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(54) Benævnelse: **Ydelsesforbedrede og opbevaringsstabile proteasevarianter**

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Storage stable protease variants with improved performance

Description

[0001] The invention is in the field of enzyme technology. The invention relates to proteases whose amino acid sequence has been altered in particular with regard to use in washing and cleaning agents, in particular with regard to liquid washing and cleaning agents in order to give them better storage stability and/or to improve their cleaning performance, and to the nucleic acids coding for them, and to the production thereof. The invention further relates to the uses of said proteases and methods in which they are used, and agents containing said proteases, in particular washing and cleaning agents, in particular liquid washing and cleaning agents.

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

[0003] Examples of subtilisin-type proteases that are preferably used in washing and cleaning agents are the subtilisins BPN′ and Carlsberg, protease PB92, subtilisins 147 and 309, the alkaline protease from *Bacillus lentus*, in particular *Bacillus lentus* DSM 5483, subtilisin DY and the enzymes thermitase, proteinase K and proteases TW3 and TW7, which are to be classified as subtilases, but no longer as subtilisins in the narrower sense, as well as variants of said proteases that have an amino acid sequence which is altered compared to the starting protease. Proteases are altered in a targeted or random manner by methods known from the prior art and are thus optimized for use in washing and cleaning agents, for example. This includes point, deletion or insertion mutagenesis, or fusion with other proteins or protein parts. Thus, appropriately optimized variants are known for most proteases known from the prior art.

[0004] In the international patent applications WO95/23221A1, WO92/21760A1, WO 2018/069158 and WO2013/060621A1 and in the German patent application DE102004027091, variants of the alkaline protease are from *Bacillus lentus* DSM 5483 are disclosed which are suitable for use in washing or cleaning agents. Furthermore, the international patent applications

WO2011/032988A1 and the European patent application EP3044302A1 disclose washing and cleaning agents which include variants of the alkaline protease from *Bacillus lentus* DSM 5483. The protease variants disclosed in these documents can, in addition to other positions, be modified at positions 3, 4, 99 and/or 199 in the numbering of the alkaline protease from *Bacillus lentus* DSM 5483 and, for example, have the amino acids 3T, 4I, 99E or 1991 at said positions. However, combinations of further modifications, as described below, cannot be found in said documents.

[0005] In general, only selected proteases are suitable for use in liquid surfactant-containing preparations. Many proteases do not exhibit sufficient catalytic performance in such preparations. For the use of proteases in washing and cleaning agents, therefore, a high catalytic activity under conditions as they are during a wash cycle and a high storage stability is particularly desirable.

[0006] 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 sufficiently storage-stable and the formulations therefore do not exhibit optimal cleaning performance on protease-sensitive stains.

[0007] Surprisingly, it has now been found that a protease of the alkaline protease type consists of *Bacillus lentus* DSM 5483 or a protease sufficiently similar thereto (in terms of the sequence identity) which, based on the numbering according to SEQ ID NO:1, has the amino acid substitutions 3T, 4I, 99E and 1991 at (i) the positions corresponding to positions 3, 4, 99 and 199, and (ii) at least one of the positions corresponding to positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267 has at least one amino acid substitution, in particular at least one amino acid substitution which is selected from the group consisting of 9C, 21F, 21W, 42S, 42H, 44S, 105V, 112V, 113A, 131D, 1371, 139R, 141S, 1451, 159L, 168V, 176E, 177D, 182C, 193M, 198D, 204L, 205D, 206A, 210C, 212D, 230E, 234A, 250N, 253C, 255Y, 259C and 267S, is improved in terms of its storage stability and/or washing performance and/or stability compared to the wild type form (SEQ ID NO:1) or a starting variant as described in WO2013060621A1 and is therefore particularly suitable for use in washing or cleaning agents.

[0008] The invention therefore relates to a protease comprising an amino acid sequence, which has at least 70% sequence identity with the amino acid sequence set forth in SEQ ID NO:1 over its entire length and has, based on the numbering according to SEQ ID NO:1, at least one amino acid substitution at (i) the positions corresponding to positions 3, 4, 99 and 199, at least the amino acid substitutions S3T, V4I, R99E and V199I, and (ii) at least one of the positions corresponding to positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267.

[0009] A further subject matter of the invention is a method for producing a protease as defined above comprising the introduction of amino acid substitutions S3T, V4I, R99E and V199I at (i) the positions which, based on the numbering according to SEQ ID NO:1, correspond to positions 3, 4, 99 and 199, and (ii) at least one of the positions which, based on the numbering according to SEQ ID NO:1, correspond to positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267, into a starting molecule which has an amino acid sequence which has at least 70% sequence identity with the amino acid sequence set forth in SEQ ID NO:1 over its entire length.

[0010] A protease within the meaning of the present patent application therefore comprises both the protease as such and a protease produced using a method according to the invention. All statements regarding the protease therefore relate both to the protease as such and to the proteases produced by means of corresponding methods.

[0011] Further aspects of the invention relate to the nucleic acids coding for these proteases, non-human host cells containing proteases or nucleic acids according to the invention, as well as agents comprising proteases according to the invention, in particular washing and cleaning agents, washing and cleaning methods, and uses of the proteases according to the invention in washing or cleaning agents for removing protein-containing stains.

[0012] These and other aspects, features and advantages of the invention will become apparent to a person skilled in the art through the study of the following detailed description and claims. Any feature from one aspect of the invention can be used in any other aspect of the invention. Furthermore, it will readily be understood that the examples contained herein are intended to describe and illustrate but not to limit the invention and that, in particular, the invention is not limited to these examples.

[0013] Numerical ranges that are indicated in the format “from x to y” also include the stated values. If several preferred numerical ranges are specified in this format, it is readily understood that any ranges resulting from the combination of the various endpoints are also included.

[0014] “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.

[0015] “Liquid,” as used herein, includes liquids and gels as well as pasty compositions. It is preferred that the liquid compositions are flowable and pourable at room temperature, but it is also possible for them to have a limit of liquidity.

[0016] The present invention is based on the surprising finding by the inventors that amino acid substitutions at the positions described herein bring about improved storage stability and/or an improved cleaning performance of this altered protease in washing and cleaning agents.

[0017] In preferred embodiments, the protease according to the invention has, at (i) the positions corresponding to positions 3, 4, 99, and 199, the amino acid substitutions selected from the group consisting of 3T, 4I, 99E, and 199I, and at (ii) at least one of the positions corresponding to positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267, at least one amino acid substitution selected from the group consisting of 9C, 21F, 21W, 42S, 42H, 44S, 105V, 112V, 113A, 131D, 137I, 139R, 141S, 145I, 159L, 168V, 176E, 177D, 182C, 193M, 198D, 204L, 205D, 206A, 210C, 212D, 230E, 234A, 250N, 253C, 255Y, 259C or267S, wherein the combination of the amino acid substitutions from group (i) and the at least one amino acid substitution from group (ii) results in improved cleaning performance of this modified protease in washing and cleaning agents on at least one protease-sensitive stain.

[0018] In preferred embodiments, the protease according to the invention has, at (i) the positions corresponding to positions 3, 4, 99, and 199, the amino acid substitutions selected from the group consisting of 3T, 4I, 99E, and 199I, and at (ii) at least one of the positions corresponding to positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267, at least one amino acid substitution which is selected from the group consisting of 9C, 21F, 21W, 42S, 42H, 44S, 105V, 112V, 113A, 131D, 137I, 139R, 141S, 145I, 159L, 168V, 176E, 177D, 182C, 193M, 198D, 204L, 205D, 206A, 210C, 212D, 230E, 234A, 250N, 253C, 255Y, 259C or267S, wherein the combination of the amino acid substitutions from group (i) and the at least one amino acid substitution from group (ii) results in an improved storage stability of this protease in washing and cleaning agents.

