

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
4 November 2010 (04.11.2010)

PCT

(10) International Publication Number
WO 2010/126156 A2(51) International Patent Classification:
C12N 9/54 (2006.01)2606, Akabane, Ichikai-machi, Haga-gun, Tochigi,
3213497 (JP).(21) International Application Number:
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(JP).(22) International Filing Date:
26 April 2010 (26.04.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2009-110792 30 April 2009 (30.04.2009) JP
2009-110793 30 April 2009 (30.04.2009) JP
2010-093307 14 April 2010 (14.04.2010) JP
2010-093339 14 April 2010 (14.04.2010) JP(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IS, KE, KG, KM, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE,
SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT,
TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.(71) Applicant (for all designated States except US): KAO
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Haga-gun, Tochigi, 3213497 (JP). SAEKI, Katsuhisa
[JP/JP]; c/o Kao Corporation Research Laboratories,(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG,
ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ,
TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Published:

- without international search report and to be republished
upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: ALKALINE PROTEASE VARIANTS

(57) Abstract: 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, which variant has mutations 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: (a) or a position corresponding thereto: Tyr, Leu, Val, Ile, Met, Tyr, Gln, Lys, Thr, Phe, Arg, Ser, Cys, Ala, or His; (b) or a position corresponding thereto: Glu, Met, Asp, Val, Gln, Arg, Cys, Trp, Ala, or Phe; (c) or a position corresponding thereto: Met, Glu, Arg, Val, Lys, Phe, Tyr, Ile, His, Asp, or Cys; (d) or a position corresponding thereto: Trp; (e) or a position corresponding thereto: His, Trp, Ser, or Leu; (f) or a position corresponding thereto: Ala, Glu, Gln, Ser, Cys, Gly, His, Lys, Arg, Met, or Asn; (g) or a position corresponding thereto: Ala, Ser, or Cys; (h) or a position corresponding thereto: Glu, Asp, Cys, Val, Thr, Pro, His, Ile, Trp, Ser, Asn, Lys, or Arg; (i) or a position corresponding thereto: Trp, Val, Thr, Leu, Ile, Cys, Glu, Lys, Tyr, Arg, Phe, Gln, Met, Pro, Asp, Asn, His, or Ser; and (j) or a position corresponding thereto: Arg, Gly, Ser, Lys, Gln, Thr, His, Ala, Cys, or Val.



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DESCRIPTION

ALKALINE PROTEASE VARIANTS

Field of the Invention

[0001]

The present invention relates to alkaline protease variants which are useful enzymes incorporated into liquid detergents, and to genes encoding the same.

Background of the Invention

[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]

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

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

Summary of the Invention

[0010]

The present invention 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:

(a) or a position corresponding thereto: tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine;

(b) or a position corresponding thereto: glutamic acid, methionine, aspartic acid, valine, glutamine, arginine, cysteine, tryptophan, alanine, or phenylalanine;

(c) or a position corresponding thereto: methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine;

(d) or a position corresponding thereto: tryptophan;

(e) or a position corresponding thereto: histidine, tryptophan, serine, or leucine;

(f) or a position corresponding thereto: alanine, glutamic acid, glutamine, serine, cysteine, glycine, histidine, lysine, arginine, methionine, or asparagine;

(g) or a position corresponding thereto: alanine, serine, or cysteine;

(h) or a position corresponding thereto: glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine or, arginine;

(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

(j) or a position corresponding thereto: arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine.

[0011]

The present invention is also directed to a gene encoding the alkaline protease variant.

The present invention is also directed to a recombinant

vector including the gene.

The present invention is also directed to a transformant including the recombinant vector.

The present invention is also directed to a detergent composition including the alkaline protease variant.

The present invention 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:

(a) or a position corresponding thereto: tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine;

(b) or a position corresponding thereto: glutamic acid, methionine, aspartic acid, valine, glutamine, arginine, cysteine, tryptophan, alanine, or phenylalanine;

(c) or a position corresponding thereto: methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine;

(d) or a position corresponding thereto: tryptophan;

(e) or a position corresponding thereto: histidine, tryptophan, serine, or leucine;

(f) or a position corresponding thereto: alanine, glutamic acid, glutamine, serine, cysteine, glycine, histidine, lysine, arginine, methionine, or asparagine;

(g) or a position corresponding thereto: alanine, serine, or cysteine;

(h) or a position corresponding thereto: glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine or, arginine;

(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

(j) or a position corresponding thereto: arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine.

