DESCRIPTION

Title of Invention: ENZYME AGENT FOR PROTEIN DEAMIDATION AND INTENDED USE THEREOF

Technical Field

[0001]

The present invention relates to an enzyme agent (enzyme agent for protein deamidation) having a protein deamidase as an active ingredient, and intended use thereof.

Background Art

[0002]

Protein deamidase is an enzyme that acts on a protein, which is a macromolecule and catalyzes a deamidation reaction. Protein deamidase deamidates glutamine residues in a protein and allows negatively charged carboxyl groups to generate, thereby bringing about various property changes to the protein. For example, an increase in hydration strength and an increase in electrostatic repulsion, which are caused by a decrease in the isoelectric point of the protein, reduce protein-protein interactions (i.e., reduce association), thus increasing the solubility and water dispersibility of the protein. Moreover, the exposure of internal hydrophobic regions due to changes in the higher-order structure of protein imparts surface activity to the protein, thereby improving the emulsifying power, emulsification stability, foaming ability, and foam stability of the protein. Since protein deamidases can significantly alter the properties of proteins in this way, they have dramatically increased the intended uses of proteins. Accordingly, protein deamidase is highly useful and has attracted considerable interest in this technological field.

[0003]

The first protein deamidase discovered is a protein glutaminase discovered in 2000 from *Chryseobacterium proteolyticum* (Non-Patent Document 1). However, although this protein glutaminase had been extremely useful and had attracted a great interest, the protein glutaminase remains an extremely special enzyme, regarding which no new enzymes were discovered for a long

period of time of approximately 20 years. While protein glutaminase was discovered in *Bacteroides helcogenes* about 20 years later (Non-Patent Document 2), as of February 2024, the only protein glutaminase used industrially is that derived from *C. proteolyticum*.

Prior Art Documents

Non-Patent Documents

[0004]

Non-Patent Document 1: A novel protein-deamidating enzyme from Chryseobacterium proteolyticum sp. nov., a newly isolated bacterium from soil. Applied and Environmental Microbiology 2000; 66(8): 3337-43

Non-Patent Document 2: A novel protein glutaminase from Bacteroides helcogenes-characterization and comparison, Applied Microbiology and Biotechnology (2020) 104: 187-199

Summary of Invention

Objects to be Solved by the Invention

[0005]

It is an object of the present invention to provide a novel enzyme agent having a protein deamidation activity.

Means for Solving the Objects

[0006]

The present inventors have newly found that a polypeptide derived from *Longimycelium tulufanense* has a protein deamidation activity. The present invention has been completed based on the above-described findings.

[0007]

According to the present invention, the following inventions are provided.

[1] An enzyme agent for protein deamidation, containing, as an active ingredient, a protein deamidase consisting of an amino acid sequence having a sequence identity of 90% or more to the amino acid sequence as set forth in SEQ ID NO: 1.

[2] The enzyme agent for protein deamidation according to [1], in which the protein deamidase is derived from Longimycelium sp.

[3] A method for deamidating a protein or a peptide, including

allowing the enzyme agent for protein deamidation according to [1] or [2] to act on the protein or the peptide.

[4] A method for deamidating protein-containing food product and beverage, including

allowing the enzyme agent for protein deamidation according to [1] or [2] to act on the protein-containing food product and beverage.

[5] A method for producing food product and beverage, including

allowing the enzyme agent for protein deamidation according to [1] or [2] to act on protein-containing food product and beverage.

[6] Food product and beverage, treated with the enzyme agent for protein deamidation according to [1] or [2].

[7] A method for producing a protein deamidase consisting of an amino acid sequence having a sequence identity of 90% or more to the amino acid sequence as set forth in SEQ ID NO: 1, including

culturing bacteria of Longimycelium sp.

[8] An enzyme agent for protein deamidation, containing, as an active ingredient, a protein deamidase consisting of an amino acid sequence having a sequence identity of 60% or more to the amino acid sequence as set forth in SEQ ID NO: 1.

[9] The enzyme agent for protein deamidation according to [8], in which the protein deamidase is derived from Longimycelium sp., Crossiella sp., or Krasilnikovia sp.

[10] A method for deamidating a protein or a peptide, including

allowing the enzyme agent for protein deamidation according to [8] or [9] to act on the protein or the peptide.

[11] A method for deamidating protein-containing food product and beverage, including

allowing the enzyme agent for protein deamidation according to [8] or [9] to act on the protein-containing food product and beverage.

[12] A method for producing food product and beverage, including

allowing the enzyme agent for protein deamidation according to [8] or [9] to act on protein-containing food product and beverage.

[13] Food product and beverage, treated with the enzyme agent for protein deamidation according to [8] or [9].

[14] A method for producing a protein deamidase consisting of an amino acid sequence having a sequence identity of 60% or more to the amino acid sequence as set forth in SEQ ID NO: 1, including

culturing bacteria of Longimycelium sp., the bacteria of Crossiella sp., or the bacteria of Krasilnikovia sp.

[15] An enzyme agent for protein deamidation, containing, as an active ingredient, a protein deamidase consisting of an amino acid sequence having a sequence identity of 50% or more to the amino acid sequence as set forth in SEQ ID NO: 1.

[16] The enzyme agent for protein deamidation according to [15], in which the protein deamidase is derived from Longimycelium sp., Crossiella sp., Krasilnikovia sp., or Longispora sp.

[17] A method for deamidating a protein or a peptide, including

allowing the enzyme agent for protein deamidation according to [15] or [16] to act on the protein or the peptide.

[18] A method for deamidating protein-containing food product and beverage, including

allowing the enzyme agent for protein deamidation according to [15] or [16] to act on the protein-containing food product and beverage.

[19] A method for producing food product and beverage, including

allowing the enzyme agent for protein deamidation according to [15] or [16] to act on protein-containing food product and beverage.

[20] Food product and beverage, treated with the enzyme agent for protein deamidation according to [15] or [16].

[21] A method for producing a protein deamidase consisting of an amino acid sequence having a sequence identity of 50% or more to the amino acid sequence as set forth in SEQ ID NO: 1, including

culturing bacteria of Longimycelium sp., Crossiella sp., Krasilnikovia sp., or Longispora

sp.

[0008]

< A > Use of an enzyme agent for protein deamidation, in which the enzyme agent contains, as an active ingredient, a protein deamidase consisting of an amino acid sequence having a sequence identity of 90% or more to the amino acid sequence as set forth in SEQ ID NO: 1.

Advantageous Effects of Invention

[0009]

The enzyme agent for protein deamidation containing a protein deamidase according to the present invention is suitable to be used in the fields of food products and medical applications.

Embodiments of Carrying out the Invention

[0010]

1. Enzyme agent for protein deamidation

The enzyme agent for protein deamidation of the present invention contains, as an active ingredient, a protein deamidase consisting of the amino acid sequence as set forth in SEQ ID NO: 1, 6, 10, or 14, or an amino acid sequence equivalent thereto.

The protein deamidase of the present invention is an enzyme exhibiting an action to decompose the amide group-containing side chains of proteins, and may further have an action to decompose the amide group-containing side chains of proteins, attended with the cleavage of peptide bonds and the crosslinking of proteins, as long as it has the above-described action. Specifically, the protein deamidase of the present invention is an enzyme (protein glutaminase) that deamidates glutamine residues in proteins and converts them to glutamic acid.

[0011]

The term "equivalent amino acid sequence" means an amino acid sequence that is partially different from the reference amino acid sequence (i.e. the amino acid sequence as set forth in SEQ ID NO: 1, 6, 10, or 14) but such difference does not substantially influence on the function of the protein (which is herein a protein deamidation activity). Accordingly, an enzyme having such an

equivalent amino acid sequence catalyzes an enzyme reaction by a protein deamidase. The degree of the activity is not particularly limited, as long as the function of a protein deamidase can be exhibited. However, the activity of the enzyme having such an equivalent amino acid sequence is preferably equivalent to or higher than the activity of an enzyme consisting of the reference amino acid sequence (i.e. having the amino acid sequence as set forth in SEQ ID NO: 1, 6, 10, or 14).
[0012]

The protein deamidase is preferably derived from Longimycelium sp., Crossiella sp., Krasilnikovia sp., or Longispora sp., and is more preferably derived from *Longimycelium tulufanense, Crossiella cryophila, Krasilnikovia cinnamomea,* or *Longispora fulva*. The amino acid sequence as set forth in SEQ ID NO: 1 is the amino acid sequence (mature body) of a protein deamidase derived from *Longimycelium tulufanense*. It is to be noted that the full-length amino acid sequence of the protein deamidase derived from *Longimycelium tulufanense* is shown in SEQ ID NO: 2. The amino acid sequence as set forth in SEQ ID NO: 6 is the amino acid sequence (mature body) of a protein deamidase derived from *Crossiella cryophila*. It is to be noted that the full-length amino acid sequence of the protein deamidase derived from *Crossiella cryophila* is shown in SEQ ID NO: 7. The amino acid sequence as set forth in SEQ ID NO: 10 is the amino acid sequence (mature body) of a protein deamidase derived from *Krasilnikovia cinnamomea*. It is to be noted that the full-length amino acid sequence of the protein deamidase derived from *Krasilnikovia cinnamomea* is shown in SEQ ID NO: 11. The amino acid sequence as set forth in SEQ ID NO: 14 is the amino acid sequence (mature body) of a protein deamidase derived from *Longispora fulva*. It is to be noted that the full-length amino acid sequence of the protein deamidase derived from *Longispora fulva* is shown in SEQ ID NO: 15.
[0013]

A "partial difference in an amino acid sequence" is produced, for example, as a result of a deletion or a substitution of one or more amino acids in the amino acids constituting the amino acid sequence, or an addition or an insertion of one or more amino acids into the amino acid sequence, or any given combination thereof. Such a partial difference in the amino acid sequence is acceptable, as long as the protein deamidation activity is retained (in which the activity may be slightly fluctuated). As far as this condition is satisfied, the position of the amino acid sequence, in which

the difference is found, is not particularly limited. In addition, such difference may be produced in multiple sites (places) on the amino acid sequence.