[0019] In preferred embodiments, the protease according to the invention has, at (i) the positions corresponding to positions 3, 4, 99, and 199, the amino acid substitutions selected from the group consisting of 3T, 4I, 99E, and 199I, and at (ii) at least one of the positions corresponding to positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267, at least one amino acid substitution which is selected from the group consisting of 9C, 21F, 21W, 42S, 42H, 44S, 105V, 112V, 113A, 131D, 137I, 139R, 141S, 145I, 159L, 168V, 176E, 177D, 182C, 193M, 198D, 204L, 205D, 206A, 210C, 212D, 230E, 234A, 250N, 253C, 255Y, 259C or 267S, wherein the combination of amino acid substitutions from group (i) and the at least one amino acid substitution from group (ii) results in both an improved cleaning performance and an improved storage stability of this modified protease in washing and cleaning agents.

[0020] Certain embodiments of the proteases according to the invention have improved storage stability. They have increased stability in washing or cleaning agents in comparison with the wild-type enzyme (SEQ ID NO:1) and in particular also with respect to the starting variant of the protease (SEQ ID NO:2 from WO2013/060621A1), 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.

[0021] Certain embodiments of the proteases according to the invention can have increased catalytic activity in washing or cleaning agents, independently of or in addition to the increased storage stability. In many embodiments, the proteases according to the invention can have proteolytic activity that is at least 101%, 102%, 103%, 104%, 105%, 106%, 107%, 108%, 109% or 110% based on the wild type (SEQ ID NO:1) and/or an already performance-enhanced starting variant of the protease (SEQ ID NO:2 from WO2013/060621A1). Such performance-enhanced proteases make improved washing results possible for proteolytically sensitive stains in different temperature ranges, in particular in a temperature range of 20 °C to 40 °C.

[0022] Furthermore, preferred embodiments of proteases according to the invention have a particular stability in washing or cleaning agents, for example compared to surfactants and/or bleaching agents and/or chelators, and/or with respect to temperature effects, in particular with respect to high temperatures of, for example, between 50 °C and 65 °C, in particular 60 °C, and/or with respect to changes in pH and/or with respect to denaturing or oxidizing agents and/or with respect to proteolytic degradation and/or with respect to a change in redox ratios. Performance-enhanced protease variants and/or protease variants with increased temperature stability are therefore provided by particularly preferred embodiments of the invention. Performance-enhanced protease variants and/or protease variants with increased temperature stability are provided by further very particularly preferred embodiments of the invention. Such advantageous embodiments of proteases according to the invention therefore allow improved washing results on protease-sensitive stains in a wide temperature range.

[0023] Cleaning performance within the scope of the invention shall be understood to mean the lightening performance on one or multiple stains, in particular on laundry or dishes. Within the scope of the invention, both the washing or cleaning agent, which comprises the protease, or the washing or cleaning liquor formed by said agent, and the protease itself have a respective cleaning performance. The cleaning performance of the enzyme thus contributes to the cleaning performance of the agent, or of the washing or cleaning liquor formed by the agent. The cleaning performance is preferably ascertained as described hereafter.

[0024] Washing liquor is understood to mean the ready-to-use solution which contains the washing or cleaning agent and acts on the textiles or fabric or hard surfaces and thus comes into

contact with the stains present on the textiles or fabrics or hard surfaces. The washing liquor is usually created when the washing or cleaning process begins and the washing or cleaning agent is diluted with water, for example in a dishwasher, a washing machine or in another suitable container.

[0025] 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 that catalyzes the hydrolysis of amide/peptide bonds in protein/peptide substrates and is thereby capable of cleaving proteins or peptides. Furthermore, a protease according to the invention is preferably a mature protease, i.e. the catalytically active molecule without a signal peptide/signal peptides and/or a propeptide/propeptides. Unless otherwise stated, the sequences indicated also refer to mature (processed) enzymes in each case.

[0026] In various embodiments of the invention, the protease is a free enzyme. This means that the protease can act directly with all components of an agent and, if the agent is a liquid agent, the protease is directly in contact with the solvent of the agent (e.g., water). In other embodiments, an agent may contain proteases that form an interaction complex with other molecules or that contain a “coating.” In this case, one or more protease molecules can be separated from the other constituents of the agent by a structure surrounding them. Such a separating structure can arise due to, but is not limited to, vesicles, such as a micelle or a liposome. However, the surrounding structure may also be a virus particle, a bacterial cell or a eukaryotic cell. In various embodiments, an agent may include cells of *Bacillus pumilus* or *Bacillus subtilis* which express the proteases according to the invention, or cell culture supernatants of such cells.

[0027] In particularly preferred embodiments of the invention, the protease comprises an amino acid sequence which has a sequence identity of at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 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%, 98.8%, 99% and 99.5% to the amino acid sequence set forth in SEQ ID NO:1 over its entire length, and based on the numbering according to SEQ ID NO:1 has, (i) the amino acid substitutions 3T, 4I, 99E and 199I and (ii) at least one amino acid substitution at at least one of the positions corresponding to the positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267, wherein the at least one amino acid substitution is preferably selected from the group consisting of 9C, 21F, 21W, 42S, 42H, 44S, 105V, 112V, 113A, 131D, 137I, 139R, 141S, 145I, 159L, 168V, 176E, 177D, 182C, 193M, 198D, 204L, 205D, 206A, 210C, 212D, 230E, 234A, 250N, 253C, 255Y, 259C and 267S.

[0028] In the context of the present invention, the feature whereby a protease has at least one of the given amino acid substitutions means that it contains one (of the given) amino acid

substitution(s) at the relevant position, i.e., at least the given positions are not otherwise mutated or deleted, for example by fragmentation of the protease.

[0029] 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. e.g., Altschul et al. (1990) "Basic local alignment search tool," J. Mol. Biol. 215:403-410, and Altschul et al. (1997): "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," Nucleic Acids Res., 25:3389-3402) and occurs in principle by similar sequences of nucleotides or amino acids in the nucleic acid or amino acid sequences being assigned to one another. A tabular assignment of the relevant positions is referred to as an alignment. A further algorithm available in the prior art is the FASTA algorithm. Sequence comparisons (alignments), in particular multiple sequence comparisons, are created using computer programs. The Clustal series (cf. e.g., Chenna et al. (2003): "Multiple sequence alignment with the Clustal series of programs," Nucleic Acids Res. 31:3497-3500), T-Coffee (cf. e.g., 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, for example, are frequently used. Also possible are sequence comparisons (alignments) using the computer program Vector NTI® Suite 10.3 (Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California, USA) with the given standard parameters, the AlignX module of which is based on ClustalW for the sequence comparisons. Unless stated otherwise, the sequence identity indicated herein is determined using the BLAST algorithm.

[0030] Such a comparison also allows a conclusion to be drawn about the similarity of the compared sequences to one another. It is usually given in percent identity, i.e., the proportion of identical nucleotides or amino acid residues at the same positions or positions corresponding to one another in an alignment. The broader concept of homology takes conserved amino acid exchanges into account in the case of amino acid sequences, i.e., amino acids having similar chemical activity, since they usually perform similar chemical activities within the protein. Therefore, the similarity of the compared sequences can also be indicated as percent homology or percent similarity. Identity and/or homology information can be provided regarding whole polypeptides or genes or only regarding individual regions. Homologous or identical regions of different nucleic acid or amino acid sequences are therefore defined by matches in the sequences. Such regions often have identical functions. They can be small and comprise only a few nucleotides or amino acids. Such small regions often perform essential functions for the overall activity of the protein. It may therefore be expedient to relate sequence matches only to individual, optionally small, regions. Unless otherwise stated, however, identity or homology information in the present application relates to the entire length of the particular nucleic acid or amino acid sequence indicated.

[0031] 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.

[0032] In a further embodiment of the invention, the protease is characterized in that its cleaning performance (after storage, e.g., over 3 weeks) compared to the wild-type enzyme (SEQ ID NO:1) or a starting variant described in WO201360621A1 is not significantly reduced, i.e., has at least 80% of the reference washing performance, preferably at least 100%, more preferably at least 110% or more. The cleaning performance can be determined in a washing system containing a washing agent in a dosage of between 4.5 and 7.0 grams per liter of washing liquor and the protease, wherein the proteases to be compared are used in the same concentration (based on active protein) and the cleaning performance with respect to a stain on cotton is determined by measuring the degree of cleaning of the washed textiles. For example, the washing process can take place for 60 minutes at a temperature of 40 °C, and the water can have a water hardness of between 15.5 and 16.5° (German hardness). The concentration of the protease in the washing agent intended for this washing system is 0.001 to 0.1 wt.%, preferably 0.01 to 0.06 wt.% based on active, purified protein.