Brief Description of the Drawings

[0012]

[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 amino acid residue at the position 337 has been substituted.

Detailed Description of the Invention

[0013]

The present invention is directed to provision of an alkaline protease variant having an enhanced stability in liquid detergents.

[0014]

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.

[0015]

The present invention 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.

[0016]

The alkaline protease variant of the present invention 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 invention may be a wild-type variant or an artificially created variant.

[0017]

In the present invention, 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).

[0018]

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.

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.

[0019]

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].

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.

[0020]

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

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) exhibiting at least 80% residual activity after treatment at 50°C and a pH of 10 for 10 minutes;

3) inhibited by diisopropylfluorophosphoric acid (DFP) or phenylmethanesulfonyl fluoride (PMSF); and

4) having a molecular weight of $43,000 \pm 2,000$ determined by SDS-PAGE.

[0021]

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.

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.

[0022]

The alkaline protease variant of the present invention 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.

[0023]

The alkaline protease variant of the present invention 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.

[0024]

For example, in the alkaline protease variant of the present invention, 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.

[0025]

Preferably, in the alkaline protease variant of the present invention, 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.

More preferably, in the alkaline protease variant of the present invention, 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 82 or 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.

[0026]

In the present invention, "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.

[0027]

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:

(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;

(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;

(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;

(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;

(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;

(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;

(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;

(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;

(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

(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.

[0028]

The alkaline protease variant of the present invention 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).

[0029]

The alkaline protease variant of the present invention 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.

[0030]

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).

[0031]

The method for producing the protease variant of the present invention 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.

[0032]

The thus-produced alkaline protease variant of the present invention 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 $\pm$ 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 invention, 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 invention, 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 invention, 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).

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

[0033]

No particular limitation is imposed on the amount of the alkaline protease variant of the present invention 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.

[0034]

The detergent composition of the present invention may contain, in addition to the alkaline protease variant of the

present invention, 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 invention, 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).

[0035]

When a protease other than the alkaline protease variant of the present invention is incorporated into a detergent composition in combination with the alkaline protease variant, 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.

[0036]

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.

[0037]

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.

[0038]

The detergent composition of the present invention may contain a known detergent component, examples of which include the following.

[0039]

(1) Surfactant

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 invention 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%.

[0040]

Examples of the surfactant to be employed in the detergent composition of the present invention 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.

[0041]

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 invention, 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.

[0042]

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.

[0043]

(2) Divalent Metal-Ion Trapping Agent

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 invention 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 .

[0044]

(3) Alkaline Agent

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.

[0045]

(4) Anti-redeposition Agent

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 invention 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.

[0046]

(5) Bleaching Agent

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 tetraacetylenediamine (TAED) or one described in JP-A-H06-316700 may be incorporated in an amount of 0.01 to 10 mass%.

[0047]

(6) Fluorescent Agent

Examples of the fluorescent agent to be employed in the detergent composition of the present invention 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%.

[0048]

(7) Other Components

The detergent composition of the present invention 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.

[0049]

The detergent composition of the present invention can be produced through a customary method using the above-obtained alkaline protease variant of the present invention 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.

[0050]

The thus-produced detergent composition of the present invention 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

[0051]

The present invention will next be described in more detail by way of examples.

Example 1

Preparation of KP43 protease

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

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.

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.

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.

[0052]

Example 2

Production of KP43 protease variants

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.

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.

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.

[0053]

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
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
S16I	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
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
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
T82I	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

[0054]

Example 3

Method for determining protease activity

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 μ L/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

$\mu\text{L}/\text{well}$), 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.

[0055]

Example 4

Evaluation of stability of variants

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 $\mu\text{L}/\text{well}$). 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 $\mu\text{L}/\text{well}$), 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 $\mu\text{L}/\text{well}$) to which a substrate solution had been added (50 $\mu\text{L}/\text{well}$). 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%.

