

SPECIFICATION

Title of the Invention: Kokumi-Imparting Agent

5 Technical Field

The present invention relates to a kokumi-imparting agent and a complex kokumi-imparting agent, which consist of a peptide showing a CaSR agonist activity. Moreover, the present invention also relates to a food composition which comprises a peptide showing a CaSR agonist activity in a concentration of not less than a predetermined level.

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Background Art

The consumers' demands on the taste and palatability of foods have recently been increased due to, for instance, the diversity of the human eating habits. In this connection, the taste and palatability of a food have conventionally been expressed by the five basic tastes, in
15 other words, sweet taste, salty taste, sour taste, bitter taste and umami, but there has correspondingly been increased the needs for the development of an excellent kokumi-imparting agent capable of imparting, to a food, a "kokumi". Kokumi means a taste that cannot be expressed only by the five basic tastes but also marginal tastes of the basic taste, such as thickness, growth (or mouthfullness), continuity and harmony.

20 On the other hand, the calcium sensing receptor (CaSR) is also referred as the calcium receptor, the signals from the receptor can control a variety of biological functions within the living bodies and the substances possessing such a CaSR agonist activity can be used as a kokumi-imparting agent (see Patent Documents 1 and 2 and Non-patent Document 3 as will be specified below).

25 There would be a variety of taste-developing patterns in the foregoing "kokumi". In this respect, there has been a need for the development of a kokumi-imparting agent capable of imparting, to a food, the kokumi of the middle/after taste type and having a high titer. Moreover, the substance for imparting the kokumi would in general be used in, for instance, foods and accordingly, it should be excellent in its stability.

30 Accordingly, there have been desired for the search for most of variational compounds

possessing the desired CaSR agonist activity to thus find out a substance capable of imparting a kokumi to other substance (foods or beverages), which shows a more excellent kokumi-imparting effect, in particular, a kokumi-imparting effect of the initial taste-imparting type one, which is excellent in the stability and which can easily be produced at a low cost and to thereby provide a kokumi-imparting agent consisting of such a substance as well as a complex kokumi-imparting agent comprising the substance and other substances possessing the CaSR agonist activities in combination.

On the other hand, regarding some γ -glutamyl peptides each carrying a γ -glutamine residue at the N-terminal thereof, there have been known those synthesized as substrates in, for instance, the studies of the enzymatic activities (see Patent Document 3 and Non-patent Documents 1 and 2, specified later), but there have not yet been known any case in which γ -Glu-Nva-Gly is actually used in a food and any case wherein it is isolated from a naturally occurring substance.

In addition, the foregoing Patent Document 1 discloses, for instance, that γ -Glu-X-Gly (wherein X represents an amino acid or a derivative thereof) is a compound possessing a CaSR agonist activity, but this Patent Document does not disclose, for instance, in Examples that γ -Glu-Nva-Gly is practically synthesized and the effects thereof are evaluated and more specifically, this Patent Document never specifically discloses this tripeptide. In this respect, the whole contents of Patent Documents 1 and 2 are incorporated herein as references as if they were, in fact, expressly disclosed in this specification.

Prior Literature

Patent Document:

Patent Document 1: International Patent Laid-Open No. 2007/055393, Pamphlet;

Patent Document 2: International Patent Laid-Open No. 2008/139945, Pamphlet;

Patent Document 3: International Patent Laid-Open No. 2007/066430, Pamphlet;

Non-Patent Documents:

Non-Patent Document 1: Molecular Pharmacology (1982), 21(3), 629-36;

Non-Patent Document 2: Boikhimiya (Moscow) (1972), 37(4), 757-61;

Non-Patent Document 3: The Journal of Biological Chemistry, (2010), 285 (2), 1016-22.

Summary of the Invention

It is an object of the present invention to search for most of variational compounds possessing the desired CaSR agonist activity to thus find out a substance capable of imparting a
5 kokumi, which shows a more excellent kokumi-imparting effect, in particular, a kokumi-imparting effect of the middle/after taste-imparting type one, which is excellent in the stability and which has a high titer and to thereby provide a kokumi-imparting agent consisting of such a substance as well as a complex kokumi-imparting agent comprising the substance and other substances possessing the CaSR agonist activities in combination. It is a further object
10 of the present invention to provide a food composition which comprises the foregoing substance in a concentration of not less than the predetermined level.

As a result of searching for a variety of compounds, the inventors of this invention have surprisingly found out that γ -Glu-Nva-Gly (L- γ -glutamyl-L-norvalyl-glycine) possesses a
15 high CaSR agonist activity and a quite excellent kokumi-imparting effect and that it can, in particular, impart, to a food, the kokumi whose taste-developing (or flavoring) pattern is middle/after taste-imparting type one. Furthermore, the inventors have found that γ -Glu-Nva-Gly thus discovered has an extremely high titer on the order of not less than 10 times that observed for γ -Glu-Val-Gly as one of tripeptides similar to γ -Glu-Nva-Gly, is excellent in the stability and has such a favorable taste-developing pattern such that it shows a stronger
20 middle/after taste-imparting ability. Moreover, the inventors have further found that γ -Glu-Nva-Gly thus discovered can serve, by itself, as a useful kokumi-imparting agent. In addition, the inventors have likewise found that a preferred food composition whose kokumi is improved can be obtained by the incorporation of γ -Glu-Nva-Gly into a food. Furthermore, a complex kokumi-imparting agent can be obtained by combining the substance with other
25 substances each showing a CaSR agonist activity. The inventors of this invention have thus completed the present invention.

More specifically, the present invention herein provides a kokumi-imparting agent consisting of γ -Glu-Nva-Gly.

Moreover, the present invention likewise provides a food composition containing γ
30 -Glu-Nva-Gly (hereunder the food composition will also referred to as "food composition of the

present invention"). According to the present invention, there is further provided a complex kokumi-imparting agent which comprises, in combination, (a) γ -Glu-Nva-Gly and (b) one or at least two amino acids or peptides selected from the group consisting of γ -Glu-X-Gly wherein X represents an amino acid or an amino acid derivative, provided that Nva is excluded, γ -Glu-Val-Y wherein Y represents an amino acid or an amino acid derivative, γ -Glu-Nva, γ -Glu-Abu, γ -Glu-Ala, γ -Glu-Gly, γ -Glu-Cys, γ -Glu-Met, γ -Glu-Thr, γ -Glu-Val, γ -Glu-Orn, Asp-Gly, Cys-Gly, Cys-Met, Glu-Cys, Gly-Cys, Leu-Asp, D-Cys, γ -Glu-Met (O), γ -Glu- γ -Glu-Val, γ -Glu-Val-NH₂, γ -Glu-Val-ol, γ -Glu-Ser, γ -Glu-Tau, γ -Glu-Cys (S-Me) (O), γ -Glu-Leu, γ -Glu-Ile, γ -Glu-t-Leu and γ -Glu-Cys (S-Me).

The present invention would be able to provide a kokumi-imparting agent which is quite excellent in its kokumi-imparting effect and, in particular, has a quite strong kokumi-imparting effect of unique middle/after taste-imparting type one represented by, for instance, a taste-developing pattern showing such a profile as shown in, for instance, Fig. 1, which is also excellent in the stability and which can easily be prepared at a low cost, as well as a complex kokumi-imparting agent containing the same. In addition, the present invention can likewise provide an excellent food composition which comprises a substance possessing an excellent kokumi-imparting effect in a concentration of not less than a predetermined level.

The use of the kokumi-imparting agent according to the present invention would permit the impartment of fat-like dense-feeling or impression and smoothness to a low fat food. Accordingly, the resulting food containing the agent of the present invention can maintain its dense impression like that observed for the original food prior to the reduction of the fat content even when the fat content of the food is reduced and therefore, the agent according to the present invention permits the production of a food highly beneficial to health. Examples of such foods include foods containing meat or products related thereto and dairy products. Especially, if eating a food containing the kokumi-imparting agent according to the present invention, the consumer can feel the fat-like dense impression and smoothness, at an instance not immediately after eating the same, but in a short time thereafter.

Brief Description of the Drawings

[Figure 1] Fig. 1 shows the taste-developing profile observed for a kokumi-imparting agent

of the middle/after taste type one.

Mode for Carrying out the Invention

The substance, γ -Glu-Nva-Gly, used in the present invention comprises L- γ -glutamyl-L-norvalyl-glycine in which three amino acids are linked to one another through peptide bonds and/or salts thereof, in particular, edible salts thereof.

The substance, γ -Glu-Nva-Gly, shows an excellent kokumi-imparting effect and therefore, it can be used as a kokumi-imparting agent. γ -Glu-Nva-Gly can be used in such a manner that a food composition to which the kokumi is imparted comprises the tripeptide in an amount ranging from 0.1 ppb by mass to 99.9% by mass, preferably 1 ppb by mass to 10% by mass and more preferably 0.01 ppm by mass to 1% by mass relative to the total mass of the food composition. In other words, another embodiment of the present invention relates to a food composition comprising γ -Glu-Nva-Gly and preferably a food composition comprising γ -Glu-Nva-Gly in an amount ranging from 0.1 ppb by mass to 99.9% by mass. More preferably, the present invention relates to a food composition comprising γ -Glu-Nva-Gly in an amount ranging from 0.01 ppm by mass to 50 ppm by mass.