[0033] A liquid reference washing agent for such a washing system may, for example, be composed as follows (all figures in wt.%): 4.4% alkyl benzene sulfonic acid, 5.6% further anionic surfactants, 2.4% C_{12}-C_{18} Na salts of fatty acids (soaps), 4.4% non-ionic surfactants, 0.2% phosphonates, 1.4% citric acid, 0.95% NaOH, 0.01% defoamer, 2% glycerol, 0.08% preservatives, 1% ethanol, and the remainder being demineralized water. The dosage of the liquid washing agent is preferably between 4.5 and 6.0 grams per liter of washing liquor, for example 4.7, 4.9 or 5.9 grams per liter of washing liquor. The washing process preferably takes place in a pH range between pH 7 and pH 10.5, preferably between pH 7.5 and pH 8.5.

[0034] In the context of the invention, the cleaning performance is determined, for example, at 20 °C or 40 °C using a liquid washing agent, for example that specified above, wherein the washing process is preferably carried out for 60 minutes at 600 rpm.

[0035] The degree of whiteness, i.e., the lightening of the stains, as a measure of the cleaning performance is determined using optical measuring methods, preferably photometrically. A suitable device for this is, for example, the Minolta CM508d spectrometer. Usually, the devices used for measurement are calibrated beforehand using a white standard, preferably a supplied white standard.

[0036] A liquid reference hand dishwashing agent for such a washing system can, for example, be composed as follows (all data in wt. %): 8-20% alkyl benzene sulfonic acid, 30-80% demineralized water, 5.4% NaOH (50%), 7.14% fatty alcohol ether sulfate, 2.0% NaCl (20%), 0.383% phosphoric acid (H_3PO_4; 34%/85%), 0.1% preservatives, 0.25% perfume, 1.0% dye, 0.04% bittering agent.

[0037] The activity-equivalent use of the relevant protease ensures that the respective enzymatic properties, i.e., for example, the cleaning performance on certain stains, are compared even if the ratio of active substance to total protein (the values of the specific activity) diverges. In general, a low specific activity can be compensated by adding a larger amount of protein.

[0038] Otherwise, methods for determining protease activity are familiar to and routinely used by a person skilled in the art in the field of enzyme technology. For example, such methods are disclosed in Tenside, volume 7 (1970), pp. 125-132. Alternatively, the protease activity can be determined via the release of the chromophore para-nitroaniline (pNA) from the substrate suc-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide (AAPF). The protease cleaves the substrate and releases pNA. The release of the pNA causes an increase in absorbance at 410 nm, the time profile of which is a measure of the enzymatic activity (cf. Del Mar et al., 1979). The measurement is carried out at a temperature of 25 °C, a pH of 8.6 and a wavelength of 410 nm. The measurement time is 5 min and the measurement interval is 20 s to 60 s. The protease activity is usually given in protease units (PE). Suitable protease activities amount to 2.25, 5 or 10 PE per mL of washing liquor, for example. However, protease activity is not equal to zero.

[0039] An alternative test for determining the proteolytic activity of the proteases according to the invention is an optical measurement method, preferably a photometric method. The test suitable for this purpose comprises protease-dependent cleavage of the substrate protein casein. The casein is cleaved by the protease into a plurality of smaller partial products. The entirety of these partial products has an increased absorption at 290 nm with respect to non-cleaved casein, wherein this increased absorption can be determined using a photometer, and thus a conclusion can be drawn regarding the enzymatic activity of the protease.

[0040] 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 (Gornall at al., J. Biol. Chem. 177 (1948): 751-766). In this regard, the active protein concentration can be determined via titration of the active centers using a suitable irreversible inhibitor and determination of the residual activity (cf. Bender et al., J. Am. Chem. Soc. 88, 24 (1966): 5890-5913).

[0041] 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 developed, for example, by targeted genetic alteration, i.e., by means of mutagenesis methods, and optimized for specific use purposes or with regard to specific properties (for example with regard to their catalytic activity, stability, etc.). Furthermore, nucleic acids according to the invention can be introduced into recombination approaches and thus used to produce completely novel proteases or other polypeptides.

[0042] The aim is to introduce targeted mutations such as substitutions, insertions or deletions into the known molecules in order, for example, to improve the cleaning performance of enzymes according to the invention. For this purpose, in particular the surface charges and/or the isoelectric point of the molecules and thus their interactions with the substrate can be altered. For example, the net charge of the enzymes can be altered in order to influence the substrate binding in particular for use in washing and cleaning agents. Alternatively or additionally, the stability or catalytic activity of the protease can be increased by one or more corresponding mutations and its cleaning performance can thereby be improved. Advantageous properties of individual mutations, e.g., individual substitutions, can complement one another. A protease already optimized with regard to certain properties, for example with regard to its stability during storage, can therefore additionally be developed in the context of the invention.

[0043] For the description of substitutions that relate to exactly one amino acid position (amino acid exchanges), the following convention is applied herein: first, the naturally present amino acid is referred to in the form of the internationally used single-letter code, followed by the associated sequence position and finally the inserted amino acid. Several exchanges within the same polypeptide chain are separated by slashes. In the case of insertions, additional amino acids are named according to the sequence position. In the case of deletions, the missing amino acid is replaced by a symbol, for example a star or a dash, or a Δ is indicated before the corresponding position. For example, A95G describes the substitution of alanine at position 95 by glycine, A95AG describes the insertion of glycine following the amino acid alanine at position 95 and A95* or ΔA59 describes the deletion of alanine at position 95. This nomenclature is known to a person skilled in the art in the field of enzyme technology.

[0044] The invention therefore also relates to a protease which is characterized in that it is obtained from a protease as described above as the starting molecule by single or multiple conservative amino acid substitution, the protease in the numbering according to SEQ ID NO:1 having at least one of the above-described amino acid substitutions. The term "conservative amino acid substitution" means the exchange (substitution) of one amino acid functional group for another amino acid functional group, with this exchange not resulting in a change to the polarity or charge at the position of the exchanged amino acid, e.g., the exchange of a nonpolar amino acid functional group for another nonpolar amino acid functional group. Conservative amino acid substitutions within the context of the invention include, for example: G=A=S, I=V=L=M, D=E, N=Q, K=R, Y=F, S=T, G=A=I=V=L=M=Y=F=W=P=S=T.

[0045] Alternatively or in addition, the protease is characterized in that it is obtained 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 190, 200, 210, 220, 230, 240, 250, 260, 261, 262, 263, 264, 265, 266, 267, 268 or 269 contiguous amino acids, wherein the protease (i) has amino acid substitution(s) at the positions according to claim 1 corresponding to positions 3, 4, 99 and 199, and (ii) at least one further amino acid substitution at at least one of positions corresponding to the positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267.

[0046] It is thus possible, for example, to delete individual amino acids at the termini or in the loops of the enzyme without the proteolytic activity being lost or reduced as a result. Furthermore, such fragmentation or deletion, insertion or substitution mutagenesis can also be used, for example, to reduce the allergenicity of the enzymes concerned and thus to improve their usability overall. Advantageously, the enzymes retain their proteolytic activity even after mutagenesis, i.e., their proteolytic activity corresponds at least to that of the starting enzyme, i.e., in a preferred embodiment, the proteolytic activity is at least 80%, preferably at least 90%, of the activity of the starting enzyme. Further substitutions can also demonstrate advantageous effects. Both individual and multiple contiguous amino acids can be replaced with other amino acids.

[0047] The 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 lentus,* as set forth in SEQ ID NO:1. Furthermore, the assignment of the positions is based on the mature protein. This assignment is also to be used in particular if the amino acid sequence of a protease according to the invention comprises a higher or lower number of amino acid functional groups than the protease from *Bacillus lentus* according to SEQ ID NO:1. Proceeding from the mentioned positions in the amino-acid sequence of the protease from *Bacillus lentus*, the modification positions in a protease according to the invention are those which are precisely assigned to these positions in an alignment.

