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(54) Benævnelse: **MIDDEL TIL FORBEDRING AF TARMFLORAEN**

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DESCRIPTION

[0001] The present invention relates to an agent for improving intestinal flora which can increase the number of beneficial bacteria such as lactic acid bacteria and Bifidobacterium to improve intestinal flora.

[0002] Recently, causal relationships between intestinal environment and various diseases are intensively investigated to reveal that improvement of intestinal environment is effective in preventing or ameliorating various diseases. In intestine, beneficial bacteria such as lactic acid bacteria and Bifidobacterium and bad bacteria such as Escherichia coli are present in a mixed manner. Forming beneficial bacteria-dominated flora is important in order to form a healthy intestinal environment.

[0003] Conventionally, probiotics, prebiotics, and the like are developed, and various materials by which beneficial bacteria become predominant to improve intestinal flora are proposed. It is also reported that administration of digestive enzymes such as amylases and proteases can also improve intestinal flora. For example, Non-Patent Document 1 discloses that in a pig which is fed with a specific enzyme blend consisting of an amylase, a protease, and a xylanase together with animal feed, the number of Lactobacillus is increased and the number of Escherichia coli is decreased in the large intestine. Non-Patent Document 2 also discloses that in a pig which is fed with a specific enzyme blend (Nopcozyme II; Diasham Resources Pte Ltd.) consisting of an amylase derived from Bacillus amyloliquefaciens, a protease derived from Bacillus subtilis, and a xylanase derived from Trichoderma together with animal feed, the number of Lactobacillus is increased and the numbers of Salmonella and Escherichia coli are decreased.

[0004] Thus, as an enzyme preparation which can improve intestinal flora, specific enzyme blends consisting of an amylase, a protease, and a xylanase are conventionally known. However, it has not been shown that which enzyme of these enzymes contributes to the improvement of intestinal flora. Further, there is a drawback that use of such enzyme blends leads to increases in cost of producing the enzymes.

PRIOR ART DOCUMENTS

[0005]

Non-Patent Document 1: Yi et al., Asian Astralas. J. Anim. Sci., 2013, 26: 1181-1188

Non-Patent Document 2: Zhang et al., J. Amin. Sci., 2014, 92: 2063-2069

Non-Patent Document 3 (Gomi Katsuya et al., Biosci. Biotech.. Biochem., vol. 57, no. 7, pages 1095 to 1100) concerns a cloning a genomic DNA sequence encoding the acid protease (PEPA) from *Aspergillus oryzae* using a 0.6-kb fragment as a probe.

Non-Patent Document 4 (Yongshou Yang et al., Biomedical Reports, vol. 3, no. 5, pages 715 to 720) discloses effects of the dietary addition of the protease preparations derived from *Aspergillus* on the colonic luminal environment in rats consuming a high-fat diet.

[0006] WO 2015/048332 A2 describes secreted nutritive polypeptides and formulations thereof, and method of production and use thereof.

[0007] WO 2014/081884 A1 describes engineered secreted proteins and methods.

[0008] Recently, in connection with increasing interest in health promotion, development of new materials by which beneficial bacteria become predominant to improve intestinal flora is desired. However, with respect to an agent for improving intestinal flora using an enzyme, no effective enzyme except for the conventionally reported enzyme preparations can be estimated even by analogy under the current circumstances.

[0009] Under the circumstances, an object of the present invention is to provide an agent for improving intestinal flora which can increase the number of beneficial bacteria such as lactic acid bacteria and Bifidobacterium to improve intestinal flora by using an enzyme.

[0010] The present inventors have made extensive investigations to solve the above problem and found that a protease consisting of a polypeptide having an amino acid sequence shown in SEQ ID NO: 1 can increase the number of beneficial bacteria such as lactic acid bacteria and Bifidobacterium in intestines to exert an excellent effect of improving intestinal flora. The present inventors have made further investigations based on the findings, leading to the completion of the invention.

[0011] Thus, the present invention includes the subject matter as defined in the claims.

[0012] The present invention can increase the number of beneficial bacteria such as lactic acid bacteria and Bifidobacterium and improve intestinal flora by using a specific protease, and thus is effective in forming healthy intestinal environment, maintaining healthy intestinal environment, and preventing or treating a disease and/or symptom due in part to unhealthy intestinal environment.

