conjugate. As shown in Fig. 3, the ratio of the Lys27-pegylated conjugate was increased based on the increase in pH, and the optimum pH was confirmed to be 7.0 to 10.0.

Example 4: Change in ratio of pegylated CA-exendin-4 (Lys27) conjugate according to pH of reaction medium containing ethanol

[0046] To investigate the change in the ratio of a polypeptide pegylated at a specific site by ethanol and pH, CA-exendin-4 and 3.4K PropionALD(2) PEG were subjected to pegylation by reacting the peptide and the PEG at 4°C for 12 hours at a molar ratio of 1:30, with a peptide concentration of 3 mg/mL. At this time, 100 mM citric acid (pH 3.0)/45% ethanol, 100 mM NaOAc (pH 4.5)/45% ethanol, 100 mM Na-P (pH 7.5)/45% ethanol, 100 mM HEPES (pH 8.0)/45% ethanol, and 100 mM Na-P (pH 8.5)/45% ethanol buffers were used as a reaction medium, respectively, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto. Each reaction mixture was purified by the method described in Example 1, followed by analyzing the ratio of the Lys27-pegylated conjugate. As shown in Fig. 4, the ratio of the Lys27-pegylated conjugate was increased based on the increase in pH in a 45% EtOH-containing reaction medium, and the optimum pH was confirmed to be 7.0 to 9.0.

Example 5: Change in ratio of pegylated CA-exendin-4 (Lys27) conjugate according to ethanol concentration in reaction medium

[0047] To investigate the change in ratio of a polypeptide pegylated at a specific site by ethanol concentration in the reaction medium, CA-exendin-4 and 3.4K PropionALD(2) PEG were subjected to pegylation by reacting the peptide and the PEG in at 4°C for 12 hours at a molar ratio of 1:30, with a peptide concentration of 3 mg/mL. At this time, 100 mM HEPES (pH 7.5)/0% ethanol, 100 mM HEPES (pH 7.5)/25% ethanol, 100 mM HEPES (pH 7.5)/35% ethanol, 100 mM

HEPES (pH 7.5)/45% ethanol, 100 mM HEPES (pH 7.5)/55% ethanol, 100 mM HEPES (pH 7.5)/75% ethanol and 100 mM HEPES (pH 7.5)/90% ethanol buffers were used as a reaction medium, respectively, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto. Each reaction mixture was purified by the method described in Example 1, followed by analyzing the ratio of the Lys27-pegylated conjugate. As shown in Fig. 5, the ratio of the Lys27-pegylated conjugate was increased until the amount of ethanol reaches about 50%, whereas decreased in more than 50% of ethanol, and the optimum amount of ethanol was confirmed to be 35% to 60%.

Example 6: Change in ratio of pegylated CA-exendin-4 (Lys27) conjugate according to isopropanol concentration in reaction medium

[0048] To investigate the change in ratio of a polypeptide pegylated at a specific site by the use of isopropanol instead of ethanol, in the reaction medium, CA-exendin-4 and 3.4K PropionALD(2) PEG were subjected to pegylation by reacting the peptide and the PEG at 4°C for 12 hours at a molar ratio of 1:30, with a peptide concentration of 3 mg/mL. At this time, 100 mM HEPES (pH 7.5)/0% isopropanol, 100 mM HEPES (pH 7.5)/30% isopropanol, 100 mM HEPES (pH 7.5)/45% isopropanol, and 100 mM HEPES (pH 7.5)/60% isopropanol buffers were used as a reaction medium, respectively, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto. Each reaction mixture was purified by the method described in Example 1, followed by analyzing the ratio of the Lys27-pegylated conjugate. As shown in Fig. 6, the ratio of the Lys27-pegylated conjugate was increased until the amount of ethanol reaches about 50%, whereas decreased in more than 50% of ethanol, and the optimum amount of ethanol was confirmed to be 35% to 60%.

Example 7: Preparation of a conjugate of CA-exendin-4 (Lys27)-PEG and immunoglobulin Fc

[0049] CA-exendin-4 (Lys27)-PEG conjugate was coupled with an immunoglobulin Fc fragment (Hanmi Pharm. Co. Ltd., Korea), by subjecting to a reaction of the conjugate and the fragment at 4°C for 16 hours at a molar ratio of 1:8, with a peptide concentration of 20 mg/mL. At this time, 100 mM K-P buffer (pH 6.0) was used as a reaction medium, and 20 mM NaCNBH3 as a reducing agent was added thereto. After the coupling reaction, the two-step purification was performed using SOURCE phe and SOURCE Q columns, under following conditions.

Column: SOURCE Phe (XK 16 mL, GE healthcare)
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Tris, pH 7.5) and B (A+1.5M NaCl); Gradient A 0→40%, 80 min

Column: SOURCE Q (XK 16 mL, GE healthcare)
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Tris, pH 7.5) and B (A+1M NaCl); Gradient A 0→40%, 80 min

[0050] The conjugate prepared above was analyzed using SDS-PAGE. As shown in Fig. 7, a single band with 60K was observed under a non-reducing condition, whereas two bands with 35K and 25K were observed under a reducing condition.

Example 8: Identification of pegylated site of CA-exendin-4 (Lys27)-PEG conjugate

[0051] To identify the binding site of PEG to CA-exendin-4, the CA-exendin-4 (Lys27)-PEG conjugates were digested with a protease enzyme, lysine-C, and the analyzed using a reverse chromatography.

[0052] Specifically, CA-exendin-4-PEG isomers were analyzed by SDS-PAGE (Fig. 8), and then a purified CA-exendin-4-PEG conjugate and CA-exendin-4 were dissolved in triethylamine-hydrochloric acid buffer (10 mmol/L; pH 7.5), 10 μL of enzyme (0.1 mg/mL) was added thereto, and reacted at 37°C for 4 hours. After the reaction was terminated, the reaction mixture was analyzed by a reverse chromatography (HPLC (Agilent), Jupiter C18 (Phenomenex)). The analysis results are shown in Figs. 9 and 10. A Lys12-pegylated CA-exendin-4 isomer was confirmed by simultaneous disappearance of #1 and #2 as shown in Fig. 9, and a Lys27-pegylated CA-exendin-4 isomer was confirmed by simultaneous **disappearance** of #2 and #3 **as** shown in **Fig.** 10.

Example 9: Identification of pegylation yield by isopropanol concentration on N-terminal pegylation

[0053] To pegylate methoxy polyethylene glycol 5K ALD (NOF Inc., Japan) to the N-terminal of calcitonin salmon (Bachem, U.S.), a calcitonin salmon and PEG was subjected to pegylation by reacting the peptide and the PEG at 4°C for 1 hour at a molar ratio of 1:1, with a peptide concentration of 1 mg/mL. At this time, 100 mM NaAc pH 5.2/0% isopropanol, 100 mM NaAc pH 5.2/45% isopropanol buffers were used as a reaction medium, respectively, and 20 mM

$NaCNBH_3$ as a reducing agent was added thereto. A mono-pegylated peptide was purified from each of the reaction mixures by SOURCE S column (XK 16 mL, GE healthcare, Korea) under the following condition. The results are shown in Table 1 below.

Column: SOURCE S
Flow rate: 2.5 mL/min
Eluting solution: A (20 mM Acetate, pH 5.2) and B (A+1M NaCl); Gradient A 0→40%, 60 min

<Table 1>

Reaction buffer solution	Yield of mono-pegylated calcitonin-5K (%)
0.1M NaAc pH 5.2	36
0.1M NaAc pH5.2/45% IPA	51.3

[0054] As shown in Table 1, the yield of a pegylated calcitonin became increased by addition of iospropanol.

Example 10: Measurement of in-vitro activity of exendin-4 by pegylation and pegylated site

[0055] To measure the efficacy of long acting preparation of exendin-4 by pegylation and pegylation site, a method for measuring the in-vitro activity was used. The measurement of in-vitro activity of GLP-1 is a method for measuring whether cAMP's in the cell was increased after treatment of GLP-1 to CHO cell lines to which GLP-1 receptors was cloned.

[0056] Specifically, CHO/GLP-1R, a cell line in which GLP-1 is cloned, was treated with GLP-1, exendin-4 and test materials described in Table 1 at varying concentrations. The occurrence of cAMP's was measured, and hence EC50 values were compared to each other. As a control, commercially available Byetta (Eli Lilly) was used. The in-vitro activities (%) according to treatment of test materials were shown in Table 2.

<Table 2>

Test material	In-vitro activity (%)
Byetta	100
CA-exendin-4	77
CA-exendin-4-PEG (Lys12)	2.1
CA-exendin-4-PEG (Lys27)	8.5

[0057] As shown in Table 2, the physiological activity of the peptide was relatively less affected when PEG is modified at Lys27, compared to Lyes12.

Example 11: Preparation and isolation of pegylated oxyntomodulin (Lys30) conjugate

<11-1> Preparation of_pegylated oxyntomodulin conjugate (Lvs30)

[0058] To prepare a pegylated oxyntomodulin conjugate, 3.4K PropionALD(2) PEG and oxyntomodulin (Anygen, Korea) were subjected to pegylation by reacting the peptide and the PEG at 4°C for 4.5 hours at a molar ratio of 1:15, with a peptide concentration of 3 mg/mL. At this time, 100 mM Na-Borate buffer (pH 9.0) containing 45% isopropanol was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto.

<11-2> Isolation of positional isomer

[0059] Lys30-pegylated Positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 11).

Column: SOURCE S
Flow rate: 2.0 mL/min

Eluting solution: A (20 mM Na-citrate, pH 3.0 + 45% ethanol) and B (A+1M KC1); Gradient A 0→3%, 1 min, Gradient B 0→40%, 222 min

Example 12: Preparation and isolation of pegylated imidazo-acetyl oxyntomodulin (Lys30) conjugate

[0060] The procedure of Example 11 was repeated except for using imidazo-acetyl-oxyntomodulin (Anygen, Korea) instead of oxyntomodulin and using 100 mM HEPES buffer (pH 7.5) containing 45% isoparopanol in Example 11 to obtain the pegylated imidazo-acetyl oxyntomodulin (Lys30) conjugate.

[0061] Isomers purified using SOURCE Q column was shown in Fig. 12, and the peptide mapping using an Asp-N protease was shown in Fig. 13. As shown Fig. 13, a part of #4:Asp(22)-(37) disappeared by PEG-modification at Lys30.

Example 13: Preparation of a conjugate of imidazo-acetyl oxyntomodulin (Lys30)-PEG and immunoglobulin Fc

[0062] The imidazo-acetyl oxyntomodulin-PEG (Lys30) conjugate and an immunoglobulin Fc (Hanmi Pharm. Co. Ltd., Korea) were subjected to a reaction at 4°C for 16 hours at a molar ratio of 1:10, with a total protein concentration of 20 mg/mL. At this time, 100 mM potassium phosphate (pH 6.0) was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto. After the reaction was terminated, the reaction mixture was purified by using SOURCE 15Q column, under the following condition.

Column: SOURCE Q
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Tris-HCl, pH 7.5) and B (A+1M NaCl); Gradient A 0→20%, 100 min

[0063] The chromatogram which is achieved by linking a Lys30-pegylated CA-oxyntomodulin with an immunoglobulin Fc and purifying the conjugate using SOURCE Q was shown in Fig. 14, and SDS-PAGE results of a conjugate of CA-oxyntomodulin (Lys30)-PEG and immunoglobulin Fc were shown in Fig. 15. As shown in Fig. 15, a single band with 60K was observed under a non-reducing condition, and two bands with 35K and 25K were observed under a reducing condition.

Example 14: Preparation and isolation of pegylated oxyntomodulin analogue (Lys27) conjugate

<14-1> Preparation of pegylated oxyntomodulin analogue (Lvs27) conjugate

[0064] To pegylate 3.4K PropionALD(2) PEG to a lysine of an oxnytomodulin analogue ([20Asp, 24Ala, 28Ser]-oxyntomodulin-[Deletion30-37]), the PEG and the oxyntomodulin analogue (Anygen, Korea) were subjected to a reaction at 4°C for 3.5 hours at a molar ratio of 1:15, with a peptide concentration of 3 mg/mL. At this time, 100 mM Na-Borate buffer (pH 9.0) containing 45% isopropanol was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto.

<14-2> Isolation of positional isomer

[0065] Lys27-pegylated positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). The conditions of purification and isolation were same with those described in Example 11. In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 16). The lysine selectivities of the purified mono-pegylated oxyntomodulin analogues were confirmed by peptide mapping method using Lys-C protease (Fig. 17). As shown in Fig. 17, a #2 part disappeared by PEG-modification at Lys27.

Example 15: Preparation and isolation of pegylated imidazo-acetyl oxyntomodulin analogue (Lys27) conjugate

<15-1> Preparation of pegylated imidazo-acetyl oxyntomodulin analogue (Lys27) conjugate

[0066] To pegylate 3.4K PropionALD(2) PEG to the Lys27 of an imidazo-acetyl oxyntomodulin analogue ([20Asp, 24Ala, 28Ser])-oxyntomodulin-[Deletion30-37]), the imidazo-acetyl oxyntomodulin analogue (Anygen, Korea) and the PEG were subjected to a reaction at 4°C for 2.5 hours at a molar ratio of 1:10, with a total protein concentration of 3 mg/mL. At this time, 100 mM HEPES (pH 7.5) containing 45% isopropanol was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto.

<15-2> Isolation of positional isomer

[0067] Lys27-pegylated positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). The conditions of purification and isolation were same with those described in Example 11. In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 18). The lysine selectivities of the purified mono-pegylated oxyntomodulin analogues were confirmed by peptide mapping method using Lys-C protease (Fig. 19). As shown in Fig. 19, a #2 part disappeared by PEG-modification at Lys27.

Example 16: Preparation and isolation of a conjugate of pegylated imidazo-acetyl oxyntomodulin analogue (Lys27) and immunoglobulin Fc

[0068] The Lys27-pegylated imidazo-acetyl oxyntomodulin analogue-PEG prepared in Example 15 and an immunoglobulin Fc were subjected to a reaction at 4°C for 16 hours at a molar ratio of 1:10, with a peptide concentration of 20 mg/mL. At this time, 100 mM potassium phosphate (pH 6.0) as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto. After the reaction was terminated, the reaction mixture was purified by using SOURCE 15Q column, under the following condition.

Column: SOURCE Q
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Tris-HCl, pH 7.5) and B (A+1M NaCl); Gradient A 0→20%, 100 min

[0069] The chromatogram which is achieved by linking a Lys27-pegylated CA-oxyntomodulin analogue with an immunoglobulin Fc and purifying the conjugate using SOURCE Q was shown in Fig. 20, and SDS-PAGE results of a conjugate of CA-oxyntomodulin analogue (Lys27)-PEG and immunoglobulin Fc were shown in Fig. 21. As shown in Fig. 21, a single band with 60K was observed under a non-reducing condition, and two bands with 35K and 25K were observed under a reducing condition.

Example 17: Preparation and isolation of pegylated GLP-1 (Lys34) conjugate

<17-1> Preparation of pegylated GLP-1 (Lys34) conjugate

[0070] To pegylate 3.4K PropionALD(2) PEG to the Lysine residue of a GLP-1, the GLP-1 and the PEG were subjected to a reaction at 4°C for 3.5 hours at a molar ratio of 1:15, with a total protein concentration of 3 mg/mL. At this time, 100 mM Na-Borate (pH 9.0) containing 45% isopropanol was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto.

<17-2> Isolation of positional isomer

[0071] Lys34-pegylated positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 22). The lysine selectivities of the purified mono-pegylated oxyntomodulin analogues were confirmed by peptide mapping method using Lys-C protease (Fig. 23).

Column: SOURCE S
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Na-citrate, pH 3.0 + 45% ethanol) and B (A+1M KCl); Gradient A 0→3%, 1 min, Gradient B 3→40%, 150 min

[0072] As shown in Fig. 23, a #2 part disappeared by PEG-modification at Lys34.

Example 18: Preparation and isolation of pegylated imidazo-acetyl GLP-1 (Lys34) conjugate

<18-1> Preparation of pegylated imidazo-acetyl GLP-1 (Lys34) conjugate

[0073] To pegylate 3.4K PropionALD(2) PEG to the Lysine residue of an imidazo-acetyl GLP-1, the imidazo-acetyl GLP-1 and the PEG were subjected to a reaction at 4°C for 4 hours at a molar ratio of 1:10, with a total protein concentration of 3 mg/mL. At this time, 100 mM HEPES (pH 7.5) containing 45% isopropanol was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto.

<18-2> Isolation of positional isomer

[0074] Lys34-pegylated positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 24). The lysine selectivities of the purified mono-pegylated oxyntomodulin analogues were confirmed by peptide mapping method using Glu-C protease (Fig. 25).

Column: SOURCE S
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Na-citrate, pH 3.0 + 45% ethanol) and B (A+1M KCl); Gradient A 0→3%, 1 min, Gradient B 3→40%, 150 min

[0075] As shown in Fig. 25, a #4 part disappeared by PEG-modification at Lys34.

Example 19: Preparation and isolation of a conjugate of pegylated imidazo-acetyl GLP-1 (Lys34) and immunoglobulin Fc

[0076] The Lys34-pegylated imidazo-acetyl GLP-1-PEG prepared in Example 18 and an immunoglobulin Fc were subjected to a reaction at 4°C for 17 hours at a molar ratio of 1:8, with a peptide concentration of 50 mg/mL. At this time, 100 mM potassium phosphate (pH 6.0) as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto. After the reaction was terminated, the reaction mixture was purified by using SOURCE Phe column, under the following condition.

Column: SOURCE Phe
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Tris-HCl, pH 7.5) and B (A+2M NaCl); Gradient A 100→0%, 100 min

[0077] The chromatogram which is achieved by linking a Lys34-pegylated CA-GLP-1 isomer with an immunoglobulin Fc and purifying the conjugate using SOURCE Phe was shown in Fig. 26, and SDS-PAGE results of a conjugate of CA-GLP-1 (Lys34)-PEG and immunoglobulin Fc were shown in Fig. 27. As shown in Fig. 27, a single band with 60K was observed under a non-reducing condition, and two bands with 35K and 25K were observed under a reducing condition.

Example 20: Preparation and isolation of pegylated GLP-2 (Lys30) conjugate

<20-1> Preparation of pegylated GLP-2 (Lys30) conjugate

[0078] To pegylate 3.4K PropionALD(2) PEG to the Lysine residue of a GLP-2 (Anygen, Korea), the GLP-2 and the PEG were subjected to a reaction at 4°C for 3 hours at a molar ratio of 1:12, with a total protein concentration of 5 mg/mL. At this time, 100 mM Na-Borate (pH 9.0) containing 45% isopropanol was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto.

<20-2> Isolation of positional isomer

[0079] Lys30-pegylated positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 28). The lysine selectivities were confirmed by peptide mapping method using a trypsin protease (Fig. 29).