[0048] Advantageous positions for sequence alterations, in particular substitutions, of the protease from *Bacillus lentus,* 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 corresponding to the positions described herein in an alignment, i.e., in the numbering according to SEQ ID NO:1. At the aforementioned positions, the following amino acid radicals are in the wild-type molecule of the protease from *Bacillus lentus* (SEQ ID NO: 1): S3, V4, S9, L21, N42, R44, R99, I105, A112, G113, A131, V137, S139, T141, V145, I159, A168, Q176, N177, S182, V193, N198, V199, P204, G205, S206, S210, N212, Q230, S234, S250, S253, N255, S259.

[0049] Further confirmation of the correct assignment of the amino acids to be altered, i.e., in particular their functional correspondence, can be provided by comparative tests, based on which

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

[0050] All of these aspects are also applicable to the methods according to the invention for producing a protease. Accordingly, a method according to the invention further comprises one or more of the following method steps:

(a) introducing one-time or multiple conservative amino acid substitution, into the protease, the protease comprising:

i) amino acid substitutions S3T, V4I, R99E and V199I at the positions corresponding to positions 3, 4, 99 and 199, and
ii) at at least one of the positions corresponding to the positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267, of at least one amino acid substitution;

(b) altering the amino acid sequence by fragmentation, deletion, insertion or substitution mutagenesis such that the protease comprises an amino acid sequence that matches the starting molecule over a length of at least 190, 200, 210, 220, 230, 240, 250, 260, 261, 262, 263, 264, 265, 266, 267, 268 or 269 contiguous amino acids, the protease comprising:

i) amino acid substitutions S3T, V4I, R99E and V199I at the positions corresponding to positions 3, 4, 99 and 199, and
ii) at at least one of the positions corresponding to the positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267, at least one amino acid substitution.

[0051] All embodiments also apply to the methods according to the invention.

[0052] In further embodiments of the invention, the protease or the protease produced by means of a method according to the invention still has a sequence identity of at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 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%, 98.8%, 99% or 99.5% to the amino acid sequence set forth in SEQ ID NO:1 over its entire length. The protease or the protease produced using a method according to the invention has (i) the amino acid substitutions 3T, 4I, 99E and 199I at the positions corresponding to positions 3, 4, 99, and 199, and (ii) at least one of the amino acid substitutions 9C, 21F, 21W, 42S, 42H, 44S, 105V, 112V, 113A, 131D, 137I, 139R, 141S, 145I, 159L, 168V, 176E, 177D, 182C, 193M, 198D, 204L, 205D, 206A, 210C, 212D, 230E, 234A, 250N, 253C, 255Y, 259C or 267S at at least one of the positions corresponding to the positions 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 or 267, each based on the numbering according to SEQ ID NO:1. Examples of this are the following amino acid substitution variants: (i) S3T + V4I + R99E + V199I + A267S + N255Y; (ii) S3T + V4I + R99E + V199I + N198D + S234A + L21F; (iii) S3T + V4I + R99E + V199I + N212D; (iv) S3T + V4I + R99E + V199I + G205D; (v) S3T + V4I + R99E + V199I + S182C; (vi) S3T + V4I + R99E + V199I + R44S + S234A; (vii) S3T + V4I + R99E + V199I + S253C; (viii) S3T + V4I + R99E + V199I + I105V; (ix) S3T + V4I + R99E + V199I + N177D; (x) S3T + V4I + R99E + V199I + V193M; (xi) S3T + V4I + R99E + V199I + S9C; (xii) S3T + V4I + R99E + V199I + N198D; (xiii) S3T + V4I + R99E + V199I + S259C; (xiv) S3T + V4. + R99E + V199I + I159L + Q230E; (xv) S3T + V4I + R99E + V199I + P204L; (xvi) S3T + V4I + R99E + V199I + L21F; (xvii) S3T + V4! + R99E + V199I + N42H; (xviii) S3T + V4I + R99E + V199I + S139R + S250N; (xix) S3T + V4I + R99E + V199I + N42S; (xx) S3T + V4I + R99E + V199I + V137I; (xxi) S3T + V4I + R99E + V199I + V145I; (xxii) S3T + V4I + R99E + V199I + S206A; (xxiii) S3T + V4I + R99E + V199I + G113A + L21W; (xxiv) S3T + V4I + R99E + V199I + A112V; (xxv) S3T + V4I + R99E + V199I + A168V; (xxvi) S3T + V4I + R99E + V199I + Q176E + A131D; (xxvii) S3T + V4I + R99E + V199I + T141S + S210C, each based on the numbering according to SEQ ID NO: 1, and the variants described in the examples.

[0053] A further subject matter of the invention is a previously described protease that is additionally stabilized, in particular by means of one or more mutations, for example substitutions, or by means of coupling to a polymer. An increase in stability during storage and/or during use, for example during the washing process, results in the enzymatic activity lasting longer and thus improves the cleaning performance. In principle, all stabilization options described and/or expedient in the prior art are conceivable. Preference is given to those stabilizations which are achieved via mutations of the enzyme itself because such stabilizations do not require any further working steps after the recovery of the enzyme. Examples of sequence alterations suitable for this purpose are specified above. Further suitable sequence alterations are known from the prior art.

[0054] Further possibilities for stabilization are, for example:

- altering the binding of metal ions, in particular the calcium binding sites, for example by exchanging one or more of the amino acid(s) involved in the calcium binding for one or more

negatively charged amino acids and/or by introducing sequence changes in at least one of the sequences of the two amino acids arginine/glycine;

- protecting against the influence of denaturing agents such as surfactants by mutations which cause an alteration of the amino acid sequence on or at the surface of the protein;
- exchanging amino acids that are close to the N-terminus for those that presumably come into contact with the rest of the molecule via non-covalent interactions and thus contribute to maintaining the globular structure.

[0055] Preferred embodiments are those in which the enzyme is stabilized in a plurality of ways because a plurality of stabilizing mutations act additively or synergistically.

[0056] The invention further relates to a protease as described above, characterized in that it has at least one chemical modification. A protease having such an alteration is referred to as a derivative, i.e., the protease is derivatized.

[0057] In the context of the present application, derivatives are thus understood to mean proteins whose pure amino acid chain has been chemically modified. Such derivatizations can, for example, be made *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. Derivatizations can also be made *in vitro*, for instance by means of chemical conversion of a side chain of an amino acid or by means of covalent bonding of another compound to the protein. For example, it is possible to couple amines to carboxyl groups of an enzyme in order to alter the isoelectric point. Another such compound can also be a further protein that is bound to a protein according to the invention via bifunctional chemical compounds, for example. Derivatization is likewise understood to mean covalent bonding to a macromolecular carrier or a non-covalent inclusion in suitable macromolecular cage structures. Derivatizations can influence, for example, the substrate specificity or the binding strength to the substrate or bring about temporary blocking of the enzymatic activity if the coupled substance is an inhibitor. This can be expedient for the period of storage, for example. Such modifications may further affect the stability or enzymatic activity. They can also serve to reduce the allergenicity and/or immunogenicity of the protein and thus to increase the skin compatibility thereof, for example. For example, couplings with macromolecular compounds, for example polyethylene glycol, can improve the protein with regard to stability and/or skin compatibility.

[0058] Derivatives of a protein according to the invention can also be understood in the broadest sense to be preparations of these proteins. A protein can, depending on the recovery, processing or preparation thereof, be combined with various other substances, for example from the culture of the producing microorganisms. A protein can also have been deliberately admixed with other substances, for example to increase its storage stability. Therefore, all preparations of a protein according to the invention are also in accordance with the invention. This is also independent of whether or not it actually exhibits this enzymatic activity in a particular preparation. This is because

it may be desirable for it to have no activity or only a small amount of activity during storage and to only exhibit its enzymatic function at the time of use. This can be controlled, for example, via corresponding accompanying substances. In particular, the joint preparation of proteases with specific inhibitors is possible in this regard.

[0059] Of all the proteases or protease variants and/or derivatives described above, particular preference is given within the context of the present invention to those of which the storage stability and/or cleaning performance is improved compared to the starting variant, with the cleaning performance in a washing system being determined as described above.

[0060] A further subject matter of invention is a nucleic acid coding for a protease according to the invention, and a vector containing such a nucleic acid, in particular a cloning vector or an expression vector.

