

Description

Title of Invention: COMPOSITION CONTAINING CYCLIC DIPEPTIDE, PURINE NUCLEOSIDE AND/OR AMINO ACID, AND CHICKEN EXTRACT, PRODUCTION METHOD THEREOF, AND USE OF CYCLIC DIPEPTIDE, PURINE NUCLEOSIDE AND/OR AMINO ACID, AND CHICKEN EXTRACT

Technical Field

[0001] The present invention relates to a composition containing a cyclic dipeptide, a purine nucleoside and/or amino acid, and chicken extract, and a production method thereof. The present invention also relates to use of a cyclic dipeptide, a purine nucleoside and/or amino acid, and chicken extract for the production of an anti-inflammatory composition.

Background Art

[0002] Inflammation is a phenomenon in which histamine, kinins, and the like are released by damaged cells, which causes vasodilation, increased capillary permeability, and aggregation of macrophages at an inflammatory site, resulting in increased blood flow at an infected site, edema, transfer of immune cells and antibodies, pain, fever, or the like.

[0003] Recently, components capable of inhibiting expression of a protein related to inflammation have been studied to provide effective relief of inflammation. While anti-inflammatory drugs having various mechanisms, such as non-steroidal anti-inflammatory drug (NSAID) and steroidal anti-inflammatory drugs (SAID), have been developed, these drugs may have side effects. Thus, there is still a demand for components that are safer and that have anti-inflammatory action.

[0004] For example, Patent Literature 1 discloses an anti-inflammatory composition containing, as an active component, a peptide derived from telomerase having anti-inflammatory activity.

Citation List

Patent Literature

[0005] PTL 1: JP2015-525768T

Summary of Invention

Technical Problem

[0006] The present invention aims to provide a novel composition containing a cyclic dipeptide, a purine nucleoside, an amino acid and/or one or more salts thereof, and

chicken extract.

The present invention also aims to provide a composition having an anti-inflammatory action.

Solution to Problem

[0007] The present inventors found that use of a combination at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract increases anti-inflammatory activity, and completed the present invention.

[0008] Specifically, the present invention is defined as follows.

(1) A composition containing at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract.

(2) The composition according to (1) above, wherein the total amount of the at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof is 0.01 to 99 wt%.

(3) The composition according to (1) or (2) above, wherein the composition contains a component derived from hydrolyzed collagen type II of chicken cartilage and having a molecular weight of less than 1100 and weight average molecular weight of 150 to 250 as determined by HPLC gel filtration.

(4) The composition according to any one of (1) to (3) above, wherein the composition contains hydrolyzed collagen type II of chicken cartilage.

(5) The composition according to any one of (1) to (4) above, wherein the chicken extract contains carnosine and/or anserine and/or one or more salts thereof.

(6) The composition according to any one of (1) to (5) above, wherein the composition is a food, beverage, or medicine.

(7) The composition according to any one of (1) to (6) above, wherein the composition is used to reduce inflammation.

(8) The composition according to any one of (1) to (7) above, wherein the composition inhibits the production of at least one cytokine selected from the group consisting of regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-9 (IL-9), and macrophage inflammatory protein-1 (MIP-1).

(9) The composition according to any one of (1) to (8) above, wherein the composition is used to prevent or alleviate inflammatory conditions or diseases.

(10) A method of producing a composition, including mixing at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof with chicken extract.

(11) The production method according to (10) above, wherein the mixing includes mixing hydrolyzed collagen type II of chicken cartilage with chicken extract, the hydrolyzed collagen type II containing at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof.

(12) The production method according to (10) or (11) above, wherein the chicken extract contains carnosine and/or anserine and/or one or more salts thereof.

(13) Use of at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract for the production of an anti-inflammatory composition.

Advantageous Effects of Invention

[0009] The present invention can provide a novel composition containing at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract. The composition of the present invention can be used as a food or beverage composition or a pharmaceutical composition to reduce inflammation and joint pain. Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, salts thereof, and chicken extract can be consumed as foods, beverages, or the like, and are also advantageous in terms of high safety.

Brief Description of Drawings

[0010] [fig.1] Fig. 1 is a flow chart summarizing a method of preparing hydrolyzed collagen type II of chicken cartilage.

[fig.2] Fig. 2 is a group of graphs each showing an effect of fraction P6 on inhibition of the production of inflammatory markers, wherein the fraction P6 is one of among seven fractions obtained in an example by fractionation of hydrolyzed collagen type II (HCII) of chicken cartilage.

[fig.3] Fig. 3 is a graph showing an effect of HCII, fraction P6 and P6 compounds (a combination of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan) on inhibition of the production of IL-6.

[fig.4] Fig. 4 is a graph showing an effect of HCII, fraction P6, P6 compounds (a combination of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan) and Cyclo(Pro-Gly)(cPG) on inhibition of the production of MCP-1.

[fig.5] Fig. 5 is a graph showing a synergistic effect of a combination of the hydrolyzed collagen type II (HCII) of chicken cartilage and the chicken extract (CE) on inhibition of the production of inflammatory marker MIP-1 β .

[fig.6] Figs. 6 (a) and (b) are graphs showing effects of HCII, CE, fraction P6, a synergistic effect of a combination of HCII and CE, and a synergistic effect of a combination of fraction P6 and CE on inhibition of the production of inflammatory markers MCP-1 and MIP-1 β .

[fig.7-1]Figs. 7-1(a) and (b) are graphs showing synergistic effects of a combination of HCII and CE, a combination of P6 compounds and CE, a combination of cPG and CE, a combination of Cyclo (Ala-Hyp)(cAH) and CE, a combination of guanosine and CE, or a combination of tryptophan and CE on inhibition of the production of inflammatory markers IL-6 and IL-8.

[fig.7-2]Figs. 7-2(c) and (d) are graphs showing synergistic effects of a combination of HCII and CE, a combination of P6 compounds and CE, a combination of cPG and CE, a combination of cAH and CE, a combination of guanosine and CE, or a combination of tryptophan and CE on inhibition of the production of inflammatory markers IL-9 and MCP-1.

[fig.7-3]Figs. 7-3(e) and (f) are graphs showing synergistic effects of a combination of HCII and CE, a combination of P6 compounds and CE, a combination of cPG and CE, a combination of cAH and CE, a combination of guanosine and CE, or a combination of tryptophan and CE on inhibition of the production of inflammatory markers MIP-1 β and RANTES.

[fig.8]Fig. 8 is a graph showing an effect of 14-day intake with hydrolyzed collagen type II of chicken cartilage (HCII) and a combination of HCII and chicken extract (CE) on VAS pain score on Days 7 and 14 in each treatment group for subjects doing less than 10th percentile of total resistance training period (n=8).

Description of Embodiments

- [0011] In an embodiment of the present invention, the composition of the present invention contains at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract.
- [0012] Cyclo (Ala-Hyp) and Cyclo (Pro-Gly) are cyclic dipeptides. Herein, the term "cyclic dipeptide" refers to a compound containing amino acids as structural units and having a diketopiperazine structure generated by dehydration condensation of an amino group of an amino acid at the N-terminal end and a carboxyl group at the C-terminal end. Herein, the order of description of amino acids in the cyclic dipeptide is not limited as long as the amino acid composition is the same. For example, (Cyclo (Ala-Hyp)) and (Cyclo (Hyp-Ala)) represent the same cyclic dipeptide.
- Guanosine is a purine nucleoside, and tryptophan is an amino acid.
- [0013] Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan may be obtained by hydrolysis of an animal/plant protein or the like, or may be artificially synthesized. Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan are preferably derived from hydrolyzed collagen type II; are more preferably derived from hydrolyzed collagen type II of chicken cartilage; and are still more preferably contained in a fraction derived from hydrolyzed collagen type II of chicken cartilage and having a

molecular weight less than 1100 and having weight average molecular weight of 150 to 250 as determined by HPLC gel filtration. Herein, the "fraction derived from hydrolyzed collagen type II of chicken cartilage and having a molecular weight less than 1100 and having weight average molecular weight of 150 to 250 as determined by HPLC gel filtration" is also referred to as "fraction 6".

[0014] Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan may be purified before use, may be contained in the form of the fraction 6 containing these components in a composition, or may be contained in the form of hydrolyzed collagen type II of chicken cartilage which contains the fraction 6 in a composition. In a preferred embodiment, the composition of the present invention contains the fraction 6 or hydrolyzed collagen type II of chicken cartilage. The composition of the present invention, which contains the fraction 6 or hydrolyzed collagen type II of chicken cartilage, exhibits higher anti-inflammatory action.

