(19) DANMARK

(10) **DK/EP 3679133 T3**

(12) Oversættelse af europæisk patentskrift

Patent- og Varemærkestyrelsen

(51) Int.Cl.: ***C 12 N 9/54 (2006.01)*** ***C 11 D 3/386 (2006.01)***

(45) Oversættelsen bekendtgjort den: **2025-02-10**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2024-11-06**

(86) Europæisk ansøgning nr.: **18765860.4**

(86) Europæisk indleveringsdag: **2018-09-05**

(87) Den europæiske ansøgnings publiceringsdag: **2020-07-15**

(86) International ansøgning nr.: **EP2018073893**

(87) Internationalt publikationsnr.: **WO2019048495**

(30) Prioritet: **2017-09-05 DE 102017215628** **2018-06-05 DE 102018208778**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **Henkel AG & Co. KGaA, Henkelstrasse 67, 40589 Düsseldorf, Tyskland**

(72) Opfinder: **Fernandez, Layla, Wiener Weg 14, 50858 Köln, Tyskland**
MUSSMANN, Nina, Am Kuhbusch 109, 47877 Willich, Tyskland
Prueser, Inken, Remscheider Str. 10, 40215 Düsseldorf, Tyskland
EGGERT, Thorsten, Im Poth 45, 45529 Hattingen, Tyskland
Griemert, Sabine, Tirat-Carmel-Str.10, 40789 Monheim am Rhein, Tyskland
HERBST, Daniela, Adlerstr. 6, 40211 Düsseldorf, Tyskland
Strauss, Daria, Rathelbeckstr. 296, 40627 Düsseldorf, Tyskland
LEGGEWIE, Christian, Kurfürstenstr. 20, 45479 Mülheim an der Ruhr, Tyskland
Wieland, Susanne, Schloßstraße 25, 41541 Zons/Dormagen, Tyskland
DEGERING, Christian, Hauptstraße 53, 40699 Erkrath, Tyskland

(74) Fuldmægtig i Danmark: **CHAS. HUDE A/S, Langebrogade 3B, 1411 København K, Danmark**

(54) Benævnelse: **Ydelsesforbedrede proteasevarianter**

(56) Fremdragne publikationer:
WO-A1-2011/036263
WO-A1-2017/046020
WO-A1-2017/162429
WO-A1-2017/198488
DE-A1- 102007 051 092
MIYAJI T ET AL: "Purification and molecular characterization of subtilisin-like alkaline protease BPP-A from Bacillus pumilus strain MS-1", LETTERS IN APPLIED MICROBIOLOGY, OXFORD, GB, vol. 42, no. 3, 1 March 2006 (2006-03-01), pages 242 - 247, XP002451280, ISSN: 1472-765X, DOI: 10.1111/J.1472-765X.2005.01851.X
"RecName: Full=Subtilisin Carlsberg; EC=3.4.21.62; Flags: Precursor", UNIPROT,, 21 July 1986 (1986-07-21), XP002769870

Fortsættes ...

PHILIP N. BRYAN: "Protein engineering of subtilisin", BIOCHIMICA ET BIOPHYSICA ACTA. PROTEIN STRUCTURE AND MOLECULAR ENZYMOLOGY, vol. 1543, no. 2, 1 December 2000 (2000-12-01), AMSTERDAM; NL, pages 203 - 222, XP055251064, ISSN: 0167-4838, DOI: 10.1016/S0167-4838(00)00235-1
VON DER OSTEN C ET AL: "Protein engineering of subtilisins to improve stability in detergent formulations", JOURNAL OF BIOTECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 28, no. 1, 1 March 1993 (1993-03-01), pages 55 - 68, XP023944106, ISSN: 0168-1656, [retrieved on 19930301], DOI: 10.1016/0168-1656(93)90125-7
LJUBICA VOJCIC ET AL: "Advances in protease engineering for laundry detergents", NEW BIOTECHNOLOGY, vol. 32, no. 6, 1 December 2015 (2015-12-01), NL, pages 629 - 634, XP055327595, ISSN: 1871-6784, DOI: 10.1016/j.nbt.2014.12.010
DATABASE Geneseq [online] 11 January 2018 (2018-01-11), "Bacillus pumilus protease mutant (P9T/S216C/Q271E/G131S), SEQ ID 12.", retrieved from EBI accession no. GSP:BEQ58494 Database accession no. BEQ58494
DATABASE Geneseq [online] 16 November 2017 (2017-11-16), "Bacillus pumilus protease mutant P9T/N187H/S216C/Q271E, SEQ 11.", retrieved from EBI accession no. GSP:BEI87382 Database accession no. BEI87382

Performance-enhanced protease variants I

Description

[0001] The invention is in the field of enzyme technology. The invention relates to proteases from *Bacillus pumilus* of which the amino acid sequences have been altered, in particular with regard to the use in washing and cleaning agents, to give them better cleaning performance at low temperatures (e.g., between 20 °C and 40 °C), and also relates to the nucleic acids coding therefor and to the production thereof. The invention further relates to the uses of said proteases and methods in which they are used, and agents containing said proteases, in particular washing and cleaning agents.

[0002] Proteases are among the technically most important enzymes. For washing and cleaning agents, they are the longest established enzymes and are contained in virtually all modern, high-performance washing and cleaning agents. They cause the degradation of protein-containing stains on the articles to be cleaned. In turn, proteases of the subtilisin type (subtilases, subtilopeptidases, EC 3.4.21.62) are particularly important, which proteases are serine proteases due to the catalytically active amino acids. They act as unspecific endopeptidases and hydrolyze any acid amide bonds within peptides or proteins. Their optimum pH is usually in the distinctly alkaline range. The article “Subtilases: Subtilisin-like Proteases” by R. Siezen, pages 75-95 in “Subtilisin enzymes,” published by R. Bott and C. Betzel, New York, 1996, gives an overview of this family, for example. Subtilases are naturally formed by microorganisms. Among these, the subtilisins formed and secreted by the Bacillus species in particular are the most significant group of subtilases.

[0003] Examples of the subtilisin proteases preferably used in washing and cleaning agents are the subtilisins BPN' and Carlsberg, the protease PB92, the subtilisins 147 and 309, the protease from *Bacillus lentus*, in particular from *Bacillus lentus* DSM 5483, subtilisin DY and the enzymes thermitase, proteinase K and the proteases TW3 and TW7, which can be classified as subtilases but no longer as subtilisins in the narrower sense, and variants of said proteases having an amino acid sequence that has been altered with respect to the starting protease. Proteases are altered in a targeted or random manner by methods known from the prior art and are thus optimized for use in washing and cleaning agents, for example. These include point mutagenesis, deletion or insertion mutagenesis or fusion with other proteins or protein parts. Thus, appropriately optimized variants are known for most proteases known from the prior art. For example, Savinase and/or BPN' variants are known from WO 2011/036263.

[0004] EP 2016175 A1, WO 2017/198488 and WO 2017/162429 disclose a protease a from *Bacillus pumilus* intended for use in washing and cleaning agents. In general, only selected proteases are suitable for use in liquid surfactant-containing preparations. Many proteases do not exhibit sufficient catalytic performance in such preparations. For the use of proteases in cleaning

agents, therefore, a high catalytic activity under conditions as they are during a wash cycle and a high storage stability is particularly desirable.

[0005] Consequently, protease and surfactant-containing liquid formulations from the prior art are disadvantageous in that the proteases contained, under standard washing conditions (e.g., in a temperature range of from 20 °C to 40 °C), do not have satisfactory proteolytic activity or are not storage-stable and the formulations therefore do not exhibit optimal cleaning performance on protease-sensitive stains.

[0006] Surprisingly, it has now been found that a protease from *Bacillus pumilus* or a sufficiently similar protease (based on the sequence identity) that has amino acid substitutions at positions corresponding to position 271 and 9, in each case based on the numbering according to SEQ ID NO:1, and at at least one of the positions corresponding to positions 18, 29, 48, 61, 92, 101, 130, 131, 133, 137, 144, 149, 156, 162, 166, 172, 192, 217, 224, 252 or 265, in each case based on the numbering according to SEQ ID NO:1, is improved in terms of proteolytic activity in standard washing conditions compared with the wild-type form and/or reference mutants and is therefore particularly suitable for use in washing or cleaning agents.

[0007] In a first aspect, the invention therefore relates to a protease according to claim 1, comprising an amino acid sequence which has at least 90% sequence identity with the amino acid sequence specified in SEQ ID NO:1 over the entire length thereof and in each case based on the numbering according to SEQ ID NO:1:

(a) at the position corresponding to position 271, the amino acid substitution 271E; and
(b) at the position corresponding to position 9, an amino acid substitution selected from 9T, 9H, 9S and 9A, preferably 9T; and
(c1) at at least one of the positions corresponding to positions 18, 61, 92, 99, 137, 149, 156, 159, 162, 166, 172, 192, 199, 217 or 265, at least one further amino acid substitution selected from 18D, 61Y, 92S, 99Y, 137K, 1491, 156G, 156Y, 1591, 162S, 166M, 172G, 172P, 192V, 199M, 217M and 265A; and/or
(c2) at at least three of the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 217, 224 and 252, in particular 130, 133, 144, 217 and 252, at least three further amino acid substitutions, selected from 29G, 48V, 101E, 130D, 130S, 130H, 131D, 131N, 131S, 131K, 133K, 133R, 133Y, 144K, 144L, 144A, 224A, 224T, 252S and 252T.

[0008] The invention also relates to a method for preparing a protease as defined above.

[0009] A protease within the meaning of the present patent application therefore comprises both the protease as such and a protease produced using a method according to the invention. All

statements regarding the protease therefore relate both to the protease as such and to the proteases produced by means of corresponding methods. The proteases described herein have features (a) and (b) and either (c1) or (c2) or (a), (b), (c1) and (c2).

[0010] Further aspects of the invention relate to the nucleic acids coding for these proteases, non-human host cells containing proteases or nucleic acids according to the invention, as well as agents comprising proteases according to the invention, in particular washing and cleaning agents, washing and cleaning methods, and uses of the proteases according to the invention in washing or cleaning agents for removing protein-containing stains.

[0011] “At least one,” as used herein, refers to one or more, i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or more.

[0012] When the protease is defined herein such that it includes "the amino acid substitutions 9T, 9H, 9S or 9A," this means that position 9 is mutated to either T, H, S or A. Thus, the phrase whereby the protease comprises “the amino acid substitutions 9T, 9H, 9S or 9A and 271E and optionally 216C" means that position 9 is mutated to either T, H, S or A, position 271 is mutated to E and position 216 is optionally mutated to C.

[0013] The present invention is based on the surprising finding by the inventors that amino acid substitutions at the positions described herein bring about improved performance of this altered protease (proteolytic activity under standard washing conditions) in washing and cleaning agents. Optionally, improved storage stability of this altered protease can additionally be brought about in washing and cleaning agents. This is surprising in particular insofar as none of the above-mentioned amino acid substitutions was previously linked to an increased catalytic activity and/or increased storage stability of the protease.

[0014] In preferred embodiments of the protease according to the invention, the protease has amino acid substitutions

(A) (i) at the positions corresponding to positions 9, 130, 133 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 131, 144, 224 or 252, in each case based on the numbering according to SEQ ID NO:1;

(B) (i) at the positions corresponding to positions 9, 130, 144 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 131, 133, 224 or 252, in each case based on the numbering according to SEQ ID NO:1;

(C) (i) at the positions corresponding to positions 9, 130, 252 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 131, 133, 144 or 224, in each case based on the numbering according to SEQ ID NO:1;

(D) (i) at the positions corresponding to positions 9, 133, 144 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 130, 131, 224 or 252, in each case based on the numbering according to SEQ ID NO:1;

(E) (i) at the positions corresponding to positions 9, 133, 252 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 130, 131, 144 or 224, in each case based on the numbering according to SEQ ID NO:1;

(F) at the positions corresponding to positions 9, 144, 252 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 130, 131, 133 or 224, in each case based on the numbering according to SEQ ID NO:1;

(G) at the positions corresponding to positions 9, 130, 144, 252 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 131, 133 or 224, in each case based on the numbering according to SEQ ID NO:1;

(H) at the positions corresponding to positions 9, 133, 144, 252 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 130, 131, or 224, in each case based on the numbering according to SEQ ID NO:1;

(I) at the positions corresponding to positions 9, 130, 133, 252 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 131, 144 or 224, in each case based on the numbering according to SEQ ID NO:1;

(J) at the positions corresponding to positions 9, 130, 133, 144 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 131, 224 or 252, in each case based on the numbering according to SEQ ID NO:1; or

(K) at the positions corresponding to positions 9, 130, 133, 144, 252 and 271, in each case based on the numbering according to SEQ ID NO:1, and optionally (ii) at at least one of the positions corresponding to positions 29, 48, 101, 131 or 224, in each case based on the numbering according to SEQ ID NO:1.

