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(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, Scharpenberg 9, 45468 Mülheim an der Ruhr, Tyskland
DEGERING, Christian, Hauptstraße 53, 40699 Erkrath, Tyskland
Wieland, Susanne, Schloßstraße 25, 41541 Zons/Dormagen, Tyskland

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

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Performance-enhanced protease variants II

[0001] The invention is in the field of enzyme technology. The invention relates to proteases from *Bacillus pumilus,* the amino acid sequences of which have been altered to give them a better storage stability, in particular with regard to the use in washing and cleaning agents, and also relates to the nucleic acids coding for said proteases and to the production thereof. The invention further relates to the uses of these proteases and to methods in which they are used, as well as to agents containing them, in particular washing and cleaning agents.

[0002] Proteases are some of the technically most important enzymes. They are the longest established enzymes for washing and cleaning agents, and are contained in virtually all modern, effective washing and cleaning agents. They bring about the decomposition of protein-containing stains on the item to be cleaned. Of these, in turn, proteases of the subtilisin type (subtilases, subtilopeptidases, EC 3.4.21.62) are particularly important, which are serine proteases due to the catalytically active amino acids. They act as non-specific endopeptidases and hydrolyze any acid amide bonds that are inside 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 from microorganisms. In particular, the subtilisins formed and secreted by *Bacillus* species 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 manner or randomly using methods known from the prior

art, and are thus optimized for use in washing and cleaning agents, for example. This includes point, deletion or insertion mutagenesis, or fusion with other proteins or protein parts. Appropriately optimized variants are therefore known for the majority of proteases known from the prior art. From WO 2011/036263, for example, savinase and/or BPN' variants are known.

[0004] EP 2016175 A1, WO 2017/198488 und WO 2017/162429 discloses a protease from *Bacillus pumilus* intended for washing and cleaning agents. In general, only selected proteases are suitable for use in liquid, surfactant-containing preparations in any case. 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 (a) at the positions corresponding to positions 9 and 271, in each case based on the numbering according to SEQ ID NO:1, and (b) at at least three 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, is improved in terms of storage stability compared with the wild-type form and/or reference mutants and is therefore particularly suitable for use in washing or cleaning agents.

[0007] The invention therefore relates, in a first aspect, to a protease according to claim 1, comprising an amino acid sequence which has at least 80% sequence identity with the amino acid sequence given in SEQ ID NO:1 over its entire length

and (i) has amino acid substitutions, preferably the amino acid substitutions 9T, 9H, 9S or 9A and 271E, at the positions corresponding to positions 9 and 271 according to SEQ ID NO:1, and (ii) has an amino acid substitution at at least three 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,

wherein

(1) the amino acid substitutions at the positions corresponding to positions 9 and 271 are the amino acid substitutions P9T, P9H, P9S or P9A and Q271E; and/or

(2) the at least three amino acid substitutions according to (b) are selected from the group consisting of A29G, A48V, D101E, N130D, N130S, N130H, G131S, G131N, G131D, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T or N252S, in each case based on the numbering according to SEQ ID NO:1.

[0008] In one embodiment of this first aspect, the invention relates to a protease comprising an amino acid sequence which has at least 80% sequence identity with the amino acid sequence given in SEQ ID NO:1 over its entire length and has, in each case based on the numbering according to SEQ ID NO:1:

(a) amino acid substitutions, in particular the amino acid substitutions 9T, 130D, 144K, 252T and 271E, at the positions corresponding to positions 9, 130, 144, 252 and 271; as well as optionally

(b1) the amino acid substitution 166M, 166Q or 166A at the position corresponding to position 166; and/or

(b2) at least one further amino acid substitution at at least one of the positions corresponding to positions 62, 133, 149, 156, 172 or 217.

[0009] In a second aspect, the invention also relates to a protease comprising an amino acid sequence which has at least 80% sequence identity with the amino acid sequence given in SEQ ID NO:1 over its entire length and, based on the numbering according to SEQ ID NO:1, has at least one amino acid substitution at at least one of the positions corresponding to positions 62, 149, 156, 166, 172 or 217, the amino

acid substitution at the position corresponding to position 166 being selected from 166M, 166Q and 166A, and the amino acid substitution at the position corresponding to position 62 being selected from 62S.

[0010] In a further aspect, the present invention relates to a protease having an amino acid sequence which has at least 80% sequence identity with the amino acid sequence given in SEQ ID NO:1 over its entire length and which comprises at least three amino acid substitutions selected from the group consisting of N130D, N130S, N130H, G131N, G131D, G131K, T133K, T133Y, N144L, N144A, S224A, or N252S, in each case based on the numbering according to SEQ ID NO:1. In such an embodiment, the protease has a substitution at one of positions 9 and 271, and optionally also a substitution at position 216.

[0011] In various embodiments of the aspects described above, the sequence identity with SEQ ID NO:1 may preferably be 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 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%.

[0012] The invention also relates to a method for producing a protease, comprising the substitution of amino acids in a starting protease which has at least 80% sequence identity with the amino acid sequence given in SEQ ID NO:1 over its entire length (i) at the positions corresponding to positions 9 and 271 in SEQ ID NO:1, preferably such that the protease comprises the amino acid substitutions 9T, 9H, 9S or 9A, in particular 9T and 271E, at the positions, and (ii) at at least three positions corresponding to position 29, 48, 101, 130, 131, 133, 144, 224 or 252 in SEQ ID NO:1, preferably such that the protease has at least three of the amino acid substitutions 29G, 48V, 101E, 130D, 130S, 130H, 131S, 131N, 131D, 131K, 133K, 133R, 133Y, 144K, 144L, 144A, 224A, 224T, 252T or 252S. The protease that can be obtained by this method preferably has at least 80% sequence identity with the amino acid sequence given in SEQ ID NO:1 over its entire length.

[0013] In various embodiments of this aspect, the invention relates to a method for

producing a protease as defined above, comprising the substitution of amino acids in a starting protease which has at least 80% sequence identity with the amino acid sequence given in SEQ ID NO:1 over its entire length (i) at the positions corresponding to positions 9, 130, 144, 252 and 271 in SEQ ID NO:1, such that the protease comprises amino acid substitutions, in particular the amino acid substitutions 9T, 130D, 144K, 252T and 271E, at the positions, and optionally (ii) at the position corresponding to position 166 in SEQ ID NO:1, such that the protease has at least one of the amino acid substitutions 166M, 166Q or 166A and/or (iii) has at least one further amino acid substitution at at least one of the positions corresponding to positions 62, 133, 149, 156, 172 or 217 in SEQ ID NO:1. The protease that can be obtained by this method has at least 80% sequence identity with the amino acid sequence given in SEQ ID NO:1 over its entire length.

[0014] A protease within the meaning of the present patent application therefore comprises both the protease as such and a protease produced by 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.

[0015] Further aspects of the invention relate to the nucleic acids coding for these proteases, to non-human host cells containing proteases or nucleic acids according to the invention, and to agents comprising proteases according to the invention, in particular washing and cleaning agents, to washing and cleaning methods, and to uses of the proteases according to the invention in washing or cleaning agents in order to remove protein-containing stains.

[0016] "At least one," as used herein, means one or more, i.e. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or more.

[0017] 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.

