

PEPTIDE WITH ANTI-OBESITY AND ANTI-DIABETES ACTIVITY AND
USE THEREOF

Technical Field

5 This application claims priority to Korean Patent Application No.10-2015-0059648, filed April 28, 2015, to the KIPO. The content of the above applications are incorporated by reference in their entirety.

10 The present invention relates to a peptide with anti-obesity and anti-diabetes activity, and use thereof.

Background Art

15 In Korea, dietary fat intake has recently increased with the growth of economy and the westernization of diet life, and onset of metabolic diseases such as obesity, diabetes, hyperlipidemia, hypertension, arteriosclerosis, and fatty liver increased due to insufficient exercise. In addition, 20 obesity is an aesthetic problem to people who generally tend to prefer to slim body types as well as being associated with various disorders.

 To date, therapeutic agents for obesity may be largely divided into drugs that act on the central 25 nervous system to affect appetite and drugs that act on the gastrointestinal tract to inhibit uptake. Drugs acting on the central nervous system were placed on the market as anti-obesity drugs which work on the serotonin (5-HT) in the nervous system such as fenfluramine, 30 dexfenfluramine and the like, on the noradrenaline nervous system such as ephedrine and caffeine, and on both the serotonin and the noradrenaline nervous system such as recently developed sibutramine, as classified by acting mechanisms. Representative of anti-obesity drugs 35 acting on the gastrointestinal tract is orlistat,

approved as a therapeutic agent for obesity, which inhibits intestinal lipase to reduce fat uptake. There are problems with some of the pre-existing drugs. For example, fenfluramine and the like have been prohibited
5 from being marketed due to the side effect of incurring primary pulmonary hypertension or valvular heart disease, and other drugs cannot be applied to patients with heart failure or kidney failure due to the occurrence of blood pressure reduction or lactic
10 acidosis.

Diabetes is a group of metabolic disorders caused when insulin is insufficiently secreted or in does that do not enable normal function (DeFronzo, 1988) and is characterized by hyperglycemia, that is, high blood
15 sugar levels over a prolonged period, which causes various symptoms and syndromes, with glucose in urine. In recent years, the prevalence of obesity, particularly, abdominal obesity has increased, leading to the explosion of the prevalence of diabetes.

20 As of 2000, diabetes patients were estimated to be 170 million worldwide and expected to increase to 370 million people in 2030. However, a 2008 analysis report showed that the number of diabetes patients may have already reached 350 million worldwide (Danaei et al.,
25 2011), with far more significant aggregation than expectation. It is reported that more than about 80 % of type 1 diabetes patients are obese whereas only less than 10 % of (non-)obese patients have diabetes (Harris et al.. 1987). The correlation between diabetes and
30 obesity is attributed to the fact that adipokines and free fatty acids are irregularly secreted to induce fatty acids to accumulate in insulin-sensitive tissues such as beta cells, kidneys, liver, heart, etc., resulting in lipotoxicity. If left without suitable
35 treatment, chronic hyperglycemia may be prone to

incurring various pathological symptoms including retinopathy, renal dysfunction, neuropathy, and vascular disorder. Indispensable for preventing such complications is effective blood sugar management.

5 Nowadays, the control of blood sugar levels is accomplished by lifestyle improvement (diet therapy, exercise therapy), and medications. However, diet therapy or exercise therapy is difficult to strictly manage and practice, with limitations of the effects
10 thereof. Hence, most patients with diabetes rely on the control of blood sugar levels by medications such as insulin, insulin secretagogues, insulin sensitizer, and hypoglycemic agents, as well as lifestyle improvement.

 Insulin produced using a recombinant method is
15 used as a drug indispensable to type 1 diabetes patients and type 2 diabetes patients which fail to control blood sugar levels, and is advantageous in blood sugar control. However, it suffers from the disadvantage of repulsion to syringe needles, difficulty in
20 administration, hypoglycemic risk, and weight gain.

 Meglitinides, a kinds of insulin secretagogues, are short-acting agents and are taken before meals. Among them are NovoNorm (repaglinide), Fastic (nateglinide), and Glufast (mitiglinide). Insulin
25 sensitizers are characterized by almost no hyperglycemic incurrence when taken alone, and may be exemplified by biguanide drugs, such as metformin, and thiazolidinedione drugs such as Avandia (rosiglitazone) and Actos (pioglitazone).

30 Recently, GLP-1 agonists have been developed using the action of glucagon-like peptide-1, which is an insulin secretion-stimulating hormone, and include exenatide and Victoza (liraglutide). In addition, DDP-4 inhibitors, which inhibit the action of DPP (dipeptidyl
35 peptidase-4), an enzyme responsible for the rapid

inactivation of GLP-1, are newly developed drugs and are representatively exemplified by Januvia (ingredient name: sitagliptin). However, those drugs are reported to have side effects of hepatotoxicity, gastrointestinal disorders, cardiovascular disorders, and carcinogenicity. Another problem with the drugs is a high annual treatment cost, which is a barrier to the treatment of diabetes. Indeed, health care costs of pre-diabetes and diabetes approached about 200 trillion won in the USA as of 2007 (Dall et al., 2010), and health care costs of obesity are also near 150 trillion won only in the USA as of 2008 (Finkelstein et al., 2009). Therefore there is an urgent need for the development of a drug that can effectively lower blood glucose levels and can be applied to both diabetes and obesity-induced diabetes, with less side effects.

For this, the present inventors have recently paid attention to energy metabolism-regulating mechanisms in order to find an improved method for the treatment of obesity, and have made research of signals responsible for lipid accumulation and proteins affecting lipid accumulation upon the intake of high-fat diets in humans, with the premise that the compound to be developed should of higher safety (lower toxicity). As a result of research on signals for suppressing the expression of proteins responsible for fat accumulation and for degrading accumulated fat and on proteins involved in the signaling, the present inventors succeeded in developing peptides that promote lipolysis. In addition, the peptides of the present invention exhibit outstanding therapeutic efficacy on diabetes and obesity-induced diabetes. The fat accumulation induced by high-fat diets, the suppression of insulin signaling attributed to fat accumulation in the liver or muscle, and resulting insulin tolerance are causes of diabetes.

