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# DESCRIPTION

**[0001]** The present disclosure relates to alkaline protease variants which are useful enzymes incorporated into liquid detergents, and to genes encoding the same.

**[0002]** Proteases have long been employed in industry for a wide variety of products, including detergents (e.g., laundry detergents), fiber-modifying agents, leather treatment agents, cosmetics, bath agents, food-modifying agents, and drugs. Among these, proteases for detergents are industrially produced in the greatest amounts. Examples of such proteases known heretofore include Alcalase®, Savinase® (Novozymes), Maxacal® (Genencor), Blap® (Henkel), and KAP (Kao Corporation).

**[0003]** Protease is incorporated into a laundry detergent for providing the detergent with the ability to degrade dirt mainly composed of protein and deposited on clothing into low-molecular-weight products, to thereby promote solubilization of the thus-degraded products with a surfactant. However, in actuality, such deposited dirt is complex dirt containing, in addition to proteins, a plurality of organic and inorganic components such as sebum-derived lipid and solid particles. Therefore, there is a continuous demand for a detergent exhibiting excellent detergency to such complex dirt.

**[0004]** In view of the foregoing, the present inventors previously discovered several alkaline proteases having a molecular weight of about 43,000, which maintain sufficient casein-degrading activity even in the presence of a fatty acid of high concentration and which exhibit excellent detergency to complex dirt containing proteins and sebum; and previously filed a patent application on the alkaline proteases (Patent Document 1). These alkaline proteases differ from conventionally known subtilisin, a serine protease derived from bacteria belonging to the genus *Bacillus,* in terms of molecular weight, primary structure, and enzymological characteristics, and having a very strong resistance to oxidizer. These alkaline proteases are suggested to be classified into a new subtilisin subfamily (Non-Patent Document 1).

**[0005]** Meanwhile, detergents can be categorized, by form thereof, into powder detergents and liquid detergents. Advantageously, liquid detergents have solubility higher than that of powder detergents, and neat liquid thereof can be directly applied to dirt. Although liquid detergents have such merits while powder detergents do not possess, liquid detergents are widely known to encounter technical difficulty in stable incorporation of an enzyme such as protease, while powder detergents do not encounter. Generally, since liquid detergents are stored at ambient temperature, the enzyme (protein) is readily denatured. In addition, liquid detergents contain a surfactant, fatty acid, solvent, etc., and the pH thereof falls within a weak alkaline range. Such conditions are very severe conditions for the enzyme. Furthermore, the protease, which is a proteolytic enzyme, undergoes problematic self-digestion, further reducing storage stability of the enzyme in liquid detergents.

**[0006]** In order to solve the aforementioned technical problems, there have been widely known

addition of an enzyme-stabilizing agent such as calcium ion, borax, boric acid, a boron compound, a carboxylic acid (e.g., formic acid), or a polyol. Some studies have been carried out to cope with the problem of self-digestion based on inhibition of protease activity. Specifically, there have been reported methods for stabilizing protease through reversible inhibition of protease activity by use of 4-substituted phenylboronic acid (Patent Document 2) or a certain peptide-aldehyde and a boron composition (Patent Document 3). Also reported is that dextran-modified protease enhances stability of protease in aqueous solution containing a surfactant (Non-Patent Document 2) .

**[0007]** However, the protease-stabilizing effect due to addition of an enzyme-stabilizing agent (e.g., calcium ion or boric acid) is insufficient, and the inhibitory effect varies depending on the type of protease. Furthermore, use of such agents increases production cost. Thus, these countermeasures are not thought to be best solutions for the problems involved in liquid detergents. Chemical modification of the enzyme also has problems in terms of production cost.

**[0008]** Generally, a surfactant, an alkaline agent, an anti-redeposition agent, solvent, perfume, a fluorescent dye, etc, are added to liquid detergents. Among these additives, a surfactant most severely impairs the stability of enzymes. Typically, an anionic surfactant and a nonionic surfactant are used in combination. Although a nonionic surfactant does not greatly damage enzymes, an anionic surfactant is thought to greatly damage enzymes, since the anionic surfactant enters the enzyme via its hydrophobic moiety and breaks hydrophobic interaction of the enzyme as well as traps divalent metal ions (e.g., calcium ions) which stabilize the enzyme (Non-Patent Document 3). Thus, enhancement of resistance of the enzyme to anionic surfactants is a very important factor for enhancing the stability of the enzyme in liquid detergents.

**[0009]** Patent Document 7 discloses JP170 like subtilases from wild-type bacteria, hybrids thereof and methods of construction and production of these proteases. Patent Document 7 also discloses to use these subtilases in detergents, such as a laundry detergent or an automatic dishwashing detergent. In an alkaline protease derived from KP43 [*Bacillus sp.* KSM-KP43 (FERM BP-6532)], the specific activity to the activity of the parent alkaline protease is known to be enhanced through substitution of the amino acid residue at the position 15 of the amino acid sequence with a histidine residue; substitution of the amino acid residue at the position 16 of the amino acid sequence with a threonine or glutamine residue (Patent Document 4); substitution of the amino acid residue at the position 65 of the amino acid sequence with a proline residue (Patent Document 5); or substitution of the amino acid residue at the position 66 of the amino acid sequence with an aspartic acid residue (Patent Document 6). However, there has never been known an alkaline protease variant which enhances the stability of an alkaline protease derived from KP43 in liquid detergents without reducing the specific activity.

Patent Document 1: WO 99/18218 pamphlet

Patent Document 2: JP-A-H11-507680

Patent Document 3: JP-A-2000-506933

Patent Document 4: JP-A-2004-305176

Patent Document 5: JP-A-2004-000122

Patent Document 6: JP-A-2002-218989

Patent Document 7: WO 2006/032278 pamphlet

Non-Patent Document 1: Saeki et al., Biochem. Biophys. Res. Commun., 279, 313-319, 2000

Non-Patent Document 2: Cosmetics & Toiletries magazine, 111, p. 79-88, 1996

Non-Patent Document 3: Detergent Enzyme: A Challenge! In Handbook of Detergents part A, New York, p. 639-690, 1999

**[0010]** The present invention relates to the embodiments as defined in the claims.

**[0011]** One aspect of the invention relates to an alkaline protease variant, wherein said alkaline protease variant

1. (i) consists of the amino acid sequence of SEQ ID NO: 2, wherein the amino acid residue at position 83 of the amino acid sequence of SEQ ID NO: 2 is substituted with the following amino acid residues: alanine, serine, or cysteine, or
2. (ii) has an identity of 90% or more with SEQ ID NO: 2, wherein the amino acid residue at the position corresponding to position 83 of the amino acid sequence of SEQ ID NO: 2 is substituted with the following amino acid residues: alanine, serine, or cysteine, and wherein the alkaline protease variant exhibits activity within an alkaline pH range.

**[0012]** The present invention also relates to a detergent composition comprising this alkaline protease variant; as well as a gene encoding this alkaline protease variant, a recombinant vector comprising said gene, and a non-human transformant comprising said recombinant vector. The invention also provides a method for producing the alkaline protease variant of the invention, which method comprises culturing this transformant.

**[0013]** Also comprised by the present invention is a method for enhancing the stability of an alkaline protease in a liquid detergent, the method comprises

1. (i) in an alkaline protease consisting of the amino acid sequence of SEQ ID NO: 2 substituting the amino acid residue at position 83 of the amino acid sequence of SEQ ID NO: 2 with the following amino acid residues: alanine, serine, or cysteine; or
2. (ii) in an alkaline protease having an identity of 90% or more with SEQ ID NO: 2,

substituting the amino acid residue at the position corresponding to position 83 of the amino acid sequence of SEQ ID NO: 2 with the following amino acid residues: alanine, serine, or cysteine, wherein the produced alkaline protease variant exhibits activity within an alkaline pH range.

**[0014]** The present disclosure is directed to an alkaline protease variant derived from an alkaline protease consisting of an amino acid sequence represented by SEQ ID NO: 2 or consisting of an amino acid sequence having an identity of 90% or more therewith, wherein one or more amino acid residues at positions selected from (a) position 6, (b) position 15, (c) position 16, (d) position 65, (e) position 66, (f) position 82, (g) position 83, (h) position 204, (i) position 319, and (j) position 337 of the amino acid sequence represented by SEQ ID NO: 2, or at positions corresponding thereto are substituted with the following amino acid residues:

1. (a) or a position corresponding thereto: tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine;
2. (b) or a position corresponding thereto: glutamic acid, methionine, aspartic acid, valine, glutamine, arginine, cysteine, tryptophan, alanine, or phenylalanine;
3. (c) or a position corresponding thereto: methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine;
4. (d) or a position corresponding thereto: tryptophan;
5. (e) or a position corresponding thereto: histidine, tryptophan, serine, or leucine;
6. (f) or a position corresponding thereto: alanine, glutamic acid, glutamine, serine, cysteine, glycine, histidine, lysine, arginine, methionine, or asparagine;
7. (g) or a position corresponding thereto: alanine, serine, or cysteine;
8. (h) or a position corresponding thereto: glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine or, arginine;
9. (i) or a position corresponding thereto: tryptophan, valine, threonine, leucine, isoleucine, cysteine, glutamic acid, lysine, tyrosine, arginine, phenylalanine, glutamine, methionine, proline, aspartic acid, asparagine, histidine, or serine; and
10. (j) or a position corresponding thereto: arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine.

**[0015]** The present disclosure is also directed to a gene encoding the alkaline protease variant.

**[0016]** The present disclosure is also directed to a recombinant vector including the gene.

**[0017]** The present disclosure is also directed to a transformant inclusding the recombinant vector.

**[0018]** The present disclosure is also directed to a detergent composition including the alkaline protease variant.

**[0019]** The present disclosure is also directed to a method for enhancing the stability of an alkaline protease in a liquid detergent, the method including, in an alkaline protease consisting of an amino acid sequence represented by SEQ ID NO: 2 or consisting of an amino acid sequence having an identity of 90% or more therewith, substituting one or more amino acid residues at positions selected from (a) position 6, (b) position 15, (c) position 16, (d) position 65, (e) position 66, (f) position 82, (g) position 83, (h) position 204, (i) position 319, and (j) position 337 of the amino acid sequence represented by SEQ ID NO: 2, or at positions corresponding thereto with the following amino acid residues:

1. (a) or a position corresponding thereto: tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine;
2. (b) or a position corresponding thereto: glutamic acid, methionine, aspartic acid, valine, glutamine, arginine, cysteine, tryptophan, alanine, or phenylalanine;
3. (c) or a position corresponding thereto: methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine;
4. (d) or a position corresponding thereto: tryptophan;
5. (e) or a position corresponding thereto: histidine, tryptophan, serine, or leucine;
6. (f) or a position corresponding thereto: alanine, glutamic acid, glutamine, serine, cysteine, glycine, histidine, lysine, arginine, methionine, or asparagine;
7. (g) or a position corresponding thereto: alanine, serine, or cysteine;
8. (h) or a position corresponding thereto: glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine or, arginine;
9. (i) or a position corresponding thereto: tryptophan, valine, threonine, leucine, isoleucine, cysteine, glutamic acid, lysine, tyrosine, arginine, phenylalanine, glutamine, methionine, proline, aspartic acid, asparagine, histidine, or serine; and
10. (j) or a position corresponding thereto: arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine.

**Brief Description of the Drawings**

**[0020]**

[Fig. 1] A graph showing relative residual activities of variants in which the amino acid residue at the position 6 has been substituted.

[Fig. 2] A graph showing relative residual activities of variants in which the amino acid residue

at the position 15 has been substituted.

[Fig. 3] A graph showing relative residual activities of variants in which the amino acid residue at the position 16 has been substituted.

[Fig. 4] A graph showing relative residual activities of variants in which the amino acid residue at the position 65 has been substituted.

[Fig. 5] A graph showing relative residual activities of variants in which the amino acid residue at the position 66 has been substituted.

[Fig. 6] A graph showing relative residual activities of variants in which the amino acid residue at the position 82 has been substituted.

[Fig. 7] A graph showing relative residual activities of variants in which the amino acid residue at the position 83 has been substituted.

[Fig. 8] A graph showing relative residual activities of variants in which the amino acid residue at the position 204 has been substituted.

[Fig. 9] A graph showing relative residual activities of variants in which the amino acid residue at the position 319 has been substituted.

[Fig. 10] A graph showing relative residual activities of variants in which the amno acid residue at the position 337 has been substituted.

**[0021]** The present disclosure is directed to provision of an alkaline protease variant having an enhanced stability in liquid detergents.

**[0022]** The present inventors have found that, through substituting specific amino acid residue(s) among the amino acid residues characteristic to alkaline protease KP43 having a molecular weight of about 43,000 by other amino acid residues, the stability of the obtained alkaline protease variant in a liquid detergent is enhanced as compared with the parent alkaline protease.

**[0023]** The present disclosure can provide an alkaline protease variant which maintains activity in a liquid detergent containing an anionic surfactant such as LAS, which has high specific activity, and which serves as a useful enzyme for detergents.