Column: SOURCE S
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Na-citrate, pH 3.0 + 45% ethanol) and B (A+1M KCl); Gradient A 0→3%, 1 min, Gradient B 3→40%, 150 min

[0080] As shown in Fig. 29, a #2 part disappeared by PEG-modification at Lys30.

Example 21: Preparation and isolation of pegylated imidazo-acetyl GLP-2 (Lys30) conjugate

<21-1> Preparation of pegylated imidazo-acetyl GLP-2 (Lys30 conjugate

[0081] To pegylate 3.4K PropionALD(2) PEG to the Lysine30 residue of an imidazo-acetyl GLP-2 (Anygen, Korea), the imidazo-acetyl GLP-2 and the PEG were subjected to a reaction at 4°C for 6 hours at a molar ratio of 1:20, with a total protein concentration of 3 mg/mL. At this time, 100 mM HEPES (pH 7.5) containing 45% isopropanol was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto.

<21-2> Isolation of positional isomer

[0082] Lys30-pegylated positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 30).

Column: SOURCE S
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Na-citrate, pH 3.0 + 45% ethanol) and B (A+1M KCl); Gradient A 0→3%, 1 min, Gradient B 3→40%, 150 min

Example 22: Preparation and isolation of a conjugate of pegylated imidazo-acetyl GLP-2 (Lys30) and immunoglobulin Fc

[0083] The Lys30-pegylated imidazo-acetyl GLP-2-PEG prepared in Example 21 and an immunoglobulin Fc were subjected to a reaction at 4°C for 16 hours at a molar ratio of 1:15, with a peptide concentration of 20 mg/mL. At this time, 100 mM potassium phosphate (pH 6.0) as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto. After the reaction was terminated, the reaction mixture was purified by using SOURCE Phe column, under the following condition.

Column: SOURCE Phe
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Tris-HCl, pH 7.5) and B (A+2M NaCl); Gradient A 100→0%, 100 min

[0084] The chromatogram which is achieved by linking a Lys30-pegylated CA-GLP-2 isomer with an immunoglobulin Fc and purifying the conjugate using SOURCE Phe was shown in Fig. 31, and SDS-PAGE results of a conjugate of CA-GLP-2 (Lys30)-PEG and immunoglobulin Fc were shown in Fig. 32. As shown in Fig. 32, a single band with 60K was observed under a non-reducing condition, and two bands with 35K and 25K were observed under a reducing condition.

Example 23: Preparation and isolation of pegylated human insulin (BIPhe) conjugate

<23-1> Preparation of pegylated human insulin (B1Phe) conjugate

[0085] To pegylate 5K PropionALD(1) methoxyPEG (PEG having one propionaldehyde group, NOF., Japan) to a N-terminal of phenylalanine which is a first amino acid of B chain in human insulin (Sigma), the PEG and the insulin hyman were subjected to a reaction at 4°C for 12 hours at a molar ratio of 1:2, with a peptide concentration of 2.3 mg/mL. At this time, 100 mM potassium phophate buffer (pH 6.0) was used as a reaction medium, and 20 mM $NaCNBH_3$ as a reducing agent was added thereto.

<23-2> Isolation of positional isomer

[0086] Positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 33). The mono-pegylations of eluted peaks were confirmed by SDS-PAGE analysis, and the lysine selectivities were confirmed by peptide mapping method using Glu-C protease (Fig. 36).

Column: SOURCE S
Flow rate: 2.0 mL/min
Eluting solution: A (20 mM Na-citrate, pH 2.0 + 60% ethanol) and B (A+0.5M KCl); Gradient A 0→3%, 1 min, Gradient

B 0→50%, 80 min

[0087] As shown in Fig. 36, a #2 part disappeared by PEG-modification at B1F.

Example 24: Preparation and isolation of pegylated human insulin (AlGly) conjugate

<24-1> Preparation of pegylated human insulin (A1Gly) conjugate

[0088] To pegylate 5K methoxyPEG-Succinimidyl Butanoate(1) (PEG having one SBA reactive group, NOF., Japan) to a N-terminal of glycine which is a first amino acid of A chain in human insulin (Sigma), the PEG and the human insulin were subjected to a reaction at 25°C for 3 hours at a molar ratio of 1:4, with a peptide concentration of 2 mg/mL. At this time, 100 mM Na-Borate buffer (pH 9.0) containing 35% isopropanol was used as a reaction medium.

<24-2> Isolation of positional isomer

[0089] Positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). The purification procedure was same with that described in Example 23. In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 34). The mono-pegylations of eluted peaks were confirmed by SDS-PAGE analysis, and the lysine selectivities were confirmed by peptide mapping method using Glu-C protease (Fig. 36).

[0090] As shown in Fig. 36, a #1 part disappeared by PEG-modification at A1G.

Example 25: Preparation and isolation of pegylated human insulin (B29Lys) conjugate

<25-1> Preparation of pegylated human insulin (B29Lys) conjugate

[0091] To pegylate 5K methoxyPEG-Succinimidyl Butanoate(1) to a lysine residue which is a 29th amino acid of B chain in human insulin (Sigma), the PEG and the human insulin were subjected to a reaction at 25°C for 1 hours at a molar ratio of 1:2, with a peptide concentration of 2 mg/mL. At this time, 100 mM Na-Borate buffer (pH 9.0) containing 45% isopropanol was used as a reaction medium.

<25-2> Isolation of positional isomer

[0092] Positional isomers were purified from the reaction mixture by using SOURCE 15S column (XK 16 mL, Amersham Bioscience). The purification procedure was same with that described in Example 23. In this process, ethanol was used in the purification solution to facilitate the isolation of isomers (Fig. 35). The mono-pegylations of eluted peaks were confirmed by SDS-PAGE analysis, and the lysine selectivities were confirmed by peptide mapping method using Glu-C protease (Fig. 36).

[0093] As shown in Fig. 36, a #4 part disappeared by PEG-modification at B29K.

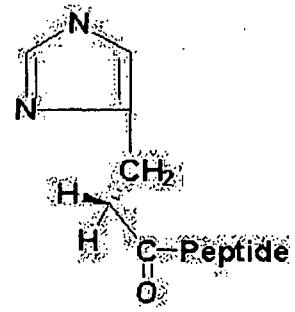
[0094] While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

Claims

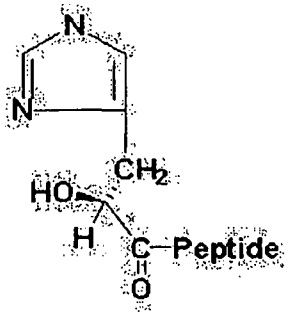
1. A method for preparing a site-specific physiologically active polypeptide conjugate, comprising the steps of:

   i) treating a physiologically active polypeptide with a non-peptidyl polymer in a reaction medium which contains a specific amount of an alcohol and has a specific pH to enable the non-peptidyl polymer to bind to a target site of the physiologically active polypeptide;
   and.
   ii) isolating and purifying the physiologically active polypeptide conjugate from the reaction mixture of step i) by ion exchange chromatography using an alcohol,

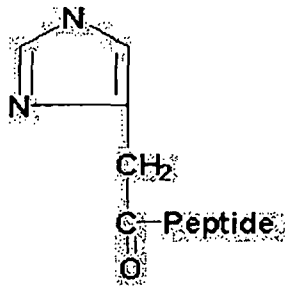
   wherein the physiologically active polypeptide is an exendin, insulin, oxyntomodulin, GLP-1, GLP-2, ghrelin, calcitonin, or one of derivatives thereof having any structure selected from Formulas (I) to (IV):



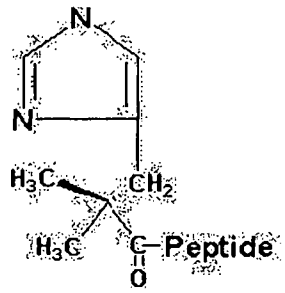
Formula I



Formula II



Formula III



Formula IV

wherein the alcohol is ethanol or isopropanol;
wherein the alcohol is present in the reaction medium in an amount of 25% to 90% by volume, based on the total volume of the reaction medium;
wherein the pH employed in step i) is 7.0 to 10.0, when the physiologically active polypeptide is an exendin, oxyntomodulin, GLP-1, GLP-2 or one of the derivatives thereof, and
wherein the pH employed in step i) is 4.0 to 10.0 when the physiologically active polypeptide is human insulin or a derivative thereof.

2. The method of claim 1, wherein the non-peptidyl polymer is polyethylene glycol.

3. The method of claim 1, wherein the alcohol is present in the reaction medium, in an amount of 35% to 60% by

volume, based on the total amount of the reaction medium.

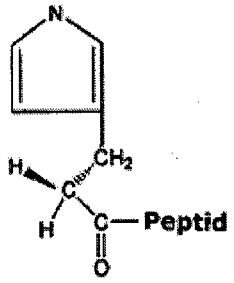
4. The method of claim 1, wherein the site-specific physiologically active polypeptide conjugate is an exendin-4 conjugate in which PEG is linked to Lys27, a calcitonin conjugate in which PEG is linked to the N-terminal, an oxyntomodulin conjugate in which PEG is linked to Lys27 or Lys30, human insulin conjugate in which PEG is linked to the N-terminal of Gly1 in A chain or is linked to Lys29 in B-chain, or a GLP-1 or GLP-2 conjugate in which PEG is linked to Lys34 or Lys30.

Patentansprüche

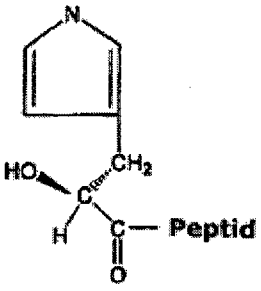
1. Verfahren zur Herstellung eines ortsspezifischen, physiologisch aktiven Polypeptid-Konjugats, umfassend die Schritte:

   i) Behandeln eines physiologisch aktiven Polypeptids mit einem Nichtpeptidyl-Polymer in einem Reaktionsmedium, das eine spezifische Menge eines Alkohols enthält und einen spezifischen pH-Wert aufweist, so dass das Nichtpeptidyl-Polymer ein einen Zielort auf dem physiologisch aktiven Polypeptid binden kann; und
   ii) Isolieren und Reinigen des physiologisch aktiven Polypeptid-Konjugats aus dem Reaktionsgemisch von Schritt i) durch Ionenaustauschchromatographie unter Verwendung eines Alkohols;

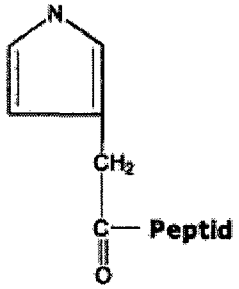
wobei das physiologisch aktive Polypeptid ein Exendin, Insulin, Oxyntomodulin, GLP-1, GLP-2, Ghrelin, Calcitonin oder eines der Derivate davon mit einer Struktur, die aus den Formeln (I) bis (IV) ausgewählt ist, ist:



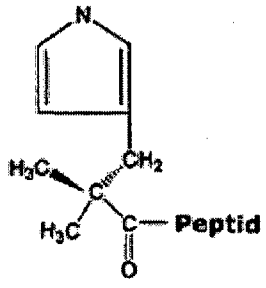
Formel I



Formel II



Formel III



Formel IV,

wobei es sich bei dem Alkohol um Ethanol oder Isopropanol handelt;
wobei der Alkohol in dem Reaktionsmedium in einer Menge von 25 bis 90 Vol.-% vorhanden ist, bezogen auf das Gesamtvolumen des Reaktionsmediums;
wobei der in Schritt i) eingesetzte pH-Wert 7,0 bis 10,0 beträgt, wenn das physiologisch aktive Polypeptid ein Exendin, Oxyntomodulin, GLP-1, GLP-2 oder eines der Derivate davon ist; und
wobei der in Schritt i) eingesetzte pH-Wert 4,0 bis 10,0 beträgt, wenn es sich bei dem physiologisch aktiven Polypeptid um Humaninsulin oder ein Derivat davon handelt.

2. Verfahren gemäß Anspruch 1, wobei es sich bei dem Nichtpeptidyl-Polymer um Polyethylenglycol handelt.

3. Verfahren gemäß Anspruch 1, wobei der Alkohol in dem Reaktionsmedium in einer Menge von 35 bis 60 Vol.-% vorhanden ist, bezogen auf die Ge-samtmenge des Reaktionsmediums.

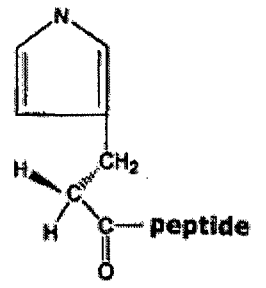
4. Verfahren gemäß Anspruch 1, wobei das ortsspezifische, physiologisch aktive Polypeptid-Konjugat ein Exendin-4-Konjugat, bei dem PEG mit Lys27 verknüpft ist, ein Calcitonin-Konjugat, bei dem PEG mit dem N-Terminus verknüpft ist, ein Oxyntomodulin-Konjugat, bei dem PEG mit Lys27 oder Lys30 verknüpft ist, ein Humaninsulin-Konjugat, bei dem PEG mit dem N-Terminus von Gly1 in der A-Kette verknüpft ist oder mit Lys29 in der B-Kette verknüpft ist, oder ein GLP-1- oder GLP-2-Konjugat, bei dem PEG mit Lys34 oder Lys30 verknüpft ist, ist.

Revendications

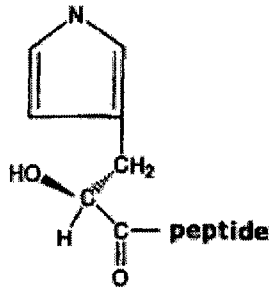
1. Procédé pour préparer un conjugué polypeptidique, physiologiquement actif, dirigé sur un site, comprenant les étapes consistant à :

   i) traiter un polypeptide physiologiquement actif avec un polymère non-peptidyle dans un milieu de réaction qui contient une quantité spécifique d'un alcool et présente un pH spécifique pour permettre la liaison du polymère non-peptidyle à une site cible dudit polypeptide physiologiquement actif, et
   ii) isoler et purifier ledit conjugué polypeptidique, physiologiquement actif, à partir du mélange réactionnel de l'étape i) par chromatographie par échange d'ions en utilisant un alcool,

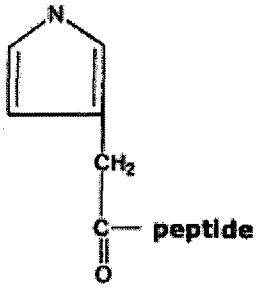
dans lequel le polypeptide physiologiquement actif est une exendine, insuline, oxyntomoduline, GLP-1, GLP-2, la ghréline, la calcitonine ou un de leurs dérivés présentant une structure quelconque choisie dans les formules (I) à (IV):



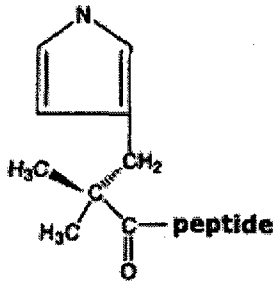
formule I



formule II



formule III



formule IV,

dans lequel ledit alcool est l'éthanol ou l'isopropanol,
dans lequel ledit alcool est présent dans le milieu de réaction en une quantité de 25 % à 90 % volumique, par rapport au volume total du milieu de réaction,
dans lequel le pH utilisé dans l'étape i) est 7,0 à 10,0 si le polypeptide physiologiquement actif est une exendine, oxyntomoduline, GLP-1, GLP-2 ou un de leurs dérivés, et
dans lequel le pH utilisé dans l'étape i) est 4,0 à 10,0 si le polypeptide physiologiquement actif est l'insuline humaine ou son dérivé.

2. Procédé selon la revendication 1, dans lequel le polymère non-peptidyle est le polyéthylène glycol.

3. Procédé selon la revendication 1, dans lequel ledit alcool est présent dans le milieu de réaction en une quantité de 35 % à 60 % volumique, par rapport à la quantité totale du milieu de réaction.

4. Procédé selon la revendication 1, dans lequel ledit conjugué polypeptidique, physiologiquement actif, dirigé sur un site, est un conjugué d'exendine-4 dans lequel PEG est relié avec Lys27, un conjugué de calcitonine dans lequel PEG est relié avec la partie N-terminale, un conjugué d'oxyntomoduline dans lequel PEG est relié avec Lys27 ou Lys30, un conjugué d'insuline hu-maine dans lequel PEG est relié avec la partie N-terminale de Gly1 dans la chaîne A ou est relié avec Lys29 dans la chaîne B, ou un conjugué de GLP-1 ou GLP-2 dans lequel PEG est relié avec Lys34 ou Lys30.