Each and complexes of the peptides according to the present invention are therapeutically effective for such diabetes and obesity-induced diabetes.

Throughout this specification, reference is made
5 to many papers and patent documents, with citations thereof indicated. The disclosures of the cited papers and patent documents are herein entirely incorporated by reference and thus the level of technical field to which the present invention belong and contents of the present
10 invention are explained more definitely.

Detailed Description of the Invention

Technical Problem

Culminating in the present invention, intensive
15 and thorough research on the development of plural outstanding peptides having biologically effective activity, conducted by the present inventors, led to the finding that peptides having the amino acid sequences of SEQ ID NOS: 1 to 7 exhibit not only anti-obesity effects
20 by suppressing high-fat diet-induced fat accumulation and degrading already accumulated fat, but also high therapeutic effects on diabetes and obesity-induced diabetes, and diabetes complications.

Accordingly, an object of the present invention is
25 to provide peptides having the amino acid sequences of SEQ ID NOS: 1 to 7.

Another object of the present invention is to provide a peptide having anti-obesity or anti-diabetes activity.

30 A further object of the present invention is to provide a peptide complex having anti-obesity or anti-diabetes activity

A still further object of the present invention is to provide a pharmaceutical composition for the
35 prevention or treatment of obesity.

Still another object of the present invention to provide a pharmaceutical composition for the prevention or treatment of diabetes.

Other purposes and advantages of the present invention will become clarified by the following detailed description of the invention, claims, and drawings.

Technical Solution

One embodiment of the present invention provides a peptide having one selected from the group consisting of the amino acid sequences of SEQ ID NOS: 1 to 7.

Another embodiment of the present invention provides a peptide of anti-obesity and anti-diabetes activity having one selected from the group consisting of the amino acid sequences of SEQ ID NOS: 1 to 7.

Provided according to another embodiment of the present invention is a peptide complex of anti-obesity and anti-diabetes activity, composed of the following peptide combination:

- (a) a peptide having the amino acid sequence of SEQ ID NO: 1;
- (b) a peptide having the amino acid sequence of SEQ ID NO: 2 or 3; and
- (c) a peptide having the amino acid sequence of SEQ ID NO: 6 or 7.

As a result of the effort of the present inventors to develop plural outstanding peptides having biologically effective activity, it was found that peptides having the amino acid sequences of SEQ ID NOS: 1 to 7 suppress high-fat diet-induced fat accumulation and degrade already accumulated fat, thus exhibiting an anti-obesity effect and a therapeutic effect on diabetes and obesity-induced diabetes, or diabetes complications.

As used herein, the term "peptide" refers to a

linear molecule of amino acid residues linked by peptide bonds. The peptides of the present invention may be prepared using chemical synthesis methods known in the art, especially solid-phase synthesis techniques
5 (Merrifield, J. Amer. Chem. Soc. 85:2149-54(1963); Stewart, et al., Solid Phase Peptide Synthesis, 2nd. ed., Pierce Chem. Co.: Rockford, 111(1984)) or a liquid-phase synthesis method (US Pat. No. 5,516,891).

In order to select regions of the amino acid
10 sequences thereof and increase the activity thereof, the peptides of the present invention may be modified at N- or C-terminals thereof. Through such modification, the peptides of the present invention may be imparted with a prolonged half-life after in vivo administration.

15 Further, C-terminals of the peptides of the present invention may be modified with a hydroxy group (-OH), an amino group (-NH₂), an azide group (-NHNH₂), etc. while N-terminals may be coupled with a protecting radical consisting of the group consisting of acetyl,
20 fluorenyl methoxy carbonyl, formyl, palmitoyl, myristyl, stearyl, and polyethylene glycol (PEG).

Through the above stated amino acid modification, the peptides of the present invention can greatly increase in stability. As used herein, the term
25 "stability" is intended to refer to both in vivo stability and storage stability (e.g., stability during storage at room temperature). The protecting group acts to protect the peptides of the present invention against the attack of proteinases in vivo.

30 According to one embodiment of the present invention, the peptides of the present invention exhibit the effect of suppressing high-fat diet-induced fat accumulation and degrading already accumulated fat, decrease the expression of the adipogenic markers PPAR γ ,
35 ACC, and aP2, increase the expression of the lipolytic

factors pHSL, AMPK- α 1, CGI-58, and ATGL, reduce the size of adipose cells, and lower blood cholesterol levels. These results indicate that the peptides of the present invention have excellent therapeutic effects on obesity,
5 diabetes, and obesity-induced diabetes.

Not only individual peptides of SEQ ID NOS: 1 to 7, but also a complex thereof exhibits excellent anti-obesity and anti-diabetes activity.

According to the present invention, the peptides
10 of SEQ ID NOS: 3, 5, and 7 correspond respectively to those of SEQ ID NOS: 2, 4, and 6, with the exception that the Cys residue is substituted with the Ser residue. The corresponding paired peptides are almost identical in terms of anti-obesity and anti-diabetes
15 activity.

In accordance with an embodiment of the present invention, the peptide complex exhibiting anti-obesity or anti-diabetes activity is composed of a peptide having the amino acid sequence of SEQ ID NO: 1; a
20 peptide having the amino acid sequence of SEQ ID NO: 2 or 3; and a peptide having the amino acid sequence of SEQ ID NO: 6 or 7.

According to another embodiment of the present invention, the peptide complex of the present invention
25 is composed of peptides having the respectively amino acid sequences of SEQ ID NOS: 1, 3, and 7.

Contemplated in accordance with another aspect of the present invention is a pharmaceutical composition comprising the peptide or peptide complex of the present
30 invention as an effective ingredient for preparing or treating obesity.

Superior in terms of anti-adipogenetic and lipolytic functions, the peptide or peptide complex of the present invention can be useful for the prophylaxis
35 or therapy of obesity.

A further aspect of the present invention provides a pharmaceutical composition comprising the peptide or peptide complex of the present invention as an effective ingredient for preventing or treating diabetes.