**[0024]** The alkaline protease variant of the present disclosure is an alkaline protease variant derived from an alkaline protease consisting of an amino acid sequence represented by SEQ ID NO: 2 or consisting of an amino acid sequence having an identity of 90% or more therewith, wherein one or more amino acid residues at a position selected from (a) position 6, (b) position 15, (c) position 16, (d) position 65, (e) position 66, (f) position 82, (g) position 83, (h) position 204, (i) position 319, and (j) position 337 of the amino acid sequence represented by SEQ ID

NO: 2, or at positions corresponding thereto are substituted with other amino acid residues. The alkaline protease variant of the present disclosure may be a wild-type variant or an artificially created variant.

**[0025]** Examples of the alkaline protease consisting of the amino acid sequence represented by SEQ ID NO: 2 include an alkaline protease derived from KP43 [*Bacillus sp.* KSM-KP43 (FERM BP-6532)] (WO 99/18218 pamphlet).

**[0026]** Examples of the alkaline protease consisting of an amino acid sequence having an identity of 90% or more with the amino acid sequence represented by SEQ ID NO: 2 include those consisting of an amino acid sequence which differs from the amino acid sequence represented by SEQ ID NO: 2 but which has an identity of 90% or more, preferably 95% or more, more preferably 96% or more, even more preferably 97% or more, even more preferably 98% or more, even more preferably 99% or more with an amino acid sequence represented by SEQ ID NO: 2; or those consisting of a amino acid sequence represented by SEQ ID NO: 2, wherein one to several amino acids are deleted, substituted or added.

**[0027]** Preferably, these alkaline proteases have a function equivalent to or higher than that of the alkaline protease consisting of the amino acid sequence represented by SEQ ID NO: 2.

**[0028]** Specific examples of the alkaline protease consisting of an amino acid sequence having an identity of 90% or more with the amino acid sequence represented by SEQ ID NO: 2 include protease KP9860 [protease derived from *Bacillus sp.* KSM-KP9860 (FERM BP-6534), WO 99/18218, GenBank accession no. AB046403] and protease 9865 [protease derived from *Bacillus sp.* KSM-9865 (FERM BP-10139), GenBank accession no. AB084155].

**[0029]** Specific examples of the alkaline protease consisting of an amino acid sequence having an identity of 90% or more with the amino acid sequence represented by SEQ ID NO: 2 also include variants such as a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residues at the positions 66 and 246 have been substituted with aspartic acid and serine, respectively, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 103 has been substituted with arginine, and a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the acid residue at the position 195 has been substituted with alanine, glutamic acid, glutamine, valine, glycine, lysine, threonine, cysteine, proline, serine, arginine, asparagine, or histidine (JP-A-2002-218989); variants such as a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 84 has been substituted with arginine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 104 has been substituted with proline, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 256 has been substituted with alanine or serine, and a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 369 has been substituted with asparagine (JP-A-2002-306176); variants such as a variant consisting of an amino acid sequence represented

by SEQ ID NO: 2 in which the amino acid residue at the position 251 has been substituted with glutamine, valine, isoleucine, or threonine, and a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 256 has been substituted with glutamine, alanine, valine, serine, or asparagine (JP-A-2003-125783); variants such as a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 65 has been substituted with proline, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 273 has been substituted with threonine or isoleucine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 320 has been substituted with phenylalanine or isoleucine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 356 has been substituted with glutamine or serine, and a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 387 has been substituted with lysine, alanine, or glutamine (JP-A-2004-000122); variants such as a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 163 has been substituted with histidine, isoleucine, leucine, threonine, valine, lysine, glutamine, aspartic acid, alanine, or phenylalanine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 170 has been substituted with valine or leucine, and a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 171 has been substituted with alanine, glycine, or threonine (JP-A-2004-057195); variants such as a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 63 has been substituted with serine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 89 has been substituted with histidine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 120 has been substituted with arginine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residues at the positions 63 and 187 have been substituted with serine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 226 has been substituted with tyrosine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 296 has been substituted with valine, and a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 304 has been substituted with serine (JP-A-2004-305175); variants such as a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 15 has been substituted with histidine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 16 has been substituted with threonine or glutamine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 166 has been substituted with glycine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 167 has been substituted with valine, a variant consisting of an amino acid sequence represented by SEQ ID NO: 2 in which the amino acid residue at the position 346 has been substituted with arginine, and a variant consisting of an amino acid sequence represented by

SEQ ID NO: 2 in which the amino acid residue at the position 405 has been substituted with aspartic acid (JP-A-2004-305176); and variants having a plurality of the aforementioned variations.

**[0030]** Among the aforementioned alkaline proteases and protease variants, preferred are those having any of the following enzymatic properties which the alkalie protease consisting of an amino acid sequence represented by SEQ ID NO:2 has:

1. 1) having oxidizer resistance and acting and being stable in an alkaline pH region (≥8). As used herein, the expression "the alkaline protease exhibits oxidizer resistance" refers to the case where, after the alkaline protease is allowed to stand at 20°C for 20 minutes in a 20 mM Britton-Robinson buffer (pH 10) containing hydrogen peroxide (50 mM) and calcium chloride (5 mM), the alkaline protease exhibits at least 50% residual activity (synthetic substrate method);
2. 2) exhibiting at least 80% residual activity after treatment at 50°C and a pH of 10 for 10 minutes;
3. 3) inhibited by diisopropylfluorophosphoric acid (DFP) or phenylmethanesulfonyl fluoride (PMSF); and
4. 4) having a molecular weight of 43,000 ± 2,000 determined by SDS-PAGE.

**[0031]** In the present specification, the identity between amino acid sequences is calculated through the Lipman-Pearson method (Science, 227, 1435, (1985)). Specifically, the identity is calculated through analysis by use of a Search homology program of genetic information processing software Genetyx-Win (Ver. 5.1.1; Software Development'Co., Ltd.), wherein the unit size to compare (ktup) is taken as 2.

**[0032]** In the present specification, the amino acid sequence in which one to several amino acids are deleted, substituted, or added is preferably an amino acid sequence in which one to ten amino acids are deleted, substituted, or added. The addition includes addition of one to several amino acids to both terminuses.

**[0033]** The alkaline protease variant of the present disclosure includes alkaline protease variants each consisting of an amino acid sequence represented by SEQ ID NO: 2 in which (a') the amino acid residue at the position 6 (glycine residue) has been substituted with tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine; (b') the amino acid residue at the position 15 (serine residue) has been substituted with glutamic acid, methionine, aspartic acid, valine, glutamine, arginine, cysteine, tryptophan, alanine, or phenylalanine; (c') the amino acid residue at the position 16 (serine residue) has been substituted with methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine; (d') the amino acid residue at the position 65 (threonine residue) has been substituted with tryptophan; (e') the amino acid residue at the position 66 (asparagine residue) has been substituted with histidine, tryptophan, serine, or leucine; (f') the amino acid residue at the

position 82 (threonine residue) has been substituted with alanine, glutamic acid, glutamine, serine, cysteine, glycine, histidine, lysine, arginine, methionine, or asparagine; (g') the amino acid residue at the position 83 (asparagine residue) has been substituted with alanine, serine, or cysteine; (h') the amino acid residue at the position 204 (glutamine residue) has been substituted with glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine, or arginine; (i') the amino acid residue at the position 319 (alanine residue) has been substituted with tryptophan, valine, threonine, leucine, isoleucine, cysteine, glutamic acid, lysine, tyrosine, arginine, phenylalanine, glutamine, methionine, proline, aspartic acid, asparagine, histidine, or serine; or (j') the amino acid residue at the position 337 (phenylalanine residue) has been substituted with arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine; and those obtained by a combination of two or more substations (a') to (j') mentioned above.

**[0034]** The alkaline protease variant of the present disclosure also includes alkaline protease variants each consisting of an amino acid sequence having an identity of 90% or more with the amino acid sequence represented by SEQ ID NO: 2 in which (a") an amino acid residue at a position corresponding to the position 6 has been substituted with tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine; (b") an amino acid residue at a position corresponding to the position 15 has been substituted with glutamic acid, methionine, aspartic acid, valine, glutamine, arginine, cysteine, tryptophan, alanine, or phenylalanine; (c") an amino acid residue at a position corresponding to the position 16 has been substituted with methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine; (d") an amino acid residue at a position corresponding to the position 65 has been substituted with tryptophan; (e") an amino acid residue at a position corresponding to the position 66 has been substituted with histidine, tryptophan, serine, or leucine; (f") an amino acid residue at a position corresponding to the position 82 has been substituted with alanine, glutamic acid, glutamine, serine, cysteine, glycine, histidine, lysine, arginine, methionine, or asparagine; (g") an amino acid residue at a position corresponding to the position 83 has been substituted with alanine, serine, or cysteine; (h") an amino acid residue at a position corresponding to the position 204 has been substituted with glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine, or arginine; (i") an amino acid residue at a position corresponding to the position 319 has been substituted with tryptophan, valine, threonine, leucine, isoleucine, cysteine, glutamic acid, lysine, tyrosine, arginine, phenylalanine, glutamine, methionine, proline, aspartic acid, asparagine, histidine, or serine; or (j") an amino acid residue at a position corresponding to the position 337 has been substituted with arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine; and those obtained by a combination of two or more substations (a") to (j") mentioned above.

**[0035]** For example, in the alkaline protease variant of the present disclosure, any one or a plurality of the amino acid residues may be simultaneously substituted at the position 6 or a position corresponding thereto, the position 15 or a position corresponding thereto, the position 16 or a position corresponding thereto, the position 65 or a position corresponding thereto, the

position 66 or a position corresponding thereto, the position 82 or a position corresponding thereto, the position 83 or a position corresponding thereto, the position 204 or a position corresponding thereto, the position 319 or a position corresponding thereto, and the position 337 or a position corresponding thereto in the amino acid sequence represented by SEQ ID NO: 2.

**[0036]** Preferably, in the alkaline protease variant of the present disclosure, the amino acid residue at the position 6 or a position corresponding thereto has been substituted with tryptophan, leucine, valine or isoleucine; the amino acid residue at the position 15 or a position corresponding thereto has been substituted with glutamic acid, methionine aspartic acid or valine; the amino acid residue at the position 16 or a position corresponding thereto has been substituted with methionine, glutamic acid, arginine or valine; the amino acid residue at the position 65 or a position corresponding thereto has been substituted with tryptophan; the amino acid residue at the position 66 or a position corresponding thereto has been substituted with histidine; the amino acid residue at the position 82 or a position corresponding thereto has been substituted with alanine, glutamic acid, glutamine or serine; the amino acid residue at the position 83 or a position corresponding thereto has been substituted with alanine or serine; the amino acid residue at the position 204 or a position corresponding thereto has been substituted with glutamic acid, aspartic acid, or tryptophan; the amino acid residue at the position 319 or a position corresponding thereto has been substituted with tryptophan, valine, threonine, leucine, isoleucine or phenylalanine; and/or the amino acid residue at the 337-position or a position corresponding thereto has been substituted with arginine or valine, in the amino acid sequence represented by SEQ ID NO: 2.

**[0037]** More preferably, in the alkaline protease variant of the present disclosure, the amino acid residue at the position 6 or a position corresponding thereto has been substituted with tryptophan; the amino acid residue at the position 15 or a position corresponding thereto has been substituted with glutamic acid; the amino acid residue at the position 16 or a position corresponding thereto has been substituted with methionine; the amino acid residue at the position 65 or a position corresponding thereto has been substituted with tryptophan; the amino acid residue at the position 66 or a position corresponding thereto has been substituted with histidine; the amino acid residue at the position 82or a position corresponding thereto has been substituted with alanine; the amino acid residue at the position 83 or a position corresponding thereto has been substituted with alanine; the amino acid residue at the position 204 or a position corresponding thereto has been substituted with glutamic acid; the amino acid residue at the position 319 or a position corresponding thereto has been substituted with tryptophan; and/or the amino acid residue at the position 337 or a position corresponding thereto has been substituted with arginine, in the amino acid sequence represented by SEQ ID NO: 2.

**[0038]** In the present disclosure, "the amino acid residue at a corresponding position" can be identified through comparison of amino acid sequences of alkaline proteases by using a known algorithm (e.g., the Lipman-Pearson method), to thereby assign maximum homology to conserved amino acid residues present in the amino acid sequences. When the amino acid

sequences of the alkaline proteases are aligned through such a method, no matter what insertion or deletion is present in the amino acid sequences, the positions of the homologous amino acid residues in each of the proteases can be determined. Conceivably, the homologous amino acid residues are located at the same positions in the three-dimensional structures of the alkaline proteases, and thus these proteases are analogous in terms of specificity-related functions.