[0061] These can be DNA or RNA molecules. They can be present as a single strand, as a single strand complementary to said single strand or as a double strand. In particular in the case of DNA molecules, the sequences of the two complementary strands must be taken into account in all three possible reading frames. Furthermore, it must be taken into account that different codons, i.e., base triplets, can code for the same amino acids such that a certain amino acid sequence can be coded by a plurality of different nucleic acids. Due to this degeneracy of the genetic code, all of the nucleic acid sequences which can code any of the proteases described above are included in this subject matter of the invention. A person skilled in the art is able to determine these nucleic acid sequences beyond a doubt because, despite the degeneracy of the genetic code, defined amino acids can be assigned to individual codons. Therefore, a person skilled in the art proceeding from said amino acid sequence can easily determine nucleic acids coding for said amino acid sequence. Furthermore, in the case of nucleic acids according to the invention, one or more codons can be replaced by synonymous codons. This aspect relates in particular to the heterologous expression of the enzymes according to the invention. Thus, each organism, for example a host cell of a production strain, has a certain codon usage. "Codon usage" is understood to mean the translation of the genetic code into amino acids by the relevant organism. Bottlenecks can occur in protein biosynthesis if the codons on the nucleic acid in the organism are faced with a comparatively small number of loaded tRNA molecules. Although coding for the same amino acid, this results in a codon being translated less efficiently in the organism than a synonymous codon coding for the same amino acid. Due to the presence of a higher number of tRNA molecules for the synonymous codon, this can be translated more efficiently in the organism.

[0062] It is possible for a person skilled in the art to use methods which are currently generally known, for example chemical synthesis or polymerase chain reaction (PCR), in conjunction with molecular biology and/or protein-chemical standard methods, to produce the corresponding nucleic

acids and even complete genes on the basis of known DNA and/or amino acid sequences. Such methods are known, for example, from Sambrook, J., Fritsch, E. F. and Maniatis, T. 2001. Molecular cloning: a laboratory manual, 3. Edition Cold Spring Laboratory Press.

[0063] For the purposes of the present invention, "vectors" are understood to mean elements consisting of nucleic acids that contain a nucleic acid according to the invention as the characteristic nucleic acid region. They are able to establish these as a stable genetic element in a species or cell line over several generations or cell divisions. Vectors are, in particular when used in bacteria, special plasmids, i.e., circular genetic elements. In the context of the present invention, a nucleic acid according to the invention is cloned into a vector. The vectors include, for example, those originating from bacterial plasmids, viruses or bacteriophages, or predominantly synthetic vectors or plasmids with elements of a wide variety of origins. With the other genetic elements present in each case, vectors are able to establish themselves as stable units in the particular host cells over several generations. They can be present extrachromosomally as separate units or can be integrated into a chromosome or chromosomal DNA.

[0064] Expression vectors comprise nucleic acid sequences that allow them to replicate in the host cells containing them, preferably microorganisms, particularly preferably bacteria, and to express a contained nucleic acid there. The expression is influenced in particular by the promoter or promoters that regulate transcription. In principle, the expression can take place by the natural promoter originally located before the nucleic acid to be expressed, but also by a promoter of the host cell provided on the expression vector or also by a modified or completely different promoter of another organism or another host cell. In the present case, at least one promoter is provided for the expression of a nucleic acid according to the invention and used for the expression thereof. Expression vectors can also be regulatable, for example by changing the cultivation conditions or when a certain cell density of the host cells containing them is reached or by adding certain substances, in particular activators of gene expression. An example of such a substance is the galactose derivative isopropyl β-D-thiogalactopyranoside (IPTG), which is used as an activator of the bacterial lactose operon (lac operon). In contrast to expression vectors, the nucleic acid contained is not expressed in cloning vectors.

[0065] The invention further relates to a non-human host cell containing a nucleic acid according to the invention or a vector according to the invention, or containing a protease according to the invention, in particular one that secretes the protease into the medium surrounding the host cell. Preferably, a nucleic acid according to the invention or a vector according to the invention is transformed into a microorganism that then represents a host cell according to the invention. Alternatively, individual components, i.e., nucleic acid parts or fragments of a nucleic acid according to the invention, can also be introduced into a host cell in such a way that the resulting host cell contains a nucleic acid according to the invention or a vector according to the invention. This

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

[0066] Further preferred embodiments are host cells that can be regulated in their activity owing to genetic regulatory elements that are provided, for example, on the vector but can also be present in these cells from the outset. Expression in said cells may be induced, for example, by controlled addition of chemical compounds used as activators, by changing the cultivation conditions or when a particular cell density is reached. This allows economic production of the proteins according to the invention. An example of such a compound is IPTG, as described above.

[0067] Prokaryotic or bacterial cells are preferred host cells. Bacteria are characterized by short generation times and low demands on cultivation conditions. This makes it possible to establish cost-effective cultivation methods or production methods. In addition, a person skilled in the art will have a wealth of experience in the case of bacteria in fermentation technology. Gram-negative or gram-positive bacteria can be suitable for a specific production for many different reasons to be determined experimentally in each individual case, such as nutrient sources, product formation rate, time needed, etc.

[0068] In gram-negative bacteria, such as *Escherichia coli*, a plurality of proteins are secreted into the periplasmic space, i.e., into the compartment between the two membranes enclosing the cells. This can be advantageous for specific applications. Furthermore, gram-negative bacteria can also be designed such that they discharge the expressed proteins not only into the periplasmic space but into the medium surrounding the bacterium. Gram-positive bacteria such as, for example, bacilli or actinomycetes or other representatives of A*ctinomycetes* in contrast do not have an outer membrane, such that secreted proteins are immediately released into the medium surrounding the bacteria, usually the nutrient medium, from which the expressed proteins can be purified. They can be isolated directly from the medium or further processed. In addition, gram-positive bacteria are

related or identical to most origin organisms for technically important enzymes and usually themselves form comparable enzymes, such that they have a similar codon usage and their protein synthesis apparatus is naturally aligned accordingly.

[0069] Host cells according to the invention may be altered in terms of their requirements for culture conditions, have different or additional selection markers, or also express different or additional proteins. In particular, this may also involve those host cells which express a plurality of proteins or enzymes.

[0070] The present invention is applicable in principle to all microorganisms, in particular to all fermentable microorganisms, particularly preferably those of the genus *Bacillus,* and leads to it being possible to produce proteins according to the invention by the use of such microorganisms. Such microorganisms then represent host cells for the purposes of the invention.

[0071] 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.*

[0072] The host cell may also be a eukaryotic cell, however, which is characterized in that it has a cell nucleus. The invention therefore further relates to a host cell that is characterized in that it has a nucleus. In contrast with prokaryotic cells, eukaryotic cells are capable of post-translationally modifying the protein formed. Examples thereof are fungi such as actinomycetes or yeasts such as *Saccharomyces* or *Kluyveromyces*. This can be particularly advantageous, for example, if the proteins are to undergo specific modifications in connection with their synthesis, which modifications make such systems possible. Modifications carried out by eukaryotic systems, in particular in connection with the protein synthesis, include, for example, the binding of low-molecular-weight compounds such as membrane anchors or oligosaccharides. Such oligosaccharide modifications can be desirable, for example, to reduce the allergenicity of an expressed protein. Coexpression with the enzymes naturally formed by such cells, such as cellulases, can also be advantageous. Furthermore, for example, thermophilic fungal expression systems can be particularly suitable for expression of temperature-resistant proteins or variants.

[0073] The host cells according to the invention are cultured and fermented in the usual manner, for example in discontinuous or continuous systems. In the first case, a suitable nutrient

medium is inoculated with the host cells and the product is harvested from the medium after a period to be determined experimentally. Continuous fermentations are characterized by achieving a flow equilibrium in which cells partially die off over a comparatively long period but also grow back and, at the same time, the protein formed can be removed from the medium.

[0074] Host cells according to the invention are preferably used to produce proteases according to the invention. The invention therefore further relates to a method for producing a protease, comprising

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

[0075] This subject matter of the invention preferably comprises fermentation processes. Fermentation processes are known per se from the prior art and represent the actual large-scale production step, generally followed by a suitable purification method for the product produced, for example for the proteases according to the invention. All fermentation processes that are based on a corresponding method for producing a protease according to the invention represent embodiments of this subject matter of the invention.