5 Functioning to effectively lower an increased blood sugar level in diabetes animal models, the peptide or peptide complex of the present invention can find applications in the prophylaxis or therapy of diabetes.

 According to some particular embodiments of the
10 present invention, the composition of the present invention is a pharmaceutical composition comprising:
 (a) a pharmaceutically effective amount of the peptide or peptide complex of the present invention; and a pharmaceutically acceptable carrier.

15 The term "pharmaceutically effective amount", as used herein, means a sufficient amount to achieve the above-stated efficacy or activity of the peptide.

 The pharmaceutically acceptable carrier contained in the pharmaceutical composition of the present
20 invention may be that commonly used in drug formulations and include, but are not limited to, lactose, dextrose, sucrose, sorbitol, mannitol, starch, acacia gum, calcium carbonate, alginate, gelatin, calcium silicate, microcrystalline cellulose, polyvinyl pyrrolidone,
25 cellulose, water, syrup, methyl cellulose, methyl hydroxybenzoate, propyl hydroxybenzoate, talc, magnesium stearate, and mineral oil. In addition to those ingredients, the pharmaceutical composition of the present invention may further comprise a lubricant, a
30 humectant, a sweetener, a flavorant, an emulsifier, a suspending agent, and a preservative. With regard to pharmaceutically acceptable carriers and agents suitable for use, reference may be made to Remington's Pharmaceutical Sciences (19th ed., 1995).

35 The pharmaceutical composition of the present

invention may be administered orally or parenterally. For parenteral administration, intramuscular, intravenous, subcutaneous, intraperitoneal, topical, or transcutaneous routes may be used.

5 The dosage of the pharmaceutical composition according to the present invention may vary depending on various factors, including dosage form, administration modality, the patient's age, weight, gender, state of health, diet, the time of administration, the route of
10 administration, excretion rate, sensitivity, etc. For example, the pharmaceutical composition according to the present invention may be administered at a daily dose in the range of 0.0001 to 1, 000 µg.

 The pharmaceutical composition according to the
15 present invention may be prepared in single-dose forms or in multi-dose packages using a pharmaceutically acceptable carrier and/or excipient according to a method that may be easily carried out by those skilled in the art. Herein, the formulation of the
20 pharmaceutical composition may be a solution, suspension or emulsion of the pharmaceutical composition in oil or aqueous medium, or an extract, powder, granule, tablet or capsule containing the pharmaceutical composition, and may further comprise a dispersing agent or a
25 stabilizer.

Advantageous Effects

 Features and advantages of the present invention are summarized as follows:

30 (i) the peptides and the peptide complex of the present invention exhibit not only an anti-obesity effect by suppressing fat accumulation and degrading already accumulated fats, but also an outstanding therapeutic effect on diabetes by effectively reducing
35 blood sugar levels.

(ii) the peptides and the peptide complex of the present invention decrease the expression of the adipogenic markers PPAR γ , ACC, and aP2, increase the expression of the lipolytic factors pHSL, AMPK- α 1, CGI-58, and ATGL, thus reducing adipocyte sizes and blood cholesterol levels.

(iii) the peptides and the peptide complex of the present invention have excellent activity and safety and thus can be advantageously applied to drugs and quasi-
10 drugs.

Brief Description of the Drawings

FIG. 1 shows lipids accumulated after treatment with peptides of the present invention, as analyzed by
15 Oil red O staining: (a) peptide of SEQ ID NO: 1, (b) peptide of SEQ ID NO: 3, and (c) peptide of SEQ ID NO: 5.

FIG. 2 shows results of lipid accumulation after treatment with the peptide complex of the present
20 invention, as analyzed by Oil red O staining.

FIG. 3 shows measurement results of the expression levels of the gene aP2, which is involved in adipogenesis, after treatment with the peptides of the present invention: (a) peptide of SEQ ID NO: 1, (b)
25 peptide of SEQ ID NO: 3, and (c) peptide of SEQ ID NO: 5.

FIG. 4 shows measurement results of expression levels of PPAR γ , ACC, and aP2 genes, which play an important role in adipogenesis, after treatment with
30 various concentrations of the peptide complex of the present invention.

FIG. 5 shows measurement results of expression levels of PPAR γ and phospho-HSL, which play an important role in adipogenesis, after various concentrations of
35 the peptide complex of the present invention.

FIG. 6 shows measurement results of expression levels of AMPK- α and CGI58 genes, which are involved in the degradation of accumulated fats, after treatment with the peptides and peptide complex of the present invention: (a) peptide of SEQ ID NO: 1, (b) peptide of SEQ ID NO: 3, (c) peptide of SEQ ID NO: 5, and (d) a complex of peptides of SEQ ID NOS: 1, 3, and 7.

FIG. 7 shows measurement results of ATGL, a protein involved in the degradation of accumulated fats, after treatment with various concentrations of the peptide complex of the present invention.

FIG. 8 shows results of expression levels of the Phospho-HSL protein involved in the degradation of accumulated fats, after treatment with the peptides of the present invention: (a) peptide of SEQ ID NO: 1, (b) peptide of SEQ ID NO: 3, (c) peptide of SEQ ID NO: 5, and (d) a complex of peptides of SEQ ID NOS: 1, 3, and 7, as measured by immunostaining.

FIG. 9 shows measurement results of glycerol produced after treatment with various concentrations of the peptides complex of the present invention.

FIG. 10a shows adipose tissues degraded in obese mouse experiment models after treatment with the peptide complex of the present invention. FIG. 10b shows sizes and numbers of the adipose tissues degraded in obese mouse experiment models after treatment with the peptide complex of the present invention.

FIG. 11 shows results of the expression levels of the Phospho-HSL protein which is involved in the degradation of accumulated fats after treatment with the peptide complex of the present invention, as measured by immunostaining.

FIG. 12 shows changes in body weight (g) and diet intake of obese mice after treatment with the peptide complex of the present invention.

FIG. 13 shows images of obese mice after treatment of the peptide complex of the present invention.

FIG. 14 shows results of fat distribution in obese mouse models induced by feeding a high-fat diet to the experimental animal model C57BL/6, as analyzed by micro-CT.

