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(54) **Titre : PRODUIT A BASE DE LAIT FERMENTE ET SON PROCEDE DE PRODUCTION**
(54) **Title: NOVEL FERMENTED MILK PRODUCT AND METHOD FOR PRODUCING THE SAME**

(57) **Abrégé/Abstract:**
The present invention addresses the problem of providing a safe and novel fermented milk product which is useful in the prevention and treatment of various bone disorders such as osteoporosis, bone fractures, rheumatism, and arthritis when taken on a daily basis. A fermented milk product containing 0.9 to 150 mg/100 g of angiogenin and/or an angiogenin degradation product, and cystatin and/or a cystatin degradation product at a mass ratio of 0.006 to 1.7 relative to the angiogenin and/or angiogenin degradation product. It is possible to strengthen bones and to prevent and treat various bone disorders such as osteoporosis, bone fractures, rheumatism, and arthritis by taking said fermented milk product.

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ABSTRACT

The invention relates to a fermented milk product includes angiogenin and/or angiogenin hydrolysate in an amount of 0.9 mg/100 g to 150 mg/100 g, and cystatin and/or cystatin hydrolysate in the mass ratio to the angiogenin and/or angiogenin hydrolysate of 0.006 to 1.7.

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NOVEL FERMENTED MILK PRODUCT AND METHOD FOR PRODUCING THE SAME

TECHNICAL FIELD

[0001]

This invention relates to a novel fermented milk product and a method for producing the same. The fermented milk product includes a specific milk component, and may be useful for prevention and treatment of various bone diseases such as osteoporosis, fracture, rheumatism, and arthritis.

BACKGROUND ART

[0002]

In recent years, various bone diseases, such as osteoporosis, fracture, and backache have increased on a global basis along with aging of society and the like, and have become a serious social problem. These diseases are caused by insufficient calcium intake, depression of calcium absorption ability, hormone imbalance after menopause, and the like. It is considered that increase the body bone mass as much as possible by activating the osteoblast and bone formation from the early stage of life, and increase the maximum bone mass and the bone strength (bone density + bone quality) is effective in preventing various bone diseases, such as osteoporosis, fracture, and backache. Note that the term "bone quality" refers to the bone microstructure, metabolic turnover, microfracture, and calcification. It is thought that various bone diseases, such as osteoporosis, fracture, and backache may be prevented by suppressing osteoclastic bone resorption. Bones are repeatedly resorbed and formed in a balanced manner (remodeling). However, various bone diseases, such as osteoporosis, fracture, and backache may occur when bone resorption exceeds bone formation due to a change in hormone balance after menopause, and the like. Therefore, bones can be

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strengthened by suppressing osteoclastic bone resorption and maintaining the bone strength at a constant level.

[0003]

In view of the above situation, a drug, food, drink, feed, or the like in which a calcium salt, such as calcium carbonate, calcium phosphate, or calcium lactate or a natural calcium product, such as whey calcium, bovine bone powder, or eggshell is added individually, has been administered in order to strengthen bones. A drug, food, drink, feed, or the like that contains such a calcium product together with a substance having a calcium absorption-promoting effect, such as casein phosphopeptide or oligosaccharide has also been used to strengthen bones. However, the calcium absorption rate is 50% or less, when a food or drink that contains a calcium salt or natural calcium product is administered, and the large part of the calcium administered may be discharged from the body without being absorbed. Moreover, even if calcium is absorbed into the body, it does not necessarily exhibit the bone metabolism-improving effect or bone-strengthening effect, since the affinity to bones may differ according to its form or the type of nutritional ingredient administered together. An estrogen product, an active vitamin D_3 product, a vitamin K_2 product, a bisphosphonate product, a calcitonin product, and the like have been known as a drug for treating osteoporosis or strengthening bones, and new drugs such as an anti-RANKL antibody have been also developed. However, these drugs may have side effects such as buzzing in the ear, a headache, or loss of appetite. Moreover, the above substances are in a situation that they cannot be added to a food or drink at present from the viewpoint of safety, cost, and the like. Therefore, in light of the nature of various bone diseases, such as osteoporosis, fracture, and backache, development of such a food or drink that can be administered orally for a long time, increases the bone strength by promoting bone formation and suppressing bone resorption, and may be expected to have the effect of preventing or treating the various bone diseases has been desired.