[0076] Fermentation processes that are characterized in that the fermentation is carried out via a feed strategy are considered in particular. In this case, the media constituents that are consumed by the continuous cultivation are added. As a result, considerable increases can be achieved both in the cell density and in the cell mass or dry mass and/or in particular in the activity of the protease of interest. Furthermore, the fermentation can also be designed such that unwanted metabolic products are filtered out or neutralized by adding buffers or suitable counterions.

[0077] The prepared protease can be harvested from the fermentation medium. Such a fermentation process is preferred over isolation of the protease from the host cell, i.e., product preparation from the cell mass (dry mass), but requires the provision of suitable host cells or one or more suitable secretion markers or mechanisms and/or transport systems so that the host cells secrete the protease into the fermentation medium. Without secretion, the isolation of the protease from the host cell, i.e., purification thereof from the cell mass, can alternatively take place, for example by means of precipitation with ammonium sulfate or ethanol or by means of chromatographic purification.

[0078] All of the above-mentioned aspects can be combined to form methods in order to produce the protease according to the invention.

[0079] The invention further provides an agent that is characterized in that it contains a protease according to the invention, as described above. The agent is preferably a washing or cleaning agent.

[0080] This subject matter of the invention covers all conceivable types of washing or cleaning agents, both concentrates and undiluted agents, 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 include, for example, dishwashing detergents for dishwashers or manual dishwashing detergents or cleaners for hard surfaces such as metal, glass, porcelain, ceramics, tiles, stone, painted surfaces, plastics, wood or leather, for which the term cleaning agent is used, i.e. in addition to manual and mechanical dishwashing detergents, also, for example, scouring agents, glass cleaners, WC toilet scenters, etc. The washing and cleaning agents according to the invention also include auxiliary washing agents which are added to the actual washing agent during manual or automatic textile washing in order to achieve a further effect. Furthermore, in the context of the invention, washing and cleaning agents also include textile pre-treatment agents and post-treatment agents, i.e., those agents with which the item of laundry is brought into contact before the actual washing, for example for dissolving stubborn stains, and also those agents which give the laundry further desirable properties, such as a pleasant feel, crease resistance or a low static charge in a step downstream of the actual textile washing. Inter alia, softeners are included in the latter agents.

[0081] The washing or cleaning agents according to the invention, which may be in the form of powdered solids, in further-compacted particulate form, as gels, 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 can contain, in particular, surfactants, builders, peroxygen compounds or bleach activators. Furthermore, they can contain water-miscible organic solvents, further enzymes, sequestering agents, electrolytes, pH regulators and/or further auxiliaries such as optical brighteners, graying inhibitors, foam regulators and dyes and fragrances and combinations thereof.

[0082] In particular, a combination of a protease according to the invention with one or more further ingredient(s) of the agent is advantageous because, in preferred embodiments according to the invention, such an agent has improved cleaning performance due to resulting synergisms. In particular, such a synergism can be achieved by combining a protease according to the invention with a surfactant and/or a builder and/or a peroxygen compound and/or a bleach activator. However, in preferred embodiments, the agent according to the invention cannot contain boric acid.

[0083] Advantageous ingredients of agents according to the invention are disclosed in the international patent application WO2009/121725A1, starting on page 5, penultimate paragraph, and ending on page 13 after the second paragraph.

[0084] An agent according to the invention advantageously contains the protease in an amount of 2 µg to 20 mg, preferably of 5 µg to 17.5 mg, particularly preferably of 20 µg to 15 mg and very particularly preferably of 50 µg to 10 mg per g of the agent. In various embodiments, the concentration of the protease (active enzyme) described herein in the agent is >0 to 1 wt.%, preferably 0.001 to 0.1 wt.%, based on the total weight of the agent or composition. Furthermore, the protease contained in the agent and/or further ingredients of the agent can be coated with a substance that is impermeable to the enzyme at room temperature or in the absence of water and becomes permeable to the enzyme under application conditions of the agent. Such an embodiment of the invention is thus characterized in that the protease is coated with a substance that is impermeable to the protease at room temperature or in the absence of water. Furthermore, the washing or cleaning agent itself can also be packaged in a container, preferably an air-permeable container, from which it is released shortly before use or during the washing process.

[0085] In further embodiments of the invention, the agent is characterized in that it:

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

[0086] These embodiments of the present invention include all solid, powdered, liquid, gel or pasty administration forms of agents according to the invention, which may optionally also consist of a plurality of phases and can be present in a compressed or uncompressed form. The agent can be present as a free-flowing powder, in particular having a bulk density of 300 g/l to 1200 g/l, in particular 500 g/l to 900 g/l or 600 g/l to 850 g/l. The solid administration forms of the agent further include extrudates, granules, tablets or pouches. Alternatively, the agent can also be in a liquid, gel or paste form, for example in the form of a non-aqueous liquid washing agent or a non-aqueous paste or in the form of an aqueous liquid washing agent or a water-containing paste. Liquid agents are generally preferred. Furthermore, the agent can be present as a single-component system. Such agents consist of one phase. Alternatively, an agent can also consist of a plurality of phases. Such an agent is accordingly divided into a plurality of components.

[0087] Washing or cleaning agents according to the invention can contain only one protease. Alternatively, they can also contain further hydrolytic enzymes or other enzymes in a concentration expedient for the effectiveness of the agent. A further embodiment of the invention is thus represented by agents that further comprise one or more further enzymes. Further enzymes which can preferably be used are all enzymes which can exhibit catalytic activity in the agent according to the invention, in particular a lipase, amylase, cellulase, hemicellulase, mannanase, tannase, xylanase, xanthanase, xyloglucanase, β-glucosidase, pectinase, carrageenase, perhydrolase, oxidase, oxidoreductase or another protease, which may be different from the proteases according to the invention, as well as mixtures thereof. Additional enzymes are contained in the agent advantageously 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 1×10^{-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.%, in each case based on active protein. Particularly preferably, the enzymes exhibit synergistic cleaning performance with respect to particular dirt or stains, i.e. the enzymes contained in the agent composition assist one another in their cleaning performance. Synergism of this kind is very particularly preferably present between the protease contained according to the invention and a further enzyme of an agent according to the invention, including in particular between said protease and an amylase and/or a lipase and/or a mannanase and/or a cellulase and/or a pectinase. Synergistic effects can occur not only between different enzymes but also between one or more enzymes and other ingredients of the agent according to the invention.

[0088] In the cleaning agents described herein, the enzymes to be used can further be formulated together with accompanying substances, for example from fermentation. In liquid formulations, the enzymes are preferably used as liquid enzyme formulation(s).

[0089] The enzymes are generally not provided in the form of the pure protein, but rather in the form of stabilized, storable and transportable preparations. These ready-made preparations include, for example, the solid preparations obtained by means of granulation, extrusion or lyophilization or, in particular in the case of liquid or gel agents, solutions of the enzymes, which are advantageously as concentrated as possible, have a low water content, and/or are admixed with stabilizers or further auxiliaries.

[0090] 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. Further active ingredients, for example, stabilizers, emulsifiers, pigments, bleaches or dyes can additionally be applied in overlaid

layers. Such capsules are made using methods that are known per se, for example by means of vibratory granulation or roll granulation or by means of fluid bed processes. Advantageously, such granules are low in dust, for example due to the application of polymeric film formers, and are stable in storage due to the coating.

[0091] Furthermore, it is possible to formulate two or more enzymes together such that a single granule exhibits a plurality of enzyme activities.

[0092] The enzymes can also be introduced into water-soluble films, such as those used in the formulation of washing and cleaning agents in a unit dosage form. Such a film allows the enzymes to be released after contact with water. As used herein, “water-soluble” refers to a film structure that is preferably completely water-soluble. Preferably, such a film consists of (completely or partially hydrolyzed) polyvinyl alcohol (PVA).

[0093] 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 or in the concentrations described herein.

[0094] 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 from 0 to 60 °C, more preferably from 20 to 40 °C and most preferably at 25 °C.