[0018] The present invention is based on the surprising finding by the inventors that amino acid substitutions at the positions corresponding to positions 9 and 271, in each case based on the numbering according to SEQ ID NO:1, such that the protease comprises the amino acid substitutions 9T, 9H, 9S or 9A and 271E at the positions, and an amino acid substitution at at least three of the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 224 or 252 of the protease from *Bacillus pumilus* according to SEQ ID NO:1, in a protease comprising an amino acid sequence that is at least 80% identical to the amino acid sequence given in SEQ ID NO:1, such that the amino acids 29G, 48V, 101E, 130D, 130S, 130H, 131S, 131N, 131D, 131K, 133K, 133R, 133Y, 144K, 144L, 144A, 224A, 224T, 252T or 252S are present at at least three of the corresponding positions, results in improved storage stability of this altered protease in washing and cleaning agents. It has further been found that this storage stability in proteases based on the sequence according to SEQ ID NO:1, which have amino acid substitutions, in particular the amino acid substitutions 9T, 130D, 144K , 252T and 271E, at the positions corresponding to positions 9, 130, 144, 252 and 271; and optionally (b1) have the amino acid substitutions 166M, 166Q or 166A at the position corresponding to position 166; and/or (b2) have at least one further amino acid substitution at at least one of the positions corresponding to positions 62, 133, 149, 156, 172 or 217, is improved in a particularly advantageous manner. This is particularly surprising insofar as none of the above-mentioned amino acid substitutions has previously been associated with increased storage stability of the protease. Optionally, improved performance of this altered protease (proteolytic activity under standard washing conditions) can additionally be brought about in washing and cleaning agents.

[0019] In various 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 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 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 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 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 based on the numbering according to SEQ ID NO:1;

(F) (i) 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 based on the numbering according to SEQ ID NO:1;

(G) (i) 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 based on the numbering according to SEQ ID NO:1;

(H) (i) 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 based on the numbering according to SEQ ID NO:1;

(I) (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 based on the numbering according to SEQ ID NO:1;

(J) (i) 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 based on the numbering according to SEQ ID NO:1;

(K) (i) at the positions corresponding to positions 9, 130, 133, 144, 252 und 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 based on the numbering according to SEQ ID NO:1; or

(L) (i) has amino acid substitutions, in particular the amino acid substitutions 9T, 130D, 144K, 252T and 271E, at the positions corresponding to positions 9, 130, 144, 252 and 271; and (ii) has one of the amino acid substitutions 62S, 149I, 156G, 156Q, 166M, 166Q, 166A, 172P, 172G or 217M at one of the positions corresponding to positions 62, 149, 156, 166, 172 or 217.

[0020] 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 + 130 and optionally at least one of 29, 48, 101, 130, 144, 224 or 252;

9 + 271 + 131 + 130 + 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;

[0021] Here, 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.

[0022] In various embodiments, the protease has

(1) amino acid substitutions, in particular the amino acid substitutions 9T, 130D, 144K, 252T and 271E, at the positions corresponding to positions 9, 130, 144, 252 and 271; and

(2) one of the amino acid substitutions 62S, 149I, 156G, 156Q, 166M, 166Q, 166A, 172P, 172G or 217M at one of the positions corresponding to positions 62, 149, 156, 166, 172 or 217;

[0023] In preferred embodiments of the protease according to the invention, the protease has substitutions selected from the amino acid substitutions 149I, 156G, 156Q, 172P, 172G and 217M at the positions corresponding to positions 149, 156, 172 or 217.

[0024] In various embodiments, the protease has amino acid substitutions, in particular the amino acid substitutions 9T, 130D, 144K, 252T and 271E, at the positions corresponding to positions 9, 130, 144, 252 and 271; and has at least one, for example 1, 2, 3, 4, 5 or 6, for example 1, 2, 3 or 4, further amino acid substitutions at one or more of the positions corresponding to positions 62, 149, 156, 166, 172 or 217, these being preferably selected from: 62S, 149I, 156G, 156Q, 166M, 166Q,

166A, 172P, 172G or 217M. Such proteases are disclosed for example as mutants 7-12 in example 1 and are thus 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 und 252; and at least one, for example 1, 2 or 3, further amino acid substitution(s) at one or more of the positions corresponding to positions 62, 166 or 217, these being preferably selected from: 62S, 166Q and 217M.

[0025] In further embodiments, the protease has amino acid substitutions at the 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
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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
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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
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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

[0026] 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 at least

one, for example 1, 2 or 3, additional amino acid substitutions at the positions corresponding to positions 62, 166 or 217, based on the numbering according to SEQ ID NO:1. In various embodiments, this additional amino acid substitution is a substitution at position 217. In various other embodiments, this additional substitution is one at position 62 and/or 166. The above-described amino acid substitution may preferably be selected from: 62S, 166Q and 217M.

[0027] Further embodiments of the invention relate to protease variants which have amino acid substitutions at the following positions (in the numbering according to SEQ ID NO:1):

9 + 130 + 144 + 252 + 271 + 62
9 + 130 + 144 + 252 + 271 + 149
9 + 130 + 144 + 252 + 271 + 156
9 + 130 + 144 + 252 + 271 + 166
9 + 130 + 144 + 252 + 271 + 172
9 + 130 + 144 + 252 + 271 + 217
9 + 130 + 144 + 252 + 271 + 62 + 149
9 + 130 + 144 + 252 + 271 + 62 + 156
9 + 130 + 144 + 252 + 271 + 62 + 166
9 + 130 + 144 + 252 + 271 + 62 + 172
9 + 130 + 144 + 252 + 271 + 62 + 217
9 + 130 + 144 + 252 + 271 + 149 + 156
9 + 130 + 144 + 252 + 271 + 149 + 166
9 + 130 + 144 + 252 + 271 + 149 + 172
9 + 130 + 144 + 252 + 271 + 149 + 217
9 + 130 + 144 + 252 + 271 + 156 + 166
9 + 130 + 144 + 252 + 271 + 156 + 172
9 + 130 + 144 + 252 + 271 + 156 + 217
9 + 130 + 144 + 252 + 271 + 166 + 172
9 + 130 + 144 + 252 + 271 + 166 + 217
9 + 130 + 144 + 252 + 271 + 172 + 217
9 + 130 + 133 + 144 + 252 + 271 + 62

9 + 130 + 133 + 144 + 252 + 271 + 149
9 + 130 + 133 + 144 + 252 + 271 + 156
9 + 130 + 133 + 144 + 252 + 271 + 166
9 + 130 + 133 + 144 + 252 + 271 + 172
9 + 130 + 133 + 144 + 252 + 271 + 217
9 + 130 + 133 + 144 + 252 + 271 + 62 + 149
9 + 130 + 133 + 144 + 252 + 271 + 62 + 156
9 + 130 + 133 + 144 + 252 + 271 + 62 + 166
9 + 130 + 133 + 144 + 252 + 271 + 62 + 172
9 + 130 + 133 + 144 + 252 + 271 + 62 + 217
9 + 130 + 133 + 144 + 252 + 271 + 149 + 156
9 + 130 + 133 + 144 + 252 + 271 + 149 + 166
9 + 130 + 133 + 144 + 252 + 271 + 149 + 172
9 + 130 + 133 + 144 + 252 + 271 + 149 + 217
9 + 130 + 133 + 144 + 252 + 271 + 156 + 166
9 + 130 + 133 + 144 + 252 + 271 + 156 + 172
9 + 130 + 133 + 144 + 252 + 271 + 156 + 217
9 + 130 + 133 + 144 + 252 + 271 + 166 + 172
9 + 130 + 133 + 144 + 252 + 271 + 166 + 217
9 + 130 + 133 + 144 + 252 + 271 + 172 + 217

[0028] 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 62, 133, 149, 156, 166, 172 and 217, if these have not yet been mentioned above.

[0029] 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.

[0030] 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 62, 149, 156, 166, 172 and 217, these being preferably selected from: 62S, 149I, 156G, 156Q, 166M, 166Q, 166A, 172P, 172G or 217M.

[0031] Such proteases are disclosed for example as mutants in example 1 and are thus 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 und 252; and at least one, for example 1, 2 or 3, further amino acid substitution(s) at one or more of the positions corresponding to positions 62, 166 or 217, these being preferably selected from: 62S, 166Q and 217M.

[0032] 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.