Figs. 1 to 10 show the stabilities of wild-type protease and KP43 protease variants.

CLAIMS

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:

(a) or a position corresponding thereto: tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine;

(b) or a position corresponding thereto: glutamic acid, methionine, aspartic acid, valine, glutamine, arginine, cysteine, tryptophan, alanine, or phenylalanine;

(c) or a position corresponding thereto: methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine;

(d) or a position corresponding thereto: tryptophan;

(e) or a position corresponding thereto: histidine, tryptophan, serine, or leucine;

(f) or a position corresponding thereto: alanine, glutamic acid, glutamine, serine, cysteine, glycine,

histidine, lysine, arginine, methionine, or asparagine;

(g) or a position corresponding thereto: alanine, serine, or cysteine;

(h) or a position corresponding thereto: glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine or, arginine;

(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

(j) or a position corresponding thereto: arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine.

2. The alkaline protease variant according to claim 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. A gene encoding the alkaline protease variant as recited in claim 1 or 2.

4. A recombinant vector comprising the gene as recited

in claim 3.

5. A transformant comprising the recombinant vector as recited in claim 4.

6. The transformant according to claim 5, whose host is a microorganism.

7. A detergent composition comprising the alkaline protease variant as recited in claim 1 or 2.

8. The detergent composition according to claim 7, which comprises an anionic surfactant.

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:

(a) or a position corresponding thereto: tryptophan, leucine, valine, isoleucine, methionine, tyrosine, glutamine, lysine, threonine, phenylalanine, arginine, serine, cysteine, alanine, or histidine;

(b) or a position corresponding thereto: glutamic acid, methionine, aspartic acid, valine, glutamine, arginine,

cysteine, tryptophan, alanine, or phenylalanine;

(c) or a position corresponding thereto: methionine, glutamic acid, arginine, valine, lysine, phenylalanine, tyrosine, isoleucine, histidine, aspartic acid, or cysteine;

(d) or a position corresponding thereto: tryptophan;

(e) or a position corresponding thereto: histidine, tryptophan, serine, or leucine;

(f) or a position corresponding thereto: alanine, glutamic acid, glutamine, serine, cysteine, glycine, histidine, lysine, arginine, methionine, or asparagine;

(g) or a position corresponding thereto: alanine, serine, or cysteine;

(h) or a position corresponding thereto: glutamic acid, aspartic acid, cysteine, valine, threonine, proline, histidine, isoleucine, tryptophan, serine, asparagine, lysine or, arginine;

(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

(j) or a position corresponding thereto: arginine, glycine, serine, lysine, glutamine, threonine, histidine, alanine, cysteine, or valine.