[0095] These include both manual and machine methods, wherein machine methods are preferred. Methods for cleaning textiles are generally characterized by the fact that, in a plurality of method steps, various cleaning-active substances are applied to the material to be cleaned and washed off after the exposure time, or in that the material to be cleaned is otherwise treated with a washing agent or a solution or dilution of this agent. The same applies to methods for cleaning all materials other than textiles, in particular hard surfaces. All conceivable washing or cleaning methods can be enhanced in at least one of the method steps by the use of a washing or cleaning agent according to the invention or a protease according to the invention, and therefore represent embodiments of the present invention. All aspects, subject matters and embodiments described for the protease according to the invention and agents containing them are also applicable to this subject matter of the invention. Therefore, reference is expressly made at this point to the disclosure at the corresponding point with the indication that this disclosure also applies to the above methods according to the invention.

[0096] Because proteases according to the invention by nature already have hydrolytic activity and also exhibit said activity in media that otherwise have no cleaning power, such as, for example, in a simple buffer, an individual and/or the single step of such a method can consist of bringing a protease according to the invention into contact with the stain as the only cleaning component, preferably in a buffer solution or in water. This represents a further embodiment of this subject matter of the invention.

[0097] Alternative embodiments of this subject matter of the invention also include methods for treating textile raw materials or for textile care, in which a protease according to the invention is active in at least one method step. Among these, methods for textile raw materials, fibers or textiles comprising natural constituents are preferred, and very particularly for those comprising wool or silk.

[0098] Finally, the invention also encompasses the use of the proteases described herein in washing or cleaning agents, for example as described above, for (improved) removal of protein-containing stains, for example from textiles or hard surfaces. In 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.

[0099] All aspects, subject matters and embodiments described for the protease according to the invention and agents containing them are also applicable to this subject matter of the invention. Therefore, reference is expressly made at this point to the disclosure at the corresponding point with the indication that this disclosure also applies to the above use according to the invention.

Examples

Example 1: Overview of the mutations

[0100] The invention relates to a subtilisin-type alkaline protease from *Bacillus lentus.* From one starting variant (protease according to SEQ ID NO:2 from WO2013/060621A1), variants were produced by random mutagenesis, which were then screened, inter alia for improved washing performance and/or enzyme stability. In this way, mutants having improved storage stability and/or improved cleaning performance were generated from said protease 27.

Variant	**Amino acid substitutions relative to SEQ ID NO:1**						
Starting variant	S3T	V4I	R99E	V199I			
Mutant 1	S3T	V4I	R99E	V199I	A267S	N255Y	
Mutant 2	S3T	V4I	R99E	V199I	N198D	S234A	L21F

Mutant 3	S3T	V4I	R99E	V199I	N212D		
Mutant 4	S3T	V4I	R99E	V199I	G205D		
Mutant 5	S3T	V4I	R99E	V199I	S182C		
Mutant 6	S3T	V4I	R99E	V199I	R44S	S234A	
Mutant 7	S3T	V4I	R99E	V199I	S253C		
Mutant 8	S3T	V4I	R99E	V199I	I105V		
Mutant 9	S3T	V4I	R99E	V199I	N177D		
Mutant 10	S3T	V4I	R99E	V199I	V193M		
Mutant 11	S3T	V4I	R99E	V199I	S9C		
Mutant 12	S3T	V4I	R99E	V199I	N198D		
Mutant 13	S3T	V4I	R99E	V199I	S259C		
Mutant 14	S3T	V4I	R99E	V199I	I159L	Q230E	
Mutant 15	S3T	V4I	R99E	V199I	P204L		
Mutant 16	S3T	V4I	R99E	V199I	L21F		
Mutant 17	S3T	V4I	R99E	V199I	N42H		
Mutant 18	S3T	V4I	R99E	V199I	S139R	S250N	
Mutant 19	S3T	V4I	R99E	V199I	N42S		
Mutant 20	S3T	V4I	R99E	V199I	V137I		
Mutant 21	S3T	V4I	R99E	V199I	V145I		
Mutant 22	S3T	V4I	R99E	V199I	S206A		
Mutant 23	S3T	V4I	R99E	V199I	G113A	L21W	
Mutant 24	S3T	V4I	R99E	V199I	A112V		
Mutant 25	S3T	V4I	R99E	V199I	A168V		
Mutant 26	S3T	V4I	R99E	V199I	Q176E	A131D	
Mutant 27	S3T	V4I	R99E	V199I	T141S	S210C	

Washing agent matrix used

[0101]

Chemical name	Wt.% of active substance in the formulation
Demineralized water	Remainder
Na-LAS	3-8
Anionic surfactants	4-6

Sodium laureth sulfate	2.0
Non-ionic surfactants	2-6
Phosphonate	0.2-0.6
Citric acid	0.2-2
NaOH	1-4
Defoamer	< 1
Glycerol	0.5-2
Preservatives	< 0.1
Without opt. brighteners, perfume, dye and enzymes. Dosage: 6 g/L	

Dishwashing detergent matrix used

[0102]

Chemical name	Wt.% of active substance in the formulation
Alkyl benzene sulfonic acid	8-20
Water	30-80
NaOH (50%)	5.4
Fatty alcohol ether sulfate	7.14
NaCl Lsg (20%)	2.0
Phosphoric acid (H_3PO_4) (34%/85%)	0.383 (34%)
Preservatives	0.1
perfume	0.25
Dye	1.0
Bittern	0.04

Example 2: Determining the storage stability

Storage

[0103] The protease variants according to the invention were expressed in *Bacillus subtilis*. From the supernatants of the *Bacillus subtilis* culture, the proteases were diluted to an identical activity level. For determining the storage stability in washing agents or dishwashing detergents, 50% washing agent matrix without boric acid or 50% rinsing agent matrix were reacted with 50% correspondingly diluted *Bacillus subtilis* culture and mixed well. The sealed vessels were each stored at 40 °C for three or 3.5

weeks. The amount of sample taken was dissolved in 0.1 M Tris/HCl (pH 8.6) by stirring at room temperature for 20 min. The AAPF assay was then carried out as described below.

Protease activity assay

[0104] The activity of the protease is determined by releasing the chromophore para-nitroaniline from the substrate succinyl alanine-alanine-proline-phenylalanine-para-nitroanilide (AAPFpNA; Bachem L-1400). The release of the pNA causes an increase in absorbance at 410 nm, the time profile of which is a measure of the enzymatic activity.

[0105] The measurement was carried out at a temperature of 25 °C, a pH of 8.6, and a wavelength of 410 nm. The measurement time was 5 min at a measuring interval of 20 to 60 seconds.

Measurement approach:

[0106]

10 µL AAPF solution (70 mg/mL)

1000 µL Tris/HCl (0.1 M; pH 8.6 with 0.1% Brij 35)

10 µL diluted protease solution

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

[0107] In the following, the improved stability compared to the stability of the starting variant after 3 weeks of storage at 40 °C in the aforementioned washing agent or dishwashing detergent matrix is shown in %:

Variant	Improved stability (%) in the aforementioned washing agent matrix	Improved stability (%) in the aforementioned dishwashing detergent matrix
Starting variant	0	0
Mutant 1	+44	+14
Mutant 2	+49	+55
Mutant 3	+93	+41
Mutant 4	+57	+5
Mutant 5	+80	+11
Mutant 6	+58	+15
Mutant 7	+9	+41

Variant	Improved stability (%) in the aforementioned washing agent matrix	Improved stability (%) in the aforementioned dishwashing detergent matrix
Mutant 8	+102	+58
Mutant 9	+65	+52
Mutant 10	+43	+35
Mutant 11	+67	+25
Mutant 12	+60	+19
Mutant 13	+54	+28
Mutant 14	+64	+9
Mutant 15	+56	+16
Mutant 16	+59	+0
Mutant 17	+64	+22
Mutant 18	+42	+2
Mutant 19	+46	+29
Mutant 20	+58	+4
Mutant 21	+73	+12
Mutant 22	+63	+33
Mutant 23	+71	+22
Mutant 24	+47	+5
Mutant 25	+38	+24
Mutant 26	+68	+62
Mutant 27	+77	+48

[0108] All variants show improved stability in washing agents and/or dishwashing detergents compared to the starting variant.