[0033] Furthermore, 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, G131S, G131N, G131D, 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 yet 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) N130D, G131S, T133Y and S224A; (ii) N130S, G131S, T133Y, N144L and S224A; (iii) N130D, G131N, T133K and N144K; (iv) N130H, G131N, N144K and S224A; (v) A29G, N130D, G131N and T133R; (vi) A29G, D101E, N130S, G131S, S224T and N252S; (vii) N130H, G131S and S224A; (viii) N130D, G131S, T133K and S224A; (ix) A29G, D101E, N130D, G131K and S224A; (x) N130D, G131N T133K, N144L and N252S; (xi) G131S, N144K and S224T; (xii) D101E, N130D, G131S, T133Y, N144A and S224A; (xiii) A29G, D101E, N130S, S224T and N252S; (xiv) A48V, G131S, T133R and S224A; or (xv) G131D, T133R and S224A, the numbering being based in each case on the numbering according to SEQ ID NO:1.

[0034] In a further embodiment, the protease according to the invention has an amino acid sequence according to SEQ ID Nos. 3-17 or 19-23 or 20-23.

[0035] The proteases according to the invention have improved storage stability. They have increased stability in washing or cleaning agents in comparison with a reference mutation variant of the protease (SEQ ID NO:18 and/or SEQ ID NO:2 and/or SEQ ID NO: 19), 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. Such stability-enhanced proteases make it possible, even after a prolonged storage time, to achieve good washing results on proteolytically sensitive stains in various temperature ranges, in particular in a temperature range of from 20 °C to 40 °C.

[0036] In addition to increased storage stability, the proteases according to the invention may also have 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:1 and/ SEQ ID NO:2 and/or SEQ ID NO: 19), 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 allow improved washing results on proteolytically sensitive stains in various temperature ranges, in particular in a temperature range of from 20 °C to 40 °C.

[0037] The proteases according to the invention exhibit enzymatic activity, i.e. they are capable of hydrolyzing peptides and proteins, in particular in a washing or cleaning agent. A protease according to the invention is therefore an enzyme which catalyzes the hydrolysis of amide/peptide bonds in protein/peptide substrates and is thus able to cleave proteins or peptides. Furthermore, a protease according to the invention is preferably a mature protease, i.e. the catalytically active molecule without signal peptide(s) and/or propeptide(s). Unless stated otherwise, the sequences given also each refer to mature (processed) enzymes.

[0038] In various embodiments of the invention, the protease is a free enzyme. This means that the protease can act directly with all the components of an agent and, if the agent is a liquid agent, that the protease is in direct 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 multiple protease molecules may be separated from the other constituents of the agent by a surrounding structure. Such a separating structure may arise from, but is not limited to, vesicles such as a micelle or a liposome. 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.

[0039] In a further embodiment of the invention, the protease comprises an amino acid sequence which, over its entire length, is at least 80%, 81%, 82%, 83%, 84%,

85%, 86%, 87%, 88%, 89%, 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 the context of 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 fragmenting of the protease. In various embodiments, it has amino acid substitutions (a) at the positions corresponding to positions 9 and 271, preferably such that the amino acid substitutions are P9T, P9H, P9S or P9A and Q271E, and (b) has three or more of the amino acid substitutions 29G, 48V, 101E, 130D, 130S, 130H, 131S, 131N, 131D, 131K, 133K, 133R, 133Y, 144K, 144L, 144A, 224A, 224T or 252S at at least three of the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 224 or 252 in the numbering according to SEQ ID NO:1. The amino acid sequences of such proteases which are preferred according to the invention are given in SEQ ID Nos: 3-17 and 19-23. 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.

[0040] 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 in principle occurs by associating similar sequences of nucleotides or amino acids in the nucleic acid or amino acid sequences. A tabular association of the positions concerned is referred to as alignment. Another 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 programs or algorithms are frequently used, for example. Sequence comparisons (alignments) using the computer program Vector NTI® Suite 10.3 (Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California, USA) with the predetermined, default parameters, and the AlignX module of which for sequence comparisons is based on ClustalW, are also possible. Unless stated otherwise, the sequence identity given herein is determined by the BLAST algorithm.

[0041] Such a comparison also allows a statement regarding the similarity of the compared sequences. It is usually given in percent identity, i.e. the proportion of identical nucleotides or amino acid residues in said sequences or in an alignment of corresponding positions. 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 of the compared sequences may also be stated 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. Often, such small regions 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 stated otherwise, however, identity or homology information in the present application relates to the entire length of the particular nucleic acid or amino acid sequence indicated.

[0042] 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.

[0043] In a further embodiment of the invention, the protease is characterized in that the cleaning performance thereof (after storage, e.g. over 4 weeks) 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:18 and/or SEQ ID NO:2 and/or SEQ ID NO:19, 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 between 4.5 and 7.0 grams per liter of washing liquor and the protease, the proteases to be compared being used in the same concentration (based on active protein) and the cleaning performance with respect to a stain on cotton being determined by measuring the degree of cleaning of the washed textiles. 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 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.

[0044] A liquid reference washing agent for such a washing system may be composed as follows (all values in wt.%): 4.4% alkyl benzene sulfonic acid, 5.6% further anionic surfactants, 2.4% C12-C18 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. Preferably, the dosage of the liquid washing agent is 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. Washing in a pH range between pH 7 and pH 10.5, preferably between pH 7.5 and pH 8.5, is preferred.

[0045] 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, the

washing process preferably being carried out for 60 minutes at 600 rpm.

[0046] The degree of whiteness, i.e. the lightening of stains, as a measure of the cleaning performance is determined by optical measuring methods, preferably photometrically. A suitable device for this purpose is for example the Minolta CM508d spectrometer. Usually, the devices used for the measurement are calibrated beforehand with a white standard, preferably a supplied white standard.

[0047] The activity-equivalent use of the relevant protease ensures that the respective enzymatic properties, 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) significantly differs. In general, a low specific activity can be compensated for by adding a larger amount of protein.

[0048] Otherwise, methods for determining protease activity are well known to, and routinely used by, a person skilled in the art of enzyme technology. For example, such methods are disclosed in Tenside, vol. 7 (1970), p. 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 temporal progression 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 measuring time is 5 min and the measuring interval is 20 s to 60 s. The protease activity is usually indicated in protease units (PE). Suitable protease activities amount for example to 2.25, 5 or 10 PE per ml of washing liquor. However, the protease activity is not equal to zero.

[0049] An alternative test for establishing the proteolytic activity of the proteases according to the invention is an optical measuring method, preferably a photometric method. The appropriate test comprises the protease-dependent cleavage of the substrate protein casein. This is cleaved by the protease into a multitude of smaller

partial products. The totality of these partial products has an increased absorption at 290 nm compared with uncleaved casein, it being possible for this increased absorption to be determined using a photometer, and thus for a conclusion to be drawn regarding the enzymatic activity of the protease.

[0050] 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), p. 751-766). The active protein concentration can be determined in this regard by titrating the active centers using a suitable irreversible inhibitor and determining the residual activity (cf. M. Bender et al., J. Am. Chem. Soc. 88, 24 (1966), p. 5890-5913).

[0051] In addition to the amino acid alterations discussed above, proteases according to the invention can have other amino acid alterations, in particular amino acid substitutions, insertions or deletions. Such proteases are, for example, further developed by targeted genetic modification, i.e. by mutagenesis methods, and optimized for specific applications 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 can thus be used to generate completely novel proteases or other polypeptides.

[0052] 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 instance, 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 in addition, one or more corresponding mutations can increase the stability or catalytic activity of the protease and thus improve its cleaning performance. Advantageous properties of individual mutations, e.g. individual substitutions, can complement one another. A

protease which has already been optimized with regard to specific properties, for example with respect to its stability during storage, can therefore also be further developed within the scope of the invention.

[0053] For the description of substitutions relating to exactly one amino acid position (amino acid exchanges), the following convention is used herein: first, the naturally occurring amino acid is designated in the form of the internationally used one-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. For insertions, additional amino acids are named following 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. This nomenclature is known to a person skilled in the field of enzyme technology.

[0054] 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 one-time 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 residue for another amino acid residue, 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 residue for another nonpolar amino acid residue. Conservative amino acid substitutions within the scope 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.