**[0039]** For example, when the amino acid sequence of SEQ ID NO: 2 is compared with that of protease KP9860 and that of protease KP9865 through the aforementioned method, the following relations can be determined:

1. (a) the amino acid residue at position 6 (glycine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the glycine residue at the position 6 of protease KP9860 and to the glycine residue at the position 6 of protease KP9865;
2. (b) the amino acid residue at position 15 (serine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the serine residue at the position 15 of protease KP9860 and to the serine residue at the position 15 of protease KP9865;
3. (c) the amino acid residue at position 16 (serine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the serine residue at the position 16 of protease KP9860 and to the serine residue at the position 16 of protease KP9865;
4. (d) the amino acid residue at position 65 (threonine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the threonine residue at the position 65 of protease KP9860 and to the threonine residue at the position 65 of protease KP9865;
5. (e) the amino acid residue at position 66 (asparagine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the asparagine residue at the position 66 of protease KP9860 and to the asparagine residue at the position 66 of protease KP9865;
6. (f) the amino acid residue at position 82 (threonine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the threonine residue at the position 82 of protease KP9860 and to the threonine residue at the position 82 of protease KP9865;
7. (g) the amino acid residue at position 83 (asparagine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the asparagine residue at the position 83 of protease KP9860 and to the asparagine residue at the position 83 of protease KP9865;
8. (h) the amino acid residue at position 204 (glutamine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the glutamine residue at the position 204 of protease KP9860 and to the glutamine residue at the position 204 of protease KP9865;
9. (i) the amino acid residue at position 319 (alanine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the alanine residue at the position 319 of protease KP9860 and to the alanine residue at the position 319 of protease KP9865; and
10. (j) the amino acid residue at position 337 (phenylalanine residue) in the amino acid sequence represented by SEQ ID NO: 2 corresponds to the phenylalanine residue at the

position 337 of protease KP9860 and to the phenylalanine residue at the position 337 of protease KP9865.

**[0040]** The alkaline protease variant of the present disclosure may be produced by incorporating a variation at a target position of a protease consisting of an amino acid sequence represented by SEQ ID NO: 2 or an alkaline protease consisting of an amino acid sequence having an identity of 90% or more with the amino acid sequence represented by SEQ ID NO: 2, which is not subjected to modification (hereinafter may be referred to as parent alkaline protease).

**[0041]** The alkaline protease variant of the present disclosure may be obtained through, for example, the following procedure. Specifically, a cloned gene encoding a parent alkaline protease (e.g., a gene having a nucleotide sequence represented by SEQ ID NO: 1) is subjected to mutation; an appropriate host is transformed with the thus-mutated gene; and the thus-transformed host is subjected to culturing, followed by recovery of the alkaline protease from the cultured product. Cloning of the gene encoding the parent alkaline protease may be performed through a generally employed genetic recombination technique, for,example, a method described in WO 99/18218 pamphlet or WO 98/56927 pamphlet.

**[0042]** Mutation of the gene encoding the parent alkaline protease may be performed through any of generally employed site-directed mutagenesis techniques. More specifically, mutation of the gene may be performed by use of, for example, a Site-Directed Mutagenesis System Mutan®-Super Express Km kit (product of Takara Bio Inc.). An arbitrary sequence fragment of the gene may be substituted with a sequence fragment of another gene that corresponds to the arbitrary sequence fragment through recombinant PCR (polymerase chain reaction) method (PCR protocols, Academic Press, New York, 1990).

**[0043]** The method for producing the protease variant of the present disclosure by use of the above-obtained mutant gene is, for example, as follows: a method in which the mutant gene is ligated into a DNA vector which can consistently amplify the gene, followed by transformation of a host bacterium; or a method in which the mutant gene is introduced into chromosomal DNA of a host bacterium which can consistently maintain the gene. Examples of the host bacterium exhibiting the aforementioned characteristics include bacteria belonging to the genus *Bacillus, Escherichia coli,* mold, yeast, and *Actinomyces.* The protease variant can be produced by inoculating the host microorganisms containing the mutant gene into a culture medium containing an assimilable carbon source, a nitrogen source, and other essential nutrients, followed by culturing through a customary method.

**[0044]** The thus-produced alkaline protease variant of the present disclosure exhibits oxidizer resistance, maintains casein-degrading activity even in the presence of a fatty acid of high concentration, has a molecular weight of 43,000 ± 2,000 as determined through SDS-PAGE, and exhibits activity within an alkaline pH range and high specific activity. In addition, the

alkaline protease variant of the present disclosure, which maintains high specific activity, is provided with excellent characteristics; i.e., the alkaline protease variant exhibits stability in liquid detergents containing an anionic surfactant such as LAS higher than that of a parent alkaline protease. Therefore, in one aspect of the present disclosure, there is provided a method for stabilizing an alkaline protease in a liquid detergent, the method including a step of substituting amino acid residue(s). In the method of the present disclosure, the alkaline protease which is subjected to substitution is the aforementioned parent alkaline protease, and the amino acid residues involved in substitution are those described in the aforementioned (a) to (j).

**[0045]** Therefore, the alkaline protease variant of the present disclosure is useful as an enzyme to be incorporated into a variety of detergent compositions. In addition, through the stability enhancing method of the present disclosure, useful enzymes to be incorporated into a variety of detergent compositions can be provided.

**[0046]** No particular limitation is imposed on the amount of the alkaline protease variant of the present disclosure to be incorporated into a detergent composition, so long as the alkaline protease variant exhibits its activity. The amount of the alkaline protease variant to be incorporated may be 0.1 to 5,000 PU on the basis of 1 kg of the detergent composition, but, from the viewpoint of economy, etc., the incorporation amount is preferably 500 PU or less.

**[0047]** The detergent composition of the present disclosure may contain, in addition to the alkaline protease variant of the present disclosure, a variety of enzymes, for example, hydrolase, oxidase, reductase, transferase, lyase, isomerase, ligase, and synthetase. Of these, protease other than the alkaline protease variant of the present disclosure, cellulase, keratinase, esterase, cutinase, amylase, lipase, pullulanase, pectinase, mannanase, glucosidase, glucanase, cholesterol oxidase, peroxidase, laccase, and the like are preferred, with protease, cellulase, amylase, and lipase being more preferred. Examples of the protease include commercially available products, such as Alcalase®, Esperase®, Savinase®, Everlase®, and Kannase® (Novozymes); Properase® and Purafect® (Genencor); and KAP (Kao Corporation). Examples of the cellulase include Celluzyme® and Carezyme® (Novozymes); and KAC (Kao Corporation), alkaline cellulase produced by *Bacillus sp.* KSM-S237 strain described in JP-A-H10-313859, and mutant alkaline cellulase described in JP-A-2003-313592. Examples of the amylase include Termamyl®, Duramyl®, and Stainzyme® (Novozymes); Purastar® (Genencor), and KAM (Kao Corporation). Examples of the lipase include Lipolase®, Lipolase® Ultra, and Lipex® (Novozymes).

**[0048]** When a protease other than the alkaline protease variant of the present disclosure is incorporated into a detergent composition in combination with the alkaline protease varient, the protease content is preferably 0.1 to 500 PU on the basis of 1 kg of the detergent composition. When cellulase is incorporated in combination with the alkaline protease variant, the cellulase content is preferably 300 to 3,000,000 KU on the basis of 1 kg of the detergent composition, wherein KU represents a unit as determined by the enzyme activity measuring method described in paragraph [0020] of JP-A-H10-313859.

**[0049]** When amylase is incorporated in combination with the alkaline protease variant, the amylase content is preferably 50 to 500,000 IU on the basis of 1 kg of the detergent composition, wherein IU represents a unit as determined by the amylase activity measuring method described in paragraph [0040] of JP-A-H11-43690.

**[0050]** When lipase is incorporated in combination with the alkaline protease variant, the lipase content is preferably 10,000 to 1,000,000 LU on the basis of 1 kg of the detergent composition, wherein LU represents a unit as determined by the lipase activity measuring method described in Example 1 of JP-A-H08-500013.

**[0051]** The detergent composition of the present disclosure may contain a known detergent component, examples of which include the following.

**(1) Surfactant**

**[0052]** A surfactant is incorporated into the detergent composition in an amount of 0.5 to 60 mass%, preferably 10 to 45 mass% in the case where the detergent composition is in a powder form, and 20 to 50 mass% in the case where the detergent composition is in a liquid form. When the detergent composition of the present disclosure is employed as a bleaching agent or a detergent for an automatic dishwasher, the amount of surfactant to be incorporated is generally 1 to 10 mass%, preferably 1 to 5 mass%.

**[0053]** Examples of the surfactant to be employed in the detergent composition of the present disclosure include one species selected from among an anionic surfactant, a nonionic surfactant, an amphoteric surfactant, and a cationic surfactant; and a combination of these surfactants. Preferably, an anionic surfactant or a nonionic surfactant is employed.

**[0054]** Examples of preferred anionic surfactants include C10-C18 alcohol sulfuric acid ester salts, C8-C20 alkoxy alcohol sulfuric acid ester salts, alkylbenzenesulfonic acid salts, paraffinsulfonic acid salts, α-olefinsulfonic acid salts, α-sulfo fatty acid salts, α-sulfo fatty acid alkyl ester salts, and fatty acid salts. In the present disclosure, preferred are linear alkylbenzenesulfonic acid salts having an alkyl chain of C10-C14, with being more preferably C12-C14. The counter ionic species is preferably an alkali metal salt or an amine salt, with being more preferably a sodium and/or a potassium; a monoethanolamine; or a diethanolamine.

**[0055]** Examples of preferred nonionic surfactants include polyoxyalkylene C8-C20 alkyl ethers, alkyl polyglycosides, polyoxyalkylene C8-C20 alkylphenyl ethers, polyoxyalkylene sorbitan C8-C22 fatty acid esters, polyoxyalkylene glycol C8-C22 fatty acid esters, and polyoxyethylene-polyoxypropylene block polymers. The nonionic surfactant is preferably a polyoxyalkylene alkyl ether obtained through addition of an alkylene oxide such as ethylene oxide or propylene oxide (4 to 20 mol) to a C10-C18 alcohol, the polyoxyalkylene alkyl ether

preferably having an HLB value (calculated by the Griffin method) of 10.5 to 15.0, more preferably 11.0 to 14.5.

**(2) Divalent Metal-Ion Trapping Agent**

**[0056]** A divalent metal-ion trapping agent is incorporated in an amount of 0.01 to 50 mass%, preferably 5 to 40 mass%. Examples of the divalent metal-ion trapping agent to be employed in the detergent composition of the present disclosure include condensed phosphoric acid salts such as tripolyphosphoric acid salts, pyrophosphoric acid salts, and orthophosphoric acid salts; aluminosilicates such as zeolite; synthetic layered crystalline silicic acid salts; nitrilotriacetic acid salts; ethylenediaminetetraacetic acid salts; citric acid salts; isocitric acid salts; and polyacetal carboxylic acid salts. Of these, crystalline aluminosilicates (synthetic zeolite) are preferred. Among A-type, X-type, and P-type zeolites, an A-type zeolite is preferred. The preferably employed synthetic zeolite has an average primary particle size of 0.1 to 10 μm, more preferably 0.1 to 5 μm.

**(3) Alkaline Agent**

**[0057]** An alkaline agent is incorporated in an amount of 0.01 to 80 mass%, preferably 1 to 40 mass%. Examples of the alkaline agent to be employed in a powder detergent include alkali metal carbonates such as sodium carbonate, which is generally called dense ash or light ash, and amorphous alkali metal silicates of JIS No. 1, 2, or 3. These inorganic alkaline agents are effective in forming particle cores upon drying of a detergent to be able to provide a comparatively hard detergent having excellent fluidity. In place of these alkaline agents, for example, sodium sesquicarbonate or sodium hydrogencarbonate may be used, and a phosphoric acid salt such as a tripolyphosphoric acid salt also acts as an alkaline agent. Examples of the alkaline agent which may be employed in a liquid detergent and act as a counter ion to a surfactant include sodium hydroxide and mono-, di-, or triethanolamine, as well as the aforementioned alkaline agents.

**(4) Anti-redeposition Agent**

**[0058]** An anti-redeposition agent is incorporated in an amount of 0.001 to 10 mass%, preferably 1 to 5 mass%. Examples of the anti-redeposition agent to be employed in the detergent composition of the present disclosure include polyethylene glycol, a carboxylic acid polymer, polyvinyl alcohol, and polyvinylpyrrolidone. Of these, a carboxylic acid polymer has metal-ion trapping ability and ability to disperse solid particulate dirt from clothes to a washing bath, as well as anti-redeposition ability. The carboxylic acid polymer is a homopolymer or copolymer formed of acrylic acid, methacrylic acid, itaconic acid, etc., and the copolymer is preferably formed through copolymerization of the aforementioned monomer with maleic acid.

The molecular weight of the copolymer is preferably some thousands to 100,000. In addition to the aforementioned carboxylic acid polymer, a polymer such as a polyglycidic acid salt, a cellulose derivative such as carboxymethyl cellulose, or an aminocarboxylic acid polymer such as polyaspartic acid is preferably employed, since these substances also have metal-ion trapping ability, dispersibility, and anti-redeposition ability.

**(5) Bleaching Agent**

**[0059]** A bleaching agent such as hydrogen peroxide or a percarbonate is preferably incorporated in an amount of 1 to 10 mass%. In the case where a bleaching agent is employed, a bleach-activator such as tetraacetylethylenediamine (TAED) or one described in JP-A-H06-316700 may be incorporated in an amount of 0.01 to 10 mass%.