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PRIOR-ART DOCUMENT

PATENT DOCUMENT

[0004]

[Patent Document 1] JP-A-H08-151331

[Patent Document 2] JP-A-H10-7585

[Patent Document 3] JP-A-2000-281587

SUMMARY OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0005]

The invention relates to provide a fermented milk product that may be useful for prevention and treatment of various bone diseases such as osteoporosis, fracture, rheumatism, and arthritis.

MEANS FOR SOLVING THE PROBLEMS

[0006]

The present inventors have found that the bone density can be effectively increased by administering a fermented milk product that includes angiogenin and/or angiogenin hydrolysate, and includes cystatin and/or cystatin hydrolysate in a specific mass ratio with respect to angiogenin and/or angiogenin hydrolysate. This finding has led to the completion of the invention.

[0007]

Specifically, the invention includes following aspects:

(1) A fermented milk product including angiogenin and/or angiogenin hydrolysate in an amount of 0.9 mg/100 g to 150 mg/100 g and cystatin and/or cystatin hydrolysate in the mass ratio to the angiogenin and/or angiogenin hydrolysate of 0.006 to 1.7.

(2) A method of preventing bone diseases including administering the fermented milk product according to (1) in an amount of 100 g/day or more.

(3) A method of producing the fermented milk product according to (1), including mixiing angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate with a milk product raw material and sterilied the obtained mixture, and then fermented.

(4) A method of producing the fermented milk product according to (1), including adding angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate to a sterilized milk raw material.

EFFECTS OF THE INVENTION

[0008]

The fermented milk product of the invention exhibits a bone-strengthening effect, and may be useful for prevention and treatment of various bone diseases such as osteoporosis, fracture, rheumatism, and arthritis.

EMBODIMENTS FOR CARRYING OUT THE INVENTION

[0009]

A fermented milk product of the invention is characterized in that the fermented milk product includes angiogenin and/or angiogenin hydrolysate in a specific amount, and further includes cystatin and/or cystatin hydrolysate in a specific mass ratio with respect to angiogenin and/or angiogenin hydrolysate.

A fermented milk product generally contains angiogenin and/or angiogenin hydrolysate in an amount of about 0.2 to 0.8 mg/100 g, and cystatin and/or cystatin hydrolysate in an amount of about 0.4 to 1.1 mg/100 g.

In contrast, the fermented milk product of the invention is added with angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate, and

the fermented milk product contains angiogenin and/or angiogenin hydrolysate in an amount of 0.9 mg/100 g to 150 mg/100 g, and cystatin and/or cystatin hydrolysate in a mass ratio with respect to angiogenin and/or angiogenin hydrolysate of 0.006 to 1.7.

[0010]

A fraction containing angiogenin and/or angiogenin hydrolysate that is prepared from milk of a mammal, such as human, cow, buffalo, goat, or sheep, a fraction containing cystatin and/or cystatin hydrolysate that is prepared from milk of a mammal, such as human, cow, buffalo, goat, or sheep, a fraction containing angiogenin and/or angiogenin hydrolysate that is produced by a genetic engineering, a fraction containing cystatin and/or cystatin hydrolysate that is produced by a genetic engineering, angiogenin and/or angiogenin hydrolysate purified from blood or an organ, cystatin and/or cystatin hydrolysate purified from blood or an organ, or the like may be used as the angiogenin and/or angiogenin hydrolysate and the cystatin and/or cystatin hydrolysate included in the fermented milk product of the invention. A commercially available purified angiogenin or cystatin reagent may also be used.

The fermented milk product of the invention may include angiogenin hydrolysate or cystatin hydrolysate obtained by digesting a fraction containing angiogenin, an angiogenin reagent, a fraction containing cystatin, a cystatin reagent, or the like using one or more proteases.