[0015] Cyclo (Ala-Hyp), Cyclo (Pro-Gly) and tryptophan can be contained in the form of a salt with an inorganic acid or an organic acid or a salt with an inorganic base or an organic base in the composition of the present invention. Such an acid or a base can be selected based on the application of the salt. In view of application to foods, beverages, and medicines, the following dietary or pharmaceutically acceptable salts are preferred. Examples of the inorganic acid salt include hydrochloride, nitrate, sulfate, methanesulfonate, and p-toluenesulfonate. Examples of the organic acid salt include salts with dicarboxylic acids such as oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, and salts with monocarboxylic acids such as acetic acid, propionic acid, and butyric acid. Examples of the inorganic base include hydroxides, carbonates, and bicarbonates of sodium, lithium, calcium, magnesium, and aluminium and ammonia. Examples of the salt with the organic base include mono-, di-, or tri-alkylamine salts such as salts of methylamine, dimethylamine, and triethylamine, mono-, di-, or tri-hydroxyalkylamine salts, guanidine salt, and N-methylglucosamine salt.

Guanosine can be phosphorylated guanosine in the composition of the present invention.

[0016] <Hydrolyzed collagen type II of chicken cartilage>

Preferably, the composition of the present invention contains hydrolyzed collagen type II of chicken cartilage.

Hydrolyzed collagen type II of chicken cartilage (hereinafter, the "hydrolyzed collagen type II of chicken cartilage" is sometimes referred to as "HCII") can be obtained by hydrolysis of collagen type II with an enzyme or the like. The collagen type II can be extracted from chicken cartilage by a known method. The hydrolyzed collagen type II of chicken cartilage for use in the present invention can be prepared from cartilage by a method usually used in this field.

For example, the hydrolyzed collagen type II can be obtained by treating the chicken cartilage with an enzyme. Specifically, the hydrolyzed collagen type II can be prepared by a pre-treatment step (1) in which chicken cartilage is heated in a liquid, and a step (2) in which the chicken cartilage after the pre-treatment step is treated with an enzyme. The enzyme for use in the step (2) is not limited as long as it is one usually used in this field. Examples include collagenase, papain, bromelain, actinidine, ficin, cathepsin, pepsin, chymosin, trypsin, protease, subtilisin, amino peptidase, endopeptidase and exopeptidase and enzyme preparations obtained by mixing these enzymes. The method of preparing hydrolyzed collagen type II is not limited to the enzyme treatment method.

[0017] The hydrolyzed collagen type II of chicken cartilage may be a solution obtained by hydrolysis of chicken cartilage, a concentrate or dry powder of the solution, or a purified product of the concentrate or dry powder. The purified product of the hydrolyzed collagen type II of chicken cartilage may be obtained by, for example, subjecting a solution obtained by hydrolysis of chicken cartilage to ultrafiltration, membrane treatment, liquid separation operation, or fraction treatment with resin or the like so as to increase the purity. After increasing the purity of the hydrolyzed collagen type II of chicken cartilage which contains at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, the purified product may be formed into powder by freeze-drying or spray-drying, for example.

[0018] Hydrolyzed collagen type II of chicken cartilage is a peptide mixture that usually contains Cyclo (Ala-Hyp), Cyclo (Pro-Gly), and/or one or more salts thereof, and can be regarded as a collagen peptide derived from the collagen type II.

The weight average molecular weight of the hydrolyzed collagen type II of chicken cartilage is preferably 100 to 20,000, more preferably 2,000 to 8,000, still more preferably 3,000 to 7,000. The molecular weight and weight average molecular weight can be measured by Eurofins HPAEC-PAD method.

[0019] In an embodiment, one fraction or a combination of two or more fractions obtained by fractionation of hydrolyzed collagen type II of chicken cartilage by molecular weight by a method such as gel filtration can be used in the composition of the present invention.

The fraction obtained by fractionation of hydrolyzed collagen type II of chicken cartilage for use in the composition of the present invention is preferably the fraction 6 described above. In the present embodiment, fraction 6 and a fraction other than fraction 6 may be used in combination in the composition.

[0020] <Chicken extract>

The chicken extract (hereinafter sometimes referred to as "CE") for use in the present

invention may be an extract that can be obtained by heating chicken meat used as a raw material in a liquid, or a commercial product. The raw material may contain bone, cartilage, legs, or the like, but preferably, the raw material does not contain the head or internal organs.

[0021] Examples of the commercial product of the chicken extract (CE) include "Brand's Essence of Chicken (BEC) (produced by Suntory Beverage & Food Asia Pte Ltd)", "Scotch™ Essence of Chicken (produced by Scotch Industrial (Thailand) Co., Ltd.)", "Quaker Essence of chicken (produced by Standard Foods Corporation (Taiwan) Co., Ltd.)", "Chicken stock and broth of SWANSON™ Produced by Campbell Soup Company (NYSE:CPB)", "Drip Chicken Essence produced by Eu Yan Sang International Ltd. (Singapore)", "Boned Chicken Tonic produced by Eu Yan Sang International Ltd. (Singapore)", "Boiled Essence of Chicken produced by Lao Xie Zhen Co. Ltd. (Taiwan)". Any of such commercial products may be used, but use of Brand's Essence of Chicken (BEC) is preferred.

[0022] When producing chicken extract for use in the present invention by hot water extraction, the chicken extract can be produced by a method that is usually used in this field. For example, normal pressure extraction and/or pressurized extraction is performed using a liquid at a temperature of 100°C or higher, preferably 125°C or higher, and the resulting extract is treated with a membrane or filtered, whereby chicken extract can be produced. Specifically, the extract is obtained by a pre-treatment step (3) in which chicken meat is heated in a liquid, and a step (4) in which the liquid is replaced with a fresh liquid after the pre-treatment and the chicken meat is heated again. The heat treatment in each of the step (3) and the step (4) is preferably performed in a solvent. The solvent is preferably water, ethanol, or a mixture of these, for example.

The chicken extract encompasses a liquid extract obtained by the method described above; a diluted solution, concentrate, or dry powder of the liquid extract; and purified products of these. The purified products may be obtained by, for example, subjecting a chicken extract liquid to ultrafiltration, membrane treatment, liquid separation operation, or fraction treatment with resin or the like so as to increase the purity. After increasing the purity of the chicken extract, the purified product may be formed into powder by freeze-drying or spray-drying, for example.

[0023] Preferably, the chicken extract for use in the present invention contains carnosine and/or anserine and/or one or more salts thereof. Carnosine is β -alanyl $\cdot$ histidine, which is a dipeptide of β -alanine and histidine. Anserine is β -alanyl $\cdot$ 1-methylhistidine in which histidine is methylated.

Examples of the carnosine salts and anserine salts include the same salts as those described above for Cyclo (Ala-Hyp), Cyclo (Pro-Gly), and tryptophan.

