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(54) **LONG-ACTING BLOOD SUGAR
DECREASING FUSION PROTEIN**

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(57) **ABSTRACT**

The present invention provides an anthropogenic glucagon-like peptide-1 (GLP-1) recombinant protein molecule fused with an anthropogenic immunoglobulin subtype (IgG2) Fc section and a preparation method and purpose thereof. The fusion protein has the biological activity of GLP-1, and also has a significantly prolonged half-life in vivo. The fusion protein can be used to treat type II diabetes, obesity, and other diseases that are treated by decreasing serum glucose, suppressing gastrointestinal motility, and emptying or suppressing food intake.

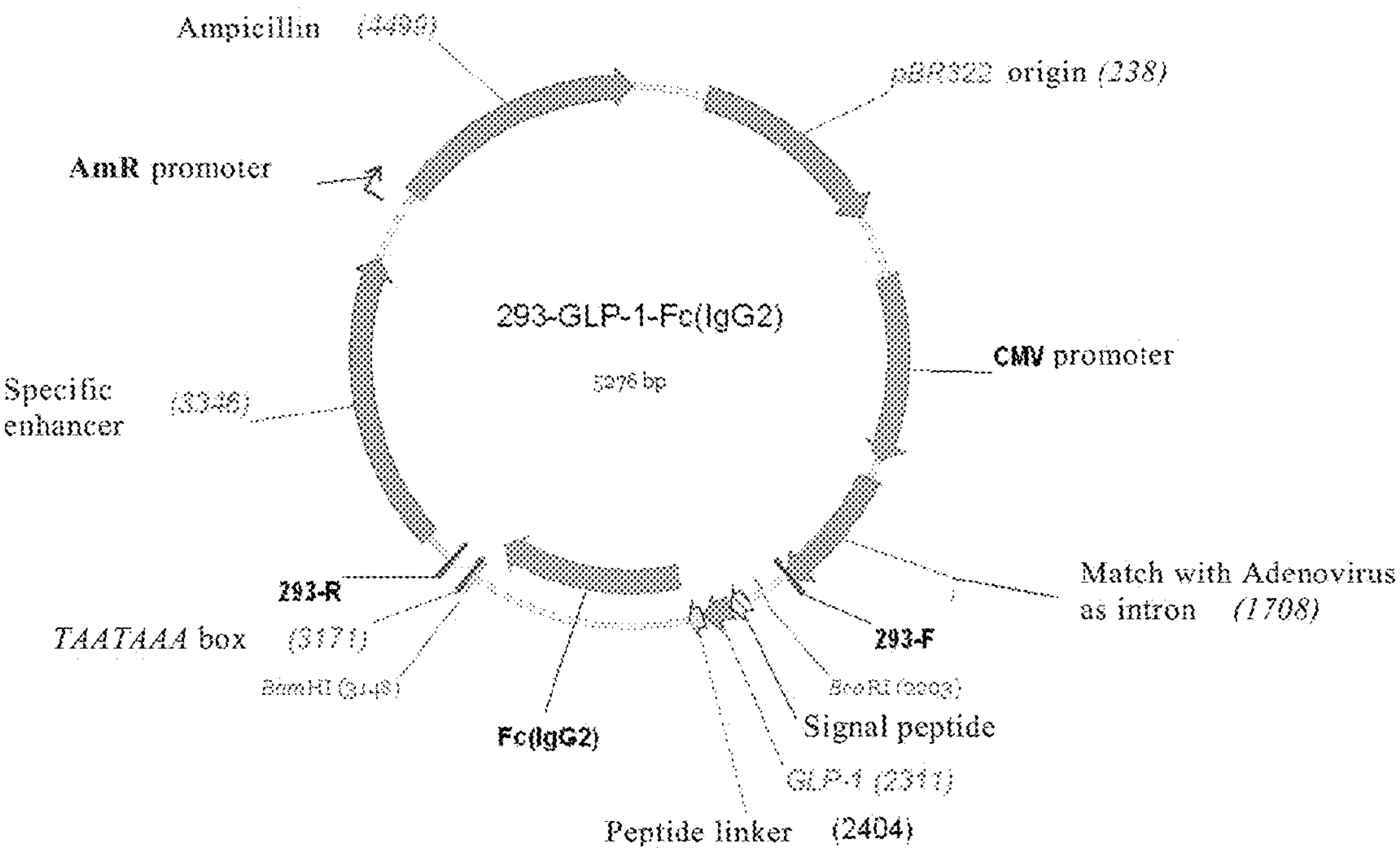


Figure 1

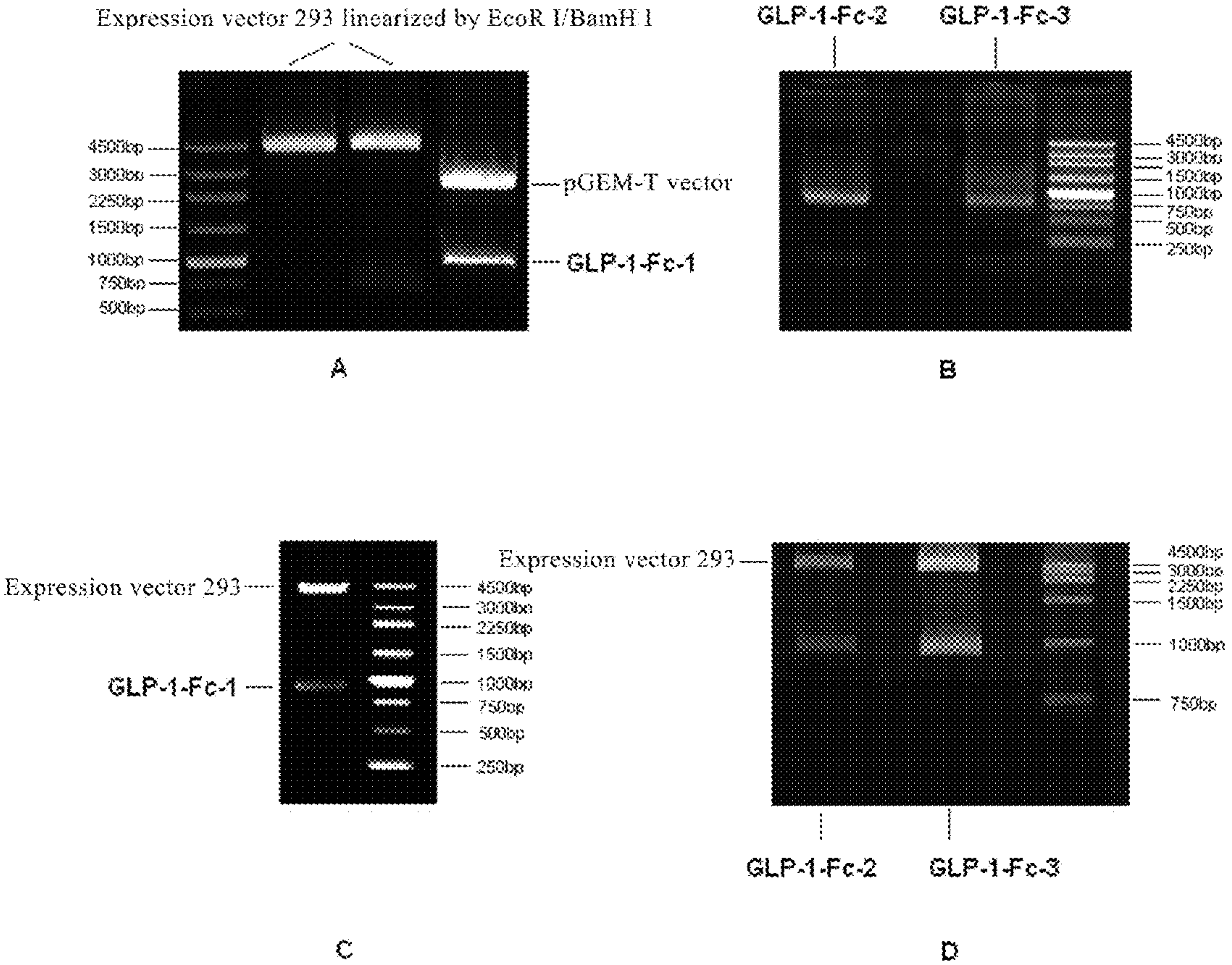


Figure 2

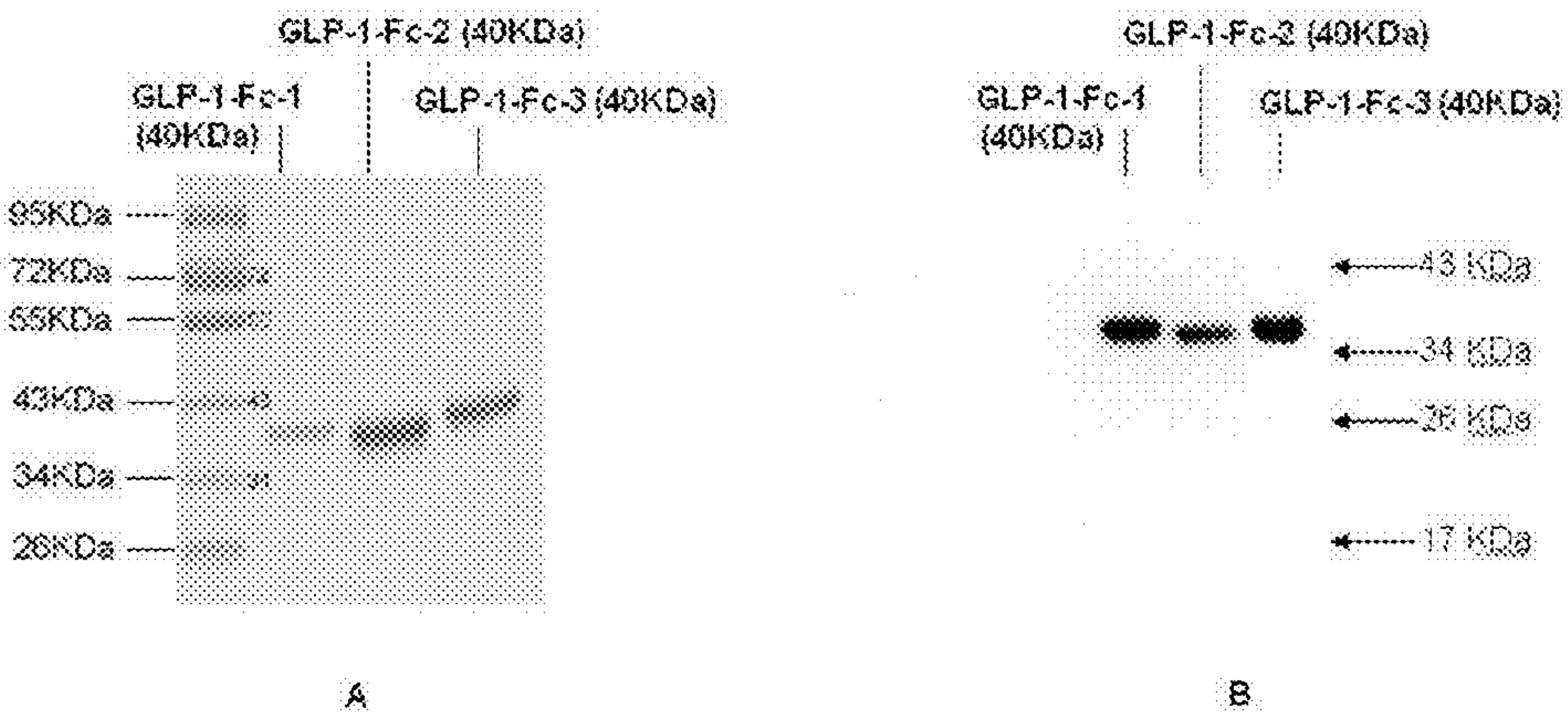


Figure 3

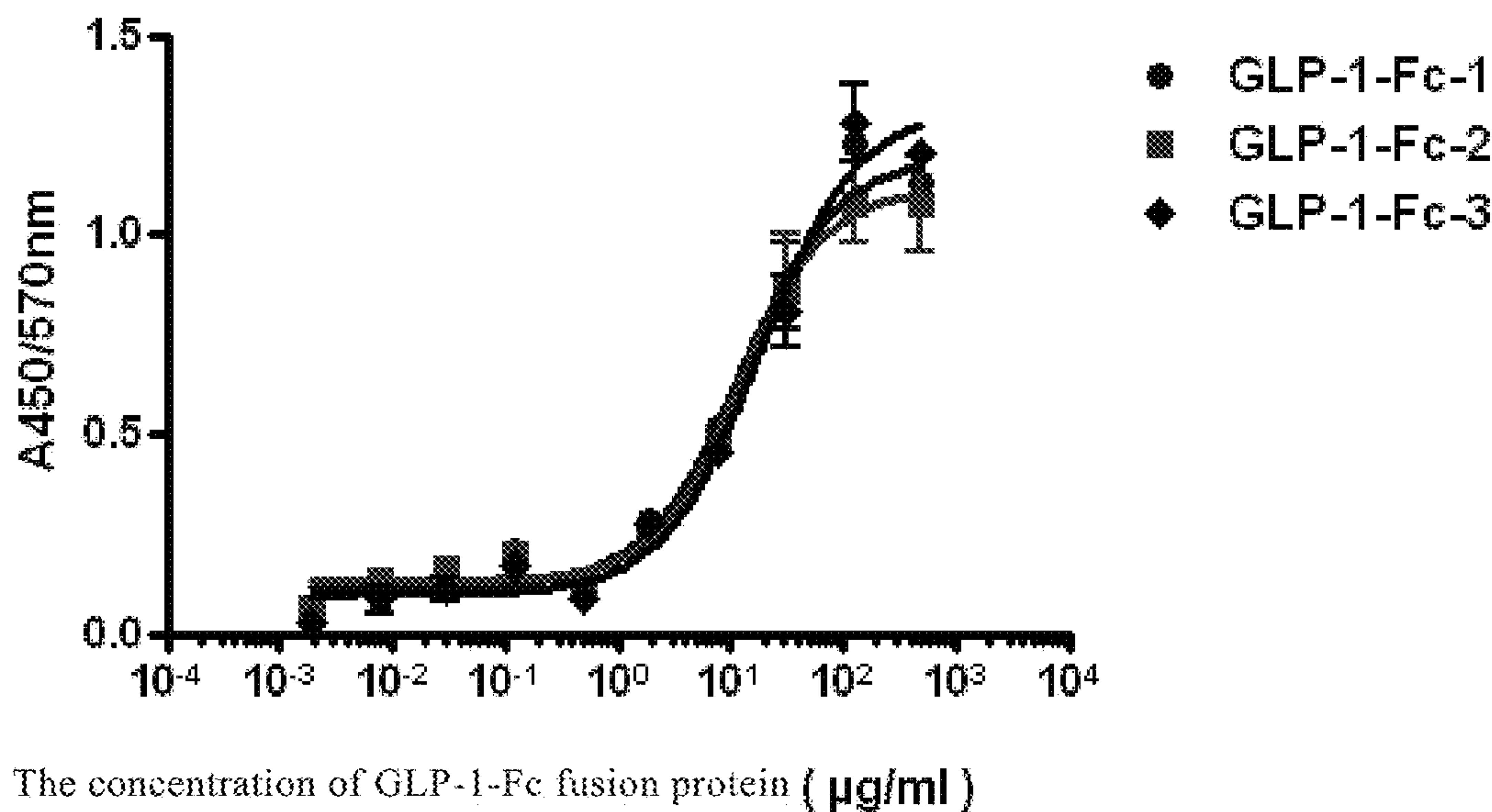


Figure 4

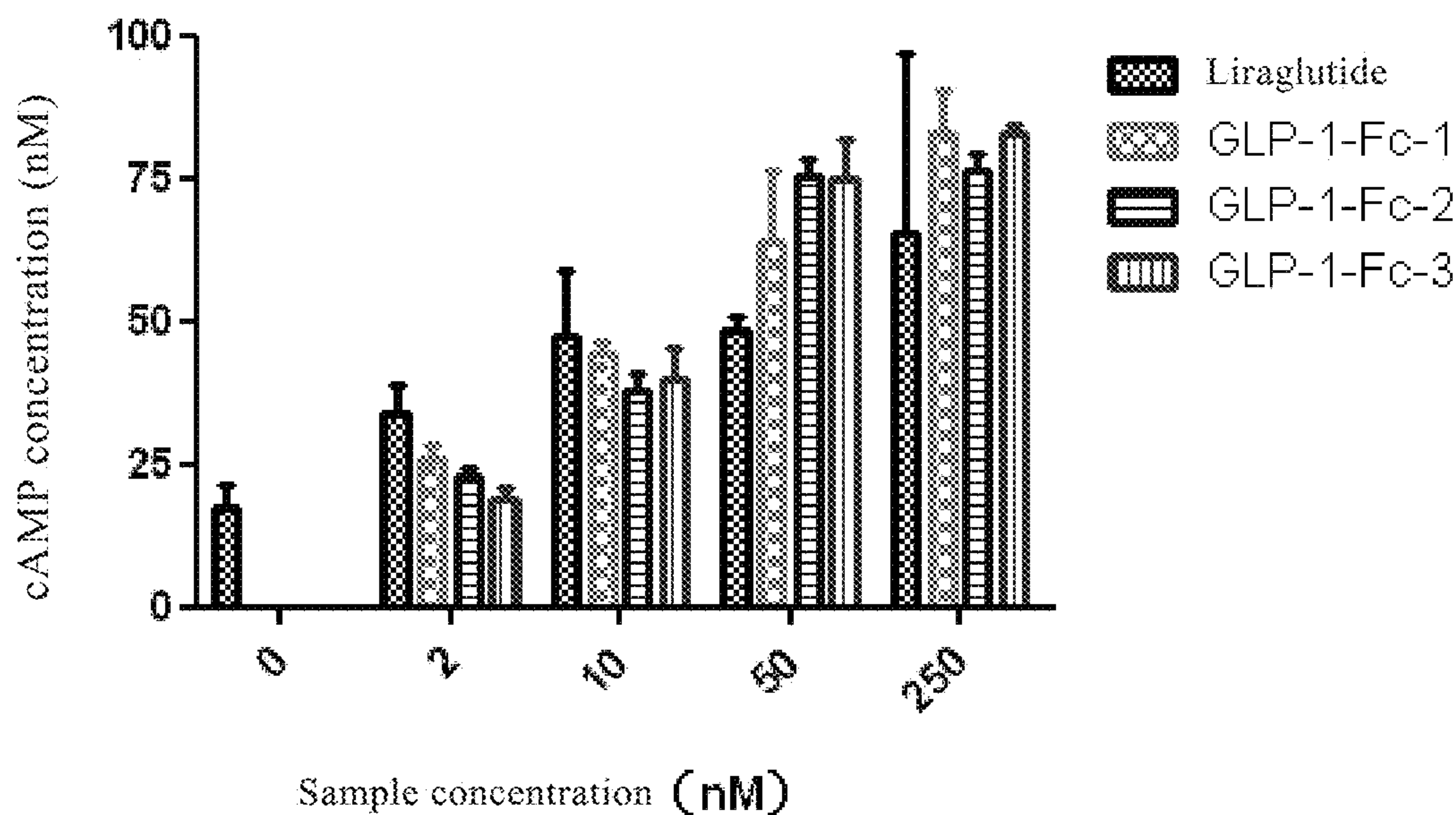


Figure 5

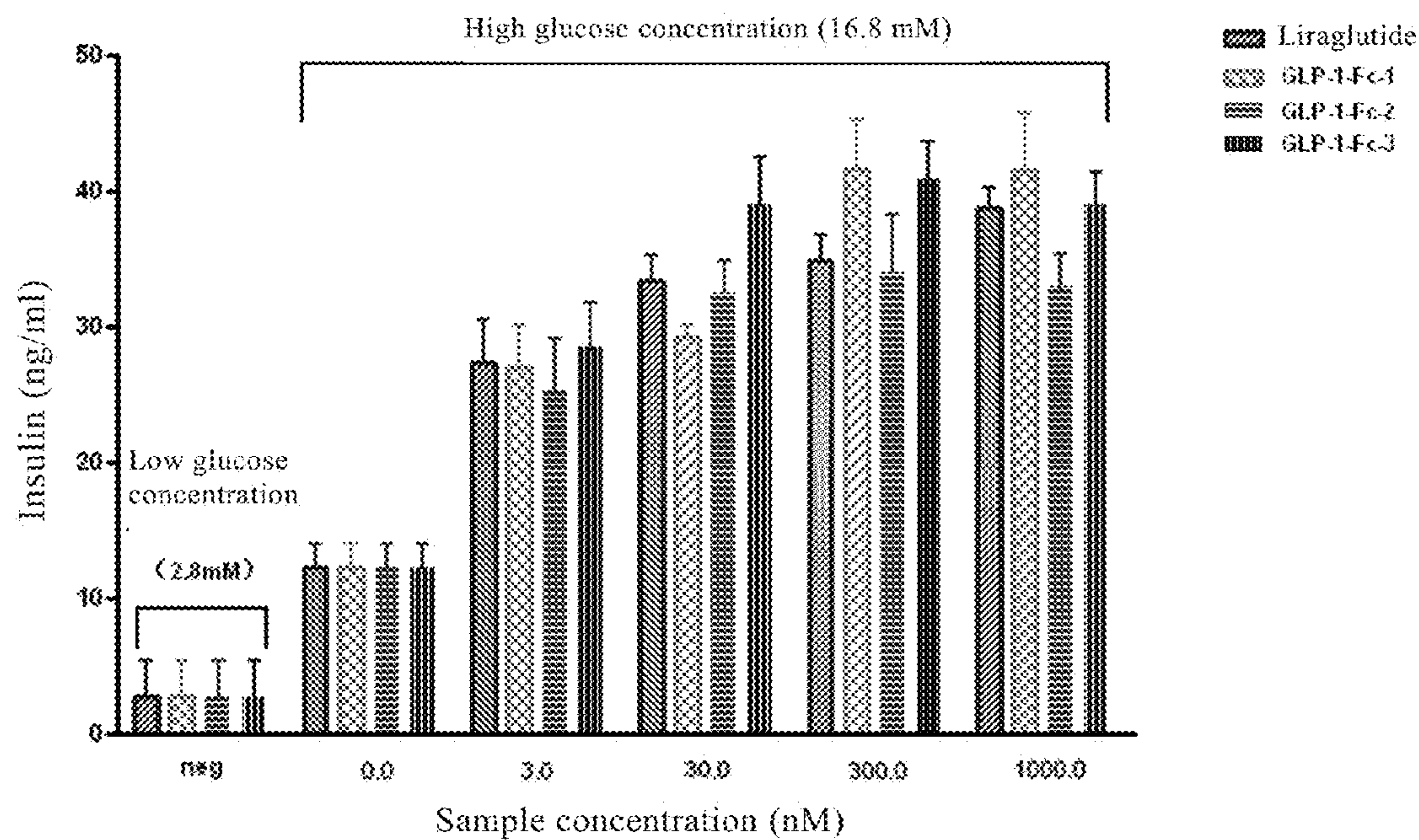


Figure 6

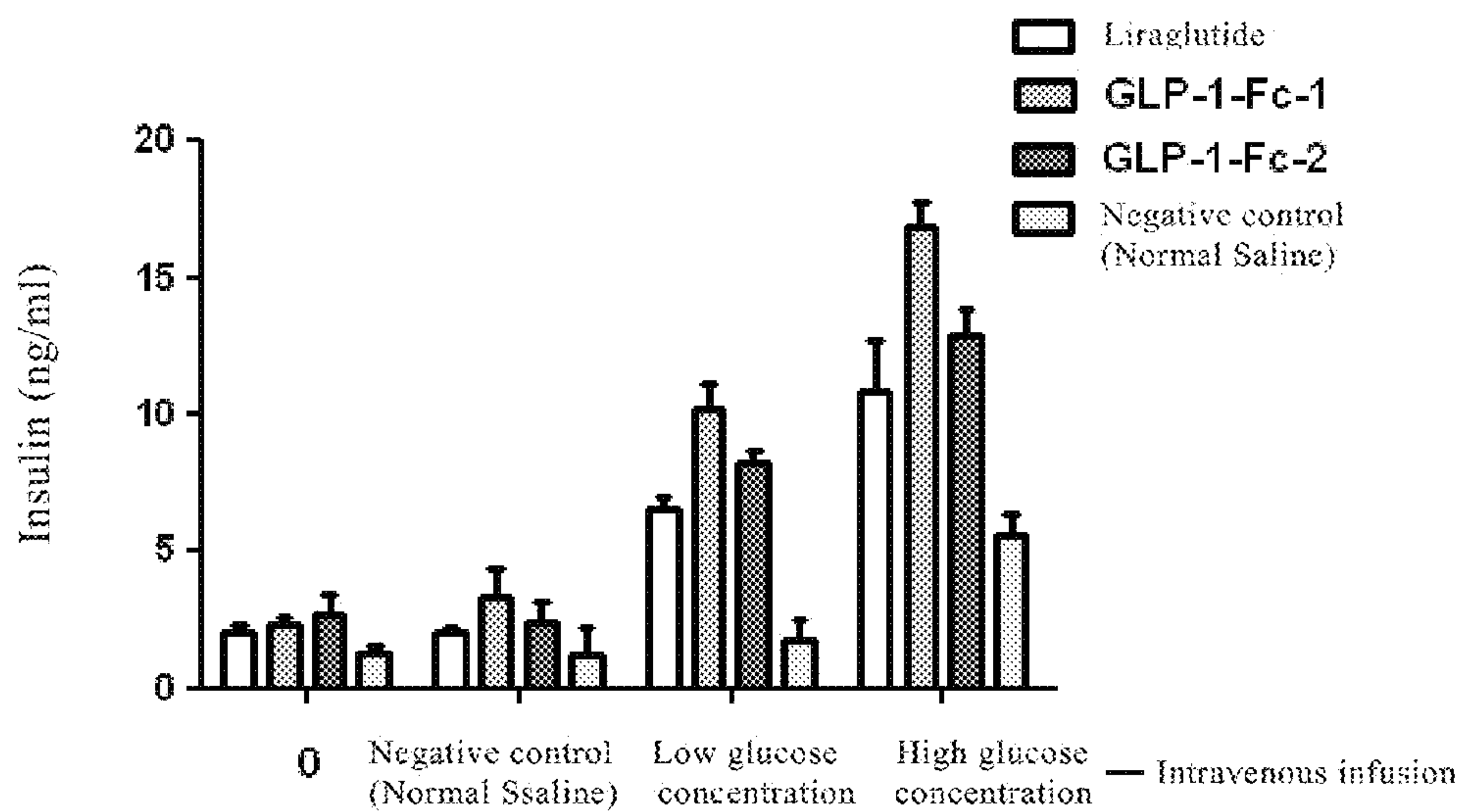


Figure 7A

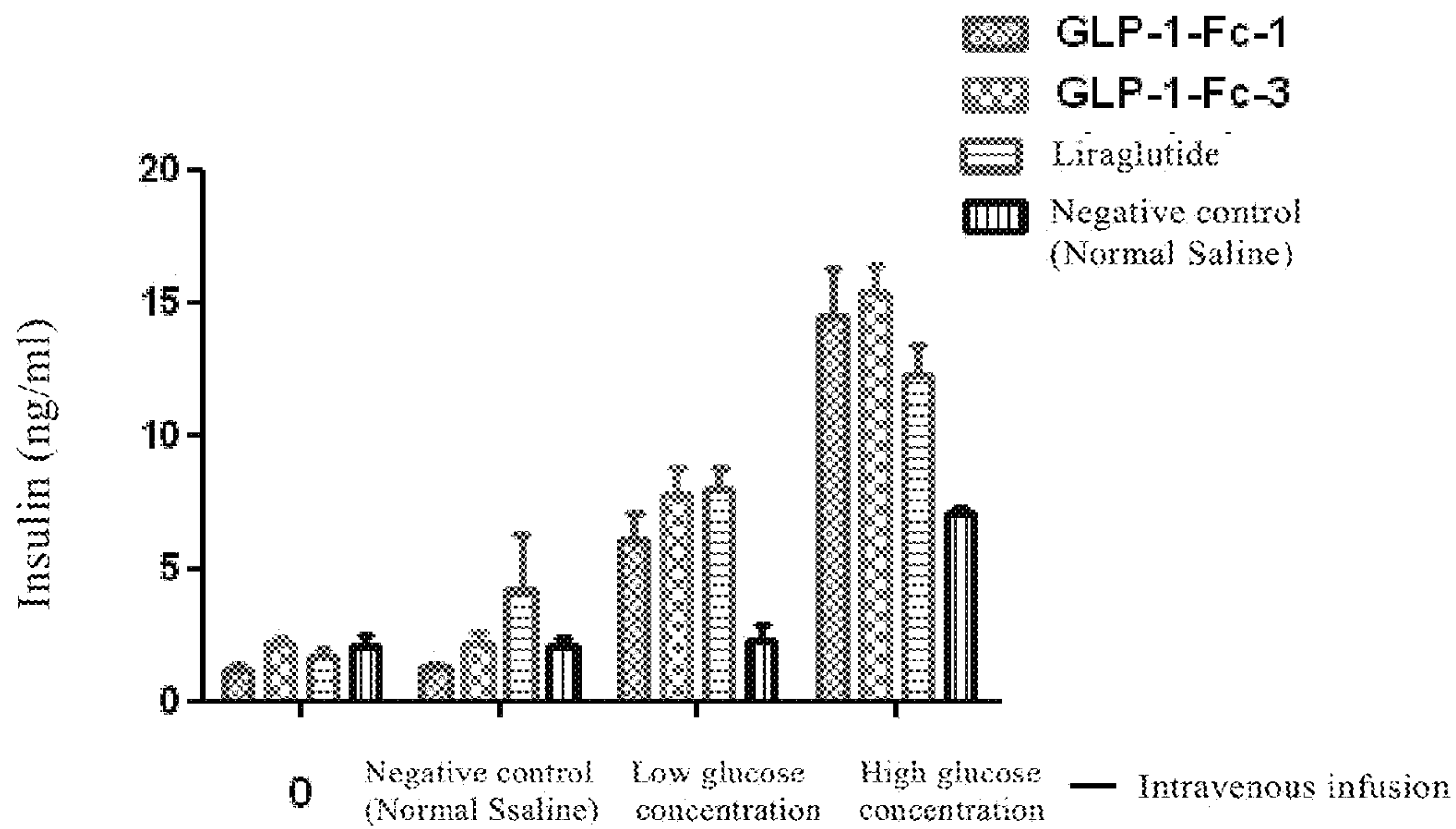


Figure 7B

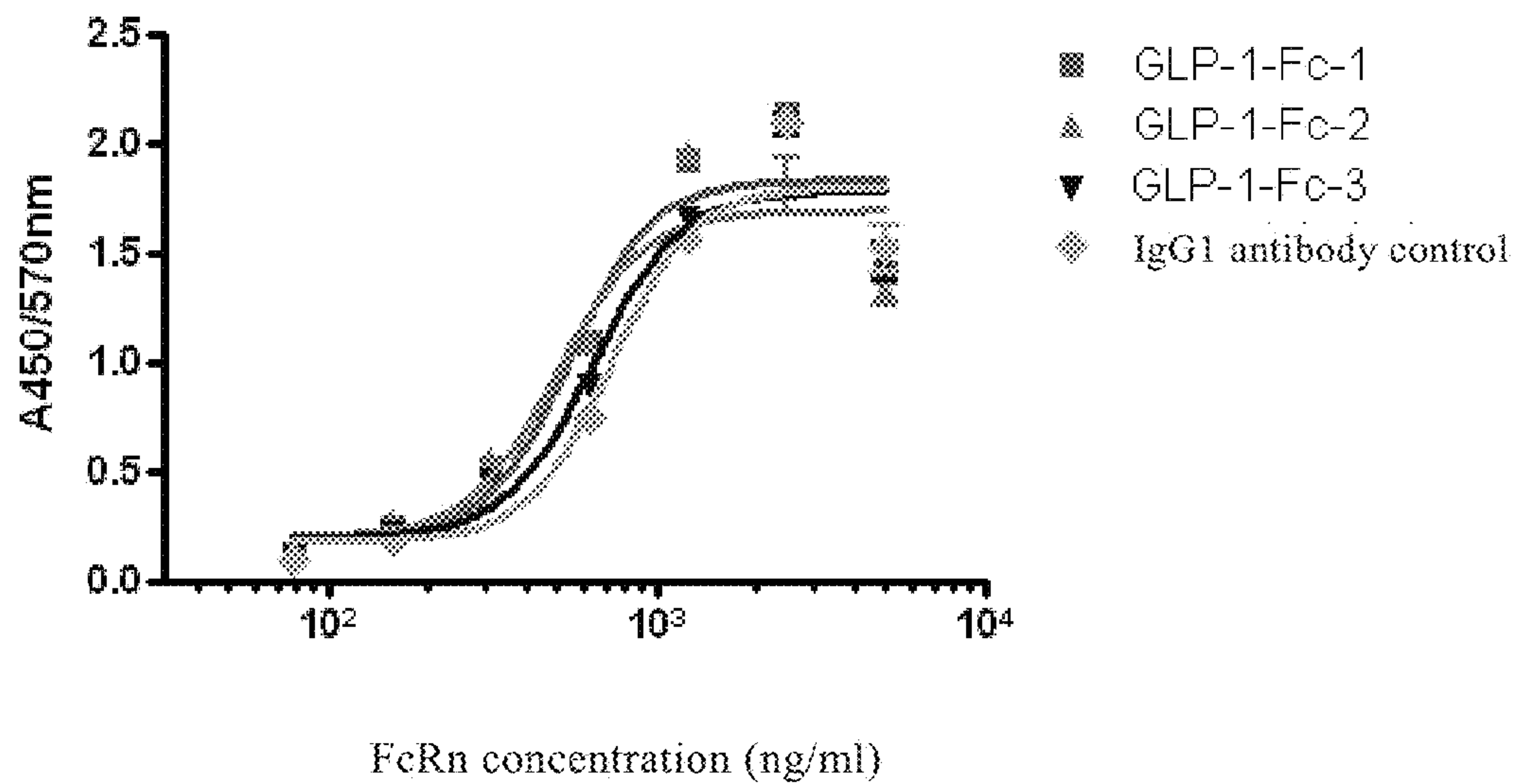


Figure 8

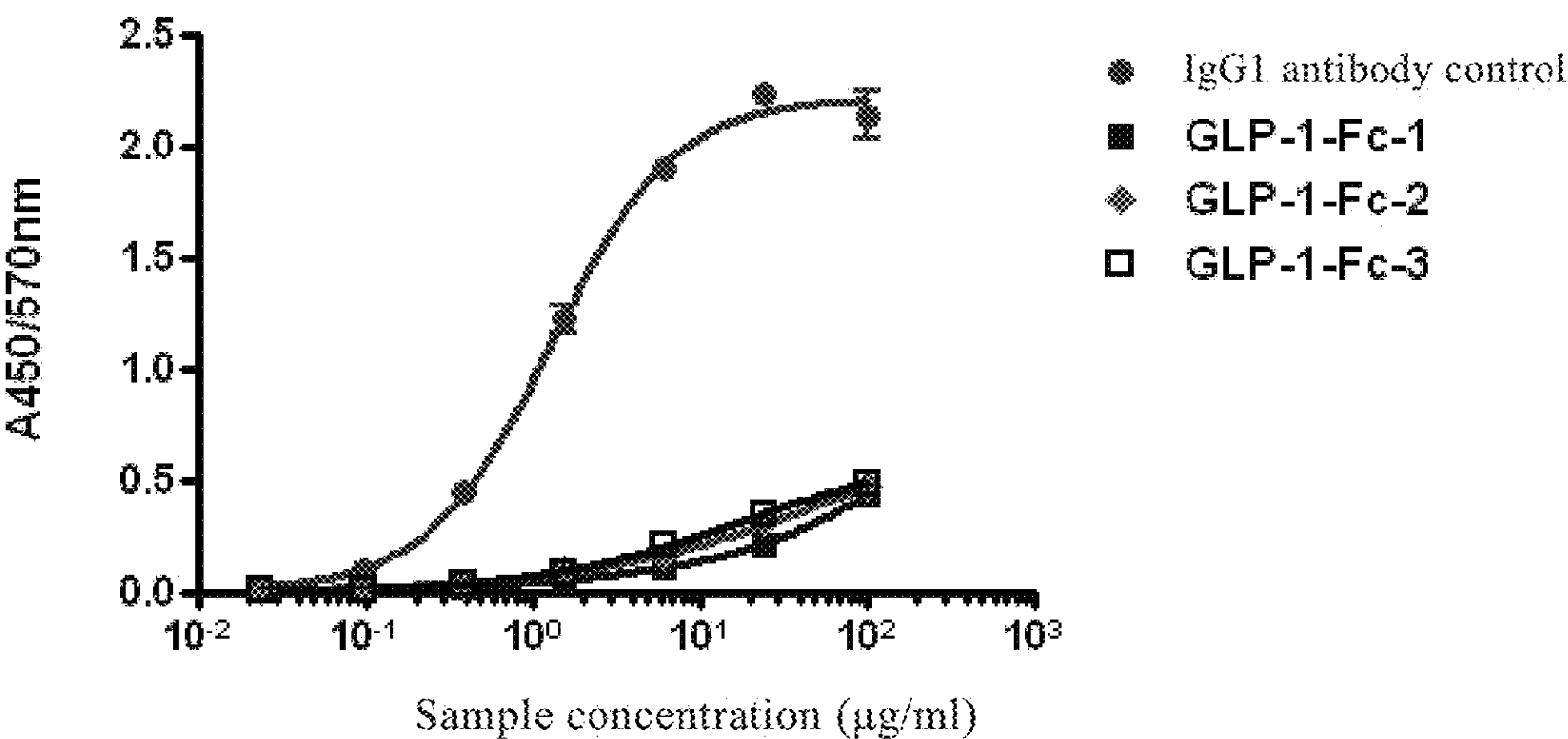


Figure 9

LONG-ACTING BLOOD SUGAR DECREASING FUSION PROTEIN

FIELD OF THE INVENTION

[0001] The present invention belongs to the field of biological pharmaceuticals, particularly to the field of therapeutic biomacromolecule medicament. More particularly, the present invention discloses a fusion protein, and the preparation method and use thereof.

BACKGROUND OF THE INVENTION

[0002] Patients with type II diabetes (diabetes mellitus II, DM II, insulin resistant diabetes) account for 90-95% of all the patients with diabetes, and their amount increases by 6% annually. By 2025, the amount of the patients with type II diabetes is expected to reach 380 million across the world. Asia now is already the region with the most patients with diabetes. The amount of patients with diabetes in developing countries, such as China and India, increases most rapidly across the world. The latest large-scale epidemiological survey about diabetes in China was conducted in 2002 among 100,000 people according to the diagnostic criteria published by WHO in 1999, showing that the prevalence rates in people older than 18 years in urban and rural areas are 4.5% and 1.8%, respectively. In the past two decades, the prevalence rate of diabetes has a nearly 4-fold increase in China.