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

Fig. 1

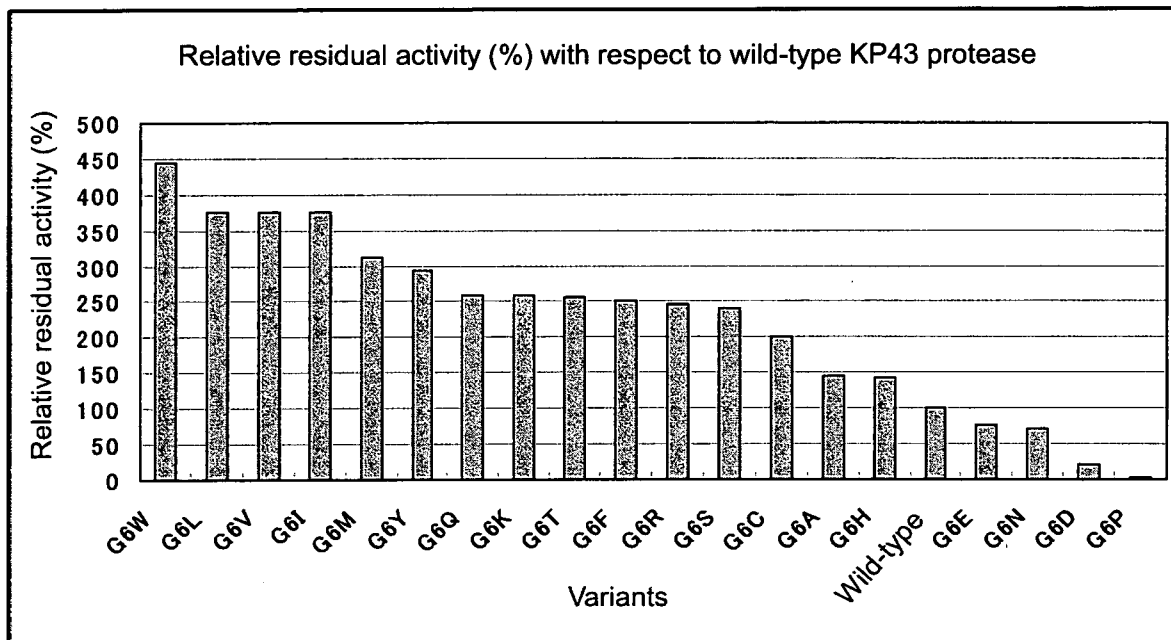