[0014]

The number of amino acids providing such a partial difference in the amino acid sequence is a number corresponding to, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, or less than about 10%, a number preferably corresponding to less than about 8%, a number more preferably corresponding to about 6%, a number even more preferably corresponding to less than about 4%, a number further preferably corresponding to less than about 2%, and a number most preferably corresponding to less than about 1%. Accordingly, the equivalent protein has an identity of, for example, about 50% or more, about 55% or more, about 60% or more, about 65% or more, about 70% or more, about 75% or more, about 80% or more, about 81% or more, about 82% or more, about 83% or more, about 84% or more, about 85% or more, about 86% or more, about 87% or more, about 88% or more, about 89% or more, about 90% or more, about 91% or more, about 92% or more, preferably about 93% or more, more preferably about 94% or more, even more preferably about 95% or more, further preferably about 96% or more, even further preferably about 97% or more, still further preferably about 98% or more, and most preferably about 99% or more, with respect to the reference amino acid sequence.

[0015]

A typical example of a "partial difference in the amino acid sequence" is that a mutation (a change) is produced in the amino acid sequence, as a result of a deletion or a substitution of 1 to 40 (preferably 1 to 30, more preferably 1 to 10, even more preferably 1 to 7, further preferably 1 to 5, and still further preferably 1 to 3) amino acids in the amino acids constituting the amino acid sequence, or an addition or an insertion of 1 to 40 (preferably 1 to 30, more preferably 1 to 10, even more preferably 1 to 7, further preferably 1 to 5, and still further preferably 1 to 3) amino acids into an amino acid sequence, or any given combination thereof.

[0016]

Preferably, an equivalent amino acid sequence is obtained by the occurrence of conservative amino acid substitution in amino acid residues that are not essential for protein deamidation activity. The term "conservative amino acid substitution" is used herein to mean that a

certain amino acid residue is substituted with an amino acid residue having a side chain with similar properties. Amino acid residues are classified into several families, depending on the side chain thereof; namely, basic side chains (for example, lysine, arginine, and histidine), acidic side chains (for example, aspartic acid and glutamic acid), uncharged polar side chains (for example, glycine, asparagine, glutamine, serine, threonine, tyrosine, and cysteine), non-polar side chains (for example, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan), β-branched side chains (for example, threonine, valine, and isoleucine), aromatic side chains (for example, tyrosine, phenylalanine, tryptophan, and histidine), etc. The conservative amino acid substitution is preferably a substitution occurring between amino acid residues in an identical family.

[0017]

The identity (%) of two amino acid sequences can be determined, for example, by the following procedures. First, two sequences are aligned such that an optimal comparison can be made (for example, it may also be possible that a gap is introduced into a first sequence, so that the alignment with a second sequence may be optimized). When a molecule (amino acid residue) in a specific position of the first sequence is identical to a molecule in a corresponding position in the second sequence, it can be said that the molecules at the positions are identical to each other. The identity of the two sequences is a function of the number of identical positions common in the two sequences (i.e. identity (%) = the number of identical positions / total number of positions x 100), and preferably, the number and size of gaps used for optimization of the alignment are also taken into consideration.

[0018]

Comparison of two sequences and determination of identity can be realized using mathematical algorithms. A specific example of the mathematical algorithm that can be utilized in comparison of sequences may be an algorithm that is described in Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87: 2264-68 and is modified in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-77, but the example of the mathematical algorithm is not limited thereto. The identity of amino acid sequences can be obtained, for example, by blastp (protein-protein BLAST) of National Center for Biotechnology Information (NCBI). As parameters, default parameters may be used. For example, using BLOSUM62 matrix, Gap Costs may be set to be Existence: 11 and

Extension: 1.

[0019]

The protein deamidase that is an active ingredient of the enzyme agent of the present invention may be a part of a larger protein (for example, a fusion protein). Examples of a sequence to be added to such a fusion protein may include sequences that are useful for purification, such as multiple histidine residues, and additional sequences that ensure stability during recombinant production.

[0020]

The protein deamidase in the present invention (hereinafter also referred to as the present enzyme) can be obtained by culturing microorganisms that produce the protein deamidase (i.e., a protein deamidase-producing strain), for example, the bacteria of Longimycelium sp., the bacteria of Crossiella sp., the bacteria of Krasilnikovia sp., or the bacteria of Longispora sp. As an example, the present enzyme can be obtained by culturing *Longimycelium tulufanense*, *Crossiella cryophila*, *Krasilnikovia cinnamomea*, or *Longispora fulva*. That is to say, the present invention may relate to a method for producing a protein deamidase consisting of an amino acid sequence having a sequence identity of 50% or more (preferably, 60% or more, 70% or more, 80% or more, or 90% or more) to the amino acid sequence as set forth in SEQ ID NO: 1, in which the method includes a step of culturing bacteria of Longimycelium sp., the bacteria of Crossiella sp., the bacteria of Krasilnikovia sp., or the bacteria of Longispora sp. The protein deamidase-producing strain may be either a wild-type strain or a mutant strain (for example, a mutant strain is obtained by UV irradiation). A specific example of the protein deamidase-producing strain may be *Longimycelium tulufanense* (NBRC107726). *Longimycelium tulufanense* (NBRC107726) is a bacteria strain stored at the NBRC (National Institute of Technology and Evaluation, Biological Resource Center), and it can be obtained by following the prescribed procedures.

[0021]

The present enzyme can be prepared using a culture solution and/or a cell mass of microorganisms that produce the present enzyme. The culture conditions or the culture methods are not particularly limited, as long as the present enzyme can be produced thereby. That is to say, the methods or the culture conditions that are adapted to the culture of the used microorganisms can

be determined, as appropriate, under conditions in which the present enzyme is produced. Regarding the culture method, either liquid culture or solid culture may be applied, and liquid culture is preferably utilized. Taking the liquid culture as an example, the culture conditions will be described below.

[0022]

The medium is not particularly limited, as long as it is a medium in which the used microorganisms can grow. Examples of the medium that can be used herein may include those to which the following substances are added: carbon sources, such as glucose, sucrose, gentiobiose, soluble starch, glycerin, dextrin, molasses, and organic acids; nitrogen sources, such as ammonium sulfate, ammonium carbonate, ammonium phosphate, ammonium acetate, or gelatin, peptone, yeast extract, corn steep liquor, casein hydrolysate, bran, and meat extract; and further, inorganic salts, such as potassium salts, magnesium salts, sodium salts, phosphates, manganese salts, iron salts and zinc salts. In order to promote the growth of the used microorganisms, vitamins, amino acids and the like may be added to the medium. The pH of the medium is adjusted to, for example, about 3 to 8, preferably about 4 to 7. The culture temperature is usually about 20°C to 40°C, preferably about 25°C to 35°C, and the microorganisms are cultured for 1 to 20 days, and preferably 3 to 10 days, under aerobic conditions. As a culture method applied herein, for example, a shaking culture method, or an aerobic deep culture method using a jar fermenter, can be utilized.

[0023]

After completion of the culture performed under the above-described conditions, the enzyme of interest is recovered from the culture solution or the culture mass. When the enzyme is recovered from the culture solution, for example, the culture supernatant is filtered, centrifuged, etc. to remove insoluble matters, and thereafter, the resultant is separated and purified by appropriately combining concentration using an ultrafiltration membrane, salting-out such as ammonium sulfate precipitation, dialysis, and various types of chromatography using ion exchange resin, etc., thereby obtaining the present enzyme. On the other hand, when the enzyme is recovered from the cell mass, for example, the cell mass is crushed by a pressure treatment, an ultrasonic treatment, etc., and is then separated and purified in the same manner as that described above, so as to obtain the present enzyme. Besides, the cell mass may be previously recovered from the culture solution by filtration,

a centrifugation treatment, etc., and thereafter, the above-described series of steps (crushing, separation, and purification of the cell mass) may be carried out.