[0015] In further embodiments, preferred proteases are those having amino acid substitutions at the positions:

9 and 271 and at least one of 131, 133, 224, 130 and 144, and optionally at least one of 29, 48, 101 and 252;

9 + 271 + 131 + 224 and optionally at least one of 29, 48, 101, 130, 133, 144 or 252;

9 + 271 + 131 + 133 and optionally at least one of 29, 48, 101, 130, 144, 224 or 252;

9 + 271 +131 + 133 + 224 and optionally at least one of 29, 48, 101, 130, 144 or 252; or

9 + 271 + 131 + 133 + 224 + 130 and optionally at least one of 29, 48, 101, 144 or 252;

[0016] In various embodiments, the protease has

(1) at the position corresponding to position 271, an amino acid substitution, in particular the amino acid substitution 271E; and

(2–1) at the positions corresponding to positions 9, 130, 144 and 252, the amino acid substitutions 9T, 130D, 144K and 252T; and/oder

(2–2) at one or more of the positions corresponding to positions 18, 61, 92, 99, 137, 149, 156, 159, 162, 166, 172, 192, 199, 217 or 265, one or more amino acid substitutions selected from: 18D, 61Y, 92S, 99Y, 137K, 1491, 156G, 156Y, 159I, 162S, 166M, 172G, 172P, 192V, 199M, 217M and 265A

. Feature (1) can be combined with feature (2–1), feature (2–2) or both.

[0017] In different embodiments, the protease has an amino acid substitution, in particular the amino acid substitution 271E, at the position corresponding to position 271; and has the amino acid substitutions 9T, 130D, 144K and 252T at the positions corresponding to positions 9, 130, 144 and 252; and optionally has at least one, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13, for example 1, 2, 3 or 4, further amino acid substitution(s) at one or more of the positions corresponding to positions 62, 99, 133, 137, 149, 156, 162, 166, 172, 192, 199, 217, 224 or 265, these being selected from: 62S, 99Y, 133R, 133K, 133A, 137K, 149I, 156G, 156Y, 162S, 166M, 172G, 172P, 192V, 199M, 217M, 224A and 265A. Such proteases are disclosed, for example, in example 1 and are thus part of the subject matter of the invention. Particularly preferred are those proteases which have an amino acid substitution, in particular the amino acid substitution 271E, at the position corresponding to position 271; and have the amino acid substitutions 9T, 130D, 144K and 252T at the positions corresponding to positions 9, 130, 144 and 252; and optionally have at least one, for example 1, 2, 3, 4, 5, 6 or 7, for example 1, 2, 3 or 4, further amino acid substitution(s) at one or more of the positions corresponding to positions 62, 133, 137, 162, 192, 217 or 265, these being preferably selected from: 62S, 133R, 133K, 133A, 137K, 162S, 192V, 217M and 224A.

[0018] In further embodiments, the protease has amino acid substitutions at the following positions (in the numbering according to SEQ ID NO:1):

9 + 271 + 29 + 48 + 101
9 + 271 + 29 + 48 + 130
9 + 271 + 29 + 48 + 131
9 + 271 + 29 + 48 + 133
9 + 271 + 29 + 48 + 144
9 + 271 + 29 + 48 + 224
9 + 271 + 29 + 48 + 252
9 + 271 + 29 + 101 + 130
9 + 271 + 29 + 101 + 131
9 + 271 + 29 + 101 + 133
9 + 271 + 29 + 101 + 144
9 + 271 + 29 + 101 + 224
9 + 271 + 29 + 101 + 252
9 + 271 + 29 + 130 + 131
9 + 271 + 29 + 130 + 133
9 + 271 + 29 + 130 + 144
9 + 271 + 29 + 130 + 224
9 + 271 + 29 + 130 + 252
9 + 271 + 29 + 131 + 133
9 + 271 + 29 + 131 + 144
9 + 271 + 29 + 131 + 224
9 + 271 + 29 + 131 + 252

are selected from: 62S, 99Y, 133R, 133K, 133A, 137K, 149I, 156G, 156Y, 162S, 166M, 172G, 172P, 192V, 199M, 217M, 224A and 265A. Such proteases are disclosed, for example, in example 1 and are thus part of the subject matter of the invention. Particularly preferred are those proteases which have an amino acid substitution, in particular the amino acid substitution 271E, at the position corresponding to position 271; and have the amino acid substitutions 9T, 130D, 144K and 252T at the positions corresponding to positions 9, 130, 144 and 252; and optionally have at least one, for example 1, 2, 3, 4, 5, 6 or 7, for example 1, 2, 3 or 4, further amino acid substitution(s) at one or more of the positions corresponding to positions 62, 133, 137, 162, 192, 217 or 265, these being preferably selected from: 62S, 133R, 133K, 133A, 137K, 162S, 192V, 217M and 224A.

[0019] In further embodiments, the protease has amino acid substitutions at the following positions (in the numbering according to SEQ ID NO:1):

9 + 271 + 29
9 + 271 + 48

9 + 271 + 101
9 + 271 + 130
9 + 271 + 131
9 + 271 + 133
9 + 271 + 144
9 + 271 + 224
9 + 271 + 252
9 + 271 + 29 + 48
9 + 271 + 29 + 101
9 + 271 + 29 + 130
9 + 271 + 29 + 131
9 + 271 + 29 + 133
9 + 271 + 29 + 144
9 + 271 + 29 + 224
9 + 271 + 29 + 252
9 + 271 + 48 + 101
9 + 271 + 48 + 130
9 + 271 + 48 + 131
9 + 271 + 48 + 133
9 + 271 + 48 + 144
9 + 271 + 48 + 224
9 + 271 + 48 + 252
9 + 271 + 101 + 130
9 + 271 + 101 + 131
9 + 271 + 101 + 133
9 + 271 + 101 + 144
9 + 271 + 101 + 224
9 + 271 + 101 + 252
9 + 271 + 130 + 131
9 + 271 + 130 + 133
9 + 271 + 130 + 144
9 + 271 + 130 + 224
9 + 271 + 130 + 252
9 + 271 + 131 + 133
9 + 271 + 131 + 144
9 + 271 + 131 + 224
9 + 271 + 131 + 252
9 + 271 + 133 + 144
9 + 271 + 133 + 224

9 + 271 + 133 + 252
9 + 271 + 144 + 224
9 + 271 + 144 + 252
9 + 271 + 224 + 252
9 + 271 + 29 + 48 + 101
9 + 271 + 29 + 48 + 130
9 + 271 + 29 + 48 + 131
9 + 271 + 29 + 48 + 133
9 + 271 + 29 + 48 + 144
9 + 271 + 29 + 48 + 224
9 + 271 + 29 + 48 + 252
9 + 271 + 29 + 101 + 130
9 + 271 + 29 + 101 + 131
9 + 271 + 29 + 101 + 133
9 + 271 + 29 + 101 + 144
9 + 271 + 29 + 101 + 224
9 + 271 + 29 + 101 + +252
9+271 + 29 + 130 + 131
9 + 271 + 29 + 130 + 133
9 + 271 + 29 + 130 + 144
9 + 271 + 29 + 130 + 224
9 + 271 + 29 + 130 + 252
9 + 271 + 29 + 131 + 133
9 + 271 + 29 + 131 + 144
9 + 271 + 29 + 131 + 224
9 + 271 + 29 + 131 + +252
9 + 271 + 29 + 133 + 144
9 + 271 + 29 + 133 + 224
9 + 271 + 29 + 133 + 252
9 + 271 + 29 + 144 + 224
9 + 271 + 29 + 144 + 252
9 + 271 + 29 + 224 + 252
9 + 271 + 48 + 101 + 130
9 + 271 + 48 + 101 + 131
9 + 271 + 48 + 101 + 133
9 + 271 + 48 + 101 + 144
9 + 271 + 48 + 101 + 224
9 + 271 + 48 + 101 + 252
9 + 271 + 48 + 130 + 131

9 + 271 + 48 + 130 + 133
9 + 271 + 48 + 130 + 144
9 + 271 + 48 + 130 + 224
9 + 271 + 48 + 130 + 252
9 + 271 + 48 + 131 + 133
9 + 271 + 48 + 131 + 144
9 + 271 + 48 + 131 + 224
9 + 271 + 48 + 131 + 252
9 + 271 + 48 + 133 + 144
9 + 271 + 48 + 133 + 224
9 + 271 + 48 + 133 + 252
9 + 271 + 48 + 144 + 224
9 + 271 + 48 + 144 + 252
9 + 271 + 48 + 224 + 252
9 + 271 + 101 + 130 + 131
9 + 271 + 101 + 130 + 133
9 + 271 + 101 + 130 + 144
9 + 271 + 101 + 130 + 224
9 + 271 + 101 + 130 + 252
9 + 271 + 101 + 131 + 133
9 + 271 + 101 + 131 + 144
9 + 271 + 101 + 131 + 224
9 + 271 + 101 + 131 + 252
9 + 271 + 101 + 133 + 144
9 + 271 + 101 + 133 + 224
9 + 271 + 101 + 133 + 252
9 + 271 + 101 + 144 + 224
9 + 271 + 101 + 144 + 252
9 + 271 + 101 + 224 + 252
9 + 271 + 130 + 131 + 133
9 + 271 + 130 + 131 + 144
9 + 271 + 130 + 131 + 224
9 + 271 + 130 + 131 + 252
9 + 271 + 130 + 133 + 144
9 + 271 + 130 + 133 + 224
9 + 271 + 130 + 133 + 252
9 + 271 + 130 + 144 + 224
9 + 271 + 130 + 144 + 252
9 + 271 + 130 + 224 + 252

9 + 271 + 131 + 133 + 144
9 + 271 + 131 + 133 + 224
9 + 271 + 131 + 133 + 252
9 + 271 + 131 + 144 + 224
9 + 271 + 131 + 144 + 252
9 + 271 + 131 + 224 + 252
9 + 271 + 133 + 144 + 224
9 + 271 + 133 + 144 + 252
9 + 271 + 133 + 224 + 252
9 + 271 + 144 + 224 + 252
9 + 271 + 29 + 48 + 101 + 130
9 + 271 + 29 + 48 + 101 + 131
9 + 271 + 29 + 48 + 101 + 133
9 + 271 + 29 + 48 + 101 + 144
9 + 271 + 29 +48 + 101 + 224
9 + 271 + 29 +48 + 101 + 252
9 + 271 + 29 + 48 + 130 + 131
9 + 271 + 29 + 48 + 130 + 133
9 + 271 + 29 + 48 + 130 + 144
9 + 271 + 29 + 48 + 130 + 224
9 + 271 + 29 + 48 + 130 + 252
9 + 271 + 29 + 48 + 131 + 133
9 + 271 + 29 + 48 + 131 + 144
9 + 271 + 29 + 48 + 131 + 224
9 + 271 + 29 + 48 + 131 + 252
9 + 271 + 29 + 48 + 133 + 144
9 + 271 + 29 + 48 + 133 + 224
9 + 271 + 29 + 48 + 133 + 252
9 + 271 + 29 + 101 + 130 + 131
9 + 271 + 29 + 101 + 130 + 133
9 + 271 + 29 + 101 + 130 + 144
9 + 271 + 29 + 101 + 130 + 224
9 + 271 + 29 + 101 + 130 + 252
9 + 271 + 29 + 101 + 131 + 133
9 + 271 + 29 + 101 + 131 + 144
9 + 271 + 29 + 101 + 131 + 224
9 + 271 + 29 + 101 + 131 + 252
9 + 271 + 29 + 101 + 133 + 144
9 + 271 + 29 + 101 + 133 + 224

9 + 271 + 29 + 101 + 133 + 252
9 + 271 + 29 + 101 + 144 + 224
9 + 271 + 29 + 101 + 144 + 252
9 + 271 + 29 + 101 + 224 + 252
9 + 271 + 29 + 130 + 131 + 133
9 + 271 + 29 + 130 + 131 + 144
9 + 271 + 29 + 130 + 131 + 224
9 + 271 + 29 + 130 + 131 + 252
9 + 271 + 29 + 130 + 133 + 144
9 + 271 + 29 + 130 + 133 + 224
9 + 271 + 29 + 130 + 133 +252
9 + 271 + 29 + 130 + 144 + 224
9 + 271 + 29 + 130 + 144 + 252
9 + 271 + 29 + 130 + 224 + 252
9 + 271 + 29 + 131 + 133 + 144
9 + 271 + 29 + 131 + 133 + 224
9 + 271 + 29 + 131 + 133 + 252
9 + 271 + 29 + 131 + 144 + 224
9 + 271 + 29 + 131 + 144 + 252
9 + 271 + 29 + 131 + 224 + 252
9 + 271 + 29 + 133 + 144 + 224
9 + 271 + 29 + 133 + 144 + 252
9 + 271 + 29 + 133 + 224 + 252
9 + 271 + 29 + 144 + 224 + 252
9 + 271 + 48 + 101 + 130 + 131
9 + 271 + 48 + 101 + 130 + 133
9 + 271 + 48 + 101 + 130 + 144
9 + 271 + 48 + 101 + 130 + 224
9 + 271 + 48 + 101 + 130 + 252
9 + 271 + 48 + 101 + 131 + 133
9 + 271 + 48 + 101 + 131 + 144
9 + 271 + 48 + 101 + 131 + 224
9 + 271 + 48 + 101 + 131 + 252
9 + 271 + 48 + 101 + 133 + 144
9 + 271 + 48 + 101 + 133 + 224
9 + 271 + 48 + 101 + 133 + 252
9 + 271 + 48 + 101 + 144 + 224
9 + 271 + 48 + 101 + 144 + 252
9 + 271 + 48 + 101 + 224 +252