[0011]

The fermented milk product of the invention may include a protein material prepared by extracting a fraction containing angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate directly from milk or a material derived from milk, such as skim milk or whey. Such a protein material may be prepared as follows, for example. Specifically, milk or a material derived from milk is brought into contact with a cation-exchange resin, and milk-derived proteins adsorbed on the resin is eluted at a salt concentration of 0.1 to 2.0 M, desalted and concentrated using a reverse

osmosis membrane, an electrodialysis membrane, an ultrafiltration membrane, a microfiltration membrane, or the like, and optionally subjected to proteolysis to a molecular weight of 8000 or less using a protease, such as trypsin, pancreatin, chymotrypsin, pepsin, papain, kallikrein, cathepsin, thermolysin, or V8 protease. When subjecting to proteolysis using a protease, the lower limit of the molecular weight is preferably 500 or more. The protein material thus obtained may be dried by freeze-drying, spray drying, or the like, and the dried product may be added in the fermented milk product.

[0012]

The fermented milk product of the invention is produced by adding angiogenin and/or angiogenin hydrolysate, and cystatin and/or cystatin hydrolysate and a protein material that contains angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate, or the like to a fermented milk product raw material so that the fermented milk product includes angiogenin and/or angiogenin hydrolysate in an amount of 0.9 mg/100 g to 150 mg/100 g, and includes cystatin and/or cystatin hydrolysate in a mass ratio with respect to angiogenin and/or angiogenin hydrolysate of 0.006 to 1.7.

As shown in the test examples described below, when the fermented milk product includes angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate as described above, the bone-strengthening effect can be obtained more effectively than the case of administering angiogenin and/or angiogenin hydrolysate or cystatin and/or cystatin hydrolysate separately.

[0013]

The fermented milk product of the invention may be produced in the usual manner as long as the fermented milk product includes the angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate in specific amounts, respectively. The fermented milk product produced according to the invention may

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include all fermented milk product, such as a fermented milk product, a dairy lactic acid bacteria beverage, a lactic acid bacteria beverage, and the like. For example, the fermented milk product of the invention is produced by optionally mixing a milk raw material, adding angiogenin and/or angiogenin hydrolysate thereto so that the fermented milk product includes angiogenin and/or angiogenin hydrolysate in a specific amount, and adding cystatin and/or cystatin hydrolysate to the mixture so that the fermented milk product includes cystatin and/or cystatin hydrolysate in the specific range of the mass ratio to angiogenin and/or angiogenin hydrolysate. Note that as the milk raw material, cow milk, concentrated skim milk, skim milk powder, whey, butter, cream, or the like, in addition to a milk-based drink, processed milk, composition-modified milk, low-fat milk, fat-free milk, or the like that is obtained by appropriately or optionally mixed the above cow milk, concentrated skim milk, skim milk powder, whey, butter, cream, or the like can be given, for example. After that, An appropriate amount of a starter culture prepared from lactic acid bacteria such as *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, or *Lactobacillus kefiri*, or yeast such as *Kluyveromyces marxianus* or *Saccharomyces unisporus* is added to the milk raw material, and the resulting mixture is fermented in the usual manner to prepare a fermented milk product of the invention.

[0014]

When adding angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate to a milk raw material, angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate may be added to either unsterilized milk raw material, or a sterilized milk raw material. When adding to an unsterilized milk raw material, sterilization may be conducted after the addition. In this instance, heat sterilization is preferable. When sterilizing the mixture after mixing the angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate with the milk raw material, it is preferable to sterilize the mixture at 130°C for 2 seconds or less.

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It may be possible that the fermented milk product of the invention may be added with a raw material or the like that is commonly used for a food or drink, such as a saccharide, a lipid, a protein, a vitamin, a mineral, or a flavor, in addition to angiogenin and/or angiogenin hydrolysate, cystatin and/or cystatin hydrolysate, other than the above milk raw material, and may also be added with another bone-strengthening component such as calcium, vitamin D, vitamin K, or isoflavone.

[0015]

The fermented milk product of the invention can strengthen bones when administered orally in an amount of 100 g or more per kg of body weight, as shown in the animal experiments described below. Since the intake for the experiment animal corresponds to the intake for adults in terms of blood drug concentration (see Mitsuyoshi Nakajima (1993), "*Yakkou Hyoka* Vol. 8", Hirokawa-Shoten Ltd., pp. 2-18), it is expected that bones can be strengthened, and especially various bone diseases, such as osteoporosis, fracture, rheumatism, and arthritis can be prevented or treated by ingesting the fermented milk product of the invention in an amount of 100 g/day or more per adult.