[0055] Alternatively or in addition, the protease is characterized in that it is obtainable from a protease according to the invention as a starting molecule by fragmentation or 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 or 275 contiguous amino acids, the amino acid substitution(s) according to the invention which is/are described above and possibly contained in the starting molecule, i.e. the substitutions at the positions corresponding to positions 9, 29, 48, 101, 130, 131, 133, 144, 216, 224, 252 and 271 in SEQ ID NO:1, and optionally the amino acid substitution 166M, 166Q or 166A at the position corresponding to position 166, and/or at least one further amino acid substitution at at least one of positions corresponding to positions 62, 133, 149, 156, 172 or 217, still being present.

[0056] For instance, it is possible to delete individual amino acids at the termini or in the loops of the enzyme without the proteolytic activity being lost or diminished in the process. Furthermore, such fragmentation or deletion, insertion or substitution mutagenesis can also, for example, reduce the allergenicity of the enzymes concerned and thus improve their overall applicability. 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. Other substitutions can also exhibit advantageous effects. Both single and multiple contiguous amino acids can be exchanged for other amino acids.

[0057] Alternatively or in addition, the protease is characterized in that it is obtainable from a protease according to the invention as the starting molecule by one-time or multiple conservative amino acid substitution, the protease having (i) the amino acid substitutions P9T, P9H, P9S or P9A and Q271E at the positions corresponding to positions 9 and 271 according to SEQ ID NO:1, and (ii) at least three of the amino acid substitutions A29G, A48V, D101E, N130D, N130S, N130H, G131S, G131N, G131D, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T or N252S at the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 224 and 252 according to SEQ ID NO:1, and optionally the

amino acid substitution 166M, 166Q or 166A at the position corresponding to position 166, and/or at least one further amino acid substitution at at least one of the positions corresponding to positions 62, 133, 149, 156, 172 or 217.

[0058] In further embodiments, the protease is characterized in that it is obtainable from a protease according to the invention as the starting molecule by fragmentation or 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 or 275 contiguous amino acids, the protease comprising (i) the amino acid substitutions P9T, P9H, P9S or P9A and Q271E at the positions corresponding to positions 9 and 271 according to SEQ ID NO:1, and (ii) at least three of the amino acid substitutions A29G, A48V, D101E, N130D, N130S, N130H, G131S, G131N, G131D, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T or N252S at the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 224 and 252 according to SEQ ID NO:1, and optionally the amino acid substitution 166M, 166Q or 166A at the position corresponding to position 166, and/or at least one further amino acid substitution at at least one of the positions corresponding to positions 62, 133, 149, 156, 172 or 217.

[0059] 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 depends on the mature protein. This assignment is in particular also to be used if the amino acid sequence of a protease according to the invention comprises a higher number of amino acid residues than the protease from *Bacillus pumilus* according to SEQ ID NO:1. Proceeding from the above-mentioned 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 precisely these positions in an alignment.

[0060] 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 positions 9, 29, 48 , 62, 101, 130, 131, 133, 144, 149, 156, 166, 172, 216, 217, 224, 252 and 271 in SEQ ID NO:1 in an alignment, i.e. in the numbering according to SEQ ID NO:1. At the positions mentioned, the following amino acid residues are present in the wild-type molecule of the protease from *Bacillus pumilus*: P9, A29, A48, Q62, D101, N130, G131, T133, N144, V149, S156, G166, D172, S216, Y217, S224, N252 and Q271.

[0061] 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 experiments, according to which the two positions assigned to one another on the basis of an alignment are altered in the same way in both compared proteases, and observations are made as to whether the enzymatic activity is altered in the same way in both cases. If, for example, an amino acid exchange in a specific position of the protease from *Bacillus pumilus* according to SEQ ID NO:1 is accompanied by an alteration of an enzymatic parameter, for example an increase in the K_M value, and a corresponding alteration of the enzymatic parameter, for example likewise an increase in the K_M value, is observed in a protease variant according to the invention of which the amino acid exchange has been achieved by the same introduced amino acid, this can therefore be considered to be confirmation of the correct assignment.

[0062] All of these aspects are also applicable to the method for producing a protease according to the invention. Accordingly, a method according to the invention further comprises one or more of the following method steps:

a) Introducing one-time or multiple conservative amino acid substitution, the protease comprising

(i) the amino acid substitutions P9T, P9H, P9S or P9A and Q271E at the positions corresponding to positions 9 and 271 according to SEQ ID NO:1, and

(ii) at least three of the amino acid substitutions A29G, A48V, D101E, N130D, N130S, N130H, G131S, G131N, G131D, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T or N252S at the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 224 and 252 according to SEQ ID NO:1;

b) Altering the amino acid sequence by fragmentation or deletion, insertion or substitution mutagenesis such that the protease 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 or 275 contiguous amino acids, the protease comprising

(i) the amino acid substitutions P9T, P9H, P9S or P9A and Q271E at the positions corresponding to positions 9 and 271 according to SEQ ID NO:1, and

(ii) at least three of the amino acid substitutions A29G, A48V, D101E, N130D, N130S, N130H, G131S, G131N, G131D, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T or N252S at the positions corresponding to positions 29, 48, 101, 130, 131, 133, 144, 224 and 252 according to SEQ ID NO:1.

[0063] In various embodiments of this method, this comprises one or more of the following steps:

a) Introducing one-time or multiple conservative amino acid substitution into the protease, the protease comprising the substitutions 9T, 130D, 144K, 252T and 271E at the positions corresponding to positions 9, 130, 144, 252 and 271; and optionally the amino acid substitution 166M, 166Q or 166A at the position corresponding to position 166, and/or at least one further amino acid substitution at at least one of the positions corresponding to positions 62, 133, 149, 156, 172 or 217;

b) Altering the amino acid sequence by fragmentation or deletion, insertion or substitution mutagenesis such that the protease 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, the protease comprising the substitutions 9T, 130D, 144K, 252T and 271E at the positions corresponding to positions 9, 130, 144, 252 and 271; and optionally the amino acid substitution 166M, 166Q or 166A at the position corresponding to position 166, and/or at least one further amino acid substitution at at least one of the positions corresponding to positions 62, 133, 149, 156, 172 or 217.

[0064] All embodiments also apply to the method according to the invention.

[0065] In further embodiments of the invention, the protease or the protease produced by means of a method according to the invention is still at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 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 its entire length. Alternatively, the protease or the protease produced by means of a method according to the invention is still at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 90.5%, 91%, 91.5%, 92%, 92.5%, 93%, 93.5%, 94%, 94.5%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, or 98% identical to one of the amino acid sequences given in SEQ ID Nos: 3-17 or 19-23 over its entire length. The protease or the protease produced by means of a method according to the invention has an amino acid substitution at positions 9 and 271 and at least three 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. In more preferred embodiments, the amino acid substitution is at least one selected from the group consisting of P9T, P9H, P9S, P9A, A29G, A48V, D101E, N130D, N130S, N130H, G131S, G131N, G131D, 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. In further embodiments, the protease further comprises a substitution at the position 216, in particular S216C; in various embodiments it may

be preferred for this position to not be substituted. The following amino acid substitution variants are most particularly preferred: P9T, P9H, P9S or P9A, in particular P9T, and Q271E and optionally also S216C combined with one of (i) N130D, G131S, T133Y and S224A; (ii) N130S, G131S, T133Y, N144L and S224A; (iii) N130D, G131N, T133K and N144K; (iv) N130H, G131N, N144K and S224A; (v) A29G, N130D, G131N and T133R; (vi) A29G, D101E, N130S, G131S,

[0066] S224T and N252S; (vii) N130H, G131S and S224A; (viii) N130D, G131S, T133K and S224A; (ix) A29G, D101E, N130D, G131K and S224A; (x) N130D, G131N T133K, N144L and N252S; (xi) G131S, N144K and S224T; (xii) D101E, N130D, G131S, T133Y, N144A and S224A; (xiii) A29G, D101E, N130S, S224T and N252S; (xiv) A48V, G131S, T133R and S224A; or (xv) G131D, T133R and S224A, the numbering being based in each case on the numbering according to SEQ ID NO:1.