- [0024] When the composition of the present invention contains carnosine and/or a salt thereof, for example, the amount of carnosine and/or a salt thereof in the composition is preferably 0.00001 wt% or more, more preferably 0.0001 wt% or more, and is preferably 10 wt% or less, more preferably 1 wt% or less in terms of carnosine. In an embodiment, for example, the amount of carnosine and/or a salt thereof in the composition is preferably 0.00001 to 10 wt%, more preferably 0.0001 to 1 wt% in terms of carnosine.
- [0025] When the composition of the present invention contains anserine and/or a salt thereof, for example, the amount of anserine and/or a salt thereof in the composition is preferably 0.00001 wt% or more, more preferably 0.0001 wt% or more, and also preferably 10 wt% or less, more preferably 1 wt% or less in terms of anserine. In an embodiment, for example, the amount of anserine and/or a salt thereof in the composition is preferably 0.00001 to 10 wt%, more preferably 0.0001 to 1 wt% in terms of anserine.
- [0026] Carnosine, anserine and salts thereof can be quantitated by HPLC, for example. More preferably, the food or beverage composition of the present invention contains carnosine and/or a salt thereof, and anserine and/or a salt thereof.
- [0027] Preferably, the weight ratio of the total weight of the carnosine, anserine and salts thereof in terms of carnosine and anserine to the total weight of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof in terms of free form (total of carnosine and anserine/total in terms of free form) is 1/15000 to 200/1. The weight ratio is more preferably 1/200 to 200/1, still more preferably 1/50 to 50/1.
- [0028] In the expression "Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof in terms of free form", a free form from Cyclo (Ala-Hyp) salt is Cyclo (Ala-Hyp), a free form from Cyclo (Pro-Gly) salt is Cyclo (Pro-Gly), a free form from guanosine salt is guanosine, and a free form from tryptophan salt is tryptophan.
- [0029] The weight ratio of the fraction 6 to the total of carnosine, anserine and salts thereof in terms of carnosine and anserine (fraction 6/total of carnosine and anserine) is preferably 1/100 to 10/1. The weight ratio is more preferably 1/100 to 5/1.
- [0030] The weight ratio of the hydrolyzed collagen type II of chicken cartilage (in terms of solids) to the total of carnosine, anserine and salts thereof in terms of carnosine and anserine (HCII/total of carnosine and anserine) is preferably 10000/1 to 10/1. The weight ratio is more preferably 1000/1 to 100/1.
- [0031] The amount of each component of the composition of the present invention is not limited, and can be set according to the form or the like of the composition.
- In an embodiment, for example, the total amount of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof in the composition of the present invention is preferably 0.01 wt% or more, more preferably 0.1 wt% or more, and

preferably 99 wt% or less, more preferably 90 wt% or less. In an embodiment, for example, the total amount of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof in the composition is preferably 0.01 to 99 wt%, more preferably 0.1 to 90 wt%.

[0032] In an embodiment, for example, the amount of fraction 6 in the composition of the present invention is preferably 0.01 wt% or more, more preferably 0.1 wt% or more, and preferably 99 wt% or less, more preferably 90 wt% or less. In an embodiment, for example, the amount of fraction 6 in the composition is preferably 0.01 to 99 wt%, more preferably 0.1 to 90 wt%. Herein, the amount of fraction 6 contains the amounts of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof.

[0033] In an embodiment, for example, the amount of hydrolyzed collagen type II of chicken cartilage (in terms of solids) in the composition of the present invention is preferably 0.1 wt% or more, more preferably 0.5 wt% or more, and preferably 99 wt% or less, more preferably 90 wt% or less. In an embodiment, for example, the amount of hydrolyzed collagen type II of chicken cartilage (in terms of solids) in the composition is preferably 0.1 to 99 wt%, more preferably 0.5 to 90 wt%.

Herein, the amount of hydrolyzed collagen type II of chicken cartilage contains the amounts of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and fraction 6.

[0034] In an embodiment, for example, the amount of chicken extract (in terms of solids) in the composition of the present invention is preferably 0.1 wt% or more, more preferably 0.5 wt% or more, and preferably 99 wt% or less, more preferably 90 wt% or less. In an embodiment, the amount of chicken extract (in terms of solids) in the composition is preferably 0.1 to 99 wt%, more preferably 0.5 to 90 wt%. Herein, the amount of chicken extract (in terms of solids) contains the amounts of carnosine and anserine.

[0035] Preferably, the composition of the present invention is used as a food, beverage, or medicine. Examples of the food or beverage include general foods or beverages, foods with function claims, health-promoting foods, foods for special dietary uses, dietary supplements, health supplements, and general supplements. The form of the food or beverage is not limited. For example, it may be a solid food or a liquid food. A beverage is preferred.

The form of the medicine is not limited. Non-limiting examples include preparations for internal administration such as capsules, tablets, powder, granules, and dry syrups; preparations for external administration such as ointment, adhesive skin patches, eye drops, and suppositories; and injections. The medicine is preferably a preparation for internal administration (oral medicine).

[0036] The composition of the present invention may contain pharmaceutically or dietary acceptable additives such as various carriers, excipients, diluents, acidulants, an-

tioxidants, stabilizers, preservatives, flavouring or masking agents, emulsifiers, pigments, seasonings, pH adjusters, and nutritional enhancers.

[0037] The composition of the present invention is applicable to both therapeutic use (medical use) and non-therapeutic use (non-medical use). The "non-therapeutic" is a concept that does not include medical activities, i.e., a concept that does not include methods of surgery, therapy or diagnosis of humans.

[0038] <Anti-inflammatory composition>

In an embodiment, the composition of the present invention can be used to reduce inflammation. The composition of the present invention may be an anti-inflammatory composition. The composition may be an anti-inflammatory composition containing at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract, as active components.

[0039] In order to achieve an anti-inflammatory effect contemplated by the present invention, at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract are added to a composition, in the same manner as described above for the above composition. The hydrolyzed collagen type II of chicken cartilage which contains at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract may be used directly, or a concentrate, dry powder, or purified product thereof may be added as described above, as long as the effect of the present invention is not impaired. The same additives as described above can be used. The same additives as described above can be used.

[0040] Preferably, the composition of the present invention is orally fed (orally administered). The dose (which can also be described as "intake") of the composition of the present invention is not limited. The dose of the composition of the present invention may be suitably set according to the body weight of the subject and the like, as long as the inflammation reducing effect can be achieved.

[0041] In an embodiment, when the composition of the present invention is orally fed or administered to a human (adult), the total dose of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof in terms of free form is preferably 0.01 mg or more, more preferably 0.1 mg or more, and preferably 2000 mg or less, more preferably 1000 mg or less, per 60 kg body weight per day. In an embodiment, the total dose of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof in terms of free form to a human (adult) is preferably 0.01 to 2000 mg, more preferably 0.1 to 1000 mg, per 60 kg body weight per day.

[0042] In an embodiment, when the composition of the present invention is orally fed or administered to a human (adult), the dose of fraction 6 is preferably 0.01 mg or more, more preferably 0.1 mg or more, and preferably 2000 mg or less, more preferably 1000

mg or less, per 60 kg body weight per day. In an embodiment, the dose of fraction 6 to a human (adult) is preferably 0.01 to 2000 mg, more preferably 0.1 to 1000 mg, per 60 kg body weight per day.

Herein, the dose of fraction 6 contains the amounts of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof.

[0043] In an embodiment, when the composition of the present invention is orally fed or administered to a human (adult), the dose of hydrolyzed collagen type II of chicken cartilage (in terms of solids) is preferably 0.01 mg or more, more preferably 0.1 mg or more, and also preferably 4000 mg or less, more preferably 3000 mg or less, per 60 kg body weight per day. In an embodiment, the dose of hydrolyzed collagen type II of chicken cartilage (in terms of solids) by a human (adult) is preferably 0.01 to 4000 mg, more preferably 0.1 to 3000 mg, per 60 kg body weight per day.

Herein, the dose of hydrolyzed collagen type II of chicken cartilage contains the amounts of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof or the amount of fraction 6.

[0044] In an embodiment, when the composition of the present invention is orally fed or administered to a human (adult), the dose of chicken extract (in terms of solids) is preferably 0.1 mg or more, more preferably 1 mg or more, and also preferably 15000 mg or less, more preferably 13000 mg or less, per 60 kg body weight per day. In an embodiment, the dose of chicken extract (in terms of solids) by a human (adult) is preferably 0.1 to 15000 mg, more preferably 1 to 13000 mg, per 60 kg body weight per day. The dose of chicken extract includes the amounts of carnosine, anserine and salts thereof.

[0045] In an embodiment, when the composition of the present invention is orally fed or administered to a human (adult), the total dose of carnosine, anserine and salts thereof is preferably 0.001 mg or more, more preferably 0.01 mg or more, and also preferably 500 mg or less, more preferably 400 mg or less, per 60 kg body weight per day in terms of carnosine and anserine. In an embodiment, the total dose of carnosine, anserine and salts thereof fed to a human (adult) is preferably 0.001 to 500 mg, more preferably 0.01 to 400 mg, per 60 kg body weight per day in terms of carnosine and anserine.

[0046] In an embodiment, the above amount of hydrolyzed collagen type II of chicken cartilage which contains Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof and the above amount of chicken extract are preferably fed or administered at least once per day, for example, at once or in several times (e.g., two or three times) per day. In an embodiment, preferably, the above amount of hydrolyzed collagen type II of chicken cartilage which contains Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof

and the above amount of chicken extract are orally fed or administered to a human. In an embodiment, the composition of the present invention can be used to feed or administer the above amount of hydrolyzed collagen type II of chicken cartilage which contains Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof and the above amount of chicken extract to a human, per 60 kg body weight per day.