[0024]

Also, the present enzyme can be easily prepared by a genetic engineering technique. For example, the present enzyme can be prepared by transforming suitable host cells (for example, *Escherichia coli*) with DNA encoding the present enzyme, and then recovering the protein expressed in the transformant. The recovered protein is appropriately purified, depending on the purpose. As such, if the enzyme of interest is obtained as a recombinant protein, various modifications can be carried out thereon. For example, a DNA encoding the present enzyme and another appropriate DNA are inserted into an identical vector, and a recombinant protein is produced using the vector, so that the present enzyme consisting of a recombinant protein, in which any given peptides or proteins are linked to each other, can be obtained. In addition, modification may be carried out, so that addition of a sugar chain and/or a lipid or the processing of the N-terminus or the C-terminus may occur. By performing the aforementioned modification, simplification of the extraction and purification of a recombinant protein, addition of biological functions, etc. can be carried out.

[0025]

Generally, the expression of a gene and the recovery of a gene expression product (the present enzyme) are carried out, utilizing an appropriate host-vector system, as described above. However, a cell-free synthesis system may also be utilized. Herein, the term "cell-free synthesis system (a cell-free transcription system or a cell-free transcription/translation system)" means that, not using living cells, but using a ribosome, a transcription/translation factor or the like derived from living cells (or obtained by a genetic engineering technique), from a nucleic acid (DNA or mRNA) used as a template, mRNA or a protein encoded by the nucleic acid is synthesized *in vitro*. In general, in the cell-free synthesis system, a cell extract obtained by purifying, as necessary, a cell-disintegrated solution is used. The cell extract generally contains a ribosome, various types of factors such as an initiation factor, and various types of enzymes such as tRNA, which are necessary for protein synthesis. When a protein is synthesized, various types of amino acids, energy sources such as ATP or GTP, and other substances necessary for protein synthesis, such as creatine phosphate, are added to the aforementioned cell extract. As a matter of course, upon the synthesis

of a protein, a ribosome, various types of factors, and/or various types of enzymes, and the like, which are prepared separately, may also be added to the aforementioned cell extract, as necessary.

[0026]

The development of a transcription/translation system, in which various molecules (factors) necessary for protein synthesis have been reconstructed, has also been reported (Shimizu, Y. et al.: Nature Biotech., 19, 751-755, 2001). In this synthesis system, genes of 31 types of factors composed of: 3 types of initiation factors, 3 types of elongation factors, 4 types of factors associated with termination, 20 types of aminoacyl tRNA synthesis enzymes that allow each amino acid to bind to tRNA, and a methionyl tRNA formyl transfer enzyme, which constitute a protein synthesis system of bacteria, are amplified from an *Escherichia coli* genome, and then, using these amplified products, a protein synthesis system is reconstructed *in vitro*. In the present invention, such a reconstructed synthesis system may be utilized.

[0027]

The term "cell-free transcription/translation system" is exchangeably used with the term "cell-free protein synthesis system," "*in vitro* translation system" or "*in vitro* transcription/translation system." In the *in vitro* translation system, RNA is used as a template to synthesize a protein. As such template RNA, total RNA, mRNA, an *in vitro* transcriptional product or the like is used. On the other hand, in the *in vitro* transcription/translation system, DNA is used as a template. The template DNA should contain a ribosome-binding region, and preferably contains an appropriate terminator sequence. In addition, in the *in vitro* transcription/translation system, in order to promote continuous progression of the transcription reaction and the translation reaction, conditions, in which factors necessary for each reaction are added, are established.

[0028]

The purified enzyme obtained as described above is pulverized, for example, by freeze drying, vacuum drying, or spray drying, so that the enzyme can be provided in the form of powders. At that time, the purified enzyme may be previously dissolved in an acetate buffer, a phosphate buffer, a triethanolamine buffer, a Tris-HCl buffer, or a GOOD buffer. Preferably, an acetate buffer, a phosphate buffer, or a triethanolamine buffer can be used. Besides, examples of the GOOD buffer used herein may include PIPES, MES, and MOPS.

[0029]

The purification degree of the enzyme is not particularly limited. In addition, the final form of the enzyme may be either a liquid or a solid (including powders).

[0030]

The present enzyme is an enzyme that deamidates the γ-amide groups of glutamine residues in proteins. The content of the active ingredient (the present enzyme) in the enzyme agent of the present invention is not particularly limited, and for example, the content of the active ingredient can be set or can be adjusted, so that the protein deamidation activity per gram of the present enzyme agent can become 0.01 U to 5000 U, preferably 0.1 U to 2000 U, and more preferably 1 U to 1000 U.

[0031]

As a method for measuring a protein deamidation activity, the following method using a synthetic substrate can be employed. That is, 0.1 mL of a sample solution containing a protein deamidase is added to 1 mL of a 0.2 M phosphate buffer (pH 6.5) containing 30 mM Z-Gln-Gly, the obtained mixture is then left at 37°C for 10 minutes, and 1 mL of a 0.4 M TCA (trichloroacetic acid) solution is then added to stop the reaction. As a blank, 1 mL of a 0.4 M TCA solution is added to 1 mL of a 0.2 M phosphate buffer (pH 6.5) containing 30 mM Z-Gln-Gly, and 0.1 mL of a sample solution containing a protein deamidase is further added thereto, followed by leaving the obtained mixture at 37°C for 10 minutes.

[0032]

"Ammonia Test Wako" (FUJIFILM Wako Pure Chemical Corporation) was used for the solution obtained above, and the amount of ammonia generated in the reaction solution was measured. The ammonia concentration in the reaction solution is determined from a calibration curve showing the relationship between the ammonia concentration and the absorbance (630 nm) prepared using an ammonia standard solution (ammonium chloride).

[0033]

The activity of the protein deamidase is calculated using the formula shown below, in which the amount of enzyme that produces 1 μmol of ammonia per minute is defined as 1 unit (1 U). In the formula, the amount of the reaction solution is 2.1, the amount of the enzyme solution is 0.1,

and Df is the dilution magnification of the enzyme solution. Also, 17.03 is the molecular weight of ammonia.

[0034]

[Expression 1]

Protein deamidase activity (U/mL)

= Ammonia concentration (mg/L) in reaction solution x (1 / 17.03) x (reaction solution amount / enzyme solution amount) x (1/10) x Df

[0035]

On the other hand, the protein deamidation activity with respect to the protein substrate can be calculated from the amount of deamidated insulin B chain produced when insulin B chain is used as a substrate. Details of the measurement conditions and measurement procedures are shown in the Examples later.

[0036]

The protein deamidation activity in the enzyme agent of the present invention is not particularly limited, and for example, the lower limit value of the deamidation percentage in the evaluation method using insulin B chain is 4% or more, 5% or more, or 7% or more, and it is preferably 10% or more, and more preferably 15% or more. On the other hand, the upper limit value of the deamidation percentage is 99% or less, 90% or less, 80% or less, 70% or less, 60% or less, or 50% or less, and it is preferably 40% or less, more preferably 30% or less, and further preferably 20% or less.

[0037]

Moreover, as described above, the protein deamidation activity of the enzyme agent of the present invention can also be evaluated by analyzing the reaction stop solution using HPLC, after the reaction has been carried out with the synthetic substrate, and detecting the peak transition. Details of the measurement conditions and measurement procedures are shown in the Examples described later.

[0038]

The enzyme agent of the present invention may also contain an excipient, a buffer agent, a suspending agent, a stabilizer, a preservative, an antiseptic, a normal saline, various types of proteins, degradation products of various types of proteins, various types of extracts, various types of salts, various types of antioxidants, cysteine, glutathione, sodium glutamate, sodium inosinate, sodium guanylate, calcined shell calcium, silicon dioxide, and the like, as well as the active ingredient (the protein deamidase of the present invention). Examples of the excipient that can be used herein may include starch, dextrin, maltose, trehalose, lactose, D-glucose, sorbitol, D-mannitol, white sugar, and glycerol. Examples of the buffer agent that can be used herein may include phosphate, citrate, and acetate. Examples of the stabilizer that can be used herein may include propylene glycol and ascorbic acid. Examples of the preservative that can be used herein may include phenol, benzalkonium chloride, benzyl alcohol, chlorobutanol, and methylparaben. Examples of the antiseptic that can be used herein may include ethanol, benzalkonium chloride, paraoxybenzoic acid, and chlorobutanol. Examples of the protein used herein may include a soybean protein, a wheat protein, a corn protein, a milk protein, and an animal-derived protein. Examples of the extracts used herein may include a meat extract, a plant extract, and a yeast extract. Examples of the salts used herein may include chloride, phosphate, polyphosphate, pyrophosphate, citrate, lactate, and carbonate. Examples of the antioxidant used herein may include L-ascorbate and sodium hydrogen sulfite. The shape of the enzyme agent of the present invention is not particularly limited, and it is, for example, powders, granules, a liquid, a capsule, or the like.