[0016]

The invention is further described below in more detail by way of reference examples, examples, and test examples. Note that the following examples are intended for illustration purposes only, and should not be construed as limiting the invention.

[0017]

Reference Example 1

Preparation (1) of angiogenin fraction

A column filled with 30 kg of cation-exchange resin (Sulfonated Chitopearl; manufactured by Fuji Spinning Co., Ltd.) was thoroughly washed with deionized water, and 1000 liters of unpasteurized skim milk (pH 6.7) was then applied to the column. After thoroughly wash the column with deionized water, the absorbed protein was

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eluted with a linear gradient of 0.1 to 2.0 M sodium chloride. The eluted fraction containing angiogenin was fractionated using an S-Sepharose cation-exchange chromatography (manufactured by Amersham Bioscientific), and the resulted angiogenin-containing fraction was heat-treated at 90°C for 10 minutes, and centrifuged to remove a precipitate. The angiogenin-containing fraction was further subjected to gel filtration chromatography (column: Superose 12). The eluate obtained was desalted using a reverse osmosis membrane, and the desalted eluate was freeze-dried to obtain 16.5 g of an angiogenin fraction having an angiogenin purity of 90%. These successive operations were repeated 30 times.

[0018]

Reference Example 2

Preparation (2) of angiogenin fraction

A column filled with 10 kg of Heparin Sepharose (manufactured by GE Healthcare) was thoroughly washed with deionized water, and 500 liters of unpasteurized skim milk (pH 6.7) was then applied to the column. After thoroughly wash the column with a 0.5 M sodium chloride solution, the absorbed protein was eluted with a 1.5 M sodium chloride solution. The eluate was desalted using a reverse osmosis membrane, and the desalted eluate was freeze-dried to obtain 18 g of an angiogenin fraction having an angiogenin purity of 5%. The above successive operations were repeated 50 times.

[0019]

Reference Example 3

Preparation of cystatin fraction

One hundred thousand liters (100,000 liters) of a 5% whey protein solution was heat-treated at 90°C for 10 minutes, and a precipitate was removed by centrifugation. A column was filled with a carrier prepared by binding carboxymethylated papain to Tresyl-Toyopearl (manufactured by Tosoh Corporation). After equilibration with a 0.5

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M sodium chloride solution, the above whey protein solution was applied to the column. The column was then sequentially washed with a 0.5 M sodium chloride solution and a 0.5 M sodium chloride solution containing Tween 20 (0.1%). After that, a cystatin-containing fraction was eluted with a 20 mM acetic acid-0.5 M sodium chloride solution. The eluted fraction was immediately neutralized with a 1 M sodium hydroxide solution. The eluate was then desalted using a reverse osmosis membrane, and the desalted eluate was freeze-dried to obtain 9.6 g of a cystatin fraction having a cystatin purity of 90%. The above successive operations were repeated 20 times.

[0020]

Measurement of angiogenin and cystatin contained in fermented milk product

The content of angiogenin, angiogenin hydrolysate, cystatin and cystatin hydrolysate in the fermented milk product was measured according to the method described in JP-A-2008-164511 with modification. Specifically, 86 μl of the fermented milk product was added to 5 ml of ultrapure water, and a 1/1000-equivalent amount of formic acid was added to the mixture to prepare a sample solution. Ten microliter (10 μl) of the sample solution was dried up, and dissolved in 20 μl of 0.1 M ammonium bicarbonate containing 8 M urea and 1 mM tris(carboxyethyl)phosphine (TCEP). The solution was heated at 56°C for 30 minutes. After returning the solution to room temperature, 5 μl of 100 mM iodoacetamide solution was added to the solution, and the mixture was reacted for 30 minutes in the dark. After the addition of 54 μl of ultrapure water, 10 μl of 0.1 μg/ml trypsin and 10 μl of 0.1 μg/ml Lysyl Endopeptidase were added to the mixture. The mixture was reacted at 37°C for 16 hours. The reaction was then terminated by adding 3 μl of formic acid and used as the sample peptide solution for measurement. The sample solution was diluted 6-fold with 10 fmol/μl internal standard peptide solution containing 0.1% formic acid, 0.02% trifluoroacetic acid (TFA), and 2% acetonitrile, and 2.5 μl of the diluted solution was subjected to LC/MS/MS analysis.