9 + 271 + 48 + 130 + 131 + 133
9 + 271 + 48 + 130 + 131 + 144
9 + 271 + 48 + 130 + 131 + 224
9 + 271 + 48 + 130 + 131 + 252
9 + 271 + 48 + 130 + 133 + 144
9 + 271 + 48 + 130 + 133 + 224
9 + 271 + 48 + 130 + 133 + 252
9 + 271 + 48 + 130 + 144 + 224
9 + 271 + 48 + 130 + 144 + 252
9 + 271 + 48 + 130 + 224 + 252
9 + 271 + 48 + 131 + 133 + 144
9 + 271 + 48 + 131 + 133 + 224
9 + 271 + 48 + 131 + 133 + 252
9 + 271 + 48 + 131 + 144 + 224
9 + 271 + 48 + 131 + 144 + 252
9 + 271 + 48 + 131 + 224 + 252
9 + 271 + 48 + 133 + 144 + 224
9 + 271 + 48 + 133 + 144 + 252
9 + 271 + 48 + 133 + 224 + 252
9 + 271 + 48 + 144 + 224 + 252
9 + 271 + 101 + 130 + 131 + 133
9 + 271 + 101 + 130 + 131 + 144
9 + 271 + 101 + 130 + 131 + 224
9 + 271 + 101 + 130 + 131 + 252
9 + 271 + 101 + 130 + 133 + 144
9 + 271 + 101 + 130 + 133 + 224
9 + 271 + 101 + 130 + 133 + 252
9 + 271 + 101 + 130 + 144 + 224
9 + 271 + 101 + 130 + 144 + 252
9 + 271 + 101 + 130 + 224 + 252
9 + 271 + 101 + 131 + 133 + 144
9 + 271 + 101 + 131 + 133 + 224
9 + 271 + 101 + 131 + 133 + 252
9 + 271 + 101 + 131 + 144 + 224
9 + 271 + 101 + 131 + 144 + 252
9 + 271 + 101 + 131 + 224 + 252
9 + 271 + 101 + 133 + 144 + 224
9 + 271 + 101 + 133 + 144 + 252
9 + 271 + 101 + 133 + 224 + 252

9 + 271 + 101 + 144 + 224 + 252
9 + 271 + 130 + 131 + 133 + 144
9 + 271 + 130 + 131 + 133 + 224
9 + 271 + 130 + 131 + 133 + 252
9 + 271 + 130 + 131 + 144 + 224
9 + 271 + 130 + 131 + 144 + 252
9 + 271 + 130 + 131 + 224 + 252
9 + 271 + 130 + 133 + 144 + 224
9 + 271 + 130 + 133 + 144 + 252
9 + 271 + 130 + 133 + 224 + 252
9 + 271 + 130 + 144 + 224 + 252
9 + 271 + 131 + 133 + 144 + 224
9 + 271 + 131 + 133 + 144 + 252
9 + 271 + 131 + 133 + 224 + 252
9 + 271 + 131 + 144 + 224 + 252
9 + 271 + 133 + 144 + 224 + 252

[0020] In various embodiments, the aforementioned variants do not have further substitutions or have only one or more additional substitutions in the positions from the group of positions of 29, 48, 101, 130, 131, 133, 144, 224 and 252, if these have not yet been mentioned above. In further embodiments, in particular in all the embodiments described above, the protease according to the invention has an additional amino acid substitution at the position corresponding to position 217, based on the numbering according to SEQ ID NO:1. This amino acid substitution may be the amino acid substitution 217M.

271 + 92
271 + 99
271 + 137
271 + 149
271 + 156
271 + 159
271 + 162
271 + 166
271 + 172
271 + 192
271 + 199
271 + 217
271 + 265
271 + 18 + 61

271 + 18 + 92
271 + 18 + 99
271 + 18 + 137
271 + 18 + 149
271 + 18 + 156
271 + 18 + 159
271 + 18 + 162
271 + 18 + 166
271 + 18 + 172
271 + 18 + 192
271 + 18 + 199
271 + 18 + 217
271 + 18 + 265
271 + 61 + 92
271 + 61 + 99
271 + 61 + 137
271 + 61 + 149
271 + 61 + 156
271 + 61 + 159
271 + 61 + 162
271 + 61 + 166
271 + 61 + 172
271 + 61 + 192
271 + 61 + 199
271 + 61 + 217
271 + 61 + 265
271 + 92 + 99
271 + 92 + 137
271 + 92 + 149
271 + 92 + 156
271 + 92 + 159
271 + 92 + 162
271 + 92 + 166
271 + 92 + 172
271 + 92 + 192
271 + 92 + 199
271 + 92 + 217
271 + 92 + 265
271 + 99 + 137

271 + 99 + 149
271 + 99 + 156
271 + 99 + 159
271 + 99 + 162
271 + 99 + 166
271 + 99 + 172
271 + 99 + 192
271 + 99 + 199
271 + 99 + 217
271 + 99 + 265
271 + 137 + 149
271 + 137 + 156
271 + 137 + 159
271 + 137 + 162
271 + 137 + 166
271 + 137 + 172
271 + 137 + 192
271 + 137 + 199
271 + 137 + 217
271 + 137 + 265
271 + 149 + 156
271 + 149 + 159
271 + 149 + 162
271 + 149 + 166
271 + 149 + 172
271 + 149 + 192
271 + 149 + 199
271 + 149 + 217
271 + 149 + 265
271 + 156 + 159
271 + 156 + 162
271 + 156 + 166
271 + 156 + 172
271 + 156 + 192
271 + 156 + 199
271 + 156 + 217
271 + 156 + 265
271 + 159 + 162
271 + 159 + 166

271 + 159 + 172
271 + 159 + 192
271 + 159 + 199
271 + 159 + 217
271 + 159 + 265
271 + 162 + 166
271 + 162 + 172
271 + 162 + 192
271 + 162 + 199
271 + 162 + 217
271 + 162 + 265
271 + 166 + 172
271 + 166 + 192
271 + 166 + 199
271 + 166 + 217
271 + 166 + 265
271 + 172 + 192
271 + 172 + 199
271 + 172 + 217
271 + 172 + 265
271 + 192 + 199
271 + 192 + 217
271 + 192 + 265
271 + 199 + 217
271 + 199 + 265
271 + 217 + 265

[0021] In various embodiments, the aforementioned variants do not have further substitutions or have only one or more additional substitutions in the positions from the group of positions of 18, 61, 92, 99, 137, 149, 156, 159, 162, 166, 172, 192, 199, 217 and 265, if these have not yet been mentioned above.

[0022] In all the aforementioned variants, the corresponding exchanges are in particular those mentioned above, i.e., 9T, 9H, 9S, 9A, 29G, 48V, 101E, 130D, 130S, 130H, 131D, 131N, 131S, 131K, 133K, 133R, 133Y, 144K, 144L, 144A, 224A, 224T, 252S, 252T and/or 271E. 9T, 130D, 133R/K, 144K, 252T/S and/or 271E, in particular 9T, 130D, 133R/K, 144K, 252T and 271E or 9T, 130D, 133K, 144K, 252T and 271E, are most particularly preferred. Accordingly, in particular the above-mentioned variants in which the aforementioned substitutions occur are preferred. A variant

which has the substitutions 9T, 130D, 144K, 252T and 271E, and optionally also 133R/K and/or 217M, is further preferred.

[0023] In preferred embodiments of the protease according to the invention, i.e., in particular the variants listed above, the protease has amino acid substitutions at the positions corresponding to positions 18, 61, 92, 99, 137, 149, 156, 159, 162, 166, 172, 192, 199, 217 or 265, which amino acid substitutions are selected from: 18D, 61Y, 92S, 99Y, 137K, 149I, 156G, 156Y, 159I, 162S, 166M, 172G, 172P, 192V, 199M, 217M and 265A.

[0024] In further embodiments, in particular in all the embodiments described above, the protease according to the invention has an additional amino acid substitution at the position corresponding to position 216, based on the numbering according to SEQ ID NO:1. This amino acid substitution may be the amino acid substitution S216C. In various preferred embodiments, however, the application described herein also relates to protease variants which do not have the substitution 216C, in particular do not have a substitution in the position 216 in the numbering according to SEQ ID NO:1.

[0025] In various embodiments, the protease according to the invention contains at least one amino acid substitution selected from the group consisting of A29G, A48V, D101E, N130D, N130S, N130H, G131D, G131N, G131S, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T or N252S, in each case based on the numbering according to SEQ ID NO:1. In still further preferred embodiments, the protease according to the invention contains the amino acid substitutions (1) P9T, P9H, P9S or P9A and (2) Q271E and optionally also (3) S216C and additionally one of the following amino acid substitution variants: (I) A48V, G131S, T133R and S224A; (II) G131D, T133R and S224A; (III) N130D, G131N, T133K and N144K; (IV) A29G, N130D, G131N and T133R; (V) N130D, G131S, T133K and S224A; (VI) N130D, G131N, T133K, N144L and N252S; (VII) G131S, N144K and S224T; (VIII) N130S, G131S, T133Y, N144L and S224A; (IX) D101E, G131N and S224A; or (X) N130H, G131S and S224A, the numbering being based in each case on the numbering according to SEQ ID NO:1.

[0026] In various embodiments, the proteases have an amino acid sequence according to one of SEQ ID Nos. 3–12, 16–25 or 15–25.

[0027] The proteases according to the invention typically have improved cleaning performance, i.e., increased catalytic activity in washing or cleaning agents. In various embodiments, the proteases according to the invention may have a proteolytic activity which, based on the wild type (SEQ ID NO:1), is at least 101%, preferably at least 102% or more. In various embodiments, the proteases according to the invention have a proteolytic activity which, based on a reference mutation variant of the protease (SEQ ID NO:13 and/or SEQ ID NO:2 and/or SEQ ID NO: 14), is at least 110%, at least

115%, at least 120%, at least 125%, at least 130%, at least 135%, at least 140%, at least 145%, at least 150%, at least 155% or at least 160%. Such performance-enhanced proteases make improved washing results possible for proteolytically sensitive stains in different temperature ranges, in particular in a temperature range of 20 °C to 40 °C.

[0028] Independently of or in addition to increased cleaning performance, the proteases according to the invention may also have increased storage stability in washing or cleaning agents. This means that they have increased stability in washing or cleaning agents in comparison with the wild-type enzyme and in particular in contrast to the starting variant or a reference mutation variant of the protease (SEQ ID NO:2, 13 or 14), in particular when stored for 3 or more days, 4 or more days, 7 or more days, 10 or more days, 12 or more days, 14 or more days, 21 or more days or 28 or more days.

[0029] The proteases according to the invention exhibit enzymatic activity, i.e., they are capable of hydrolysis of peptides and proteins, in particular in a washing or cleaning agent. A protease according to the invention is therefore an enzyme that catalyzes the hydrolysis of amide/peptide bonds in protein/peptide substrates and is thereby capable of cleaving proteins or peptides. Furthermore, a protease according to the invention is preferably a mature protease, i.e., the catalytically active molecule without a signal peptide/signal peptides and/or a propeptide/propeptides. Unless otherwise stated, the sequences indicated also refer to mature (processed) enzymes in each case.

[0030] In various embodiments of the invention, the protease is a free enzyme. This means that the protease can act directly with all components of an agent and, if the agent is a liquid agent, the protease is directly in contact with the solvent of the agent (e.g., water). In other embodiments, an agent may contain proteases that form an interaction complex with other molecules or that contain a “coating.” In this case, an individual protease molecule or a plurality of protease molecules may be separated from the other constituents of the agent by a surrounding structure. Such a separating structure can arise due to, but is not limited to, vesicles, such as a micelle or a liposome. However, the surrounding structure may also be a virus particle, a bacterial cell or a eukaryotic cell. In various embodiments, an agent may include cells of *Bacillus pumilus* or *Bacillus subtilis* which express the proteases according to the invention, or cell culture supernatants of such cells.

[0031] In various embodiments of the invention, the protease comprises an amino acid sequence which, over its entire length, is at least 90%, 90.5%, 91%, 91.5%, 92%, 92.5%, 93%, 93.5%, 94%, 94.5%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5% and 98.8% identical to the amino acid sequence given in SEQ ID NO:1, and has the amino acid substitutions given above in each case based on the numbering according to SEQ ID NO:1. In connection with the present invention, the feature whereby a protease has the given substitutions means that it contains one (of

the given) substitution(s) at the relevant position, i.e., at least the given positions are not otherwise mutated or deleted - for example, by fragmentation of the protease. The amino acid sequences of exemplary proteases which are encompassed by the invention are given in SEQ ID Nos.: 3–12 and 16–25 or 17–25. In various embodiments, the proteases described herein, with the exception of the explicitly mentioned substitutions, have the sequence of SEQ ID NO:1, i.e., apart from the substituted positions, they are 100% identical to the sequence according to SEQ ID NO:1.