[0039]

2. Gene

The gene encoding the present enzyme consists of DNA encoding a protein containing the amino acid sequence as set forth in SEQ ID NO: 1, 6, 10, or 14. Specific examples of the present aspect may include DNA consisting of the nucleotide sequence as set forth in SEQ ID NO: 3, 8, 12, or 16, and DNA consisting of the nucleotide sequence as set forth in SEQ ID NO: 4, 9, 13, or 17. The DNA of SEQ ID NO: 3 encodes only the amino acid sequence of a mature body (SEQ ID NO: 1), whereas the DNA of SEQ ID NO: 4 encodes a signal peptide and a pro-sequence, in addition to the mature body (the amino acid sequence as set forth in SEQ ID NO: 1). The DNA of SEQ ID NO: 8 encodes only the amino acid sequence of a mature body (SEQ ID NO: 6), whereas the DNA

of SEQ ID NO: 9 encodes a signal peptide and a pro-sequence, in addition to the mature body (the amino acid sequence as set forth in SEQ ID NO: 6). The DNA of SEQ ID NO: 12 encodes only the amino acid sequence of a mature body (SEQ ID NO: 10), whereas the DNA of SEQ ID NO: 13 encodes a signal peptide and a pro-sequence, in addition to the mature body (the amino acid sequence as set forth in SEQ ID NO: 10). The DNA of SEQ ID NO: 16 encodes only the amino acid sequence of a mature body (SEQ ID NO: 14), whereas the DNA of SEQ ID NO: 17 encodes a signal peptide and a pro-sequence, in addition to the mature body (the amino acid sequence as set forth in SEQ ID NO: 14).

[0040]

The gene encoding the present enzyme is typically utilized in preparation of the present enzyme. According to a genetic engineering preparation method using the gene encoding the present enzyme, it is possible to obtain the present enzyme in a more homogeneous state. In addition, it is said that this method is a method preferably used in the case of preparing a large amount of the present enzyme. Besides, the intended use of the gene encoding the present enzyme is not limited to the preparation of the present enzyme. For example, the present nucleic acid can also be used as an experimental tool for elucidating the action mechanism of the present enzyme, or as a tool for designing or preparing a mutant (a modified body) of the present enzyme.

[0041]

In the present description, the phrase "the gene encoding the present enzyme" means a nucleic acid for providing the present enzyme, when the nucleic acid is allowed to express. Thus, the gene encoding the present enzyme includes not only a nucleic acid having a nucleotide sequence corresponding to the amino acid sequence of the present enzyme, but also a nucleic acid formed by adding a sequence that does not encode the amino acid sequence to the aforementioned nucleic acid. Moreover, codon degeneracy is also taken into consideration.

[0042]

The nucleic acid can be prepared in an isolated state according to a standard genetic engineering technique, a molecular biological technique, a biochemical technique, chemical synthesis, a PCR method (for example, overlap PCR), or a combination thereof, with reference to the present description or the sequence information disclosed in the sequence listing attached herewith.

[0043]

There may be used a nucleic acid, in which when the nucleic acid is compared with the nucleotide sequence of the gene encoding the present enzyme, the function of a protein encoded thereby is equivalent, but the nucleotide sequence thereof is partially different from the nucleotide sequence of the gene encoding the present enzyme (which is hereinafter also referred to as an "equivalent nucleic acid," and the nucleotide sequence of the equivalent nucleic acid is also referred to as an "equivalent nucleotide sequence"). An example of such an equivalent nucleic acid may be DNA that consists of a nucleotide sequence having a substitution, deletion, insertion, addition or inversion of one or more nucleotides, based on the nucleotide sequence of the nucleic acid encoding the present enzyme, and encodes a protein having an enzyme activity characteristic of the present enzyme (i.e. protein deamidation activity). Substitution, deletion, etc. of a nucleotide(s) may occur in multiple sites. The term "multiple" is used herein to mean for example, 2 to 40 nucleotides, preferably 2 to 20 nucleotides, and more preferably 2 to 10 nucleotides, although the number of nucleotides is different depending on the positions or types of amino acid residues in the three-dimensional structure of a protein encoded by the nucleic acid. The equivalent nucleic acid has a sequence identity of, for example, 50% or more, 60% or more, 70% or more, 80% or more, 81% or more, 82% or more, 83% or more, 84% or more, 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, or 92% or more, preferably 93% or more, more preferably 94% or more, even more preferably 95% or more, further preferably 96% or more, even further preferably 97% or more, still further preferably 98% or more, and most preferably 99% or more, with respect to the nucleotide sequence serving as a reference (SEQ ID NO: 3, 4, 8, 9, 12, 13, 16, or 17).

[0044]

Such an equivalent nucleic acid as described above can be obtained by, for example, a restriction enzyme treatment, a treatment with exonuclease, DNA ligase, etc., introduction of a mutation according to a site-directed mutagenesis (Molecular Cloning, Third Edition, Chapter 13, Cold Spring Harbor Laboratory Press, New York) or a random mutation introduction method (Molecular Cloning, Third Edition, Chapter 13, Cold Spring Harbor Laboratory Press, New York),

etc. Moreover, the equivalent nucleic acid can also be obtained by other methods such as ultraviolet irradiation.

[0045]

There may be used a nucleic acid having a nucleotide sequence complementary to the nucleotide sequence of the gene that encodes the present enzyme. There may also be used a nucleic acid having a nucleotide sequence that has a sequence identity of, at least, about 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, to the nucleotide sequence of the gene that encodes the present enzyme, or to a nucleotide sequence complementary to the nucleotide sequence of the gene that encodes the present enzyme.

[0046]

There may also be used a nucleic acid having a nucleotide sequence that hybridizes, under stringent conditions, to a nucleotide sequence complementary to the nucleotide sequence of the gene that encodes the present enzyme or complementary to a nucleotide sequence equivalent thereto. The term "stringent conditions" is used herein to mean conditions under which, what is called, a specific hybrid is formed, but a non-specific hybrid is not formed. Such stringent conditions are known to those skilled in the art, and can be determined, for example, with reference to Molecular Cloning (Third Edition, Cold Spring Harbor Laboratory Press, New York) or Current protocols in molecular biology (edited by Frederick M. Ausubel et al., 1987). The stringent conditions may be, for example, conditions in which incubation is performed at about 50℃, using a hybridization solution (50% formamide, 10 x SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5 x Denhardt solution, 1% SDS, 10% dextran sulfate, 10 μg/ml denatured salmon sperm DNA, and 50 mM phosphate buffer (pH 7.5)), and thereafter, washing is performed at about 65℃, using 0.1 x SSC and 0.1% SDS. More preferred stringent conditions may be, for example, conditions of using, as a hybridization solution, 50% formamide and 5 x SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 1 x Denhardt solution, 1% SDS, 10% dextran sulfate, 10 μg/ml denatured salmon sperm DNA, 50 mM phosphate buffer (pH 7.5)).

[0047]

There may be used recombinant DNA containing the gene that encodes the present

enzyme. The recombinant DNA is provided in the form of, for example, a vector. The term "vector" is used in the present description to mean a nucleic acid molecule capable of transporting a nucleic acid inserted in the nucleic acid molecule into a target such as a cell.

[0048]

Depending on intended purpose (cloning or the expression of a protein), or taking into consideration of the type of a host cell, a suitable vector is selected. Examples of the vector having *Escherichia coli* as a host may include M13 phage or a modified body thereof, λ phage or a modified body thereof, and pBR322 or a modified body thereof (pB325, pAT153, pUC8, etc.). Examples of the vector having yeast as a host may include pYepSec1, pMFa, and pYES2. Examples of the vector having an insect cell as a host may include pAc and pVL. Examples of the vector having a mammalian cell as a host may include pCDM8 and pMT2PC.

[0049]

The vector is preferably an expression vector. The term "expression vector" means a vector that is capable of introducing a nucleic acid inserted in the vector into a cell of interest (a host cell) and that is also capable of allowing the nucleic acid to express in the host cell. The expression vector usually contains a promoter sequence necessary for the expression of the inserted nucleic acid, an enhancer sequence for promoting the expression, and the like. An expression vector containing a selective marker can also be used. In the case of using such an expression vector, the presence or absence of the introduction of the expression vector (and the degree thereof) can be confirmed by utilizing the selective marker.

[0050]

Insertion of the nucleic acid into the vector, insertion of a selective marker (if necessary), insertion of a promoter (if necessary), and the like can be carried out by using a standard recombinant DNA technique (for example, a publicly known method using restriction enzyme and DNA ligase, which can refer to Molecular Cloning, Third Edition, 1.84, Cold Spring Harbor Laboratory Press, New York).

[0051]

In terms of the ease of handling, microorganisms such as *Escherichia coli*, Filamentous fungi (*Aspergillus oryzae*), Actinomycetes (Streptomyces sp., *Streptomyces lividans*, and

Streptomyces mobaraensis), *Bacillus subtilis*, and budding yeast (*Saccharomyces cerevisiae*) are preferably used as host cells. However, any host cells can be utilized, as long as they are capable of replicating recombinant DNA and expressing the gene of the present enzyme. In the case of utilizing a T7 promoter, the used *Escherichia coli* may be, for example, the *Escherichia coli* BL21(DE3)pLysS. In the case of not using a T7 promoter, the used *Escherichia coli* may be, for example, the *Escherichia coli* JM109. In addition, examples of the budding yeast may include the budding yeast SHY2, the budding yeast AH22, and the budding yeast INVSc1 (Invitrogen).