Example 3: Determining the cleaning performance

Mini wash test

[0109] Washing test with *Bacillus subtilis* culture supernatants containing the screened protease mutants by heterologous expression. The supernatants are used in washing agents in the equivalent activity to the benchmark = starting variants with at a market-standard concentration for

proteases. In contrast to the determination of the storage stability, the samples are not stored, but instead the cleaning performance is determined directly. The mutants are all based on the wash performance of the starting variant, which is set equal to 100% (sum of the 7 stains, corrected by the performance of the washing agent alone).

Conditions: 40 °C, 16° dH water, 1 h

Stains:

[0110]

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

[0111] Punched-out pieces of fabric (diameter = 10 mm) were placed in microtiter plates, washing liquor was preheated to 40 °C, with a final concentration of 3.17 g/L, the liquor and enzyme were added to the stain and incubated for 1 h at 40 °C and 600 rpm, then the stain was rinsed several times with clear water and left to dry and the brightness was determined using a color-measuring device. The lighter the fabric, the better the cleaning performance. The L value = brightness is measured here, the higher the brighter. The sum of the 7 stains is given in % based on the starting variant corrected by the performance of the washing agent without protease.

Variant	**Performance in washing test at 40 °C** (based on the performance of the output variant)
Starting variant	100%
Mutant 10	104%
Mutant 13	104%

[0112] Variants 10 and 13 show increased washing performance compared to the starting variant.

[0113] Proteases according to the invention, in particular variants 10 and 13, consequently exhibit not only improved storage stability but also improved cleaning performance.

PATENTKRAV

1. Protease, omfattende en aminosyresekvens, der har mindst 70% sekvensidentitet med den i SEQ ID NO:1 angivne aminosyresekvens over hele dens længde, og som på basis af nummereringen ifølge SEQ ID NO:1 på

   I) positionerne, der svarer til positionerne 3, 4, 99 og 199, som omfatter aminosyresubstitutionerne S3T, V41, R99E og V199I, og

   II) på mindst én af positionerne, der svarer til positionerne 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 eller 267, omfatter mindst en aminosyresubstitution.

2. Protease ifølge krav 1, hvorved
   den mindst ene aminosyresubstitution på mindst én af positionerne, der svarer til positionerne 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 eller 267, er udvalgt fra gruppen, som består af 9C, 21F, 21W, 42S, 42H, 44S, 105V, 112V, 113A, 131D, 137I, 139R, 141S, 145I, 159L, 168V, 176E, 177D, 182C, 193M, 198D, 204L, 205D, 206A, 210C, 212D, 230E, 234A, 250N, 253C, 255Y, 259C og 267S.
3. Protease ifølge et af kravene 1 til 2, hvorved proteasen omfatter, baseret henholdsvis på nummereringer ifølge SEQ ID NO:1:

   I) aminosyresubstitutionerne S3T + V41 + RDDE + V199I, og

   (II) en af de følgende aminosyresubstitutionskombinationer: (i) A267S + N255Y; (ii) N198D + S234A + L21F; (iii) N212D; (iv) G205D; (v) S182C; (vi) R44S + S234A; (vii) S253C; (viii) I105V; (ix) N177D; (x) V193M; (xi) S9c; (xii) N198D; (xiii) S259C; (xiv) I159L + Q230E; (xv) P204L; (xvi) L21F; (xvii) N42H; (xviii) S139R + S250N; (xix) N42S; (xx) V137I; (xxi) V145L; (xxii) S206A; (xxiii)

G113A + L21W; (xxiv) A112V; (xxv) A168V; (xxvi) Q176E + A131D; (xxvii) T141S + S210C.

4. Protease, **kendetegnet ved, at**

(a) den kan opnås ud fra en protease ifølge et af kravene 1 til 3 som udgangsmolekyle ved hjælp af enkelt eller multipel, konservativ aminosyresubstitution, hvorved proteasen omfatter:

I) aminosyresubstitutioner S3T, V4I, R99E og V199I på positionerne, der svarer til positionerne 3, 4, 99 og 199, og

II) på mindst én af positionerne, som svarer til positionerne 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 eller 267, mindst én aminosyresubstitution; eller

(b) de kan opnås ud fra en protease ifølge et af kravene 1 til 3 ved fragmentering, deletions-, indsætnings- eller substitutionsmutagenese, og omfatter en aminosyresekvens, som over en længde på mindst 190, 200, 210, 220, 230, 240, 250, 260, 261, 262, 263, 264, 265, 266, 267, 268 eller 269 sammenhængende aminosyrer stemmer overens med udgangsmolekylet, hvorved proteinasen omfatter:

I) aminosyresubstitutioner S3T, V4I, RDDE og V199I på positionerne, der svarer til positionerne 3, 4, 99 og 199, og

II) på mindst én af positionerne, som svarer til positionerne 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 eller 267, mindst én aminosyresubstitution.

5. Fremgangsmåde til fremstilling af en protease, omfattende indsætning

I) af aminosyresubstitutioner S3T, V4I, R99E og V199I på positionerne, der svarer til positionerne 3, 4, 99 og 199, baseret på nummerering ifølge SEQ ID NO: 1, og

II) mindst én aminosyresubstitution på mindst én af positionerne, som svarer til positionerne 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 eller 267,

i et udgangsmolekyle med en aminosyresekvens, som har mindst 70% sekvensidentitet med aminosyresekvensen, der er angivet i SEQ ID NO:1, over hele dens længde.

6. Fremgangsmåde ifølge krav 5, omfattende et eller flere af de følgende fremgangsmådetrin:

(a) indsætning af en enkelt eller multipel, konservativ aminosyresubstitution, hvorved proteasen omfatter:

I) aminosyresubstitutioner S3T, V4I, R99E og V199I på positionerne, der svarer til positionerne 3, 4, 99 og 199, og

II) på mindst én af positionerne, som svarer til positionerne 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 eller 267, mindst én aminosyresubstitution;

(b) ændring af aminosyresekvensen ved fragmentering, deletions-, eller substitutionsmutagenese, således at proteasen omfatter en amino-

syresekvens, som over en længde på mindst 190, 200, 210, 220, 230, 240, 250, 260, 261, 262, 263, 264, 265, 266, 267, 268 eller 269 sammenhængende aminosyrer stemmer overens med udgangsmolekylet, hvorved proteinasen omfatter:

I) aminosyresubstitutioner S3T, V4I, RDDE og V199I på positionerne, der svarer til positionerne 3, 4, 99 og 199, og

II) på mindst én af positionerne, som svarer til positionerne 9, 21, 42, 44, 105, 112, 113, 131, 137, 139, 141, 145, 159, 168, 176, 177, 182, 193, 198, 204, 205, 206, 210, 212, 230, 234, 250, 253, 255, 259 eller 267, mindst én aminosyresubstitution.

7. Nukleinsyre, kodende for en protease ifølge et af kravene 1 til 4 eller kodende for en protease, der er opnået ved en fremgangsmåde ifølge et af kravene 5 til 6.

8. Vektor, indeholdende en nukleinsyre ifølge krav 7, navnlig en kloningsvektor eller en expressionsvektor.

9. Ikke-human værtscelle, der omfatter en nukleinsyre ifølge krav 7 eller en vektor ifølge krav 8, eller som omfatter en protease ifølge et af kravene 1 til 4, eller som omfatter en protease, der er opnået ved en fremgangsmåde ifølge et af kravene 5 til 6, og som udskiller proteasen i mediet, der omgiver værtscellen.

10. Fremgangsmåde til fremstilling af en protease, omfattende

a) dyrkning af en værtscelle ifølge krav 9; og
b) isolering af proteasen fra dyrkningsmediet eller fra værtscellen.

11. Middel, navnlig et vaske- eller rensemiddel, især et flydende vaskemiddel eller et håndopvaskemiddel,

kendetegnet ved, at det mindst indeholder en protease ifølge et af kravene 1 til 4 eller en protease, der er opnået ved en fremgangsmåde ifølge et af kravene 5 til 6.

12. Fremgangsmåde, til rensning af tekstiler eller hårde overflader, **kendetegnet ved, at** der i mindst et fremgangsmådetrin anvendes et middel ifølge krav 11.

13. Anvendelse af en protease ifølge et af kravene 1 til 4, eller en protease, som er opnået ved en fremgangsmåde ifølge et af kravene 5 til 6, i et vaske- eller rensemiddel til fjernelse af peptid- eller proteinholdige tilsmudsninger.

SEKVENSLISTE

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