FIG. 1

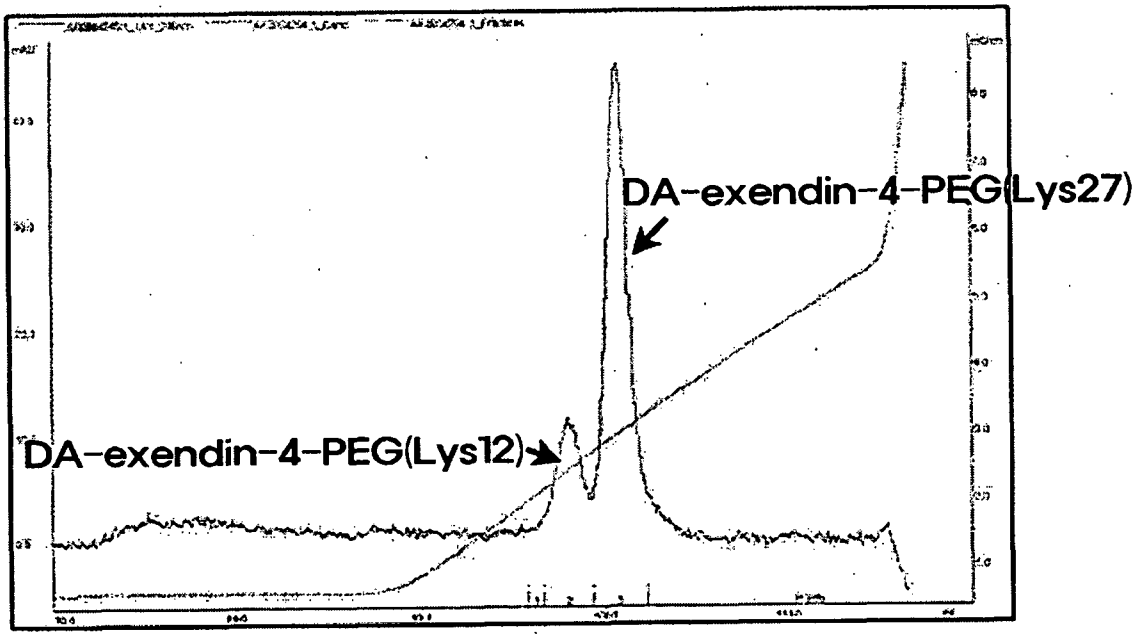

DA-exendin-4-PEG(Lys27)
DA-exendin-4-PEG(Lys12)

FIG. 2

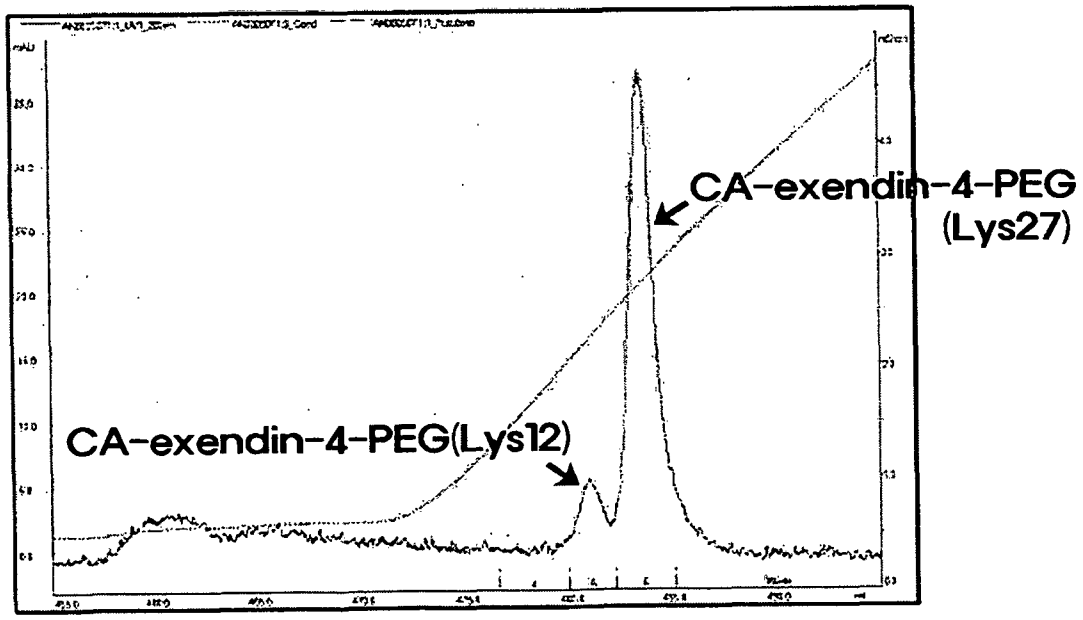

CA-exendin-4-PEG
(Lys27)
CA-exendin-4-PEG(Lys12)

FIG. 3

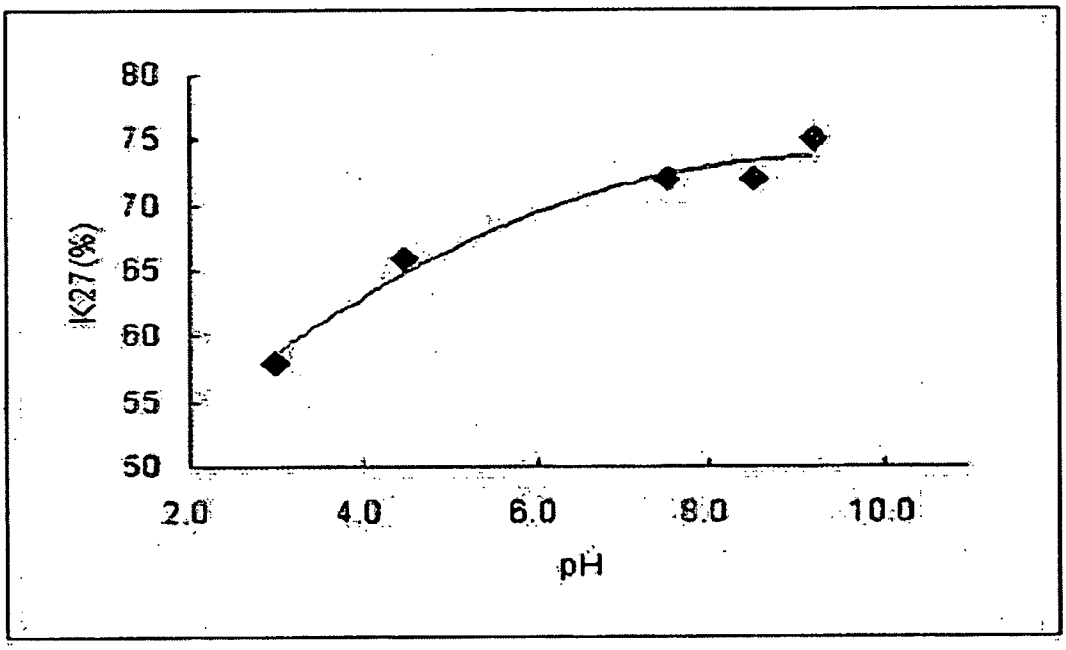

80
75
70
65
60
55
50
K27(%)
2.0
4.0
6.0
8.0
10.0
pH

FIG. 4

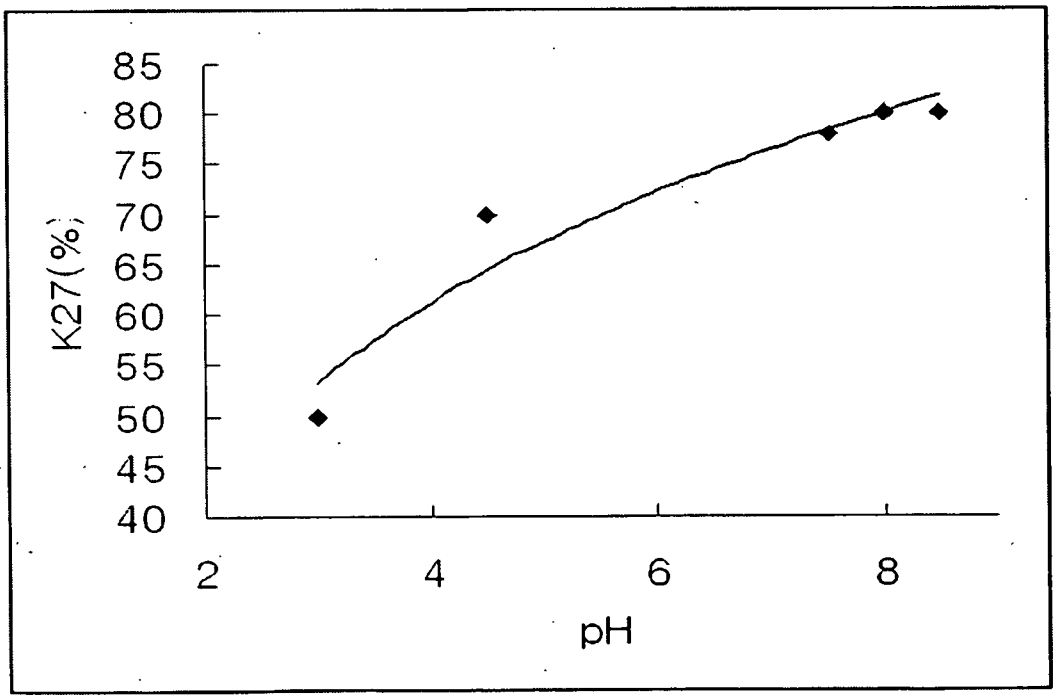

85
80
75
70
65
60
55
50
45
40
K27(%)
2
4
6
8
pH

FIG. 5

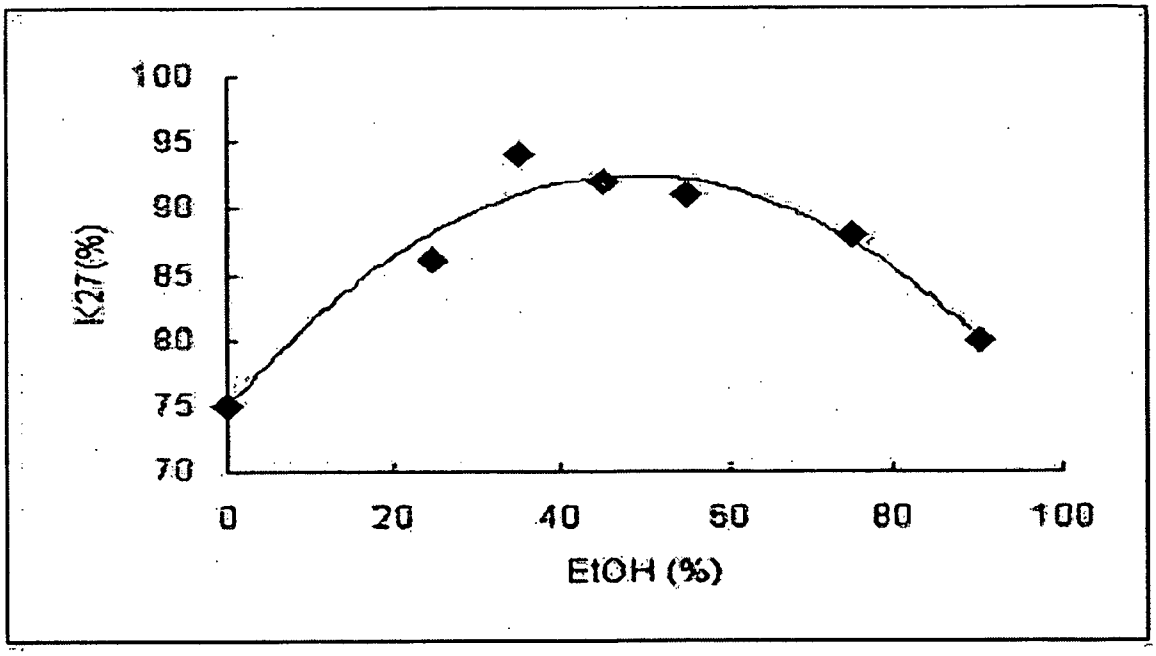

100
95
90
85
80
75
70
K27(%)
0
20
40
60
80
100
EtOH (%)

FIG. 6

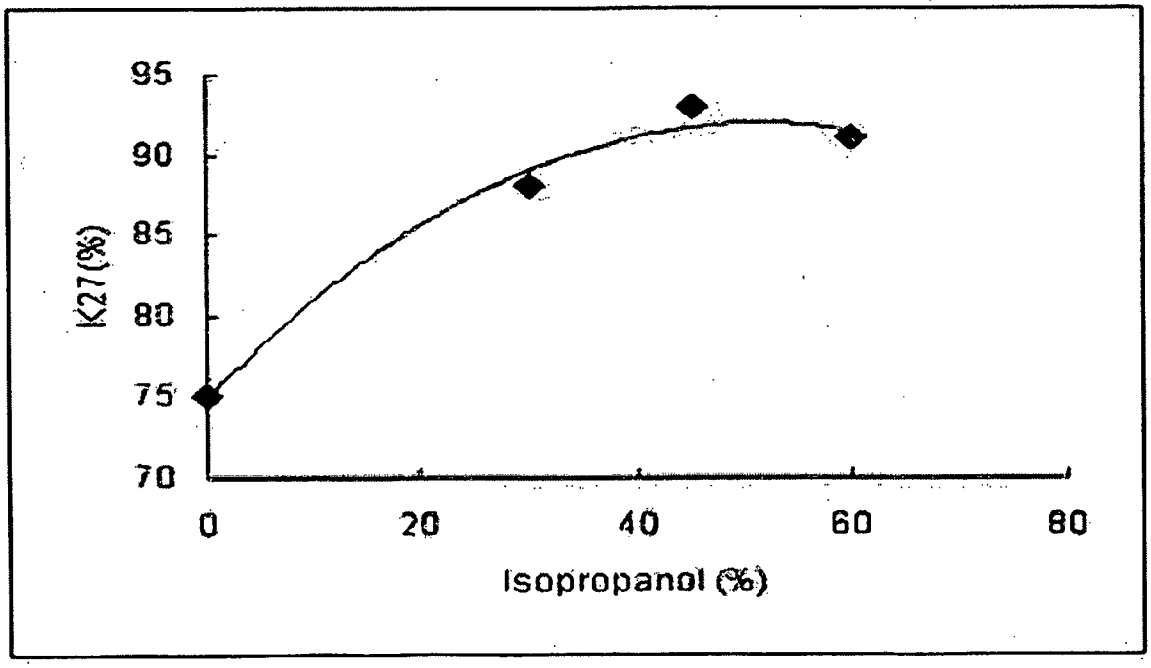

95
90
85
80
75
70
K27(%)
0
20
40
60
80
Isopropanol (%)

FIG. 7

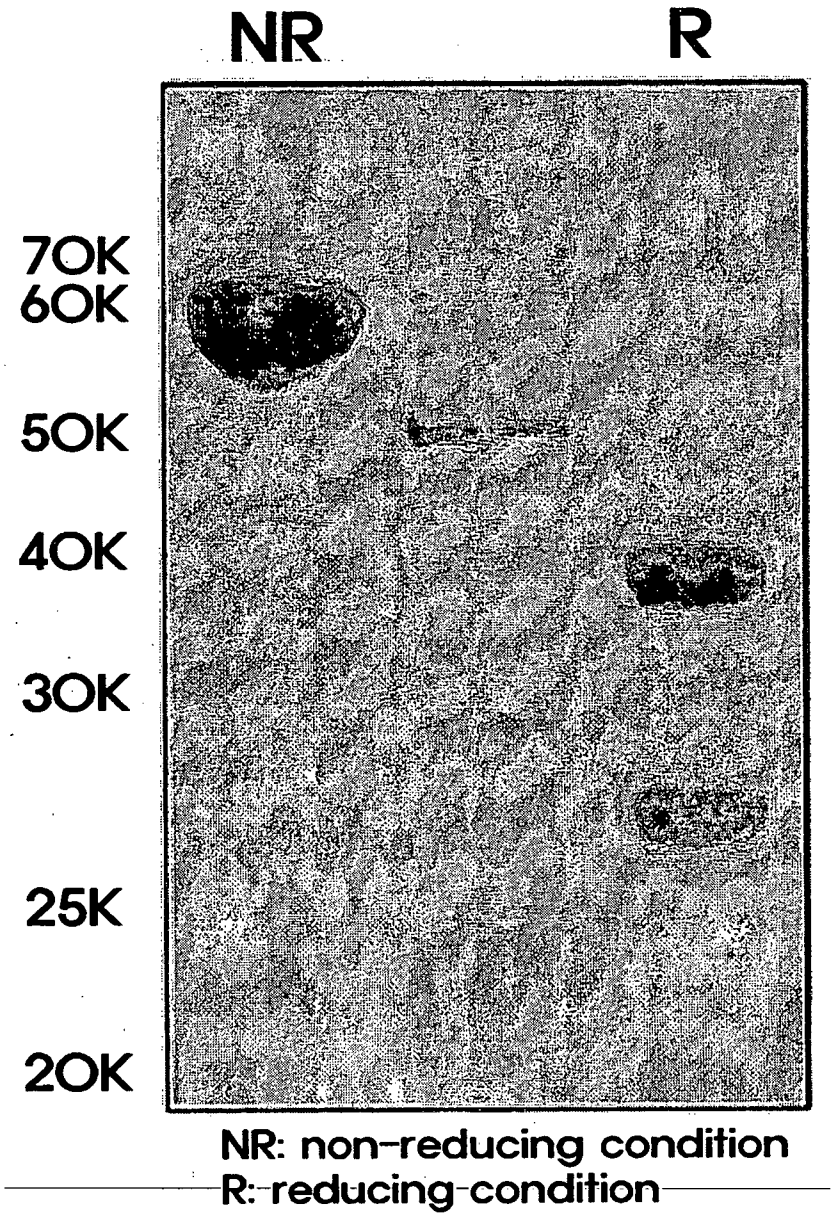
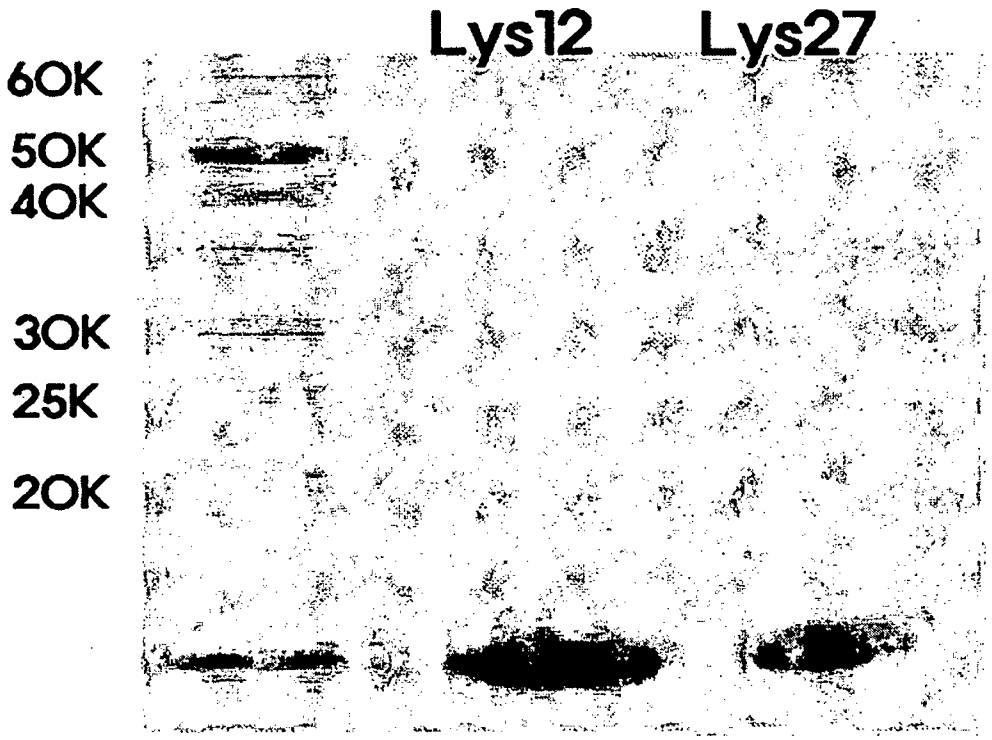

NR
R
70K
60K
50K
40K
30K
25K
20K
NR: non-reducing condition
R: reducing condition

FIG. 8



Lys12: CA-exendin-4-PEG (Lys12)
Lys27: CA-exendin-4-PEG (Lys27)