[0052]

A microorganism containing the recombinant DNA (i.e. a transformant) can be used. The microorganism can be obtained by transfection or transformation using the above-described vector of the present invention. Such transfection or transformation can be carried out, for example, by a calcium chloride method (Journal of Molecular Biology (J. Mol. Biol.), Vol. 53, p. 159 (1970)), a Hanahan method (J. Mol. Biol., Vol. 166, p. 557 (1983)), an SEM method (Gene, Vol. 96, p. 23 (1990)), a method of Chung, et al. (Proceedings of the National Academy of Sciences of the USA. Vol. 86, p. 2172 (1989)), a calcium phosphate coprecipitation method, electroporation (Potter, H. et al., Proc. Natl. Acad. Sci. U.S.A. 81, 7161-7165 (1984)), lipofection (Felgner, P. L. et al., Proc. Natl. Acad. Sci. U.S.A. 84, 7413-7417 (1984)), a conjugative transfer method, and the like. The above-described microorganism can be utilized to produce the present enzyme.

[0053]

3. Intended use of enzyme agent

The present invention further relates to an intended use of the enzyme agent of the present invention.

That is to say, by allowing the enzyme agent of the present invention to act on a substrate (a protein or a peptide), the protein or the peptide can be modified. Specifically, by allowing the enzyme agent of the present invention to act on a protein or a peptide containing glutamine residues, the glutamine residues of the protein can be deamidated. That is, the present invention may relate to a method for deamidating a protein or a peptide, including allowing an enzyme agent for protein deamidation to act on the protein or the peptide. In addition, even when the enzyme agent of the present invention is allowed to act on a protein or a peptide containing both glutamine residues and

lysine residues, the present enzyme agent is able to deamidate glutamine residues of the protein. A conventional general transglutaminase is known to hardly catalyze deamidation reactions, when the conventional general transglutaminase is allowed to act on a protein or a peptide containing both glutamine residues and lysine residues (i.e., when the conventional general transglutaminase is allowed to act thereon in the presence of an acyl receptor). However, the high-performance material of the present invention can deamidate glutamine residues in the protein, even when lysine residues are present as acyl receptors.

[0054]

The protein or the peptide is preferably contained in protein-containing food product and beverage. The present invention preferably relates to a method for deamidating protein-containing food product and beverage, including allowing an enzyme agent for protein deamidation to act on the protein-containing food product and beverage.

[0055]

The present invention may relate to a method for producing food product and beverage, including allowing an enzyme agent for protein deamidation to act on protein-containing food product and beverage. For example, by allowing the enzyme agent of the present invention to act on food product and beverage or a food product and beverage material, which contains a protein or a peptide (protein-containing food product and beverage, etc.), food product and beverage or a food product and beverage material, which contains a deamidated protein (deamidated protein-containing food product and beverage, etc.), can be produced. By allowing the enzyme agent of the present invention to act on protein-containing food product and beverage, the quality of the protein-containing food product and beverage can be improved. According to the present invention, food product and beverage (deamidated protein-containing food product and beverage), which is treated with the enzyme agent of the present invention, is provided. Moreover, the enzyme agent of the present invention can also be used for production of industrial materials and pharmaceutical raw materials, other than food products and beverages or food product and beverage materials (protein-containing food product and beverage, etc.).

[0056]

Specific examples of the improvement of protein-containing food product and beverage

may include, but are not particularly limited to, intended use for improving the solubility, dispersibility, and emulsification of animal proteins and plant proteins (e.g., intended use for production of dairy products (milk, yogurt, etc.), plant-based milk, plant-based yogurt, coffee whiteners, juices and other beverages, dressings, mayonnaise, and cream); increasing the solubility and dispersibility of poorly soluble plant proteins (e.g., intended use for production of *tempura* flour, etc. using wheat gluten); intended use for modifying dough in bread and confectionery (e.g., intended use for production of cracker, biscuit, cookie, pizza, or pie crusts); intended use for removing or reducing allergens from allergenic proteins in food products and beverages (e.g., intended use for production of food products for patients suffering from wheat allergy); intended use for reducing the mineral sensitivity of proteins, increasing the soluble mineral content in liquids containing proteins and minerals, and improving the absorption of minerals into the human body (e.g., intended use for production of high-mineral (e.g., calcium)-containing beverages and mineral (e.g., calcium) absorption enhancers); and intended use for reducing bitterness, intended use for improving the protein degradation rate of proteases, and/or intended use for enhancing glutamic acid content (e.g., intended use for production of amino acid-based seasonings (hydrolyzed animal protein (HAP), hydrolyzed plant protein (HVP)), and *miso* and soy sauce).

[0057]

The method for producing food product and beverage using the present enzyme agent is not particularly limited, and it is, for example, a method including the following step (a). Furthermore, the method may also include a step (b) and/or a step (c), after the step (a):

(a) a step of allowing the present enzyme agent to act on a food product or a food product material that contains a protein (protein-containing food product and beverage),

(b) a step of recovering the food product or the food product material, which contains the deamidated protein, and

(c) a step of deactivating or removing the present enzyme contained in the obtained enzyme-treated food product or enzyme-treated food product material.

Examples

[0058]

Example 1: Preparation of protein deamidase derived from *Longimycelium tulufanense*

A cell mass of *Longimycelium tulufanense* (obtained from the National Institute of Technology and Evaluation (NITE)) (NBRC107726) was cultured in Trypto-Sawyer Broth Medium (manufactured by Nissui Pharmaceutical Co., Ltd.) at 37°C for 4 days, and the supernatant was then recovered. The obtained supernatant was dialyzed with 20 mM phosphate buffer (pH 6) according to an ordinary method. The dialyzed sample was subjected to ion exchange chromatography using a cation exchange column (SP Sepharose FF, manufactured by GE HealthCare) equilibrated with the same buffer as described above. After desalting and concentrating the active fraction, the resultant was further subjected to gel filtration using Superdex 200 HR to obtain the present enzyme sample.

[0059]

The amino acid sequences and nucleotide sequences of the present enzyme are shown below.

Amino acid sequence (only mature body sequence) (SEQ ID NO: 1)

ATSLPPIAPPLPRGVQSKSWSVPDYIAAWEKQHGRPMTAEERYHLARGCIGVTVVNLDRE
DAPNPPLNLSFGTYQRAMEVQAALNEIVATRPSPREYAEQVRKHPALQGVQNLVRAFPT
FIDPANLHAAIFSKRFYSKQDPNWTDEQAAEMYRPNPRTGQVDMSTYRYRARPGYVNFD
YGWYDEQTNNWWHANHAEPGMKVYQSTLRYYSRPLLDFDEQVFTVAFARVA

[0060]

Amino acid sequence (full length) * The underlined part indicates the mature body sequence. (SEQ ID NO: 2)

MKKWLPRALVALFVLLGLPAGLAGTAHAAVVAHAAVATSLPPIAPPLPRGVQSKSWSVP
DYIAAWEKQHGRPMTAEERYHLARGCIGVTVVNLDREDAPNPPLNLSFGTYQRAMEVQ
AALNEIVATRPSPREYAEQVRKHPALQGVQNLVRAFPTFIDPANLHAAIFSKRFYSKQDPN
WTDEQAAEMYRPNPRTGQVDMSTYRYRARPGYVNFDYGWYDEQTNNWWHANHAEP
GMKVYQSTLRYYSRPLLDFDEQVFTVAFARVA

[0061]

Nucleotide sequence (mature body) (SEQ ID NO: 3)

gccacgtcgctgccaccgatcgcaccgccgcttccccggggcgtgcagagcaagagctggtcggtgccggactacattgccgcctgggaga
agcaacacggtaggccgatgacggccgaggagcggtaccacctcgcccggggctgcatcggcgtcaccgtggtcaacctcgaccgggagg

acgcgccgaacccgccgctcaacctgtcgttcggcacttaccagcgggccatggaagtgcaggccgcgctgaacgagatcgtcgcgacccg
gccgtcgccgcgggagtacgccgagcaggtgcgcaagcacccggcgctgcagggcgtgcagaacctggtgcgggcgttccccacgttcat
cgacccggcgaacctgcacgccgccatcttctccaagcggttctactcgaagcaggacccgaactggaccgacgagcaggcggccgagatg
taccggccgaatccgcggaccggccaggtcgacatgagcacctaccgctaccgggcgcggccgggttacgtgaacttcgactacggttggta
cgacgagcagacgaacaactggtggcacgccaaccacgcggagccgggcatgaaggtctaccagagcacgttgcggtactactcccggcc
gctgctggatttcgacgagcaggttttcaccgtggcgttcgcgcgggtcgcctga

[0062]