[0003] Currently, the major therapies for type II diabetes include insulin replacement therapy (insulin and insulin analogue) and oral administration of chemical hypoglycemics (insulin secretagogues which can directly stimulate insulin secretion, such as sulfonylureas and glinides; non-insulin secretagogues such as biguanides, thiazolidinediones and alpha glucosidase inhibitors, wherein the biguanides mainly reduce hepatic glucose output, the thiazolidinediones can improve insulin resistance, and the alpha glucosidase inhibitors mainly delay carbohydrate absorption in the intestines). Although the above-mentioned medicaments can decrease blood sugar, they may cause side effects such as hypoglycemia and weight gain, and progressive loss of the function of pancreatic beta-cells (Nature 2001, 414: 821-827).

[0004] GLP-1 (Glucagon-Like Peptide-1), as one of the incretins, simulates the "incretin effect" that decreases blood sugar under physiological conditions, and targets two major pathogenesis of diabetes (insufficient insulin secretion and insulin resistance) with unique therapeutic mechanism. At present, GLP-1 has been approved as the second-line medicament in developed countries (Diabetes Care. 2009, 32: 193-203).

[0005] GLP-1 promotes insulin secretion and inhibits glucagon secretion in a blood sugar concentration-dependent manner; that is, when blood sugar concentration is higher than normal level, GLP-1 produces an insulin secretion-promoting effect; and when blood sugar concentration is normal, this insulin secretion-promoting effect diminishes. Therefore, the treatment by exogenous GLP-1 will not induce hypoglycemia side effect due to overdose, which is the most prominent feature of GLP-1 analogues over other insulin secretagogues, as well as insulin and insulin analogues (Diabetologia. 1986, 29: 46-52; J Clin Invest. 1993, 91: 301-307; and J Clin Endocrinol Metab. 2001, 86: 3717-3723).

[0006] GLP-1 could control postprandial glucagon secretion by binding to the receptors on pancreatic alpha cells; promote proliferation of pancreatic beta cells, inhibit their

apoptosis, and increase their sensitivity to glucose by interacting with pancreatic beta cells, thereby increasing glucose-dependent insulin secretion; reduce hepatic glycogen output by acting on liver; delay gastric emptying and reduce food intake by acting on stomach; and increase satiety and reduce appetite by acting on hypothalamus, thereby resulting in weight loss (Diabetes Care. 2003, 26: 2929-2940; Gastroenterology. 2007, 132: 2131-2157; Proc Natl Acad Sci. 1982, 79(2): 345-349; Diabetologia. 1996, 39: 1546-1553; Endocrinology. 2003, 144: 5149-5158; Diabetes. 2002, 51: 5434-5442; Diabetologia. 1993, 36: 741-744; and Lancet. 2002, 359: 824-830).