[0032] The identity of nucleic acid or amino acid sequences is determined by a sequence comparison. This sequence comparison is based on the BLAST algorithm established and commonly used in the prior art (cf., for example, Altschul et al. (1990) "Basic local alignment search tool," J. Mol. Biol. 215:403–410, and Altschul et al. (1997): "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," Nucleic Acids Res., 25:3389–3402) and occurs in principle by similar sequences of nucleotides or amino acids in the nucleic acid or amino acid sequences being assigned to one another. A tabular assignment of the relevant positions is referred to as an alignment. A further algorithm available in the prior art is the FASTA algorithm. Sequence comparisons (alignments), in particular multiple sequence comparisons, are created using computer programs. The Clustal series (cf., for example, Chenna et al. (2003): "Multiple sequence alignment with the Clustal series of programs," Nucleic Acid Res. 31:3497–3500), T-Coffee (cf., for example, Notredame et al. (2000): "T-Coffee: A novel method for multiple sequence alignments," J. Mol. Biol. 302:205–217) or programs based on these

[0033] programs or algorithms are frequently used, for example. Also possible are sequence comparisons (alignments) using the computer program Vector NTI® Suite 10.3 (Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California, USA) with the given standard parameters, the AlignX module of which is based on ClustalW for the sequence comparisons. Unless stated otherwise, the sequence identity indicated herein is determined using the BLAST algorithm.

[0034] Such a comparison also allows a conclusion to be drawn about the similarity of the compared sequences to one another. It is usually given in percent identity, i.e., the proportion of identical nucleotides or amino acid functional groups at the same positions or positions corresponding to one another in an alignment. The broader concept of homology takes conserved amino acid exchanges into account in the case of amino acid sequences, i.e., amino acids having similar chemical activity, since they usually perform similar chemical activities within the protein. Therefore, the similarity between the compared sequences can also be expressed as percent homology or percent similarity. Identity and/or homology information can be provided regarding whole polypeptides or genes or only regarding individual regions. Homologous or identical regions of different nucleic acid or amino acid sequences are therefore defined by matches in the sequences. Such regions often have identical functions. They can be small and comprise only a few nucleotides or amino acids. Such small regions often perform essential functions for the overall activity of the

protein. It may therefore be expedient to relate sequence matches only to individual, optionally small, regions. Unless otherwise stated, however, identity or homology information in the present application relates to the entire length of the particular nucleic acid or amino acid sequence indicated.

[0035] In the context of the present invention, the indication that an amino acid position corresponds to a numerically designated position in SEQ ID NO:1 therefore means that the corresponding position is associated with the numerically designated position in SEQ ID NO:1 in an alignment as defined above.

[0036] In a further embodiment of the invention, the protease is characterized in that the cleaning performance thereof is not significantly reduced compared with that of a protease comprising an amino acid sequence that corresponds to the amino acid sequence given in SEQ ID NO:13 and/or SEQ ID NO:2 and/or SEQ ID NO:14, i.e., has at least 80% of the reference washing performance, preferably at least 100%, more preferably at least 110% or more. The cleaning performance can be determined in a washing system containing a washing agent in a dosage of between 4.5 and 7.0 grams per liter of washing liquor and the protease, wherein the proteases to be compared are used in the same concentration (based on active protein) and the cleaning performance with respect to a stain on cotton is determined by measuring the degree of cleaning of the washed textiles.

[0037] For example, the washing process can take place for 60 minutes at a temperature of 40 °C, and the water can have a water hardness of between 15.5 and 16.5° (German hardness). The concentration of the protease in the washing agent intended for this washing system is 0.001 to 0.1 wt.%, preferably 0.01 to 0.06 wt.% based on active, purified protein.

[0038] A liquid reference washing agent for such a washing system may be composed as follows (all figures in wt.%): 4.4% alkyl benzene sulfonic acid, 5.6% further anionic surfactants, 2.4% C_{12}-C_{18} Na salts of fatty acids (soaps), 4.4% non-ionic surfactants, 0.2% phosphonates, 1.4% citric acid, 0.95% NaOH, 0.01% defoamer, 2% glycerol, 0.08% preservatives, 1% ethanol, and the remainder being demineralized water. The dosage of the liquid washing agent is preferably between 4.5 and 6.0 grams per liter of washing liquor, for example 4.7, 4.9 or 5.9 grams per liter of washing liquor. The washing process preferably takes place in a pH range between pH 7 and pH 10.5, preferably between pH 7.5 and pH 8.5.

[0039] In the context of the invention, the cleaning performance is determined, for example, at 20 °C or 40 °C using a liquid washing agent as specified above, wherein the washing process is preferably carried out for 60 minutes at 600 rpm.

[0040] The degree of whiteness, i.e., the lightening of the stains, as a measure of the cleaning performance is determined using optical measuring methods, preferably photometrically. A suitable device for this is, for example, the Minolta CM508d spectrometer. Usually, the devices used for measurement are calibrated beforehand using a white standard, preferably a supplied white standard.

[0041] The activity-equivalent use of the relevant protease ensures that the respective enzymatic properties, i.e., for example, the cleaning performance on certain stains, are compared even if the ratio of active substance to total protein (the values of the specific activity) diverges. In general, a low specific activity can be compensated by adding a larger amount of protein.

[0042] Otherwise, methods for determining protease activity are familiar to and routinely used by a person skilled in the art in the field of enzyme technology. For example, such methods are disclosed in Tenside [Surfactants], volume 7 (1970), pp. 125-132. Alternatively, the protease activity can be determined by the release of the chromophore para-nitroaniline (pNA) from the substrate suc-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide (AAPF). The protease cleaves the substrate and releases pNA. The release of the pNA causes an increase in absorbance at 410 nm, the time profile of which is a measure of the enzymatic activity (cf. Del Mar et al., 1979). The measurement is carried out at a temperature of 25 °C, a pH of 8.6 and a wavelength of 410 nm. The measurement time is 5 min and the measurement interval is 20 s to 60 s. The protease activity is usually given in protease units (PE). Suitable protease activities are, for example, 2.25, 5 or 10 PE per ml of washing liquor. However, protease activity is not equal to zero.

[0043] An alternative test for determining the proteolytic activity of the proteases according to the invention is an optical measurement method, preferably a photometric method. The test suitable for this purpose comprises protease-dependent cleavage of the substrate protein casein. The casein is cleaved by the protease into a plurality of smaller partial products. The entirety of these partial products has an increased absorption at 290 nm with respect to non-cleaved casein, wherein this increased absorption can be determined using a photometer, and thus a conclusion can be drawn regarding the enzymatic activity of the protease.

[0044] The protein concentration can be determined using known methods, for example the BCA method (bicinchoninic acid; 2,2'-bichinolyl-4,4'-dicarboxylic acid) or the Biuret method (A. G. Gornall C. S. Bardawill and M. M. David, J. Biol. Chem., 177 (1948), pp. 751-766). In this regard, the active protein concentration can be determined via titration of the active centers using a suitable irreversible inhibitor and determination of the residual activity (cf. M. Bender et al., J. Am. Chem. Soc. 88, 24 (1966), pp. 5890-5913).

[0045] In addition to the amino acid alterations explained above, proteases according to the invention can have further amino acid alterations, in particular amino acid substitutions, insertions or deletions. Such proteases are developed, for example, by targeted genetic alteration, i.e., by means of mutagenesis methods, and optimized for specific use purposes or with regard to specific properties (for example with regard to their catalytic activity, stability, etc.). Furthermore, nucleic acids according to the invention can be introduced into recombination approaches and thus used to produce completely novel proteases or other polypeptides.

[0046] The aim is to introduce targeted mutations such as substitutions, insertions or deletions into the known molecules in order, for example, to improve the cleaning performance of enzymes according to the invention. For this purpose, in particular the surface charges and/or the isoelectric point of the molecules and thus their interactions with the substrate can be altered. For example, the net charge of the enzymes can be altered in order to influence the substrate binding in particular for use in washing and cleaning agents. Alternatively or additionally, the stability or catalytic activity of the protease can be increased by one or more corresponding mutations and its cleaning performance can thereby be improved. Advantageous properties of individual mutations, e.g., individual substitutions, can complement one another. A protease already optimized with regard to certain properties, for example with regard to its stability during storage, can therefore additionally be developed in the context of the invention.

[0047] For the description of substitutions that relate to exactly one amino acid position (amino acid exchanges), the following convention is applied herein: first, the naturally present amino acid is referred to in the form of the internationally used single-letter code, followed by the associated sequence position and finally the inserted amino acid. Several exchanges within the same polypeptide chain are separated by slashes. In the case of insertions, additional amino acids are named according to the sequence position. In the case of deletions, the missing amino acid is replaced by a symbol, for example a star or a dash, or a Δ is indicated before the corresponding position. For example, P9T describes the substitution of proline at position 9 by threonine, P9TH describes the insertion of histidine following the amino acid threonine at position 9 and P9* or ΔP9 describes the deletion of proline at position 9 and 130R/K describes the substitution at position 130 by lysine or arginine. This nomenclature is known to a person skilled in the art in the field of enzyme technology.

[0048] The invention therefore also relates to a protease which is characterized in that it is obtainable from a protease as described above as the starting molecule by single or multiple conservative amino acid substitution, the protease in the numbering according to SEQ ID NO:1 having the above-described amino acid substitutions. The term "conservative amino acid substitution" means the exchange (substitution) of one amino acid functional group for another amino acid functional group, with this exchange not resulting in a change to the polarity or charge at the

position of the exchanged amino acid, e.g., the exchange of a nonpolar amino acid functional group for another nonpolar amino acid functional group. Conservative amino acid substitutions within the context of the invention include, for example: G=A=S, I=V=L=M, D=E, N=Q, K=R, Y=F, S=T, G=A=I=V=L=M=Y=F=W=P=S=T.

[0049] Alternatively or in addition, the protease is characterized in that it can be obtained from a protease according to the invention as the starting molecule by fragmentation, deletion, insertion or substitution mutagenesis and comprises an amino acid sequence which matches the starting molecule over a length of at least 200, 210, 220, 230, 240, 250, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, or 274 contiguous amino acids, wherein the amino acid substitution(s) which may be contained in the starting molecule and which are described herein in accordance with the invention are still present. This means that if the proteases described herein are modified, the modification takes place in such a way that the substitutions according to the invention are retained.

[0050] Alternatively or in addition, the protease is characterized in that it can be obtained from a protease according to the invention as the starting molecule by fragmentation, deletion, insertion or substitution mutagenesis and comprises an amino acid sequence which matches the starting molecule over a length of at least 200, 210, 220, 230, 240, 250, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, or 274 contiguous amino acids, wherein the amino acid substitution(s) which may be contained in the starting molecule and which are described herein in accordance with the invention are still present.

[0051] It is thus possible, for example, to delete individual amino acids at the termini or in the loops of the enzyme without the proteolytic activity being lost or reduced as a result. Furthermore, such fragmentation or deletion, insertion or substitution mutagenesis can also be used, for example, to reduce the allergenicity of the enzymes concerned and thus to improve their usability overall. Advantageously, the enzymes retain their proteolytic activity even after mutagenesis, i.e., their proteolytic activity corresponds at least to that of the starting enzyme, i.e., in a preferred embodiment, the proteolytic activity is at least 80%, preferably at least 90%, of the activity of the starting enzyme. Further substitutions can also demonstrate advantageous effects. Both individual and multiple contiguous amino acids can be replaced with other amino acids.

[0052] Alternatively or additionally, the protease is characterized in that it is obtainable from a protease according to the invention as a starting molecule by single or multiple conservative amino acid substitution, wherein amino acid substitutions according to the invention remain unchanged.

[0053] The further amino acid positions are in this case defined by an alignment of the amino acid sequence of a protease according to the invention with the amino acid sequence of the protease

from *Bacillus pumilus,* as given in SEQ ID NO:1. Furthermore, the assignment of the positions is based on the mature protein. This assignment is also to be used in particular when the amino acid sequence of a protease according to the invention comprises a higher number of amino acid functional groups than the protease from *Bacillus pumilus* according to SEQ ID NO:1. Proceeding from the stated positions in the amino acid sequence of the protease from *Bacillus pumilus*, the alteration positions in a protease according to the invention are those which are assigned to said positions in an alignment.

[0054] Advantageous positions for sequence alterations, in particular substitutions, of the protease from *Bacillus pumilus,* which are of particular significance when transferred to homologous positions of the proteases according to the invention and which impart advantageous functional properties to the protease, are therefore the positions which correspond to the positions described herein in an alignment, i.e., in the numbering according to SEQ ID NO:1. At the positions mentioned, the following amino acid functional groups are present in the wild-type molecule of the protease from *Bacillus pumilus*: P9, A18, A29, A48, F61, A92, N99, D101, N130, G131, T133, N137, N144, V149, S156, T159, T162, G166, D172, A192, S199, S216, Y217, S224, N252, K265 and Q271.