1. Agent for improving intestinal flora

[0013] An agent for improving intestinal flora in accordance with the present invention is characterized by containing a specific protease as an active ingredient. The agent for improving intestinal flora in accordance with the present invention is described in detail below.

[Protease]

[0014] In the agent for improving intestinal flora in accordance with the present invention, a protease comprising at least one of the following polypeptides (1) to (3) is used as an active ingredient. By selecting and using the specific protease, beneficial bacteria such as lactic acid bacteria and Bifidobacterium in intestines can be increased to improve intestinal flora effectively.

1. (1) a polypeptide consisting of an amino acid sequence shown in SEQ ID NO: 1,
2. (2) a polypeptide comprising an amino acid sequence in which 1 to 16 of amino acids are substituted, added, inserted, or deleted in the amino acid sequence shown in SEQ ID NO: 1, and having a protease activity equivalent to that of a polypeptide consisting of an amino acid sequence shown in SEQ ID NO: 1, and
3. (3) a polypeptide comprising an amino acid sequence having 95% or more sequence identity to an amino acid sequence shown in SEQ ID NO: 1, and having a protease activity equivalent to that of a polypeptide consisting of an amino acid sequence shown in SEQ ID NO: 1.

[0015] The polypeptide set forth in the above (1) is an acid protease derived from Aspergillus oryzae, and polypeptides set forth in the above (2) and (3) are variants of the polypeptide set forth in the above (1).

[0016] In the polypeptide of the above (2), amino acid modifications introduced may comprise any one of the modifications including substitution, addition, insertion, and deletion alone (e.g., substitution alone) or comprise two or more of the modifications (e.g., substitution and insertion). In the polypeptide of the above (2), the number of amino acids which is substituted, added, inserted, or deleted is 1 to 16, preferably 1 to 10, or 1 to 8, even more preferably 1 to 3, 1 or 2, or 1.

[0017] In the polypeptide of the above (3), sequence identity to the amino acid sequence shown in SEQ ID NO: 1 is 95% or more, preferably 99% or more.

[0018] Herein, in the a polypeptide of the above (3), the sequence identity to the amino acid sequence shown in SEQ ID NO: 1 refers to a sequence identity calculated by comparison with the amino acid sequence shown in SEQ ID NO: 1. The "sequence identity" refers to a value of amino acid sequence identity obtained by bl2seq program (Tatiana A. Tatsusova, Thomas L. Madden, FEMS Microbiol.Lett., Vol.174, p247-250, 1999) in BLAST PACKAGE [sgi32 bit edition, Version 2.0.12; available from National Center for Biotechnology Information (NCBI)]. Parameter settings may be as follows: Gap insertion Cost value: 11 and Gap extension Cost value: 1.

[0019] In polypeptides of the above (2) and (3), when an amino acid substitution is introduced

in the amino acid sequence shown in SEQ ID NO: 1, examples of the amino acid substitution introduced include a conservative substitution according to a preferred aspect. That is, examples of the substitution in the polypeptides of the above (2) and (3) include the following substitutions: when an amino acid to be substituted is a non-polar amino acid, a substitution with other non-polar amino acids; when an amino acid to be substituted is a non-charged amino acid, a substitution with other non-charged amino acids; when an amino acid to be substituted is an acidic amino acid, a substitution with other acidic amino acids; and when an amino acid to be substituted is a basic amino acid, a substitution with other basic amino acids.

[0020] In the polypeptides of the above (2) and (3), the phrase "having a protease activity equivalent to that of a polypeptide consisting of an amino acid sequence shown in SEQ ID NO: 1" refers to showing a protease activity equivalent to that of the polypeptide of the above (1) when the protease activities are measured under the following conditions (i.e., showing a protease activity of about 80 to 120% as compared to the protease activity (100%) of the polypeptide of the above (1)).