Nucleotide sequence (full length) (SEQ ID NO: 4)

gtgaagaagtggttgccgcgcgccctggtcgcgctgttcgtgctcctggggttacccgccggcctggccggaacggcacatgccgcggtcgtc
gcgcacgccgccgtcgccacgtcgctgccaccgatcgcaccgccgcttccccggggcgtgcagagcaagagctggtcggtgccggactaca
ttgccgcctgggagaagcaacacggtaggccgatgacggccgaggagcggtaccacctcgcccggggctgcatcggcgtcaccgtggtcaa
cctcgaccgggaggacgcgccgaacccgccgctcaacctgtcgttcggcacttaccagcgggccatggaagtgcaggccgcgctgaacgag
atcgtcgcgacccggccgtcgccgcgggagtacgccgagcaggtgcgcaagcacccggcgctgcagggcgtgcagaacctggtgcgggc
gttccccacgttcatcgacccggcgaacctgcacgccgccatcttctccaagcggttctactcgaagcaggacccgaactggaccgacgagca
ggcggccgagatgtaccggccgaatccgcggaccggccaggtcgacatgagcacctaccgctaccgggcgcggccgggttacgtgaacttc
gactacggttggtacgacgagcagacgaacaactggtggcacgccaaccacgcggagccgggcatgaaggtctaccagagcacgttgcggt
actactcccggccgctgctggatttcgacgagcaggttttcaccgtggcgttcgcgcgggtcgcctga

[0063]

Example 2: Confirmation of protein deamidation activity

The protein deamidation activity of the present enzyme sample was confirmed by generation of a deamidated insulin B chain, using an insulin B chain (a peptide containing glutamine residues and lysine residues) as a substrate.

(Method)

5 mg of Insulin B chain (insulin B chain, oxidized form, derived from bovine pancreas, Sigma-Aldrich, sequence: Phe-Val-Asn-Gln-His-Leu-Cys(SO_3H)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys(SO_3H)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala (SEQ ID NO: 5)) was dissolved in 2 mL of ultrapure water to prepare a substrate solution. Thereafter, 10 μL or 20 μL of a 1 mg/mL purified enzyme solution and a 0.2 M potassium dihydrogen phosphate/disodium hydrogen phosphate buffer (pH 6.5) were added to 90 μL of the substrate solution to a total volume

of 180 μL, so as to prepare a reaction solution. The reaction solution was heated to 40°C in an incubator and was then left at rest for 1 hour. Thereafter, 20 μL of 1 N HCl was added to the reaction solution to stop the reaction. The described reaction stop solution was analyzed using HPLC. Deamidation activity was detected by the peak transition from the insulin B chain to the deamidated insulin B chain. As a comparative example, deamidation activity was similarly detected using Streptomyces-derived transglutaminase (manufactured by Amano Enzyme Inc.).

(Evaluation of deamidation)

The amount of the deamidated insulin B chain produced was calculated from the peak areas of the insulin B chain and the deamidated insulin B chain that were calculated from the chromatogram, and was then evaluated using the deamidation percentage (amount of deamidated insulin B chain produced ÷ amount of substrate insulin B chain x 100).

[0064]

(Results)

The results are shown in Table 1.

[Table 1]

	Comparative example		Example	
Enzyme used	Streptomyces-derived transglutaminase		Longimycelium-derived protein deamidase	
Amount of enzyme added (μL)	10	20	10	20
Deamidation percentage (%)	3.3	3.4	10.8	18.9

[0065]

(Consideration)

In the results shown in Table 1, the transglutaminase of the comparative examples generated only a small amount of deamidated insulin B chain, and the amount of the deamidated insulin B chain generated did not change even when the amount of the enzyme added was increased. From these results, it could be confirmed that the transglutaminase does not catalyze the deamidation reaction for peptides containing glutamine residues and lysine residues. In contrast, the protein deamidase of the present invention generated a deamidated insulin B chain, and the amount of the deamidated insulin B chain generated increased by increasing the amount of the enzyme added, indicating that the present protein deamidase catalyzes the deamidation reaction.

[0066]

Example 3: Preparation of protein deamidase derived from *Crossiella cryophila* and protein deamidase derived from *Krasilnikovia cinnamomea*

1. Production of transformants

A sequence of interest was incorporated using an expression vector for microorganisms of Streptomyces sp. Amplification was performed by PCR, and transformants (a heterologous expression strain of *C. cryophila* -derived protein deamidase and a heterologous expression strain of *K. cinnamomea*-derived protein deamidase) were obtained using an ordinary method. Sequence analysis of the obtained transformants was performed to confirm whether the sequence of interest had been incorporated.

[0067]

2. *C. cryophila*-derived protein deamidase heterologous expression strain, and *K. cinnamomea*-derived protein deamidase heterologous expression strain

The obtained transformants were each cultured in Trypto-Sawyer Broth Medium (manufactured by Nissui Pharmaceutical Co., Ltd.) at 32°C for 4 days, and the supernatant was then recovered. The obtained supernatant was dialyzed with 20 mM phosphate buffer (pH 6) according to an ordinary method. The dialyzed sample was subjected to ion exchange chromatography using a cation exchange column (SP Sepharose FF, manufactured by GE HealthCare) equilibrated with the same buffer as described above. Thereafter, the resulting sample was further purified using a His tag protein purification column (His trap HP, manufactured by Cytiva) to obtain each enzyme sample.

[0068]

The amino acid sequences and nucleotide sequences of a protein deamidase derived from *C. cryophila* are shown below.

Amino acid sequence (only mature body sequence) (SEQ ID NO: 6)

GLPIAPPLRPGVESKTWKVADYIKAWETYHGRPMTADERENLARGCIGVTVVNLERGDV
GNPPLGLSFGTFGKAREVQAALNEIIKSKPSAAQYAAAVRSHPLLSKLENVQRALPADLN
TGELTAAIFSKRFYSKQNPNWTDEFAEKMYRANARGQVDMNSYRYVARPGYVNFDYG
WYDENTRNWWHANHAEPGMKVYQSTFDYYSRDLLDFDRQVFTVAFAKKV

[0069]

Amino acid sequence (full length) * The underlined part indicates the mature body sequence. (SEQ ID NO: 7)

MRNSVLIRTLTTAALGLGLLIPGAAAATAAPAPSTAAAVSIVGLPIAPPLRPGVESKTWKV
ADYIKAWETYHGRPMTADERENLARGCIGVTVVNLERGDVGNPPLGLSFGTFGKAREV
QAALNEIIKSKPSAAQYAAAVRSHPLLSKLENVQRALPADLNTGELTAAIFSKRFYSKQNP
NWTDEFAEKMYRANARGQVDMNSYRYVARPGYVNFDYGWYDENTRNWWHANHAEP
GMKVYQSTFDYYSRDLLDFDRQVFTVAFAKKV

[0070]

Nucleotide sequence (mature body) (SEQ ID NO: 8)

ggcctccccatcgccccccgctgcgcccgggcgtcgagtcgaagacgtggaaggtcgcggactacatcaaggcgtgggagacgtaccatg
gccgcccgatgaccgcggacgaacgggagaacctcgcgcggggctgcatcggggtcaccgtggtcaacctcgagcggggcgacgtgggg
aatccgccgctcggcctctcgttcggcacgttcggcaaagcgcgggaggtccaagcggcgctcaacgagatcatcaagtcgaagccgtcggc
ggcgcagtacgcggccgcggtccgctcgcacccccctgctctcgaagctcgagaacgtgcagcgggccctcccggcggatctcaacacgggc
gagctcacggcggcgatcttctcgaagcggttctactcgaagcagaacccgaactggacggacgagttcgcggagaagatgtaccgggcgaa
cgcgcggggccaagtcgacatgaactcgtaccggtacgtcgcgcggccgggctacgtcaacttcgactacggctggtacgacgagaacacg
cggaactggtggcacgcgaatcacgcggagccgggcatgaaggtctatcagtcgacgttcgactactactcgcgggatctcctcgacttcgacc
ggcaagtcttcacggtcgcgttcgcgaagaaggtc

[0071]

Nucleotide sequence (full length) (SEQ ID NO: 9)

atgcggaactccgtgctcatccgcacgctgaccaccgccgccctcggcctgggcctcctgatccccggggcggcggccgcgacggcggccc
cggcgccctccaccgcggcggccgtctccattgtcggcctccccatcgcccccccgctgcgcccgggcgtcgagtcgaagacgtggaaggtc
gcggactacatcaaggcgtgggagacgtaccatggccgcccgatgaccgcggacgaacgggagaacctcgcgcggggctgcatcggggtc
accgtggtcaacctcgagcggggcgacgtggggaatccgccgctcggcctctcgttcggcacgttcggcaaagcgcgggaggtccaagcgg
cgctcaacgagatcatcaagtcgaagccgtcggcggcgcagtacgcggccgcggtccgctcgcacccccctgctctcgaagctcgagaacgtg
cagcgggccctcccggcggatctcaacacgggcgagctcacggcggcgatcttctcgaagcggttctactcgaagcagaacccgaactggac
ggacgagttcgcggagaagatgtaccgggcgaacgcgcggggccaagtcgacatgaactcgtaccggtacgtcgcgcggccgggctacgt
caacttcgactacggctggtacgacgagaacacgcggaactggtggcacgcgaatcacgcggagccgggcatgaaggtctatcagtcgacgt
tcgactactactcgcgggatctcctcgacttcgaccggcaagtcttcacggtcgcgttcgcgaagaaggtc

[0072]

The amino acid sequences and nucleotide sequences of a protein deamidase derived from *K. cinnamomea* are shown below.