FIG. 15 shows images of the adipocyte tissues extracted from obese mouse models induced by feeding a high-fat diet to the experimental animal model C57BL/6, after treatment with the peptide complex of the present invention.

FIG. 16a shows morphological images of the adipocytes in adipose tissues taken from obese mouse models induced by feeding a high-fat diet to the experimental animal model C57BL/6, after treatment with the peptide complex of the present invention. FIG. 16b shows size results of the adipocytes in the adipose tissues taken from obese mouse models induced by feeding a high-fat diet to the experimental animal model C57BL/6, after treatment with the peptide complex of the present invention.

FIG. 17 shows measurement results of the expression levels of the phosphor-HSL protein, which is involved in lipolysis, in adipocytes of adipose tissues taken from obese mouse models induced by feeding a high-fat diet to the experimental animal model C57BL/6, after treatment with the peptide complex of the present invention.

FIG. 18 shows measurement results of cholesterol levels in blood samples taken from obese mouse models induced by feeding a high-fat diet to the experimental animal model C57BL/6, after treatment with the peptide complex of the present invention.

FIG. 19 shows measurement results of glucose levels in blood samples taken from obese mouse models

induced by feeding a high-fat diet to the experimental animal model C57BL/6, after treatment with the peptide complex of the present invention.

5 FIG. 20 shows changes in blood sugar level in obesity-induced DB/DB mouse models after treatment with the peptide complex of the present invention.

FIG. 21 shows changes in blood cholesterol level in obesity-induced DB/DB mouse models after treatment with the peptide complex of the present invention.

10 FIG. 22 shows changes in blood sugar level in obesity-induced DB/DB mouse models after treatment with the peptides of the present invention: (a) peptide of SEQ ID NO: 1, (b) peptide of SEQ ID NO: 3, and (c) peptide of SEQ ID NO: 5.

15 FIG. 23 shows measurement results of expression levels of IGF-1 and insulin after treatment with the peptide of SEQ ID NO: 7.

20 FIG. 24 shows changes in blood sugar level in obesity-induced DB/Db mouse model after treatment with the peptide of SEQ ID NO: 7.

FIGS. 25a-25d show changes in blood sugar levels in diabetes patients having high blood glucose levels after treatment with the peptide complex of the present invention.

25

Mode for Carrying Out the Invention

Hereinafter, the present invention will be described in further detail with reference to examples. It will be obvious to a person having ordinary skill in the art that these examples are illustrative purposes only and are not to be construed to limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

35

EXAMPLES

SYNTHESIS EXAMPLE 1: Peptide Synthesis

In a reactor, 700 mg of chlorotrityl chloride resins (CTL resins, Nova biochem Cat No. 01-64-0021) was added with 10 ml of methylene chloride (MC) and stirred for 3 min. After removal of the solvent, 10 ml of dimethyl formamide (DMF) was added. The solution was stirred again for 3 min, and then the solvent was removed. To the reactor was added 10 ml of a dichloromethane solution, followed by 200 mmole of Fmoc-Asn(Trt)-OH (Bachem, Swiss) and 400 mmole of diisopropyl ethylamine (DIEA). The reactants were well dissolved and reacted while stirring for 1 hour. Thereafter, the solution was washed, and reacted with a solution of methanol and DIEA (2:1) in DCM (dichloromethane) for 10 min. Subsequent to washing with an excess of DCM/DMF (1:1), the solvent was removed. Then, 10 ml of dimethyl formamide (DMF) was added, followed by stirring for 3 min. After removal of the solvent, 10 ml of a deprotecting solution (20 % piperidine/DMF) was added to the reactor. Stirring at room temperature for 10 min was precedent to the removal of the solvent. The deprotecting solution was added in the same amount and then removed after 10 min of reaction. Washing was performed twice with DMF, once with MC, and once with DMG for 3 min each wash to afford Asn-CTL resins. In another reactor, 200 mmole of Fmoc-Arg(Pbf)-OH (Bachem, Swiss), 200 mmole of HoBt, and 200 mmole of Bop were added to 10 ml of a DMF solution and well dissolved by stirring. To the reactor, 400 mmole of DIEA was added in two aliquots, followed by stirring for at least 5 min to the complete dissolution of the solid. The dissolved amino acid mixture solution was introduced into the reactor in which the deprotected resins were placed, followed by stirring for 1 hour at room temperature for

reaction. After the reaction liquid was removed, stirring was carried three times for 3 min each time, together with a DMF solution which was then removed. A small amount of the reaction resins was taken and used
5 in a Kaiser test (ninhydrin test) for examining an extent of the reaction. The same deprotection reaction was performed twice with the deprotecting solution to give Arg-Asn-CTL resins. The resins were sufficiently washed with DMF and MC before an additional Kaiser test.
10 The following amino acid attachment experiments were carried out in the same manner as described above. According to selected amino acid sequences, reactions were sequentially induced with Fmoc-Thr(tBu)-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Leu-OH in that order. The Fmoc-
15 protecting group was removed by reacting twice with a deprotecting solution for 10 min for each reaction and then well washing. Acetic anhydride, DIEA, and HoBt were added and subjected to acetylation for 1 hour. The peptidyl resins thus obtained were washed with DMF, MC,
20 and methanol three times each. The resins were dried with nitrogen gas slowly flowed and then were completely vacuum-dried under a P2O5 atmosphere. The resins were reacted for 2 hours at room temperature with 30 ml of a leaving solution (trifluoroacetic acid 81.5%, distilled
25 water 5%, thioanisole 5%, phenol 5%, EDT 2.5%, and TIS 1%) while intermittently agitating. The resins were filtered and washed with a small volume of TFA solution, after which the filtrate was combined with the mother liquid. After distillation at a reduced pressure to
30 reduce the total volume by two, 50 ml of cold ether was used to induce precipitation, and the precipitates thus formed were collected by centrifugation and washed twice with cold ether. After removal of the mother liquid, the remainder was sufficiently dried under a nitrogen
35 atmosphere to afford 0.85 g of the unpurified peptide of