(Method for measuring protease activity)

[0021] In a test tube, 5 mL of a 0.6 wt% casein solution (pH 3.0) is placed and maintained at 37°C. Then, 1 mL of an enzyme solution (in water as a solvent) containing a sample which is to be measured for a protease activity is added and allowed to stand at 37°C for exactly 10 minutes, and then 5 mL of a 0.44 mol/L trichloroacetic acid solution is added to stop the reaction. The mixture is allowed to stand at 37°C for 30 minutes followed by filtration with filter paper to obtain 2 mL of filtrate, which is transferred into another test tube, and then 5 mL of 0.55 mol/L sodium carbonate and 1 mL of 3-fold diluted Folin's reagent are added thereto in this order. The mixture is allowed to stand at 37°C for 30 minutes followed by measurement of absorbance at a wavelength of 660 nm. In blank, the enzyme solution is added after the addition of the trichloroacetic acid solution. Separately, a standard curve for tyrosine is constructed using 10 to 40 µg/mL of tyrosine solutions by the same procedure as the above-described procedure for filtrate. Under the above conditions, an amount of an enzyme which causes an increase in colored materials by Folin's reagent corresponding to 1 µg of tyrosine per minute is defined as 1 U. The following equations are used for the calculation.

[Equation 1]

$$\text{Protease activity per 1 g or 1 mL of sample (U/g or U/mL)} = (AT - AB) \times F \times (11/2) \times (1/10) \times 1/W$$

AT: Absorbance at wavelength of 660 nm

AB: Absorbance at wavelength of 660 nm in blank

F: Amount of tyrosine (µg) corresponding to difference in absorbance of 1 as determined by standard curve for tyrosine

11/2: Factor of conversion after completion of reaction into total liquid volume

1/10: Factor of conversion into per minute of reaction

W: Amount of sample (g or mL) in 1 mL of enzyme solution

[0022] The polypeptide of the above (1) to (3) can be obtained by a method of culturing Aspergillus oryzae which produces the polypeptide, and also obtained by a publicly-known genetic engineering technique.

[Dose of protease]

[0023] The agent for improving intestinal flora in accordance with the present invention may be applied at a suitable dose as determined according to, for example, types of products in which the agent is used, applications, expected effects, and dosage forms. Examples of a daily dose for an adult human as an amount of the above protease ingested or administered include 0.1 to 3,000 mg, preferably 1 to 2000 mg, more preferably 1 to 1000 mg, even more preferably 2 to 500 mg, still more preferably 2 to 150 mg, most preferably 5 to 100 mg.

[Use of agent for improving intestinal flora]

[0024] The agent for improving intestinal flora in accordance with the present invention can increase the number of beneficial bacteria such as Bifidobacterium and lactic acid bacteria in intestines by the effect of the above protease to form a beneficial bacteria-dominated flora, and thus is used for the purpose of forming healthy intestinal environment, maintaining healthy intestinal environment, and the like. Specifically, the agent for improving intestinal flora in accordance with the present invention is superior in an effect of increasing the number of bacteria of Bifidobacterium spp. and Lactobacillus spp. in intestines, and thus can also be used as an agent for increasing the number of enteric bacteria of Bifidobacterium spp. and Lactobacillus spp. in intestines.

[0025] In addition, the agent for improving intestinal flora in accordance with the present invention can form a beneficial bacteria-dominated flora to form a healthy intestinal environment, and thus can also be used for the purpose of preventing or treating a disease and/or symptom due in part to unhealthy intestinal environment. Examples of the disease and/or symptom include decreased immunity, colorectal cancer, allergic disease, nonalcoholic steatohepatitis, obesity, and inflammatory bowel disease.

[Form for using agent for improving intestinal flora]

[0026] The agent for improving intestinal flora in accordance with the present invention is orally applied by oral ingestion or oral administration. The agent for improving intestinal flora in accordance with the present invention is orally ingested or orally administered to exert an effect of improving intestinal flora after arriving at intestines, and thus can be blended for use with various products such as food and drink, drugs for oral administration, animal feed, and pet food.

[0027] When the agent for improving intestinal flora in accordance with the present invention is blended with the above various products, the products may contain probiotics and/or prebiotics as required together with the agent for improving intestinal flora in accordance with the present invention.