Amino acid sequence (only mature body sequence) (SEQ ID NO: 10)

TESDPRSPTPARTATQAPIAEVRPAERSLANANAATATELTPLAPALPPGVSTRTWSVEDF
VELFERKYGRPMTQDERNALARGCIGVTTVNLERGNINPPLGMSFGTFATSRDVQNAIND
ILATNPSRTQFVAAVAQHPLLSRIDNVTDSLPGGPTSQWTAVIFSKRFYSKQDPSWTDEQA
DQAFRPDPATGQVDMTDYRYRAKPGYVNFDYGWLDEGSGNWWHANHAEPGMKVYQ
STLRHYSRPLLDFDRQVFSVTFGRVHP

[0073]

Amino acid sequence (full length) * The underlined part indicates the mature body sequence. (SEQ ID NO: 11)

MPKRLLRLFVIFVAGLSISTMVAAPALA<u>TESDPRSPTPARTATQAPIAEVRPAERSLANAN
AATATELTPLAPALPPGVSTRTWSVEDFVELFERKYGRPMTQDERNALARGCIGVTTVNL
ERGNINPPLGMSFGTFATSRDVQNAINDILATNPSRTQFVAAVAQHPLLSRIDNVTDSLPG
GPTSQWTAVIFSKRFYSKQDPSWTDEQADQAFRPDPATGQVDMTDYRYRAKPGYVNFD
YGWLDEGSGNWWHANHAEPGMKVYQSTLRHYSRPLLDFDRQVFSVTFGRVHP</u>

[0074]

Nucleotide sequence (mature body) (SEQ ID NO: 12)

accgaatccgacccgcggtcccccaccccgcgcgcacggccacgcaagcgccgatcgcggaagtgcggccggcggaacgctcgctggc
gaacgccaatgccgccaccgccaccgaactgacccccctcgcccccgccctccccccgggggtgtcgacgcggacgtggtcggtcgagga
cttcgtcgagctcttcgagcggaagtacgggcggccgatgacccaagacgaacgcaatgccctcgcgcggggctgcatcggcgtcacgacg
gtcaacctcgagcggggcaacatcaacccgccgctcggcatgtcgttcggcacgttcgcgacgtcgcgggacgtgcagaacgcgatcaacga
catcctcgcgaccaatccctcccggacccaatttgtggcggccgtggcgcagcacccgctcctctcgcggatcgacaacgtcacggactcgctc
ccgggcggcccgacgtcgcagtggacggcggtcatcttctcgaagcggttctactcgaagcaagacccgtcgtggacggacgagcaagcgg
atcaagcgttccgccccgatccggcgacgggccaagtcgacatgacggactaccggtaccgggcgaagccgggctacgtcaacttcgactac
ggctggctcgacgagggctcgggcaactggtggcacgcgaaccacgcggagccggggatgaaggtgtatcagtcgacgctccggcactact
cgcggccgctcctcgacttcgaccggcaagtcttctcggtcacgttcggccgggtccacccg

[0075]

Nucleotide sequence (full length) (SEQ ID NO: 13)

atgccgaagcggctcctccggctcttcgtcatcttcgtcgcgggcctctcgatttcgaccatggtggccgccccggccctcgcgaccgaatccga
cccgcggtcccccaccccgcgcgcacggccacgcaagcgccgatcgcggaagtgcggccggcggaacgctcgctggcgaacgccaatg
ccgccaccgccaccgaactgacccccctcgcccccgcccctcccccgggggtgtcgacgcggacgtggtcggtcgaggacttcgtcgagct
cttcgagcggaagtacgggcggccgatgacccaagacgaacgcaatgccctcgcgcggggctgcatcggcgtcacgacggtcaacctcga
gcggggcaacatcaacccgccgctcggcatgtcgttcggcacgttcgcgacgtcgcgggacgtgcagaacgcgatcaacgacatcctcgcga
ccaatccctcccggacccaatttgtggcggccgtggcgcagcacccgctcctctcgcggatcgacaacgtcacggactcgctcccgggcggc
ccgacgtcgcagtggacggcggtcatcttctcgaagcggttctactcgaagcaagacccgtcgtggacggacgagcaagcggatcaagcgttc
cgccccgatccggcgacgggccaagtcgacatgacggactaccggtaccgggcgaagccgggctacgtcaacttcgactacggctggctcg
acgagggctcgggcaactggtggcacgcgaaccacgcggagccggggatgaaggtgtatcagtcgacgctccggcactactcgcggccgc
tcctcgacttcgaccggcaagtcttctcggtcacgttcggccgggtccacccg

[0076]

Example 4: Confirmation of protein deamidation activity

(Method)

5 mg of Insulin B chain (insulin B chain, oxidized form, derived from bovine pancreas, Sigma-Aldrich, sequence: Phe-Val-Asn-Gln-His-Leu-Cys(SO_3H)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys(SO_3H)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala (SEQ ID NO: 5)) was dissolved in 2 mL of ultrapure water to prepare a substrate solution. Thereafter, 10 μL of a 0.2 mg/mL each purified enzyme solution, 25 μL of a 0.1 M sodium phosphate buffer (pH 6.5), and 0.9 μL of protease inhibitor (ProteoGuard EDTA-Free Protease Inhibitor Cocktail (TaKaRa)) were added to 22.5 μL of the substrate solution to a total volume of 58.4 μL, so as to prepare a reaction solution. The reaction solution was heated to 40°C in an incubator and was then left at rest overnight. Thereafter, the reaction solution was heated at 100°C for 10 minutes to stop the reaction. The described reaction stop solution was analyzed using HPLC. Deamidation activity was detected by the peak transition from the insulin B chain to the deamidated insulin B chain.

(Evaluation of deamidation)

The amount of the deamidated insulin B chain produced was calculated from the peak areas of the insulin B chain and the deamidated insulin B chain that were calculated from the chromatogram, and the deamidation activity was then evaluated using the deamidation percentage (amount of deamidated insulin B chain produced ÷ amount of substrate insulin B chain x 100).

[0077]

(Results)

The results are shown in Table 2.

[Table 2]

	Example 2	Example 3
	K. cinnamomea-derived protein deamidase	*C. cryophila* -derived protein deamidase
Protein deamidation percentage (%)	20	44

(Consideration)

In the results shown in Table 2, since the *K. cinnamomea*-derived protein deamidase and *C. cryophila*-derived protein deamidase of the present invention generated deamidated insulin B chains, these protein deamidases were demonstrated to catalyze deamidation reactions.

[0078]

Example 5: Confirmation of protein deamidation activity

Preparation of protein deamidase derived from *Longispora fulva*

A cell mass of *Longispora fulva* was cultured in Trypto-Sawyer Broth Medium (manufactured by Nissui Pharmaceutical Co., Ltd.) at 28°C for 6 days, and the supernatant was then recovered. The obtained supernatant was dialyzed with 20 mM phosphate buffer (pH 6) according to an ordinary method. The dialyzed sample was subjected to ion exchange chromatography using a cation exchange column (SP Sepharose FF, manufactured by GE HealthCare) equilibrated with the same buffer as described above. The active fraction was desalted and concentrated to obtain a crude purified enzyme sample.

[0079]

The amino acid sequences and nucleotide sequences of a protein deamidase derived from *Longispora fulva* are shown below.