**(6) Fluorescent Agent**

**[0060]** Examples of the fluorescent agent to be employed in the detergent composition of the present disclosure include biphenyl fluorescent agents (e.g., Tinopal® CBS-X) and stilbene fluorescent agents (e.g., DM-type fluorescent dyes). Such a fluorescent agent is preferably incorporated in an amount of 0.001 to 2 mass%.

**(7) Other Components**

**[0061]** The detergent composition of the present disclosure may further contain a builder, a softening agent, a reducing agent (e.g., a sulfurous acid salt), a defoaming agent (e.g., silicone), or a perfume, which are known in the laundry detergent field; or other additives.

**[0062]** The detergent composition of the present disclosure can be produced through a customary method using the above-obtained alkaline protease variant of the present disclosure in combination with the aforementioned other enzymes and/or the aforementioned known detergent components, if needed. The form of the detergent composition may be appropriately selected in accordance with use thereof, and the detergent may assume the form of, for example, liquid, powder, granule, paste, or solid.

**[0063]** The thus-produced detergent composition of the present disclosure can be employed as, for example, a laundry detergent, a bleaching agent, a detergent for cleaning hard surfaces, a detergent for drainpipes, a denture-cleaning agent, and a detergent for sterilizing medical instruments.

**Examples**

**[0064]** The present disclosure will next be described in more detail by way of examples.

**Example 1**

**Preparation of KP43 protease**

**[0065]** Next will be described a method of preparing a protease employed in enzyme stability evaluation, taking a wild-type KP43 protease as an example.

**[0066]** A plasmid pHA64 (Japanese Patent No. 349293, having a *Bam*HI site and an *Xba*I site on the downstream side of the expression promoter) was digested simultaneously with restriction enzymes *Bam*HI and *Xba*I (Roche), and the product was employed as a vector for gene insertion and gene expression.

**[0067]** A DNA fragment represented by SEQ ID NO: 1 and including a wild-type KP43 protease gene (having *Bam*HI site at the 5'-terminus on the upstream side of the gene, and *Xba*I site at the 3'-terminus on the downstream side of the gene) was digested simultaneously with restriction enzymes *Bam*HI and *Xba*I and mixed with the above-prepared insertion and expression vector. The mixture was subjected to ligation by use of Ligation High (product of Toyobo). The ligation product was purified through ethanol precipitation, and *Bacillus sp.* KSM-9865 (FERM BP-10139) serving as a host was transformed with the purified product through electroporation. The product was applied to a skimmed milk-containing alkaline LB agar medium (containing 1% bactotrypton, 0.5% yeast extract, 1% sodium chloride, 1% skimmed milk, 1.5% agar, 0.05% sodium carbonate, and 15 ppm tetracycline). From the colonies appeared in the agar medium several days after, a transformant transfected with a protease gene was selected by confirming the presence of skimmed milk dissolution spots. Plasmid DNA was extracted from the transformant, and correct insertion of the protease gene represented by SEQ ID NO: 1 was confirmed. The thus-obtained plasmid was employed as plasmid pHA64TSA.

**[0068]** A KSM-9865 transformant harboring pHA64TSA was inoculated to a seed medium (6.0% (w/v) polypeptone S, 0.1% yeast extract, 1.0% maltose, 0.02% magnesium sulfate heptahydrate, 0.1% potassium dihydrogenphosphate, 0.3% anhydrous sodium carbonate, 30 ppm tetracycline) (5 mL), and shake-cultured at 30°C for 16 hours. Subsequently, the seed culture medium was inoculated (1% (v/v)) to a culture medium (8% polypeptone S, 0.3% yeast extract, 10% maltose, 0.04% magnesium sulfate heptahydrate, 0.2% potassium dihydrogenphosphate, 1.5% anhydrous sodium carbonate, 30 ppm tetracycline) (30 mL), and shake-cultured at 30°C for three days. The culture liquid containing KP43 protease obtained through culturing was centrifuged, and the stability of the recovered pellet in a liquid detergent was evaluated.

**Example 2**

**Production of KP43 protease variants**

**[0069]** A method of producing KP43 protease variants will next be described, taking a variant "G6A" as an example. In G6A, the 6-position glycine (G6) in the amino acid sequence (SEQ ID NO: 2) of a wild-type mature KP43 protease region was mutated to alanine.

**[0070]** PCR was performed by use of sufficiently diluted plasmid pHA64TSA as a template, primer KG24S2 (SEQ ID NO: 3, having *Bam*HI site) complementary to the upstream region of the initiation codon, and primer G6_R (SEQ ID NO: 4) complementary to the upstream region adjacent to the G6 codon, to thereby amplify a DNA sequence encoding the N-terminal portion of the KP43 protease. Separately, PCR was performed by use of plasmid pHA64TSA as a template, primer G6A_F (SEQ ID NO: 5, a 5'-terminal portion thereof being complementary to primer G6_R) for substituting the codon of G6 by the codon of alanine, and primer KG11S (SEQ ID NO: 6, having *Xba*I site) on the downstream side of the termination codon, to thereby amplify a DNA sequence encoding the C-terminal portion of the KP43 protease. Subsequently, the thus-obtained PCR products encoding the N-terminal and C-terminal portions were mixed and the mixture was employed as a template. PCR was performed by use of the primer KG24S2 and primer KG11S, to thereby obtain a PCR product containing the full-length of a KP43 protease variant gene in which the G6 codon had been substituted by the codon of alanine. The PCR product was purified through ethanol precipitation, and the purified product was digested simultaneously with restriction enzymes *Bam*HI and *Xba*I. The digested product was mixed with the vector for insertion and expression of Example 1, and the mixture was subjected to ligation by use of Ligation High (product of Toyobo). The ligation product was purified through ethanol precipitation, and *Bacillus sp.* KSM-9865 (FERM BP-10139) serving as a host was transformed with the purified product through electroporation. The product was applied to a skimmed milk-containing alkaline LB agar medium. From the colonies appeared in the agar medium several days after, a transformant transfected with a protease gene was selected by confirming the presence of skimmed milk dissolution spots. Thus, a transformant which produces a KP43 protease variant "G6A" in which G6 was mutated to alanine was produced.

**[0071]** The above procedure was repeated, except that primers represented by SEQ ID NOs. listed in the column "Mutation primer ·R" of the following Tables 1 to 10 were used instead of primer G6R, and that primers represented by SEQ ID NOs. listed in the column "Mutation primer ·F" of the following Tables 1 to 10 were used instead of primer G6A_F, to thereby produce transformants which produce KP43 protease variants having mutations listed in the column "KP43 protease mutation" of the following Tables 1 to 10. Each of the thus-obtained transformants was cultured through the method described in Example 1, to thereby recover a culture supernatant containing a protease variant of interest. The stability of the protease

variant in a liquid detergent was evaluated.

Table 1

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| G6A | G6_R | SEQ ID NO: 4 | G6A_F | SEQ ID NO: 5 |
| G6C | G6_R | SEQ ID NO: 4 | G6C_F | SEQ ID NO: 7 |
| G6D | G6_R | SEQ ID NO: 4 | G6D_F | SEQ ID NO: 8 |
| G6E | G6_R | SEQ ID NO: 4 | G6E_F | SEQ ID NO: 9 |
| G6F | G6_R | SEQ ID NO: 4 | G6F_F | SEQ ID NO: 10 |
| G6H | G6_R | SEQ ID NO: 4 | G6H_F | SEQ ID NO: 11 |
| G6I | G6_R | SEQ ID NO: 4 | G6I_F | SEQ ID NO: 12 |
| G6K | G6_R | SEQ ID NO: 4 | G6K_F | SEQ ID NO: 13 |
| G6L | G6_R | SEQ ID NO: 4 | G6L_F | SEQ ID NO: 14 |
| G6M | G6_R | SEQ ID NO: 4 | G6M_F | SEQ ID NO: 15 |
| G6N | G6_R | SEQ ID NO: 4 | G6N_F | SEQ ID NO: 16 |
| G6P | G6_R | SEQ ID NO: 4 | G6P_F | SEQ ID NO: 17 |
| G6Q | G6_R | SEQ ID NO: 4 | G6Q_F | SEQ ID NO: 18 |
| G6R | G6_R | SEQ ID NO: 4 | G6R_F | SEQ ID NO: 19 |
| G6S | G6_R | SEQ ID NO: 4 | G6S_F | SEQ ID NO: 20 |
| G6T | G6_R | SEQ ID NO: 4 | G6T_F | SEQ ID NO: 21 |
| G6V | G6_R | SEQ ID NO: 4 | G6V_F | SEQ ID NO: 22 |
| G6W | G6_R | SEQ ID NO: 4 | G6W_F | SEQ ID NO: 23 |
| G6Y | G6_R | SEQ ID NO: 4 | G6Y_F | SEQ ID NO: 24 |

Table 2

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| S15A | S15_R | SEQ ID NO: 25 | S15A_F | SEQ ID NO: 26 |
| S15C | S15_R | SEQ ID NO: 25 | S15C_F | SEQ ID NO: 27 |
| S15D | S15_R | SEQ ID NO: 25 | S15D_F | SEQ ID NO: 28 |
| S15E | S15_R | SEQ ID NO: 25 | S15E_F | SEQ ID NO: 29 |
| S15F | S15_R | SEQ ID NO: 25 | S15F_F | SEQ ID NO: 30 |
| S15G | S15_R | SEQ ID NO: 25 | S15G_F | SEQ ID NO: 31 |
| S15I | S15_R | SEQ ID NO: 25 | S15I_F | SEQ ID NO: 32 |
| S15K | S15_R | SEQ ID NO: 25 | S15K_F | SEQ ID NO: 33 |
| S15L | S15_R | SEQ ID NO: 25 | S15L_F | SEQ ID NO: 34 |
| S15M | S15_R | SEQ ID NO: 25 | S15M_F | SEQ ID NO: 35 |

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| S15N | S15_R | SEQ ID NO: 25 | S15N_F | SEQ ID NO: 36 |
| S15P | S15_R | SEQ ID NO: 25 | S15P_F | SEQ ID NO: 37 |
| S15Q | S15_R | SEQ ID NO: 25 | S15Q_F | SEQ ID NO: 38 |
| S15R | S15_R | SEQ ID NO: 25 | S15R_F | SEQ ID NO: 39 |
| S15T | S15_R | SEQ ID NO: 25 | S15T_F | SEQ ID NO: 40 |
| S15V | S15_R | SEQ ID NO: 25 | S15V_F | SEQ ID NO: 41 |
| S15W | S15_R | SEQ ID NO: 25 | S15W_F | SEQ ID NO: 42 |
| S15Y | S15_R | SEQ ID NO: 25 | S15Y_F | SEQ ID NO: 43 |

Table 3

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| S16A | S16_R | SEQ ID NO: 44 | S16A_F | SEQ ID NO: 45 |
| S16C | S16_R | SEQ ID NO: 44 | S16C_F | SEQ ID NO: 46 |
| S16D | S16_R | SEQ ID NO: 44 | S16D_F | SEQ ID NO: 47 |
| S16E | S16_R | SEQ ID NO: 44 | S16E_F | SEQ ID NO: 48 |
| S16F | S16_R | SEQ ID NO: 44 | S16F_F | SEQ ID NO: 49 |
| S16G | S16_R | SEQ ID NO: 44 | S16G_F | SEQ ID NO: 50 |
| S16H | S16_R | SEQ ID NO: 44 | S16H_F | SEQ ID NO: 51 |
| S26I | S16_R | SEQ ID NO: 44 | S16I_F | SEQ ID NO: 52 |
| S16K | S16_R | SEQ ID NO: 44 | S16K_F | SEQ ID NO: 53 |
| S16L | S16_R | SEQ ID NO: 44 | S16L_F | SEQ ID NO: 54 |
| S16M | S16_R | SEQ ID NO: 44 | S16M_F | SEQ ID NO: 55 |
| S16N | S16_R | SEQ ID NO: 44 | S16N_F | SEQ ID NO: 56 |
| S16P | S16_R | SEQ ID NO: 44 | S16P_F | SEQ ID NO: 57 |
| S16R | S16_R | SEQ ID NO: 44 | S16R_F | SEQ ID NO: 58 |
| S16V | S16_R | SEQ ID NO: 44 | S16V_F | SEQ ID NO: 59 |
| S16W | S16_R | SEQ ID NO: 44 | S16W_F | SEQ ID NO: 60 |
| S16Y | S16_R | SEQ ID NO: 44 | S16Y_F | SEQ ID NO: 61 |

Table 4

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| T65A | T65_R | SEQ ID NO: 62 | T65A_F | SEQ ID NO: 63 |
| T65C | T65_R | SEQ ID NO: 62 | T65C_F | SEQ ID NO: 64 |
| T65D | T65_R | SEQ ID NO: 62 | T65D_F | SEQ ID NO: 65 |