[0028] Examples of a microorganism used as the probiotics include lactic acid bacteria, Bifidobacterium, and Bacillus subtilis var natto. Specific examples of the lactic acid bacteria include lactic acid bacteria of Lactobacillus spp. such as Lactobacillus casei, Lactobacillus acidophilus, and Lactobacillus plantarum; lactic acid bacteria of Enterococcus spp. such as Enterococcus faecalis, Enterococcus faecium, and Enterococcus hirae; and lactic acid bacteria of Streptococcus spp. such as Streptococcus bovis and Streptococcus thermophilus. Specific examples of Bifidobacterium include Bifidobacterium adolescentis, Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium pseudolongum, and Bifidobacterium thermophilum. These probiotics may be used alone or in combination of two or more.

[0029] Examples of the prebiotics include xylooligosaccharide, fructooligosaccharide, soybean oligosaccharides, isomaltooligosaccharide, lacto-fructo-oligosaccharides, galactooligosaccharides, and lactulose. These prebiotics may be used alone or in combination of two or more.

[0030] A formulation of a product with which the agent for improving intestinal flora in accordance with the present invention is blended may be any one of solid form, semi-solid form, liquid form, and the like, and is suitably selected according to types and applications of the product.

[0031] When the agent for improving intestinal flora in accordance with the present invention is used for food and drink, the above protease is provided, as food and drink for improving intestinal flora, solely or in combination with other food materials or additives to be prepared in a desired form. Examples of the food and drink include a food for specified health uses, a nutritional supplement, a functional food, and a food for patients in addition to common food and drink. Forms of these foods and drinks are not specifically limited. Specific examples include supplements such as tablets, granules, powders, capsules, and soft capsules; and drinks such as energy drinks, fruit drinks, carbonated beverages, and lactic acid drinks.

[0032] When the agent for improving intestinal flora in accordance with the present invention is used for food and drink, an amount of the agent blended into the food and drink varies according to, for example, forms of the food and drink. In supplements, the agent may be

blended, for example, so that an amount of the above protease is in a range of 0.03 to 1,000 mg/g, preferably 0.3 to 700 mg/g, more preferably 0.3 to 350 mg/g, even more preferably 0.6 to 170 mg/g, still more preferably 0.6 to 80 mg/g, most preferably 1.5 to 50 mg/g. In drinks, the agent may be blended, for example, so that an amount of the above protease is in a range of 0.0003 to 10 mg/mL, preferably 0.003 to 7 mg/mL, more preferably 0.003 to 3.5 mg/mL, even more preferably 0.006 to 1.7 mg/mL, still more preferably 0.006 to 0.8 mg/mL, most preferably 0.015 to 0.5 mg/mL.

[0033] When the agent for improving intestinal flora in accordance with the present invention is used in the food and drink field, the agent for improving intestinal flora of the present invention can be provided, as a food additive, solely or in combination with other ingredients.

[0034] When the agent for improving intestinal flora in accordance with the present invention is used for drugs for oral administration, the agent for improving intestinal flora of the present invention is provided, as drugs for oral administration which exert an effect of improving intestinal flora, solely or in combination with, for example, other pharmacologically active ingredients, pharmaceutically acceptable bases, or additives to be prepared in a desired form. Forms of the drugs are not specifically limited. Specific examples include formulations for oral administration such as tablets, granules, powders, capsules, soft capsules, syrups, and liquids.

[0035] Examples of the bases and the additives used for manufacturing the drugs for oral administration include aqueous bases such as water and alcohol, oil-based bases, diluents, binders, filling agents, disintegrants, lubricants, algefacients, pH-adjusting agents, thickeners, antioxidants, sequestering agents, surfactants, emulsifiers, solubilizers, solubilizing agents, flavoring agents, and antiseptics.

[0036] When the agent for improving intestinal flora in accordance with the present invention is used as drugs for oral administration, ratio of the agent blended into the drugs for oral administration can be suitably determined according to, for example, forms of the drugs for oral administration within a range satisfying the above described dose. In drugs for oral administration in solid form or semi-solid form, the agent may be blended, for example, so that an amount of the above protease is in a range of 0.03 to 1,000 mg/g, preferably 0.3 to 700 mg/g, more preferably 0.3 to 350 mg/g, even more preferably 0.6 to 170 mg/g, still more preferably 0.6 to 80 mg/g, most preferably 1.5 to 50 mg/g. In drugs for oral administration in liquid form, the agent may be blended, for example, so that an amount of the above protease is in a range of 0.0003 to 10 mg/mL, preferably 0.003 to 7 mg/mL, more preferably 0.003 to 3.5 mg/mL, even more preferably 0.006 to 1.7 mg/mL, still more preferably 0.006 to 0.8 mg/mL, most preferably 0.015 to 0.5 mg/mL.