Moreover, the kokumi-imparting agent of the present invention or γ -Glu-Nva-Gly can also be used in combination with at least one additional ingredient for seasonings selected from the group consisting of amino acids such as sodium glutamate (MSG), nucleic acids such as inosine monophosphate (IMP), inorganic salts such as sodium chloride, organic acids such as citric acid, and various kinds of yeast extracts to thus provide a favorable seasoning which is improved in its kokumi, as compared with those obtained by the use of such additional ingredients for seasonings individually.

In the present invention, the term "kokumi" means the taste which cannot be expressed by the five basic tastes or the sweet taste, the salty taste, the sour taste, the bitter taste and the umami and more specifically means the taste in which the marginal tastes of the basic ones such as the thickness, the growth (mouthfullness), the continuity and the harmony are enhanced in addition to the basic tastes. In addition, the term "kokumi-impartment" herein used means that the five basic tastes expressed by the sweet taste, the salty taste, the sour taste, the bitter taste and the umami are enhanced, while the marginal tastes of the basic tastes such as the thickness,

the growth (mouthfullness) and the harmony associated with the former are simultaneously imparted to a subject. Moreover, this can also be referred to as the flavor-enhancing effect. Therefore, γ -Glu-Nva- Gly serving as the kokumi-imparting agent of the present invention can likewise be referred to as a "flavor enhancer". The kokumi-imparting agent according to the present invention or γ -Glu-Nva-Gly may also be used as a sweet taste enhancer, a salty taste enhancer, a sour taste enhancer, a bitter taste enhancer or an umami enhancer.

Moreover, the taste of a food may vary with the elapse of time after eating the same and the taste of a food after eating the same is in general referred to as the initial taste, the middle taste and the after taste, in order, immediately after eating the food. Although they are indeed the relative concept, but the initial taste, the middle taste and the after taste of a subject are, as a whole, defined to be the taste observed within the term extending from 0 to 2 seconds, 2 to 5 seconds and not less than 5 seconds after eating the same, respectively. The taste observed during the term extending from 0 to 5 seconds is herein referred to as the "initial/middle taste" and that observed during the term extending from 2 to about 30 seconds is referred to as the "middle/after taste" (see the data plotted on Fig. 1). Regarding the evaluation of the taste in case where the taste is divided into three subdivisions, it is difficult for the panelists (persons who eat the food to be examined) to concentrate their attention on the evaluation and the habitually used test comprises the evaluation of the taste while dividing it into two subdivisions.

The effects of a substance showing a CaSR activity on the kokumi and the taste-developing pattern can be confirmed by a method, for instance, a human sensory test for the evaluation of taste. An example of such a human sensory test for the evaluation of taste is one illustrated in Examples of this patent application, but the sensory test for the evaluation of taste usable herein is not restricted to this specific one.

The term "CaSR" used in this specification means the calcium sensing receptor which belongs to the class C of the 7-time transmembrane receptor and which is thus also referred to as "calcium receptor". The term "CaSR agonist" used in this specification means a substance which is linked with the foregoing CaSR to thus activate the receptor CaSR. In addition, the term "activate CaSR" used herein means that a ligand is linked with CaSR to thus activate a protein linked with guanine nucleotide and to transmit signals outputted from the same. Moreover, the ability of a substance to form a linkage with CaSR to thus activate the same is

referred to as "CaSR agonist activity".

Now, a method for screening a compound having a CaSR agonist activity will specifically be presented hereunder, but the steps for screening the compound are not limited to those specified below at all.

- 5 1) A step for adding a test substance to a CaSR activity-measuring system used for determining the CaSR activity and a step for the determination of the CaSR activity;
- 2) A step for comparing the CaSR activity observed when adding the test substance to the activity-measuring system with that observed for the system prior to the addition of the substance; and
- 10 3) A step for selecting a substance which shows the CaSR agonist activity when it is added to the CaSR activity-measuring system.

The determination of the CaSR activity can likewise be carried out while using, for instance, a measuring system which makes use of cells having an ability of expressing CaSR. The foregoing cells may be those endogeneously expressing CaSR or genetically recombinant
15 cells into which a CaSR-expression gene is exogeneously introduced. The aforementioned CaSR activity-measuring system is not restricted to any specific one inasmuch as it permits the detection of the linkage (or a reaction) between the CaSR-activation substance and CaSR, or it can emit or output a detectable signal in response to the formation of the linkage (or a reaction) between the CaSR-activation substance and CaSR within the cells, when adding an extracellular
20 ligand (activating substance) specific to CaSR to the foregoing cells having an ability of expressing CaSR. If the CaSR activity is detected through the reaction with a test substance, it would be judged that the test substance shows the desired CaSR-stimulation activity.

An example of the foregoing CaSR preferably used herein is human CaSR encoded by the human CaSR gene registered under the GenBank Accession Number of NM_000388. In
25 this respect, however, the CaSR is not restricted to the protein encoded by the gene having the foregoing sequence of the registered CaSR and it may be any protein capable of being encoded by a gene having not less than 60%, preferably not less than 80% and more preferably not less than 90% of the sequence homology with the foregoing gene sequence, inasmuch as the protein encoded by such a gene possesses the desired CaSR function. In this connection, the CaSR
30 function can be examined by making cells express these genes and then determining any change

of an electric current and/or any change in the calcium ion concentration within the cells observed when calcium is added to the system containing the cells.

Regarding the foregoing CaSR, the source thereof is not restricted to any particular one and it may be those derived from the whole kinds of animals including mice, rats and canines, in addition to the foregoing human CaSR.

As has been discussed above, the CaSR activity can be confirmed through the use of the living cells capable of expressing CaSR or a fragment thereof, the cell membranes which can express CaSR or a fragment thereof, or an in vitro system including CaSR or a protein as a fragment thereof.

An example which makes use of such living cells will be given below, but the present invention is by no means limited to this example at all.

CaSR is expressed in cultured cells such as oocytes of xenopus, ovarian cells derived from hamsters, and human fetal renal cells. This expression of CaSR can be carried out by introducing, into a plasmid possessing an exogenous gene, the CaSR gene which has been subjected to the cloning treatment in the form of a plasmid or cRNA obtained using the same as a template. Usable for the detection of such a reaction may be an electrophysiological means or a fluorescent indicator for the detection of any increase in the calcium concentration within the cells.

The expression of CaSR is initially confirmed by the presence of any response observed when adding calcium or an activator having the specificity thereto. More specifically, usable herein as the desired cells are those in which an intracellular electric current is detected when adding calcium in a concentration of about 5 mM or those in which the emission of fluorescent rays are observed upon the addition of a fluorescent indicator. In this connection, the concentration of calcium added to the cells is variously changed to determine any calcium concentration-dependency of the intensity of the intracellular electric current. Then a test substance is diluted to a concentration ranging from about 1 μ M to 1 mM, the resulting dispersion is added to oocytes or cultivated cells and subsequently the CaSR activity in the presence of the foregoing test substance is measured to thus determine the CaSR agonist activity of the test substance.

More specifically, usable herein as such a test for the determination of the CaSR agonist

activity is, for instance, the test illustrated in Test Examples disclosed in this specification, but the activity-determining test is not limited to such a specific one at all.

The amino acids or peptides used in the complex kokumi-imparting agent according to the present invention in combination with γ -Glu-Nva-Gly include, for instance, one or at least
5 two amino acids or peptides selected from the group consisting of γ -Glu-X-Gly (wherein X represents an amino acid or an amino acid derivative other than Nva), γ -Glu-Val-Y (wherein Y represents an amino acid or an amino acid derivative), γ -Glu-Nva, γ -Glu-Abu, γ -Glu-Ala, γ -Glu-Gly, γ -Glu-Cys, γ -Glu-Met, γ -Glu-Thr, γ -Glu-Val, γ -Glu-Orn, Asp-Gly, Cys-Gly, Cys-Met, Glu-Cys, Gly-Cys, Leu-Asp, D-Cys, γ -Glu-Met (O), γ -Glu- γ
10 -Glu-Val, γ -Glu-Val-NH₂, γ -Glu-Val-ol, γ -Glu-Ser, γ -Glu-Tau, γ -Glu-Cys (S-Me) (O), γ -Glu-Leu, γ -Glu-Ile, γ -Glu-t-Leu and γ -Glu-Cys (S-Me). In this respect, the amino acids may likewise include, for instance, neutral amino acids such as Gly, Ala, Val, Leu, Ile, Ser, Thr, Cys, Met, Asn, Gln, Pro, Hyp and t-Leu; acidic amino acids such as Asp and Glu; basic amino acids such as Lys, Arg and His; aromatic amino acids such as Phe, Tyr and Trp; as well as
15 homoserine, citrulline, ornithine, α -aminobutyric acid, norvaline, norleucine and taurine. Moreover, the amino acids used herein may also be artificial amino acids (having non-proteinaceous construction) such as tert-leucine, cyclo-leucine, α -amino-isobutyric acid and L-penicillamine. In this connection, in the peptide: γ -Glu-X-Gly, X may be the aforementioned amino acid or derivative thereof, but preferably used herein are amino acids or
20 derivatives thereof other than Cys. Among them, preferably used herein in combination with γ -Glu-Nva-Gly include, for instance, γ -Glu-Val-Gly, γ -Glu-Abu-Gly, γ -Glu-tLeu-Gly, γ -Glu-Nva and γ -Glu-Abu.