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[0021]

The peptides were separated by gradient elution using an HPLC system. More specifically, the peptides were separated using a column (MAGIC C18, 0.2 mm (ID) × 50 mm) equipped with a 5 μl-peptide trap on a MAGIC 2002 HPLC system at a flow rate of 2 μl/min. A solution A (2% acetonitrile-0.05% formic acid) and a solution B (90% acetonitrile-0.05% formic acid) were used as eluant for HPLC. Gradient elution was conducted under the elution condition from 2 to 65% the solution B over 20 minutes.

As object ions for measuring cystatin, parent ion was NH_2-QVVSGMNYFLDVELGR-COOH (m/z 914.4), and the MS/MS target ion was NH_2-FLDVELGR-COOH (m/z 948.7). As object ions for measuring angiogenin, parent ion was NH_2-YIHFLTQHYDAK-COOH (m/z 768.8), and the MS/MS target ion was NH_2-FLTQHYDAK-COOH (m/z 1122.8). Regarding the internal standard peptide parent ion was NH_2-ETTVFENLPEK-COOH (wherein, P was labeled with ^{13}C and ^{15}N) (m/z 656.9.), and the MS/MS target ion was NH_2-FENLPEK-COOH (wherein, P was labeled with ^{13}C and ^{15}N) (m/z 882.4).

A system "LCQ Advantage" was used for MS. The peak area of each protein was calculated from the resulting chromatogram, and the concentration was calculated from the ratio with respect to the internal standard peptide.

Example 1

[0022]

One hundred and sixty six milligrams (166 mg) of the angiogenin fraction obtained in Reference Example 1 and 0.5 mg of the cystatin fraction obtained in Reference Example 3 were mixed with100 g of a mixture prepared by adding a starter culture to a 10% reduced skim milk powder that had been sterilized at 100°C for 10 minutes, the resulting mixture was fermented in the usual manner to obtain a fermented milk product (example product 1). The obtained fermented milk product contained

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angiogenin and/or angiogenin hydrolysate in an amount of 150 mg/100 g, and the mass ratio of cystatin and/or cystatin hydrolysate to angiogenin and/or angiogenin hydrolysate in the fermented milk product was 0.006.

Example 2

[0023]

Thirteen point five milligrams (13.5 mg) of the angiogenin fraction obtained in Reference Example 2 and 1.2 mg of the cystatin fraction obtained in Reference Example 3 were mixed with 100 g of a mixture prepared by adding a starter culture to a 10% reduced skim milk powder that had been sterilized at 100°C for 10 minutes, and the resulting mixture was fermented in the usual manner to obtain a fermented milk product (example product 2). The obtained fermented milk product contained angiogenin and/or angiogenin hydrolysate in an amount of 0.9 mg/100 g, and the mass ratio of cystatin and/or cystatin hydrolysate to angiogenin and/or angiogenin hydrolysate in the fermented milk product was 1.7.

Example 3

[0024]

Thirteen point five milligrams (13.5 mg) of the angiogenin fraction obtained in Reference Example 1 and 1.2 mg of the cystatin fraction obtained in Reference Example 3 were mixed with 100 g of a mixture prepared by adding a starter culture to a 10% reduced skim milk powder that had been sterilized at 100°C for 10 minutes, and the resulting mixture was fermented in the usual manner to obtain a fermented milk product (example product 3). The obtained fermented milk product contained angiogenin and/or angiogenin hydrolysate in an amount of 12.4 mg/100 ml, and the mass ratio of cystatin and/or cystatin hydrolysate to angiogenin and/or angiogenin hydrolysate in the fermented milk product was 0.12.