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| T65E | T65_R | SEQ ID NO: 62 | T65E_F | SEQ ID NO: 66 |
| T65F | T65_R | SEQ ID NO: 62 | T65F_F | SEQ ID NO: 67 |
| T65G | T65_R | SEQ ID NO: 62 | T65G_F | SEQ ID NO: 68 |
| T65H | T65_R | SEQ ID NO: 62 | T65H_F | SEQ ID NO: 69 |
| T65I | T65_R | SEQ ID NO: 62 | T65I_F | SEQ ID NO: 70 |
| T65K | T65_R | SEQ ID NO: 62 | T65K_F | SEQ ID NO: 71 |
| T65L | T65_R | SEQ ID NO: 62 | T65L_F | SEQ ID NO: 72 |
| T65M | T65_R | SEQ ID NO: 62 | T65M_F | SEQ ID NO: 73 |
| T65N | T65_R | SEQ ID NO: 62 | T65N_F | SEQ ID NO: 74 |
| T65Q | T65_R | SEQ ID NO: 62 | T65Q_F | SEQ ID NO: 75 |
| T65R | T65_R | SEQ ID NO: 62 | T65R_F | SEQ ID NO: 76 |
| T65S | T65_R | SEQ ID NO: 62 | T65S_F | SEQ ID NO: 77 |
| T65V | T65_R | SEQ ID NO: 62 | T65V_F | SEQ ID NO: 78 |
| T65W | T65_R | SEQ ID NO: 62 | T65W_F | SEQ ID NO: 79 |
| T65Y | T65_R | SEQ ID NO: 62 | T65Y_F | SEQ ID NO: 80 |

Table 5

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| N66A | N66_R | SEQ ID NO: 81 | N66A_F | SEQ ID NO: 82 |
| N66C | N66_R | SEQ ID NO: 81 | N66C_F | SEQ ID NO: 83 |
| N66D | N66_R | SEQ ID NO: 81 | N66D_F | SEQ ID NO: 84 |
| N66E | N66_R | SEQ ID NO: 81 | N66E_F | SEQ ID NO: 85 |
| N66F | N66_R | SEQ ID NO: 81 | N66F_F | SEQ ID NO: 86 |
| N66G | N66_R | SEQ ID NO: 81 | N66G_F | SEQ ID NO: 87 |
| N66H | N66_R | SEQ ID NO: 81 | N66H_F | SEQ ID NO: 88 |
| N66I | N66_R | SEQ ID NO: 81 | N66I_F | SEQ ID NO: 89 |
| N66K | N66_R | SEQ ID NO: 81 | N66K_F | SEQ ID NO: 90 |
| N66L | N66_R | SEQ ID NO: 81 | N66L_F | SEQ ID NO: 91 |
| N66M | N66_R | SEQ ID NO: 81 | N66M_F | SEQ ID NO: 92 |
| N66P | N66_R | SEQ ID NO: 81 | N66P_F | SEQ ID NO: 93 |
| N66Q | N66_R | SEQ ID NO: 81 | N66Q_F | SEQ ID NO: 94 |
| N66R | N66_R | SEQ ID NO: 81 | N66R_F | SEQ ID NO: 95 |
| N66S | N66_R | SEQ ID NO: 81 | N66S_F | SEQ ID NO: 96 |
| N66T | N66_R | SEQ ID NO: 81 | N66T_F | SEQ ID NO: 97 |

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| N66V | N66_R | SEQ ID NO: 81 | N66V_F | SEQ ID NO: 98 |
| N66W | N66_R | SEQ ID NO: 81 | N66W_F | SEQ ID NO: 99 |
| N66Y | N66_R | SEQ ID NO: 81 | N66Y_F | SEQ ID NO: 100 |

Table 6

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| T82A | T82_R | SEQ ID NO: 101 | T82A_F | SEQ ID NO: 102 |
| T82C | T82_R | SEQ ID NO: 101 | T82C_F | SEQ ID NO: 103 |
| T82D | T82_R | SEQ ID NO: 101 | T82D_F | SEQ ID NO: 104 |
| T82E | T82_R | SEQ ID NO: 101 | T82E_F | SEQ ID NO: 105 |
| T82F | T82_R | SEQ ID NO: 101 | T82F_F | SEQ ID NO: 106 |
| T82G | T82_R | SEQ ID NO: 101 | T82G_F | SEQ ID NO: 107 |
| T82H | T82_R | SEQ ID NO: 101 | T82H_F | SEQ ID NO: 108 |
| T821 | T82_R | SEQ ID NO: 101 | T82I_F | SEQ ID NO: 109 |
| T82K | T82_R | SEQ ID NO: 101 | T82K_F | SEQ ID NO: 110 |
| T82L | T82_R | SEQ ID NO: 101 | T82L_F | SEQ ID NO: 111 |
| T82M | T82_R | SEQ ID NO: 101 | T82M_F | SEQ ID NO: 112 |
| T82N | T82_R | SEQ ID NO: 101 | T82N_F | SEQ ID NO: 113 |
| T82P | T82_R | SEQ ID NO: 101 | T82P_F | SEQ ID NO: 114 |
| T82Q | T82_R | SEQ ID NO: 101 | T82Q_F | SEQ ID NO: 115 |
| T82R | T82_R | SEQ ID NO: 101 | T82R_F | SEQ ID NO: 116 |
| T82S | T82_R | SEQ ID NO: 101 | T82S_F | SEQ ID NO: 117 |
| T82V | T82_R | SEQ ID NO: 101 | T82V_F | SEQ ID NO: 118 |
| T82W | T82_R | SEQ ID NO: 101 | T82W_F | SEQ ID NO: 119 |
| T82Y | T82_R | SEQ ID NO: 101 | T82Y_F | SEQ ID NO: 120 |

Table 7

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| N83A | N83_R | SEQ ID NO: 121 | N83A_F | SEQ ID NO: 122 |
| N83C | N83_R | SEQ ID NO: 121 | N83C_F | SEQ ID NO: 123 |
| N83D | N83_R | SEQ ID NO: 121 | N83D_F | SEQ ID NO: 124 |
| N83E | N83_R | SEQ ID NO: 121 | N83E_F | SEQ ID NO: 125 |
| N83F | N83_R | SEQ ID NO: 121 | N83F_F | SEQ ID NO: 126 |
| N83G | N83_R | SEQ ID NO: 121 | N83G_F | SEQ ID NO: 127 |
| N83H | N83_R | SEQ ID NO: 121 | N83H_F | SEQ ID NO: 128 |
| N83I | N83_R | SEQ ID NO: 121 | N83I_F | SEQ ID NO: 129 |
| N83K | N83_R | SEQ ID NO: 121 | N83K_F | SEQ ID NO: 130 |
| N83L | N83_R | SEQ ID NO: 121 | N83L_F | SEQ ID NO: 131 |
| N83M | N83_R | SEQ ID NO: 121 | N83M_F | SEQ ID NO: 132 |
| N83P | N83_R | SEQ ID NO: 121 | N83P_F | SEQ ID NO: 133 |
| N83Q | N83_R | SEQ ID NO: 121 | N83Q_F | SEQ ID NO: 134 |
| N83R | N83_R | SEQ ID NO: 121 | N83R_F | SEQ ID NO: 135 |
| N83S | N83_R | SEQ ID NO: 121 | N83S_F | SEQ ID NO: 136 |
| N83T | N83_R | SEQ ID NO: 121 | N83T_F | SEQ ID NO: 137 |
| N83V | N83_R | SEQ ID NO: 121 | N83V_F | SEQ ID NO: 138 |
| N83W | N83_R | SEQ ID NO: 121 | N83W_F | SEQ ID NO: 139 |
| N83Y | N83_R | SEQ ID NO: 121 | N83Y_F | SEQ ID NO: 140 |

Table 8

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| Q204A | Q204_R | SEQ ID NO: 141 | Q204A_F | SEQ ID NO: 142 |
| Q204C | Q204_R | SEQ ID NO: 141 | Q204C_F | SEQ ID NO: 143 |
| Q204D | Q204_R | SEQ ID NO: 141 | Q204D_F | SEQ ID NO: 144 |
| Q204E | Q204_R | SEQ ID NO: 141 | Q204E_F | SEQ ID NO: 145 |
| Q204F | Q204_R | SEQ ID NO: 141 | Q204F_F | SEQ ID NO: 146 |
| Q204G | Q204_R | SEQ ID NO: 141 | Q204G_F | SEQ ID NO: 147 |
| Q204H | Q204_R | SEQ ID NO: 141 | Q204H_F | SEQ ID NO: 148 |
| Q204I | Q204_R | SEQ ID NO: 141 | Q204I_F | SEQ ID NO: 149 |
| Q204K | Q204_R | SEQ ID NO: 141 | Q204K_F | SEQ ID NO: 150 |
| Q204L | Q204_R | SEQ ID NO: 141 | Q204L_F | SEQ ID NO: 151 |
| Q204M | Q204_R | SEQ ID NO: 141 | Q204M_F | SEQ ID NO: 152 |
| Q204N | Q204_R | SEQ ID NO: 141 | Q204N_F | SEQ ID NO: 153 |
| Q204P | Q204_R | SEQ ID NO: 141 | Q204P_F | SEQ ID NO: 154 |
| Q204R | Q204_R | SEQ ID NO: 141 | Q204R_F | SEQ ID NO: 155 |
| Q204S | Q204_R | SEQ ID NO: 141 | Q204S_F | SEQ ID NO: 156 |
| Q204T | Q204_R | SEQ ID NO: 141 | Q204T_F | SEQ ID NO: 157 |
| Q204V | Q204_R | SEQ ID NO: 141 | Q204V_F | SEQ ID NO: 158 |
| Q204W | Q204_R | SEQ ID NO: 141 | Q204W_F | SEQ ID NO: 159 |
| Q204Y | Q204_R | SEQ ID NO: 141 | Q204Y_F | SEQ ID NO: 160 |

Table 9

| KP43 protease mutation | Mutation primer ·R | | Mutation primer ·F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| A319C | A319_R | SEQ ID NO: 161 | A319C_F | SEQ ID NO: 162 |
| A319D | A319_R | SEQ ID NO: 161 | A319D_F | SEQ ID NO: 163 |
| A319E | A319_R | SEQ ID NO: 161 | A319E_F | SEQ ID NO: 164 |
| A319F | A319_R | SEQ ID NO: 161 | A319F_F | SEQ ID NO: 165 |
| A319G | A319_R | SEQ ID NO: 161 | A319G_F | SEQ ID NO: 166 |
| A319H | A319_R | SEQ ID NO: 161 | A319H_F | SEQ ID NO: 167 |
| A319I | A319_R | SEQ ID NO: 161 | A319I_F | SEQ ID NO: 168 |
| A319K | A319_R | SEQ ID NO: 161 | A319K_F | SEQ ID NO: 169 |
| A319L | A319_R | SEQ ID NO: 161 | A319L_F | SEQ ID NO: 170 |
| A319M | A319_R | SEQ ID NO: 161 | A319M_F | SEQ ID NO: 171 |
| A319N | A319_R | SEQ ID NO: 161 | A319N_F | SEQ ID NO: 172 |
| A319P | A319_R | SEQ ID NO: 161 | A319P_F | SEQ ID NO: 173 |
| A319Q | A319_R | SEQ ID NO: 161 | A319Q_F | SEQ ID NO: 174 |
| A319R | A319_R | SEQ ID NO: 161 | A319R_F | SEQ ID NO: 175 |
| A319S | A319_R | SEQ ID NO: 161 | A319S_F | SEQ ID NO: 176 |
| A319T | A319_R | SEQ ID NO: 161 | A319T_F | SEQ ID NO: 177 |
| A319V | A319_R | SEQ ID NO: 161 | A319V_F | SEQ ID NO: 178 |
| A319W | A319_R | SEQ ID NO: 161 | A319W_F | SEQ ID NO: 179 |
| A319Y | A319_R | SEQ ID NO: 161 | A319Y_F | SEQ ID NO: 180 |

Table 10

| KP43 protease mutation | Mutation primer R | | Mutation primer F | |
|---|---|---|---|---|
| | Primer | SEQ ID NO | Primer | SEQ ID NO |
| F337A | F337_R | SEQ ID NO: 181 | F337A_F | SEQ ID NO: 182 |
| F337C | F337_R | SEQ ID NO: 181 | F337C_F | SEQ ID NO: 183 |
| F337D | F337_R | SEQ ID NO: 181 | F337D_F | SEQ ID NO: 184 |
| F337E | F337_R | SEQ ID NO: 181 | F337E_F | SEQ ID NO: 185 |
| F337G | F337_R | SEQ ID NO: 181 | F337G_F | SEQ ID NO: 186 |
| F337H | F337_R | SEQ ID NO: 181 | F337H_F | SEQ ID NO: 187 |
| F337I | F337_R | SEQ ID NO: 181 | F337I_F | SEQ ID NO: 188 |
| F337K | F337_R | SEQ ID NO: 181 | F337K_F | SEQ ID NO: 189 |
| F337L | F337_R | SEQ ID NO: 181 | F337L_F | SEQ ID NO: 190 |
| F337M | F337_R | SEQ ID NO: 181 | F337M_F | SEQ ID NO: 191 |
| F337N | F337_R | SEQ ID NO: 181 | F337N_F | SEQ ID NO: 192 |
| F337P | F337_R | SEQ ID NO: 181 | F337P_F | SEQ ID NO: 193 |
| F337Q | F337_R | SEQ ID NO: 181 | F337Q_F | SEQ ID NO: 194 |
| F337R | F337_R | SEQ ID NO: 181 | F337R_F | SEQ ID NO: 195 |
| F337S | F337_R | SEQ ID NO: 181 | F337S_F | SEQ ID NO: 196 |
| F337T | F337_R | SEQ ID NO: 181 | F337T_F | SEQ ID NO: 197 |
| F337V | F337_R | SEQ ID NO: 181 | F337V_F | SEQ ID NO: 198 |
| F337W | F337_R | SEQ ID NO: 181 | F337W_F | SEQ ID NO: 199 |
| F337Y | F337_R | SEQ ID NO: 181 | F337Y_F | SEQ ID NO: 200 |

**Example 3**

**Method for determining protease activity**

**[0072]** Protease activity was determined in the following manner. Specifically, 40mM Glt-Ala-Ala-Pro-Leu-pNA·H2O (AAPL) (product of Peptide Laboratory) dissolved in dimethyl sulfoxide (3 parts by volume), 200mM borate buffer (pH: 10.5) (10 parts by volume), and ion-exchange water (7 parts by volume) were mixed, to thereby prepare a substrate solution. The substrate solution was dispensed in a 96-well assay plate (50 μL/well). Each protease-containing solution was diluted with ion-exchange water to an appropriate concentration, and the diluted protease was added to the assay plate (50 μL/well), whereby reaction was initiated. Immediately after start of reaction, the plate was placed into a chamber (VersaMax™, product of Molecular Device) maintained at 30°C. The change in absorbance at 420 nm was monitored in the kinetic

mode for 10 minutes. The measurements were processed by analysis software (Softmax®Pro, product of Molecular Device), and the output of absorbance change rate (mOD/min) was employed as a provisional activity value of the protease.