SEQ ID NO: 1 NH₂-Leu-Lys-Thr-Arg-Asn-COOH (yield: 92%). Synthesis was made of NH₂-Lys-Gly-Ala-Cys(Ser)-Thr-Gly-Trp-Met-Ala-COOH in an amount of 0.78 g as peptides of SEQ ID NOS: 2 and 3 (yield: 82%), NH₂-Ala-Cys(Ser)Thr-Leu-Pro-His-Pro-Trp-Phe-Cys(Ser)-COOH in an amount of 0.92 g as peptides of SEQ ID NOS: 4 and 5 (yield: 85%), and NH₂-Cys(Ser)-Asp-Leu-Arg-Arg-Leu-Glu-Met-Tyr-Cys(Ser)-COOH in an amount of 0.76 g as peptides of SEQ ID NOS: 6 and 7 (yield: 88%). The peptides of SEQ ID NOS: 1, 2, 4, and 6 were found to have molecular weights of 630.7 (calc.: 630.7), 924.5 (calc.: 924.1), 1236 (calc.: 1236.5), and 1301.5 (calc.: 1301.5), respectively, as measured by mass spectrometry.

15

TABLE 1

Peptide	Amino Acid Sequence	Analysis (Mass spectrometry)	
		Measured	Calculated
SEQ ID NO: 1		630.7	630.7
SEQ ID NO: 2	KGACTGWMA	924.5	924.1 (908.0)
SEQ ID NO: 3	KGASTGWMA		
SEQ ID NO: 4	ACYLPHPWFC	1236	1236.5 (1269.4)
SEQ ID NO: 5	ASYLPHPWFS		
SEQ ID NO: 6	CDLRRLEMYC	1301.5	1301.5
SEQ ID NO: 7	SDLRRLEMYS		

Meanwhile, peptides of SEQ ID NOS: 1, 3, and 7 were mixed in equal amounts to give a peptide complex which was evaluated for efficacy.

20

EXAMPLE 1: Assay for Inhibitory Activity against Adipogenesis

1-1. Assay for suppression of lipid accumulation by use of pre-adipocyte (Oil red O staining)

25

The pre-adipocytes 3T3-L1 cells were grown to

confluence and then incubated for two days with various concentrations of the peptides in a differentiation medium containing 10 µg/ml insulin, 0.1 µM dexamethasone, and 0.5 µM IBMX. The medium was exchanged
5 every two days with a medium containing 10 µg/ml insulin. After differentiation was induced for 10 days, the generation of droplet in the cells was examined by Oil Red O staining. The prepared 3T3-L1 adipocytes were washed with PBS, fixed with 3.7% formalin for one hour,
10 and washed with 60% isopropanol. The resulting cells were dyed with Oil Red O reagent at room temperature for 20 min. After removal of the Oil Red O reagent, the cells were washed three times with distilled water, and observed under a phase-contrast microscope. For
15 quantitative analysis, fats were extracted from the cells using 100 % isopropanol, and the cells were transferred in an amount of 200 µl/well into 96-well plates and measured for optical density at 500 nm using an ELISA reader.

20 Experimental data showed that treatment with peptides of SEQ ID NOS: 1, 3, and 5 decreased extents of fat accumulation in cells, as measured by Oil red O staining (FIGS. 1a-c).

An extent of lipid accumulation in cells was also
25 reduced when a complex of peptides of SEQ ID NOS: 1, 3, and 7 was applied by concentrations (FIG. 2).

1-2. Suppression of expression of genes involved in adipogenesis

30 3T3-L1 cells (pre-adipocytes) were seeded at a density of 3×10^5 cells/well into 6-well plates. After 24 hours of culturing, the cells were incubated at with predetermined concentrations (0.1, 1, and 10 µg/ml) of the peptides for 14 days in a 37°C incubator.
35 Thereafter, the cells were harvested and treated with an

RNA extraction solution (Easy Blue, Intron) to prepare RNA from which cDNA was then synthesized using an RT premix (Intron). PCR was performed using primers for antigenic markers (PPAR γ , ACC, and aP2), and a PCR
5 premix (Intron).

Target-specific primer sequences for PCR of adipogenic markers were as follows: PPAR γ forward primer sequence, 5'-TTTTCAAGGGTGCCAGTTTC-3' and PPAR γ reverse primer, 5'-AATCCTTGGCCCTCTGAGAT-3' (annealing
10 temperature, 60°C); ACC forward primer sequence, 5'-ACCTTACTGCCATCCCATGTGCTA-3' and ACC reverse primer, 5'-GTGCCTGATGATCGCACGAACAAA-3' (annealing temperature, 60°C); aP2 forward primer sequence, 5'-CATCAGCGTAAATGGGGATT-3' and aP2 reverse primer, 5'-
15 ACACATTCCACCACAGCTT-3' (annealing temperature, 60°C).

PCR products were each loaded in a volume of 5 μ l into a 1 % agarose gel, and electrophoresed, followed by identifying bands in a Gel-Doc.

In the mouse osteoblast cell line 3T3-L1 which was
20 incubated with the peptide of SEQ ID NO: 1, 3, or 5 for three days, decreased expression levels of the adipogenic marker aP2 were observed (FIGS. 3a-c).

Also, when incubated for three days with concentrations of 0.1 μ g/ml, 1 μ g/ml, and 10 μ g/ml of a
25 complex of peptides of SEQ IS Nos: 1, 3 and 7, the mouse osteoblast cell line was observed to decrease in the expression of the adipogenic markers PPAR γ , ACC, and aP2, like the positive control cells treated with 100 ng/ml TNF α (FIG. 4).

30

1-3. Expression observation of adipogenesis and lipolysis inducing proteins by use of pre-adipocyte

3T3-L1 cells (pre-adipocytes) were seeded at a density of 3x10⁵ cells/well into 6-well plates. After 24
35 hours of culturing, the cells were incubated for 14 days

with predetermined concentrations (0.1, 1, and 10 µg/ml) of the peptide complex in a 37°C incubator. Cell lysates obtained by treatment with a cell lysis buffer were used for protein quantitation, followed by Western blotting
5 with an anti-PPAR γ antibody (Santa Cruz Biotechnology, USA), which is an antibody against an adipogenic marker, and an anti-pHSL antibody (Santa Cruz Biotechnology, USA), which is an antibody against an lipolytic marker.