[0025]

Comparative Example 1

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Twelve milligrams (12 mg) of the angiogenin fraction obtained in Reference Example 2 and 2.7 mg of the cystatin fraction obtained in Reference Example 3 were mixed with100 g of a mixture prepared by adding a starter culture to a 10% reduced skim milk powder that had been sterilized at 100°C for 10 minutes, and the resulting mixture was fermented in the usual manner to obtain a fermented milk product (comparative example product 1). The obtained fermented milk product contained angiogenin and/or angiogenin hydrolysate in an amount of 0.9mg/100 g, and the mass ratio of cystatin and/or cystatin hydrolysate to angiogenin and/or angiogenin hydrolysate in the fermented milk product was 3.2.

[0026]

Comparative Example 2

One hundred and sixty six milligrams (166 mg) of the angiogenin fraction obtained in Reference Example 1 and 0.25 mg of the cystatin fraction obtained in Reference Example 3 were mixed with 100 g of a mixture prepared by adding a starter culture to a 10% reduced skim milk powder that had been sterilized at 100°C for 10 minutes, and the resulting mixture was fermented in the usual manner to obtain a fermented milk product (comparative example product 2). The obtained fermented milk product contained angiogenin and/or angiogenin hydrolysate in an amount of 150 mg/100 ml, and the mass ratio of cystatin and/or cystatin hydrolysate to angiogenin and/or angiogenin hydrolysate in the fermented milk product was 0.0045.

[0027]

Test Example 1

The bone-strengthening effects of the example products 1 to 3 and the comparative example products 1 and 2 were determined by animal experiments. C3H/HeJ mice (5 weeks old, male) were used for the animal experiments. After 1 week acclimation, the mice were divided into six groups (10 mice/group). The mice were orally administered each product of the example products 1 to 3 and the

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comparative example products 1 and 2 in an amount of 100 g per 1 kg of mouse weight once a day for 2 weeks using a tube. The control group was not administrated any example products 1 to 3 and the comparative example products 1 and 2. After completion of administration (second week), the bone density of the right tibia of each mouse was measured using a micro-CT (manufactured by Rigaku Corporation). The results are shown in Table 1. As shown in Table 1, the groups that were orally administered the example products 1 to 3 showed a significant increase in bone density compared with the control group and the comparative example groups that were orally administered the comparative example product 1 or 2.

[0028]

TABLE 1

	Bone density (mg/cm^3)
Control group	1235 ± 9
Example product 1	1268 ± 12
Example product 2	1273 ± 13
Example product 3	1267 ± 11
Comparative example product 1	1241 ± 7
Comparative example product 2	1240 ± 5

[0029]

Reference Example 4

A column (diameter: 4 cm, height: 30 cm) filled with 400 g of cation-exchange resin (Sulfonated Chitopearl; manufactured by Fuji Spinning Co., Ltd.) was thoroughly washed with deionized water, and 40 liters of unpasteurized skim milk (pH 6.7) was applied to the column at a flow rate of 25 ml/min. After thoroughly washing the column with deionized water, proteins adsorbed on the resin were eluted using a 0.02 M carbonate buffer (pH 7.0) containing 0.78 M sodium chloride. The eluate was desalted

using a reverse osmosis membrane, and the desalted eluate was freeze-dried to obtain 18 g of a powdery protein material (reference example product 4).

[0030]

Reference Example 5

Four grams (4 g) of protein material of the reference example product 4 was dissolved in 800 ml of water. After the addition of trypsin (manufactured by Sigma), which is a protease, at the final concentration of 0.03 wt%, the mixture was subjected to enzymatic treatment at 37°C for 8 hours. After inactivating the protease through heat-treatment at 90°C for 5 minutes, the mixture was freeze-dried to obtain 3.0 g of a powdery protein material (reference example product 5).

Example 4

[0031]

Forty milligrams (40 mg) of the reference example product 4 was mixed with 97 g of a 10% reduced skim milk powder, and the mixture was sterilized at 93°C for 6 minutes, followed by adding 3 g of a starter culture, the resulting mixture was fermented in the usual manner to obtain a fermented milk product (example product 4). The obtained fermented milk product contained angiogenin and/or angiogenin hydrolysate in an amount of 2.4 mg/100 g, and the mass ratio of cystatin and/or cystatin hydrolysate to angiogenin and/or angiogenin hydrolysate in the fermented milk product was 0.39.