[0067] In further embodiments, the protease or the protease produced by means of a method according to the invention has the amino acid substitutions 9T, 130D, 144K, 252T and 271E at the positions corresponding to positions 9, 130, 144, 252 and 271; and optionally the amino acid substitution 166M, 166Q or 166A at the position corresponding to position 166, and/or at least one further amino acid substitution at at least one of the positions corresponding to positions 62, 133, 149, 156, 172 or 217, in each case based on the numbering according to SEQ ID NO:1. Examples thereof are the following amino acid substitution variants: P9T and Q271E combined with one of (i) N130D, N144K, N252T and G166M; (ii) N130D, N144K, N252T and G166Q; (iii) N130D, N144K, N252T and G166A; (iv) N130D, N144K, N252T and S156G; or (v) N130D, N144K, N252T and Y217M, the numbering being based in each case on the numbering according to SEQ ID NO:1 and the variants described in the examples.

[0068] The invention also relates to a protease described above which is additionally stabilized, in particular by one or more mutations, for example substitutions, or by coupling to a polymer. An increase in stability during storage and/or during use, for example in the washing process, leads to longer enzymatic activity and thus

improves the cleaning performance. In principle, all stabilization options which are described in the prior art and/or are appropriate are considered. Those stabilizations are preferred which are achieved by mutations of the enzyme itself, since such stabilizations do not require any further work steps following the recovery of the enzyme. Examples of sequence alterations suitable for this purpose are mentioned above. Further suitable sequence alterations are known from the prior art.

[0069] 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 acids involved in the calcium binding with one or more negatively charged amino acids and/or by introducing sequence alterations into at least one of the results of the two amino acids arginine/glycine;
- Protecting against the influence of denaturing agents such as surfactants by mutations that cause an alteration of the amino acid sequence on or at the surface of the protein;
- Replacing amino acids which are close to the N-terminus with those likely to contact the rest of the molecule via non-covalent interactions, thus contributing to the maintenance of the globular structure.

[0070] Preferred embodiments are those in which the enzyme is stabilized in several ways, since several stabilizing mutations have an additive or synergistic effect.

[0071] The invention also relates to a protease as described above, which is characterized in that it has at least one chemical modification. A protease having such an alteration is called a derivative, i.e. the protease is derivatized.

[0072] In the context of the present application, derivatives are thus understood to mean those proteins of which the 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 may also be another protein that is bound to a protein according to the invention via bifunctional chemical compounds, for example. Derivatization is also understood to mean the covalent bonding to a macromolecular carrier or a non-covalent inclusion in suitable macromolecular cage structures. Derivatizations may, for example, affect the substrate specificity or bonding strength to the substrate or cause a temporary blockage of the enzymatic activity when the coupled substance is an inhibitor. This can be expedient, for example, for the period of the storage. Such modifications may further affect the stability or enzymatic activity. They can also be used to reduce the allergenicity and/or immunogenicity of the protein and thus, for example, increase its skin compatibility. For example, couplings with macromolecular compounds, for example polyethylene glycol, can improve the protein in terms of stability and/or skin compatibility.

[0073] Derivatives of a protein according to the invention can also be understood in the broadest sense to mean preparations of these proteins. Depending on the recovery, processing or preparation, a protein can be combined with various other substances, for example from the culture of the producing microorganisms. A protein may also have been deliberately added to 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 irrespective of whether or not it actually exhibits this enzymatic activity in a particular preparation. This is because it may be desired that it has no or only low activity during storage, and exhibits its enzymatic function only at the time of use. This can be controlled via appropriate accompanying substances, for example. In particular, the joint preparation of proteases with specific inhibitors is possible in this regard.

[0074] Of all the proteases or protease variants and/or derivatives described above, in the context of the present invention those of which the storage stability

corresponds to at least one of those of the proteases according to SEQ ID Nos: 3-17 or 19-23, and/or of which the cleaning performance corresponds to at least one of those of the proteases according to SEQ ID Nos: 3-17 or 19-23, are particularly preferred, the cleaning performance being determined in a washing system as described above.

[0075] The invention also relates to a nucleic acid which codes for a protease according to the invention, as well as to a vector containing such a nucleic acid, in particular a cloning vector or an expression vector.

[0076] These may be DNA or RNA molecules. They can be present as a single strand, as a single strand that is complementary to this 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 should be noted that different codons, i.e. base triplets, can code for the same amino acids such that a particular 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 unequivocally since, 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 an 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. For instance, every organism, for example a host cell of a production strain, has a particular codon usage. Codon usage is understood to mean the translation of the genetic code into amino acids by the particular organism. Bottlenecks can occur in the 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, it can be translated more efficiently in the organism.

[0077] Using methods which are currently generally known, such as chemical synthesis or the polymerase chain reaction (PCR), in conjunction with molecular-biological and/or protein-chemical standard methods, it is possible for a person skilled in the art 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.

[0078] Within the meaning of the present invention, vectors are understood to mean elements consisting of nucleic acids, which elements 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 special plasmids, i.e. circular genetic elements, in particular when used in bacteria. 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 additional genetic elements present in each case, vectors are able to establish themselves as stable units in the corresponding host cells over several generations. They may be present as separate units in an extrachromosomal manner or integrated into a chromosome or chromosomal DNA.

[0079] Expression vectors comprise nucleic acid sequences which enable 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 in particular influenced by the promoter(s) that regulate the 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. Furthermore, expression vectors can be regulatable, for example by changing the cultivation conditions or when a specific cell density of the host cells containing them is reached or by addition of specific 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 with expression vectors, the nucleic acid contained is not expressed in cloning vectors.

[0080] The invention also relates to a non-human host cell which contains a nucleic acid according to the invention or a vector according to the invention or which contains a protease according to the invention, in particular one which 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, which 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 be introduced into a host cell such 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 that can be managed in a genetically advantageous manner, for example in terms of the transformation with the nucleic acid or the vector and the stable establishment thereof, are preferred, for example unicellular fungi or bacteria. Furthermore, preferred host cells are characterized 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 attachment of sugar molecules, formylations, aminations, etc. Such post-translational modifications can functionally influence the protease.

[0081] Other preferred embodiments are those host cells of which the activity can be regulated on account of genetic regulatory elements, which are, for example, made available on the vector but may also be present in these cells from the outset. These host cells may be induced to express for example by the controlled addition of chemical compounds which are used as activators, by changing the cultivation conditions, or upon reaching a specific cell density. This enables an economical production of the proteins according to the invention. An example of such a compound is IPTG as described above.

[0082] Prokaryotic or bacterial cells are preferred host cells. Bacteria are characterized by short generation times and low demands on cultivation conditions. As a result, cost-effective cultivation methods or production methods can be established. In addition, a person skilled in the art has a wealth of experience in the case of bacteria in fermentation technology. For a specific production, gram-negative or gram-positive bacteria may be suitable for a wide variety of reasons to be determined experimentally in individual cases, such as nutrient sources, product formation rate, time requirement, etc.

[0083] In the case of gram-negative bacteria, such as *Escherichia coli*, a large number of proteins are secreted into the periplasmic space, i.e. into the compartment between the two membranes enclosing the cells. This may be advantageous for particular applications. Furthermore, gram-negative bacteria can also be designed such that they eject the expressed proteins not only into the periplasmic space, but into the medium surrounding the bacterium. In contrast,

gram-positive bacteria such as bacilli or actinomycetes or other representatives of *Actinomycetales* have no outer membrane, and therefore secreted proteins are released immediately 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 of the origin organisms for technically significant enzymes and usually even form comparable enzymes, meaning that they have a similar codon usage and the protein synthesis apparatus is naturally aligned accordingly.