**Example 4**

**Evaluation of stability of variants**

**[0073]** Composition A (8% Softanol®, 70H, 14% Emulgen®, 120, 8% acid precursor for linear sodium alkylbenzenesulfonate liquid (LAS-S), 4% Lunac® L-55, 2% ethanol, 4% butoxydiglycol, 3.5% monoethanolamine, 0.1% sodium sulfite, 0.55% citric acid, pH: 9.0) was added to a 96-well plate (90 µL/well). Then, a culture supernatant containing a wild-type KP43 protease or each of the KP43 protease variants was added to a 96-well plate (10 µL/well), followed by sufficient stirring. Immediately after stirring, a portion (10 µL) of the liquid mixture was collected and diluted with ion-exchange water (250 µL), followed by sufficient stirring. The dilution was performed to a dilution factor of 26 folds. The thus-obtained diluted liquid was added to the 96-well assay plate (50 µL/well) to which a substrate solution had been added (50 µL/well). The plate was placed into a microplate-reader (VersaMax™, product of Molecular Device) and the protease activity of the sample was determined. The initial activity value was determined for evaluation of storage stability of the sample. The 96-well assay plate to which each evaluation liquid (composition A and culture supernatant) had been added was maintained in a sealed container at 40°C. After storage for 72 hours, the plate was removed from the container, and the residual activity was determined through the same procedure as employed for the determination of the initial activity. The residual activity (relative value) of each KP43 protease variant was calculated, with respect to the residual activity of the wild-type KP43 protease as 100%.

**[0074]** Figs. 1 to 10 show the stabilities of wild-type protease and KP43 protease variants.
The disclosure comprises the following:

1. 1. An alkaline protease variant derived from an alkaline protease consisting of an amino acid sequence represented by SEQ ID NO: 2 or consisting of an amino acid sequence having an identity of 90% or more therewith, wherein one or more amino acid residues at positions selected from (a) position 6, (b) position 15, (c) position 16, (d) position 65, (e) position 66, (f) position 82, (g) position 83, (h) position 204, (i) position 319, and (j) position 337 of the amino acid sequence represented by SEQ ID NO: 2, or at positions corresponding thereto are substituted with the following amino acid residues:
    1. (a) or a position corresponding thereto: tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine;
    2. (b) or a position corresponding thereto: glutamic acid, methionine, aspartic acid, valine, glutamine, arginine, cysteine, tryptophan, alanine, or phenylalanine;

3. (c) or a position corresponding thereto: methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine;
4. (d) or a position corresponding thereto: tryptophan;
5. (e) or a position corresponding thereto: histidine, tryptophan, serine, or leucine;
6. (f) or a position corresponding thereto: alanine, glutamic acid, glutamine, serine, cysteine, glycine, histidine, lysine, arginine, methionine, or asparagine;
7. (g) or a position corresponding thereto: alanine, serine, or cysteine;
8. (h) or a position corresponding thereto: glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine or, arginine;
9. (i) or a position corresponding thereto: tryptophan, valine, threonine, leucine, isoleucine, cysteine, glutamic acid, lysine, tyrosine, arginine, phenylalanine, glutamine, methionine, proline, aspartic acid, asparagine, histidine, or serine; and
10. (j) or a position corresponding thereto: arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine.

2. 2. The alkaline protease variant according to item 1, which is derived from the alkaline protease consisting of the amino acid sequence represented by SEQ ID NO:2, wherein one or more amino acid residues at positions selected from (a) position 6, (b) position 15, (c) position 16, (d) position 65, (e) position 66, (f) position 82, (g) position 83, (h) position 204, (i) position 319, and (j) position 337 of the amino acid sequence, or at positions corresponding thereto are substituted with other amino acid residues.
3. 3. A gene encoding the alkaline protease variant as recited in item 1 or 2.
4. 4. A recombinant vector comprising the gene as recited in item 3.
5. 5. A transformant comprising the recombinant vector as recited in item 4.
6. 6. The transformant according to item 5, whose host is a microorganism.
7. 7. A detergent composition comprising the alkaline protease variant as recited in item 1 or 2.
8. 8. The detergent composition according to item 7, which comprises an anionic surfactant.
9. 9. A method for enhancing the stability of an alkaline protease in a liquid detergent, the method comprising, in an alkaline protease consisting of an amino acid sequence represented by SEQ ID NO: 2 or consisting of an amino acid sequence having an identity of 90% or more therewith, substituting one or more amino acid residues at positions selected from (a) position 6, (b) position 15, (c) position 16, (d) position 65, (e) position 66, (f) position 82, (g) position 83, (h) position 204, (i) position 319, and (j) position 337 of the amino acid sequence represented by SEQ ID NO: 2, or at positions corresponding thereto with the following amino acid residues:
    1. (a) or a position corresponding thereto: tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine;
    2. (b) or a position corresponding thereto: glutamic acid, methionine, aspartic acid, valine, glutamine, arginine, cysteine, tryptophan, alanine, or phenylalanine;
    3. (c) or a position corresponding thereto: methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine;

4. (d) or a position corresponding thereto: tryptophan;
5. (e) or a position corresponding thereto: histidine, tryptophan, serine, or leucine;
6. (f) or a position corresponding thereto: alanine, glutamic acid, glutamine, serine, cysteine, glycine, histidine, lysine, arginine, methionine, or asparagine;
7. (g) or a position corresponding thereto: alanine, serine, or cysteine;
8. (h) or a position corresponding thereto: glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine or, arginine;
9. (i) or a position corresponding thereto: tryptophan, valine, threonine, leucine, isoleucine, cysteine, glutamic acid, lysine, tyrosine, arginine, phenylalanine, glutamine, methionine, proline, aspartic acid, asparagine, histidine, or serine; and
10. (j) or a position corresponding thereto: arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine.

10. 10. A method for producing the alkaline protease variant as recited in item 1 or 2, which method comprises culturing the transformant as recited in item 5.

**SEQUENCE LISTING**

**[0075]**

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Tyr Gly Leu Tyr Gly Gln Gly Gln Ile Val Ala Val Ala Asp Thr Gly
            20                  25                  30

Leu Asp Thr Gly Arg Asn Asp Ser Ser Met His Glu Ala Phe Arg Gly
        35                  40                  45

Lys Ile Thr Ala Leu Tyr Ala Leu Gly Arg Thr Asn Asn Ala Asn Asp
    50                  55                  60

Thr Asn Gly His Gly Thr His Val Ala Gly Ser Val Leu Gly Asn Gly
65                  70                  75                  80

Ser Thr Asn Lys Gly Met Ala Pro Gln Ala Asn Leu Val Phe Gln Ser
                85                  90                  95

Ile Met Asp Ser Gly Gly Gly Leu Gly Gly Leu Pro Ser Asn Leu Gln
            100                 105                 110

Thr Leu Phe Ser Gln Ala Tyr Ser Ala Gly Ala Arg Ile His Thr Asn
        115                 120                 125

Ser Trp Gly Ala Ala Val Asn Gly Ala Tyr Thr Thr Asp Ser Arg Asn
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Val Asp Asp Tyr Val Arg Lys Asn Asp Met Thr Ile Leu Phe Ala Ala
145                 150                 155                 160

Gly Asn Glu Gly Pro Asn Gly Gly Thr Ile Ser Ala Pro Gly Thr Ala
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Lys Asn Ala Ile Thr Val Gly Ala Thr Glu Asn Leu Arg Pro Ser Phe
            180                 185                 190

Gly Ser Tyr Ala Asp Asn Ile Asn His Val Ala Gln Phe Ser Ser Arg
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Gly Pro Thr Lys Asp Gly Arg Ile Lys Pro Asp Val Met Ala Pro Gly
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Thr Phe Ile Leu Ser Ala Arg Ser Ser Leu Ala Pro Asp Ser Ser Phe
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Trp Ala Asn His Asp Ser Lys Tyr Ala Tyr Met Gly Gly Thr Ser Met
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Ala Thr Pro Ile Val Ala Glu Asp Val Ala Gln Leu Arg Glu His Phe
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Ala Thr Pro Ile Val Ala Gly Asn Val Ala Gln Leu Arg Glu His Phe
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Val Lys Asn Arg Gly Ile Thr Pro Lys Pro Ser Leu Leu Lys Ala Ala
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Leu Ile Ala Gly Ala Ala Asp Ile Gly Leu Gly Tyr Pro Asn Gly Asn
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Gln Gly Trp Gly Arg Val Thr Leu Asp Lys Ser Leu Asn Val Ala Tyr
305                 310                 315                 320

Val Asn Glu Ser Ser Ser Leu Ser Thr Ser Gln Lys Ala Thr Tyr Ser
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Phe Thr Ala Thr Ala Gly Lys Pro Leu Lys Ile Ser Leu Val Trp Ser
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Asp Ala Pro Ala Ser Thr Thr Ala Ser Val Thr Leu Val Asn Asp Leu
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Asp Leu Val Ile Thr Ala Pro Asn Gly Thr Gln Tyr Val Gly Asn Asp
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Phe Thr Ser Pro Tyr Asn Asp Asn Trp Asp Gly Arg Asn Asn Val Glu
385                 390                 395                 400

Asn Val Phe Ile Asn Ala Pro Gln Ser Gly Thr Tyr Thr Ile Glu Val
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<220>
<223> Oligonucleotide as PCR primer F337T_F

<400> 197
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<210> 198
<211> 45

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<210> 199
<211> 45
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<210> 200
<211> 45
<212> DNA
<213> Artificial

<220>
<223> Oligonucleotide as PCR primer F337Y_F

<400> 200
agccaaaaag cgacgtactc gtatactgct actgccggca agcct 45

# REFERENCES CITED IN THE DESCRIPTION

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**Patent documents cited in the description**


- JP170A [0009]

- WO9918218A [0009] [0025] [0028] [0041]
- JPH11507680A [0009]
- JP2000506933A [0009]
- JP2004305176A [0009] [0029]
- JP2004000122A [0009] [0029]
- JP2002218989A [0009] [0029]
- WO2006032278A [0009]
- JP2002306176A [0029]
- JP2003125783A [0029]
- JP2004057195A [0029]
- JP2004305175A [0029]
- WO9856927A [0041]
- JPH10313859A [0047] [0048]
- JP2003313592A [0047]
- JPH1143690A [0049]
- JPH08500013A [0050]
- JPH06316700A [0059]
- JP349293A [0066]
- JP2009110792A [0075]
- JP2009110793A [0075]
- JP2010093307A [0075]
- JP2010093339A [0075]


**Non-patent literature cited in the description**


- **SAEKI et al.**Biochem. Biophys. Res. Commun., 2000, vol. 279, 313-319 [0009]
- Cosmetics & Toiletries magazine, 1996, vol. 111, 79-88 [0009]
- Detergent Enzyme: A Challenge!In Handbook of Detergents19990000639-690 [0009]
- Science, 1985, vol. 227, 1435- [0031]
- PCR protocolsAcademic Press19900000 [0042]

## PATENTKRAV

**1.** Alkalisk proteasevariant, hvor den alkaliske proteasevariant

(i) består af aminosyresekvensen ifølge SEQ ID NO: 2, hvor aminosyreresten ved position 83 i aminosyresekvensen ifølge SEQ ID NO: 2 er substitueret med følgende aminosyrerester: alanin, serin eller cystein, eller
(ii) har en identitet på 90% eller mere med SEQ ID NO: 2, hvor aminosyreresten ved positionen svarende til position 83 i aminosyresekvensen ifølge SEQ ID NO: 2 er substitueret med følgende aminosyrerester: alanin, serin eller cystein, og hvor den alkaliske proteasevariant udviser aktivitet i et alkalisk pH område.