[0007] In addition, GLP-1 may improve pathological defects in patients with type II diabetes, protects pancreatic beta cells and cardiovascular system, and has nerve protection effect. Therefore, GLP-1 can reduce occurrence of complications in patients with diabetes, and its advantages and comprehensive effects in decreasing blood sugar, losing weight, and protecting pancreatic cells and cardiovascular system, will certainly improve its position in the future treatment of type II diabetes (Diabetes Care. 1998, 21: 1925-1931; Diabetes Spectrum. 2004, 17: 183-190; Lancet. 2006, 368: 1696-1705; and PLoS ONE. 2011, 6(8): e23570).

[0008] Natural GLP-1 has no druggability, since it can be easily inactivated in vivo by endogenous DPP-4 (Dipeptidyl peptidase-4) that removes the N-terminal histidine (His) and alanine (Ala) residues of GLP-1, and has a half-life of less than 2 minutes. Therefore, the medicaments under development need to overcome this problem through various ways. At present, there are mainly two classes of GLP-1-targeting medicaments that are marketed or under development: one is small-molecule medicament that can inhibit the degradation effect of DPP-4 in vivo, the other is modified GLP-1 or GLP-1 analogue that has extended half-life without losing the biological function of GLP-1 (J Biol Chem. 1992, 267: 7402-7405; Drug Dev Res. 2001, 53: 260-267; Diabetes. 2007, 56: 1475-1480; Clin Ther. 2008, 30: 858-867; Diabetes Obes Metab. 2008, 10: 82-90; and Curr Med Res Opin. 2008, 24: 275-286).

[0009] GLP-1 analogues, GLP-1 mutants, GLP-1 long-acting formulations or DPP-4 inhibitors that have been successfully marketed or under development, are all originally developed to extend the in vivo half-life of active substances. At present, GLP-1 and most of analogues thereof developed at home and abroad possess similar therapeutic effects, and mainly differ in the action time and immunogenicity. Among those medicaments, the first marketed GLP-1 analogue, Exenatide, is developed by Eli Lilly and marketed in the United States in April, 2005, which is derived from the saliva of Gila monster (*Heloderma suspectum*) and needs to be administered twice a day by subcutaneous injection. The subsequently marketed Liraglutide, a human GLP-1 mutant developed by Novo Nordisk, is marketed in Europe in April, 2009 and in China in October, 2011, which needs to be administered once a day by subcutaneous injection. The human GLP-1 mutant that binds to a human immunoglobulin IgG Fc section, e.g., Dulaglutide under development by Eli Lilly, which takes advantage of the long circulating half-life of IgG and can be administered once a week, is the optimal one among current similar products (Diabetes Obes Metab. 2011, 13: 302-312).