Fig. 2

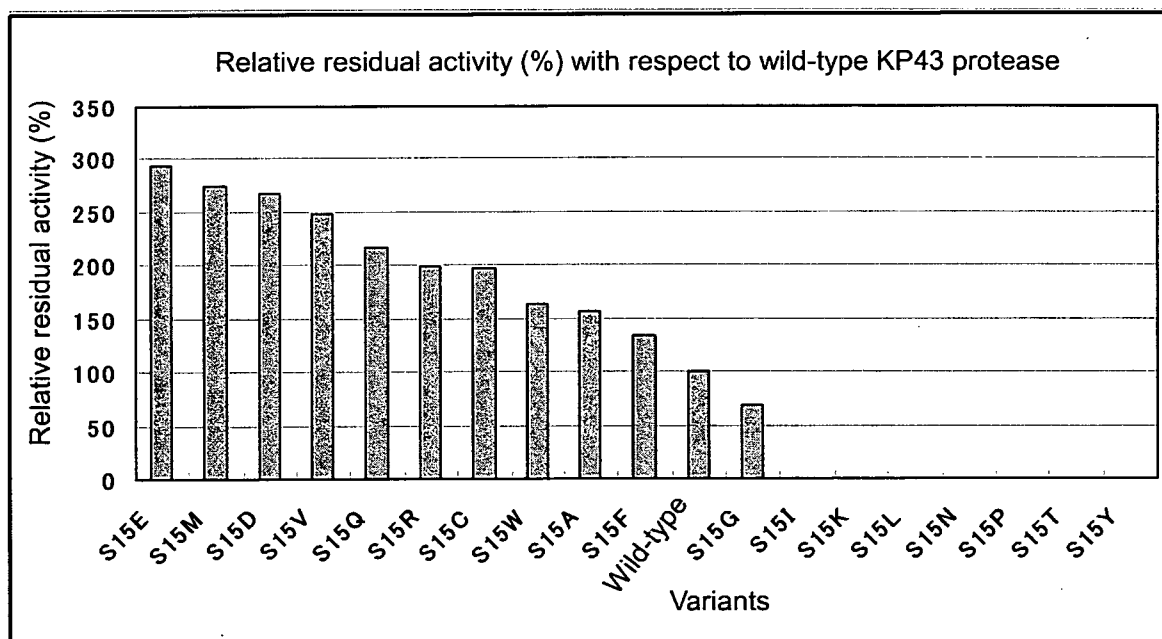


Fig. 3

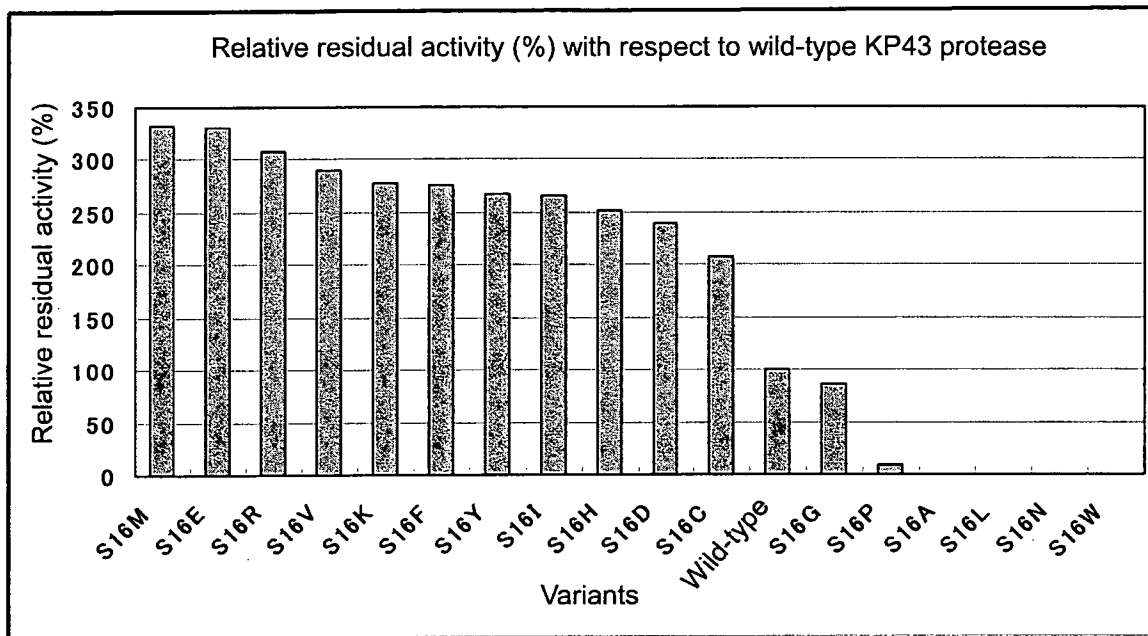