When treated with the peptide complex by
10 concentration, the cells were observed to decrease in the expression of the adipogenic marker PPAR γ in a dose-dependent manner while all increasing in the expression of the lipolysis marker pHSL (FIG. 5).

15 EXAMPLE 2: Assay for Lipolytic Activity

2-1. Increased expression of genes involved in lipolysis

3T3-L1 cells (pre-adipocytes) were seeded at a density of 3x10⁵ cells/well into 6-well plates. After 24
20 hours of culturing, the cells were incubated for 14 days with predetermined concentrations (0.1, 1, and 10 µg/ml) of the peptides in a 37°C incubator (positive control: 100 ng/ml TNF α (SIGMA)). The cells were harvested and treated with an RNA extraction solution (Easy Blue,
25 Intron) to prepare RNA from which cDNA was then synthesized using an RT premix (Intron). PCR was performed using primers for markers (AMPK- α 1 and CGI58), and a PCR premix (Intron).

Target-specific primer sequences for PCR of
30 lipolytic markers were as follows: AMPK- α 1 forward primer sequence, 5'-TGACCGGACATAAAGTGGCTGTGA-3' and AMPK- α 1 reverse primer, 5'-TGATGATGTGAGGGTGCCTGAACA-3' (annealing temperature, 60°C); CGI58 forward primer sequence, 5'-TGTGCAGGACTCTTACTTGGCAGT-3' and CGI58
35 reverse primer, 5'-GTTTCTTTGGGCAGACCGGTTTCT-3' (annealing

temperature, 60°C).

PCR products were each loaded in a volume of 5 µl into a 1 % agarose gel, and electrophoresed, followed by identifying bands in a Gel-Doc.

5 In all of the pre-adipocytes (3T3-L1) which were incubated with the peptides, increased expression levels of the lipolytic markers AMPK-α1 and CGI-58 were detected (FIGS. 6a-c). In addition, treatment with the peptide complex was observed to increase the expression
10 of AMPK-α1 and CGI-58 in dose-dependent manners and to higher levels compared to the positive control TNFα 100 ng/ml treatment (FIG. 6d).

2-2. Expression observation of lipolysis inducing
15 proteins by use of pre-adipocyte

3T3-L1 cells (pre-adipocytes) were seeded at a density of 3x10⁵ cells/well into 6-well plates. After 24 hours of culturing, the cells were incubated for 14 days with predetermined concentrations (0.1, 1, and 10 µg/ml)
20 of the peptide complex in a 37°C incubator (positive control: 100 ng/ml TNFα(SIGMA)). Cell lysates obtained by treatment with a cell lysis buffer were used for protein quantitation, followed by Western blotting with an anti-ATGL antibody (Santa Cruz Biotechnology, USA),
25 which is an antibody against an lipolytic marker.

The expression of the lipolytic marker ATGL was increased by treatment with the peptide complex (FIG. 7).

30 2-3. Fluorescence microscopic observation of expression of lipolysis inducing protein by use of pre-adipocyte

3T3-L1 cells (pre-adipocytes) were seeded at a density of 3x10⁵ cells/well into 6-well plates. After 24
35 hours of culturing, the cells were incubated for 14 days

with the individual peptides or the peptide complex (1 µg/ml) in a 37°C incubator (positive control: 100 ng/ml TNFα (SIGMA)). Thereafter, the cells were fixed with 70 % ethanol and then subjected to immunostaining with
5 an anti-phospho-HSL antibody (Santa Cruz Biotechnology, USA) to observe the cellular expression of phospho-HSL, a lipolytic marker.

From the experimental data, the peptides alone (FIGS. 8a-c) and the peptide complex (FIG. 8d) were both
10 observed to increase the expression of the lipolytic marker phospho-HSL.

2-4. Quantitation of lipolysis product glycerol

After being taken from the abdomens of obesity-
15 induced mice, adipose tissues were plated at a density of 100 mg/well into 24-well culture plates and cultured in a culture medium (1 ml Krebs-Ringer buffer containing 25 mM HEPES, 5.5 mM glucose, and 2 % (w/v) bovine serum albumin). In this regard, the tissues were incubated for
20 48 hours with 0.1 µg/ml, 1 µg/ml, and 10 µg/ml of the peptide complex whereas 100 ng/ml of TNFα was used as a positive control. Glycerol produced during lipolysis was quantitatively analyzed.

As is understood from the experimental data, the
25 amount of glycerol resulting from lipolysis by treatment with the peptide complex was increased in a dose-dependent manner and greater than that produced upon treatment with the positive control TNFα (FIG. 9).

30 2-5. Lysis effect on adipose tissue isolated from obese mouse

Adipose tissues are divided into white fat and brown fat by color and into subcutaneous fat, abdominal fat, mesentery fat (visceral fat), and epididymal fat by
35 tissue. After body anatomization, lipoectomy was

performed on the tissues. White fats were isolated, plated in an amount of 100 mg/well into 24-well plates, and then incubated for 72 hours with concentrations of the peptide complex in a culture medium (1 ml Krebs-
5 Ringer buffer containing 25 mM HEPES, 5.5 mM glucose, and 2 % (w/v) bovine serum albumin). The fats were sectioned into slices which were then dyed with hematoxylin and eosin. Sizes of adipocytes were compared under a microscope (TS100 Nikon) with 100x
10 magnification.

Compared to the control, the fats treated with various concentrations of the peptides decreased in size (FIG. 10a). In addition, when treated with the peptide complex, adipose tissues having distinct cell membrane
15 compartments were observed in cell size, as measured by a program (FIG. 10b).

2-6. Observation of lipolytic marker in adipose tissue

20 An adipose tissue taken from the abdomen of an obesity-induced mouse was plated in an amount of 100 mg per well into 24-well culture plates and incubated for 48 hours with the peptide complex while $\text{TNF}\alpha$ 100 ng/ml was used as a positive control. The labeled lipolytic
25 marker phospho-HSL (green fluorescent) was detected.