[0047] In an embodiment, when the composition of the present invention is fed to achieve the anti-inflammatory effect, preferably, the above amount of hydrolyzed collagen type II of chicken cartilage which contains Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan and/or one or more salts thereof and the above amount of chicken extract are fed.

[0048] The composition of the present invention inhibits the production of cytokines such as regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-9 (IL-9), and macrophage inflammatory protein-1 (MIP-1). The composition of the present invention is particularly highly effective in inhibiting the production of RANTES, MCP-1, IL-6, IL-8, IL-9 and MIP-1 β .

Inflammation in a living body can be reduced by inhibiting the production of the above cytokines.

[0049] The composition of the present invention can be used to prevent or alleviate inflammatory conditions or diseases. The inflammatory conditions or diseases are, for example, conditions or diseases caused by inflammation or conditions or diseases accompanied by inflammation. Examples of such conditions or diseases include collagen diseases such as arthritis and rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, tendonitis, sciatica, intervertebral hernia, stenosis, myelopathy, back pain, facet joint pain, carpal tunnel syndrome, tarsal tunnel syndrome, post-lumbar surgery pain syndrome, AIDS, arteriosclerosis, asthma, arthritis, diabetes, hepatitis, stroke, dementia, muscle wasting, viral infection, skin aging including photoaging, cancer, aging, allergic diseases, Parkinson's disease, cerebral infarction, cataract, epilepsy, spinal cord injury, retinopathy of prematurity, nephropathy, peptic ulcer, pancreatitis, ulcerative colitis, myocardial infarction, adult respiratory distress syndrome, emphysema, vasculitis, edema, diabetes complications, UV damage, altitude sickness, porphyria, burns, frostbite, contact dermatitis, shock, multiple organ failure, DIC, fatigue, sarcopenia (muscle weakness), mitochondrial dysfunction, Alzheimer's disease, psoriatic arthritis, ankylosing spondylitis, juvenile idiopathic arthritis and systemic lupus erythematosus (lupus). The composition of the present invention is preferably used to prevent or alleviate these diseases. In particular, the food or beverage composition is preferably used to prevent or alleviate diseases such as os-

teoarthritis, heumatoid arthritis and psoriatic arthritis.

Herein, prevention of conditions or diseases encompasses prevention of disease onset, delay of disease onset, reduction in disease incidence, reduction of risk of disease onset, and the like. Alleviation of conditions or diseases encompasses recovery of the subject from conditions or diseases, alleviation of conditions or disease symptoms, improvement of conditions or disease symptoms, delay or prevention of progress of conditions or diseases, and the like.

[0050] The subject to which the composition of the present invention is fed or administered (which can also be referred to as a "subject") is not limited. The subject is preferably a human or non-human mammal, more preferably a human.

In an embodiment, the subject may be one needing or wanting inhibition of inflammation. Such a subject may be, for example, one needing or wanting prevention or alleviation of inflammation or one needing or wanting prevention or alleviation of inflammatory conditions or diseases. In an embodiment, the subject in the present invention may be a middle-aged or older person. The composition of the present invention can also be used by a healthy person, for example, for the purpose of prevention of conditions that can be prevented or alleviated by reducing inflammation.

[0051] The composition of the present invention may be labeled with function claims stating that the effect is exerted by reducing inflammation. Such a label is also referred to as a label with function claims, the contents of the label are not limited. Examples of such function claims on the label include "relief of joint pain", "reduction of joint pain", "management of knee conditions", "maintenance of knee health", "improvement of joint health", "improvement of joint mobility" and other function claims equivalent to those mentioned above.

In an embodiment of the present invention, the composition of the present invention is preferably a food or beverage labeled with the function claims described above, and a beverage is more preferred. The label may describe use of the composition of the present invention to achieve the above functions. The label may be added to the composition itself or a container or a package of the food or beverage composition.

[0052] <Production method>

The present invention also relates to a method of producing a composition, the method including mixing at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, with chicken extract.

In the mixing, the Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof can be directly mixed with chicken extract. Yet, it is preferred to mix hydrolyzed collagen type II of chicken cartilage which contains Cyclo (Ala-Hyp) and the like with chicken extract to produce a composition. In this case, for

example, a hydrolysate obtained by hydrolysis of collagen type II of chicken cartilage with an enzyme or the like can be directly used for mixing with the chicken extract. As described above, the fraction 6 derived from hydrolyzed collagen type II of chicken cartilage and containing Cyclo (Ala-Hyp) and the like may be added, or a concentrate, dry powder, or a purified product of hydrolyzed collagen type II of chicken cartilage may be added, as long as the effect of the present invention is not impaired.

[0053] In the production method of the present invention, the order of adding raw materials is not limited. For example, Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof, or the fraction 6 or hydrolyzed collagen type II of chicken cartilage which contains these components may be first placed in a container, followed by addition of chicken extract. Alternatively, chicken extract may be first placed in a container, followed by addition of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof, or the fraction 6 or hydrolyzed collagen type II of chicken cartilage which contains these components.

In the production method of the present invention, a preferred embodiment of the hydrolyzed collagen type II of chicken cartilage is as described above. A preferred embodiment of the chicken extract is as described above.

In the production method of the present invention, preferably, the chicken extract contains carnosine and/or anserine and/or one or more salts thereof.

[0054] In the production method of the present invention, commercially available chicken extract or chicken extract produced by hot water extraction can be directly mixed with Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof, or the fraction 6 or hydrolyzed collagen type II of chicken cartilage which contains these components. Yet, when the chicken extract is in the form of a concentrate, dry powder, or a purified product of the concentrate or the dry powder, the chicken extract may be diluted, dissolved, or the like in a liquid such as water, ethanol, or a mixture of water and ethanol, and then mixed with Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof, or the fraction 6 or hydrolyzed collagen type II of chicken cartilage which contains these components, for example. When the fraction 6 of hydrolyzed collagen type II of chicken cartilage which contains Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan and/or one or more salts thereof is in the form of a concentrate, dry powder, or a purified product thereof, such a product can be similarly diluted, dissolved, or the like in the liquid or the like before adding. Alternatively, such a product may be added without being diluted, dissolved, or the like in advance, and then blended with a liquid so as to be diluted, dissolved, or the like in the liquid.

[0055] The composition produced in the production method of the present invention can contain the above-described additives, in addition to Cyclo (Ala-Hyp), Cyclo

(Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof as well as hydrolyzed collagen type II of chicken cartilage and chicken extract.

[0056] The present invention also relates to use of at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract for the production of an anti-inflammatory composition.

Examples of the anti-inflammatory composition include a composition similar to the above-described composition containing Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof, and chicken extract. Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and/or one or more salts thereof can be used in the form of the fraction 6 or hydrolyzed collagen type II of chicken cartilage which contains these components. The same chicken extract and the same additives as described above can be used.

Examples

[0057] The present invention is more specifically described below with reference to examples. The present invention is not limited to these examples.

[0058] Raw materials, reagents, and the like used in the following examples and comparative examples are as follows.

<Preparation of hydrolyzed collagen type II of chicken cartilage>

Fig. 1 shows a flow chart briefly describing a HCII preparation method. First, frozen chicken cartilage was thawed in water at 40°C, and cleansed in water at 40°C (1 hour). Next, the wash water was discarded, and fresh water was poured into a 1200L pot as to be 3 times amount to the chicken cartilage. The water was heated to the optimal working temperature for enzyme treatment, and the washed chicken cartilage was immersed therein to perform enzyme treatment for several hours. After the enzyme treatment, the pot containing the chicken cartilage was heated to 90°C or higher, and the temperature was held at 90°C or higher for 30 minutes, whereby the enzyme used in the earlier enzyme treatment was inactivated. The resulting mixture (the liquid and the enzyme-treated chicken cartilage) was filtered. The resulting liquid was concentrated. Lastly, the resulting concentrate was spray-dried at 200°C, whereby HCII powder was prepared.

[0059] <Measuring for the molecular weight of the HCII powder>

The molecular weight distribution of the HCII obtained was measured by Eurofins HPAEC-PAD method. Table 1 below shows the molecular weight distribution of the HCII obtained. The HCII obtained had a weight average molecular weight of 4582 which was obtained by calculating with usually used method in this field.