Fig. 4

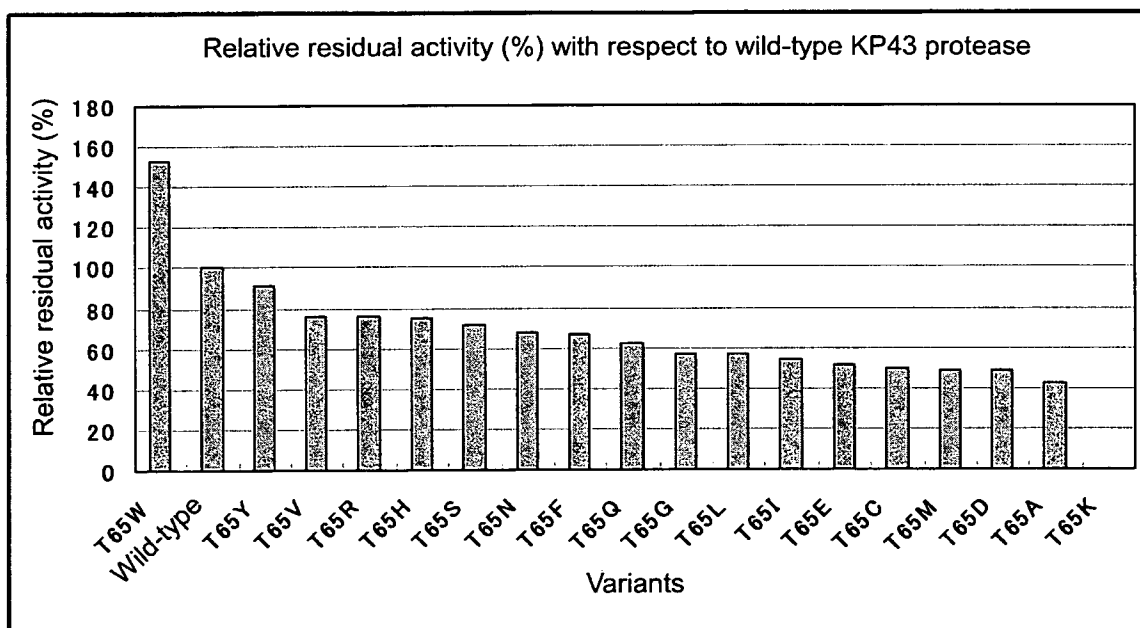


Fig. 5

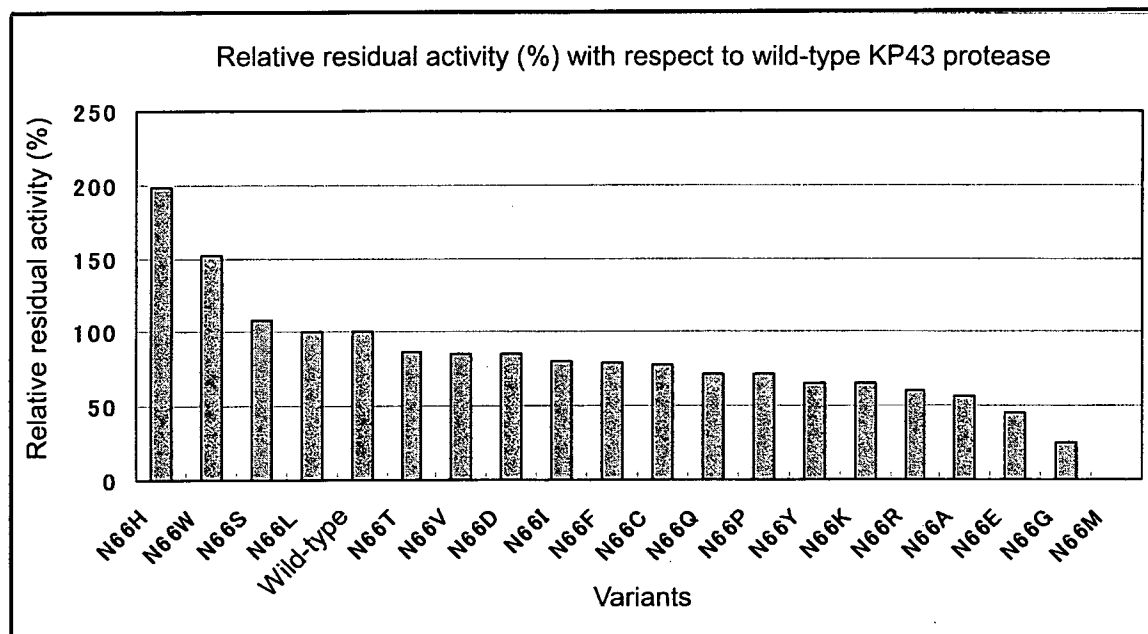