[0084] Host cells according to the invention may be altered in terms of their requirements for the culture conditions, may have different or additional selection markers or may express other or additional proteins. In particular, this may also involve those host cells which transgenically express several proteins or enzymes.

[0085] 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 within the meaning of the invention.

[0086] 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.*

[0087] The host cell may also be a eukaryotic cell, however, which is characterized in that it has a cell nucleus. The invention therefore also relates to a host cell which is characterized in that it has a cell 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 that 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 may be desirable, for example, to lower the allergenicity of an expressed protein. Co-expression with the enzymes naturally formed by such cells, such as cellulases, may be advantageous. Furthermore, for example, thermophilic fungal expression systems may be particularly suitable for the expression of temperature-resistant proteins or variants.

[0088] The host cells according to the invention are cultivated and fermented in the conventional way, 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 the achievement of a flow equilibrium, in which cells partially die over a comparatively long period of time but also grow back and the protein formed can be removed from the medium at the same time.

[0089] Host cells according to the invention are preferably used to produce proteases according to the invention. The invention therefore also relates to a method for preparing 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.

[0090] 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, usually followed by a suitable purification method of the produced product, for example the proteases according to the invention. All fermentation processes which are based on a corresponding method for producing a protease according to the invention constitute embodiments of this subject matter of the invention.

[0091] Fermentation processes which are characterized in that the fermentation is carried out via a feed strategy shall be considered in particular. In this case, the media components 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 in such a way that undesired metabolic products are filtered out or neutralized by adding buffers or suitable counter ions.

[0092] The protease produced can be harvested from the fermentation medium. Such a fermentation process is preferable to isolation of the protease from the host cell, i.e. product preparation from the cell mass (dry matter), but requires the provision of suitable host cells or one or more suitable secretion markers or mechanisms and/or transport systems for the host cells to secrete the protease into the fermentation medium. Without secretion, the protease can alternatively be isolated from the host cell, i.e. purified from the cell mass, for example by precipitation with ammonium sulphate or ethanol, or by chromatographic purification.

[0093] All of the above-mentioned aspects can be combined into methods in order to produce protease according to the invention.

[0094] The invention also relates to an agent which is characterized in that it contains a protease according to the invention as described above. The agent is preferably a washing or cleaning agent.

[0095] This subject matter of the invention covers all conceivable types of washing or cleaning agents, both concentrates and agents to be used undiluted, for use on a commercial scale, in washing machines or for 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 also 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, toilet rim blocks, 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, washing and cleaning agents according to the invention also include textile pre-treatment and post-treatment agents, i.e. those agents with which the item of laundry is brought into contact before the actual washing cycle, for example to loosen stubborn soiling, and also those agents which give the laundry further desirable properties such as a pleasant feel, crease resistance or low static charge in a step subsequent to the actual textile wash. Inter alia, softeners are included in the last-mentioned agents.

[0096] The washing or cleaning agents according to invention, which may be in the form of powdered solids, in further-compacted particulate form, homogeneous solutions or suspensions, may contain, in addition to a protease according to the invention, all known ingredients conventional in such agents, with preferably at least one other ingredient being present in the agent. The agents according to the invention may in particular contain surfactants, builders, peroxygen compounds or bleach activators. They may also contain water-miscible organic solvents, further enzymes, sequestering agents, electrolytes, pH regulators and/or further auxiliaries such as optical brighteners, graying inhibitors, foam regulators, as well as dyes and fragrances, and combinations thereof.

[0097] In particular, a combination of a protease according to the invention with one or more further ingredients of the agent is advantageous, since, in preferred embodiments according to the invention, such an agent has improved cleaning performance by virtue of resulting synergisms. In particular, combining a protease according to the invention with a surfactant and/or a builder and/or a peroxygen compound and/or a bleach activator can result in such a synergism. However, in preferred embodiments, the agent according the invention may not contain boric acid.

[0098] Advantageous ingredients of agents according to the invention are disclosed in international patent application WO2009/121725, starting at the penultimate paragraph of page 5 and ending after the second paragraph on page 13. Reference is expressly made to this disclosure and the disclosure therein is incorporated in the present patent application by reference.

[0099] An agent according to the invention advantageously contains the protease in an amount of from 2 µg to 20 mg, preferably from 5 µg to 17.5 mg, more preferably from 20 µg to 15 mg and most particularly preferably from 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. Further, the protease contained in the agent, and/or other ingredients of the agent, may be coated with a substance which is impermeable to the enzyme at room temperature or in the absence of water, and which becomes permeable to the enzyme under conditions of use of the agent. Such an embodiment of the invention is thus characterized in that the protease is coated with a substance which is impermeable to the protease at room temperature or in the absence of water. Furthermore, the washing or cleaning agent itself can be packed in a container, preferably an air-permeable container, from which it is released shortly before use or during the washing process.

[0100] 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 paste 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.

[0101] These embodiments of the present invention include all solid, powdered, liquid, gel or paste administration forms of agents according to the invention, which may optionally also consist of a plurality of phases and can be present in compressed or uncompressed form. The agent may be present as a flowable powder, in particular having a bulk density of from 300 g/l to 1200 g/l, in particular from 500 g/l to 900 g/l or from 600 g/l to 850 g/l. The solid administration forms of the agent also include extrudates, granules, tablets or pouches. Alternatively, the agent may also be in 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. The agent may also be present as a one-component system. Such agents consist of one phase. Alternatively, an agent may also consist of a plurality of phases. Such an agent is therefore divided into a plurality of components.

[0102] Washing or cleaning agents according to the invention may contain only one protease. Alternatively, they may also contain other hydrolytic enzymes or other enzymes in a concentration that is expedient for the effectiveness of the agent. A further embodiment of the invention is therefore represented by agents which 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 proteases according to the invention, as well as mixtures thereof. Further enzymes are advantageously contained in the agent in an amount of from 1×10^{-8} to 5 wt.% based on active protein. Increasingly preferably, each further

enzyme is contained in agents according to the invention in an amount of from 1 x 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 against specific stains or spots, i.e. the enzymes contained in the agent composition support one another in their cleaning performance. Very particularly preferably, there is such synergism between the protease 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 arise not only between different enzymes, but also between one or more enzymes and other ingredients of the agent according to the invention.

[0103] In the cleaning agents described herein, the enzymes to be used may furthermore be formulated together with accompanying substances, for example from fermentation. In liquid formulations, the enzymes are preferably used as enzyme liquid formulations.

[0104] The enzymes are generally not provided in the form of pure protein, but rather in the form of stabilized, storable and transportable preparations. These pre-formulated preparations include, for example, the solid preparations obtained through granulation, extrusion, or lyophilization or, in particular in the case of liquid or gel agents, solutions of the enzymes, advantageously maximally concentrated, low-water, and/or supplemented with stabilizers or other auxiliaries.

[0105] Alternatively, the enzymes can also be encapsulated, for both the solid and the liquid administration form, for example by 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 in a set gel, or in those of the core-shell type, in which an enzyme-containing core is coated with a water-, air-, and/or chemical-impermeable protective layer. Other active ingredients such as stabilizers, emulsifiers, pigments, bleaching agents, or dyes can additionally

be applied in overlaid layers. Such capsules are applied using inherently known methods, for example by shaking or roll granulation or in fluidized bed processes. Such granules are advantageously low in dust, for example due to the application of polymeric film-formers, and are stable in storage due to the coating.

[0106] Moreover, it is possible to formulate two or more enzymes together, such that a single granule exhibits a plurality of enzyme activities.

[0107] The enzymes can also be incorporated in 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 release of the enzymes following 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 (fully or partially hydrolyzed) polyvinyl alcohol (PVA).

[0108] The invention also relates to a method for cleaning textiles or hard surfaces, which is characterized in that an agent according to the invention is used in at least one method step, or in that a protease according to the invention becomes catalytically active in at least one method step, in particular such that the protease is used in an amount of from 40 µg to 4 g, preferably from 50 µg to 3 g, particularly preferably from 100 µg to 2 g, and most particularly preferably from 200 µg to 1 g.