In particular, the kokumi-imparting agent according to the present invention consists of γ -Glu-Nva-Gly and this agent has a unique and excellent middle/after taste type
25 kokumi-imparting effect and the taste-developing (flavoring) profile as shown in Fig. 1. Accordingly, it is preferred that γ -Glu-Nva-Gly is used in combination with a peptide such as initial taste type γ -Glu-Abu-Gly and γ -Glu-Abu which show a taste-developing profile different from that of the former.

In the specification of this patent application, the following abbreviated forms of amino
30 acids (residues) mean the following amino acids, respectively:

- (1) Gly: glycine;
(2) Ala: alanine;
(3) Val: valine;
(4) Leu: leucine;
5 (5) Ile: isoleucine;
(6) Met: methionine;
(7) Phe: phenylalanine;
(8) Tyr: tyrosine;
(9) Trp: tryptophane;
10 (10) His: histidine;
(11) Lys: lysine;
(12) Arg: arginine;
(13) Ser: serine;
(14) Thr: threonine;
15 (15) Asp: aspartic acid;
(16) Glu: glutamic acid;
(17) Asn: asparagine;
(18) Gln: glutamine;
(19) Cys: cysteine;
20 (20) Pro: proline;
(21) Orn: ornithine;
(22) Sar: sarcosine;
(23) Cit: citrulline;
(24) N-Val: (or Nva): norvaline (2-aminovaleric acid);
25 (25) N-Leu (or Nle): norleucine;
(26) Abu: α -aminobutyric acid;
(27) Tau: taurine;
(28) Hyp: hydroxy-proline;
(29) t-Leu: tert-leucine;
30 (30) Cle: cyclo-leucine;

(31) Aib: α -amino-isobutyric acid (2-methyl-alanine);

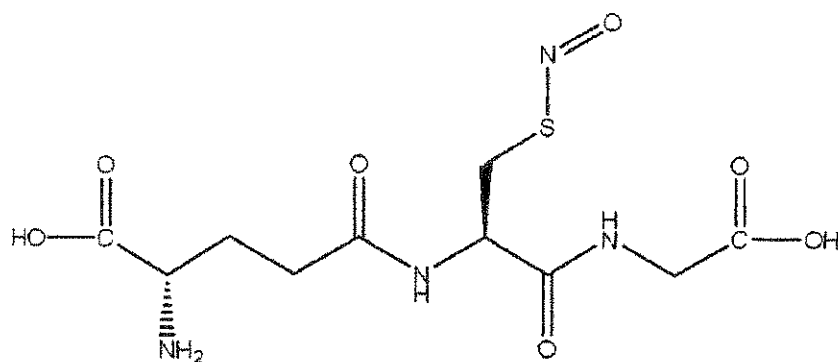
(32) Pen: L-penicillamine;

(33) allo-Thr: allothreonine;

(34) allo-Ile: allo-isoleucine.

5 Furthermore, the term "amino acid derivative" used herein means a variety of derivatives of the foregoing amino acids and specific examples thereof include special amino acids and artificial amino acids, amino alcohols, or amino acids whose side chains of amino acids such as terminal carboxyl groups, amino groups and/or thiol group of cysteine are substituted with a variety of substituents. Examples of such substituents include an alkyl
10 group, an acyl group, a hydroxyl group, an amino group, an alkylamino group, a nitro group, a sulfonyl group or a variety of protective groups. Thus, examples of such amino acid derivatives include Arg (NO₂): N- γ -nitro-alanine, Cys (SNO): S-nitro-cysteine, Cys (S-Me): S-methyl cysteine, Cys (S-allyl): S-allyl cysteine, Val-NH₂: valine-amide, and Val-ol: valinol (2-amino-3-methyl-1-butanol). In the meantime, the peptide: γ -Glu-Cys(SNO)-Gly used
15 herein is one represented by the following structural formula and the symbol: "(O)" appearing in the foregoing formulas: γ -Glu-Met (O) and γ -Glu-Cys (S-Me) (O) means that these peptides each have a sulfoxide structure. The symbol "(γ)" of γ -Glu means that another amino acid is linked with the glutamic acid through the carboxyl group situating at the γ -position of the latter.

20 [Chemical Formula 1]



S-Nitrosoglutathione (GNSO)

The γ -Glu-Nva-Gly and the amino acids and peptides used in combination in the present invention may be commercially available ones if they can be purchased on the market, or can be obtained according to any known technique such as (1) chemical synthetic methods or (2) a method which makes use of an enzyme reaction, but it would be more convenient to use a chemical synthetic technique. The γ -Glu-Nva-Gly used in the present invention is very short in its length since it includes only three amino acid residues and therefore, it is more convenient to adopt a chemical synthetic technique. For this reason, the use of this tripeptide is quite advantageous from the industrial standpoint. In addition, when chemically synthesizing the γ -Glu-Nva-Gly used in the present invention and the amino acids and peptides used in combination therewith, the preparation thereof may be carried out by synthesizing or semi-synthesizing these oligo-peptides while using a peptide-synthesis device. As such a method for chemically synthesizing these peptides, usable herein may be, for instance, the solid phase peptide-synthesis technique. The peptide thus produced can subsequently be purified according to the usual techniques such as the ion-exchange chromatography technique, the reversed phase high performance liquid chromatography technique, or the affinity chromatography technique. Such a solid phase peptide-synthesis technique and the subsequently used peptide-purification technique are well known in the field of this art.

Alternatively, when producing the γ -Glu-Nva-Gly used in the present invention and the amino acids and peptides used in combination therewith, while making use of an enzyme reaction, the preparation thereof may be carried out by the use of the method disclosed in the pamphlet of International Patent Laid-Open WO 2004/ 011653. More specifically, they can be prepared by reacting an amino acid or a peptide, in which the terminal carboxyl group thereof is esterified or aminated, with another amino acid whose amino group is in its free state (for instance, an amino acid whose carboxyl group is protected) in the presence of a peptide-forming enzyme and then purifying the resulting dipeptide or tripeptide. Examples of such peptide-forming enzymes are cultures of microorganisms showing the ability of producing a peptide; the cell bodies of microorganisms isolated from the culture; or a product obtained by processing the cell bodies of the microorganisms; or a peptide-forming enzyme originated from the microorganisms. In the meantime, it is herein construed that the disclosure of WO 2004/011653 is included in the description of this specification.

In addition to the foregoing enzymatic production methods and chemical synthesis methods, the peptides used in the present invention are often included in naturally occurring products, for instance, plants such as vegetables and fruits, microorganisms such as yeast and other natural resources. When they are present in naturally occurring substances, they can be isolated from the same and the resulting isolated products can likewise be used in the present invention.

The kokumi-imparting agent or the complex kokumi-imparting agent according to the present invention can be used as a seasoning without any particular post-treatment or can be mixed with carriers acceptable for foods and beverages or ingredients for other seasonings to thus give various seasonings. Examples of such other ingredients for seasonings include spices, saccharides, sweeteners, edible fibers, vitamins, amino acids such as sodium glutamate (MSG), nucleic acids such as inosine monophosphate (IMP), inorganic salts such as sodium chloride, and organic acids such as citric acid as well as various kinds of yeast extracts.

Incidentally, low fat foods preferred as food compositions each comprising the kokumi-imparting agent or the complex kokumi-imparting agent according to the present invention are, by nature, those containing fats and, in particular, foods whose fat content is reduced. In this respect, the term "fat(s)" is synonymous with the term "oil and fat", the fats include those in the both solid and liquid forms and they may be either animal fats or vegetable fats.

Examples of such low fat foods include dairy products such as milk, yoghurt, butter and cream; animal oils and fats-containing and/or vegetable oils and fats-containing foods such as margarine, milk for coffee, sauces and roux; emulsified foods such as dressings and mayonnaise; various kinds of curry and stew containing processed or cooked meat or the like; and a variety of soups containing meat extracts or essences of meat. Moreover, such low fat foods likewise include, for instance, steak, grilled meat or the like each consisting of low fat meat, and baked snack foods different from those subjected to the usual frying treatment. Among them, preferred as such low fat foods include, for instance, those each having a fat content of 1/2 to 1/3 time that of the usual foods.