[0055] Further confirmation of the correct assignment of the amino acids to be altered, i.e., in particular their functional correspondence, can be provided by comparative tests, based on which the two positions assigned to one another on the basis of an alignment in the two proteases compared with one another are altered in the same way and observation is carried out to determine whether the enzymatic activity is altered in the same way in the two proteases. If, for example, an amino acid substitution in a certain position of the protease from *Bacillus pumilus* according to SEQ ID NO:1 is accompanied by an alteration of an enzymatic parameter, for example with an increase in the K_M value, and a corresponding alteration of the enzymatic parameter, for example also an increase in the K_M value, is observed in a protease variant according to the invention, the amino acid exchange of which was achieved by the same introduced amino acid, this can be seen as confirmation of the correct assignment.

[0056] All of these aspects are also applicable to the methods according to the invention for producing a protease. Accordingly, a method according to the invention further comprises one or more of the following method steps:

a) introducing a single or multiple conservative amino acid substitution into the protease, the protease comprising:

(a) at the position corresponding to position 271, the amino acid substitution 271E; and
(b) at the position corresponding to position 9, an amino acid substitution selected from 9T, 9H, 9S and 9A, preferably 9T; and

(c1) at at least one of the positions corresponding to positions 18, 61, 92, 99, 137, 149, 156, 159, 162, 166, 172, 192, 199, 217 or 265, at least one further amino acid substitution selected from 18D, 61Y, 92S, 99Y, 137K, 149I, 156G, 156Y, 159I, 162S, 166M, 172G, 172P, 192V, 199M, 217M and 265A; and/or

(c2) at at least three of the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 217, 224 and 252, in particular 130, 133, 144, 217 and 252, at least three further amino acid substitutions selected from A29G, A48V, D101E, N130D, N130S, N130H, G131D, G131N, G131S, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T and N252S;

b) altering the amino acid sequence by fragmentation or deletion, insertion or substitution mutagenesis such that the protease has an amino acid sequence which matches the starting molecule over a length of at least 200, 210, 220, 230, 240, 250, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, or 274 contiguous amino acids, the protease comprising

(a) at the position corresponding to position 271, the amino acid substitution 271E; and

(b) at the position corresponding to position 9, an amino acid substitution selected from 9T, 9H, 9S and 9A, preferably 9T; and

(c1) at at least one of the positions corresponding to positions 18, 61, 92, 99, 137, 149, 156, 159, 162, 166, 172, 192, 199, 217 or 265, at least one further amino acid substitution selected from 18D, 61Y, 92S, 99Y, 137K, 149I, 156G, 156Y, 159I, 162S, 166M, 172G, 172P, 192V, 199M, 217M and 265A; and/or

(b2) at at least three of the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 217, 224 and 252, in particular 130, 133, 144, 217 and 252, at least three further amino acid substitutions selected from A29G, A48V, D101E, N130D, N130S, N130H, G131D, G131N, G131S, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T and N252S.

[0057] All embodiments also apply to the methods according to the invention. In particular, each of the aforementioned proteases may additionally comprise an amino acid substitution at position 216 and/or 217 in the numbering according to SEQ ID NO:1, in particular 216C and/or 217M. However, in various embodiments it may be preferred that the protease has no substitution in position 216.

[0058] In further embodiments of the invention, the protease or the protease produced using a method according to the invention is still at least 90%, 90.5%, 91%, 91.5%, 92%, 92.5%, 93%, 93.5%, 94%, 94.5%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, or 98.8% identical to the amino acid sequence given in SEQ ID NO:1 over the entire length thereof. Alternatively, the protease or the protease prepared by a method according to the invention is still at least 90%, 90.5%, 91%, 91.5%,

92%, 92.5%, 93%, 93.5%, 94%, 94.5%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, or 98.8% identical to one of the amino acid sequences given in SEQ ID NOS: 3–12, 16–25 over the entire length thereof. The protease or the protease prepared by a method according to the invention has an amino acid substitution at position 271, in particular 271E, and (i) in particular one or more of the amino acid substitutions 9T, 130D, 144K and 252T at position 9 and at least one of the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 224 or 252, in each case based on the numbering according to SEQ ID NO:1, and/or (ii) at least one further amino acid substitution at at least one of the positions corresponding to positions 18, 61, 92, 99, 137, 149, 156, 159, 162, 166, 172, 192, 199, 217 or 265, in each case based on the numbering according to SEQ ID NO:1.

[0059] In more preferred embodiments, the amino acid substitution is at least one selected from the group consisting of P9T, P9H, P9T, P9A, A29G, A48V, D101E, N130D, N130S, N130H, G131D, G131N, G131S, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T, N252S and Q271E, in each case based on the numbering according to SEQ ID NO:1.

[0060] By way of example, the following amino acid substitution variants may be mentioned: P9T, P9H, P9S or P9A, in particular P9T, and Q271E and optionally also S216C combined with one of (I) A48V, G131S, T133R and S224A; (II) G131D, T133R and S224A; (III) N130D, G131N, T133K and N144K; (IV) A29G, N130D, G131N and T133R; (V) N130D, G131S, T133K and S224A; (VI) N130D, G131N, T133K, N144L and N252S; (VII) G131S, N144K and S224T; (VIII) N130S, G131S, T133Y, N144L and S224A; (IX) D101E, G131N and S224A; or (X) N130H, G131S and S224A, the numbering being based in each case on the numbering according to SEQ ID NO:1.

[0061] In various embodiments, the invention relates to proteases according to SEQ ID NO:1 having the following amino acid substitution variants: Q271E combined with one of (i) P9T, N130D, N144K and N252T; (ii) P9T, N130D, N144K, N252T and S156G; (iii) P9T, N130D, N144K, N252T and S156Y; (iv) P9T, N130D, N144K, N252T and Y217M; (v) P9T, N130D, N144K, N252T and N137K; (vi) N130D, N144K, N252T, P9H, A18D and T159I; or (vii) P9T and D172G, the numbering being based in each case on the numbering according to SEQ ID NO:1. Further examples are the variants described above and in the examples.

[0062] A further subject matter of the invention is a previously described protease that is additionally stabilized, in particular by means of one or more mutations, for example substitutions, or by means of coupling to a polymer. An increase in stability during storage and/or during use, for example during the washing process, results in the enzymatic activity lasting longer and thus improves the cleaning performance. In principle, all stabilization options described and/or expedient in the prior art are conceivable. Preference is given to those stabilizations which are achieved via mutations of the enzyme itself because such stabilizations do not require any further working steps

after the recovery of the enzyme. Examples of sequence alterations suitable for this purpose are specified above. Further suitable sequence alterations are known from the prior art.

[0063] Further possibilities for stabilization are, for example:

- altering the binding of metal ions, in particular the calcium binding sites, for example by exchanging one or more of the amino acid(s) involved in the calcium binding for one or more negatively charged amino acids and/or by introducing sequence changes in at least one of the sequences of the two amino acids arginine/glycine;
- protecting against the influence of denaturing agents such as surfactants by mutations which cause an alteration of the amino acid sequence on or at the surface of the protein;
- exchanging amino acids that are close to the N-terminus for those that presumably come into contact with the rest of the molecule via non-covalent interactions and thus contribute to maintaining the globular structure.

[0064] Preferred embodiments are those in which the enzyme is stabilized in a plurality of ways because a plurality of stabilizing mutations act additively or synergistically.

[0065] The invention further relates to a protease as described above, characterized in that it has at least one chemical modification. A protease having such an alteration is referred to as a derivative, i.e., the protease is derivatized.

[0066] In the context of the present application, derivatives are thus understood to mean proteins whose pure amino acid chain has been chemically modified. Such derivatizations can be achieved, for example, *in vivo* by the host cell that expresses the protein. In this regard, couplings of low-molecular-weight compounds such as lipids or oligosaccharides are particularly noteworthy. However, the derivatizations may also be carried out *in vitro*, for example by the chemical conversion of a side chain of an amino acid or by covalent bonding of another compound to the protein. For example, it is possible to couple amines to carboxyl groups of an enzyme in order to alter the isoelectric point. Another such compound can also be a further protein that is bound to a protein according to the invention via bifunctional chemical compounds, for example. Derivatization is likewise understood to mean covalent bonding to a macromolecular carrier or a non-covalent inclusion in suitable macromolecular cage structures. Derivatizations can influence, for example, the substrate specificity or the binding strength to the substrate or bring about temporary blocking of the enzymatic activity if the coupled substance is an inhibitor. This can be expedient for the period of storage, for example. Such modifications may further affect the stability or enzymatic activity. They can also serve to reduce the allergenicity and/or immunogenicity of the protein and thus to increase the skin compatibility thereof, for example. For example, couplings with macromolecular compounds, for example polyethylene glycol, can improve the protein with regard to stability and/or skin compatibility.

[0067] Derivatives of a protein according to the invention can also be understood in the broadest sense to be preparations of these proteins. A protein can, depending on the recovery, processing or preparation thereof, be combined with various other substances, for example from the culture of the producing microorganisms. A protein can also have been deliberately admixed with other substances, for example to increase its storage stability. Therefore, all preparations of a protein according to the invention are also in accordance with the invention. This is also independent of whether or not it actually exhibits this enzymatic activity in a particular preparation. This is because it may be desirable for it to have no activity or only a small amount of activity during storage and to only exhibit its enzymatic function at the time of use. This can be controlled, for example, via corresponding accompanying substances. In particular, the joint preparation of proteases with specific inhibitors is possible in this regard.

[0068] Of all the proteases or protease variants and/or derivatives described above, in the context of the present invention those of which the catalytic activity corresponds to at least one of those of the proteases according to SEQ ID Nos.: 3–12 and 16–25, and/or of which the cleaning performance corresponds to at least one of those of the proteases according to SEQ ID Nos.: 3–12 or 16–25, are particularly preferred, the cleaning performance being determined in a washing system as described above.

[0069] A further subject matter of invention is a nucleic acid coding for a protease according to the invention, and a vector containing such a nucleic acid, in particular a cloning vector or an expression vector.

[0070] These can be DNA or RNA molecules. They can be present as a single strand, as a single strand complementary to said single strand or as a double strand. In particular in the case of DNA molecules, the sequences of the two complementary strands must be taken into account in all three possible reading frames. Furthermore, it must be taken into account that different codons, i.e., base triplets, can code for the same amino acids such that a certain amino acid sequence can be coded by a plurality of different nucleic acids. Due to this degeneracy of the genetic code, all of the nucleic acid sequences which can code any of the proteases described above are included in this subject matter of the invention. A person skilled in the art is able to determine these nucleic acid sequences beyond a doubt because, despite the degeneracy of the genetic code, defined amino acids can be assigned to individual codons. Therefore, a person skilled in the art proceeding from said amino acid sequence can easily determine nucleic acids coding for said amino acid sequence. Furthermore, in the case of nucleic acids according to the invention, one or more codons may be replaced by synonymous codons. This aspect relates in particular to the heterologous expression of the enzymes according to the invention. Thus, each organism, for example a host cell of a production strain, has a certain codon usage. "Codon usage" is understood to mean the translation of the genetic

code into amino acids by the relevant organism. Bottlenecks can occur in protein biosynthesis if the codons on the nucleic acid in the organism are faced with a comparatively small number of loaded tRNA molecules. Although coding for the same amino acid, this results in a codon being translated less efficiently in the organism than a synonymous codon coding for the same amino acid. Due to the presence of a higher number of tRNA molecules for the synonymous codon, this can be translated more efficiently in the organism.

[0071] It is possible for a person skilled in the art to use methods which are currently generally known, for example chemical synthesis or polymerase chain reaction (PCR), in conjunction with molecular biology and/or protein-chemical standard methods, to produce the corresponding nucleic acids and even complete genes on the basis of known DNA and/or amino acid sequences. Such methods are known, for example, from Sambrook, J., Fritsch, E. F. and Maniatis, T. 2001. Molecular cloning: a laboratory manual, 3. Edition Cold Spring Laboratory Press.

[0072] For the purposes of the present invention, "vectors" are understood to mean elements consisting of nucleic acids that contain a nucleic acid according to the invention as the characteristic nucleic acid region. They are able to establish these as a stable genetic element in a species or cell line over several generations or cell divisions. Vectors are, in particular when used in bacteria, special plasmids, i.e., circular genetic elements. In the context of the present invention, a nucleic acid according to the invention is cloned into a vector. The vectors include, for example, those originating from bacterial plasmids, viruses or bacteriophages, or predominantly synthetic vectors or plasmids with elements of a wide variety of origins. With the other genetic elements present in each case, vectors are able to establish themselves as stable units in the particular host cells over several generations. They can be present extrachromosomally as separate units or can be integrated into a chromosome or chromosomal DNA.