Fig. 6

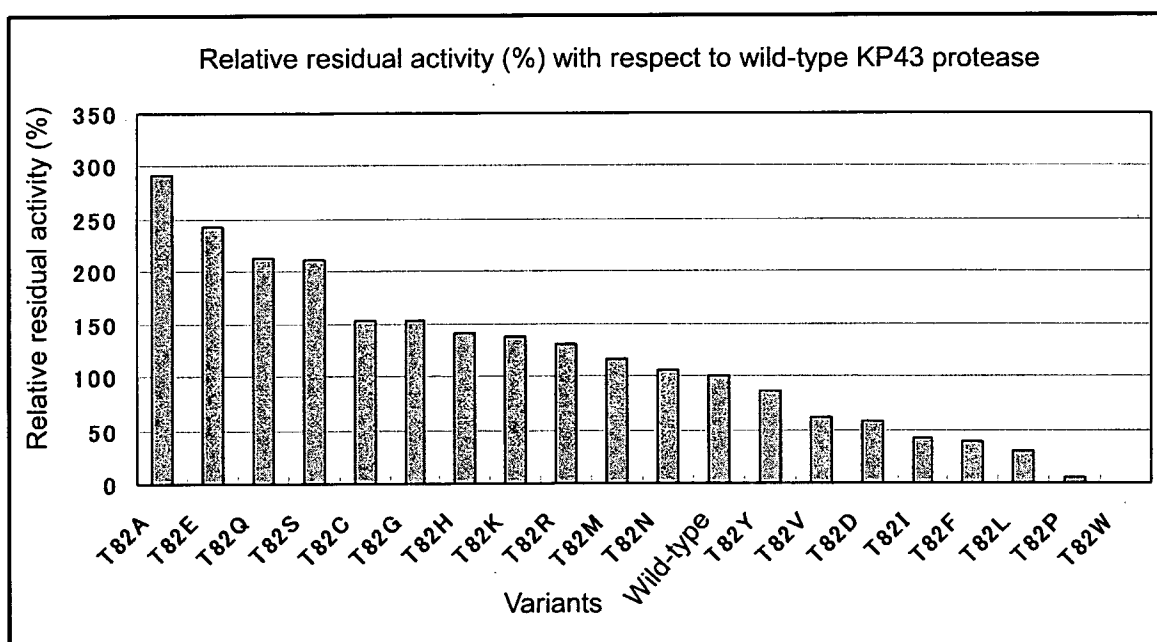


Fig. 7

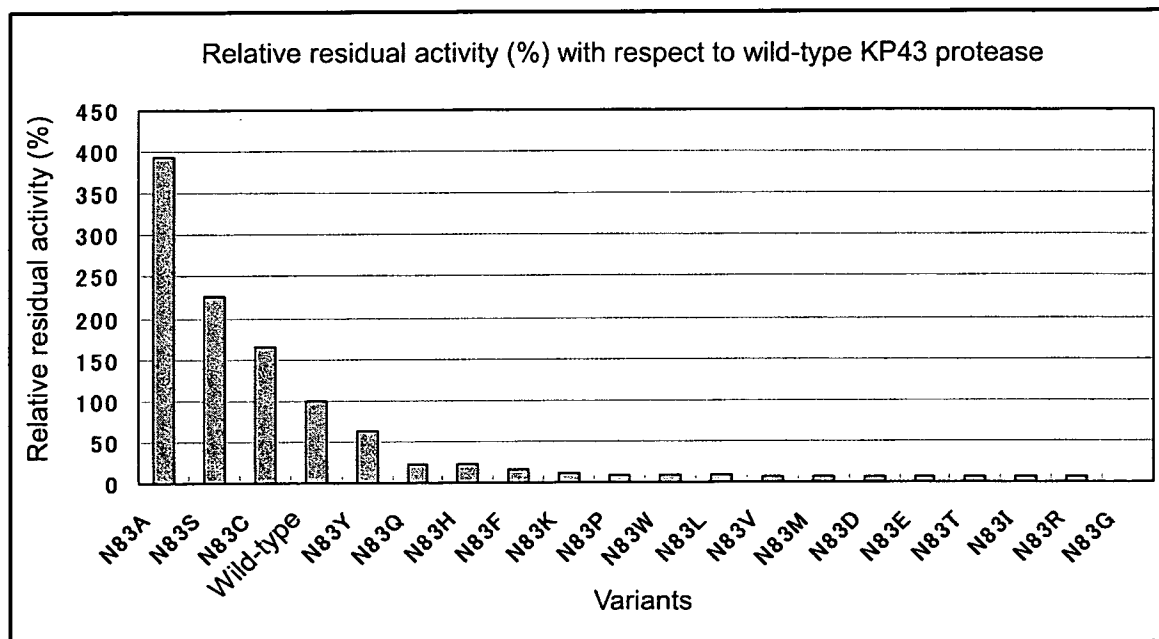


Fig. 8