If the kokumi-imparting agent of the present invention is incorporated into the foregoing low fat foods, the foods would be able to give, to the consumers, a fat-like dense impression and

smoothness not at the initial stage, but at the stage subsequent to the same, when they eat these low fat foods.

In this connection, the usual products of milk and yoghurt have a fat content ranging from 3 to 4%, but there have also been known, as such milk and yoghurt, those free of any fat (fat content of about 0.1%). The kokumi-imparting agent or the complex kokumi-imparting agent of the present invention is likewise effective for those free of any fat.

In addition, the kokumi-imparting agent or the complex kokumi- imparting agent of the present invention is particularly preferably added to a food containing an ingredient derived from pork. More specifically, the present invention herein provides a food composition comprising γ -Glu-Nva-Gly and an ingredient derived from pork. The food containing the ingredient derived from pork is not restricted to any particular one, but there can be listed, for instance, extracts of pork, sausage, and soups for convenience noodles. In this respect, the content of the ingredient derived from pork to be incorporated into these foods is not limited to any specific range, but examples of such foods containing the ingredient derived from pork include those having a content thereof on the order of 0.005 to 80% by mass.

Moreover, it is also preferred that the kokumi-imparting agent or the complex kokumi-imparting agent of the present invention is incorporated into a food containing an ingredient derived from beef. More specifically, the present invention also provides a food composition comprising γ -Glu-Nva-Gly and an ingredient derived from beef. The food containing the ingredient derived from beef is not restricted to any particular one, but there can be listed, for instance, extracts of beef, corned beef, soups prepared using beef and sauces which make use of beef. In this respect, the content of the ingredient derived from beef to be incorporated into these foods is not limited to any specific range, but examples of such foods containing the ingredient derived from beef include those having a content thereof on the order of 0.005 to 80% by mass.

The γ -Glu-Nva-Gly used in the present invention and the amino acids or peptides used in combination therewith may likewise include those in their salt form. If the γ -Glu-Nva-Gly used in the present invention and the amino acids or peptides used in combination therewith are present in their salt form, the salts are not restricted to specific ones inasmuch as they are pharmacologically acceptable and soluble ones, and specific examples

thereof include ammonium salts, salts with alkali metals such as sodium and potassium, salts with alkaline earth metals such as calcium and magnesium, aluminum salts, zinc salts, salts with organic amines such as triethylamine, ethanolamine, morpholine, pyrrolidine, piperidine, piperazine and dicyclo-hexylamine, and salts with basic amino acids such as alginine and lysine, for the acidic groups such as carboxyl groups. Moreover, examples of the foregoing compounds likewise include salts with inorganic acids such as hydrochloric acid, sulfuric acid, phosphoric acid, nitric acid and hydrobromic acid; salts with organic carboxylic acids such as acetic acid, citric acid, benzoic acid, maleic acid, fumaric acid, tartaric acid, succinic acid, tannic acid, butyric acid, hibenzoic acid, pamoic acid, enanthic acid, decanoic acid, theoclic acid, salicylic acid, lactic acid, oxalic acid, mandelic acid and malic acid; and salts with organic sulfonic acids such as methanesulfonic acid, benzenesulfonic acid and p-toluenesulfonic acid, for the basic groups of the compounds.

The kokumi-imparting agent, the food composition or the complex kokumi-imparting agent according to the present invention can be used in any form such as dried powders, pastes, and solutions, without any restriction in the physical properties thereof.

The kokumi-imparting agent, the food composition or the complex kokumi-imparting agent according to the present invention can be used for incorporating into, for instance, a variety of foods and beverages such as foods, beverages and seasonings.

If the kokumi-imparting agent, the food composition or the complex kokumi-imparting agent according to the present invention is used for incorporating into, for instance, a variety of foods and beverages such as foods, beverages and seasonings, the ultimate amount of γ -Glu-Nva-Gly and that of the amino acid or peptide used in combination therewith are not restricted to any specific one inasmuch as they can ensure the achievement of the desired effects of the present invention, but the amount of γ -Glu-Nva-Gly and/or that of the amino acid or peptide each fall within the range of from about 0.1 ppb by mass to 99.9% by mass and preferably about 1 ppb by mass to 10% by mass and more preferably about 0.01 ppm by mass to 1% by mass on the basis of the total mass of each corresponding food, beverage, seasoning or the like.

A variety of foods such as foods, beverages or seasonings, which comprise the incorporated kokumi-imparting agent, food composition or complex kokumi-imparting agent

according to the present invention, may further comprise, for instance, any solid or liquid carrier and/or appropriate seasoning ingredients, which are acceptable for foods and beverages.

As the foregoing carriers, there can be listed, for instance, glucose, lactose, saccharose, starch, mannitol, dextrin, fatty acid glycerides, polyethylene glycol, hydroxyethyl starch,
5 ethylene glycol, polyoxyethylene sorbitan fatty acid esters, gelatine, albumin, amino acids, water, and physiological saline.

The aforementioned ingredients for seasoning are not restricted to any particular one and they may be any ones currently used in this field, but specific examples thereof are those already described above.

10 The contents of the foregoing carriers, other seasoning ingredients or the like are not restricted to specific ones.

Among the foregoing seasoning ingredients, the yeast extract is not particularly restricted in any of the cell bodies of microorganisms, from which the extract is originated, the conditions of cultivating the microorganisms and the methods for the extraction thereof and
15 accordingly, any yeast extract can be used in the products of the present invention. Furthermore, these yeast extracts may be ones which are subjected to, for instance, a heat-treatment, a treatment with an enzyme, a concentration treatment and/or a treatment for converting the same into powder.

The present invention also provides a method for the preparation of a variety of foods
20 and beverages, which comprises the step of incorporating γ -Glu-Nva-Gly into an intermediate used for preparing a variety of foods and beverages in such a manner that the resulting product contains γ -Glu-Nva-Gly in an amount ranging from 1 ppb by mass to 99.9% by mass. In this respect, the foods, beverages or the like are preferably those having a low content of fats.

The present invention also provides a method for the preparation of a variety of foods or
25 beverages, which comprises the step of incorporating the food composition of the present invention into intermediates used for preparing various kinds of foods or beverages. In this connection, the various kinds of foods or beverages are preferably low fat foods.

Regarding the method, according to the present invention, for preparing an intermediate used for the preparation of other foods or beverages, which makes use of the kokumi-imparting
30 agent, it is preferred that the method comprises the steps of adding the flavor enhancer

consisting of γ -Glu-Nva-Gly to food ingredients (such as umami-imparting ingredients, hydrolyzates of proteins, or extracts of meat) while admixing them together and, depending on necessity, further cooking the resulting mixture of the food ingredients to thus form foods or beverages or intermediates for preparing the same.

5 In this respect, the step for the addition of the flavor enhancer consisting of γ -Glu-Nva-Gly to the food ingredients preferably comprises the step of controlling the concentration of γ -Glu-Nva-Gly in the intermediate used for preparing a food or beverage to a level ranging from 0.01 to 999,900 ppm by mass and preferably 0.1 to 200,000 ppm by mass.

10 In addition, it is also preferred that the method further comprises the step of adding an intermediate for the preparation of foods or beverages to other ingredients for foods or beverages (such as agricultural products, marine products, meat, dairy products, or processed products thereof) while controlling the concentration of γ -Glu-Nva-Gly in the resulting foods or beverages to a level of from 0.01 to 50 ppm by mass and preferably 0.05 to 20 ppm by mass.

15 Moreover, the step for adding the flavor enhancer consisting of γ -Glu-Nva-Gly to the food ingredients, while mixing them together preferably comprises the step of controlling the concentration of γ -Glu-Nva-Gly in the resulting foods or beverages to a level of from 0.01 to 50 ppm by mass and preferably 0.05 to 20 ppm by mass.

In the aforementioned production method, it is preferred that the foods and beverages are those containing ingredients derived from pork. In this case, each of the foods and beverages 20 is preferably one comprising 0.01 to 50 ppm by mass of γ -Glu-Nva-Gly, 0.005 to 80% by mass of the ingredient derived from pork, and other ingredients of each corresponding food.

In the foregoing production method, the foods and beverages are preferably those containing ingredients derived from beef. In this case, each of the foods and beverages is preferably one comprising 0.01 to 50 ppm by mass of γ -Glu-Nva-Gly, 0.005 to 80% by mass 25 of the ingredient derived from beef, and other ingredients of each corresponding food.

Moreover, the foods intended for the present invention preferably include, in addition to the aforementioned foods, foods (sweet type ones) belonging to the category of the dessert and the confectionary, which are mainly intended for sweet taste such as ice cream, honey, marmalade, and strawberry jam; and foods (savory type ones), for instance, processed foods 30 and/or daily dishes which are mainly intended for salty taste such as chicken soups.