[0060]

[Table 1]

Molecular weight Mass Range	Percentage of Molecules	Assumed Mass of Molecule	Total Mass Type of Molecule	Weight Fraction Type of Molecule (NiMi)/Σ(NiMi)	Weight Molecular Weight
	Ni(%)	Mi(Da)	NiMi		WiMi
<500 D	57.0	500	285	0.221	110.47
500 - 1000 D	23.0	750	172.5	0.134	100.29
1000 - 3000 D	14.0	2000	280	0.217	434.11
3000 - 5000 D	1.0	4000	40	0.031	124.03
5000 - 10000 D	1.1	7500	82.5	0.064	479.65
> 10000 D	4.3	10000	430	0.333	3333.33
			Σ(NiMi)=1290		Σ(WiMi)=4581.88

[0061] <Fractionation of HCII fractions>
 (Material and method for HCII fractionation and bioactive identification)
 (Materials)

Formic acid was purchased from Tokyo Chemical Industry Co. Ltd. Ultrapure water was obtained from Merck Milli-Q water purification system.

[0062] (Preparative Gel Permeation Chromatography (GPC))

Fractionation of HCII was carried out using a preparative VERITY 271 HPLC system (Gilson). HCII was dissolved in ultrapure water to prepare a 10 mg/mL (w/v) solution. After centrifugation at 14000 rpm for 5 min, 1000 μ L aliquot was injected onto a preparative GPC column (BioSep 5 μ m SEC-s2000 145Å LC column 300 $\times$ 21.2 mm (Phenomenex)) attached to a guard column (SecurityGuard PREP Cartridge C12 15 $\times$ 21.2 mm ID (Phenomenex)) and eluted isocratically with eluent A (ultrapure water: formic acid = 100 0.1 (v/v)) for 30 min at the rate of 5 mL/min. The chromatogram was observed at 214 nm.

Fractions were collected every 0.5 min between 8 min to 28 min using a GX series fraction collector (Gilson), the resulted 40 individual fractions were subsequently pooled into 7 fractions, P1 to P7 (P1: 8.5-12.0 min, P2: 12.0-13.5 min, P3: 13.5-14.5 min, P4: 14.5-16.0 min, P5: 16.0-17.5 min, P6: 17.5-19.0 min, P7: 19.0-27.5 min). The pooled fractions were evaporated to dryness in a freeze dryer (ScanVac) and the dried samples were stored at -20°C until further use.

[0063] In addition, Table 2 shows the molecular weights of the fractions obtained above. In Table 2, “Mw” and “Mp” indicate weight average molecular weight and peak molecular weight, respectively. As shown in Table 2, fraction 6 (P6) was composed of general small molecules having a molecular weight of less than 300.

The molecular weights of fractions of HCII were measured by HPLC Gel filtration method under the following conditions.

Instrument: Agilent 1100 Series

Detection: UV 214 nm

Flow rate: 1mL/min

Mobile phase: Isocratic 0.1 mM Sodium phosphate buffer at pH 6.8

Running time: 20 minutes

Column: Biosep™ 5 μ m SEC-s2000 145Å LC Column 300 $\times$ 7.8 mm

[0064] [Table 2]

Fraction	Mw	Mp
P1	1359	1626
P2	1092	1494
P3	1035	956
P4	784	783
P5	422	213
P6	211	225

[0065] <Identification of main component of fraction P6>

Major components in fraction P6 were separated by Phenomenex, Luna™ Omega 5 µm PS C18 100A 250 × 4.6 mm on Agilent HPLC, 1100 series, eluted with eluent A (ultrapure water:formic acid = 100:0.1 (v/v)) and eluent B (acetonitrile:formic acid = 100:0.1 (v/v)), with the following linear gradient: 0-4.0 min: 100% eluent A; 4.0-12.0 min: 0-90% eluent A; 12.0-15.5 min: 90-80% eluent A; 15.5-17.0 min: 80-15% eluent A; 17.0-18.0 min: linear 15% eluent A; 18.0-19.0 min: 15-100% eluent A; and 19.0-21.0 min: 100% eluent A. The injection volume was 10 µL at the flow rate of 1 mL/min and UV absorbance of the eluents was monitored at 214 nm. The eluates were collected with equipped fraction collector. Four fractions were then freeze-dried and then reconstituted in purified water, which submitted to LC-MS analysis on an Agilent HPLC 1290 series-coupled TripleTOF 5600 (AB Sciex) with a Duo Spray Turbo V ion source and a gas generator (Peak Scientific). Some fractions were also submitted to proton-NMR experiment, conducted on Bruker ECA 400 for characterization. The identity of the major components in the fraction P6 was confirmed by commercially available chemicals.

Free amino acids in the fraction HCII were detected and quantified according to AOAC 999.13 method.

[0066] According to the results of the analysis, the major components in the fraction P6 were Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan.

[0067] <Quantification of Cyclo (Ala-Hyp) and Cyclo (Pro-Gly)>

Cyclo (Ala-Hyp) was reconstituted in ultrapure water and injected onto a Zorbax Eclipse Plus C18 RRHD 1.8 µm 2.1 × 50 mm column (Agilent), with UHPLC Guard Zorbax Eclipse Plus C18 2.1 × 5 mm 1.8 µm (Agilent), and eluted with eluent A (ultrapure water:formic acid = 100:0.1 (v/v)) and eluent B (acetonitrile:formic acid = 100:0.1 (v/v)), with the following linear gradient: 0-0.5 min: 100% eluent A; 0.5-7.5 min: 100-65% eluent A; 7.5-10.0 min: 65-0% eluent A; 11.0-13.0 min: 0% eluent A; 13.0-13.1 min: 0-100% eluent A; and 13.1-15.0 min: 100% eluent A.

The injection volume was 10 µL at the flow rate of 300 µL/min and UV absorbance of the eluents was monitored at 214 nm. The collision energy (CE) and declustering potential (DP) of the MS were optimized as 27.0 V and 100 V respectively. Cyclo (Ala-Hyp) was detected using Q1/Q3 ion transitions at m/z 185.1/86.1 with the retention time of 2.45 min as quantifier and qualifier. A calibration curve was constructed by infusing 31.25 ng/mL, 62.5 ng/mL, 125 ng/mL, or 250 ng/mL of standard Cyclo (Ala-Hyp) into the mass spectrometer. The fraction P6 was reconstituted as 100 µg/mL solution as the working solution. The quantification of the Cyclo (Ala-Hyp) in the fraction P6 was done by Sciex OS (AB Sciex).

[0068] Cyclo (Pro-Gly) was reconstituted in ultrapure water and injected onto a Zorbax

Eclipse Plus C18 RRHD 1.8 μm 2.1 $\times$ 50 mm column (Agilent), with UHPLC Guard Zorbax Eclipse Plus C18 2.1 $\times$ 5 mm 1.8 μm (Agilent), and eluted with eluent A (ultrapure water:formic acid = 100:0.1 (v/v)) and eluent B (acetonitrile:formic acid = 100:0.1 (v/v)), with the following linear gradient: 0-0.5 min: 100% eluent A; 0.5-7.5 min: 100-65% eluent A; 7.5-10.0 min: 65-0% eluent A; 11.0-13.0 min: 0% eluent A; 13.0-13.1 min: 0-100% eluent A; and 13.1-15.0 min: 100% eluent A.

The injection volume was 10 μL at the flow rate of 300 $\mu\text{L}/\text{min}$ and UV absorbance of the eluents was monitored at 214 nm. The collision energy (CE) and declustering potential (DP) of the MS were optimized as 30.0 V and 100 V respectively. Cyclo (Pro-Gly) was detected using Q1/Q3 ion transitions at m/z 70.1/127.1 Th with the retention time of 2.6 min as quantifier and qualifier. A calibration curve was constructed by infusing 70 ng/mL, 140 ng/mL, 280 ng/mL, or 560 ng/mL of standard Cyclo (Pro-Gly) into the mass spectrometer. The fraction P6 was reconstituted as 100 $\mu\text{g}/\text{mL}$ solution as the working solution. The quantification of the Cyclo (Pro-Gly) in the fraction P6 was done by Sciex OS (AB Sciex).