[0010] The present invention relates to a fusion protein in which the amino acid sequence of positions 7 to 37 of a human GLP-1 is fused with a human IgG Fc section, which

differs from Dulaglutide in that the human IgG Fc section used in this protein is IgG2 Fc section. From a safety perspective, it offers the following advantages:

[0011] 1) The human GLP-1 polypeptide has low immunogenicity, and thus does not likely generate antibody during long-term use; and

[0012] 2) The Fc section of certain IgG subtypes (such as IgG1) may bind to the Fc receptors on the surfaces of macrophages and NK cells, having ADCC (Antibody-Dependent Cell Cytotoxicity) and regulation effects. The Fc section of human IgG2 cannot bind to high-affinity Fc receptor, CD64, or to low-affinity Fc receptors, CD32 and CD16, and thus can reduce its ADCC effect.

[0013] In view of the above two advantages, the fusion protein of the present invention can not only reduce immunogenicity, but also avoid the effector function of the Fc section that is not associated with the GLP-1 treatment.

[0014] Neonatal Fc receptor (FcRn) can extend the half-life of IgG in blood, maintain a high level of IgG concentration in blood circulation, and keep the dynamic balance of antibody level. FcRn is expressed by vascular endothelial cells in normal adults, and can bind to IgG Fc section. Vascular endothelium is an important position where FcRn protects IgG from being degraded and metabolized. FcRn, which depends on endocytosis, not only absorbs IgG from the extracellular acidic environment, but also involves in IgG circulation and homeostatic regulation within cells. Under physiological conditions, when IgG concentration in serum is lower than normal level, more FcRns bind to Fc and decrease IgG degradation, such that IgG concentration can be maintained, when IgG concentration in serum is higher than normal level, the FcRns on the surface of endothelial cells are saturated, and thus cannot bind to more IgG, thereby enhancing IgG degradation and decreasing IgG concentration in serum. By binding FcRn to Fc to protect the Fc-containing protein from being degraded, the high fusion protein concentration in serum can be maintained in a dynamic balance, thereby extending its in vivo half-life.