**2.** Alkalisk proteasevariant ifølge krav 1, hvor yderligere én eller flere aminosyrerester med positioner valgt blandt (a) position 6, (b) position 15, (c) position 16, (d) position 65, (e) position 66, (f) position 82, (g) position 204, (h) position 319 og (i) position 337 i aminosyresekvensen ifølge SEQ ID NO: 2, eller ved positioner svarende dertil, er substituerede med følgende aminosyrerester:

(a) tryptophan, leucin, valin, isoleucin, methionin, tyrosin, glutamin, lysin, threonin, phenylalanin, arginin, serin, cystein, alanin eller histidin;
(b) glutaminsyre, methionin, aspartansyre, valin, glutamin, arginin, cystein, tryptophan, alanin eller phenylalanin;
(c) methionin, glutaminsyre, arginin, valin, lysin, phenylalanin, tyrosin, isoleucin, histidin, aspartansyre eller cystein;
(d) tryptophan;
(e) histidin, tryptophan, serin eller leucin;
(f) alanin, glutaminsyre, glutamin, serin, cystein, glycin, histidin, lysin, arginin, methionin eller asparagin;
(g) glutaminsyre, aspartansyre, cystein, valin, threonin, prolin, histidin, isoleucin, tryptophan, serin, asparagin, lysin eller arginin;
(h) tryptophan, valin, threonin, leucin, isoleucin, cystein, glutaminsyre, lysin, tyrosin, arginin, phenylalanin, glutamin, methionin, prolin, aspartansyre, asparagin, histidin eller serin; og
(i) arginin, glycin, serin, lysin, glutamin, threonin, histidin, alanin, cystein eller valin.

**3.** Gen, som koder for den alkaliske proteasevariant ifølge krav 1 eller 2.

**4.** Rekombinant vektor omfattende genet ifølge krav 3.

**5.** Ikke-human transformant, omfattende den rekombinante vektor ifølge krav 4.

**6.** Den ikke-humane transformant ifølge krav 5, hvis vært er en mikroorganisme.

**7.** Vaskemiddelsammensætning omfattende den alkaliske proteasevariant ifølge krav 1 eller 2.

**8.** Vaskemiddelsammensætning ifølge krav 7, som omfatter et anionisk overfladeaktivt stof.

**9.** Fremgangsmåde til at fremme stabiliteten af en alkalisk protease i et flydende vaskemiddel, hvilken fremgangsmåde omfatter

(i) i en alkalisk protease bestående af aminosyresekvensen ifølge SEQ ID NO: 2, at substituere aminosyreresten ved position 83 i aminosyresekvensen ifølge SEQ ID NO: 2 med følgende aminosyrerester: alanin, serin eller cystein; eller
(ii) i en alkalisk protease med en identitet på 90% eller mere med SEQ ID NO: 2, at substituere aminosyreresten ved positionen svarende til position 83 i aminosyresekvensen ifølge SEQ ID NO: 2 med følgende aminosyrerester: alanin, serin eller cystein, hvor den fremstillede alkaliske proteasevariant udviser aktivitet i et alkalisk pH område.

**10.** Fremgangsmåde ifølge krav 9, hvor, i den alkaliske protease, én eller flere aminosyrerester yderligere er substituerede ved positioner valgt blandt (a) position 6, (b) position 15, (c) position 16, (d) position 65, (e) position 66, (f) position 82, (g) position 204, (h) position 319, and (i) position 337 i aminosyresekvensen ifølge SEQ ID NO: 2, eller ved positioner svarende dertil, med følgende aminosyrerester:

(a) tryptophan, leucin, valin, isoleucin, methionin, tyrosin, glutamin, lysin, threonin, phenylalanin, arginin, serin, cystein, alanin eller histidin;
(b) glutaminsyre, methionin, aspartansyre, valin, glutamin, arginin, cystein, tryptophan, alanin eller phenylalanin;

(c) methionin, glutaminsyre, arginin, valin, lysin, phenylalanin, tyrosin, isoleucin, histidin, aspartansyre eller cystein;
(d) tryptophan;
(e) histidin, tryptophan, serin eller leucin;
(f) alanin, glutaminsyre, glutamin, serin, cystein, glycin, histidin, lysin, arginin, methionin eller asparagin;
(g) glutaminsyre, aspartansyre, cystein, valin, threonin, prolin, histidin, isoleucin, tryptophan, serin, asparagin, lysin eller arginin;
(h) tryptophan, valin, threonin, leucin, isoleucin, cystein, glutaminsyre, lysin, tyrosin, arginin, phenylalanin, glutamin, methionin, prolin, aspartansyre, asparagin, histidin eller serin; og
(i) arginin, glycin, serin, lysin, glutamin, threonin, histidin, alanin, cystein eller valin.

**11.** Fremgangsmåde til fremstilling af den alkaliske proteasevariant ifølge krav 1 eller 2, hvilken fremgangsmåde omfatter dyrkning af transformanten ifølge krav 5.

# DRAWINGS

Fig. 1

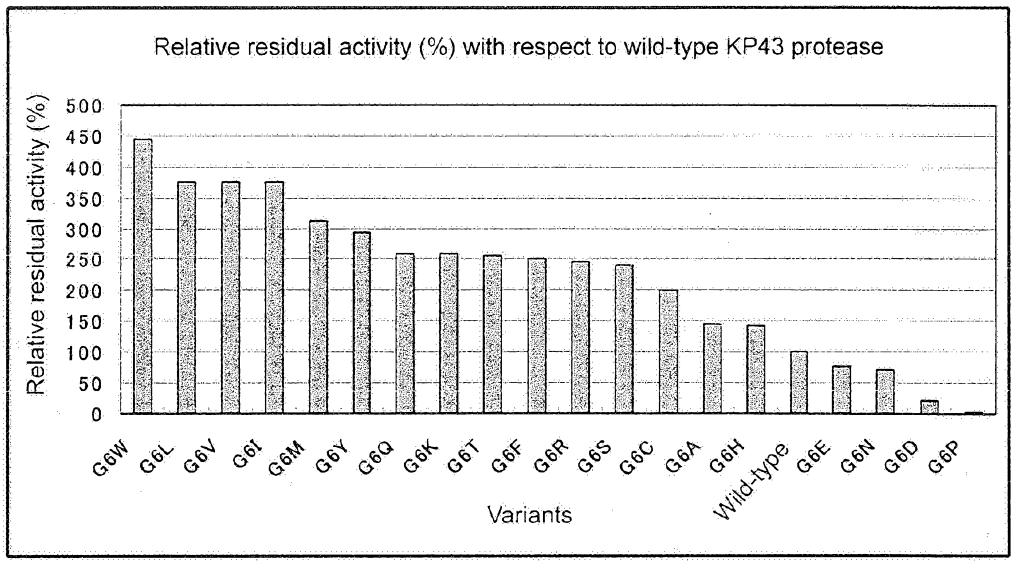
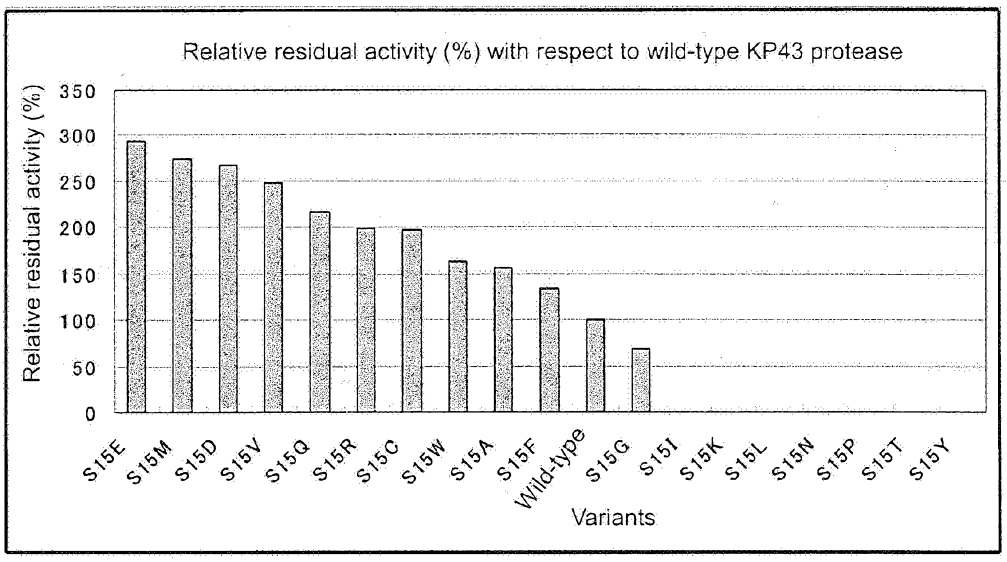



Fig. 2



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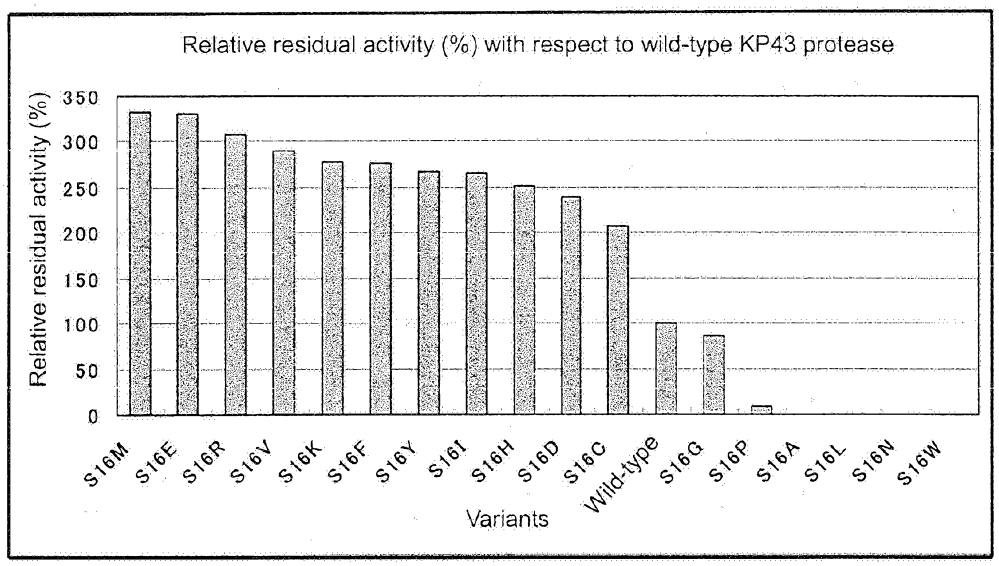
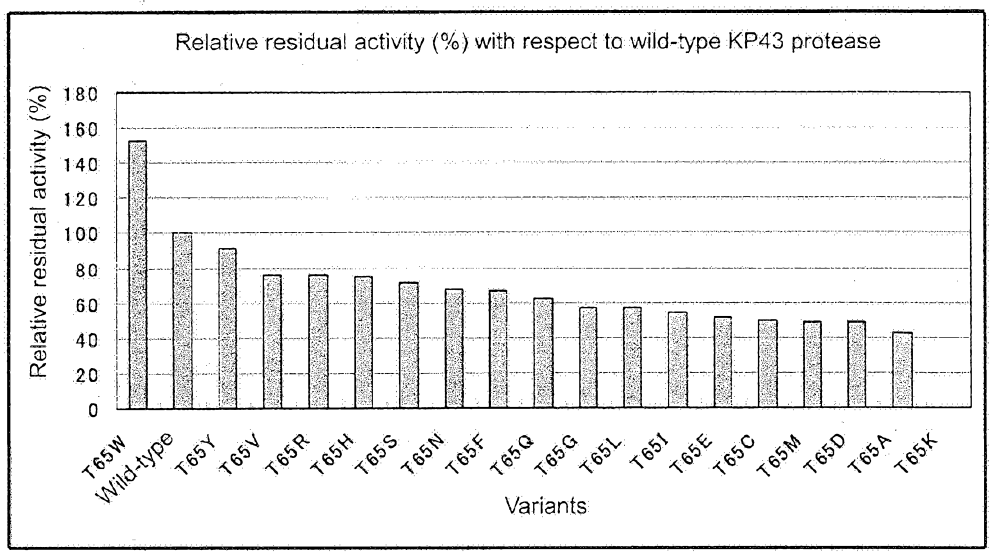

Relative residual activity (%) with respect to wild-type KP43 protease
Relative residual activity (%)
350
300
250
200
150
100
50
0
S16M
S16E
S16R
S16V
S16K
S16F
S16Y
S16I
S16H
S16D
S16C
Wild-type
S16G
S16P
S16A
S16L
S16N
S16W
Variants