[0069] <Quantification of guanosine>

Guanosine was reconstituted in ultrapure water and injected onto a Zorbax Eclipse Plus C18 RRHD 1.8 μm 2.1 $\times$ 50 mm column (Agilent), with UHPLC Guard Zorbax Eclipse Plus C18 2.1 $\times$ 5 mm 1.8 μm (Agilent), and eluted with eluent A (ultrapure water:formic acid = 100:0.1 (v/v)) and eluent B (acetonitrile:formic acid = 100:0.1 (v/v)), with the following linear gradient: 0-0.5 min: 100% eluent A; 0.5-10.5 min: 100-0% eluent A; 10.5-14.0 min: 0% eluent A; 14.0-16.1 min: 0-100% eluent A; and 16.1-18.0 min: 100% eluent A. The injection volume was 10 μL at the flow rate of 300 $\mu\text{L}/\text{min}$ and UV absorbance of the eluents was monitored at 214 nm. The collision energy (CE) and declustering potential (DP) of the MS were optimized as 10.0 V and 80 V respectively. Guanosine was detected using Q1/Q3 ion transitions at m/z 152.1/284.1 Th with the retention time of 2.17 min as quantifier and qualifier. A calibration curve was constructed by infusing 31.25 ng/mL, 62.5 ng/mL, 125 ng/mL, or 250 ng/mL of standard guanosine into the mass spectrometer. P6 was reconstituted as 100 $\mu\text{g}/\text{mL}$ solution as the working solution. The quantification of the guanosine in P6 was done by Sciex OS (AB Sciex).

[0070] HCII contained 0.23wt% of Cyclo (Ala-Hyp), 0.30wt% of Cyclo (Pro-Gly), 0.10wt% of guanosine, and 0.132wt% of tryptophan, assuming the total weight of the HCII as 100 wt%.

[0071] <Quantification of carnosine and anserine>

Quantification of carnosine and anserine in the chicken extract was performed by HPLC under the following conditions.

Carnosine standard stock was prepared by adding and dissolving carnosine powder in

deionized water to a carnosine concentration of 2.50 mg/ml. Anserine standard stock was prepared by adding and dissolving L-anserine nitrate powder in deionized water to an L-anserine concentration of 3.96 mg/ml.

The weight of L-anserine was calculated by the following formula.

$$\text{L-anserine (g)} = 0.792 \times \text{L-anserine nitrate (g)}$$

<Analysis conditions of HPLC>

Device: High performance liquid chromatography system with UV detector (Agilent 1100 produced by Agilent).

Column: Zorbax 300-SCX 4.6 mm ID × 250 mm (Agilent)

Mobile phase: 50 mM potassium dihydrogen phosphate

Flow rate: 1.0 mL/min

Flow channel: channel A (50 mM potassium dihydrogen phosphate), channel B (acetonitrile), channel D (deionized water)

UV detector wavelength: 210 nm

Sample injection volume: 10 μL

[0072] <Evaluation method of anti-inflammatory activity>

In Examples and Comparative Examples, the effect of inhibiting the production of inflammatory markers was evaluated by the following method.

(Raw materials and reagents)

Chicken extract (CE): Brand's Essence of Chicken (Suntory Beverage & Food Asia Pte Ltd, carnosine content in 1 ml: 0.94 mg/ml; anserine content in 1 ml: 1.9 mg/ml)

Chondrocyte medium (CM), fetal bovine serum (FBS), chondrocyte growth supplement (CGS), and penicillin/streptomycin (P/S): ScienCell Research Laboratories
IL-1β: R&D Systems

Poly-L-lysine (PLL) coated 96-well plates (Corning)

[0073] <Cell culture and pre-treatments>

Human chondrocytes (HC-a, ScienCell Research Laboratories) were isolated from human articular cartilage and were maintained in CM supplemented with 5% FBS, 1% CGS and 1% P/S. Cells were seeded in PLL-coated 96-well plate at a cell density of 4000 cells/well and incubated overnight at 37°C in a humidified gas chamber containing 5% CO₂. The chondrocytes were washed once with PBS, and treated with chicken extract (CE), HCII, fraction P6 fractionated from HCII, a combination of the fraction P6 and CE, P6 compounds containing Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan, a combination of P6 compounds and CE, Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, or a combination of CE and any one of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine or tryptophan. After treating for 24 hours, the chondrocytes were further treated for 24 hours by adding 25 ng/ml of IL-1β. The cells were centrifuged at 1100 g for 5 minutes and the supernatant was then used for

cytokine analysis. For comparison, cells treated with IL-1 β (IL-1 β -treated cells) without being pre-treated with CE, HCII, fraction P6, P6 compounds, Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan were prepared.

[0074] (Multiplex cytokine analysis)

The Pro-Human Cytokine Multiplex Assays (Bio-Rad) was used to analyze the cytokines in the culture media. The following 27-plex analyses were performed: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin (CCL11), macrophage colony-stimulating factor (M-CSF), interferon (IFN)- γ , monocyte chemotactic protein 1 (MCP-1; CCL2), macrophage inflammatory protein-1 α (MIP-1 α ; CCL3), MIP-1 β (CCL4), regulated on activation, normal T cell expressed and secreted (RANTES) (CCL5), TNF- α , and vascular endothelial growth factor (VEGF). Multiplex assays were carried out according to the manufacturers' instructions and run on the Luminex xPONENT for MAGPIX platform. Bio-Plex Manager version 6.0 was used for data processing. Cytokine and chemokine concentrations were calculated by reference to the standard curve. The sensitivity of the multiplex kit was < 5 pg/mL.

[0075] (Statistical analyses)

Statistical analysis was conducted using GraphPad Prism version 5.0 (GraphPad). All results were expressed as mean $\pm$ standard deviation. Statistical analysis was performed by analysis of variance (ANOVA) followed by posthoc Tukey's multiple comparison test. Data was considered to be significant when $p < 0.05$.

[0076] (Comparative Example 1) Effect of fraction P6 on inhibition of the production of inflammatory markers

To assess the influence of HCII and fraction P6 fractionated from HCII on the inflammatory markers (RANTES, MCP-1, IL-6, IL-8, IL-9, and MIP-1 β) produced by the human chondrocytes, the following four different conditions were compared: cells before treatment (untreated control), IL-1 β -treated cells, IL-1 β + HCII-treated cells (Comparative Example 1), and IL-1 β + fraction P6-treated cells (Comparative Example 1). HCII-treated cells and fraction P6-treated cells were pre-treated with HCII or fraction P6. In the pre-treatment, the HCII concentration was 5 mg/ml, and the fraction P6 concentration was 5 mg/ml.

As shown in Fig. 2, IL-1 β 25 ng/mL induced inflammation in human chondrocytes leading to a significant increase in level of all the inflammatory markers as compared to untreated cells (control). Pre-treatment of the cells with HCII or the fraction P6 reduced the level of all the inflammatory markers induced by IL-1 β . Data was represented as means $\pm$ SD ($n = 3$). * denotes significant difference with $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$ against IL-1 β (IL-1 β -treated cells) unless otherwise indicated.

[0077] (Comparative Example 2) Effect of a combination of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan (P6 compounds) on inhibition of the production of inflammatory markers IL-6

To assess the influence of P6 compounds containing Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan (the ratio of these components were the same as in HCII) on the inflammatory marker IL-6 produced by the human chondrocytes, the following five different conditions were compared: cells before treatment (untreated control), IL-1 β -treated cells, IL-1 β + HCII-treated cells (Comparative Example 2), IL-1 β + fraction P6-treated cells (Comparative Example 2) and IL-1 β + P6 compounds-treated cells (Comparative Example 2). HCII-treated cells, fraction P6-treated cells and P6 compounds-treated cells were pre-treated with HCII, fraction P6 or P6 compounds. In the pre-treatment, the HCII concentration was 5 mg/ml, the fraction P6 concentration was 5 mg/ml and the P6 compounds concentration was 100 mg/ml.

As shown in Fig. 3, IL-1 β 25 ng/mL induced inflammation in human chondrocytes leading to a significant increase in level of IL-6 as compared to untreated cells (control). Pre-treatment of the cells with HCII, P6, or P6 compounds reduced the level of the inflammatory marker IL-6 induced by IL-1 β . Data was represented as means $\pm$ SD (n = 3). * denotes significant difference with p < 0.05, ** denotes p < 0.01, and *** denotes p < 0.001 against IL-1 β .