[0015] The present invention, by means of the unique metabolic pathway of immunoglobulin IgG with slow clearance, use a method of fusing a human GLP-1 polypeptide with an Fc section of the human immunoglobulin IgG2 for expression to produce a fusion protein in which GLP-1 has an in vivo half-life close to that of IgG, while maintaining its biological activity.

[0016] The fusion protein can be absorbed by subcutaneous injection, and can be administered once every 1 to 2 weeks by subcutaneous injection and maintained an effective in vivo blood drug concentration for a long time due to its prolonged in vivo half-life (the Fc section of IgG2 type having a longer in vivo half-life than those of IgG4 and IgG1 types) (Nature Biotechnology. 2007, 25(12): 1369-1372). Thus, this fusion protein relieves the patients from the pain regarding frequent injections, improves the therapeutic compliance, and reduces the treatment cost.

[0017] Although this approach is feasible for GLP-1 therapy, an antibody would be generated when a fusion protein is administered repeatedly over a prolonged period. Furthermore, in view of the fact that the patients with diabetes have to receive treatment during their lifetime after final diagnosis, if the Fc section of the GLP-1-Fc fusion protein retains undesirable effector function, the resultant GLP-1-Fc fusion protein therapy may have a safety concern. The present invention attempts to overcome the problems of potential

immunogenicity and effector activity associated with the use of a GLP-1-Fc fusion protein. The fusion protein of the present invention has various amino acid residue substitutions in both the GLP-1 section and the Fc section. The substitutions provide greater potential to increase in vivo stability, reduce immunogenicity and eliminate effector function.

Contents of the Invention

[0018] The present invention provides a recombinant fusion protein in which the amino acid sequence of positions 7 to 37 of a human glucagon-like peptide-1 (GLP-1) is fused with an Fc section of the human immunoglobulin subtype IgG2, and the preparation method and use thereof, wherein the GLP-1 comprises a C-terminus linked to the IgG2 Fc section via a glycine (Gly)-rich peptide linker (Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Gly-Ser, SEQ ID NO: 11). The fusion protein has the biological activity of GLP-1, and also has a significantly extended in vivo half-life. The fusion protein can be used for the treatment and/or prevention of type II diabetes, obesity, and other diseases that can benefit from decreasing serum glucose, suppressing gastrointestinal motility, and emptying or inhibiting food intake.

[0019] The present invention constructs a fusion protein in which GLP-1 is fused with an IgG2 Fc section through gene engineering technology. Particularly, the present invention discloses:

[0020] 1. A fusion protein, which is obtained by fusing the C-terminus of a glucagon-like peptide-1 with the N-terminus of an IgG2 Fc section via a peptide linker, and comprises an amino acid sequence shown in SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6.

[0021] 2. A gene encoding the fusion protein described under item 1. In one embodiment, the gene comprises a nucleotide sequence shown in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

[0022] 3. A method for preparing the fusion protein described under item 1, comprising:

[0023] a) constructing the gene described under item 2;

[0024] b) cloning the gene obtained in step a) into a eukaryotic expression vector, to obtain a eukaryotic expression vector that can express the fusion protein described under item 1; and

[0025] c) transfecting cells with the expression vector obtained in step b), to express the recombinant fusion protein, and then isolating and purifying the fusion protein.

[0026] 4. A formulation, comprising the fusion protein described under item 1 as an active component, and optionally further comprising one or more pharmaceutically acceptable carriers well-known in the art.

[0027] 5. Use of the fusion protein described under item 1 or a formulation comprising the same, in the manufacture of a medicament for the treatment and/or prevention of type II diabetes, obesity, and other diseases that can benefit from decreasing serum glucose, suppressing gastrointestinal motility, and emptying or suppressing food intake.

[0028] In one embodiment, a gene encoding the fusion protein of the present invention is cloned into a eukaryotic expression vector 293.

[0029] In one embodiment, FreeStyle 293F cells are transfected with a eukaryotic expression vector comprising the gene which encodes the fusion protein of the present invention, to express the fusion protein of the present invention.

DESCRIPTION OF FIGURES

[0030] FIG. 1 shows the structure of the recombinant eukaryotic expression vector 293-GLP-1-Fc (IgG2) for expressing the fusion protein of the present invention. Meanwhile, the structures of the recombinant eukaryotic expression vectors for the three fusion proteins of this application, GLP-1-Fc-1, GLP-1-Fc-2 and GLP-1-Fc-3, share the same gene insertion sites.

[0031] FIG. 2A shows the DNA agarose electropherograms of the fragment obtained by double digesting and linearizing the expression vector 293 with the enzymes EcoR I and BamH I, and of the gene fragment that encodes the fusion protein GLP-1-Fc-1 obtained by enzyme digestion of pGEM-T plasmid vector (synthesized by Generay™). FIG. 2B shows the gene fragments encoding the fusion proteins GLP-1-Fc-2 and GLP-1-Fc-3 obtained by polymerase chain reaction (PCR). FIGS. 2C and 2D respectively show DNA agarose electropherograms for identifying the constructed expression vectors 293 encoding the three fusion proteins by double digested with enzymes EcoR I and BamH I.

[0032] FIG. 3A shows polyacrylamide gel electropherograms (SDS-PAGE) of the three fusion proteins. FIG. 3B is a graph showing the western blot results of the three fusion proteins.

[0033] FIG. 4 is a graph showing the experimental results of the binding of the three fusion proteins on β -TC-6 cells.

[0034] FIG. 5 is a graph showing the experimental results that the three fusion proteins could promote cAMP (adenosine 3',5'-cyclic monophosphate) release in β -TC-6 cells.

[0035] FIG. 6 is a graph showing the experimental results that the three fusion proteins could promote insulin secretion in β -TC-6 cells.

[0036] FIGS. 7A and 7B are graphs showing the experimental results that the three fusion proteins could improve serum insulin level in a rat model of high-dose glucose infusion, respectively.

[0037] FIG. 8 is a graph showing the experimental results of the binding between the three fusion proteins and FcRn protein.

[0038] FIG. 9 is a graph showing the experimental results of the binding between the three fusion proteins and Fc γ RIIIa (CD16a) protein.

SPECIFIC MODE FOR CARRYING OUT THE INVENTION

[0039] Unless otherwise indicated, all technical and scientific terms used herein have the same meanings as those generally understood by those skilled in the art to which the present invention pertains.

[0040] In one embodiment, the specific technical solution for producing the fusion protein of the present invention is set out as follows:

[0041] I. Construction of the Expression Vector Encoding the Fusion Protein of the Present Invention

[0042] Based on the disclosed GLP-1 (7-37) sequence (Diabetes Metab Res Rev. 2010, 26: 287-296.) and the Fc sequence of IgG2 (AJ250170) disclosed by Pubmed, we synthesized the cDNA sequences which encode a human GLP-1 (7-37), a peptide linker of 15 amino acids, and an IgG2 type Fc section, respectively. The cDNA sequences were used to obtain the fusion gene sequence encoding the fusion protein of the present invention by linking the C-terminus of the gene encoding the human GLP-1 (7-37) to the gene encoding the

human IgG2 type Fc section via the peptide linker gene sequence. We made some modifications to the GLP-1 (7-37) amino acid sequence, such that alanine (Ala) at position 8 was replaced with glycine (Gly), glycine (Gly) at position 22 was replaced with glutamic acid (Glu), and arginine (Arg) at position 36 was replaced with glycine (Gly).

[0043] The human IgG2 type Fc section in the fusion protein of the present invention has three different modification forms in respect of amino acid sequence, corresponding to three fusion proteins having different sequences which are referred herein as GLP-1-Fc-1, GLP-1-Fc-2 and GLP-1-Fc-3, respectively; the amino acid sequences thereof are shown in SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6, respectively, and the gene sequences thereof are shown in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3, respectively. In the amino acid sequence (SEQ ID NO: 4) of the GLP-1-Fc-1, it is a secreting signal peptide sequence (the secreting signal peptide refers to the amino acid sequence that generally occurs in the N-terminal region of a large polypeptide, with the functions of initiating the binding of the polypeptide and cellular endoplasmic reticulum, and secreting the polypeptide across plasma membrane) at positions 1-19, a GLP-1 (7-37) sequence at positions 20-50, a peptide linker sequence at positions 51-65, and an IgG2 Fc sequence at positions 66-288 (its hinge region is VECPPCP, SEQ ID NO: 12). In the amino acid sequence (SEQ ID NO: 5) of the GLP-1-Fc-2, it is a signal peptide sequence at positions 1-19, a GLP-1 (7-37) sequence at positions 20-50, a peptide linker sequence at positions 51-65, and an IgG2 Fc sequence at positions 66-292 (its hinge region is ERKCCVECP (SEQ ID NO: 13), and the amino acid K at the C-terminal is deleted). In the amino acid sequence (SEQ ID NO: 6) of the GLP-1-Fc-3, it is a signal peptide sequence at positions 1-19, a GLP-1 (7-37) sequence at positions 20-50, a peptide linker sequence at positions 51-65, and an IgG2 Fc sequence at positions 66-287 (its hinge region is VECPPCP (SEQ ID NO: 12), and the amino acid K at the C-terminal is deleted). In the gene sequence (SEQ ID NO: 1) of the GLP-1-Fc-1, it is a signal peptide gene sequence at positions 1-57, a GLP-1 (7-37) gene sequence at positions 58-150, a peptide linker gene sequence at positions 151-195, and an IgG2 Fc gene sequence at positions 196-864. In the gene sequence (SEQ ID NO: 2) of the GLP-1-Fc-2, it is a signal peptide gene sequence at positions 1-57, a GLP-1 (7-37) gene sequence at positions 58-150, a peptide linker gene sequence at positions 151-195, and an IgG2 Fc gene sequence at positions 196-876. In the gene sequence (SEQ ID NO: 3) of the GLP-1-Fc-3, it is a signal peptide gene sequence at positions 1-57, a GLP-1 (7-37) gene sequence at positions 58-150, a peptide linker gene sequence at positions 151-195, and an IgG2 Fc gene sequence at positions 196-861.

[0044] After the three fusion genes encoding the above-mentioned fusion proteins were obtained, molecular cloning technique was employed for further cloning said fusion genes into a eukaryotic expression vector 293 so as to construct the eukaryotic expression vector 293-GLP-1-Fc (IgG2).

[0045] For the purpose of the present invention, any suitable eukaryotic expression vector can be used.

[0046] II. General Method for Expressing the Fusion Protein of the Present Invention

[0047] Eukaryotic host cell FreeStyle 293F was transfected with the expression vector 293-GLP-1-Fc (IgG2) obtained as

mentioned above, to generate a fusion protein. The technique for transfecting a host cell using a recombinant DNA is well-known in the art.

[0048] III. Collection and Purification of the Recombinant Fusion Protein

[0049] The non-antigen binding regions, i.e. the Fc sections, of the three IgG subtypes (IgG1, IgG2 and IgG4), can bind to staphylococci Protein A (SPA), which can be used to purify the antibodies of the above subtypes or the fusion proteins containing the Fc sections of the corresponding subtypes, providing a convenient purification method for the industrial preparation thereof. The Fc-containing fusion protein was obtained by separation and purification through Protein A affinity column. The fusion protein was identified by SDS-PAGE and Western blot.