Amino acid sequence (only mature body sequence) (SEQ ID NO: 14)

APPTSADRSGTPVAGLRVPSSLSAVRGAARFDAVAPEATPLPVGQKTRTWTIARYAAAW
RTANGRAMTDRERDALARGCIGVTTVNIERGNINPPLGLSFGTFEKATAVRDALDAVLA

GAAGRTDYAARVGADPLLSTLDNVTESLPAGDPDQWAAVVFSKRFHSGQDSTWTAAET
DRAFRPDARGQVDMSAYHYRAKPGYVNFDYGWYDPATGSWWHANHAEPGMEIYQST
LKYYSRPLLDFDRQVYSVTFARKAAPAWRSSTN

[0080]

Amino acid sequence (full length) * The underlined part indicates the mature body sequence. (SEQ ID NO: 15)

MRNRLLRITMAAVLGVGATVGLAATTGYAAPPTSADRSGTPVAGLRVPSSLSAVRGAAR
FDAVAPEATPLPVGQKTRTWTIARYAAAWRTANGRAMTDRERDALARGCIGVTTVNIE
RGNINPPLGLSFGTFEKATAVRDALDAVLAGAAGRTDYAARVGADPLLSTLDNVTESLP
AGDPDQWAAVVFSKRFHSGQDSTWTAAETDRAFRPDARGQVDMSAYHYRAKPGYVNF
DYGWYDPATGSWWHANHAEPGMEIYQSTLKYYSRPLLDFDRQVYSVTFARKAAPAWR
SSTN

[0081]

Nucleotide sequence (mature body) (SEQ ID NO: 16)

gccccgccgacgtccgccgaccgctcggggacccccgtggcggggctgcgggtcccgtcgtccctctccgccgtgcgcggcgccgcccgg
tttgacgccgtggcgccggaagccaccccgctcccggtcgggcaaaagacccgcacgtggacgattgcccgctacgccgcggcctggcgg
accgcgaacggccgcgccatgaccgatcgggagcgcgatgccctcgcccggggctgcatcggcgtcacgacggtcaacatcgagcgggg
caacatcaacccgccgctcggcctctccttcggcacgtttgaaaaagcgaccgcggtccgcgacgcgctggacgccgtcctcgccggggccg
cgggccgcaccgactacgccgcgcgcgtcggcgcggatccgctcctctccacgctcgataatgtcacggaatcgctcccggcgggggaccc
ggatcagtgggcggcggtcgtcttctcgaagcgctttcattccgggcaagattccacctggaccgccgcggagaccgaccgggcgtttcggcc
cgatgcgcgcggccaagtcgacatgtcggcgtaccactaccgggcgaagccgggctacgtcaacttcgactacggctggtacgacccggcg
acgggctcgtggtggcacgcgaaccacgcggagccgggcatggagatctatcagtcgacgctcaagtactactcgcggccgctcctcgacttc
gaccggcaagtctactcggtcacgttcgcgcggaaggcggcgccggcgtggcggtcgtcgacgaac

[0082]

Nucleotide sequence (full length) (SEQ ID NO: 17)

atgcggaaccgcctgctccggatcacgatggccgcggtcctgggggtgggcgccaccgtggggctcgccgcgaccacgggctatgcggcc
ccgccgacgtccgccgaccgctcggggacccccgtggcggggctgcgggtcccgtcgtccctctccgccgtgcgcggcgccgcccggtttg
acgccgtggcgccggaagccaccccgctcccggtcgggcaaaagacccgcacgtggacgattgcccgctacgccgcggcctggcggacc
gcgaacggccgcgccatgaccgatcgggagcgcgatgccctcgcccggggctgcatcggcgtcacgacggtcaacatcgagcggggcaa

catcaacccgccgctcggcctctccttcggcacgtttgaaaaagcgaccgcggtccgcgacgcgctggacgccgtcctcgccggggccgcgg
gccgcaccgactacgccgcgcgcgtcggcgcggatccgctcctctccacgctcgataatgtcacggaatcgctcccggcgggggacccggat
cagtgggcggcggtcgtcttctcgaagcgctttcattccgggcaagattccacctggaccgccgcggagaccgaccgggcgtttcggcccgat
gcgcgcggccaagtcgacatgtcggcgtaccactaccgggcgaagccgggctacgtcaacttcgactacggctggtacgacccggcgacgg
gctcgtggtggcacgcgaaccacgcggagccgggcatggagatctatcagtcgacgctcaagtactactcgcggccgctcctcgacttcgacc
ggcaagtctactcggtcacgttcgcgcggaaggcggcgccggcgtggcggtcgtcgacgaac

[0083]

Example 6: Evaluation of protein deamidation activity to protein substrate

(Method)

5 mg of Insulin B chain (insulin B chain, oxidized form, derived from bovine pancreas, Sigma-Aldrich, sequence: Phe-Val-Asn-Gln-His-Leu-Cys(SO_3H)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys(SO_3H)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala (SEQ ID NO: 5)) was dissolved in 2 mL of ultrapure water to prepare a substrate solution. Thereafter, 40 μL of a 1 mg/mL purified enzyme solution and a 0.1 M potassium dihydrogen phosphate/disodium hydrogen phosphate buffer (pH 6.5) were added to 90 μL of the substrate solution to a total volume of 180 μL, so as to prepare a reaction solution. The reaction solution was heated to 40°C in an incubator and was then left at rest for 5 hours. Thereafter, the reaction solution was heated at 100°C for 10 minutes to stop the reaction. The reaction stop solution was analyzed using HPLC. Deamidation activity was detected by the peak transition from the insulin B chain to the deamidated insulin B chain.

[0084]

(Results)

As a result of the analysis using HPLC, a peak based on the deamidation reaction was confirmed in the case of using the *Longispora fulva*-derived protein deamidase.

Industrial Applicability

[0085]

The enzyme agent of the present invention contains a protein deamidase as an active ingredient. Therefore, the present enzyme agent is suitable to be used in the fields of food products

and medical applications, and is industrially highly useful.

The present invention is not limited in any way to the above-described embodiments and examples of the present invention. Various modifications are also included in the present invention, in such a range that those skilled in the art can readily conceive of them, and that the modifications are not deviated from the scope of the claims. The contents of the study papers, laid-open patent publications, patent publications, etc., which are explicitly mentioned in the present description, are cited herein by reference in their entirety.

CLAIMS

[Claim 1]

An enzyme agent for protein deamidation, containing, as an active ingredient, a protein deamidase consisting of an amino acid sequence having a sequence identity of 90% or more to the amino acid sequence as set forth in SEQ ID NO: 1.

[Claim 2]

The enzyme agent for protein deamidation according to claim 1, in which the protein deamidase is derived from Longimycelium sp.

[Claim 3]

A method for deamidating a protein or a peptide, including

allowing the enzyme agent for protein deamidation according to claim 1 or 2 to act on the protein or the peptide.

[Claim 4]

A method for deamidating protein-containing food product and beverage, including

allowing the enzyme agent for protein deamidation according to claim 1 or 2 to act on the protein-containing food product and beverage.

[Claim 5]

A method for producing food product and beverage, including

allowing the enzyme agent for protein deamidation according to claim 1 or 2 to act on protein-containing food product and beverage.

[Claim 6]

Food product and beverage, treated with the enzyme agent for protein deamidation according to claim 1 or 2.

[Claim 7]

A method for producing a protein deamidase consisting of an amino acid sequence having a sequence identity of 90% or more to the amino acid sequence as set forth in SEQ ID NO: 1, including

culturing bacteria of Longimycelium sp.

[Claim 8]

An enzyme agent for protein deamidation, containing, as an active ingredient, a protein deamidase consisting of an amino acid sequence having a sequence identity of 60% or more to the amino acid sequence as set forth in SEQ ID NO: 1.

[Claim 9]

The enzyme agent for protein deamidation according to claim 8, in which the protein deamidase is derived from Longimycelium sp., Crossiella sp., or Krasilnikovia sp.

[Claim 10]

A method for deamidating a protein or a peptide, including

allowing the enzyme agent for protein deamidation according to claim 8 or 9 to act on the protein or the peptide.

[Claim 11]

A method for deamidating protein-containing food product and beverage, including

allowing the enzyme agent for protein deamidation according to claim 8 or 9 to act on the protein-containing food product and beverage.

[Claim 12]

A method for producing food product and beverage, including

allowing the enzyme agent for protein deamidation according to claim 8 or 9 to act on protein-containing food product and beverage.

[Claim 13]

Food product and beverage, treated with the enzyme agent for protein deamidation according to claim 8 or 9.

[Claim 14]

A method for producing a protein deamidase consisting of an amino acid sequence having a sequence identity of 60% or more to the amino acid sequence as set forth in SEQ ID NO: 1, including

culturing bacteria of Longimycelium sp., the bacteria of Crossiella sp., or the bacteria of Krasilnikovia sp.

[Claim 15]

An enzyme agent for protein deamidation, containing, as an active ingredient, a protein deamidase consisting of an amino acid sequence having a sequence identity of 50% or more to the amino acid sequence as set forth in SEQ ID NO: 1.

[Claim 16]

The enzyme agent for protein deamidation according to claim 15, in which the protein deamidase is derived from Longimycelium sp., Crossiella sp., Krasilnikovia sp., or Longispora sp.

[Claim 17]

A method for deamidating a protein or a peptide, including

allowing the enzyme agent for protein deamidation according to claim 15 or 16 to act on the protein or the peptide.

[Claim 18]

A method for deamidating protein-containing food product and beverage, including

allowing the enzyme agent for protein deamidation according to claim 15 or 16 to act on the protein-containing food product and beverage.

[Claim 19]

A method for producing food product and beverage, including

allowing the enzyme agent for protein deamidation according to claim 15 or 16 to act on protein-containing food product and beverage.

[Claim 20]

Food product and beverage, treated with the enzyme agent for protein deamidation according to claim 15 or 16.

[Claim 21]

A method for producing a protein deamidase consisting of an amino acid sequence having a sequence identity of 50% or more to the amino acid sequence as set forth in SEQ ID NO: 1, including

culturing bacteria of Longimycelium sp., Crossiella sp., Krasilnikovia sp., or Longispora sp.