The present invention will hereunder be described in more detail with reference to the following Examples, but the present invention is not restricted to these specific Examples at all.

Example

5 (Synthetic Example 1): Synthesis of γ -Glu-Nva-Gly (γ -L-glutamyl-L-norvalyl-glycine):

There were dissolved, in methylene chloride (CH_2Cl_2 , 100 mL), Boc-Nva (t-butoxycarbonyl-L-norvaline, 4.44 g, 20.4mM) and Gly-OBzl $\cdot$ HCl (glycine benzyl ester hydrochloride, 4.12 g, 20.4mM). There were then added, to the reaction solution, triethylamine (Et_3N , 3.13 g, 1.1 eq., 22.4mM), HOBt $\cdot$ H_2O (1-hydroxy-benzotriazole hydrate, 10 3.44 g, 1.1 eq., 22.4mM) and WSC $\cdot$ HCl (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, 4.30 g, 1.1 eq., 22.4mM), while maintaining the solution at 0°C . The temperature of the reaction solution was gradually raised and the solution was stirred at room temperature overnight (16 hours). The reaction solution was concentrated under reduced pressure, ethyl acetate (150 mL) was added to the resulting residue, the organic phase was 15 washed with water (50mL), twice with a 5% aqueous solution of citric acid (50mL), with a saturated common salt solution (50mL), twice with a 5% aqueous solution of sodium hydrogen carbonate (50mL) and with a saturated common salt solution (50mL), and then the organic phase was dried over anhydrous magnesium sulfate. The magnesium sulfate was removed through filtration and the filtrate was concentrated under reduced pressure. When n-hexane 20 was added to the residue, crystals were formed and they were then recovered through filtration and dried under reduced pressure to thus obtain Boc-Nva-Gly-OBzl (6.88 g, 18.9mM) as crystals.

To Boc-Nva-Gly-OBzl (6.88 g, 18.9mM), there was added a 4N HCl/dioxane solution (94.5 mL) and the resulting mixture was stirred at room temperature for one hour. The 25 dioxane was removed through the concentration of the mixture under reduced pressure and n-hexane (30 mL) was added to the residue and then the resulting mixture was concentrated under reduced pressure. In this respect, the latter two steps were repeated 3 times to give H-Nva-Gly-OBzl $\cdot$ HCl at a quantitative yield.

The product of the foregoing step: H-Nva-Gly-OBzl $\cdot$ HCl was dissolved in methylene 30 chloride (130 mL) and the reaction solution was maintained at 0°C . To the reaction solution,

there were added Z-Glu-OBzl (N- α -carbobenzoxy-L-glutamic acid α -benzyl ester, 7.03 g, 18.9mM), triethylamine (2.90mL, 1.1 eq., 20.8mM), HOBt $\cdot$ H₂O (3.20 g, 1.1 eq., 20.8mM) and WSC $\cdot$ HCl (3.98 g, 1.1 eq., 20.8mM). The temperature of the reaction solution was gradually raised and the solution was stirred at room temperature overnight (16 hours). Then the reaction mixture was concentrated under reduced pressure, ethyl acetate (1000mL) was added to the resulting residue, and the organic phase was then washed with water (100mL), twice with a 5% aqueous solution of citric acid (100mL), with a saturated common salt solution (100mL), twice with a 5% aqueous solution of sodium hydrogen carbonate (100mL) and with a saturated common salt solution (100mL), and then the organic phase was dried over anhydrous magnesium sulfate. After the solution was heated to 50°C, the magnesium sulfate was removed through filtration and the filtrate was concentrated under reduced pressure. When the crystallization was initiated, n-hexane was added to the resulting concentrate so that the crystals were sufficiently separated. The crystals were collected through filtration and then dried under reduced pressure to thus give Z-Glu(Nva-Gly-OBzl)-OBzl (10.16 g, 16.4mM) in the form of crystals.

To a mixed solution of ethanol (250mL) and water (30mL), there were added Z-Glu(Nva-Gly-OBzl)-OBzl (10.16 g, 16.4mM) and 5% palladium on carbon (5% palladium/carbon, 1.20 g) and a catalytic reducing reaction was carried out at 50°C overnight (14 hours) in a hydrogen gas atmosphere. Water (100mL) was added to the reaction system in small portions during the reaction. The palladium/carbon was removed from the reaction system through filtration and the resulting filtrate was concentrated under reduced pressure. The residue was recrystallized from a small amount of water and ethanol to thus give γ -Glu-Nva-Gly (4.59 g, 15.1mM) as white crystals. The characteristic values thereof will be given below:

ESI-MS: (M+H)⁺ = 304.1

¹H-NMR (400 MHz, D₂O): δ (ppm): 0.82 (3H, t, J=7.4Hz), 1.23-1.37 (2H, m), 1.55-1.75 (2H, m), 2.01-2.09 (2H, m), 2.38-2.48 (2H, m), 3.72 (1H, t, J=6.4Hz), 3.87 (1H, dd, J=17.8 and 20.9Hz), 4.21 (1H, dd, J=4.4 and 8.9Hz).

(Test Example 1): Preparation of CaSR-Expression Plasmid

A CaSR-expression plasmid was prepared according to the following procedures:

There were synthesized synthetic oligo DNA used for the PCR procedures (i.e., forward primer (Sequence No. 3: ACTAATACGACTCACTATAGGGACCATGGCA-TTTTATAGCTGCTGCTGG) and reverse primer (Sequence No. 4: TTATGAATT-CACTACGTTTTCTGTAAACAG) on the basis of the DNA sequence registered at NCBI (CaSR (calcium receptor): NM_000388, Sequence Nos. 1 and 2).

The PCR procedures were carried out under the conditions specified below using cDNA (available from Clontech Company) derived from human kidney as a material, while using the foregoing primers and Pfu Ultra DNA Polymerase (available from Stratagene Company): The reaction system was processed at 94°C for 3 minutes and then at 94°C for 30 seconds, at 55°C for 30 seconds and at 72°C for 2 minutes, wherein these steps were repeated over 35 times and the system was finally reacted at 72°C for 7 minutes. The reaction system was then subjected to electrophoresis treatment through agarose as a support, the agarose was stained with a DNA-staining agent and then it was irradiated with UV light rays to detect whether the cDNA was surely amplified by the PCR procedures or not. At the same time, the electrophoresis pattern was compared with the DNA marker whose electrophoretic size had been known to thus confirm the chain lengths of the PCR products.

Plasmid vector pBR322 was cleaved with a restriction enzyme EcoRV (available from Takara Co., Ltd) and the gene fragment amplified by the PCR procedures was ligated with the plasmid vector at the cleaved site, using Ligation Kit (available from Promega Company). The cells of Escherichia coli DH5 α strain were transformed with this reaction solution, followed by the selection of the transformants containing the plasmid in which the PCR-amplified product had been cloned and further the PCR-amplified product was confirmed by the DNA-base sequence analysis.

This recombinant plasmid was used for the establishment of a human CaSR-expression plasmid hCaSR/pcDNA3.1.

(Test Example 2): Evaluation of CaSR Agonist Activity

293E Cells (EBNA1-expression HEK293 cells, ATCC No. CRL-10852) were cultivated in DMEN/Ham's-F12 (3.15/mL glucose-containing Dulbecco's modified Eagle medium, available from Nakalai Tesque) supplemented with 10% fetal calf serum, in the presence of 200 μ g/mL of G418 (Geneticin). The cultured cells were inoculated in F25 flask at a density of

3x10⁶ cells/10 mL, the flask was allowed to stand over 24 hours in a CO₂ incubator (5% CO₂, 37°C), and then the human CaSR-expression plasmid hCaSR/pcDNA3.1 was transformed or transfected with a reagent for transfection Fugene6 (available from Roche Company). The transfected plasmid was maintained in a CO₂ incubator over 6 to 7 hours, then the cells were recovered using a 10% fetal calf serum-containing DMEM/ Ham's-F12 and the cells were inoculated in each well of a poly-D-lysine coat 96-well plate (BD-Biocoat) at a density of 70,000 cells/well.

The 96-well plate was allowed to stand over 24 hours in a CO₂ incubator, then the culture medium was removed from each well of the 96-well plate in which the cells were inoculated, followed by the addition, to each well, of a Ca²⁺ fluorescent indicator Calcium 4 Assay Kit (available from Molecular Devices Company) dissolved in Assay Buffer (containing 146mM of NaCl, 5mM of KCl, 1mM of MgSO₄, 1 mg/mL of glucose, 20mM of HEPES (pH 7.2) and 0.75 to 1.25mM of CaCl₂) in an amount of 200 μ l/well, and subsequently allowing the 96-well plate to stand at 37°C for one hour and then at room temperature for 10 minutes so that the indicator was incorporated into the cells.

To each well of the 96-well plate, there was added each test compound dissolved in a 0.1% BSA-containing Assay Buffer in an amount of 50 μ l/well and then any change of the intensity of fluorescence was monitored over 3 minutes using FLEX Station (available from Molecular Devices Company).