Treatment with the peptide complex was observed to increase the expression level of the lipolytic marker phospho-HSL in adipose tissues (FIG. 11).

30 EXAMPLE 3: Adipogenesis-Suppressive and Lipolysis-Promotive Effect in Experimental Animal

Weight loss and adipogenesis suppression in high-fat diet-fed animal

Models DIO (diets induced obesity), which had
35 become obese by feeding high-fat diets thereto, were

used for anti-obesity experiments in which TNF α 5 μ g/ml was used as a positive control. For a control, a general diet, not a high-fat diet, was fed. In the experiment, a high-fat diet was fed for 12 weeks while the peptide complex or the positive control was applied. During the experiment, the weight was monitored.

TNF α and the anti-obesity compounds were intraperitoneally injected at PM 3 - 4 o'clock every week for 12 weeks. Weights and meal sizes were measured just before the initial injection and then regularly at intervals of one week. Blood samples were taken from tail veins after the experiments of drug injection and measured for blood sugar levels, using Accu-Check Active (Roche) and analyzed for cholesterol levels, using Cholesterol calculation Kit (BioVision). Adipose tissues are divided into white fat and brown fat by color and into subcutaneous fat, abdominal fat, mesentery fat (visceral fat), and epididymal fat by tissue. After lipoectomy, the fats thus obtained from the tissues were observed. For histological examination, the fats were fixed with 10 % neutral buffered formalin, embedded in paraffin blocks, cut into 5 μ m-thick sections, and dyed with hematoxylin and eosin. To analyze the phosphorylation of the lipolytic marker HSL, fluorescent staining was carried out with an anti-pHSL antibody. A tissue sample was made, mounted on glycerine jell mounting media, and covered with a cover glass. The tissues were observed under a microscope (Nikon, TS100), with a built-in digital camera taking images thereof.

Over 12 weeks from the initial stage to the final stage of the experiment, mice were measured to increase in weight from 20.9 g to 28.74 g when fed with a general diet and from 20.99 g to 49.5 g when fed with a high-fat diet. In the mice fed with a high-fat diet with the peptide complex injected thereto, the weight gain was

reached only to 36.76 g after 12 weeks from the initial weight of 21.1 g, indicating a significant reduction of weight gain (174.2 %), compared to the high-fat diet-fed control (235.8%) (Table 2 and FIG. 12).

5

TABLE 2

Weight of Obese Mouse Model after Treatment with Peptide Complex

	General diet (control)	high fat diet (control)	H.F+P/C	H.F+P.Complex	Weight (g)
0w	20.09	20.99	22.41	21.1	
1w	20.75	22.32	23	21.26	
2w	21.99	25.25	26.12	23.72	
3w	18.23	27.35	27.45	24.36	
4w	23.26	30.2	30.51	25.29	
5w	23.16	32.76	32.76	28.65	
6w	23.28	36.78	33.49	28.79	
7w	24.71	38.31	35.14	30.37	
8w	25.84	40.12	37.15	31.53	
9w	25.59	42.14	38.97	32.59	
10w	28.13	43.02	40.39	33.78	
11w	27.9	45.7	41.35	35.33	
12w	28.74	49.5	43.91	36.76	

	General diet (control)	high fat diet (control)	H.F+P/C	H.F+P.Complex	Weight (%)
0w	100	100	100	100	
1w	103.3	106.3	102.6	100.8	
2w	109.5	120.3	116.6	112.4	
3w	90.7	130.3	122.5	115.5	
4w	115.8	143.9	136.1	119.9	
5w	115.3	156.1	146.2	135.8	
6w	115.9	175.2	149.4	136.4	
7w	123.0	182.5	156.8	143.9	
8w	128.6	191.1	165.8	149.4	
9w	127.4	200.8	173.9	154.5	
10w	140.0	205.0	180.2	160.1	
11w	138.9	217.7	184.5	167.4	
12w	143.1	235.8	195.9	174.2	

10

After completion of the 12-week experiment, in addition, the mice treated with the peptide complex, were observed to maintain their body sizes in similar patterns to those of the normal mice (general diet), but not to those of the high-fat diet-fed mice, as analyzed on the images (FIG. 13).

After 12 weeks of the experiment, the mice were subjected to micro-CT to examine fat distribution across the body. As a result of the micro-CT data of fats (yellow) in the body, the fat distributed across the body was remarkably increased in the high-fat diet-fed

20

mice, compared to the general diet-fed control while a significantly reduced level of fats distributed across the body was observed in the group which were treated with the peptide complex with the high-fat diet fed thereto (FIG. 14).

The mice which completed micro-CT imaging were anatomized to extract the adipose tissues distributed across the body. Volumes of the adipose tissues were compared. As a result, the fat extracted from the high-fat diet-fed mice was greater than that from the general diet-fed mice, with a significant reduction in the fat volume in the mice treated with the peptide complex plus the high-fat diet (FIG. 15).

Fats were isolated, and dyed with H&E to visualize fat sizes. Smaller sizes of fats were observed in the mice treated with both the high-fat diet and the peptide complex than in the high-fat diet-fed control (FIG. 16a). Fat size analysis through a program showed that, when the fat size of the general diet-fed control was assumed to be 100 %, a fat size was increased to 127 % in the high-fat diet-fed group, but decreased to 97 % in the group treated with the high-fat diet and the peptide complex (FIG. 16b).

The fats were isolated and examined for the expression level of the lipolytic marker phospho-HSL in adipose tissues. The mice treated with both the high-fat diet and the peptide complex were observed to have an elevated expression level of phospho-HSL (FIG. 17).

Blood cholesterol levels in the mice after the experiment were measured. As a result, the blood cholesterol level was 2.52 $\mu\text{g/ml}$ in the general diet-fed mice, 3.5 $\mu\text{g/ml}$ in the high-fat diet-fed mice, and 2.86 $\mu\text{g/ml}$ in the mice treated with both the high-fat diet and the peptide complex, indicating that the peptide complex reduced the cholesterol level that elevated with

obesity (FIG. 18).