[0109] 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 or 25 °C.

[0110] These include both manual and mechanical methods, with mechanical methods being 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 then represent embodiments of the present invention. All aspects, objects, and embodiments described for the protease according to the invention and agents containing it are also applicable to this subject matter of the invention. Therefore, reference is expressly made at this point to the disclosure at the appropriate point with the note that this disclosure also applies to the above-described method according to the invention.

[0111] Since proteases according to the invention naturally already have hydrolytic activity and also exhibit this in media which otherwise have no cleaning power, for example in a simple buffer, a single and/or the sole step of such a method can consist in the protease, which is the only cleaning-active component according to the invention, being brought into contact with the stain, preferably in a buffer solution or in water. This represents a further embodiment of this subject matter of the invention.

[0112] Alternative embodiments of this subject matter of the invention are also represented by methods for treating textile raw materials or for textile care, in which a protease according to the invention becomes active in at least one method step. Among these, methods for textile raw materials, fibers or textiles with natural components are preferred, and especially for those with wool or silk.

[0113] Finally, the invention also encompasses the use of the proteases described herein in washing or cleaning agents, for example as described above, for the (improved) removal of protein-containing stains, for example from textiles or hard surfaces. In preferred 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.

[0114] All aspects, objects, and embodiments described for the protease according to the invention and agents containing it are also applicable to this subject matter of the invention. Therefore, reference is expressly made at this point to the disclosure at the appropriate point with the note that this disclosure also applies to the above-described use according to the invention.

Examples

Overview of the mutations:

[0115] This invention relates to a subtilisin-type alkaline protease from *Bacillus pumilus.* From this protease (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:18] and mutant 2 [SEQ ID NO:2]) were generated from the wild-type protease 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 3-17 according to SEQ ID Nos. 3-17 were generated. Therefore, all of mutants 3-17 mentioned here also carry at least some of the mutations of mutants 1 or 2. In a third round, another performance-enhanced mutant (mutant 18 [SEQ ID NO:19]) was generated by error-prone mutagenesis. This mutant was subject to a fourth error-prone round. In this fourth round of mutation, mutants 19-23 according to SEQ ID Nos. 20-24 were generated. Therefore, all of mutants 19-23 mentioned here also carry the mutations of mutant 18.

Variant	Amino acid substitutions relative to SEQ ID NO:1									SEQ ID NO:
Mutant 1	P9T	Q271E	S216C							18
Mutant 2	P9T	Q271E	S216C	T133R	S224A					2
Mutant 3	P9T	Q271E	S216C	N130D	G131S	T133Y	S224A			3
Mutant 4	P9T	Q271E	S216C	N130S	G131S	T133Y	N144L	S224A		4
Mutant 5	P9T	Q271E	S216C	N130D	G131N	T133K	N144k			5
Mutant 6	P9T	Q271E	S216C	N130H	G131N	N144K	S224A			6
Mutant 7	P9T	Q271E	S216C	A29G	N130D	G131N	T133R			7
Mutant 8	P9T	Q271E	S216C	A29G	D101E	N130S	G131S	S224T	N252S	8
Mutant 9	P9T	Q271E	S216C	N130H	G131S	S224A				9
Mutant	P9T	Q271E	S216C	N130D	G131S	T133K	S224A			10
Mutant 11	P9T	Q271E	S216C	A29G	D101E	N130D	G131K	S224A		11
Mutant 12	P9T	Q271E	S216C	N130D	G131N	T133K	N144L	N252S		12
Mutant 13	P9T	Q271E	S216C	G131S	N144K	S224T				13
Mutant 14	P9T	Q271E	S216C	D101E	N130D	G131S	T133Y	N144A	S224A	14
Mutant 15	P9T	Q271E	S216C	A29G	D101E	N130S	S224T	N252S		15
Mutant 16	P9T	Q271E	S216C	A48V	G131S	T133R	S224A			16
Mutant 17	P9T	Q271E	S216C	G131D	T133R	S224A				17
Mutant 18	P9T	Q271E		N130D	N144K	N252T				19
Mutant 19	P9T	Q271E		N130D	N144K	N252T	G166M			20
Mutant 20	P9T	Q271E		N130D	N144K	N252T	G166Q			21
Mutant 21	P9T	Q271E		N130D	N144K	N252T	G166A			22
Mutant 22	P9T	Q271E		N130D	N144K	N252T	Y217M			23
Mutant 23	P9T	Q271E		N130D	N144K	N252T	S156G			24
Mutant 24	P9T	Q271E		N130D	N144K	N252T	D172P			
Mutant 25	P9T	Q271E		N130D	N144K	N252T	V149I			
Mutant 26	P9T	Q271E		N130D	N144K	N252T	Q62S			
Mutant 27	P9T	Q271E		N130D	N144K	N252T	Y217M	T133R		
Mutant 28	P9T	Q271E			N144K	N252T	Y217M			
Mutant 29	P9T	Q271E		N130D	N144K	N252T	S156Q			

Washing agent matrix used

[0116] The following washing agent matrices (commercially available, without enzymes, opt. brighteners, perfume and dyes) were used for the washing test:

Storage stability test 1:

[0117]

Chemical name	Wt.% of active substance in the raw	Wt.% of active substance in the
Demineralized water	100	Remainder
Alkyl benzene sulfonic acid	96	4.4
Anionic surfactants	70	5.6
C12-C18 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
Preservative	100	0.08
Ethanol	93	1
Without opt. brighteners, perfume, dye and enzymes.		

Dosage 4.7 g/L

Storage stability test 2:

[0118]

Chemical name	Wt.% of active substance in the raw	Wt.% of active substance in the
Demineralized water	100	Remainder
Alkyl benzene sulfonic acid	96	12-18
Anionic surfactants	70	4-8
C12-C18 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
Without opt. brighteners, perfume, dye and enzymes.		

Dosage 3.17 g/L

Protease activity assays

[0119] The activity of the protease is determined by the release of 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 absorbance at 410 nm, the temporal progression of which is a measure of the enzymatic activity.

[0120] The measurement was carried out at a temperature of 25 °C, a pH of 8.6, and a wavelength of 410 nm. The measuring time was 5 minutes with a measuring interval of from 20 to 60 seconds.

Measurement approach:

[0121]

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

[0122] Kinetics created over 5 min at 25 °C (410 nm)

Storage stability test and results

[0123] The proteases were stirred into a washing agent matrix at the same level of activity and stored at 30 °C. By means of a conventional activity assay for proteases (hydrolysis of suc-AAPF-pNA), the starting activity and the residual activity of the protease are measured after 4 weeks' storage at 30 °C. In order to generate harsh conditions, the proteases were stored in a washing agent matrix without a stabilizer (boric acid).

[0124] The proteases were generated in shake flask supernatants from *Bacillus subtilis*. They were diluted to an equal level of activity. 50% washing agent matrix without boric acid was added to 50% of appropriately diluted *Bacillus subtilis* protease supernatant and mixed well. The sealed glasses were incubated at 30 °C. At the time of sampling, a predetermined amount of matrix/protease mixture was removed and dissolved by stirring for 20 min at RT in the sample buffer (0.1 M Tris/HCl, pH 8.6). The AAPF assay is then carried out as described above.

[0125] In the first storage stability test, 15 mutants had been found to be advantageous. The activity is shown in % of the residual activity of the starting variants (mutant 1 according to SEQ ID NO:18 or mutant 2 according to SEQ ID NO:2) after 4 weeks' storage at 30 °C. Mutants 3-15 relate to starting mutant 1 (SEQ ID NO:18), and mutants 16 and 17 relate to starting mutant 2 (SEQ ID NO:2).