(Method for the Determination of EC₅₀):

The difference (RFU (Max-Min)) between the maximum and minimum fluorescent intensities observed for each well before and after the addition of each specific test compound was determined by the automatic calculation using FLEX Station. The activity rate was calculated while the RFU (Max-Min) value observed when adding the compound at the maximum concentration was defined to be 100% and the RFU (Max-Min) value observed when using the 0.1% BSA-containing Assay Buffer free of any test compound was defined to be 0%, followed by subjecting the resulting data to the curve-fitting procedures using the spreadsheet software Xfit or Graph-Pad-Prism to thus determine the EC₅₀ which was the concentration of the compound at the activity rate of 50%. The results thus obtained are summarized in the following Table 1. In addition, the same EC₅₀-determining procedures used above were

repeated except for using other tripeptides as Comparative Examples. The results thus obtained are summarized in the following Tables 2 and 3. In this respect, the data listed in Table 3 are disclosed in Non-Patent Document 3.

Table 1

Compound	EC ₅₀ , μ M
γ -Glu-Nva-Gly	0.055
γ -Glu-Val-Gly	0.075

5

Table 2

Compound	EC ₅₀ , μ M
γ -Glu-Ala-Gly	0.016
γ -Glu-tLeu-Gly	0.09
γ -Glu-Thr-Gly	2.8

Table 3

Compound	EC ₅₀ , μ M
γ -Glu-Val-Gly	0.039
γ -Glu-Abu-Gly	0.025
γ -Glu-Cys-Gly	0.7

10 Unexpectedly, the foregoing results indicate that γ -Glu-Nva-Gly shows a high CaSR activity almost identical to those observed for other peptides each having a γ -Glu-X-Gly type structure.

Example 1: Evaluation of Kokumi-Imparting Activity

15 In this example, γ -Glu-Nva-Gly was inspected for the intensity of its kokumi-imparting activity according to a quantitative sensory evaluation test.

 This quantitative sensory evaluation test was carried out according to the following procedures: The intensity of the kokumi-imparting activity observed for each test compound was determined as a value observed when blending 0.000001 to 0.1 g/dL of the corresponding

test compound to the distilled water containing sodium glutamate (0.05 g/dL), inosinic acid monophosphate (0.05 g/dL), and sodium chloride (0.5 g/dL). In this connection, when a sample showed an acidic nature upon the dissolution of the test compound as compared with the test compound-free control, the pH value of the sample was adjusted, using NaOH, to a level of the pH value (observed for the control) ± 0.2 prior to the practical use of the sample in the evaluation. The evaluation criteria are assumed to be as follows: control: 0 point; strong: 3 points; very strong: 5 points. Moreover, to make more clear the criteria, the initial/middle taste and the after taste of γ -Glu-Cys-Gly were set at 3.0 points, respectively. The scoring or rating was carried out using the linear standard technique and more specifically, it was carried out by writing each corresponding score on a straight line on which points corresponding to -5 ~ 0 ~ 5 had been indicated. The panelists used in this test were defined to be persons who had been engaged in the development of seasonings for foods over not less than one year of the cumulative time period and who could judge that the difference in the titer between γ -Glu-Cys-Gly and γ -Glu-Val-Gly added to a solution having umami/salty taste is about 10 times (while confirming the ability of these persons at regular intervals). The evaluation was carried out at n (the number of panelists used) = 4. In this connection, the term "initial/middle taste" means the taste-development detected during the term extending from 0 to 5 seconds after the sample is introduced into the panelist's mouth, while the term "after taste" means the taste-development detected thereafter. The test compound widely showed the kokumi-imparting activity over the foregoing range of concentration thereof added. However, the results observed for typical concentrations thereof are summarized in the following Table 4.

As a result, it has been elucidated that all of the tripeptides other than γ -Glu-Nva-Gly, examined above show a CaSR activity on the order of at highest 10 times that observed for glutathione (γ -Glu-Cys-Gly), but γ -Glu-Nva-Gly surprisingly shows higher CaSR activity on the order of greater than 100 times that observed for glutathione.

Table 4

Sample	Conc. (g/dL)	Intensity of Kokumi		Comment on Evaluation
		Initial/ middle Taste	After Taste	
Control	--	0	0	--
γ -Glu- Cys-Gly	0.01	3.0	3.0	Dense-feeling, thickness, and continuity were enhanced.
γ -Glu- Val-Gly	0.001	2.5	3.0	Mainly mallowness, dense-feeling, and growth were enhanced.
	0.005	3.5	4.0	Mainly mallowness, dense-feeling and growth were enhanced.
γ -Glu- Nva-Gly	0.00005	2.7	2.9	Mainly dense-feeling and growth were enhanced at the middle/after taste stage.
	0.0001	3.0	3.3	Mainly dense-feeling and growth were enhanced at the middle/after taste stage.
	0.001	4.0	4.0	The taste was entirely thickened. The taste was too strong.
γ -Glu- Abu-Gly	0.002	3.0	2.0	The middle was enhanced as compared with γ -Glu-Val-Gly.
γ -Glu- Ala-Gly	0.001	3.3	2.3	Dense-feeling at the initial taste stage was strong, but the after taste became obscure.
γ -Glu- Abu-GlyA	0.001	2.7	3.0	Slight astringency was observed at the after taste stage.
γ -Glu- tLeu-Gly	0.001	2.5	3.0	This showed such a tendency that the after taste was rather strong. Slight astringency remained within the mouth.

The kokumi-imparting activity of γ -Glu-Nva-Gly is about 100 times higher than that observed for γ -Glu-Cys-Gly and at least about 10 times higher than that observed for γ -Glu-Val-Gly of the like. Further, γ -Glu-Nva-Gly never possesses any off-taste (such as the astringency of the after taste) unlike γ -Glu-Ala-Gly, γ -Glu-Abu-Gly and γ -Glu-tLeu-Gly and this accordingly indicates that γ -Glu-Nva-Gly is excellent as compared with other tripeptides examined.

Example 2: Evaluation of Kokumi-Imparting Activity

In this example, γ -Glu-Nva-Gly was inspected for the intensity of its kokumi-imparting activity according to a quantitative sensory evaluation test, while paying attention to a separate item for evaluation in order to make clear that this tripeptide is a middle/after taste type one.

This quantitative sensory evaluation test was carried out according to the following procedures: In this test, to make the detection of the middle/after taste easy, the use of inosinic acid monophosphate in the liquid used for the evaluation was omitted to thus reduce the umami, at the middle/after taste stage, in the liquid. More specifically, the intensity of the kokumi-imparting activity observed for each test compound was determined as a value observed when blending 0.000001 to 0.1 g/dL of the corresponding test compound as a sample to the distilled water containing sodium glutamate (0.1 g/dL), and sodium chloride (0.4 g/dL). In this connection, when a sample showed an acidic nature upon the dissolution of the test compound as compared with the test compound-free control, the pH value of the sample was adjusted, using NaOH, to a level of the pH value (observed for the control) ± 0.2 prior to the practical use of the sample in the evaluation. The evaluation criteria are assumed to be as follows: control: 0 point; strong: 3 points; very strong: 5 points. Moreover, to make the criteria more clear, the initial taste and the middle/after taste of γ -Glu-Cys-Gly were set at 3.0 points, respectively. The scoring or rating was carried out using the method of line scale and more specifically, it was carried out by writing each corresponding score on a straight line on which points corresponding to -5 ~ 0 ~ 5 had been indicated. The panelists used in this test were defined to be persons who had been engaged in the development of seasonings for foods over not less than one year of the cumulative time period and who could judge that the difference in the titer between γ -Glu-Cys-Gly and γ -Glu-Val-Gly added to a solution having umami/salty taste is

about 10 times (while confirming the ability of these persons at regular intervals). The evaluation was carried out at n (the number of panelists used) = 4. In this connection, the term “initial taste” means the taste-development detected during the term extending from 0 to 2 seconds after the sample is introduced into the panelist’s mouth, while the term “middle/after taste” means the taste-development detected thereafter. The test compound widely showed the kokumi-imparting activity over the foregoing range of concentration thereof added. However, the results observed for typical concentrations thereof are summarized in the following Table 5.

These results likewise indicate that γ -Glu-Val-Gly shows a kokumi- imparting activity of about 10 times higher than that observed for glutathione (γ -Glu-Cys- Gly), while γ -Glu-Nva-Gly shows higher kokumi-imparting activity on the order of more than 100 times that observed for glutathione.

Table 5

Sample	Conc. (g/dL)	Intensity of Kokumi		Comment on Evaluation
		Initial Taste	Middle/ after Taste	
Control	--	0	0	--
γ -Glu- Cys- Gly	0.01	3	3	Dense-feeling, thickness and continuity were enhanced.
γ -Glu- Val-Gly	0.001	2	3	Mainly, smoothness, dense-feeling and growth were enhanced.
γ -Glu- Nva-Gly	0.00005	2	3	Dense-feeling and growth were enhanced mainly at the middle/after taste stage.
	0.0001	3	4	Dense-feeling and growth were enhanced mainly at the middle/after taste stage.