[0037] When the agent for improving intestinal flora in accordance with the present invention is used for animal feed or pet food, the agent for improving intestinal flora of the present invention is provided, as animal feed or pet food which exerts an effect of improving intestinal flora, solely or in a desired form controlled in combination with other animal feed ingredients. Examples of the animal feed ingredients used for animal feed or pet food include cereal crops

such as corn, wheat, barley, and rye; brans such as wheat bran and rice bran; dregs such as corn gluten meal and corn germ meal; animal-derived feed such as skimmed milk powder, whey, fish flour, and powdered bone; yeasts such as brewer's yeast; calciums such as calcium phosphate and calcium carbonate; vitamins; amino acids; and saccharides.

[0038] When the agent for improving intestinal flora in accordance with the present invention is used as animal feed or pet food, ratio of the agent blended into the animal feed or pet food varies according to, for example, forms and types of the animal feed or pet food, and types of animals for application. For example, the agent may be blended so that an amount of the above protease is in a range of 0.00067 to 6.7 mg/g, preferably 0.0067 to 6.7 mg/g, more preferably 0.0067 to 0.67 mg/g.

EXAMPLE

[0039] The present invention is described more specifically below with reference to Example, but it should not be construed to be limited to the example.

Test Example 1: Influence of polypeptide consisting of an amino acid sequence shown in SEQ ID NO: 1 on intestinal flora

1. Preparation of protease

[0040] A protease comprising a polypeptide consisting of an amino acid sequence shown in SEQ ID NO: 1 was prepared from a koji extract obtained by culturing a microorganism which is a strain producing the protease by a solid-state fermentation. Specifically, Aspergillus oryzae was cultured on a solid culture medium comprising wheat bran at 25 ± 5°C for 3 days. The koji after the culturing was immersed in water to extract the polypeptide, followed by removal of the koji by filtration through diatomaceous earth to obtain a polypeptide solution. Then, the polypeptide solution was concentrated by using ultra filtration membrane, followed by desalting to obtain a partial purified polypeptide solution. The partial purified polypeptide solution was sterilized by filtration through a membrane filter, followed by spray drying using a spray dryer to give a power of a partially purified polypeptide. The partially purified polypeptide was subjected to a purification step comprising ion exchange column chromatography, hydrophobic column chromatography, gel filtration chromatography, desalting column chromatography, and freeze-drying in this order to yield a powder of a purified polypeptide. The resulting purified polypeptide was subjected to SDS-polyacrylamide gel electrophoresis and CBB staining to detect a band of the polypeptide, which was treated with an enzyme followed by LC-MS/MS analysis and was confirmed to be a polypeptide consisting of an amino acid sequence shown in SEQ ID NO: 1.

2. Evaluation of effect of improving intestinal flora using rat

[0041] Using SD rats (male, 3 weeks old, from Hiroshima Institute for Experimental Animals), an influence of ingestion of a protease comprising a polypeptide having an amino acid sequence shown in SEQ ID NO: 1 on intestinal flora was investigated. In the experiment, the rats were divided into 4 groups consisting of control groups 1 and 2 and working groups 1 and 2, and the number of rats in each group was as follows: control group 1: 8 rats, control group 2: 4 rats, working group 1: 4 rats, and working group 2: 4 rats. The rats of each group were fed ad libitum for 14 days with animal feeds shown in Table 1.