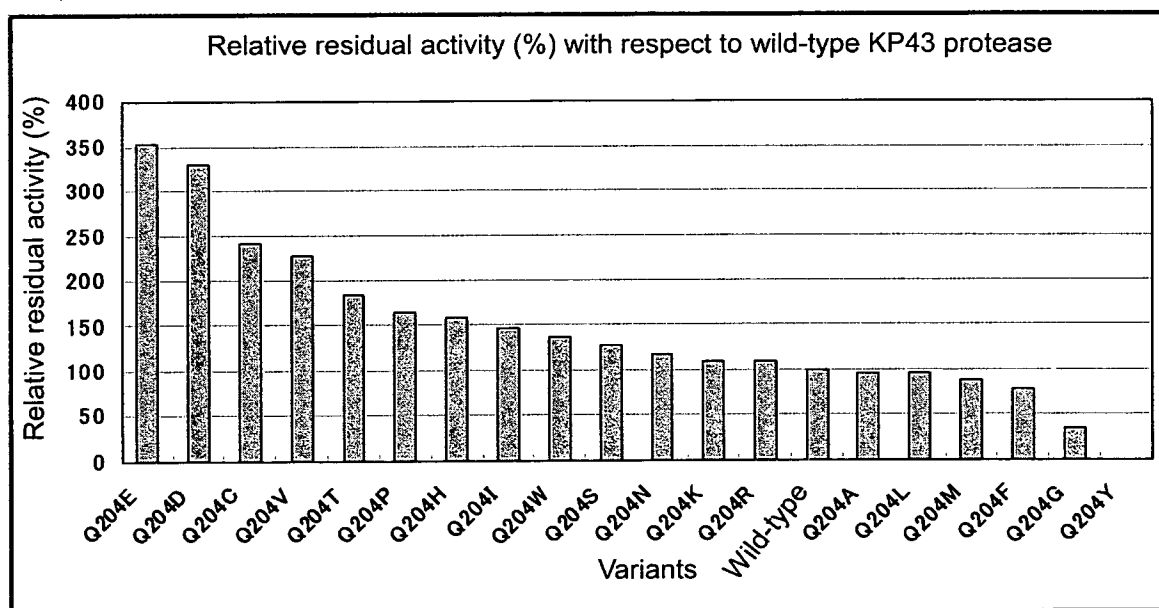


Fig. 9

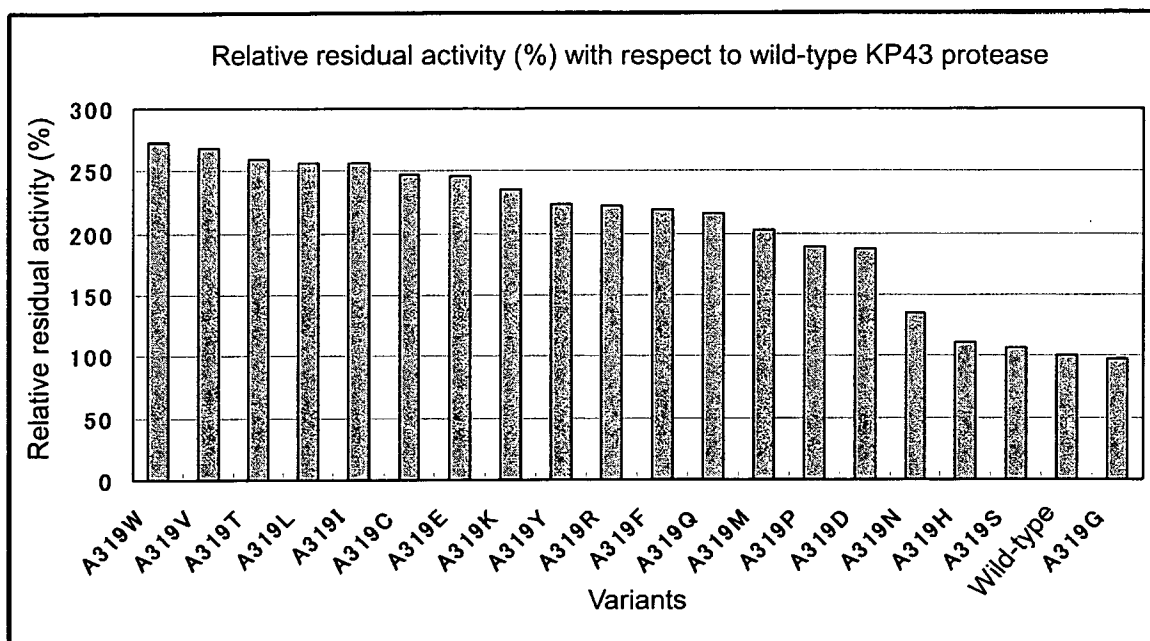


Fig. 10