[0073] Expression vectors comprise nucleic acid sequences that allow them to replicate in the host cells containing them, preferably microorganisms, particularly preferably bacteria, and to express a contained nucleic acid there. The expression is influenced in particular by the promoter or promoters that regulate transcription. In principle, the expression can take place by the natural promoter originally located before the nucleic acid to be expressed, but also by a promoter of the host cell provided on the expression vector or also by a modified or completely different promoter of another organism or another host cell. In the present case, at least one promoter is provided for the expression of a nucleic acid according to the invention and used for the expression thereof. Expression vectors can also be regulatable, for example by changing the cultivation conditions or when a certain cell density of the host cells containing them is reached or by adding certain substances, in particular activators of gene expression. An example of such a substance is the galactose derivative isopropyl β-D-thiogalactopyranoside (IPTG), which is used as an activator of the

bacterial lactose operon (lac operon). In contrast to expression vectors, the nucleic acid contained is not expressed in cloning vectors.

[0074] The invention further relates to a non-human host cell containing a nucleic acid according to the invention or a vector according to the invention, or containing a protease according to the invention, in particular one that secretes the protease into the medium surrounding the host cell. Preferably, a nucleic acid according to the invention or a vector according to the invention is transformed into a microorganism that then represents a host cell according to the invention. Alternatively, individual components, i.e., nucleic acid parts or fragments of a nucleic acid according to the invention, can also be introduced into a host cell in such a way that the resulting host cell contains a nucleic acid according to the invention or a vector according to the invention. This procedure is particularly suitable when the host cell already contains one or more constituents of a nucleic acid according to the invention or a vector according to the invention and the further constituents are then supplemented accordingly. Methods for transforming cells are established in the prior art and are well known to a person skilled in the art. In principle all cells, i.e., prokaryotic or eukaryotic cells, are suitable as host cells. Host cells which can be managed in a genetically advantageous manner, for example with regard to transformation with the nucleic acid or the vector and its stable establishment, are preferred, for example single-cell fungi or bacteria. Furthermore, preferred host cells are distinguished by good microbiological and biotechnological manageability. This relates, for example, to easy cultivation, high growth rates, low requirements for fermentation media and good production and secretion rates for foreign proteins. Preferred host cells according to the invention secrete the (transgenically) expressed protein into the medium surrounding the host cells. Furthermore, the proteases can be modified by the cells producing them after their production, for example by linking sugar molecules, formylations, aminations, etc. Such post-translational modifications can functionally influence the protease.

[0075] Further preferred embodiments are host cells that can be regulated in their activity owing to genetic regulatory elements that are provided, for example, on the vector but can also be present in these cells from the outset. Expression in said cells may be induced, for example, by controlled addition of chemical compounds used as activators, by changing the cultivation conditions or when a particular cell density is reached. This allows economic production of the proteins according to the invention. An example of such a compound is IPTG, as described above.

[0076] Prokaryotic or bacterial cells are preferred host cells. Bacteria are characterized by short generation times and low demands on cultivation conditions. This makes it possible to establish cost-effective cultivation methods or production methods. In addition, a person skilled in the art will have a wealth of experience in the case of bacteria in fermentation technology. Gram-negative or gram-positive bacteria can be suitable for a specific production for many different reasons to be determined

experimentally in each individual case, such as nutrient sources, product formation rate, time needed, etc.

[0077] In gram-negative bacteria, such as *Escherichia coli*, a plurality of proteins is secreted into the periplasmic space, i.e., into the compartment between the two membranes enclosing the cells. This can be advantageous for specific applications. Furthermore, gram-negative bacteria can also be designed such that they discharge the expressed proteins not only into the periplasmic space but into the medium surrounding the bacterium. Gram-positive bacteria such as, for example, bacilli or actinomycetes or other representatives of A*ctinomycetes* in contrast do not have an outer membrane, such that secreted proteins are immediately released into the medium surrounding the bacteria, usually the nutrient medium, from which the expressed proteins can be purified. They can be isolated directly from the medium or further processed. In addition, gram-positive bacteria are related or identical to most origin organisms for technically important enzymes and usually themselves form comparable enzymes, such that they have a similar codon usage and their protein synthesis apparatus is naturally aligned accordingly.

[0078] Host cells according to the invention may be altered in terms of their requirements for culture conditions, have different or additional selection markers, or also express different or additional proteins. In particular, this may also involve those host cells which express a plurality of proteins or enzymes.

[0079] The present invention is applicable in principle to all microorganisms, in particular to all fermentable microorganisms, particularly preferably those of the genus *Bacillus,* and leads to it being possible to produce proteins according to the invention by the use of such microorganisms. Such microorganisms then represent host cells for the purposes of the invention.

[0080] In a further embodiment of the invention, the host cell is characterized in that it is a bacterium, preferably one selected from the group of the genera of *Escherichia, Klebsiella, Bacillus, Staphylococcus, Corynebacterium, Arthrobacter, Streptomyces, Stenotrophomonas* and *Pseudomonas,* more preferably one selected from the group of *Escherichia coli, Klebsiella planticola, Bacillus licheniformis, Bacillus lentus, Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus alcalophilus, Bacillus globigii, Bacillus gibsonii, Bacillus clausii, Bacillus halodurans, Bacillus pumilus, Staphylococcus carnosus, Corynebacterium glutamicum, Arthrobacter oxidans, Streptomyces lividans, Streptomyces coelicolor* and *Stenotrophomonas maltophilia.*

[0081] The host cell may also be a eukaryotic cell, however, which is characterized in that it has a cell nucleus. The invention therefore further relates to a host cell that is characterized in that it has a nucleus. In contrast with prokaryotic cells, eukaryotic cells are capable of post-translationally modifying the protein formed. Examples thereof are fungi such as *actinomycetes* or yeasts such as

Saccharomyces or *Kluyveromyces*. This can be particularly advantageous, for example, if the proteins are to undergo specific modifications in connection with their synthesis, which modifications make such systems possible. Modifications carried out by eukaryotic systems, in particular in connection with the protein synthesis, include, for example, the binding of low-molecular-weight compounds such as membrane anchors or oligosaccharides. Such oligosaccharide modifications can be desirable, for example, to reduce the allergenicity of an expressed protein. Coexpression with the enzymes naturally formed by such cells, such as cellulases, can also be advantageous. Furthermore, for example, thermophilic fungal expression systems can be particularly suitable for expression of temperature-resistant proteins or variants.

[0082] The host cells according to the invention are cultured and fermented in the usual manner, for example in discontinuous or continuous systems. In the first case, a suitable nutrient medium is inoculated with the host cells and the product is harvested from the medium after a period to be determined experimentally. Continuous fermentations are characterized by achieving a flow equilibrium in which cells partially die off over a comparatively long period but also grow back and, at the same time, the protein formed can be removed from the medium.

[0083] Host cells according to the invention are preferably used to produce proteases according to the invention. The invention therefore further relates to a method for producing a protease, comprising

a) cultivating a host cell according to the invention, and
b) isolating the protease from the culture medium or from the host cell.

[0084] This subject matter of the invention preferably comprises fermentation processes. Fermentation processes are known per se from the prior art and represent the actual large-scale production step, generally followed by a suitable purification method for the product produced, for example for the proteases according to the invention. All fermentation processes that are based on a corresponding method for producing a protease according to the invention represent embodiments of this subject matter of the invention.

[0085] Fermentation processes that are characterized in that the fermentation is carried out via a feed strategy are considered in particular. In this case, the media constituents that are consumed by the continuous cultivation are added. As a result, considerable increases can be achieved both in the cell density and in the cell mass or dry mass and/or in particular in the activity of the protease of interest. Furthermore, the fermentation can also be designed such that unwanted metabolic products are filtered out or neutralized by adding buffers or suitable counterions.

[0086] The prepared protease can be harvested from the fermentation medium. Such a fermentation process is preferred over isolation of the protease from the host cell, i.e., product preparation from the cell mass (dry mass), but requires the provision of suitable host cells or one or more suitable secretion markers or mechanisms and/or transport systems so that the host cells secrete the protease into the fermentation medium. Without secretion, the isolation of the protease from the host cell, i.e., purification thereof from the cell mass, can alternatively take place, for example by means of precipitation with ammonium sulfate or ethanol or by means of chromatographic purification.

[0087] All of the above-mentioned aspects can be combined to form methods in order to produce the protease according to the invention.

[0088] The invention further provides an agent that is characterized in that it contains a protease according to the invention, as described above. The agent is preferably a washing or cleaning agent.

[0089] This subject matter of the invention includes all conceivable washing or cleaning agent types, both concentrates and agents to be used undiluted, for use on a commercial scale, in the washing machine or in the case of hand washing or cleaning. These include, for example, washing agents for textiles, carpets or natural fibers for which the term washing agent is used. These include, for example, dishwashing detergents for dishwashers or manual dishwashing detergents or cleaners for hard surfaces such as metal, glass, porcelain, ceramics, tiles, stone, painted surfaces, plastics, wood or leather, for which the term cleaning agent is used, i.e., in addition to manual and mechanical dishwashing detergents, also, for example, scouring agents, glass cleaners, WC toilet scenters, etc. The washing and cleaning agents according to the invention also include auxiliary washing agents which are added to the actual washing agent during manual or automatic textile washing in order to achieve a further effect. Furthermore, in the context of the invention, washing and cleaning agents also include textile pre-treatment agents and post-treatment agents, i.e., those agents with which the item of laundry is brought into contact before the actual washing, for example for dissolving stubborn stains, and also those agents which give the laundry further desirable properties, such as a pleasant feel, crease resistance or a low static charge in a step downstream of the actual textile washing. Inter alia, softeners are included in the latter agents.

[0090] In addition to a protease according to the invention, the washing or cleaning agents according to the invention, which can be present as powdered solids, in a post-compacted particle form, as homogeneous solutions or suspensions, can contain all known ingredients conventional in such agents, wherein preferably at least one further ingredient is present in the agent. The agents according to the invention can contain, in particular, surfactants, builders, peroxygen compounds or bleach activators. Furthermore, they can contain water-miscible organic solvents, further enzymes,

sequestering agents, electrolytes, pH regulators and/or further auxiliaries such as optical brighteners, graying inhibitors, foam regulators and dyes and fragrances and combinations thereof.

[0091] In particular, a combination of a protease according to the invention with one or more further ingredient(s) of the agent is advantageous because, in preferred embodiments according to the invention, such an agent has improved cleaning performance due to resulting synergisms. In particular, such a synergism can be achieved by combining a protease according to the invention with a surfactant and/or a builder and/or a peroxygen compound and/or a bleach activator. However, in preferred embodiments, the agent according to the invention cannot contain boric acid.

[0092] Advantageous ingredients of agents according to the invention are disclosed in the international patent application WO 2009/121725, starting on page 5, penultimate paragraph, and ending on page 13 after the second paragraph.

[0093] An agent according to the invention advantageously contains the protease in an amount of 2 µg to 20 mg, preferably of 5 µg to 17.5 mg, particularly preferably of 20 µg to 15 mg and very particularly preferably of 50 µg to 10 mg per g of the agent. In various embodiments, the concentration of the protease (active enzyme) described herein in the agent is >0 to 1 wt.%, preferably 0.001 to 0.1 wt.%, based on the total weight of the agent or composition. Furthermore, the protease contained in the agent, and/or further ingredients of the agent, can be coated with a substance that is impermeable to the enzyme at room temperature or in the absence of water and becomes permeable to the enzyme under application conditions of the agent. Such an embodiment of the invention is thus characterized in that the protease is coated with a substance that is impermeable to the protease at room temperature or in the absence of water. Furthermore, the washing or cleaning agent itself can also be packaged in a container, preferably an air-permeable container, from which it is released shortly before use or during the washing process.

[0094] In further embodiments of the invention, the agent is characterized in that it:

(a) is present in solid form, in particular as a flowable powder having a bulk density of from 300 g/L to 1200 g/L, in particular from 500 g/L to 900 g/L, or
(b) is present in a pasty or liquid form, and/or
(c) is present in the form of a gel or in the form of dosing pouches, and/or
(d) is present as a single-component system, or
(e) is divided into a plurality of components.

[0095] These embodiments of the present invention include all solid, powdered, liquid, gel or pasty administration forms of agents according to the invention, which may optionally also consist of a plurality of phases and can be present in a compressed or uncompressed form. The agent can be

present as a free-flowing powder, in particular having a bulk density of 300 g/l to 1200 g/l, in particular 500 g/l to 900 g/l or 600 g/l to 850 g/l. The solid administration forms of the agent further include extrudates, granules, tablets or pouches. Alternatively, the agent can also be in a liquid, gel or paste form, for example in the form of a non-aqueous liquid washing agent or a non-aqueous paste or in the form of an aqueous liquid washing agent or a water-containing paste. Furthermore, the agent can be present as a single-component system. Such agents consist of one phase. Alternatively, an agent can also consist of a plurality of phases. Such an agent is accordingly divided into a plurality of components.