Example 5

[0032]

Forty milligrams (40 mg) of the reference example product 5 was mixed with 97 g of a 10% reduced skim milk powder, and the mixture was sterilized at 93°C for 6 minutes, followed by adding 3 g of a starter culture, the mixture was fermented in the usual manner to obtain a fermented milk product (example product 5). The obtained fermented milk product contained angiogenin and/or angiogenin hydrolysate in an

amount of 2.3 mg/100 g, and the mass ratio of cystatin and/or cystatin hydrolysate to angiogenin and/or angiogenin hydrolysate in the fermented milk product was 0.4.

[0033]

Comparative Example 3

Thirty milligrams (30 mg) of the reference example product 4 and 10 mg of the cystatin fraction obtained in Reference Example 3 were mixed with 97 g of a 10% reduced skim milk powder, and the mixture was sterilized at 93°C for 6 minutes, followed by adding 3 g of a starter culture, the resulting mixture was fermented in the usual manner to obtain a fermented milk product (comparative example product 3). The obtained fermented milk product contained angiogenin and/or angiogenin hydrolysate in an amount of 1.9 mg/100 g, and the mass ratio of cystatin and/or cystatin hydrolysate to angiogenin and/or angiogenin hydrolysate in the fermented milk product was 5.2

[0034]

Test Example 2

The bone-strengthening effects of the example products 4 and 5 and the comparative example product 3 was determined by animal experiments. Forty SD female rats (51 weeks old) were used for the animal experiments. The rats were divided into five groups (8 rats/group). Four groups underwent ovariectomy, and the remaining one group sham surgery. After a 4-week recovery period, the ovariectomized rats were orally administered the example products 4 or 5 or the comparative example product 3 in an amount of 100 g per 1kg of rat weight daily in six divided dose for 16 weeks using a tube. The control group was not administrated any example products 4 and 5 and the comparative example product 3. After a 4-week recovery period, the rats underwent sham surgery were fed for 16 weeks in the same manner as the control group. After completion of administration (sixteenth week), the bone density of the right tibia of each rat was measured using a micro-CT

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(manufactured by Rigaku Corporation).

The results are shown in Table 2. As shown in Table 2, the groups that were orally administered the example products 4 and 5 showed a significant increase in bone density as compared with the control group and the group that was orally administered the comparative example product 3. Moreover, the bone density approached that of the sham surgery group.

[0035]

TABLE 2

	Bone density (mg/cm^3)
Control group	551 ± 9
Sham surgery group	602 ± 10
Example product 4	596 ± 11
Example product 5	598± 13
Comparative example product 3	556± 12

Example 6

[0036]

Fifty milligrams (50 mg) of the reference example product 4 was mixed with 98 g of 2.5% reduced skim milk powder that had been sterilized at 100°C for 10 minutes, followed by adding 2 g of a starter culture, the resulting mixture was fermented in the usual manner, sterilized at 130°C for 2 seconds, and cooled to 10°C to obtain a fermented milk product (example product 6). The obtained fermented milk product contained angiogenin and/or angiogenin hydrolysate in an amount of 2.9 mg/100 g, and the mass ratio of cystatin and/or cystatin hydrolysate to angiogenin and/or angiogenin hydrolysate in the fermented milk product was 0.32.

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CLAIMS

1. A fermented milk product comprising angiogenin and/or angiogenin hydrolysate in an amount of 0.9 mg/100 g to 150 mg/100 g and cystatin and/or cystatin hydrolysate in the mass ratio to the angiogenin and/or angiogenin hydrolysate of 0.006 to 1.7.

2. A method of preventing bone diseases comprising administering the fermented milk product according to claim 1 in an amount of 100 g/day or more.

3. A method of producing the fermented milk product according to claim 1, comprising mixing angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate with a milk product raw material and sterilized the obtained mixture, and then fermented.

4. A method of producing the fermented milk product according to claim 1, comprising adding angiogenin and/or angiogenin hydrolysate and cystatin and/or cystatin hydrolysate to a sterilized milk product raw material.