[0050] IV. Assay for In Vitro or In Vivo Activity of the Fusion Protein of the Present Invention

[0051] 1. Assay for the In Vitro Binding Activity of the Fusion Protein of the Present Invention to Mouse Beta Pancreatic Tumor Cells

[0052] An assay for the direct binding activity of the fusion protein to GLP-1 receptor (GLP-1R)-positive mouse beta pancreatic tumor cells (β -TC-6) was performed according to the method described in PLoS ONE. 2010, 5 (9): e12734. The results showed that the binding of the fusion protein of the present invention to the GLP-1R on the cells exhibited a specific concentration gradient-dependent increase, suggesting that the GLP-1 fused with the Fc section of the present invention can specifically bind to the corresponding receptor on the cell surface.

[0053] 2. The Effect of the Fusion Protein of the Present Invention on cAMP Level In Vitro

[0054] The fusion protein of the present invention was added into β -TC-6 cells, and then the cAMP level was measured according to the method described in Diabetes. 2004, 53: 2492-2500. The results showed that the fusion protein of the present invention resulted in an increase in the cAMP level in vitro with an effect comparable to Liraglutide, suggesting that after binding to the corresponding receptor on the cell surface, the GLP-1 fused with the Fc section of the present invention can activate intracellular signal transmission mediated by the receptor.

[0055] 3. The effect of the fusion protein of the present invention on insulin secretion level of mouse Beta Pancreatic Tumor Cells In Vitro

[0056] The fusion protein of the present invention was added into β -TC-6 cells, and then the insulin secretion level of the cells was measured according to the method described in the previous references (Shi-Ying Ding, et al. JBC. 2011, 286(19): 16768-16774; and PLoS ONE. 2010, 5(9): e12734). The results showed that in the medium with low glucose concentration, the three fusion proteins of the present invention or Liraglutide had no significant promotion effect on insulin secretion of mouse beta pancreatic tumor cells β -TC-6; while in the medium with high glucose concentration, the three fusion proteins at the concentrations of 3, 30, 300 and 1000 nM each can significantly increase the insulin secretion level in β -TC-6 cells to different degrees, with an effect comparable to Liraglutide, and all of them exhibited a concentration gradient-dependent increase.

[0057] 4. The Effect of the Fusion Protein of the Present Invention on Serum Insulin Level in the Rats Infused with High-Dose Glucose

[0058] According to the previous references (Diabetes Metab Res Rev. 2010, 26: 287-296; and Diabetes. 2004, 53: 2492-2500), the method that uses normal SD (Sprague-Dawley) rats to establish a high-dose glucose infusion model for measuring the effect of medicaments in promoting increased serum insulin level, is a conventional efficacy assay for detecting the GLP-1-type insulin secretagogues. Said model was used in the present invention, wherein SD rats were injected subcutaneously with the fusion protein of the present invention (3 nM/kg) or Liraglutide (3 nM/kg, as a positive control) or Normal Saline (as a negative control), subjected to overnight fasting (16-18 h), and then sequentially administered the following substances by continuous intravenous infusion: Normal Saline for 20 minutes, low concentration of glucose (50 mg/kg/min) for 30 minutes, and high concentration of glucose (150 mg/kg/min) for 30 minutes. The time point at which Normal Saline infusion is finished was taken as a zero point, and blood samples were collected at minutes -20, 0, 30 and 60, respectively. The results showed that in the healthy rats group administered with Normal Saline by subcutaneous injection, after intravenous infusion with Normal Saline or low concentration of glucose, the serum insulin level has no significant increase compared with the non-intravenous infusion group, while after intravenous infusion with high concentration of glucose, the serum insulin level increases significantly; in the healthy rats group administered with the three fusion proteins of the present invention or Liraglutide by subcutaneous injection, intravenous infusion with Normal Saline still cannot induce any increase in serum insulin level, while after intravenous infusion with low concentration or high concentration of glucose, the serum insulin level increases to different degrees compared with the insulin level induced by glucose infusion of the corresponding concentrations in the group administered with Normal Saline by subcutaneous injection. The effect in the experimental animals for promoting insulin secretion in a glucose concentration-dependent manner suggests that the fusion proteins of the present invention in which the GLP-1 (7-37) is fused with the Fc section can be absorbed through subcutaneous injection, and can exert the same pharmacological effect as Liraglutide.

[0059] 5. Assay for Binding Ability of the Fusion Protein of the Present Invention to Neonatal Receptor Protein (FcRn)

[0060] According to the method described in a previous reference (The Journal of Biological Chemistry. 2001, 276 (9): 6591-6604), the fusion protein of the present invention was coated onto an ELISA plate, and the plate was added with an FcRn protein labeled with His and then incubated under acidic condition (pH=6.0). The FcRn bound to the fusion protein was detected by a murine anti-His monoclonal antibody and a goat anti-mouse antibody labeled with horseradish peroxidase (HRP) (goat anti-human IgG-HRP). The results showed that compared with the Fc section of the control antibody of IgG1 type, the Fc section of the fusion protein of the present invention has an FcRn binding ability comparable to that of IgG1. As the endocytosis and circulation of the Fc section-containing protein mediated by FcRn on vascular endothelial cells can maintain the stable state of the protein concentration in serum, the experimental results suggested that the in vivo half-life of the GLP-1-Fc fusion protein may be comparable to the IgG1 antibody, which is far higher than that of Liraglutide (which needs to be injected once a day), and realizes an injection frequency of once every 1 to 2 weeks.

[0061] 6. Assay for the Effector Activity of the Fusion Protein of the Present Invention

[0062] According to the method described in the previous references (Angiogenesis. 2004, 7: 335-345; and Cancer Res. 2010: 4481-4489), an FcγRIIIa (CD16a) protein was coated onto an ELISA plate, and the plate was added with the fusion protein of the present invention. The binding ability of the fusion protein to the FcγRIIIa (CD16a) protein was detected. The results showed that compared with the Fc section of the control antibody of IgG1 type, the Fc section of the fusion protein of the present invention exhibited very low binding to the FcγRIIIa (CD16a) protein, thereby avoiding the effector function (such as ADCC) of the Fc section.

[0063] As mentioned above, the assay results of in vitro and in vivo biological activity show that the three fusion proteins of the present invention not only have normal biological activity of GLP-1 (7-37) polypeptide, but also possess significantly longer biological half-life than GLP-1 (7-37) polypeptide; and since the effector function of the Fc section thereof is weak, the effector function that is not associated with the treatment purpose will not be induced such that the application of the medicament is more safe.

[0064] The advantageous effects of the present invention are as follows: the GLP-1 (7-37) the fusion protein maintains the natural function; the human IgG2 type Fc section extends the half-life and facilitates the purification of the fusion protein; and the effector function that is not associated with the treatment is avoided.

[0065] The reasons why the fusion protein of the present invention has an extended half-life are as follows:

[0066] 1) The GLP-1 (7-37) section is modified by replacing alanine (Ala) at position 8 with glycine (Gly), which can reduce the degradation of the fusion protein by DPP-4, thereby extending the half-life thereof; and

[0067] 2) The Fc section of the fusion protein can bind to FcRn, thereby making the half-life of the fusion protein be comparable to those of the IgG1 and IgG2 antibodies.

[0068] The following examples are included to describe how to implement the embodiments of the present invention. These examples are intended to illustrate the present invention, but not to limit the protection scope of the present invention in any manner.

Example 1

Construction of the Expression Vector Encoding the Fusion Protein of the Present Invention

[0069] The gene (SEQ ID NO: 1) encoding the GLP-1-Fc-1 fusion protein of the present invention was synthesized and cloned into pGEM-T plasmid vector by Shanghai Generay Biotech Co., Ltd. The gene contains EcoR I, Not I and Hind III enzyme cutting sites at 5' end, and TGA termination codon and Pme I, Xho I, and BamH I enzyme cutting sites at 3' end. The pGEM-T plasmid vector was designated as GLP-1-Fc-1-T.

[0070] The plasmid vector GLP-1-Fc-1-T was double digested with enzymes EcoR I and BamH I (purchased from NEB Co.) (37° C., 4 hours) according to the instruction. 1% agarose gel electrophoresis (FIG. 2A) showed that a gene fragment encoding the GLP-1-Fc-1 fusion protein with a length of about 950 bp, and a pGEM-T plasmid vector fragment with a length of about 3000 bp were generated after double enzymatic digestion. GLP-1-Fc-1 gene fragment was extracted by a gel extraction kit according to the instruction

(the gel extraction kit is purchased from Axygen Co.). Meanwhile, an expression vector 293 (FreeStyle MAX293 Expression System, K900-20, purchased from Invitrogen Co.) was double digested with enzymes EcoR I and BamH I (37° C. 4 hours). FIG. 2A shows the presence of a 293-EcoR I/BamH I fragment with a length of about 4300 bp after double enzymatic digestion. Said fragment was recovered by the above gel extraction kit.

[0071] The above two gene fragments GLP-1-Fc-1 and 293-EcoR I/BamH I obtained from enzymatic digestion were ligated via T4 DNA ligase (purchased from NEB Co.) (16° C., 16 hours). The ligation product was transformed into *E. coli* by heat shock (42° C., 90 seconds), and plated (Amp⁺ LB medium, i.e. Amp⁺-resistant Luria-Bertani medium). The resultant clones were picked and used to extract the plasmids. The plasmids were double digested with the enzymes EcoR I/BamH I (37° C., 2 hours) for identification and screening. Positive clones obtained by screening contain the successfully constructed eukaryotic expression vector 293-GLP-1-Fc-1 (as shown in FIG. 2C).

[0072] Polymerase chain reaction (PCR) was performed using GLP-1-Fc-1-T plasmid as a template and the primers 1, 2, 3 and 4, so as to give two intermediate products of the corresponding gene GLP-1-Fc-2. Subsequently, overlap-PCR was performed using the two intermediate products as the templates and the primers 1 and 4, so as to give a gene fragment GLP-1-Fc-2.

[0073] Polymerase chain reaction (PCR) was performed using GLP-1-Fc-1-T plasmid as the template and the primers 1 and 4, so as to give a gene fragment GLP-1-Fc-3.

[0074] PCR primers are set out as follows:

1. (SEQ ID NO: 7)
5' - GCGGCCGCGAATTCATGGAGTTGGGACTGTCTTG - 3' ;
2. (SEQ ID NO: 8)
5' - CCACCGCCACCGTCGCTCGCGTTTACAACACAGCTC - 3' ;
3. (SEQ ID NO: 9)
5' - GGTGCCGCTGGCAGCGAGCGCAAATGTTGTGTCGAGTGC - 3' ;
and
4. (SEQ ID NO: 10)
5' - GTTTAAACGGATCCTCAACCCGGAGACAGGGAGAG - 3' .

[0075] FIG. 2B shows the gene fragments encoding the fusion proteins GLP-1-Fc-2 and GLP-1-Fc-3 both with a length of about 950 bp. The gene fragments GLP-1-Fc-2 and GLP-1-Fc-3 were recovered by the above-mentioned method, double digested with enzymes EcoR I and BamH I (37° C., 4 hours), and ligated to 293-EcoR I/BamH I fragment obtained by double digested with enzymes EcoR I and BamH I (37° C., 4 hours) via T4 DNA ligase (16° C., 16 hours). The ligation product was transformed into *E. coli* by heat shock (42° C., 90 seconds), and plated (Amp⁺ LB medium). The resultant clones were picked and used to extract the plasmids. The plasmids were double digested with enzymes EcoR I/BamH I (37° C., 2 hours) for identification and screening. Positive clones obtained by screening contain the successfully constructed eukaryotic expression vectors 293-GLP-1-Fc-1 and 293-GLP-1-Fc-3 (as shown in FIG. 2D).