[0096] Washing or cleaning agents according to the invention can contain only one protease. Alternatively, they can also contain further hydrolytic enzymes or other enzymes in a concentration expedient for the effectiveness of the agent. A further embodiment of the invention is thus represented by agents that further comprise one or more further enzymes. Further enzymes which can preferably be used are all enzymes which can exhibit catalytic activity in the agent according to the invention, in particular a lipase, amylase, cellulase, hemicellulase, mannanase, tannase, xylanase, xanthanase, xytoglucanase, ß-glucosidase, pectinase, carrageenase, perhydrolase, oxidase, oxidoreductase or another protease which is different from the protease according to the invention, as well as mixtures thereof. Further enzymes are advantageously contained in the agent in each case in an amount of 1×10^{-8} to 5 wt.%, based on the active protein. Increasingly preferably, each further enzyme is contained in agents according to the invention in an amount of from 1×10^{-7} to 3 wt.%, from 0.00001 to 1 wt.%, from 0.00005 to 0.5 wt.%, from 0.0001 to 0.1 wt.% and particularly preferably from 0.0001 to 0.05 wt.%, based on active protein. Particularly preferably, the enzymes exhibit synergistic cleaning performance with respect to particular dirt or stains, i.e., the enzymes contained in the agent composition assist one another in their cleaning performance. Synergism of this kind is very particularly preferably present between the protease contained according to the invention and a further enzyme of an agent according to the invention, including in particular between said protease and an amylase and/or a lipase and/or a mannanase and/or a cellulase and/or a pectinase. Synergistic effects can occur not only between different enzymes but also between one or more enzymes and other ingredients of the agent according to the invention.

[0097] In the cleaning agents described herein, the enzymes to be used can further be formulated together with accompanying substances, for example from fermentation. In liquid formulations, the enzymes are preferably used as liquid enzyme formulation(s).

[0098] The enzymes are generally not provided in the form of the pure protein, but rather in the form of stabilized, storable and transportable preparations. These ready-made preparations include, for example, the solid preparations obtained by means of granulation, extrusion or lyophilization or, in particular in the case of liquid or gel agents, solutions of the enzymes, which are advantageously as concentrated as possible, have a low water content, and/or are admixed with stabilizers or further auxiliaries.

[0099] Alternatively, the enzymes can be encapsulated both for the solid and for the liquid administration form, for example by means of spray-drying or extrusion of the enzyme solution together with a preferably natural polymer or in the form of capsules, for example those in which the enzymes are enclosed as in a solidified gel, or in those of the core-shell type, in which an enzyme-containing core is coated with a protective layer that is impermeable to water, air and/or chemicals. Further active ingredients, for example, stabilizers, emulsifiers, pigments, bleaches or dyes can additionally be applied in overlaid layers. Such capsules are made using methods that are known per se, for example by means of vibratory granulation or roll granulation or by means of fluid bed processes. Advantageously, such granules are low in dust, for example due to the application of polymeric film formers, and are stable in storage due to the coating.

[0100] Furthermore, it is possible to formulate two or more enzymes together such that a single granule exhibits a plurality of enzyme activities.

[0101] The enzymes can also be introduced into water-soluble films, such as those used in the formulation of washing and cleaning agents in a unit dosage form. Such a film allows the enzymes to be released after contact with water. As used herein, “water-soluble” refers to a film structure that is preferably completely water-soluble. Preferably, such a film consists of (completely or partially hydrolyzed) polyvinyl alcohol (PVA).

[0102] A further subject matter of the invention is a method for cleaning textiles or hard surfaces, characterized in that an agent according to the invention is used in at least one method step, or that in at least one method step a protease according to the invention becomes catalytically active, in particular such that the protease is used in an amount of 40 µg to 4 g, preferably 50 µg to 3 g, particularly preferably 100 µg to 2 g and very particularly preferably of 200 µg to 1 g.

[0103] In various embodiments, the method described above is characterized in that the protease is used at a temperature of from 0 to 100 °C, preferably 0 to 60 °C, more preferably 20 to 40 °C and most preferably at 20 °C or 25 °C.

[0104] These include both manual and machine methods, wherein machine methods are preferred. Methods for cleaning textiles are generally characterized by the fact that, in a plurality of method steps, various cleaning-active substances are applied to the material to be cleaned and washed off after the exposure time, or in that the material to be cleaned is otherwise treated with a washing agent or a solution or dilution of this agent. The same applies to methods for cleaning all materials other than textiles, in particular hard surfaces. All conceivable washing or cleaning methods can be enhanced in at least one of the method steps by the use of a washing or cleaning agent according to the invention or a protease according to the invention, and therefore represent

embodiments of the present invention. All aspects, subject matter, and embodiments described for the protease according to the invention and agents containing them are also applicable to this subject matter of the invention. Therefore, reference is expressly made at this point to the disclosure at the corresponding point with the note that this disclosure also applies to the above methods according to the invention.

[0105] Because proteases according to the invention by nature already have hydrolytic activity and also exhibit said activity in media that otherwise have no cleaning power, such as, for example, in a simple buffer, an individual and/or the single step of such a method can consist of bringing a protease according to the invention into contact with the stain as the only cleaning component, preferably in a buffer solution or in water. This represents a further embodiment of this subject matter of the invention.

[0106] Alternative embodiments of this subject matter of the invention also include methods for treating textile raw materials or for textile care, in which a protease according to the invention is active in at least one method step. Among these, methods for textile raw materials, fibers or textiles comprising natural constituents are preferred, and very particularly for those comprising wool or silk.

[0107] Finally, the invention also encompasses the use of the proteases described herein in washing or cleaning agents, for example as described above, for (improved) removal of protein-containing stains, for example from textiles or hard surfaces. In various embodiments of this use, the protease in the washing or cleaning agent is stored for 3 or more days, 4 or more days, 7 or more days, 10 or more days, 12 or more days, 14 or more days, 21 or more days or 28 or more days before a washing or cleaning process.

[0108] All aspects, subject matter, and embodiments described for the protease according to the invention and agents containing them are also applicable to this subject matter of the invention. Therefore, reference is expressly made at this point to the disclosure at the corresponding point with the indication that this disclosure also applies to the above use according to the invention.

Examples

Overview of the mutations:

[0109] This invention relates to a subtilisin-type alkaline protease from *Bacillus pumilus.* From this protease (wild-type *Bacillus pumilus* DSM18097 protease according to SEQ ID NO:1), variants were produced by random mutagenesis, which were then screened, inter alia for improved washing performance and/or enzyme stability. In this way, two performance-enhanced mutants (mutant 1 [SEQ ID NO:13] and mutant 2 [SEQ ID NO:2]) were generated from the wild-type protease (SEQ ID

NO:1) mentioned above in a first round by error-prone mutagenesis. Both of these mutants were subject to an independent, second error-prone round. In this second round of mutation, mutants 3–12 according to SEQ ID Nos. 3–12 were generated. Therefore, all of mutants 3–12 mentioned here also carry at least some of the mutations of mutants 1 or 2. In a third round, further performance-improved mutants (mutant 1 [SEQ ID NO:14], mutant 2 [SEQ ID NO:15], mutant 3 [SEQ ID NO: 16]) were generated by error prone mutagenesis. These mutants were subject to another error-prone round (fourth round) and a plurality of saturation mutagenesis processes were carried out at specific positions. Furthermore, some mutants were produced by the targeted production of synthetic genes. In this fourth round of mutation, mutants 16–24 according to SEQ ID Nos. 17–25 were generated, as well as mutants 25–52.

Variant	**Amino acid substitutions relative to SEQ ID NO:1**									**SEQ ID NO:**
Mutant 1	P9T	Q271E	S216C							13
Mutant 2	P9T	Q271E	S216C	T133R	S224A					2
Mutant 3	P9T	Q271E	S216C	A48V	G131S	T133R	S224A			3
Mutant 4	P9T	Q271E	S216C	G131D	T133R	S224A				4
Mutant 5	P9T	Q271E	S216C	N130D	G131N	T133K	N144K			5
Mutant 6	P9T	Q271E	S216C	A29G	N130D	G131N	T133R			6
Mutant 7	P9T	Q271E	S216C	N130D	G131S	T131K	S224A			7
Mutant 8	P9T	Q271E	S216C	N130D	G131N	T133K	N144L		N252S	8
Mutant 9	P9T	Q271E	S216C	G131S	N144K	S224T				9
Mutant 10	P9T	Q271E	S216C	N130S	G131S	T133Y	N144L		S224A	10
Mutant 11	P9T	Q271E	S216C	D101E	G131N	S224A				11
Mutant 12	P9T	Q271E	S216C	N130H	G131S	S224A				12
Mutant 13	P9T				Q271E					14
Mutant 14		N130D	N144K	N252T	Q271E					15
Mutant 15	P9T	N130D	N144K	N252T	Q271E					16
Mutant 16	P9T	N130D	N144K	N252T	Q271E	S156G				17
Mutant 17	P9T	N130D	N144K	N252T	Q271E	S156Y				18
Mutant 18	P9T	N130D	N144K	N252T	Q271E	Y217M				19
Mutant 19	P9T	N130D	N144K	N252T	Q271E	N137K				20
Mutant 20		N130D	N144K	N252T	Q271E	F61Y	A92S			21
Mutant 21		N130D	N144K	N252T	Q271E	T162S				22
Mutant 22		N130D	N144K	N252T	Q271E	A192V				23
Mutant 23	P9H	N130D	N144K	N252T	Q271E	A18D	T159I			24
Mutant 24	P9T				Q271E	D176G				25
Mutant 25	P9T	N130D	N144K	N252T	Q271E	T133R				
Mutant 26	P9T	N130D	N144K	N252T	Q271E	T133K				
Mutant 27	P9T	N130D	N144K	N252T	Q271E	S224A				
Mutant 28	P9T	N130D	N144K	N252T	Q271E	S224A	T133R			

Variant	Amino acid substitutions relative to SEQ ID NO:1									SEQ ID NO:
Mutant 29	P9T	N130D	N144K	N252T	Q271E	D172G				
Mutant 30	P9T	N130D	N144K	N252T	Q271E	D172P				
Mutant 31	P9T	N130D	N144K	N252T	Q271E	V149I				
Mutant 32	P9T	N130D	N144K	N252T	Q271E	N99Y				
Mutant 33	P9T	N130D	N144K	N252T	Q271E	Q62S				
Mutant 34	P9T	N130D	N144K	N252T	Q271E	N137K				
Mutant 35	P9T	N130D	N144K	N252T	Q271E	T162S				
Mutant 36	P9T	N130D	N144K	N252T	Q271E	Y217M	T133R			
Mutant 37	P9T	N130D	N144K	N252T	Q271E	Y217M				
Mutant 38	P9T	N130D	N144K	N252T	Q271E	Y217M	T133A			
Mutant 39	P9T	N130D	N144K	N252T	Q271E	Y217M	A192V			
Mutant 40	P9T	N130D	N144K	N252T	Q271E	T162S	A192V			
Mutant 41	P9T	N130D	N144K	N252T	Q271E	T162S	A192V			
Mutant 42	P9T	N130D	N144K	N252T	Q271E	T162S	Y217M			
Mutant 43	P9T	N130D	N144K	N252T	Q271E	T162S	T133A			
Mutant 44	P9T	N130D	N144K	N252T	Q271E	T162S	Y217M	A192V		
Mutant 45	P9T	N130D	N144K	N252T	Q271E	T162S	Y217M	A192V	T133A	
Mutant 46	P9T	N130D	N144K	N252T	Q271E	T162S	Y217M	T133A		
Mutant 47	P9T	N130D	N144K	N252T	Q271E	T162S	A192V	T133A		
Mutant 48	P9T	N130D	N144K	N252T	Q271E	T133A	K265A			
Mutant 49	P9T	N130D	N144K	N252T	Q271E	T133A	S199M			
Mutant 50	P9T	N130D	N144K	N252T	Q271E	T133A	G166M			
Mutant 51	P9T	N130D	N144K	N252T	Q271E	T162S	G166M			
Mutant 52	P9T	N130D	N144K	N252T	Q271E	A192V				

Washing agent matrix used

[0110] The following washing agent matrices (commercially available, without enzymes, opt. brighteners, perfume and dyes) were used for the washing tests:

Washing test 2:

Chemical name	Wt.% of active substance in the raw material	Wt.% of active substance in the formulation
Demineralized water	100	Remainder
Alkyl benzene sulfonic acid	90	12–18
Anionic surfactants	70	4–8
C_{12}-C_{18} fatty acid Na salt	30	2–4

Non-ionic surfactants	100	8–14
Phosphonate	60	0.5–2
Citric acid	100	3–5
NaOH	50	0.5–2
Defoamer	100	<1%
Glycerol	99.5	1–3
1,2-propanediol	100	8–12
Monoethanolamine	100	4–8
Soil repellent polymer	30	0.5–1
Protease stabilizer	100	0.5–1.5
Without opt. brighteners, perfume, dye and enzymes.		
Dosage 3.17 g/L		

Washing test 1:

Chemical name	Wt.% of active substance in the raw material	Wt.% of active substance in the formulation
Demineralized water	100	Remainder
Alkyl benzene sulfonic acid	96	4.4
Anionic surfactants	70	5.6
C_{12}-C_{18} fatty acid Na salt	30	2.4
Non-ionic surfactants	100	4.4
Phosphonates	40	0.2
Citric acid	100	1.4
NaOH	50	0.95
Defoamer	t.q.	0.01
Glycerol	100	2
Preservatives	100	0.08
Ethanol	93	1
Without opt. brighteners, perfume, dye and enzymes.		
Dosage 4.7 g/L		

Protease activity assays

[0111] The activity of the protease is determined by releasing the chromophore para-nitroaniline from the substrate succinyl alanine-alanine-proline-phenylalanine-para-nitroanilide (AAPFpNA; Bachem L-1400). The release of the pNA causes an increase in extinction at 410 nm, the time profile of which is a measure of the enzymatic activity.