As a result, it was found that γ -Glu-Nva-Gly was quite excellent. This is because it possesses an excellent kokumi-imparting activity, has the middle/after taste type characteristics with respect to its taste-developing pattern and is free of any off-taste (such as astringent taste). In addition, γ -Glu-Nva-Gly has a kokumi-imparting activity about 100 times higher than that observed for γ -Glu-Cys-Gly and at least about 10 times higher than that observed for, for

instance, γ -Glu-Val-Gly. For this reason, γ -Glu-Nva-Gly can be used in an extremely low concentration. Accordingly, the present invention can thus provide a kokumi-imparting agent, which can easily be prepared at a low cost and therefore, the present invention would be quite effective from the industrial standpoint.

5 Example 3: Evaluation of Kokumi-Imparting Activity in Foods

γ -Glu-Nva-Gly was examined on whether, or not, it showed a kokumi-imparting effect higher than that observed for γ -Glu-Val-Gly having a high titer, according to a sensory evaluation test, while the former was actually incorporated into a food.

The sensory evaluation test was carried out according to the following procedures: As
10 foods which were considered to be one having strong middle/after taste, commercially available ice cream, honey, marmalade and strawberry jam were used in this example as typical of foods (sweet type ones) belonging to the category of the dessert and the confectionary, which were mainly intended for sweet taste. As samples typical of the foods (savory type ones) belonging to the category of the processed foods, daily dishes and snack foods, which were mainly
15 intended for salty taste, there were used commercially available chicken soup; a paste comprising mashed potato and 0.1% by mass of commercially available powdered pepper; commercially available ginger paste; and a paste comprising mashed potato and 2% by mass of butter. The amount of γ -Glu-Val-Gly, as a subject of comparison, to be added was set at a level of 0.002% by mass at which the effect thereof was distinct. The tripeptide γ
20 -Glu-Nva-Gly was inspected for the enhancement (the intensity of the kokumi-imparting activity) of the overall taste-development when it was blended with each test food in an amount of 0.0000001 to 0.01% by mass. In this case, the control was each food free of any added tripeptide. The evaluation criteria are assumed to be as follows, so as not to cause any small difference such as the fractional portion of each score : control: $\pm$; rather strong: +; strong: ++; very strong: ++++. Moreover, the score of + was defined to be 1 point; the score of ++ was defined to be 2 points; and the score of +++ was defined to be 3 points, and if a numerical average value is, for instance, 2.2, the decimal fraction is approximated to be 2=++, through the rounding off of the number to the nearest integer. The panelists used in this test were defined to be persons who had been engaged in the development of seasonings for foods over not less
25 than one year of the cumulative time period and who could judge that the difference in the titer
30

between γ -Glu-Cys-Gly and γ -Glu-Val-Gly added to a solution having umami/ salty taste is about 10 times (while confirming the ability of these persons at regular intervals). The evaluation was carried out at n (the number of panelists used) = 4. The test compound γ -Glu-Val-Gly widely showed the kokumi-imparting activity over the foregoing range of concentration thereof added. However, only the results which can definitely be used for the purpose of comparison are summarized in the following Tables 7 and 8.

The results thus obtained likewise indicate that γ -Glu-Val-Gly shows the kokumi-imparting activity about 10 times higher than that observed for glutathione (γ -Glu-Cys-Gly), while γ -Glu-Nva-Gly shows more higher kokumi-imparting activity on the order of 5 to more than 13 times that observed for γ -Glu-Val-Gly.

Table 7

Sample	Conc. (wt%)	Enhancement of Overall Taste-Development (Flavoring)			
		Ice Cream	Honey	Marmalade	Strawberry Jam
γ -Glu-Val-Gly	0.002	+	+	+	+
γ -Glu-Val-Gly	0.00015	+	$\pm$	$\pm$	$\pm$
	0.00028	++	$\pm$	$\pm$	$\pm$
	0.0004	+++	+	+	+

Table 8

Sample	Conc. (wt%)	Enhancement of Overall Taste-Development (Flavoring)			
		Chicken soup	Ginger paste	Powdered pepper	Butter paste
γ -Glu-Val-Gly	0.002	+	+	+	+
γ -Glu-Val-Gly	0.00015	+	+	$\pm$	$\pm$
	0.00028	++	++	+	+
	0.0004	+++	+++	++	++

The foregoing test results indicate that γ -Glu-Nva-Gly shows a promising kokumi-imparting activity capable of improving the overall taste-developing ability in all of the actual foods, whose middle/after tastes are characteristic and which extend from the savory-type food to the sweet-type food. Moreover, the foregoing results also indicate that γ -Glu-Nva-Gly shows an extremely high kokumi-imparting activity, in the actual foods, 5 to more than 13 times higher than that observed for γ -Glu-Val-Gly. Accordingly, the use of γ -Glu-Nva-Gly in an extremely low amount would improve the quality of foods even when more additional ingredients cannot be incorporated into each specific food for the purpose of ensuring the intended quality stability when there is a demand for further improvement of the quality thereof. In addition, a kokumi-imparting agent can be provided at a low cost.

Example 4: Effect of γ -Glu-Nva-Gly on Extracts of Pork:

It was found that the kokumi-imparting activity of γ -Glu-Nva-Gly could be enhanced at the early stage after eating the food containing the same as compared with those observed for γ -Glu-Cys-Gly (glutathione) and γ -Glu-Val-Gly. Under such circumstances, it was then confirmed, according to a sensory evaluation test, that γ -Glu-Nva-Gly was considerably effective for the improvement of the taste-development of an extract of pork whose taste-development was not complete middle taste type one, but whose taste was enhanced slightly earlier, while comparing the effect thereof with those observed for γ -Glu-Cys-Gly (glutathione) and γ -Glu-Val-Gly.

The sensory evaluation test was carried out according to the following procedures: Commercially available pork extract (solid content: 55.1% by mass, salt content: 9.3% by mass) was dissolved in hot water such that the concentration of the extract was set at a level of 5.0% by mass to thus form a pork extract solution. There was then blended, with this pork extract solution, γ -Glu-Nva-Gly, γ -Glu-Cys-Gly or γ -Glu-Val-Gly as a sample. According to the paired comparison test, the panelists were requested to comparatively evaluate the following three samples and to judge that "either of them was preferred or favorable since it could enhance the flavor and taste of the pork extract without changing the balance between them": (1) 0.02% by mass of γ -Glu-Cys-Gly whose kokumi-imparting activity was identical to 0.0003% by mass of γ -Glu-Nva-Gly; (2) 0.0003% by mass of γ -Glu-Val-Gly, the amount of which was identical to 0.0003% by mass of γ -Glu-Nva-Gly; (3) 0.002% by mass of γ -Glu-Val-Gly

whose kokumi- imparting activity was identical to that observed for 0.0003% by mass of γ -Glu-Nva-Gly. The evaluation was carried out at N (the number of panelists used) = 9. The following Table 9 shows the number of the panelists who judged that 0.0003% by mass of γ -Glu-Nva-Gly was “preferred or favorable since it could enhance the flavor and taste of the pork extract without changing the balance between them”.

From the results thus obtained, it could be concluded as follows: γ -Glu-Nva- Gly shows such a remarkable effect that it can definitely “enhance the flavor and taste of the pork extract without changing the balance between them” even in cases where the titers of the kokumi were identical to one another like the cases of (1) and (3).

Table 9

N=9

Sample	Conc. (wt%)	Sample	Conc. (wt%)	No. of Panelists*
(1) γ -Glu-Nva-Gly	0.0003	γ -Glu-Cys-Gly	0.02	8/9**
(2) γ -Glu-Nva-Gly	0.0003	γ -Glu-Val-Gly	0.0003	8/9**
(3) γ -Glu-Nva-Gly	0.0003	γ -Glu-Val-Gly	0.002	8/9**

*: The number of panelists who judged that γ -Glu-Nva-Gly was rather preferred since it could enhance the flavor and taste of the pork extract, without changing the balance between them.

**: The result indicates that γ -Glu-Nva-Gly is rather preferred since it can enhance the flavor and taste of the pork extract, without changing the balance between them, at a significant level of 5%.

The foregoing results clearly indicate that γ -Glu-Nva-Gly shows the following quite remarkable effect: it can “enhance and make, more favorable, the flavor and taste of pork extract without changing the balance between them”, when comparing them with those observed for γ -Glu-Cys-Gly and γ -Glu-Val-Gly used at concentrations capable of exhibiting the same kokumi-imparting activities. Ingredients derived from pork have widely been used throughout the world in, for instance, seasonings, soup, processed meat products, cooked and processed products, confectionery and snack foods. As has been discussed above, γ -Glu-Nva-Gly would permit the improvement of the flavor and taste of foods, at a low cost, while using the same only in a trace amount and therefore, the use thereof is quite advantageous

from the industrial standpoint.