FIG. 9

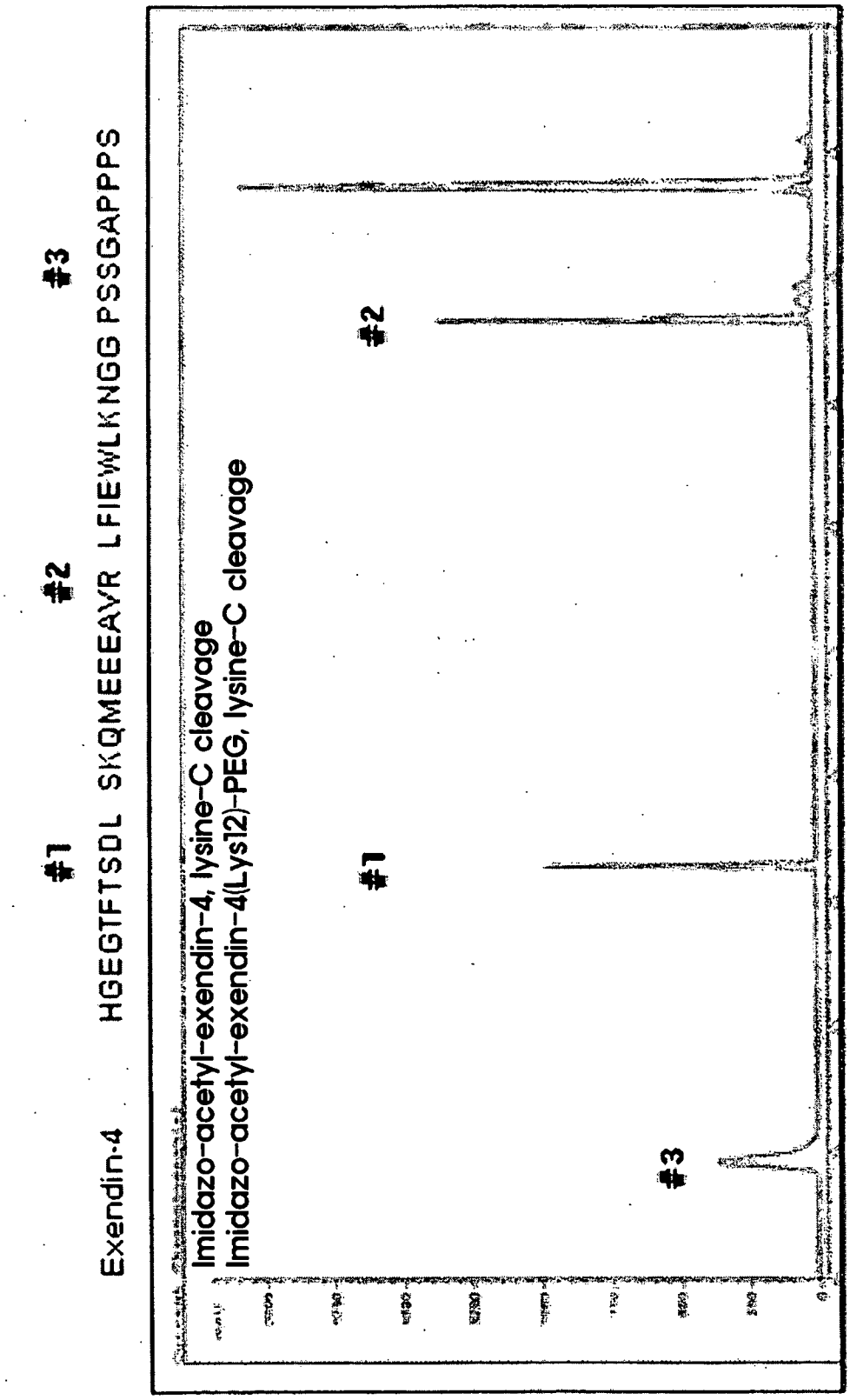

#1
#2
#3
Exendin-4 HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS
Imidazo-acetyl-exendin-4, lysine-C cleavage
Imidazo-acetyl-exendin-4(Lys12)-PEG, lysine-C cleavage
#1
#2
#3

FIG. 10

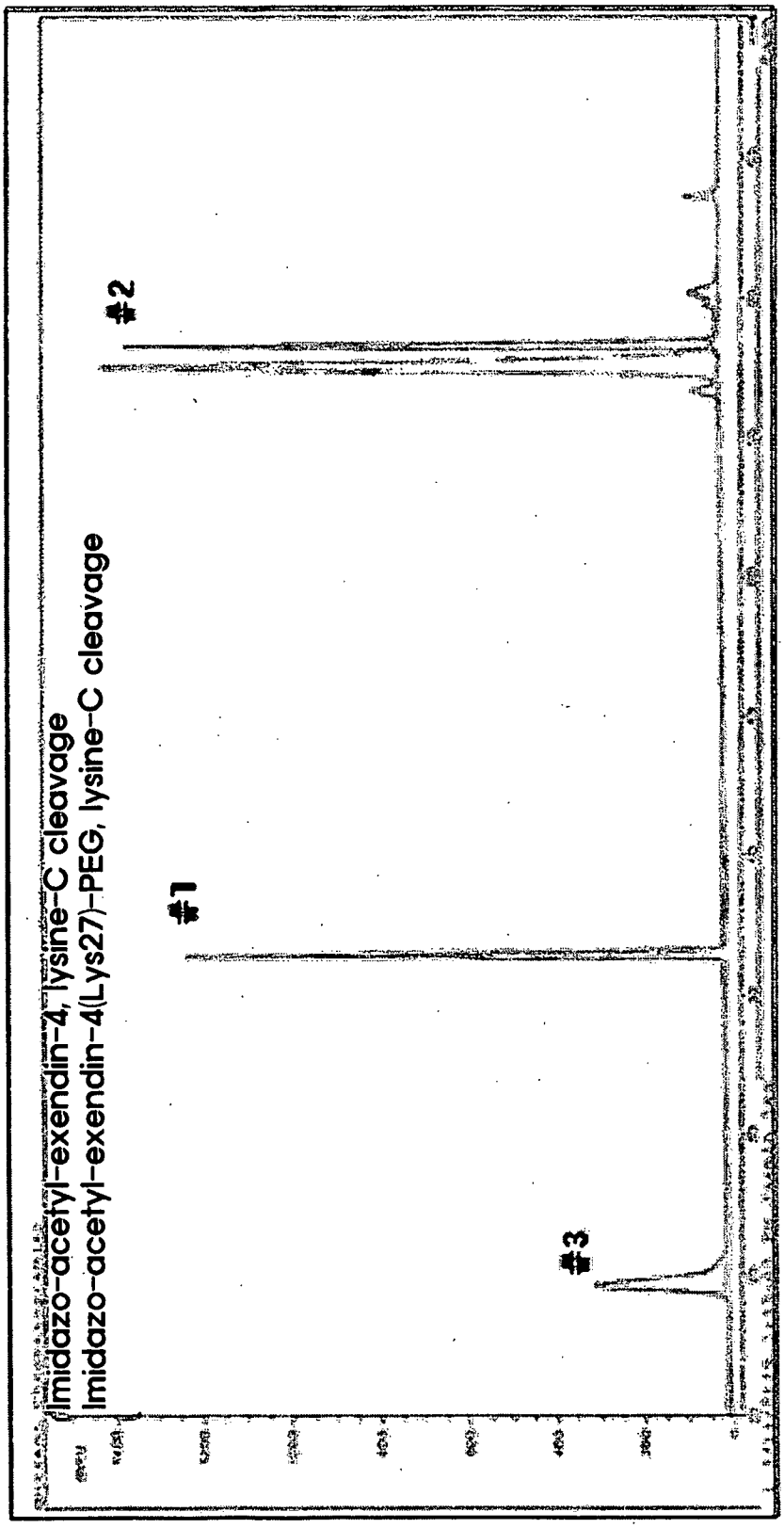

#1
#2
#3
Exendin-4 HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS
Imidazo-acetyl-exendin-4, lysine-C cleavage
Imidazo-acetyl-exendin-4(Lys27)-PEG, lysine-C cleavage
#1
#2
#3

FIG. 11

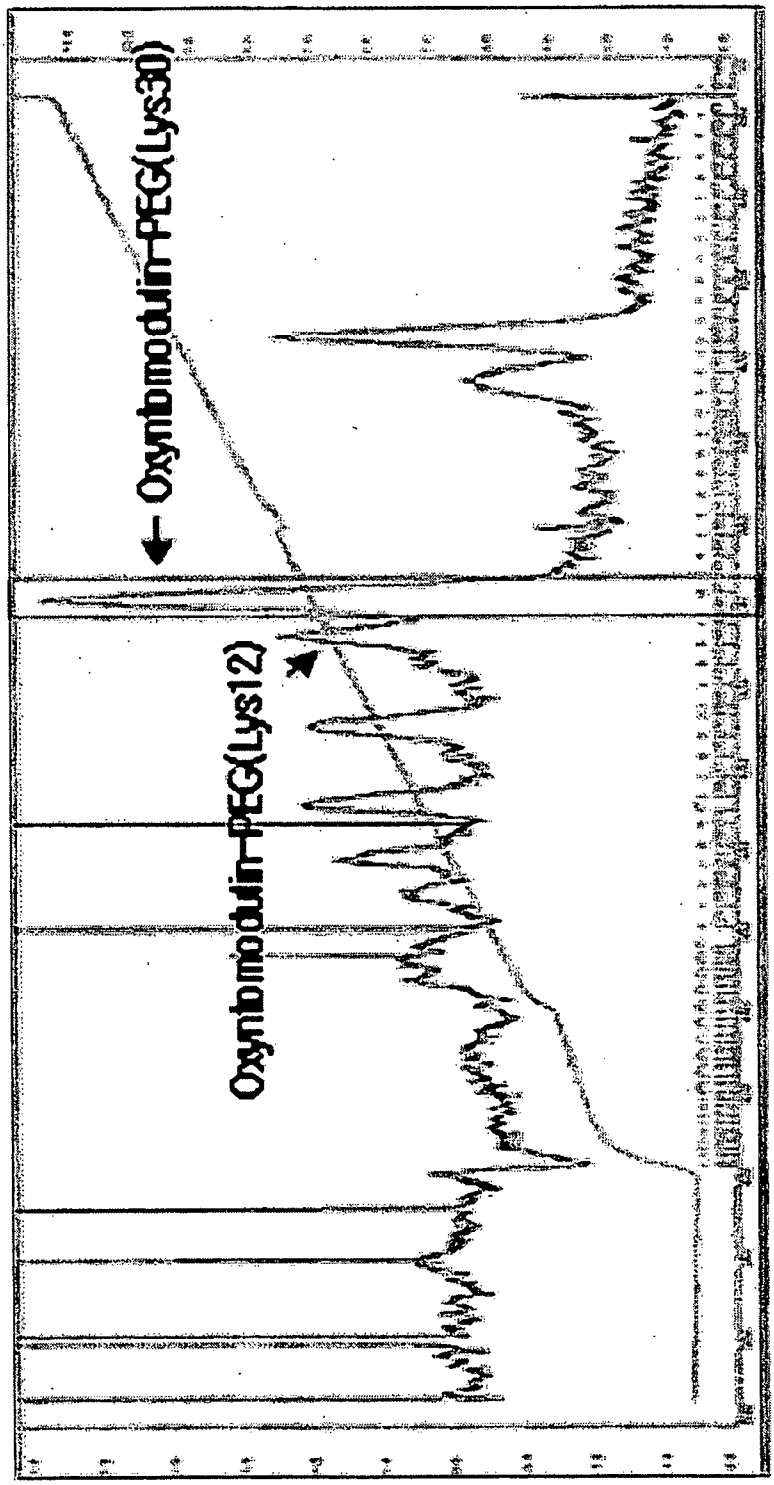


FIG. 12

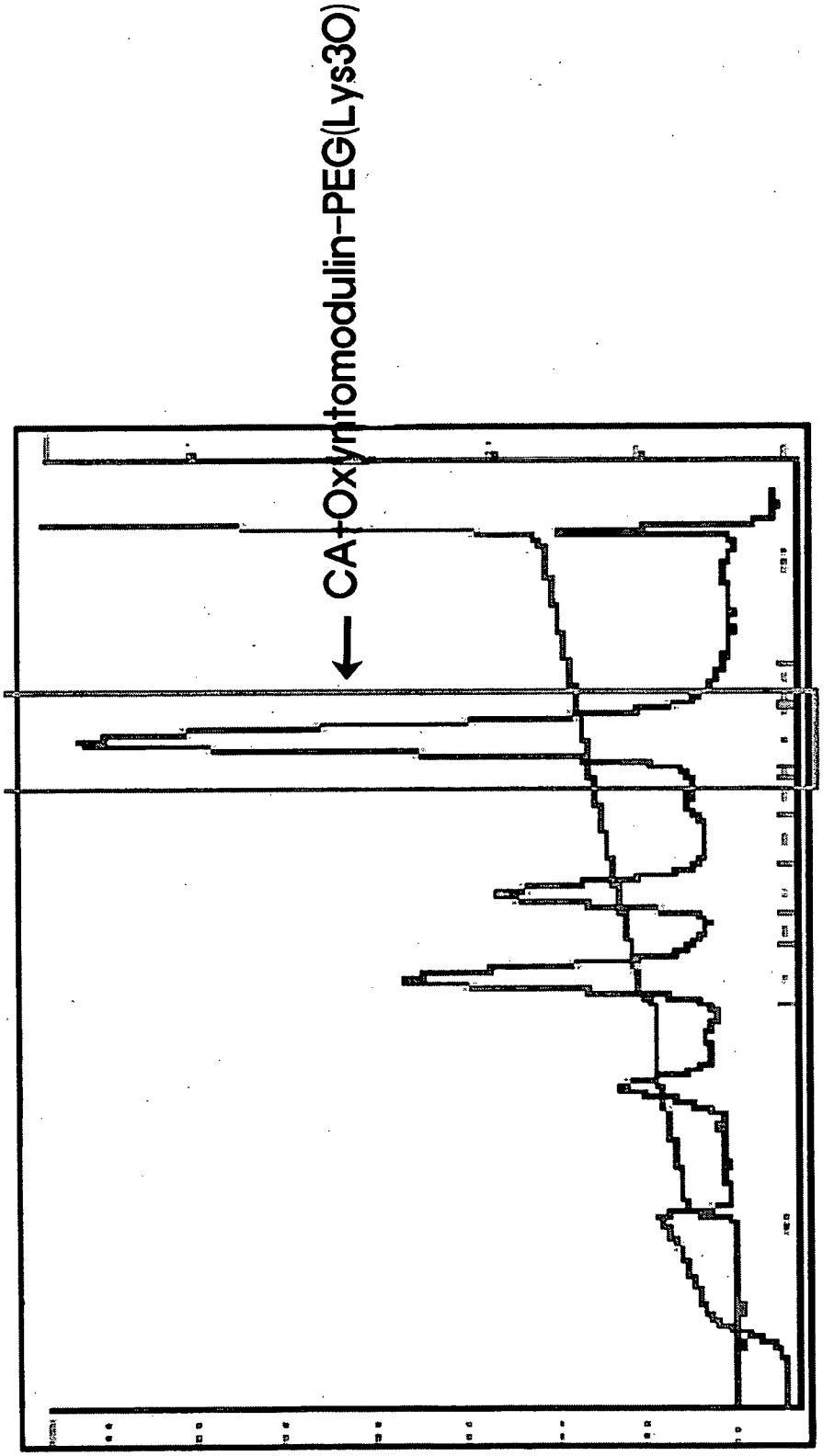
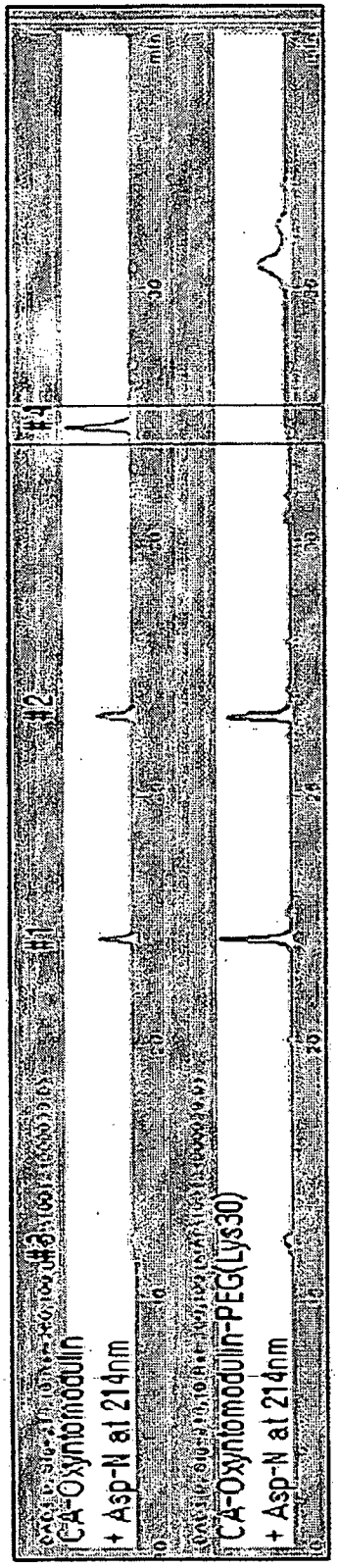


FIG. 13



#1 : His(1)-Ser(8)
#2 : Asp(9)-Leu(14)
#3 : Asp(16)-Gln(20)
#4 : Asp(22)-(37)