[0112] The measurement was carried out at a temperature of 25 °C, a pH of 8.6 and a wavelength of 410 nm. The measurement time was 5 min at a measuring interval of 20 to 60 seconds.

Measurement approach:

[0113]

10 µL AAPF solution (70 mg/mL)
1000 µl Tris/HCl (0.1 M; pH 8.6 with 0.1% Brij 35)
10 µl diluted protease solution

Kinetics produced over 5 min at 25 °C (410 nm)

Mini washing test and results

Washing test 1:

[0114] Washing test with *Bacillus subtilis* culture supernatants containing the screened protease mutants through heterologous expression. The supernatants were used in washing agents in the equivalent activity to the benchmark = starting molecules for this mutagenesis round (mutants 1 and 2 according to SEQ ID Nos. 1 and 2) at a market-standard concentration for proteases.
Conditions: 40 °C, 16 °dH water, 1 h

Stains:

[0115]

1. CFT CS038
2. CFT PC-10
3. WfK 10N
4. CFT C-03
5. EMPA 112
6. CFT C-05

[0116] Punched-out pieces of fabric (diameter = 10 mm) were placed in a microtiter plate, washing liquor was preheated to 40 °C, with a final concentration of 4.7 g/L, the liquor and enzyme were applied to the stain and incubated for 1 h at 40 °C and 600 rpm, then the stain was rinsed repeatedly with clear water and left to dry and the brightness was determined using a color measuring device. The lighter the fabric, the better the cleaning performance. The L value = brightness was

measured here; the higher the brighter. The sum of the 6 stains is given in % based on the mutant according to SEQ ID NO:13 or the mutant according to SEQ ID NO:2.

Variant	Catalytic activity (based on catalytic activity of mutant 2 (SEQ ID NO:2))	
	40 °C	**20 °C**
Mutant 2	100%	100%
Mutant 3	112%	117%
Mutant 4	112%	110%

[0117] Both variants exhibit increased washing performance compared to the starting variant (mutant 2 according to SEQ ID NO:2). The improvements can be seen at 40 °C and 20 °C.

Variant	Catalytic activity (based on catalytic activity of mutant 1 (SEQ ID NO:13))	
	40 °C	**20 °C**
Mutant 1	100%	100%
Mutant 5	129%	138%
Mutant 6	113%	148%
Mutant 7	129%	124%
Mutant 8	126%	121%
Mutant 9	134%	117%
Mutant 10	118%	124%
Mutant 11	118%	117%
Mutant 12	121%	131%

[0118] All variants exhibit increased washing performance compared to the starting variant (mutant 1 according to SEQ ID NO:13). The improvements can be seen at 40 °C and 20 °C.

Washing test 2:

[0119] Washing test with *Bacillus subtilis* culture supernatants containing the screened protease mutants by heterologous expression. The supernatants are used in washing agents in the equivalent activity to the benchmark = wild type (according to SEQ ID NO:1) at a market-standard concentration for proteases. The mutants are all based on the washing performance of the wild type, which is set at 100% (sum of the 7 stains, corrected by the performance of the washing agent alone). Conditions: 40 °C, 16 °dH water, 1 h

Stains:

[0120]

1. CFT CS038
2. CFT PC-10
3. WfK 10N
4. CFT C-03
5. EMPA 112
6. CFT C-05
7. H-MR-B

[0121] Punched-out pieces of fabric (diameter = 10 mm) were placed in microtiter plates, washing liquor was preheated to 40 °C, with a final concentration of 3.17 g/L, the liquor and enzyme were added to the stain and incubated for 1 h at 40 °C and 600 rpm, then the stain was rinsed a plurality of times with clear water and left to dry and the brightness was determined using a color-measuring device. The lighter the fabric, the better the cleaning performance. The L value = brightness was measured here; the higher the brighter. The sum of the 7 stains is given in % based on the wild type according to SEQ ID NO:1 corrected by the performance of the washing agent without protease.

Variant	**Performance in washing test at 40 °C** (based on performance of wild type (SEQ ID NO: 1))
Wild type	100%
Mutant 13	103%
Mutant 15	105%
Mutant 16	109%
Mutant 17	110%
Mutant 18	106.5%
Mutant 19	106%
Mutant 20	113%
Mutant 21	109%
Mutant 22	107.5%
Mutant 23	101.5%
Mutant 24	101.5%

[0122] All variants exhibit increased washing performance in comparison with the wild type according to SEQ ID NO:1.

[0123] The same washing test was performed again at 20 °C, using mutant 3 as a reference.

Variant	**Performance in washing test at 20 °C** (based on performance of mutant 3 (SEQ ID NO: 4))
Mutant 15	100%
Mutant 25	105%
Mutant 26	107%
Mutant 27	107%
Mutant 28	113%

[0124] A further washing test was carried out as described above at 40 °C with the stain CFT PC–10, using mutant 15 as a reference.

Variant	**Performance in washing test at 40 °C** (based on performance of mutant 3 (SEQ ID NO: 4))
Mutant 15	100%
Mutant 29	117%
Mutant 30	118%
Mutant 31	108%
Mutant 32	108%
Mutant 33	117%
Mutant 34	121%
Mutant 35	116%
Mutant 36	118%
Mutant 37	122%

[0125] All variants exhibited increased washing performance compared to the variant according to SEQ ID NO:16.

PATENTKRAV

1. Protease med en aminosyresekvens, som i det mindste udviser 90% sekvensidentitet med den i SEQ ID NO:1 angivne aminosyresekvens over hele dennes længde og i hvert tilfælde relateret til nummereringen i henhold til SEQ ID NO:1:

(a) På positionen svarende til position 271, aminosyresubstitutionen Q271E; og

(b) på positionen svarende til position 9, en aminosyresubstitution, der er udvalgt fra P9T, P9H, P9S og P9A, fortrinsvis P9T; og

(c1) på mindst én af positionerne, svarende til positionerne 18, 61, 92, 99, 137, 149, 156, 159, 162, 166, 172, 192, 199, 217 eller 265, mindst én yderligere aminosyresubstitution, udvalgt fra A18D, F61Y, A92S, N99Y, N137K, V149L, S156G, S156Y, T159L, T162S, G166M, D172P, A198V, S199M, Y217M og K265A;

(c2) på mindst tre af positionerne, svarende til positionerne 29, 48, 101, 130, 131, 133, 144, 217, 224 og 252, navnlig 130, 133, 144, 217, 224 og 252, i det mindste tre yderligere aminosyresubstitutioner, udvalgt fra A29G, A48V, D101E, N130D, N130S, N130H, G131D, G131N, G131S, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T, N252S og N252T.

2. Protease ifølge krav 1, omfattende en aminosyresekvens, som har mindst 90% sekvensidentitet med aminosyresekvensen, der er specificeret i SEQ ID NO:1 over hele dens længde og i hvert tilfælde i relation til nummereringen ifølge SEQ ID NO:1:

(a) På positionen, svarende til position 271, aminosyresubstitionen Q271E; og

(b) på positionen, svarende til position 9, en aminosyresubstitution udvalgt fra P9T, P9H, P9S og P9A, fortrinsvis P9T; og

(c2) på mindst tre af positionerne, svarende til positioner 29, 48, 101, 130, 131, 133, 144, 224 og 252, navnlig 130, 133, 144, 224 og 252, i det mindste tre yderligere aminosyresubstitutioner, udvalgt fra A29G, A48V, D101E, N130D, N130S, N130H, G131D, G131N, G131S, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T, N252S og N252T.

3. Protease ifølge krav 1, omfattende en aminosyresekvens, som har mindst 90% sekvensidentitet med aminosyresekvensen, der er specificeret i SEQ ID NO:1 over hele dens længde og i hvert tilfælde i relation til nummereringen ifølge SEQ ID NO:1:

(a) På positionen, svarende til position 271, aminosyresubstitionen Q271E;
(b) på positionen, der svarer til positionen 9, aminosyresubstitutionen P9T; og
(c1) på mindst én af positionerne svarende til positionerne 18, 61, 92, 99, 137, 149, 156, 159, 162, 166, 172, 192, 199, 217 eller 265, mindst én yderligere aminosyresubstitution, udvalgt fra A18D, F61Y, A92S, N99Y, N137K, V149L, S156G, S156Y, T159L, T162S, G166M, D172P, A198V, S199M, Y217M og K265A; og
(c2) på positionerne svarende til positioner 130, 144 og 252, aminosyresubstitutionerne N130D, N144K og N252T.

4. Protease ifølge krav 1, hvorved proteasen omfatter aminosyresubstitutioner
(1) på positionerne svarende til positioner 9 og 271 samt mindst én blandt 130, 131, 133 og 224, i hvert tilfælde baseret på nummereringen ifølge SEQ ID NO:1, og
(2) eventuelt på mindst én af positionerne svarende til henholdsvis positioner 29, 48, 101, 144 og 252 i relation til nummereringen ifølge SEQ ID NO:1.

5. Protease ifølge et af de foregående krav, hvorved proteasen omfatter en af de respektive aminosyresubstitutionsvarianter, baseret på nummereringen ifølge SEQ ID NO:1:

P9T, P9H, P9S eller P9A, navnlig P9T, og Q271E samt eventuelt S216C og desuden én af

(I) A48V, G131S, T133R og S224A;
(II) G131D, T133R og S224A;
(III) N130D, G131N, T133K og N144K;
(IV) A29G, N130D, G131N og T133R;
(V) N130D, G131S, T133K og S224A;
(VI) N130D, G131N, T1K, N144L og N252S;
(VII) G131S, N144K og S224T;
(VIII) N130S, G131S, T133Y, N144L og S224A;
(IX) D101E, G131N og S224A; eller
(X) N130H, G131S og S224A.

6. Protease ifølge et af kravene 1-3, hvorved proteasen omfatter

(1) på positionerne, der svarer til positionerne 9, 130, 144, 252 og 271, aminosyresubstitutionerne P9T, N130D, N144K, N252T og Q271E; og
(2) på mindst én af positionerne, som svarer til positionerne 62, 99, 133, 137, 149, 156,162,166,172,192,199, 217, 224 eller 265, mindst én yderligere aminosyresubstitution.

7. Protease ifølge krav 1 eller krav 3, hvorved proteasen

(1) på positionerne, der svarer til positionerne 9 og 271, omfatter aminosyresubstitutionerne P9T og Q271E; og
(2) på positionen, som svarer til positionen 172, omfatter mindst én yderligere aminosyresubstitution;

8. Protease ifølge et af de foregående krav, hvorved proteasen omfatter en af de følgende aminosyresubstitutionsvarianter, hver for sig relateret til nummereringen i henhold til SEQ ID NO:1:

Q271E og desuden en af:

(I) P9T, N130D, N144K og N252T;
(II) P9T, N130D, N144K, S156G og N252T;
(III) P9T, N130D, N144K, S156Y og N252T;
(IV) P9T, N130D, N144K, Y217M og N252T;
(V) P9T, N130D, N137K, N144K og N252T;
(VI) P9H, A18D, N130D, N144K, T159L og N252T; eller
(VII) P9T og D172G.

9. Protease ifølge et af de foregående krav, hvorved proteasen omfatter en aminosyresekvens ifølge et af SEQ ID NO. 3-12 og 16-25.

10. Nukleinsyre kodende for en protease ifølge et af kravene 1-9.

11. Vektor, indeholdende en nukleinsyre ifølge krav 10, navnlig en kloningsvektor eller en ekspressionsvektor.

12. Ikke-human værtscelle, som indeholder en nukleinsyre ifølge krav 11 eller en vektor ifølge krav 14, eller omfattende en protease ifølge et af kravene 1-9.

13. Fremgangsmåde til fremstilling af en protease, omfattende

a) dyrkning af en værtscelle ifølge krav 12; og
b) isolation af proteasen fra dyrkningsmediet eller fra værtscellen.

14. Middel, navnlig af vaske- eller rengøringsmiddel,
kendetegnet ved, at det mindst indeholder en protease ifølge et af kravene 1-9.

15. Fremgangsmåde til rengøring af tekstiler eller hårde overflader,

kendetegnet ved, at der i mindst eet fremgangsmådetrin anvendes et middel ifølge krav 14, eller at der i mindst eet fremgangsmådetrin anvendes en protease ifølge et af kravene 1-9.

16. Anvendelse af en protease ifølge et af kravene 1-9 i et vaske- eller rengøringsmiddel til fjernelse af peptid- eller proteinholdigt smuds.