Example 5: Effect of γ -Glu-Nva-Gly on Extracts of Beef

It was found that the kokumi-imparting effect and activity of γ -Glu-Nva-Gly could be enhanced at the early stage after eating the food containing the same as compared with those observed for γ -Glu-Cys-Gly (glutathione) and γ -Glu-Val-Gly. Under such circumstances, it was then confirmed, according to a sensory evaluation test, that γ -Glu-Nva-Gly was considerably effective for the improvement of the taste-development of an extract of beef whose taste-development was not complete middle taste type one, but whose taste was enhanced slightly earlier and sustained even in the after taste stage, when comparing the effect thereof with those observed for γ -Glu-Cys-Gly (glutathione) and γ -Glu-Val-Gly.

The sensory evaluation test was carried out according to the following procedures: Commercially available beef extract (solid content: 61.2% by mass, salt content: 12.2% by mass) was dissolved in hot water such that the concentration of the extract was set at a level of 3.0% by mass to thus form a beef extract solution. There was then blended, with this beef extract solution, γ -Glu-Nva-Gly, γ -Glu-Cys-Gly or γ -Glu-Val-Gly as a sample. According to the two point-distinguishing test method (pair test), the panelists were requested to comparatively evaluate the following three samples and to judge that "either of them was preferred or favorable since it could enhance the flavor and taste of the beef extract without changing the balance between them": (1) 0.02% by mass of γ -Glu-Cys-Gly whose kokumi-imparting activity was identical to 0.0003% by mass of γ -Glu-Nva-Gly; (2) 0.0003% by mass of γ -Glu-Val-Gly, the amount of which was identical to 0.0003% by mass of γ -Glu-Nva-Gly; (3) 0.002% by mass of γ -Glu-Val-Gly whose kokumi-imparting activity was identical to that observed for 0.0003% by mass of γ -Glu-Nva-Gly. The evaluation was carried out at N (the number of panelists used) = 9. The following Table 10 shows the number of the panelists who judged that 0.0003% by mass of γ -Glu-Nva-Gly was "preferred or favorable since it could enhance the flavor and taste of the beef extract without changing the balance between them".

From the results thus obtained, it could be concluded as follows: γ -Glu-Nva-Gly shows such a remarkable effect that it can definitely "enhance the taste and flavor of the beef extract without changing the balance between them" even in cases where the titers of the

kokumi were identical to one another like the cases of (1) and (3).

Table 10

N=9

Sample	Conc. (wt%)	Sample	Conc. (wt%)	No. of Panelists*
(1) γ -Glu-Nva-Gly	0.0003	γ -Glu-Cys-Gly	0.02	9/9**
(2) γ -Glu-Nva-Gly	0.0003	γ -Glu-Val-Gly	0.0003	8/9***
(3) γ -Glu-Nva-Gly	0.0003	γ -Glu-Val-Gly	0.002	8/9***

*: The number of panelists who judged that γ -Glu-Nva-Gly was rather preferred since it could enhance the taste and flavor of the beef extract, without changing the balance between them.

**: The result indicates that γ -Glu-Nva is rather preferred since it can enhance the taste and flavor of the beef extract, without changing the balance between them, at a significant level of 1%.

***: The result indicates that γ -Glu-Nva-Gly is rather preferred since it can enhance the flavor and taste of the beef extract, without changing the balance between them, at a significant level of 5%.

The foregoing results clearly indicate that γ -Glu-Nva-Gly shows the following quite remarkable effect: it can "enhance and make, more favorable, the flavor and taste of beef extract without changing the balance between them", when comparing them with those observed for γ -Glu-Cys-Gly and γ -Glu-Val-Gly used at concentrations capable of exhibiting the same kokumi-imparting activities. Ingredients derived from beef have widely been used throughout the world in, for instance, seasonings, soup, processed meat products, cooked and processed products, confectionery and snack foods. As has been discussed above, γ -Glu-Nva-Gly would permit the improvement of the flavor and taste of foods, at a low cost, while using the same only in a trace amount and therefore, the use thereof is quite advantageous from the industrial standpoint.

What is claimed is:

1. A kokumi-imparting agent consisting essentially of γ -Glu-Nva-Gly.
2. A complex kokumi-imparting agent comprising, in combination, (a) γ -Glu-Nva-Gly and (b)
5 one or at least two amino acids or peptides selected from the group consisting of γ -Glu-X-Gly
wherein X represents an amino acid or an amino acid derivative, γ -Glu-Val-Y wherein Y
represents an amino acid or an amino acid derivative, γ -Glu-Nva, γ -Glu-Abu, γ -Glu-Ala,
 γ -Glu-Gly, γ -Glu-Cys, γ -Glu-Met, γ -Glu-Thr, γ -Glu-Val, γ -Glu-Orn, Asp-Gly,
Cys-Gly, Cys-Met, Glu-Cys, Gly-Cys, Leu-Asp, D-Cys, γ -Glu-Met (O), γ -Glu- γ -Glu-Val,
10 γ -Glu-Val-NH₂, γ -Glu-Val-ol, γ -Glu-Ser, γ -Glu-Tau, γ -Glu-Cys (S-Me) (O), γ -
Glu-Leu, γ -Glu-Ile, γ -Glu-t-Leu and γ -Glu-Cys (S-Me).
3. A food composition comprising 0.1 ppb by mass to 99.9% by mass of γ -Glu-Nva-Gly.
4. The food composition as set forth in claim 3, wherein it comprises 0.01 to 50 ppm by mass of
 γ -Glu-Nva-Gly, 0.005 to 80% by mass of an ingredient derived from pork, and other food
15 ingredients.
5. The food composition as set forth in claim 3, wherein it comprises 0.01 to 50 ppm by mass of
 γ -Glu-Nva-Gly, 0.005 to 80% by mass of an ingredient derived from beef, and other food
ingredients.
6. A method for preparing a food or beverage, or an intermediate used for preparing a food or
20 beverage, comprising the steps of:
 adding a flavor enhancer consisting essentially of γ -Glu-Nva-Gly to food ingredients,
 while mixing them together; and
 optionally cooking the resulting mixture of the food ingredients.
7. The method for preparing a food or beverage, or an intermediate used for preparing a food or
25 beverage as set forth in claim 6, wherein the step for adding the flavor enhancer consisting
essentially of γ -Glu-Nva-Gly to the food ingredients, while mixing them together, further
comprises controlling the concentration of γ -Glu-Nva-Gly present in the intermediate used for
preparing a food or beverage to a level ranging from 0.01 to 999,900 ppm by mass.
8. The method for preparing a food or beverage as set forth in claim 7, wherein it further
30 comprises the step of adding an intermediate for preparing a food or beverage to other food

ingredients while controlling the concentration of γ -Glu-Nva-Gly present in the resulting food or beverage to a level ranging from 0.01 to 50 ppm by mass.

9. The method for preparing a food or beverage as set forth in claim 7, wherein the step for adding the flavor enhancer consisting essentially of γ -Glu-Nva-Gly to the food ingredients,
5 while mixing them together, comprises the step of controlling the concentration of γ -Glu-Nva-Gly present in the resulting food or beverage to a level ranging from 0.01 to 50 ppm by mass.

10. The method for preparing a food or beverage as set forth in any one of claims 6 to 9, wherein the food or beverage is a food containing an ingredient derived from pork.

10 11. The method for preparing a food or beverage as set forth in any one of claims 6 to 10, wherein the food or beverage is a food containing an ingredient derived from beef.

12. A food or beverage, or an intermediate used for preparing a food or beverage characterized in that it is a product prepared according to the method as set forth in any one of claims 6 to 11.

13. A method for enhancing the flavor and/or taste of a food or beverage comprising the step of
15 adding a composition containing γ -Glu-Nva-Gly to a food or beverage.

14. The method as set forth in claim 13, wherein the flavor-enhancing step is a kokumi-imparting step.

Kokumi-Imparting Agent

Abstract

The present invention intends to search for most of variational compounds possessing the desired CaSR agonist activity and to thus find out a substance capable of imparting a kokumi to a food, which shows a more excellent kokumi-imparting effect, in particular, a
5 kokumi-imparting effect of the middle/after taste-imparting type one, which is excellent in the stability and which has a high titer and more specifically the present invention intends to provide a kokumi-imparting agent consisting of such a substance as well as a complex kokumi-imparting agent comprising the substance and other substances each possessing a CaSR
10 agonist activity.

The present invention thus provides a kokumi-imparting agent consisting of γ -Glu-Nva-Gly (L- γ -glutamyl-L-norvalyl-glycine), and a complex kokumi-imparting agent comprising the substance and another substance having a CaSR agonist activity, in combination.

(No suitable figure)