Fig. 4


Relative residual activity (%) with respect to wild-type KP43 protease
Relative residual activity (%)
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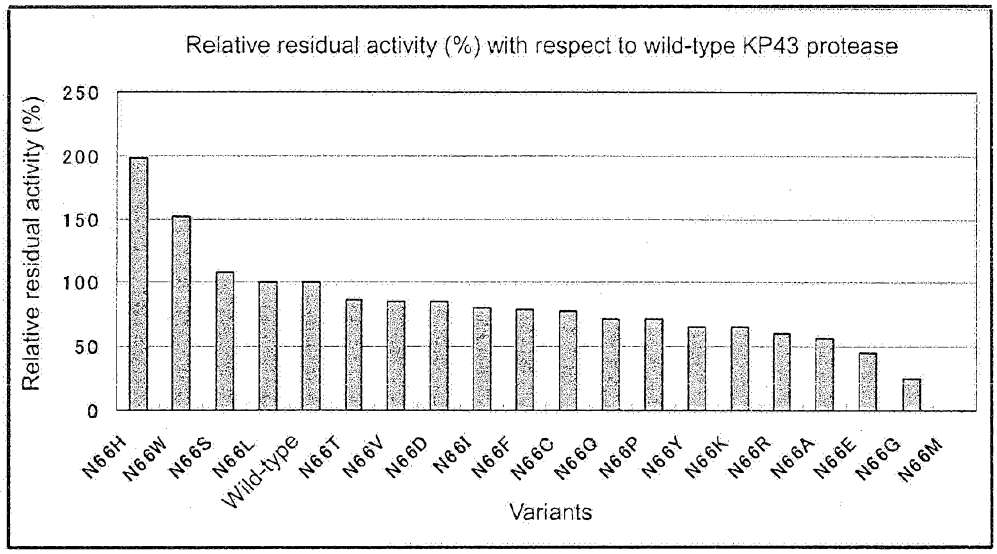
160
140
120
100
80
60
40
20
0
T65W
Wild-type
T65Y
T65V
T65R
T65H
T65S
T65N
T65F
T65Q
T65G
T65L
T65I
T65E
T65C
T65M
T65D
T65A
T65K
Variants

Fig. 5


Relative residual activity (%) with respect to wild-type KP43 protease
Relative residual activity (%)
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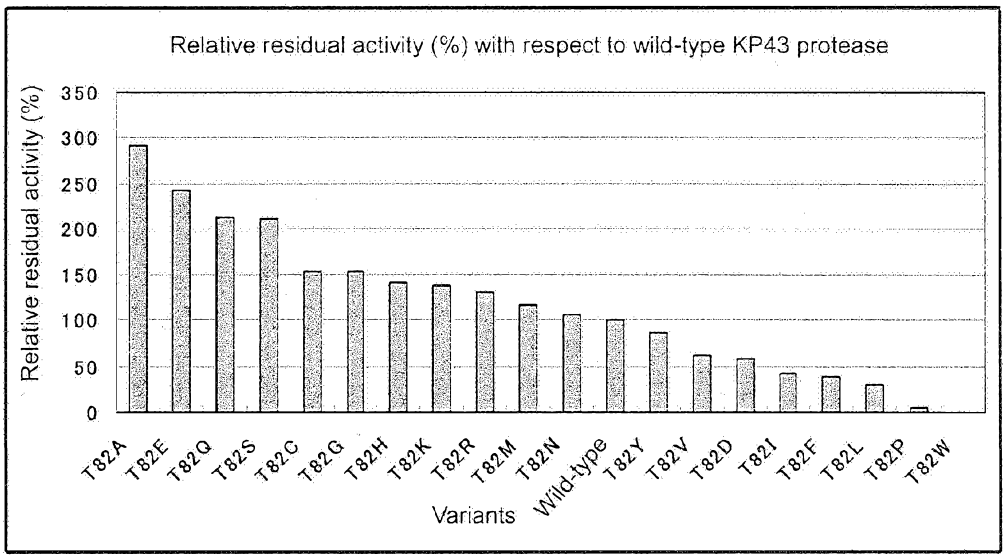
200
150
100
50
0
N66H
N66W
N66S
N66L
Wild-type
N66T
N66V
N66D
N66I
N66F
N66C
N66Q
N66P
N66Y
N66K
N66R
N66A
N66E
N66G
N66M
Variants

Fig. 6


Relative residual activity (%) with respect to wild-type KP43 protease
Relative residual activity (%)
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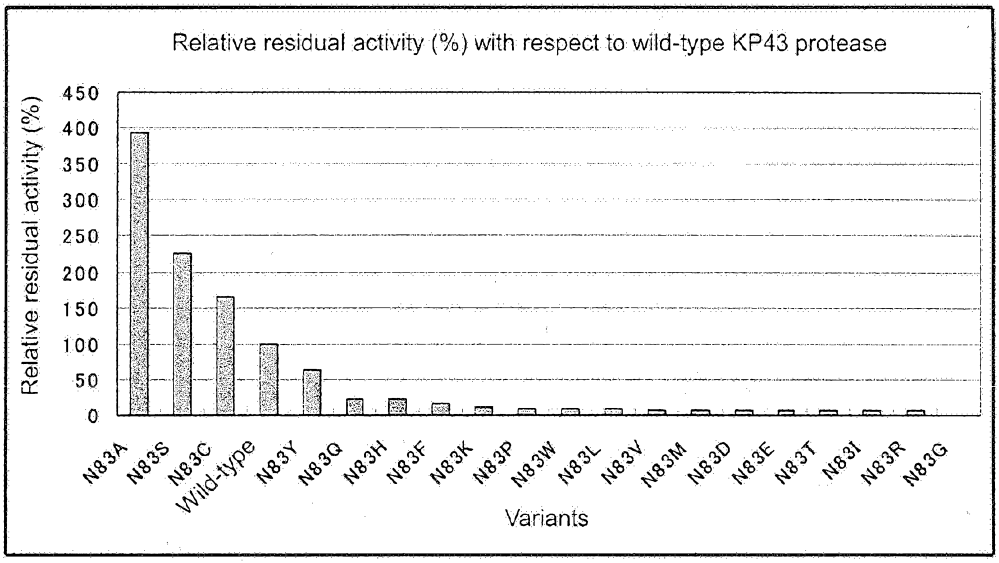
300
250
200
150
100
50
0
T82A
T82E
T82Q
T82S
T82C
T82G
T82H
T82K
T82R
T82M
T82N
Wild-type
T82Y
T82V
T82D
T82I
T82F
T82L
T82P
T82W
Variants

Fig. 7


Relative residual activity (%) with respect to wild-type KP43 protease
Relative residual activity (%)
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300
250
200
150
100
50
0
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N83S
N83C
Wild-type
N83Y
N83Q
N83H
N83F
N83K
N83P
N83W
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N83V
N83M
N83D
N83E
N83T
N83I
N83R
N83G
Variants

Fig. 8

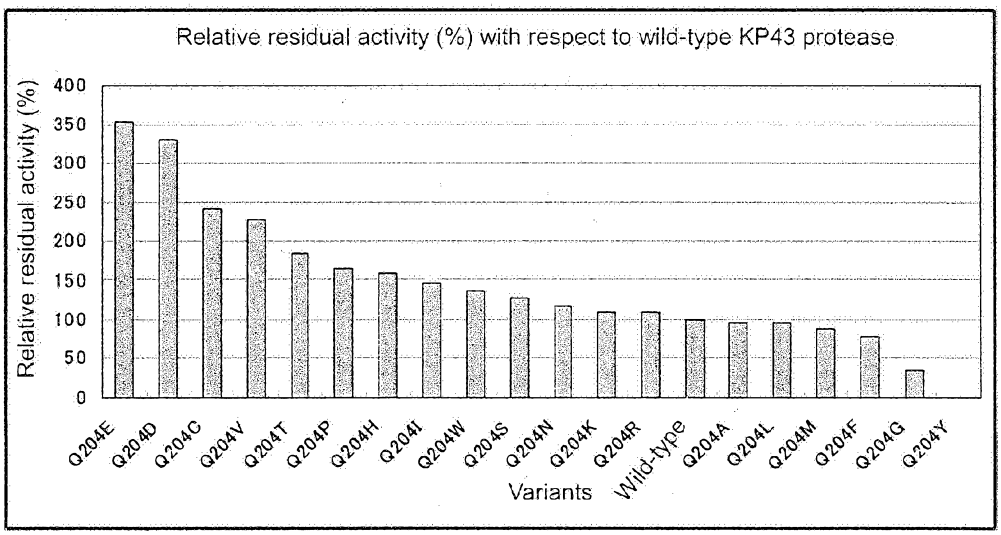
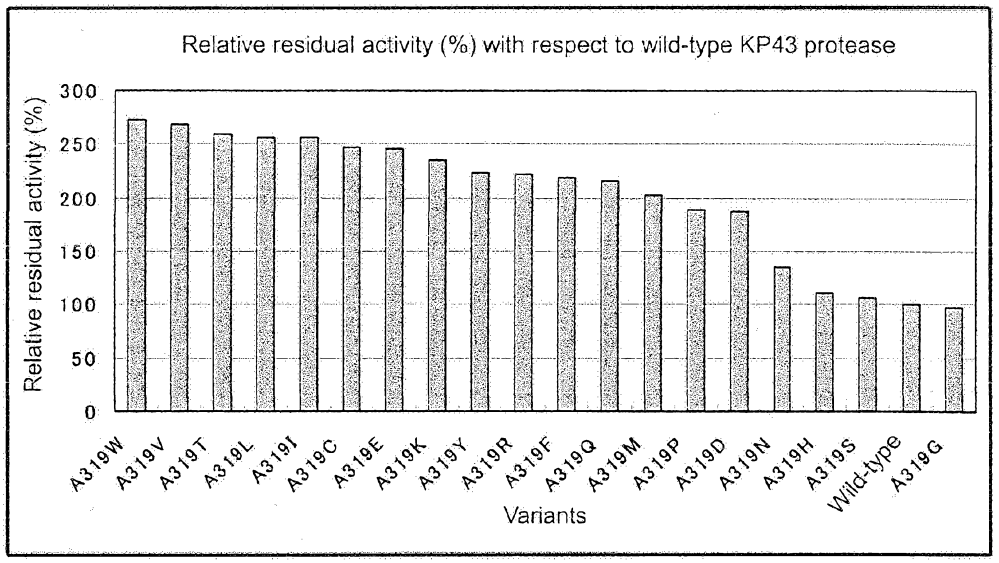

Relative residual activity (%) with respect to wild-type KP43 protease
Relative residual activity (%)
400
350
300
250
200
150
100
50
0
Q204E
Q204D
Q204C
Q204V
Q204T
Q204P
Q204H
Q204I
Q204W
Q204S
Q204N
Q204K
Q204R
Wild-type
Q204A
Q204L
Q204M
Q204F
Q204G
Q204Y
Variants

Fig. 9


Relative residual activity (%) with respect to wild-type KP43 protease
Relative residual activity (%)
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250
200
150
100
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0
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A319V
A319T
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A319I
A319C
A319E
A319K
A319Y
A319R
A319F
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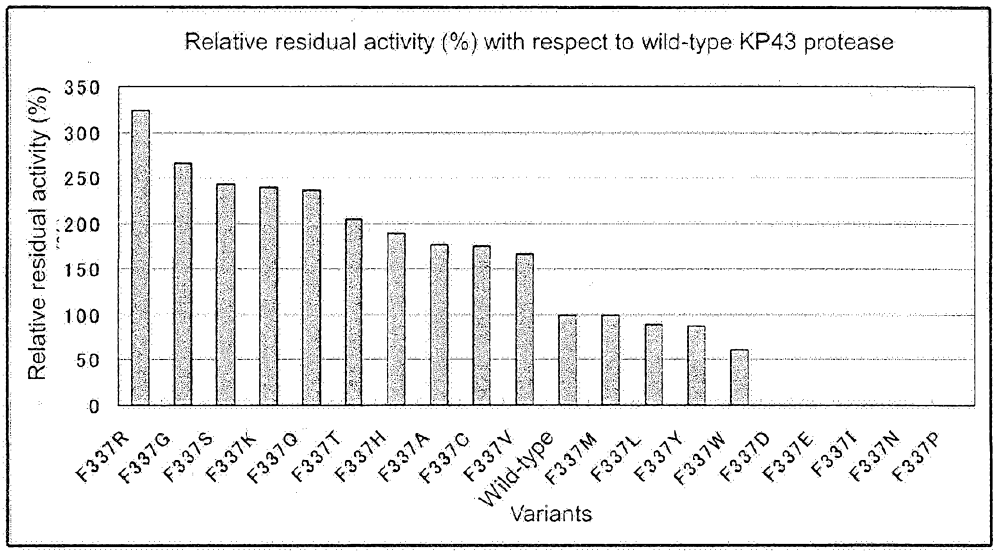
A319P
A319D
A319N
A319H
A319S
Wild-type
A319G
Variants

Fig. 10


Relative residual activity (%) with respect to wild-type KP43 protease
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200
150
100
50
0
F337R
F337G
F337S
F337K
F337Q
F337T
F337H
F337A
F337C
F337V
Wild-type
F337M
F337L
F337Y
F337W
F337D
F337E
F337I
F337N
F337P
Variants