Blood sugar levels after completion of the experiment were 174 mg/dL in the general diet-fed mice, and increased to 235 mg/dL in the high-fat diet-fed mice. However, a blood sugar level of 183 mg/dL was measured in the mice treated with both the high-fat diet and the peptide complex, with a significant reduction therein (FIG. 19).

10 EXAMPLE 4: Blood Sugar Control

Effect on blood sugar control

In this animal experiment, C57BL/6 (normal mouse) (purchased from Samtako Inc.) and female C57BLKS/JLepr (diabetes model mouse, db/db mouse) (purchased from Central Lab. Animal Inc.) were used, together with the peptide complex as an anti-diabetes and/or anti-obesity effective material, and sitagliptin as a positive control drug. In this Example, the anti-diabetes and/or anti-obesity effective complex was evaluated for acute anti-diabetes efficacy (single administration) in a normal mouse model and a genetically potential-diabetic model, using GTT (glucose tolerance test), which is a representative diagnostic method for diabetes. Mice were bred per cage at a temperature of 22-24°C and a relative humidity of 50-30%, with four per cage. The mice was under 150-300 Lux light from AM 8 o'clock to PM 8 o'clock with 12 light /12 dark cycles. They were given free access to a general diet (18 % protein, manufactured in 2018, Harlan Laboratories Inc, USA). To begin with, the mice were starved for 4 hours or longer before ITT experiment and for 12 hours before GTT experiment. The complex was orally administered by force with the aid of a disposable oral administration syringe one hour before GTT experiment. For GTT experiment, the mice were allowed to freely access to a high-fat diet on

0 (zero) hour after experiment started. After 40 min of free access to a high-fat diet, blood samples for use in examining blood glucose levels were taken from the tail vein at intervals of 0, 30, 60, 90, 120, and 180 min. Blood glucose levels were measured using Accu-Chek active (Roche). Sitagliptin, used as a therapeutic agent for diabetes, was selected as a positive control drug, and administered at a dose of 100 mg/kg. The complex selected as an anti-diabetes and/or anti-obesity effective candidate was administered at a dose of 100 mg/kg to experimental groups of four mice.

As a result, the peptide complex exhibited a reductive effect on blood sugar levels in which the blood sugar level increased by the high-fat diet was reduced by treatment with the peptide complex. In the diabetes-induced mouse models, the high blood sugar level was decreased by the complex (FIGS. 20a and 20b). Further, lower blood cholesterol levels were detected in the group treated with both the high-fat diet and the peptide complex than the high-fat diet-fed control (FIG. 21).

In addition, after starvation for 16 hours, DB/DB diabetes-induced mice were fed for 30 min and then administered with the peptides. Blood sugar levels were measured over times.

The blood sugar levels in the groups respectively treated with the peptides of SEQ ID NOS: 1, 3, and 5 were observed to decrease in a time-dependent manner (FIGS. 22a-22c).

30

EXAMPLE 5: Promotion of Expression of Insulin and Insulin-like Growth Factor

Promotion of expression of insulin and insulin-like growth factor

35 3T3-L1 cells (pre-adipocytes) were seeded at a

density of 3×10^5 cells/well into 6-well plates and grown for 24 hours. Subsequently, the cells were incubated with various concentrations (10 ng - 1 μ g/ml) of the peptides for 14 days in a 37°C incubator. Proteins were
5 extracted from cell lysates which were obtained by treatment with cell lysis buffer, quantitatively analyzed, and subjected to Western blotting using an anti-IGF-1 antibody, which is an antibody against the lipolytic marker, and an insulin antibody (Santa Cruz
10 Biotechnology, USA).

From the data, it was observed that the peptide of SEQ ID NO: 7 increased the expression of IGF-1 and insulin in dose-dependent manners (FIG. 23).

15 **EXAMPLE 6: Observation of Blood Sugar Level Reducing Effect in Clinical Experiment**

Reduction of blood sugar level by intake of the complex.

A brief clinical test was performed on persons 45-
20 65 years old who had a fasting blood glucose level of 170 mg/dL or higher. They were ingested with a complex formulation 30 min after meals. Blood samples were taken at intervals of 30, 60, 90, 120, 150, and 180 min from the persons, and measured for glucose level, using Accu-
25 Chek active (Roche).

A reduction of blood sugar level by the complex formulation was observed in all the tested persons (FIGS. 25a - 25d).

30 Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the
35 substantial scope of the present invention will be

defined by the appended claims and equivalents thereof.

Claims

1. A peptide consisting of the amino acid sequence of SEQ ID NO: 2 or 3.

5

2. A peptide consisting of the amino acid sequence of SEQ ID NO: 2 or 3 and exhibiting anti-obesity or anti-Type II diabetes activity.

10 3. The peptide of claim 2, wherein the peptide suppresses adipogenesis.

4. The peptide of claim 2, wherein the peptide reduces expression of PPAR γ (peroxisome proliferator-activated receptor gamma), ACC (acetyl-CoA carboxylase),
15 or aP2 (adipose-specific fatty acid-binding protein 2).

5. The peptide of claim 2, wherein the peptide promotes lipolysis.

20

6. The peptide of claim 2, wherein the peptide increases expression of pHSL (phospho-hormone-sensitive lipase), AMPK- α 1 (AMP-activated protein kinase α 1), CGI-58 (comparative gene identification-58), or ATGL (adipose
25 triglyceride lipase).

7. The peptide of claim 2, wherein the peptide reduces a blood sugar level.

30 8. A pharmaceutical composition, comprising the peptide of any one of claims 2 to 7 as an effective ingredient for preventing or treating obesity.

9. A pharmaceutical composition, comprising the peptide of any one of claims 2 to 7 as an effective ingredient for preventing or treating Type II diabetes.

5 10. Use of a peptide of any one of claims 2 to 7 in the manufacture of a medicament for preventing or treating obesity or Type II diabetes.

10 11. The peptide of any one of claims 1 to 7 for use in medicine.

12. A pharmaceutical composition comprising the peptide of any one of claims 2 to 7 for use in medicine.

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