[0078] (Comparative Example 3) Effect of Cyclo (Pro-Gly) and a combination of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan (P6 compounds) on inhibition of the production of inflammatory markers MCP-1(MCAF)

To assess the influence of P6 compounds containing Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, and tryptophan (the ratio of these components were the same as in HCII) and Cyclo (Pro-Gly) (cPG) on the inflammatory marker MCP-1(MCAF) produced by the human chondrocytes, the following six different conditions were compared: cells before treatment (untreated control), IL-1 β -treated cells, IL-1 β + HCII-treated cells (Comparative Example 3), IL-1 β + fraction P6-treated cells (Comparative Example 3), IL-1 β + P6 compounds-treated cells (Comparative Example 3) and IL-1 β + cPG compounds-treated cells (Comparative Example 3). HCII-treated cells, fraction P6-treated cells, P6 compounds-treated cells and cPG-treated cells were pre-treated with HCII, fraction P6, P6 compounds or cPG. In the pre-treatment, the HCII concentration was 5 mg/ml, the fraction P6 concentration was 5 mg/ml, the P6 compounds concentration was 100 mg/ml and the cPG concentration was 50 mg/ml, 100 mg/ml or 250 mg/ml.

As shown in Fig. 4, IL-1 β 25 ng/mL induced inflammation in human chondrocytes leading to a significant increase in level of MCP-1(MCAF) as compared to untreated cells (control). Pre-treatment of the cells with HCII, P6, Cyclo (Pro-Gly) or P6

compounds reduced the level of the inflammatory marker MCP-1(MCAF) induced by IL-1 β . Data was represented as means $\pm$ SD (n = 3). * denotes significant difference with $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$ against IL-1 β .

[0079] <Example 1> Synergistic effect of combination of HCII and CE

To assess a synergistic effect of a combination of HCII and CE on inhibition of the production of an inflammatory marker MIP-1 β , pre-treatment was performed with HCII alone, CE alone, or a combination of HCII and CE. In the pre-treatment, the HCII concentration was 0.5 mg/ml and the CE concentration was 1.25 mg/ml.

As shown in Fig. 5, a combination of HCII 0.5 mg/mL and CE 1.25 mg/mL provided a synergistic effect of reducing inflammation, as compared to treatment with HCII or CE alone. Data is represented as means $\pm$ SD (n = 3). * denotes significant difference with $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$ against IL-1 β (IL-1 β -treated cells) unless otherwise indicated.

[0080] <Example 2> Synergistic effect of combination of HCII and CE and a synergistic effect of a combination of fraction P6 and CE on inhibition of the production of inflammatory markers MCP-1 and MIP-1 β

To assess a synergistic effect of a combination of HCII and CE and a synergistic effect of a combination of fraction P6 and CE on inhibition of the production of inflammatory markers MCP-1 and MIP-1 β , pre-treatment was performed with IL-1 β and HCII alone, CE alone, P6 alone, a combination of HCII and CE, or a combination of P6 and CE as in Example 1. In the pre-treatment, HCII concentration was 2.5mg/mL or 5mg/mL, P6 concentration was 2.5 mg/ml or 5mg/mL, and the CE concentration was 6.25 mg/ml.

Fig. 6(a) and Fig. 6(b) show the results. Data is represented as means $\pm$ SD (n=3). * denotes significant difference with $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$ against IL-1 β (IL-1 β -treated cells) unless otherwise indicated.

[0081] <Example 3> Synergistic effect of combination of HCII and CE, a combination of P6 compound and CE, a combination of Cyclo(Pro-Gly) and CE, a combination of Cyclo (Ala-Hyp) and CE, a combination of guanosine and CE, and a combination of tryptophan and CE on inhibition of the production of inflammatory markers

To assess a synergistic effect of a combination of HCII and CE, a combination of P6 compound and CE, a combination of Cyclo(Pro-Gly)(cPG) and CE, a combination of Cyclo (Ala-Hyp) (cAH) and CE, a combination of guanosine and CE, and a combination of tryptophan and CE on inhibition of the production of inflammatory markers IL-6, IL-8, IL-9, MCP-1, MIP-1 β and RANTES, pre-treatment was performed as in Example 1. In the pre-treatment, HCII concentration was 2.5mg/mL, P6 compounds concentration was 25 mg/ml, 125 mg/ml or 250mg/mL, cPG concentration was 25 mg/ml, 125 mg/ml or 250mg/mL, cAH concentration was 25 mg/ml, 125 mg/ml or

250mg/mL, guanosine concentration was 25 mg/ml, 125 mg/ml or 250mg/mL, tryptophan concentration was 25 mg/ml, 125 mg/ml or 250mg/mL, and CE concentration was 6.25 mg/ml.

Fig. 7-1(a), Fig. 7-1(b), Fig. 7-2(c), Fig. 7-2(d), Fig. 7-3(e) and Fig. 7-3(f) show the results. Data is represented as means $\pm$ SD (n = 3). * denotes significant difference with $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$ against IL-1 β (IL-1 β -treated cells) unless otherwise indicated.

[0082] <Example 4> A Randomized, Double-blind, Four-arm Pilot Study to Evaluate the Effects of HCII and CE on knee pain

This pilot study was carried out in a single-center, and as double-blind, randomized, placebo-controlled study.

[0083] (Subject enrolment and randomization)

A total of 160 subjects between the ages of 45 to 75 were selected using inclusion and exclusion criteria summarized in Table 3. Subjects were randomly assigned to four groups i.e. "Placebo", "Glucosamine", "HCII" and "HCII and CE". Each group included 40 subjects.

[0084]

[Table 3]

Criteria	Description
Inclusion	Male and female at 45 to 75 years of age
	Subject has a body mass index range of 18.0 to 30.0 kg/m ² , inclusive, and weighs at least 40 kg at screening visit
	Subject agrees not to participate in another interventional study while participating in the present study, defined as signing the informed consent form until completion of the study
	Experience knee pain $\geq$ 3 months
	WOMAC total pain score $\geq$ 6 evaluated by walking on a flat surface, descending and/or ascending stairs
	Subject has mild-to-moderate knee osteoarthritis (OA) at grade 1 to 3 per Kellgren-Lawrence grading system
	Experience loss in muscular strength or physical performance greater than 1 year
	Be willing to refrain from hormone therapy and dietary supplements such as calcium, vitamin D, supplements enriched with proteins, omega-3, omega-6, glucosamine, or chondroitin during the entire study
Exclusion	Active viral infection or bacterial infection based on clinical observations
	Subjects with history of rheumatoid arthritis or any other inflammatory arthritis
	Chronic medical conditions including renal dysfunctions, psychiatric disorders, or diabetes (or HbA1c > 6.5%)
	History of stroke or myocardial infarction, mental retardation, or schizophrenia
	Gout
	Paget disease of bone or spinal disc herniation
	Subjects intolerant or allergic to protein-based food or supplement
	Anti-osteoporotic therapy in the past year
	Intra-articular injection during previous 3 months (6 months for hyaluronic acid) applied at target knee joint (most painful knee joint at Screening visit)
	Current corticosteroid therapy
	Those expecting knee arthroscopy or arthroplasty
	Life-threatening pathology (such as cancer) in remission for less than 1 year or still ongoing
	Psychological or linguistic incapability to sign the informed consent
	History of allergy to chicken meat
	Pregnant or lactating women
	Suspected or known alcohol abuse or addiction (consume $\geq$ 14 drinks of alcohol per week; 1 drink: 355mL beer or 150mL wine)
	Subjects with any other conditions or diseases that investigator considers not appropriate to be entered in the study
	Subjects with active pathology that could confound interpretation of knee pain or any conditions that interfere with adherence

[0085] (Investigational product)

“Placebo” group took a mixture of 6.8g of maltodextrin and 7mg of xanthan gum.

“Glucosamine” group took 1.5g of glucosamine hydrochloride. Glucosamine hydrochloride acted as an active comparator.

“HCII” group took BRAND’S Collagen Hydrolysate (Suntory Beverage & Food Asia Pte Ltd), a hydrolyzed chicken sternal cartilage extract composed of a naturally occurring matrix of hydrolyzed collagen type II (HCII) and low molecular weight chondroitin sulfate and hyaluronic acid (HA). This product included HCII containing Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine and tryptophan. Each bottle with a volume of 68mL contained 2g of HCII providing naturally occurring composition of

HCII (66.5%), depolymerized chondroitin sulfate (18%) and HA (11%). Uncharacterized components of sternal cartilage account for the remaining 4.5%.