[0076] FIG. 1 is a gene structure diagram of the successfully constructed expression vector 293-GLP-1-Fc (IgG2).

The three expression vectors 293-GLP-1-Fc-1, 293-GLP-1-Fc-2 and 293-GLP-1-Fc-3 have substantially the same gene structure, except that their genes of about 950 bp between the enzyme cutting sites of EcoR I and BamH I respectively encode GLP-1-Fc-1, GLP-1-Fc-2 and GLP-1-Fc-3 fusion proteins, which respectively have the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3, and the amino acid sequences shown in SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6. FIGS. 2C and 2D are the identification results of the eukaryotic expression vectors 293-GLP-1-Fc-1, 293-GLP-1-Fc-2 and 293-GLP-1-Fc-3 by double digestion with enzymes EcoR I/BamH I, showing that they have correct fusion protein gene fragments and 293-EcoR I/BamH I fragments respectively having a length of about 950 bp and 4300 bp.

Example 2

Expression of the Fusion Protein of the Present Invention

[0077] The expression of the recombinant expression vectors 293-GLP-1-Fc-1, 293-GLP-1-Fc-2 and 293-GLP-1-Fc-3 constructed in Example 1 can be carried out by the method of transiently transfecting FreeStyle 293F cells (R790-07, purchased from Invitrogen Co.). 24 hours prior to transfection, FreeStyle 293F cells were subcultured at a concentration of 6×10^5 cells/ml, and cultured in a constant temperature shaker at 135 rpm under the condition of 37° C. and 8% CO₂, such that the cell density is about $1.2-1.5 \times 10^6$ cells/ml on the day of transfection. The cells were diluted with the FreeStyle 293F culture medium (12338-018, purchased from Invitrogen Co.) to the density of 1×10^6 cells/ml. To ensure the optimal transfection effect, the cell viability should be more than 95%.

[0078] The transfection agent FreeStyle Max Reagent (16447-500, purchased from Invitrogen Co.) was mixed well by gentle reverse mixing for 4 times. 625 µg of 293-GLP-1-Fc-1, 293-GLP-1-Fc-2 and 293-GLP-1-Fc-3 vector plasmids were added respectively to the transfection nutrient solution OptiPRO SFM (12309-050, purchased from Invitrogen Co.), and the mixture was further supplemented with OptiPRO SFM to reach a volume of 10 ml and mixed well. In another centrifuge tube, 625 µl of the FreeStyle Max Reagent was diluted with OptiPRO SFM to the volume of 10 ml, and mixed well by gentle reverse mixing. The diluted plasmid and the diluted FreeStyle Max Reagent were mixed well and incubated at room temperature for 15 minutes. 20 ml of the mixed solution was added slowly to a shake flask containing 500 ml FreeStyle 293F culture medium. The shake flask was incubated in a constant temperature shaker 135 rpm for 7 days (37° C. and 8% CO₂).

[0079] After 7 days, the cells respectively expressing the three fusion proteins of GLP-1-Fc-1, GLP-1-Fc-2 and GLP-1-Fc-3 were centrifuged at 9,000 rpm for 20 minutes in a refrigerated centrifuge. The supernate was harvested for the subsequent protein purification.

Example 3

Purification of the Fusion Protein of the Present Invention

[0080] The supernates of the FreeStyle 293F cells respectively containing the three fusion proteins of the present invention obtained in the above Example 2 were applied to a Protein A column (71-5000-09 AD, purchased from GE

Healthcare Bio-Sciences Co.) on an AKTA instrument (purchased from GE Healthcare Bio-Sciences Co.), to capture the three fusion proteins, respectively. The three fusion proteins were eluted with 50 mM citric acid-sodium citrate buffer (pH=3.3) to collect the eluates, respectively (each about 0.5 ml); and 100 µl of 1 M tris(hydroxymethyl)aminomethane-hydrochloric acid (Tris-HCL) buffer (pH=11.0) was added to neutralize the eluates to be neutral. The protein contents were determined at OD280 nm respectively, and then the proteins were dialyzed against phosphate buffer PBS (0.01M Na₂HPO₄·12H₂O+0.002M KH₂PO₄+0.14M NaCl+0.002M KCl, PH=7.2) through a 10 K dialysis membrane, filtered and sterilized through a 0.22 µm filter (purchased from Millipore Co.), and stored at -80° C.

Example 4

SDS-PAGE and Western-Blot of the Fusion Protein of the Present Invention

[0081] 10% polyacrylamide gel electrophoresis was used to determine the purity and the molecular weight of the purified three fusion proteins which have been reduced by 50 mM of DTT (DL-dithiothreitol); and Western blot was used to further identify the property and the molecular weight thereof. The resultant electrophoresis gel is transferred onto PVDF (polyvinylidene fluoride) membrane via an electrotransfer method (300 mA, 80 minutes). After the membrane was blocked with 10% skimmed milk, 1 µg/ml mouse anti-human GLP-1 (7-37) monoclonal antibody (purchased from BioPorto Co.) was added. The membrane was incubated at 4° C. overnight, and washed with PBST (PBS buffer containing 0.02% Tween-20) twice. Goat anti-mouse IgG (H+L) antibody labeled with HRP (1 µg/ml, purchased from R&D Co.) was added. The membrane was washed again with PBST twice after incubating at room temperature for 45 minutes, and finally treated by electrochemiluminescence (ECL) method for color development. The results of polyacrylamide gel electrophoresis (FIG. 3A) and Western blot (FIG. 3B) both show that under reduction conditions, all of the three fusion proteins GLP-1-Fc-1, GLP-1-Fc-2 and GLP-1-Fc-3 have a band with a molecular weight of about 40 KDa, being consistent with the theoretical molecular weight thereof. These results demonstrated that the three fusion proteins constructed by the present invention have correct structures and properties.

Example 5

Assay for In Vitro Binding Activity of the Fusion Protein of the Present Invention to the β-TC-6 Cells

[0082] 8.5×10^6 of mouse beta pancreatic tumor cells β-TC-6 (purchased from ATCC) were collected, centrifuged and fixed with 8.5 ml of fixative (IC Fixation buffer, purchased from Invitrogen Co.) (4° C., 10 min). The resultant cells were centrifuged again, resuspended with 3.4 ml of PBS, and seeded at a density of 2.5×10^5 cells/well (100 µl) in a 96-well U-shaped plate. In the meantime, the three fusion proteins of the present invention were diluted 4-fold with PBS starting from 500 µg/ml to obtain a total of 10 gradients, respectively.

[0083] The cells in the 96-well U-shaped plate were centrifuged and the supernate was removed. The diluted fusion proteins were added at 100 µl/well into the plate, and then the plate was incubated at 4° C. for 1 hour. The cells were cen-

trifuged again to remove the supernate, and washed with 200 μ l/well of PBS twice. 1 μ g/ml (100 μ l/well) of goat anti-human antibody IgG labeled with HRP (purchased from Bethyl laboratories Co.) was added, and then the plate was incubated at 4° C. for 45 minutes. After centrifugation, the cells were washed with 200 μ l/well of PBS for three times. 100 μ l/well of color developing solution (9 ml of substrate buffer+1 ml of substrate color developing solution+10 μ l of 0.3% H₂O₂ solution, wherein the substrate buffer is 0.02 M citric acid+0.01 M Na₂HPO₄·12H₂O, and the substrate color developing solution is 2 mg/ml of TMB, i.e. 3,3',5,5'-tetramethyl benzidine) were added. The plate was incubated at room temperature for color development for 15 minutes, and then 50 μ l/well of stop buffer (1 M sulfuric acid) was added. The absorbance at a wavelength of 450/570 nm was read on a M5 multifunctional microplate reader (purchased from Molecular Devices Co.), and the results are shown in FIG. 4.

[0084] As shown in FIG. 4, all of the three fusion proteins GLP-1-Fc-1, GLP-1-Fc-2 and GLP-1-Fc-3 of the present invention show a specific binding activity to GLP-1R on the surface of β -TC-6 cells.

Example 6

The Effect of the Fusion Protein of the Present Invention on cAMP Level in β -TC-6 Cells In Vitro

[0085] The β -TC-6 cells were seeded at a density of 1×10^4 cells/well (5 μ l/well, DMEM medium without serum and glucose, purchased from Gibco Co.) into a 384-well plate, to which 5 μ l/well of 5000 μ M cAMP inhibitor IBMX (3-isobutyl-1-methylxanthine, purchased from Sigma Co.) in DMEM medium without serum and glucose was added such that the final concentration of IBMX is 2500 μ M. The cells were starved in an incubator at 37° C. for 4 to 5 hours.

[0086] The 384-well plate was centrifuged 800 rpm for 1 minute. After the supernate was removed at 5 μ l/well, 250 mM glucose and 25 mM IBMX were added each at 1 μ l/well, and then the three fusion proteins of the present invention and the control medicament Liraglutide (purchased from Novo Nordisk Co.) were added each at 1 μ l/well, such that the three fusion proteins and Liraglutide all have four different final concentrations of 2, 10, 50 and 250 nM. Either non-protein group or Liraglutide group (0 nM) is used as control. Finally, DMEM medium without serum and glucose was added at 1 μ l/well, and the resultant mixture was mixed well by gently shaking the 384-well plate, and reacted at room temperature for 30 minutes.

[0087] A control curve and a standard curve were established according to the experimental procedures in the cAMP detection kit (Dynamic2 Kit, purchased from Cisbio Co.). The values were read on a M5 multifunctional microplate reader (Flu668/620 nm), and the results are shown in FIG. 5.

[0088] The results of FIG. 5 show that the three fusion proteins of the present invention at different concentrations can increase the cAMP level in mouse beta pancreatic tumor cells β -TC-6 in vitro to different degrees, and their effects are equivalent to that of Liraglutide, all showing a concentration gradient-dependent increase. The results suggest that after binding to the corresponding receptor on the cell surface, the GLP-1 fused with the Fc section can activate the intracellular signal transmission mediated by the receptor.

Example 7

The Effect of the Fusion Protein of the Present Invention on Insulin Secretion Level in β -TC-6 Cells In Vitro

[0089] The β -TC-6 cells grown on DMEM medium containing 10% FBS (fetal bovine serum, purchased from Gibco Co.) were seeded at a density of 2.5×10^5 cells/well (500 μ l) into a 24-well plate and cultured at 37° C. overnight. The supernate was removed. The cells were washed once with Krebs-Ringer Buffer (KRB buffer, 125 mM NaCl+5.9 mM KCl+1.28 mM CaCl₂+1.2 mM MgCl₂+25 mM HEPES+0.1% BSA, pH7.4), added with the KRB buffer again, and starved at 37° C. for 2 hours. The supernate was removed. The three fusion proteins and the control medicament Liraglutide at different concentrations were added, such that the four samples all have final concentrations of 0, 3, 30, 300 and 1000 nM (the diluents are KRB buffer+16.8 mM glucose). The low glucose concentration group without the four samples (containing KRB buffer+2.8 mM glucose) is used as a negative control group (neg). The reaction was carried out at 37° C. for 1 hour.

[0090] A control curve and a standard curve were established according to the experimental procedures in the insulin detection kit (Insulin Kit, purchased from Cisbio Co.). The insulin standard has an initial concentration of 20 ng/ml, and diluted into 7 gradients by two-fold dilution. In the meantime, the samples (the cell supernates) were diluted to 0-20 ng/ml (being diluted 10-fold) with KRB buffer. To a 384-well plate, the KRB buffer, the diluted standard and the diluted samples were added, each 10 μ l. Two fluorescence-labeled antibodies, anti-insulin Ab-cryptate and anti-insulin Ab-XL665, were further added, each 5 μ l/well (both of them are from Insulin Kit, purchased from Cisbio Co.). The resultant mixture was mixed well by gently shaking the 384-well plate and incubated at room temperature for 2 hours. The values were read on a M5 multifunctional microplate reader (wavelength 1: excitation/emission=314 nm/668 nm; wavelength 2: excitation/emission=314 nm/620 nm).