FIG. 14

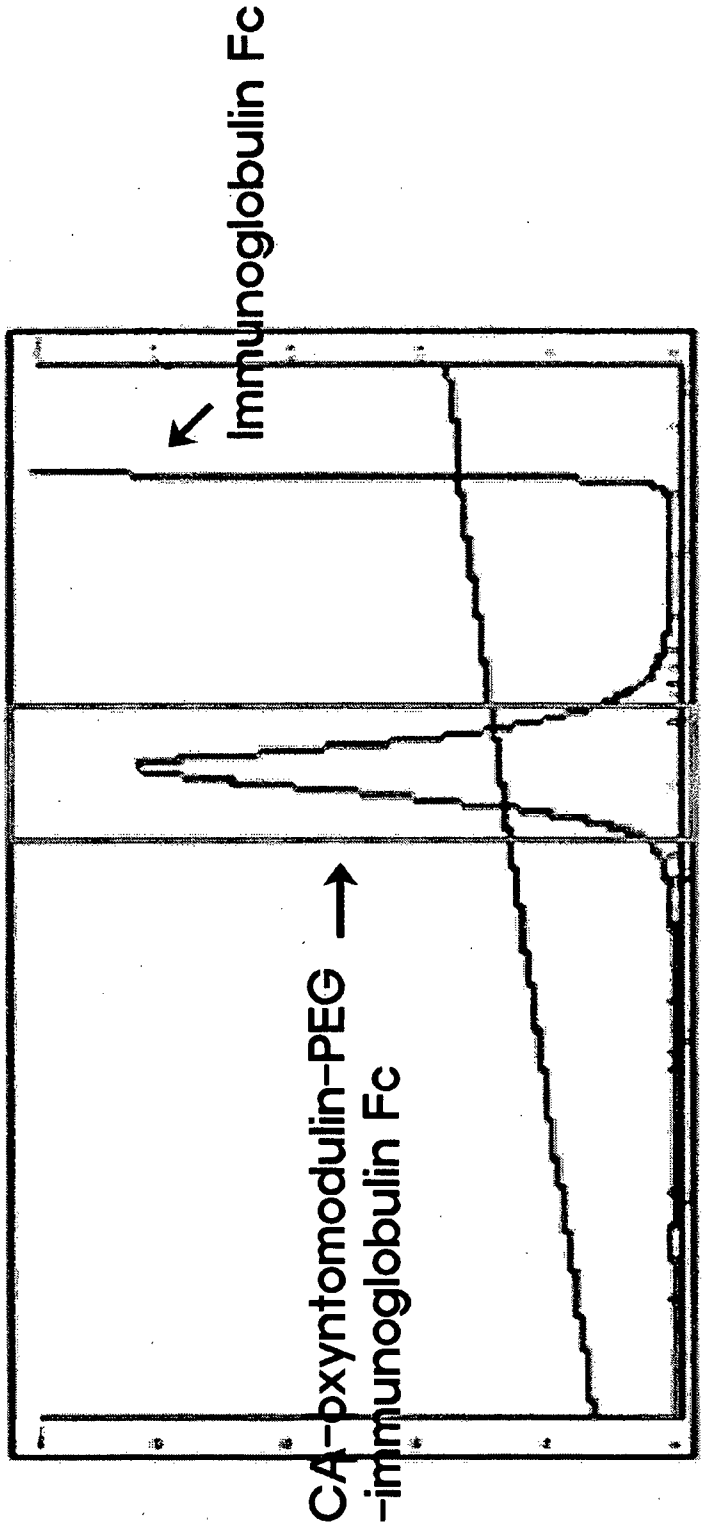
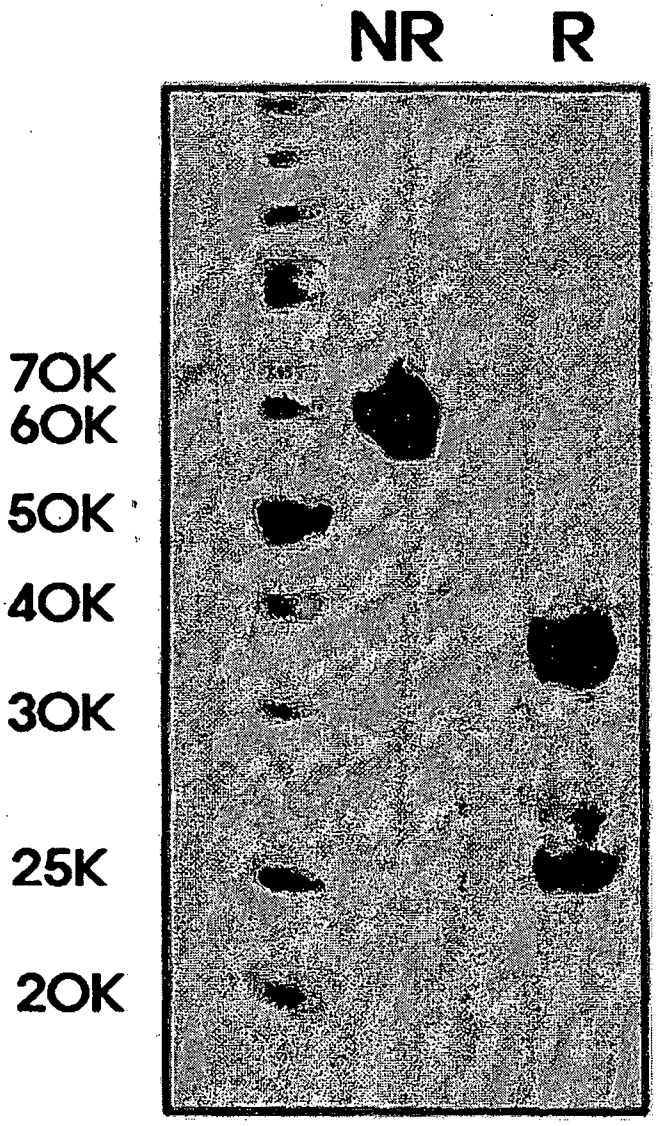

Immunoglobulin Fc
CA-oxyntomodulin-PEG
-immunoglobulin Fc

FIG. 15



NR: non-reducing condition
R: reducing condition

FIG. 16

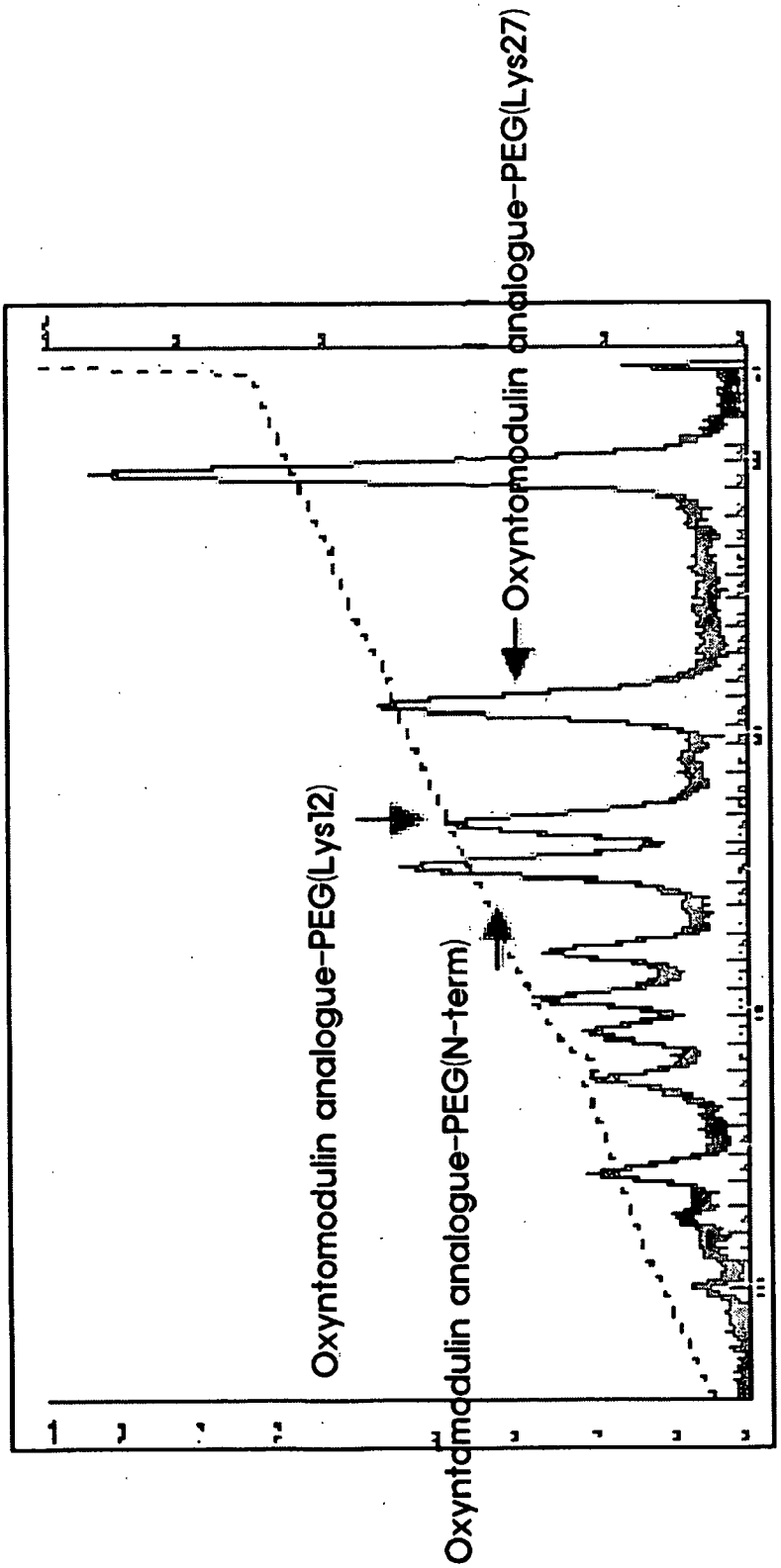

Oxyntomodulin analogue-PEG(Lys27)
Oxyntomodulin analogue-PEG(Lys12)
Oxyntomodulin analogue-PEG(N-term)

FIG. 17

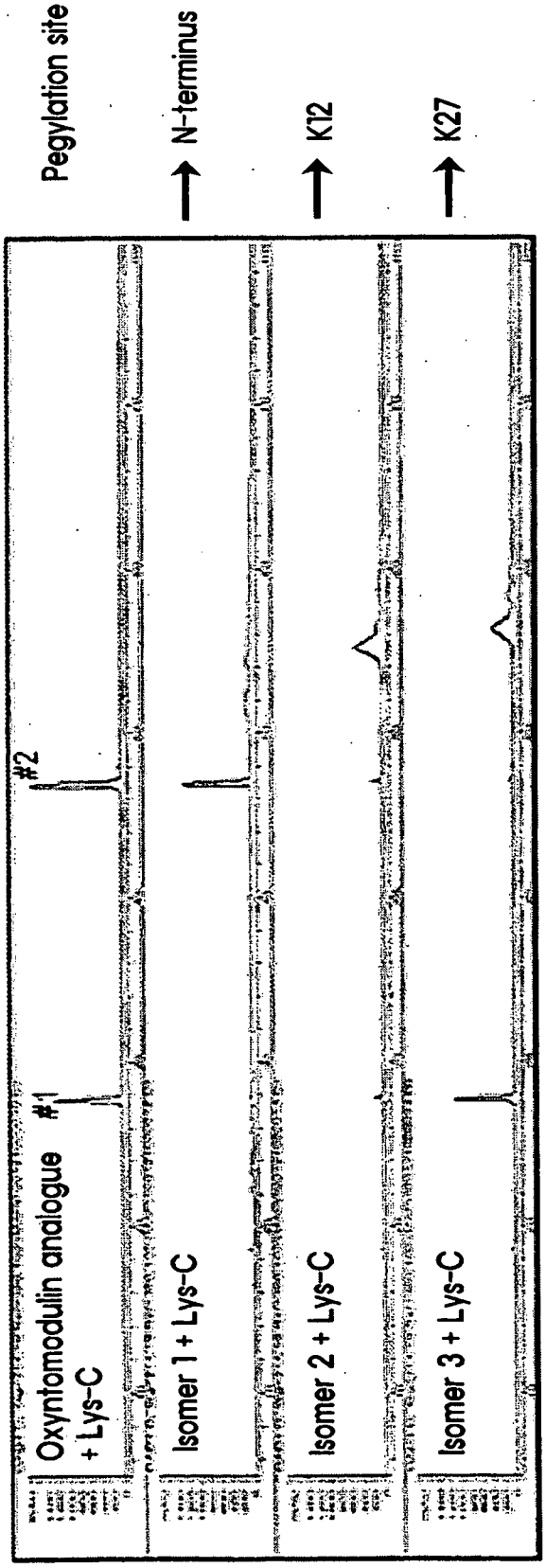

Pegylation site
N-terminus
K12
K27
Oxyntomodulin analogue + Lys-C
#1
#2
Isomer 1 + Lys-C
Isomer 2 + Lys-C
Isomer 3 + Lys-C

FIG. 18

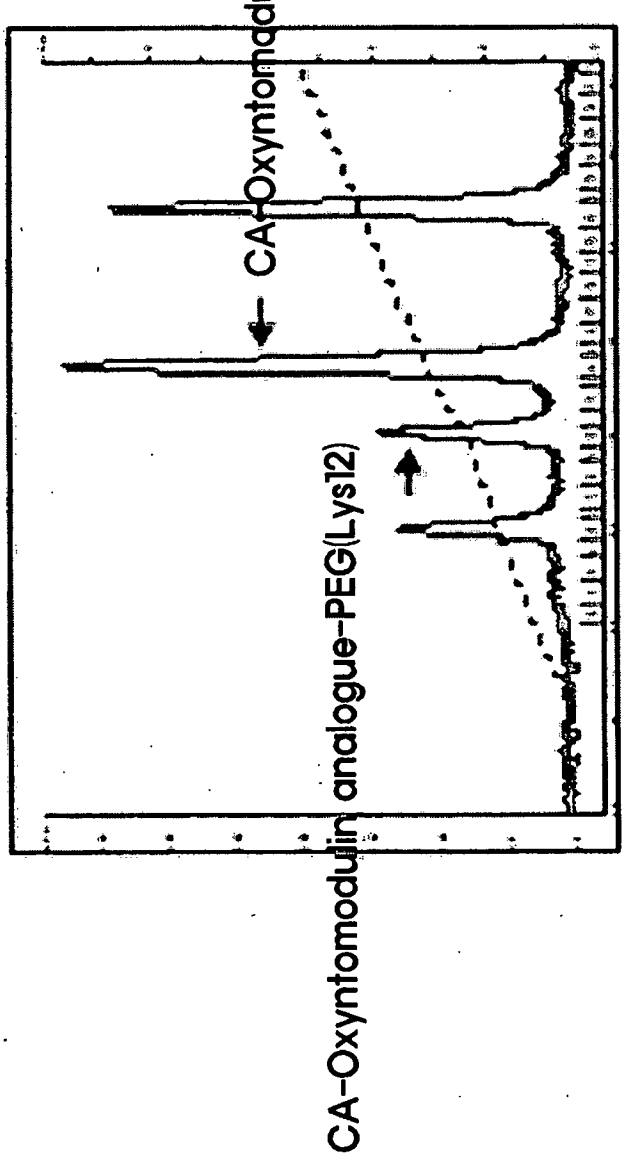

CA-Oxyntomodulin analogue-PEG(Lys27)
CA-Oxyntomodulin analogue-PEG(Lys12)

FIG. 19

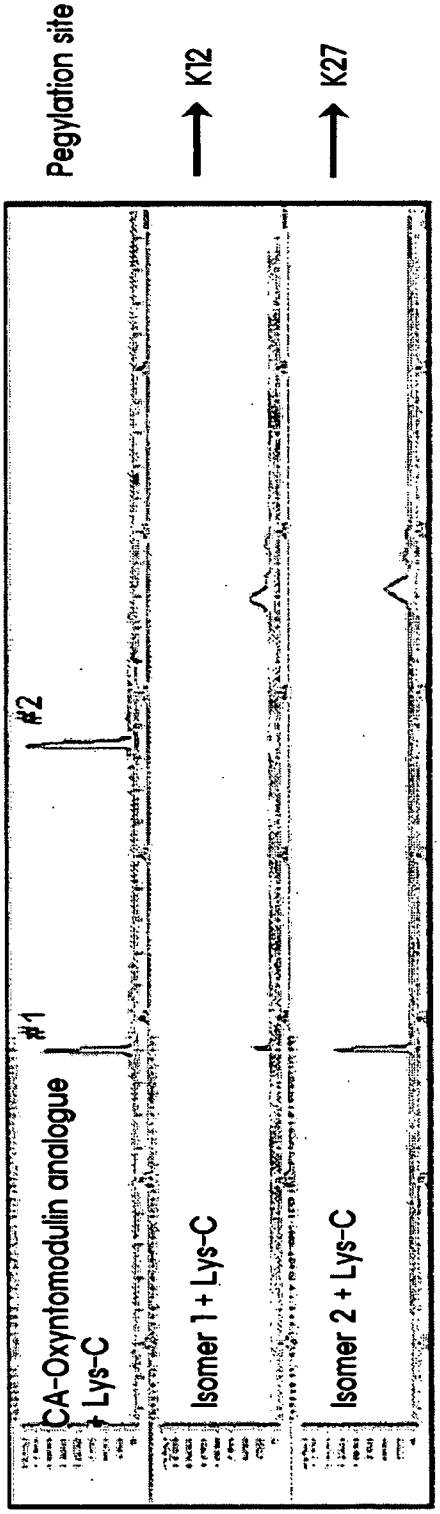

Pegylation site
K12
K27
#1
#2
CA-Oxyntomodulin analogue + Lys-C
Isomer 1 + Lys-C
Isomer 2 + Lys-C