“HCII and CE” group took a mixture of 70g of BRAND’S Essence of chicken (BEC) (dry weight: 5-6g) and 2g of HCII in 68mL. All test products were prepared as liquid products in glass bottles, which were isocaloric, identical in appearance and equivalent in flavour and texture. The participants had to take the investigational product once daily in the morning after meal.

Subjects were allowed to continue on concomitant medication or supplements deemed not to affect the outcome of the study. Analgesics or painkillers were allowed as a rescue medication under prescription. Proportion of days covered (PDC) of painkillers was calculated based on the records in the Concomitant Medication of Painkiller section and defined as the percentage of painkillers covered days over the total number of days in the interval between visits. Any treatment or dietary supplement that could support joint, bone and muscle health including hormone therapy (growth hormone, progesterone, estrogen, or testosterone), calcium and vitamin D, supplements enriched with amino acids, peptides, proteins, omega-3, omega-6, glucosamine or chondroitin were prohibited throughout the study duration.

[0086] (Procedure)

The study consisted of a screening visit (28 days before baseline visit), followed by a baseline visit (Baseline visit was Day 0. Screening and baseline visits can be on the same day), and 3 follow-up visits (Week 8, 16, and 24). Subjects were screened from Day -28 to Day 0 to determine the eligibility for the study. Intake of test product was taken starting from the day following the baseline visit for 168 days (24 weeks) consecutively. For 168 days (24 weeks) consecutively, subjects took one bottle of test product daily in the morning (after meal). Intake compliance was recorded on a diary card.

The visual analogue scale (VAS) of knee pain was scored at Day 0, Day 7 and Day 14 post-intake.

During the study period, subjects were recommended to do the resistance training (not mandatory) twice a week at home, 30 minutes each time following the training schedule. Training was recorded on a diary card. Food dietary in the prior week before visits was recorded using a food questionnaire.

[0087] (Treatment compliance)

Compliance, regarding the intake of the investigational product, was checked by the collection of unused products and the daily records of the consumption kept by the subjects. Compliance was defined by the percentage of assigned doses that were actually consumed over number of days between visits. A 70% of consumption was considered as compliant.

[0088] (Exercise programme)

All subjects were encouraged to do the resistance training for 30 minutes twice a week at home following the training manual provided at baseline visit. Compliance on the training programme were recorded on a diary card and were calculated using the following formula:

[0089] [Math.1]

$$\text{Compliance(\%)} = \frac{\text{Sum of minutes of training (actual)}}{\text{Sum of minutes of training (plan)}} \times 100$$

[0090] A compliance rate of $\geq 50\%$ was considered as compliant.

[0091] (Visual Analogue scale (VAS) of knee pain)

The visual analogue scale (VAS) was scored from 0 to 100 mm, where 0 indicates no pain and 100 the worst pain ever. At Day 0, Day 7 and Day 14 post-intake, respondents were asked to specify the level of pain felt by indicating a position along a continuous line by drawing a straight line on the scale.

[0092] (Tolerability and safety)

Spontaneous reported adverse events were recorded throughout the study. Vital signs were monitored at every visit. For assessment of safety of the investigational products, serum and urine of all participants were evaluated at each visit of the study duration.

[0093] (Statistical analyses)

Per-protocol statistical analyses were performed, according to the a priori statistical analysis plan. Randomized subjects with intake compliance rate $\geq 70\%$ were included in the per-protocol analyses. Analyses of safety parameters were performed based on the subjects who have taken at least 28 bottles of the study product. Dichotomous variables were reported using percentages, whereas continuous variables were reported as mean and SD. Comparisons of categorical variables were performed with chi-square test, whereas the Kruskal-Wallis test was used to compare differences between groups for continuous variables.

Repeated measures analysis of variance (ANOVA) was performed using mixed-effect models used for testing mean difference in changes between the study groups, with the intake-by-visit interaction as fixed effect factors, for continuous primary and secondary endpoints. All results were considered to be statistically significant if the corresponding p-value was below 0.05. If the intake-by-visit interaction term was significant, post-hoc, pairwise comparisons between treatment groups were performed and adjusted p-values reported. Variables which were expected to influence endpoints were included in the models as factors. Gender and gender*visit interaction term was included as a factor in joint health analysis.

In addition, subgroup analysis was done for subjects doing resistance training less than 10th percentile of the total training period. An independent statistician performed

the analyses using SAS version 9.4 (Cary, USA).

[0094] <Results>

(Baseline characteristics and treatment compliance)

A total of 160 subjects were enrolled and 151 subjects completed the study and were included in the statistical analysis (PP). Nine subjects dropped out during the study; there were no significant differences in the number of the drop-outs between the groups. None of the drop-outs were related to any side effects caused by the intake of the investigational products or placebo. No adverse events were noted. No clinically significant changes were observed in the serum biochemistry markers and urinalysis.

Overall, there was no statistically difference in the compliance rate between all four groups (Table 4). To evaluate the homogeneity of the data, all parameters were compared between four arms. There was no statistically significant difference between the study groups at baseline (Table 4).

[0095]

[Table 4]

	HCII+CE (n=37)	HCII (n=38)	Glucosamine (n=38)	Placebo (n=38)
Age (years)	57.3 ± 7.14	59.8 ± 7.66	59.1 ± 8.22	56.4 ± 6.42
Male/female ratio (female %)	4:33 (89.2)	8:30 (78.9)	6:32 (84.2)	5:33 (86.8)
Menopause (%)	87.9	73.3	75	60.6
Kellgren-Lawrence grading system for knee OA (%)	Probable	81.1	57.9	68.4
	Mild	10.8	34.2	28.9
	Moderate	8.1	7.9	2.6
Treatment compliance(%)	98.8	98.9	98.9	99.9

[0096] (Changes in VAS of knee pain)

After 14 days of intake, sub-group analysis for subjects doing less than 10th percentile of total resistance training period showed that placebo group experienced significantly increased pain score of 200% from baseline at Day 14 compared to HCII+CE (p=0.047) group, with and 42.9% from Day 0(Fig. 8, Table 5).

In Table 5, “P-value (\$)” means p value between groups; “(a)” denotes method used to determine the p value((a)= One-way ANOVA); “95%C.I.” denotes 95% Confidence Interval.

In Fig. 8, two numerical values in parentheses for each data are “mean - standard deviation” and “mean + standard deviation”.

[0097]

[Table 5]

	HCII+CE (n=2)	Placebo (n=2)	P-value(\$)
Day 0 (Baseline)			
Number	2	2	0.135(a)
Mean±SD	2.8 ± 1.99	1.9 ± 1.25	
Median	2.8	1.9	
Range	(1.4, 4.2)	(1.0, 2.8)	
95% C.I.	(-15.11, 20.63)	(-9.32, 13.18)	
Day 7			
Number	2	2	0.395(a)
Mean±SD	4.0 ± 1.77	5.7 ± 0.74	
Median	4	5.7	
Range	(2.7, 5.2)	(5.2, 6.3)	
95% C.I.	(-11.92, 19.84)	(-0.89, 12.35)	
Day 14			
Number	2	2	0.155(a)
Mean±SD	4.0 ± 1.77	5.7 ± 0.74	
Median	4	5.7	
Range	(2.7, 5.2)	(5.2, 6.3)	
95% C.I.	(-11.92, 19.84)	(-0.89, 12.35)	

[0098] (Conclusion)

In conclusion, HCII and CE were well-tolerated and provided a quick and significant symptomatic relief in patients suffering from osteoarthritic pain. Compared to placebo, combination of HCII and CE significantly reduced knee joint pain in just 14 days in subjects who did minimal amount of resistance training.

In vitro studies suggest that mechanism of action may be through modification of underlying disease processes, particularly inhibition of inflammation that leads to localized pain sensation. Taken together, a combination of HCII and CE may be considered as a safe and efficacious complement to current medical and dietary options in the management of OA symptoms.

Industrial Applicability

[0099] The present invention can provide a novel composition containing at least one selected from the group consisting of Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, and salts thereof, and chicken extract. The composition of the present invention can be used as a food or beverage composition or a pharmaceutical composition to reduce inflammation and joint pain. Cyclo (Ala-Hyp), Cyclo (Pro-Gly), guanosine, tryptophan, salts thereof, and chicken extract can be consumed as foods, beverages, or the like, and are also advantageous in terms of high safety.