[0091] The data were processed as follows: Ratio=A₆₆₈ nm/A₆₂₀ nm \times 104; Delta F=(standard ratio or sample ratio-KRB ratio)/KRB ratio; a standard curve was plotted; and the insulin value of the sample was calculated.

[0092] The results of FIG. 6 show that in in-vitro normal cell medium (results not shown) or in the medium containing low concentration (2.8 mM) of glucose (neg), the three fusion proteins of the present invention or Liraglutide have no significant promotion effect on insulin secretion in mouse beta pancreatic tumor cells β -TC-6 (used as a control); while in the medium containing high concentration (16.8 mM) of glucose, the negative control group containing no sample (0.0) has somewhat increased promotion effect on insulin secretion in β -TC-6 cells, and the three fusion proteins at the concentrations of 3, 30, 300 and 1000 nM each can significantly increase insulin secretion level in β -TC-6 cells to different degrees, and their effects are comparable to Liraglutide, all showing a concentration gradient-dependent increase. The results suggest that the final effect of the GLP-1 fused with the Fc section resulted from activating the corresponding receptor on the cell surface is to promote insulin secretion in a glucose concentration-dependent manner.

Example 8

The Effect of the Fusion Protein of the Present Invention on Serum Insulin Level in the Rats Infused with High-Dose Glucose

[0093] SD (Sprague-Dawley) rats were injected subcutaneously with the three fusion proteins of the present invention (3 nM/kg) or Liraglutide (3 nM/kg) or Normal Saline (as a negative control), subjected to overnight fasting, and then sequentially administered the following substances by continuous intravenous infusion (16-18 h): Normal Saline for 20 minutes, low concentration of glucose (50 mg/kg/min) for 30 minutes, and high concentration of glucose (150 mg/kg/min) for 30 minutes. The time point at which Normal Saline infusion is finished was taken as a zero point, and blood samples were collected at -20, 0, 30 and 60 min, respectively. The blood samples were centrifuged at 4000 rpm for 10 minutes to isolate the serum. The insulin level in serum was measured by the method described in the previous references (Diabetes Metab Res Rev. 2010, 26: 287-296; and Diabetes. 2004, 53: 2492-2500).

[0094] The results in FIGS. 7A and 7B show that in the healthy rats group administered with Normal Saline by subcutaneous injection (the negative control group), after intravenous infusion with Normal Saline or low concentration of glucose, the serum insulin level has no significant increase compared with the non-intravenous infusion group, while after intravenous infusion with high concentration of glucose, the serum insulin level increases significantly; in the healthy rats group administered with the three fusion proteins of the present invention or Liraglutide by subcutaneous injection, intravenous infusion with Normal Saline still cannot induce any increase in serum insulin level, while after intravenous infusions with low concentration or high concentration of glucose, the serum insulin level increases to different degrees compared with the insulin level induced by glucose infusion of the corresponding concentrations in the group administered with Normal Saline by subcutaneous injection. The effect in experimental animal for promoting insulin secretion in a glucose concentration-dependent manner suggests that the fusion proteins of the present invention can be absorbed through subcutaneous injection, and can exert the same pharmacological effect as Liraglutide.

Example 9

Assay for Binding Ability of the Fusion Protein of the Present Invention to FcRn

[0095] The three fusion proteins of the present invention and a control IgG1 antibody (Remicade, purchased from Xi'an Janssen Pharmaceutical Ltd.) were respectively diluted to 5 µg/ml with PBS, and then added to an ELISA plate at 100 µl/well. The plate was coated at 4° C. overnight. After washing with PBST for 4 times, the plate was added with 1% BSA (Bovine Serum Albumin) at 300 µl/well, and then blocked at room temperature for 1 hour. An FcRn protein (purchased from Sino Biological Co.) was diluted into 7 gradients by two-fold dilution starting from 5 µg/ml. After the plate was washed with PBST (pH=6.0) for 4 times, the above FcRn protein was added at 100 µl/well into the ELISA plate, and the plate was incubated at room temperature for 1 hour under acidic condition (pH=6.0). After the plate was washed with PBST (pH=6.0) for 4 times, a murine anti-His monoclonal

antibody (purchased from R&D Co.) was diluted to 1 µg/ml with PBS buffer and added at 100 µl/well to the ELISA plate, and the plate was incubated at room temperature for 1 hour. After the plate was washed with PBST (pH=6.0) for 4 times, a goat anti-mouse antibody labeled with HRP (goat anti-mouse IgG-HRP, purchased from R&D Co.) was diluted to 1 µg/ml with PBS buffer and added at 100 µl/well to the ELISA plate, and the plate was incubated at room temperature for 1 hour. After the plate was washed with PBST (pH=6.0) for 4 times, TMB color developing solution was added to the plate at 100 µl/well, and the plate was incubated at room temperature for 15 minutes. After color development, a stop buffer was added at 50 µl/well. The absorbance at a wavelength of 450/570 nm was read on a M5 multifunctional microplate reader, and the results are shown in FIG. 8.

[0096] The results in FIG. 8 show that the Fc sections of the fusion proteins of the present invention all have an FcRn binding ability comparable to the Fc section of control antibody of IgG1 type. As the endocytosis and circulation of the Fc section-containing protein mediated by FcRn on vascular endothelial cells can maintain the stable state of the protein concentration in serum, the experimental results suggest that the in vivo half-life of the GLP-1-Fc fusion protein may be comparable to the antibody of IgG1 type, which is far higher than that of Liraglutide (which needs to be injected once a day), and realizes an injection frequency of once every 1-2 weeks.

Example 10

Assay for Effector Activity of the Fc Section of the Fusion Protein of the Present Invention

[0097] An FcγRIIIa (CD16a) protein (purchased from Sino Biological Co.) was diluted to 0.25 µg/ml with PBS buffer, and added to an ELISA plate at 100 µl/well. The plate was coated at 4° C. overnight. After washing with PBST for 4 times, the plate was added with 1% BSA at 300 µl/well, and then blocked at room temperature for 1 hour. The three fusion proteins of the present invention and a control antibody of IgG1 type (Herceptin, purchased from Roche Co.) were diluted into 7 gradients by 4-fold dilution starting from 200 µg/ml, and a rabbit anti-human κchain antibody (purchased from R&D Co.) was diluted into 7 gradients by 4-fold dilution starting from 100 µg/ml. Then, each fusion protein dilution was mixed well with a rabbit anti-human κchain antibody dilution in a ratio of 1:1, and the mixtures were incubated at room temperature for 1 hour. After washing with PBST for 4 times, the ELISA plate coated with FcγRIIIa (CD16a) protein was added with the incubated mixtures at 100 µl/well, and incubated at room temperature for 2 hours. After washing with PBST for 4 times, the plate was added with 1 µg/ml F(ab')₂ antibody fragment of the goat anti-human IgG H&L chain labeled with HRP (goat anti-human IgG H&L chain F(ab')₂ fragment-HRP, purchased from CalBiochem Co.) diluted with PBS at 100 µl/well, and incubated at room temperature for 1 hour. After washing with PBST for 4 times, the plate was added with TMB color developing solution at 100 µl/well, and incubated at room temperature for 15 minutes for color development. After color development, a stop buffer was added at 50 µl/well. The absorbance at a wave length of 450/570 nm was read on a M5 multifunctional microplate reader, and the results are shown in FIG. 9.

[0098] The results in FIG. 9 show that compared with the Fc section of the control antibody of IgG1 type, the Fc sections

of the three fusion proteins of the present invention exhibit very low binding to FcγRIIIa (CD16a) protein, thereby avoid- ing the effector function (such as ADCC) of the Fc section thereof and minimizing the side effects of the medicaments.

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<400> SEQUENCE: 13

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Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro
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1-10. (canceled)

11. A fusion protein obtained by fusing the C-terminus of a glucagon-like peptide-1 with the N-terminus of an IgG2 Fc section via a peptide linker.

12. The fusion protein according to claim **11**, comprising an amino acid sequence shown in SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6.

13. A gene encoding the fusion protein according to claim **11**.

14. A gene encoding the fusion protein according to claim **12**.

15. The gene according to claim **13**, comprising a nucleotide sequence shown in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

16. The gene according to claim **14**, comprising a nucleotide sequence shown in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

17. A method for preparing the fusion protein according to claim **11**, comprising:

- a) constructing a gene encoding the fusion protein according to claim **11**;
- b) cloning the gene obtained in step a) into a eukaryotic expression vector, to obtain a eukaryotic expression vector that can express the fusion protein according to claim **11**; and
- c) using the expression vector obtained in step b) to transfect cells, so as to express the recombinant fusion protein in the cells, and then isolating and purifying the fusion protein.

18. A method for preparing the fusion protein according to claim **12**, comprising:

- a) constructing a gene encoding the fusion protein according to claim **12**;
- b) cloning the gene obtained in step a) into a eukaryotic expression vector, to obtain a eukaryotic expression vector that can express the fusion protein according to claim **12**; and

c) using the expression vector obtained in step b) to transfect cells, so as to express the recombinant fusion protein in the cells, and then isolating and purifying the fusion protein.

19. The method according to claim **17**, wherein the eukaryotic expression vector is the eukaryotic expression vector 293.

20. The method according to claim **18**, wherein the eukaryotic expression vector is the eukaryotic expression vector 293.

21. The method according to claim **17**, wherein the cells to be used are FreeStyle 293F cells.

22. The method according to claim **18**, wherein the cells to be used are FreeStyle 293F cells.

23. A formulation, comprising the fusion protein according to claim **11** as an active component, and optionally further comprising one or more pharmaceutically acceptable carriers.

24. A formulation, comprising the fusion protein according to claim **12** as an active component, and optionally further comprising one or more pharmaceutically acceptable carriers.

25. Use of the fusion protein according to claim **11**, or a formulation comprising the same, in the manufacture of a medicament for the treatment and/or prevention of type II diabetes, obesity, and other diseases that can benefit from decreasing serum glucose, suppressing gastrointestinal motility, and emptying or suppressing food intake.

26. Use of the fusion protein according to claim **12**, or a formulation comprising the same, in the manufacture of a medicament for the treatment and/or prevention of type II diabetes, obesity, and other diseases that can benefit from decreasing serum glucose, suppressing gastrointestinal motility, and emptying or suppressing food intake.

27. Use of the gene according to claim **13** in the manufacture of a medicament for the treatment and/or prevention of type II diabetes, obesity, and other diseases that can benefit from decreasing serum glucose, suppressing gastrointestinal motility, and emptying or suppressing food intake.

28. Use of the gene according to claim **14** in the manufacture of a medicament for the treatment and/or prevention of type II diabetes, obesity, and other diseases that can benefit from decreasing serum glucose, suppressing gastrointestinal motility, and emptying or suppressing food intake.

29. Use of the gene according to claim **15** in the manufacture of a medicament for the treatment and/or prevention of type II diabetes, obesity, and other diseases that can benefit from decreasing serum glucose, suppressing gastrointestinal motility, and emptying or suppressing food intake.

30. Use of the gene according to claim **16** in the manufacture of a medicament for the treatment and/or prevention of type II diabetes, obesity, and other diseases that can benefit from decreasing serum glucose, suppressing gastrointestinal motility, and emptying or suppressing food intake.

* * * * *