FIG. 20

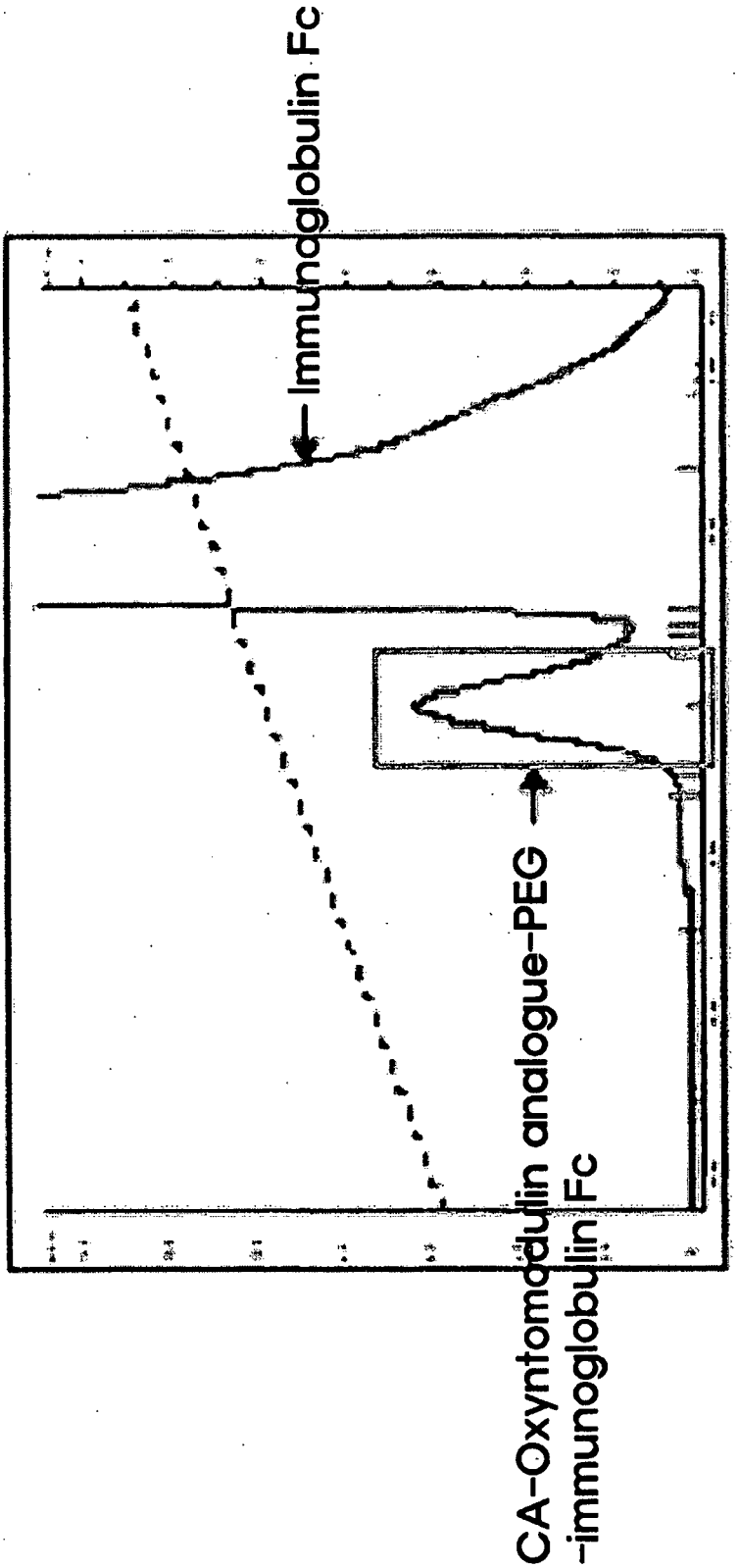
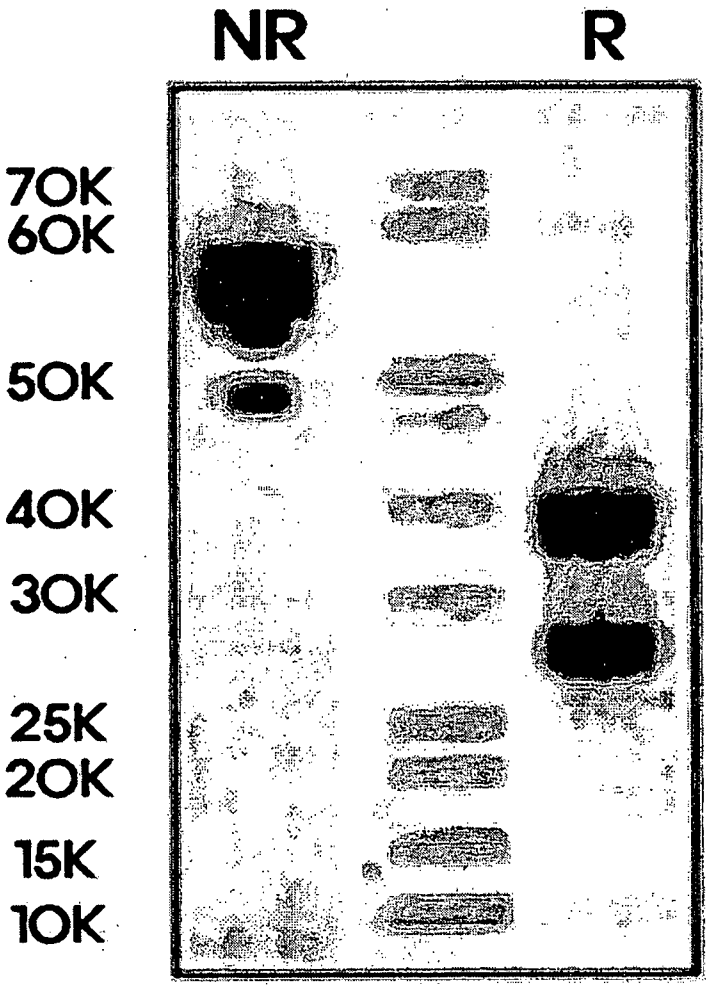

Immunoglobulin Fc
CA-Oxyntomodulin analogue-PEG
-immunoglobulin Fc

FIG. 21



NR: non-reducing condition
R: reducing condition

FIG. 22

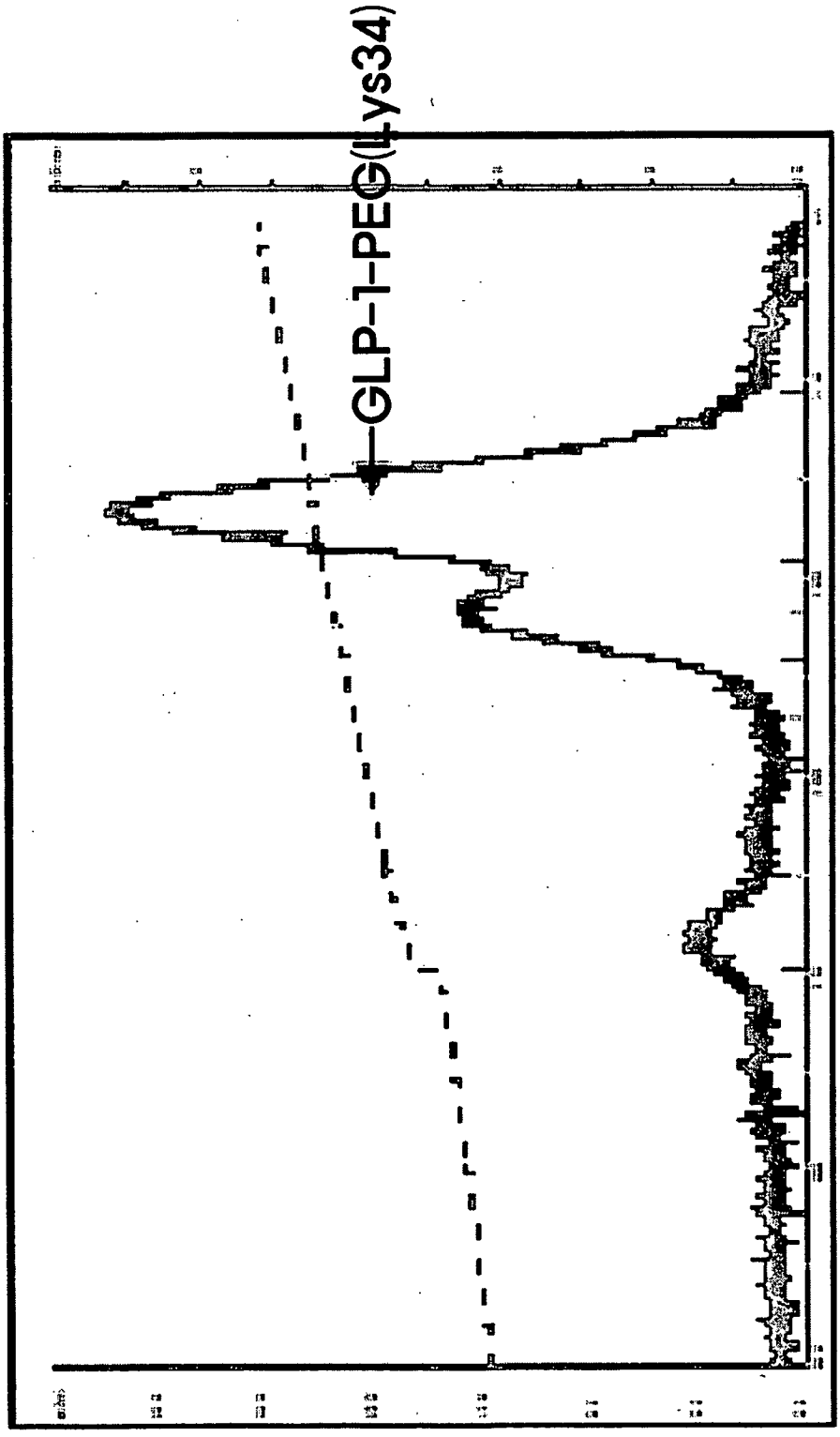


FIG. 23

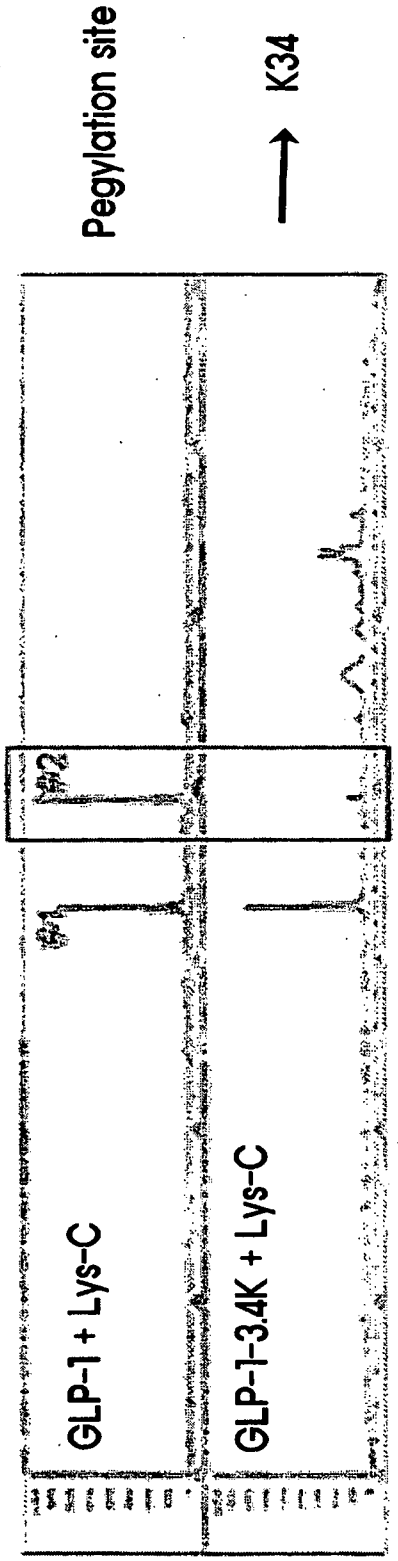


FIG. 24

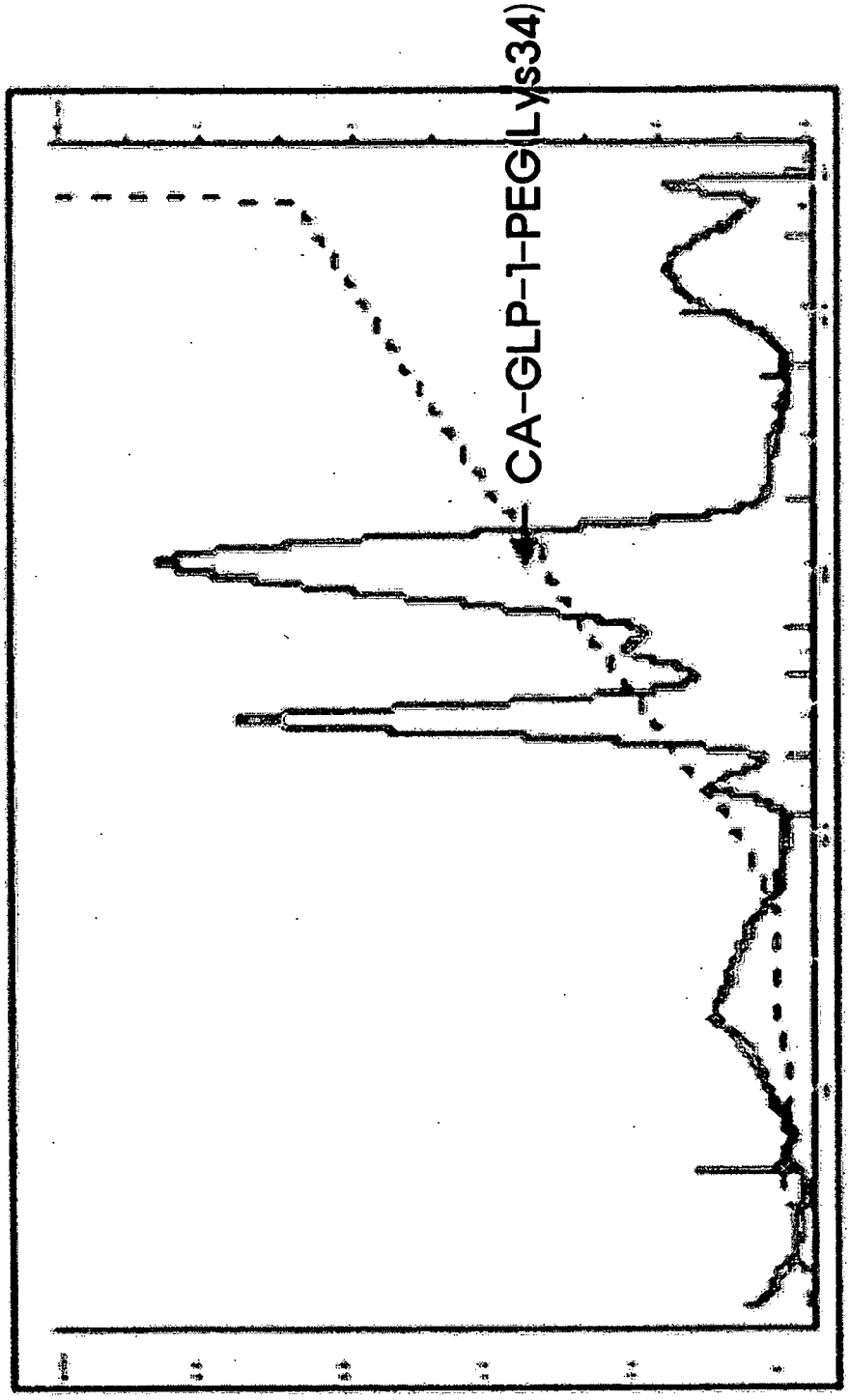


FIG. 25

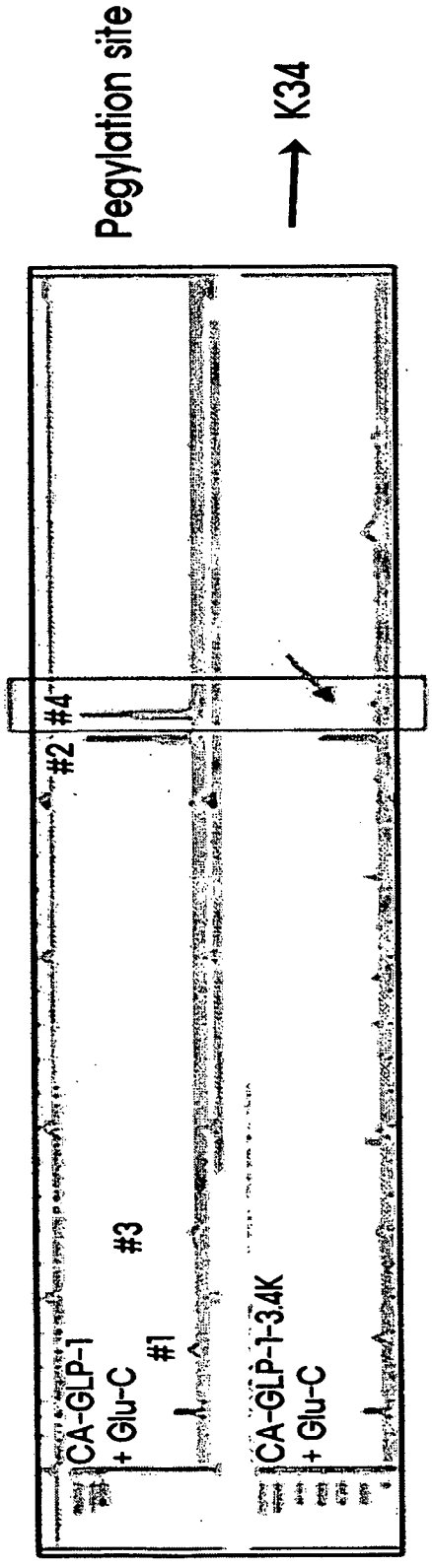


FIG. 26

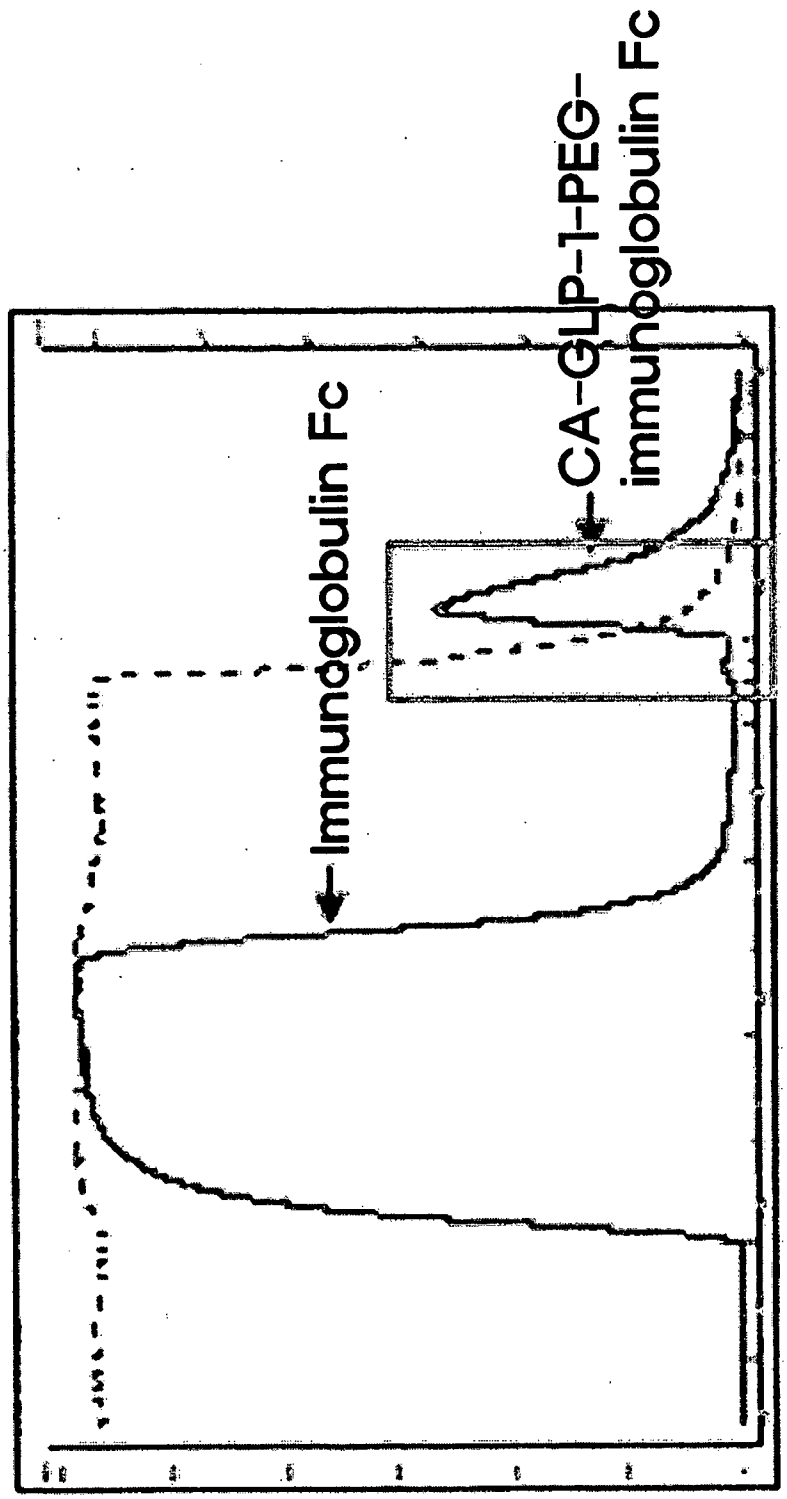
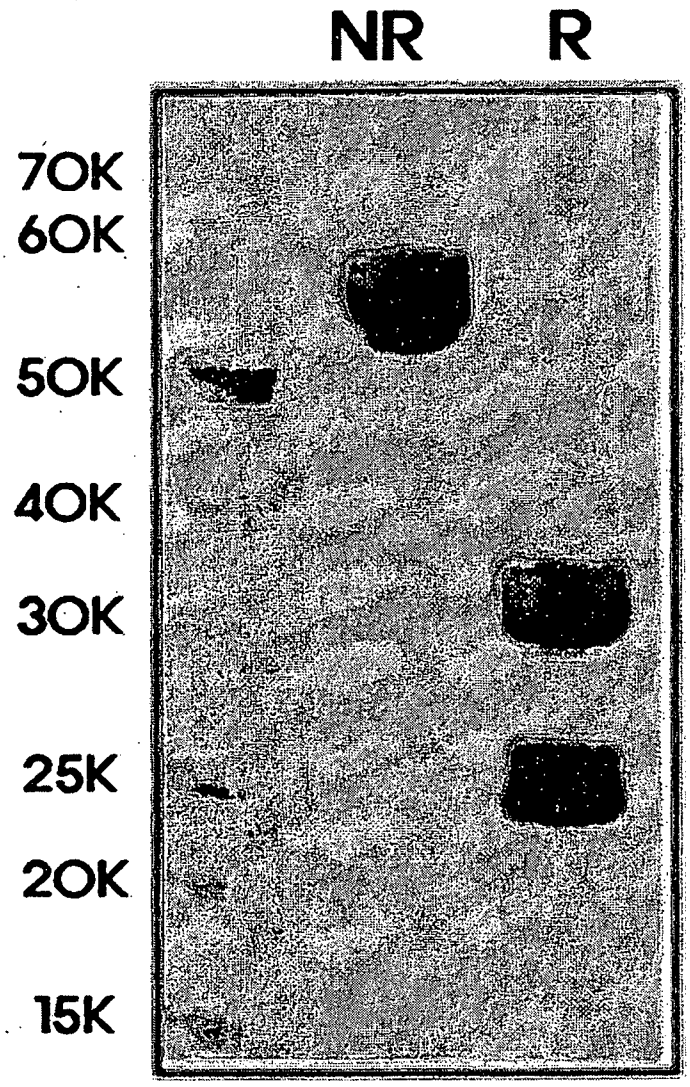