Variant	SEQ ID NO:	Residual activity
Mutant 1	18	100%
Mutant 3	3	153%
Mutant 4	4	148%
Mutant 5	5	150%
Mutant 6	6	133%
Mutant 7	7	154%
Mutant 8	8	132%
Mutant 9	9	147%
Mutant 10	10	146%
Mutant 11	11	132%
Mutant 12	12	144%
Mutant 13	13	136%
Mutant 14	14	138%
Mutant 15	15	132%

Variant	SEQ ID NO:	Residual activity
Mutant 2	2	100%
Mutant 16	16	121%
Mutant 17	17	110%

[0126] It can be seen that all the mutants exhibit greatly improved stability without the addition of boric acid in comparison with the respective starting mutants according to SEQ ID Nos. 18 and 2. All the mutants exhibit a washing performance that is comparable to the wild type according to SEQ ID NO:1, i.e. they are at most 10% worse in terms of washing performance, which is within the measurement fluctuation (results not shown).

[0127] In the second storage stability test, 6 mutants had been found to be advantageous. The activity is shown in % of the residual activity of the starting variant (mutant 18 according to SEQ ID NO:19) - which already has significantly improved stability compared to the wild-type enzyme - after 4 weeks' storage at 30 °C.

Variant	SEQ ID NO:	Residual activity
Mutant 18	19	100%
Mutant 19	20	117%
Mutant 20	21	118%
Mutant 21	22	107%
Mutant 22	23	109%
Mutant 23	24	109%
Mutant 29		117%

[0128] It can be seen that all the mutants, without the addition of boric acid, exhibit improved stability in comparison with the starting mutant according to SEQ ID NO:19. All the mutants exhibit a washing performance that is comparable to the wild type according to SEQ ID NO:1, i.e. they are at most 10% worse in terms of washing performance, which is within the measurement fluctuation (results not shown).

[0129] In another round of testing, additional mutants were tested for stability, with incubation and storage taking place as described above, but at 40 °C.

[0130] The following mutants have been found to be advantageous. The activity is shown as % of the residual activity with respect to the initial value. The starting variant (mutant 18 according to SEQ ID NO:19) already has markedly improved stability compared to the wild-type enzyme, and was stored for 4 weeks at 40 °C.

Variant	SEQ ID NO:	Residual activity
Mutant 18	19	10%
Mutant 24		19%
Mutant 25		22%
Mutant 26		39%
Mutant 27		28%
Mutant 28		16%

[0131] All the mutants exhibited a further improvement in stability.

PATENTKRAV

1. Protease, omfattende en aminosyresekvens, som har i det mindste 80 % sekvensidentitet med aminosyresekvensen, der er specificeret i SEQ ID NO:1 over hele dens længde og aminosyresubstitutioner, hver for sig baseret på nummereringen i henhold til SEQ ID NO:1,

(a) på positionerne, der svarer til positionerne 9 og 271, og
(b) på mindst tre af positionerne, som svarer til positionerne 29, 48, 101, 130, 131, 133, 144, 224 eller 252, i hvert tilfælde baseret på nummereringen i henhold til SEQ ID NO:1,

hvorved

(1) aminosyresubstitutionerne på de positioner, som svarer til positionerne 9 og 271, er aminosyresubstitutionerne P9T, P9H, P9S eller P9A og Q271E; og
(2) de mindst tre aminosyresubstitutioner ifølge (b) er udvalgt fra gruppen, der består af henholdsvis A29G, A48V, D101E, N130D, N130S, N130H, G131S, G131N, G131D, G131K, T133K, T133R, T133Y, N144K, N144L, N144A, S224A, S224T eller N252S, i henhold til nummereringen ifølge SEQ ID NO:1.

2. Protease ifølge krav 1, hvorved proteasen omfatter aminosyresubstitutioner

(1) på positionerne, der svarer til positionerne 9 og 271 såvel som mindst tre af henholdsvis 130, 131, 133, 144 og 224, baseret på nummereringen ifølge SEQ ID NO:1, og
(2) eventuelt på mindst én af positionerne, der svarer til henholdsvis positioner 29, 48, 101 og 252, baseret på nummereringen ifølge SEQ ID NO:1.

3. Protease ifølge et af kravene 1-2, hvorved proteasen omfatter en af de følgende respektive aminosyresubstitutionsvarianter, baseret på nummereringen ifølge SEQ ID NO:1:

P9T, P9H, P9S eller P9A, navnlig P9T og Q271E samt eventuelt S216C såvel som ydermere en af

(I) N130D, G131S, T133Y og S224A;
(II) N130S, G131S, T133Y, N144L og S224A;
(III) N130D, G131N, T133K og N144K;
(IV) N130H, G131N, N144K og S224A;
(V) A29G, N130D, G131N og T133R;
(VI) A29G, D101E, N130S, G131S, S224T og N252S;
(VII) N130H, G131S og S224A;
(VIII) N130D, G131S, T133K og S224A;
(IX) A29G, D101E, N130D, G131K og S224A;
(X) N130D, G131N, T133K, N144L og N252S;
(XI) G131S, N144K og S224T;
(XII) D101E, N130D, G131S, T133Y, N144A og S224A;
(XIII) A29G, D101E, N130S, S224T og N252S;
(XIV) A48V, G131S, T133R og S224A; eller
(XV) G131D, T133R og S224A.

4. Protease ifølge et af kravene 1 til 2, hvorved proteasen, baseret på nummereringen ifølge SEQ ID NO:1 omfatter

(a) på positionerne, der svarer til positionerne 9, 130, 144, 252 og 271, omfatter aminosyresubstitutionerne 9T, 130D, 144K, 252T og 271E; tillige med eventuelt
(b1) på positionen, der svarer til positionen 166, aminosyresubstitutionen 166, 166Q eller 166A;
og/eller

(b2) på mindst én af positionerne, der svarer til positionerne 62, 133, 149, 156, 172 eller 217, mindst én yderligere aminosyresubstitution.

5. Protease ifølge krav 4, hvorved aminosyresubstitutionerne på de positioner, som svarer til positionerne 62, 133, 149, 156, 172 eller 217, er udvalgt fra aminosyresubstitutionerne 62S, 133R, 133A, 133K, 149I, 156G, 156Q, 172P, 172G og 217M.

6. Protease ifølge et af kravene 4-5, hvorved proteasen omfatter

(1) på positionerne, der svarer til positionerne 9, 139, 144, 252 og 271, aminosyresubstitutionerne 9T, 130D, 144K, 252T og 271E; og
(2) på en af positionerne, som svarer til positionerne 62, 133, 149, 156, 166, 172 eller 217, en af aminosyresubstitutionerne 62S, 133R, 133A, 133K, 149I, 156G, 156Q, 166M, 166Q, 166A, 172P 172G eller 217M.

7. Protease ifølge et af kravene 1-6, hvorved proteasen omfatter en aminosyresekvens ifølge SEQ ID Nos. 3-17 eller 19-23.

8. Nukleinsyre, kodende for en protease ifølge et af kravene 1-7.

9. Vektor, indeholdende en nukleinsyre ifølge krav 8, navnlig en kloningsvektor eller en ekspressionsvektor.

10. Ikke-human værtscelle, omfattende en nukleinsyre ifølge krav 8 eller en vektor ifølge krav 9, eller som omfatter en protease ifølge et af kravene 1-7.

11. Fremgangsmåde til fremstilling af en protease, omfattende

a) dyrkning af en værtscelle ifølge krav 10; og
b) isolation af proteasen fra dyrkningsmediet eller fra værtscellen.

12. Middel, navnlig et vaske- eller rengøringsmiddel,

kendetegnet ved, at det i det mindste indeholder en protease ifølge et af kravene 1-7.

13. Fremgangsmåde til rengøring af tekstiler eller hårde overflader, **kendetegnet ved, at** der i det mindste i et fremgangsmådetrin anvendes et middel ifølge krav 12, eller **ved, at** der i mindst et fremgangsmådetrin anvendes en protease ifølge et af kravene 1-7.

14. Anvendelse af en protease ifølge et af kravene 1-7 i et vaske- eller rengøringsmiddel til fjernelse af peptid- eller proteinholdige tilsmudsninger.