[Table 1]

Ingredient	Control group 1 % w/w	Working group 1 % w/w	Working group 2 % w/w	Control group 2 % w/w
Beef tallow	30.00	30.00	30.00	30.00
Casein	20.00 (Net protein : 17.40)	20.00 (Net protein : 17.40)	20.00 (Net protein : 17.40)	20.00 (Net protein : 17.40)
L-Cysteine	0.30	0.30	0.30	0.30
Cellulose	5.00	5.00	5.00	5.00
Vitamin Mix #1	1.00	1.00	1.00	1.00
Mineral Mix #1	3.50	3.50	3.50	3.50
Sucrose	20.00	20.00	20.00	20.00
Corn starch	20.20	20.1904	20.1616	20.1616
Polypeptide #2	-	0.0096	0.0384	-
Inactivated polypeptide #3	-	-	-	0.0384

#1 refers to a standard purified diet for mice and rats according to AIN-93 (American Institute of Nutrition (AIN)). #2 refers to a protease consisting of a polypeptide of an amino acid sequence shown in SEQ ID NO: 1.
#3 refers to a polypeptide, which is a polypeptide of an amino acid sequence shown in SEQ ID NO: 1 inactivated under conditions of the trichloroacetic acid treatment.

[0042] Bacteria in cecal contents of the SD rats were analyzed after 14-day feeding with the animal feed by real-time PCR using primers shown in Table 2 to calculate the numbers of Bifidobacterium (Bifidobacterium spp.) and lactic acid bacteria (Lactobacillus spp.) in intestines.

[Table 2]

	Base sequences of primers used
For detecting Bifidobacterium spp.	Forward : CGCGTCYGGTGTGAAAG
	Reverse: CCCCACATCCAGCATCCA
For detecting Lactobacillus spp.	Forward : GAGGCAGCAGTAGGGAATCTTC
	Reverse : GGCCAGTTACTACCTCTATCCTTCTTC

3. Results

[0043] The results are shown in Table 3. In the working groups 1 and 2, the numbers of Bifidobacterium and lactic acid bacteria in intestines were both higher than those in the control groups 1 and 2. That is, it was confirmed that ingestion of the protease having the amino acid sequence shown in SEQ ID NO: 1 promoted proliferations of Bifidobacterium and lactic acid bacteria, leading to improvement of intestinal flora. It was also confirmed that ingestion of a protease having an enzyme activity, which is not merely an administration of a peptide, improved intestinal flora.

[Table 3]

		Control group 1	Working group 1	Working group 2	Control group 2
Intestinal flora	Bifidobacterium spp.	0.001 ± 0.000	0.282 ± 0.045	1.826 ± 0.214	0.005 ± 0.002
(% of total bacteria)	Lactobacillus spp.	0.90 ± 0.21	2.85 ± 0.68	3.05 ± 0.60	1.21 ± 0.53

REFERENCES CITED IN THE DESCRIPTION

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P A T E N T K R A V

1. Middel til anvendelse som et medikament til forbedring af tarmfloraen, omfattende en protease omfattende mindst én af følgende polypeptider (1) til (3) som aktiv ingrediens:

(1) et polypeptid bestående af en aminosyresekvens vist i SEQ ID NO: 1,

(2) et polypeptid omfattende en aminosyresekvens, hvor 1 til 16 af aminosyrer er substitueret, tilføjet, indsat eller slettet i aminosyresekvensen vist i SEQ ID NO: 1, og som har en proteaseaktivitet på ca. 80 til 120 % i forhold til proteaseaktiviteten (100 %) af et polypeptid bestående af en aminosyresekvens vist i SEQ ID NO: 1, hvor proteaseaktiviteten måles under de forhold, som er angivet i beskrivelsen, og polypeptidet er en variant af en syreprotease afledt af Aspergillus oryzae, og

(3) et polypeptid omfattende en aminosyresekvens med 95 % eller mere sekvensidentitet med en aminosyresekvens vist i SEQ ID NO: 1, og som har en proteaseaktivitet på ca. 80 til 120 % i forhold til proteaseaktiviteten (100 %) af et polypeptid bestående af en aminosyresekvens vist i SEQ ID NO: 1, hvor proteaseaktiviteten måles under de forhold, der er angivet i beskrivelsen, og polypeptidet er en variant af en syreprotease afledt af Aspergillus oryzae.

2. Middel til anvendelse som medikament til forbedring af tarmfloraen ifølge krav 1, som er i form af et lægemiddel til oral administration.

3. Middel til anvendelse som medikament til forbedring af tarmfloraen ifølge krav 1, som er i form af et fødevaretilsætningsstof.

4. Middel til anvendelse som medikament til forbedring af tarmfloraen ifølge krav 1, som er i form af en fødevare eller drikkevare.