FIG. 27



NR: non-reducing condition
R: reducing condition

FIG. 28

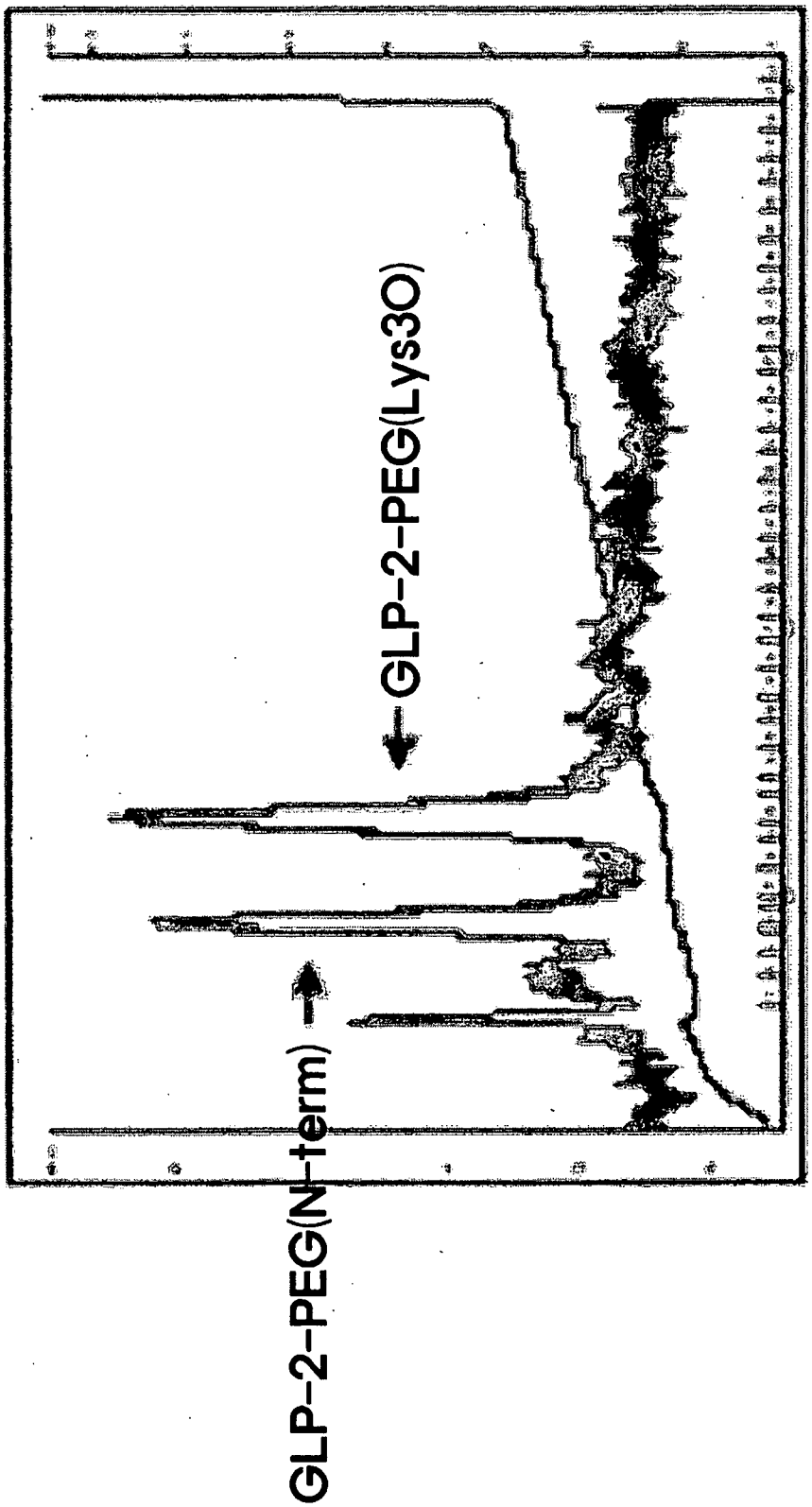


FIG. 29

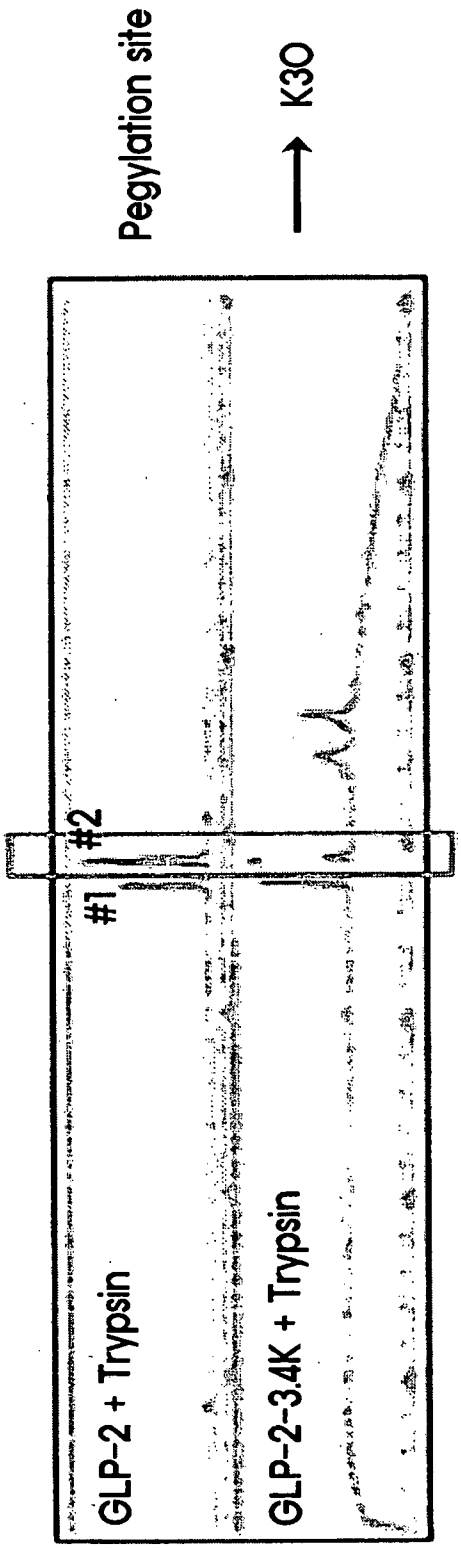


FIG. 30

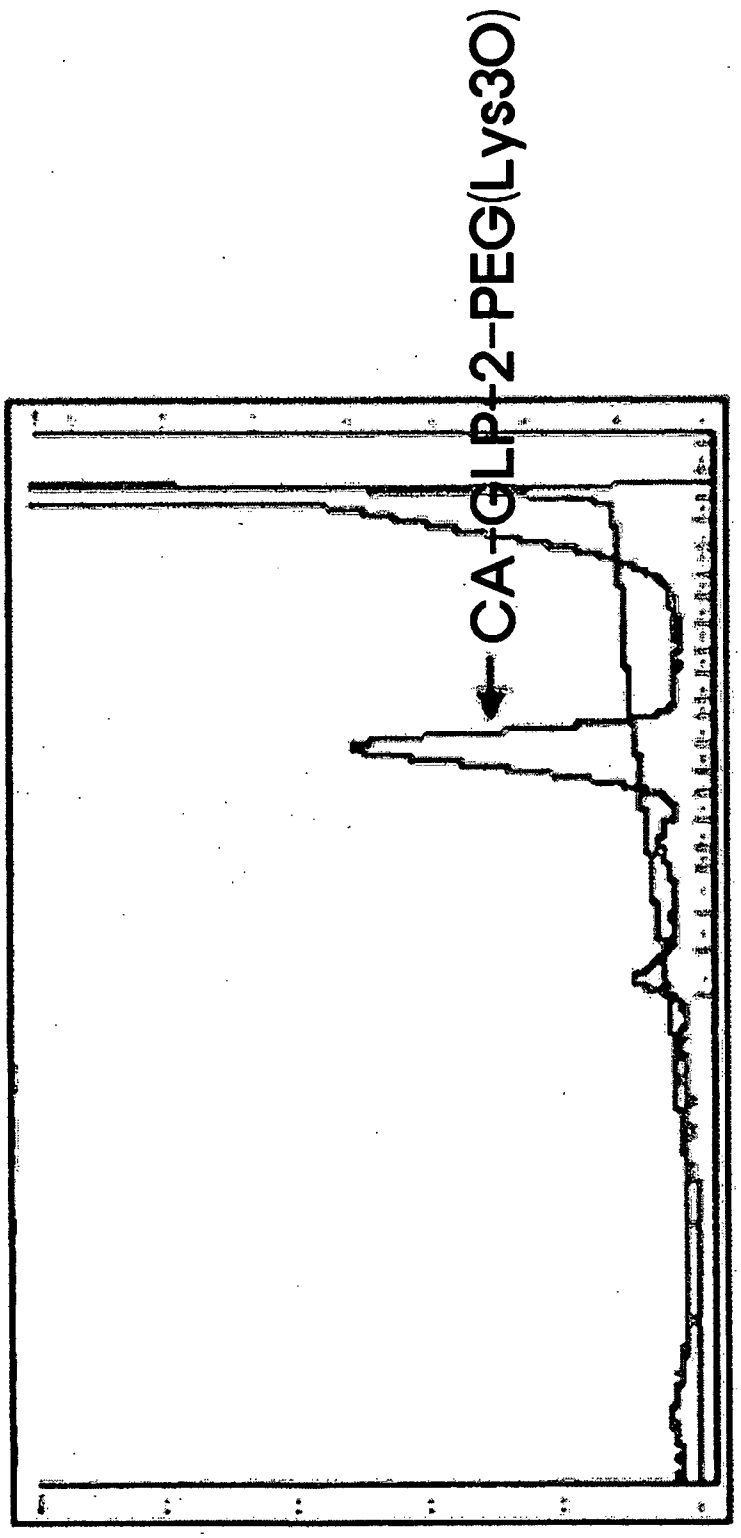


FIG. 31

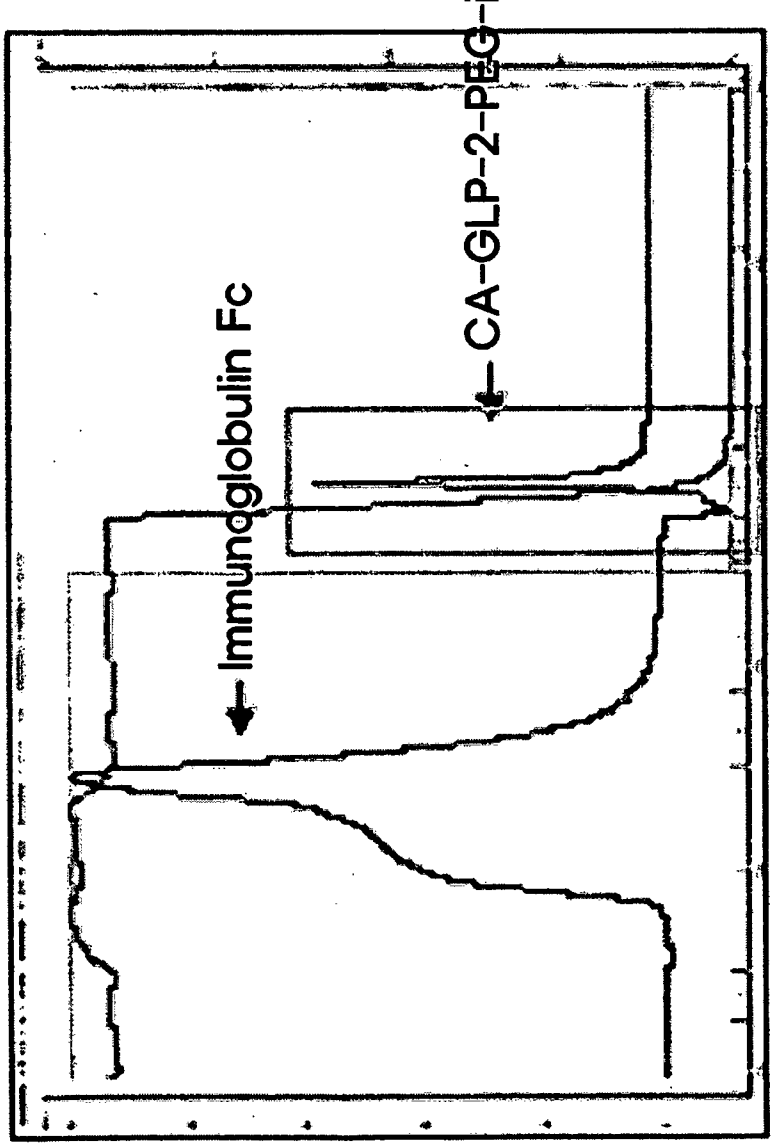
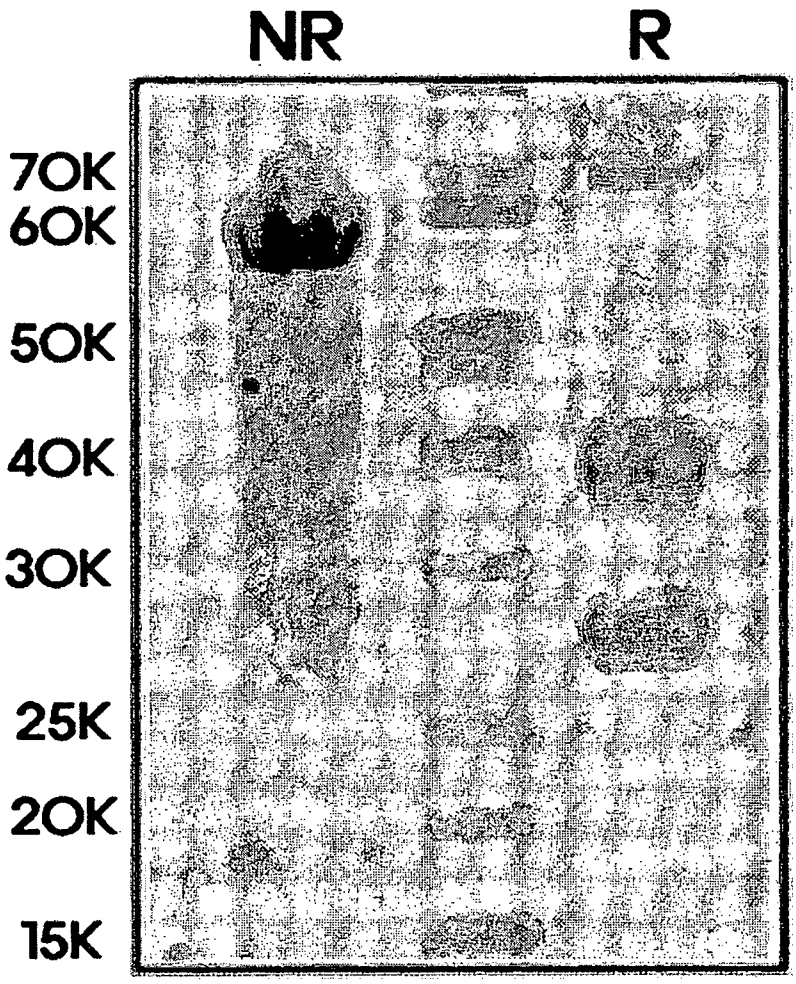


FIG. 32



NR: non-reducing condition
R: reducing condition

FIG. 33

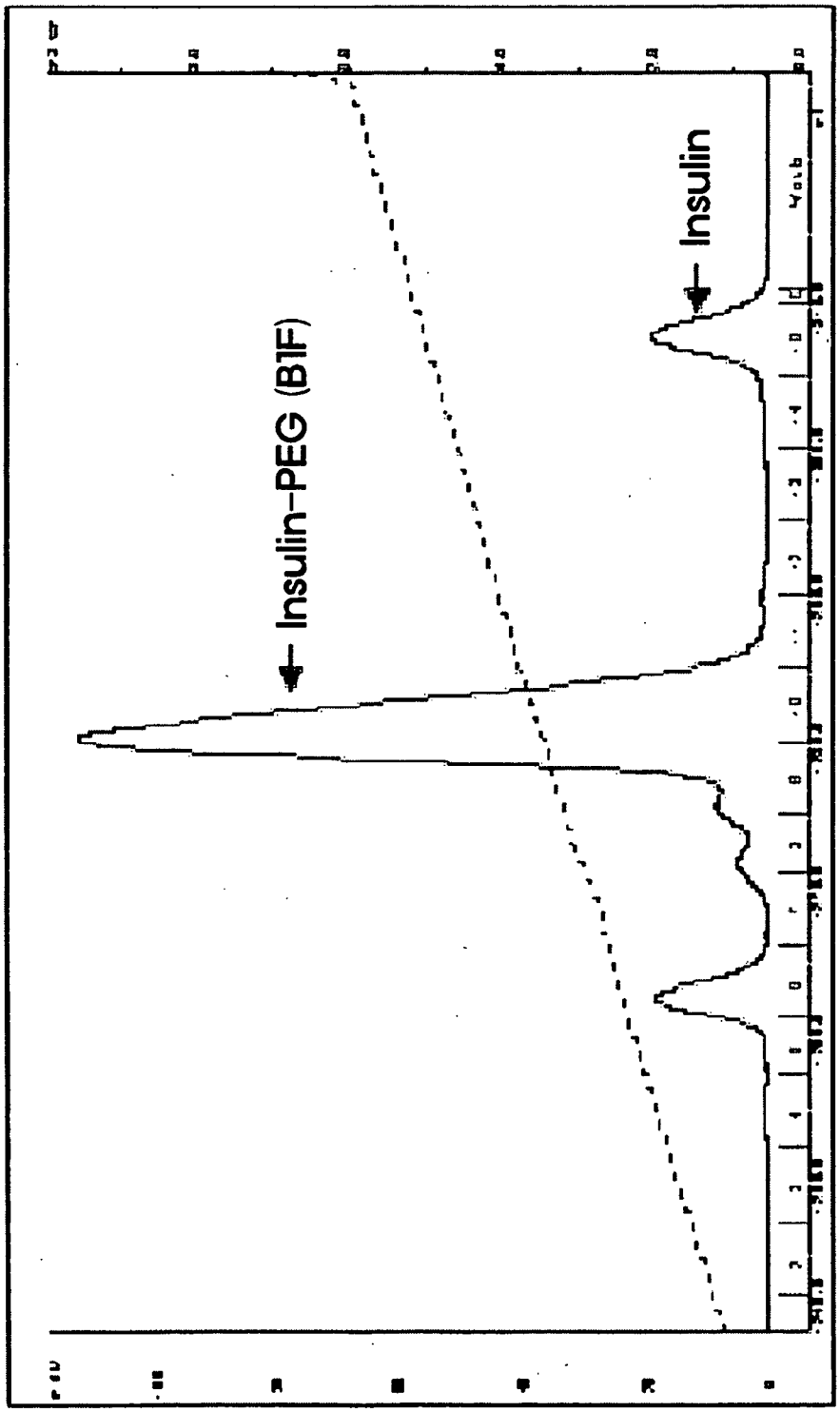


FIG. 34

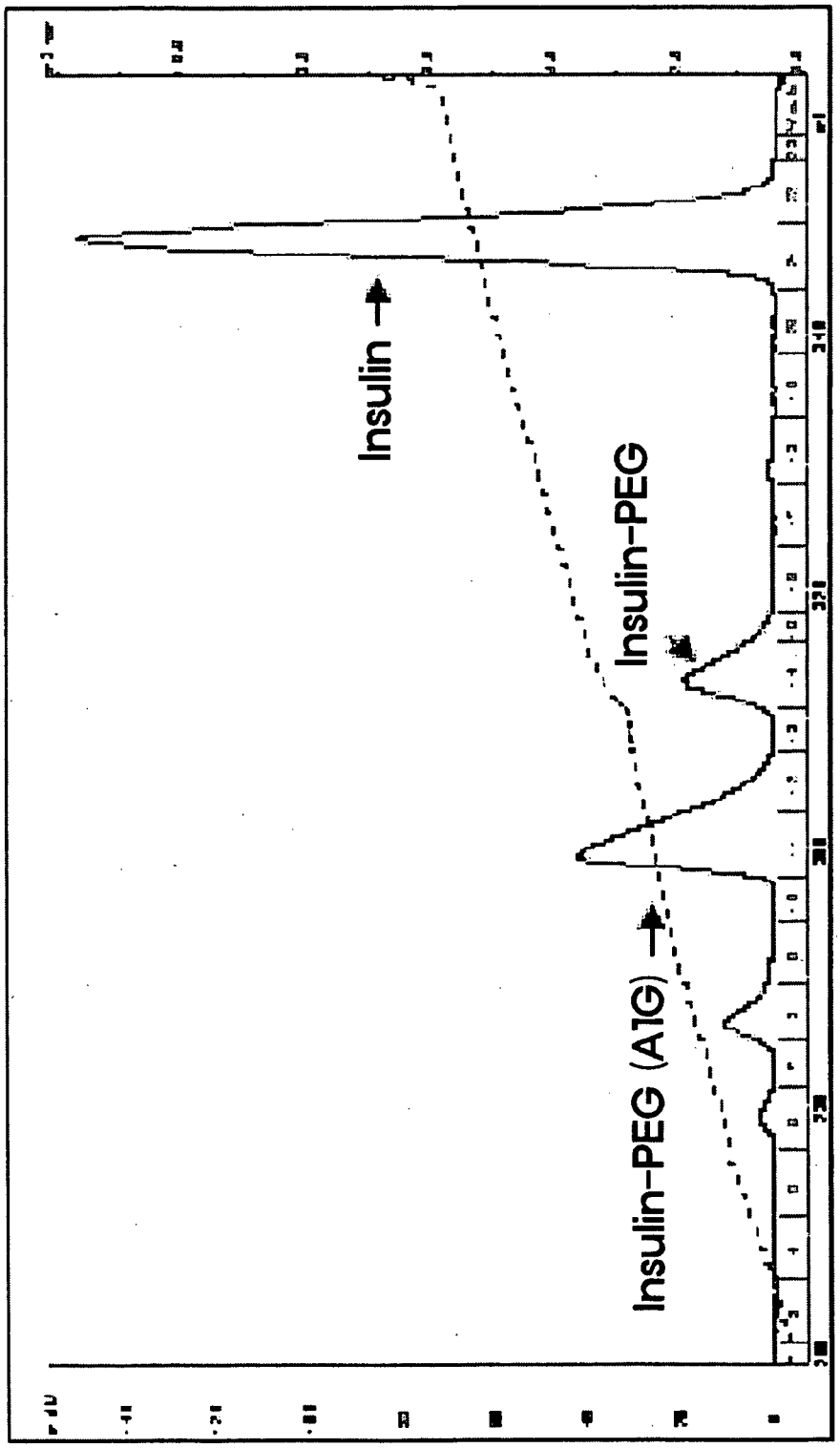


FIG. 35

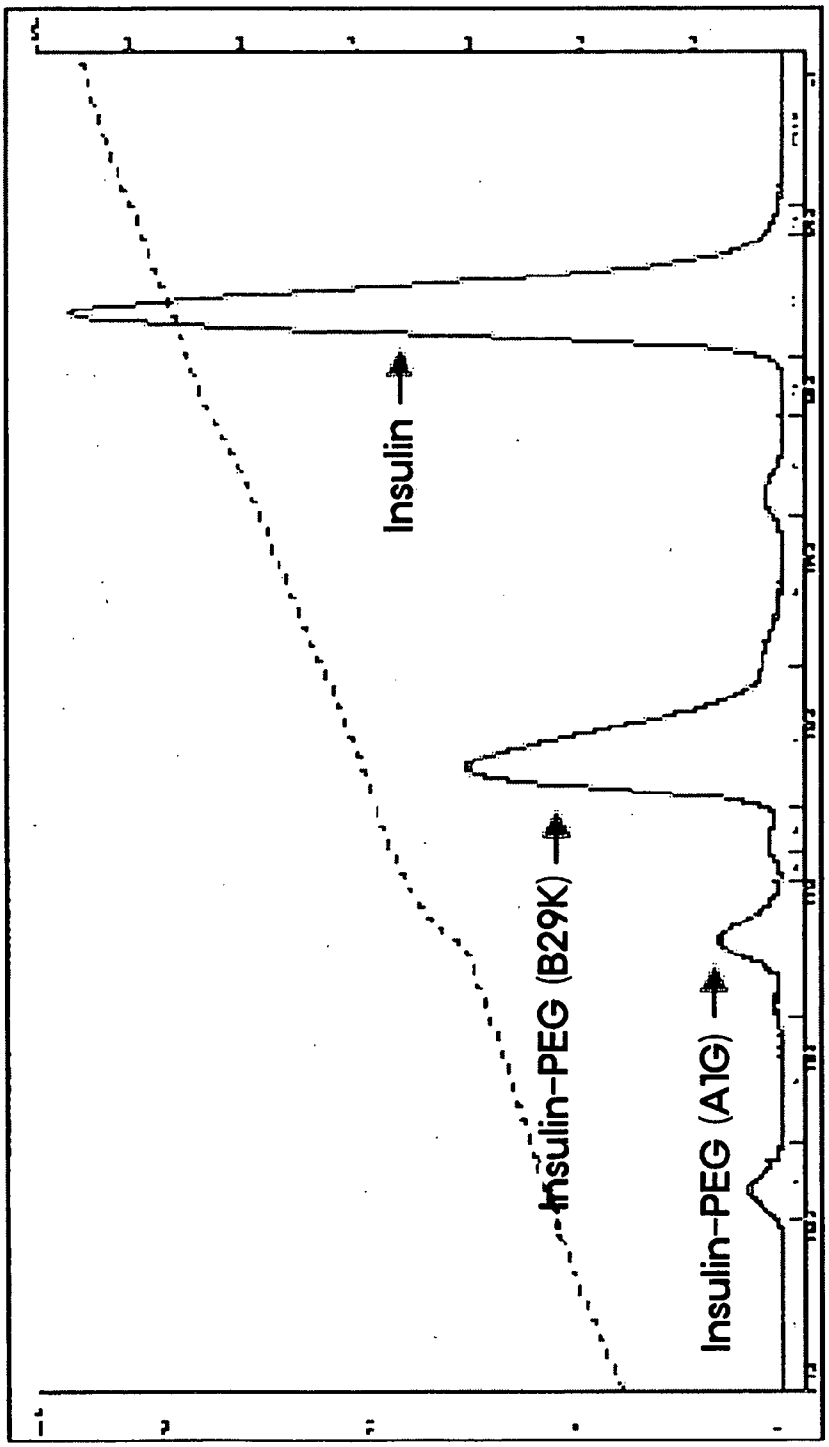
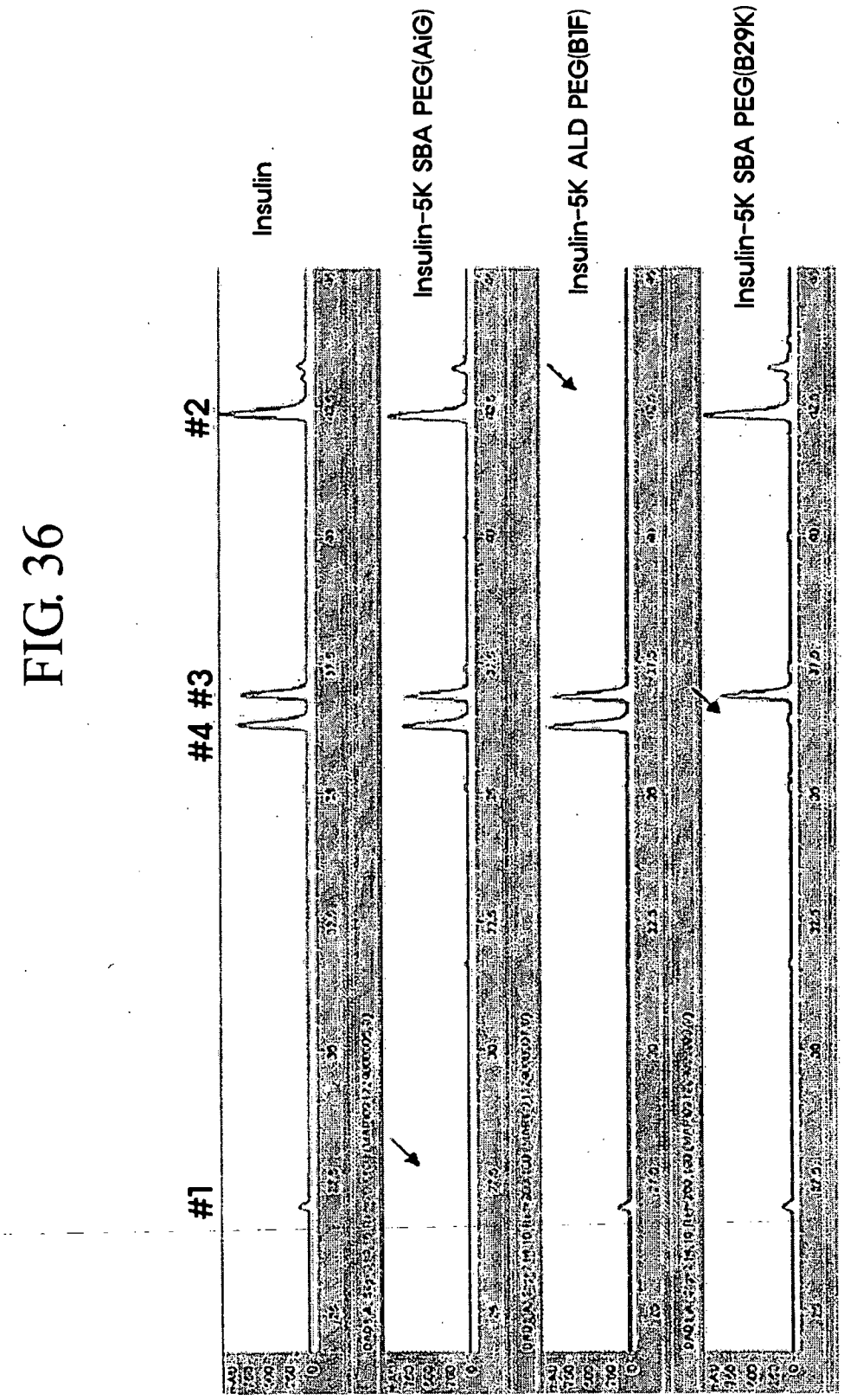


FIG. 36


Insulin
Insulin-5K SBA PEG(AiG)
Insulin-5K ALD PEG(B1F)
Insulin-5K SBA PEG(B29K)
#1
#2
#3
#4

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

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- US 6924264 B **[0003]**
- KR 200869234 **[0014]**
- KR 20090008151 **[0028]**
- KR 775343 **[0038]**
- KR 725314 **[0038]**
- KR 725315 **[0038]**
- KR 824505 **[0038]**

Szabadalmi igénypontok

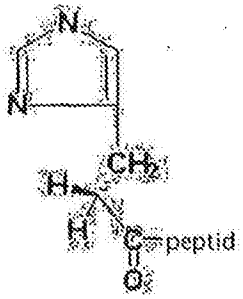
1. Eljárás helyspecifikus, fiziológiásan aktív polipeptid-konjugátum előállítására, amely magában foglalja a következő lépéseket:

i. fiziológiásan aktív polipeptid kezelése nem peptidil polimerrel olyan reakcióközegben, amely tartalmaz adott mennyiségű alkoholt és adott pH-ja van, ezáltal lehetővé teszi a nem peptidil polimer kötődését a fiziológiásan aktív polipeptid célhelyéhez

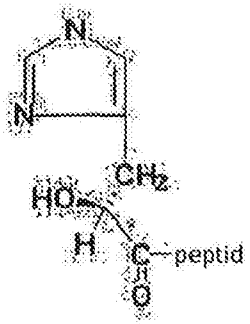
és

ii) a fiziológiásan aktív polipeptid-konjugátum izolálása és tisztítása az i) lépés reakciókeverékéből ioncserélő kromatográfiával, alkohol alkalmazásával,

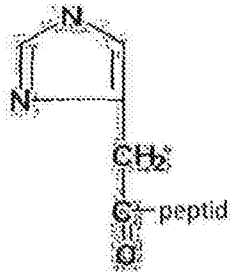
ahol a fiziológiásan aktív polipeptid exendin, inzulin, oxintomodulin, GLP-1, GLP-2, grelin, calcitonin vagy ezek valamely származéka, amelynek képlete a (I)-(IV) képlet közül van kiválasztva:



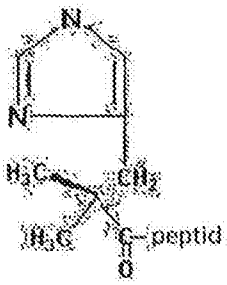
I képlet



II képlet



III képlet



IV képlet

ahol az alkohol etanol vagy izopropanol,

ahol az alkohol a reakcióközeg teljes térfogatára vonatkoztatva 25-90 térfogat% mennyiségben van jelen a reakcióközegben,

ahol az i) lépésben alkalmazott pH 7-10, amennyiben a fiziológiásan aktív polipeptid exendin, oxintomodulin, GLP-1, GLP-2 vagy ezek valamely származéka, és

ahol az i) lépésben alkalmazott pH 4-10, amennyiben a fiziológiásan aktív polipeptid humán inzulin vagy származéka.

2. Az 1. igénypont szerinti eljárás, ahol a nem-peptidil polimer polietilén-glikol.

3. Az 1. igénypont szerinti eljárás, ahol a reakcióközeg teljes mennyiségére vonatkoztatva az alkohol a reakcióközegben 35-60 térfogat% mennyiségben van jelen.

4. Az 1. igénypont szerinti eljárás, ahol a helyspecifikus, fiziológiásan aktív polipeptid-konjugátum exendin-4 konjugátum, amelyben PEG kapcsolódik Lys27-hez, calcitonin-konjugátum, amelyben PEG kapcsolódik az N-terminálishoz, oxintomodulin-konjugátum, amelyben PEG kapcsolódik Lys27-hez vagy Lys30-hoz, humáninzulin-konjugátum, amelyben PEG kapcsolódik az A láncban a Gly1 N-terminálisához vagy a B-láncban Lys29-hez, vagy GLP-1 vagy GLP-2 konjugátum, amelyben PEG kapcsolódik Lys27-hez vagy Lys30-hoz.

A meghatalmazott:
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Szabadalmi és Jogi Iroda Kft.

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szabadalmi ügyvivő