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Toscano et al.(10) **Pub. No.: US 2017/0342349 A1**(43) **Pub. Date: Nov. 30, 2017**(54) **STABILIZED ENZYME COMPOSITIONS****Publication Classification**(71) Applicant: **NOVOZYMES A/S**, Bagsvaerd (DK)(51) **Int. Cl.**(72) Inventors: **Miguel Duarte Guilherme Toscano**,
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ABSTRACT(30) **Foreign Application Priority Data**Dec. 19, 2014 (EP) 14199399.8
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The present invention relates to methods for obtaining protease variants with improved properties. The present invention also relates to protease variants, compositions comprising the protease variants and methods of using the protease variants.

STABILIZED ENZYME COMPOSITIONS

REFERENCE TO A SEQUENCE LISTING

[0001] This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention relates to method for improve binding of an enzyme to an inhibitor of the enzyme. The enzyme variants generated by the method of the invention are suitable for use in cleaning processes and detergent compositions, such as laundry compositions and dish wash compositions, including hand wash and automatic dish wash compositions.

Description of the Related Art

[0003] Enzymes have been used within the detergent industry as part of detergent formulations for many decades. Proteases are from a commercial perspective the most relevant enzyme in such formulations, but other enzymes including lipases, amylases, cellulases, hemicellulases or mixtures of enzymes are also commonly used. One family of proteases, which are widely used in detergents, are the subtilases. A generally encountered problem using proteases with or without other enzymes in particular liquid detergent compositions, is that its needed to stabilize the protease in order to minimize loss due to proteolysis or auto-proteolysis during storage in the detergent in order not to get reduced enzyme wash performance when finally washed. The storage stability of enzymes in liquid cleaning compositions has been improved for example by adding various protease inhibitors or stabilizers. The use of a reversible peptide aldehyde protease inhibitor to stabilize liquid detergent compositions is described in WO2007/141736, WO2007/145963 and WO2007/145964 (Procter and Gamble Co.) and compositions containing enzymes stabilized with inhibitors is described in US2003/157088 (Procter and Gamble Co.), WO96/41638 (Sanofi Winthrop Inc.) and WO2005/105826 (Zaidan Hojin Biseibutsu) disclose peptide aldehydes and ketones. Boric acid and boronic acids are known to reversibly inhibit proteases and are described e.g. in WO1996041859 (Novozymes NS). The use of peptide aldehydes or hydrosulfite derivatives thereof for stabilizing certain proteases in detergents is described in e.g. WO2009/118375, WO2013/004636 and WO11036153 (Novozymes NS). An enzyme and its inhibitor interact in a specific manner and the inhibitors are designed to fit the enzyme similar to a key and a lock. Consequently, not all inhibitors will bind equally strong to all enzymes and a specific protease inhibitor may interact with optimal efficiency to a limited number of proteases which will be relatively similar in the binding affinity to the specific inhibitor. An optimal interaction of protease and protease inhibitor is necessary for obtaining stabilized compositions where the enzymes of the compositions are stabilized and do not adversely affect each other. Enzymes formulated in cleaning compositions are often modified to increase their cleaning performance i.e. proteases are modified by introducing specific alterations in the back bone (protease parent) and tested for performance in a relevant assay. The optimized protease is subsequently

formulated in a detergent and in particular, when the protease formulated in compositions comprising additional enzymes it may be necessary to stabilize the protease to maintain optimal effect of the cleaning composition until finally used in the wash. The optimized protease may vary in the three dimensional structure from the parent protease and thus may vary in the interaction with a specific inhibitor for the parent protease. Further, proteases used in the detergent industry are very different in structure and sequences. Thus there is a need for ways to optimize the enzyme inhibitor interaction in particular for methods to improve proteases toward stronger binding to specific protease inhibitors.

SUMMARY OF THE INVENTION

[0004] The present invention relates to a method for improve binding of a protease to an inhibitor of the protease, comprising the steps of:

- [0005] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,
- [0006] b) modify the protease by modifying the amino acid residues identified in step a)
- [0007] c) determine the binding constant Ki
- [0008] d) select modified protease generated in step b)

[0009] The invention further relates to a protease obtained by the method of the invention.

Overview of Sequences Listing

SEQ ID NO: 1 Savinase

SEQ ID NO: 2 BPN'

SEQ ID NO: 3 TY145

SEQ ID NO: 4 BLAP

SEQ ID NO: 5 Protease P

SEQ ID NO: 6 Protease C

Definitions

[0010] The term "protease" is defined herein as an enzyme that hydrolyses peptide bonds. It includes any enzyme belonging to the EC 3.4 enzyme group (including each of the thirteen subclasses thereof). The EC number refers to Enzyme Nomenclature 1992 from NC-IUBMB, Academic Press, San Diego, Calif., including supplements 1-5 published in Eur. J. Biochem. 1994, 223, 1-5; Eur. J. Biochem. 1995, 232, 1-6; Eur. J. Biochem. 1996, 237, 1-5; Eur. J. Biochem. 1997, 250, 1-6; and Eur. J. Biochem. 1999, 264, 610-650; respectively. The term "subtilases" refer to a sub-group of serine protease according to Siezen et al., Protein Engng. 4 (1991) 719-737 and Siezen et al. Protein Science 6 (1997) 501-523. Serine proteases or serine peptidases is a subgroup of proteases characterized by having a serine in the active site, which forms a covalent adduct with the substrate. Further, the subtilases (and the serine proteases) are characterized by having two active site amino acid residues apart from the serine, namely a histidine and an aspartic acid residue. The subtilases may be divided into 6 sub-divisions, i.e. the Subtilisin family, the Thermitase family, the Proteinase K family, the Lantibiotic peptidase family, the Kexin family and the Pyrolysins family. The term

“protease activity” means a proteolytic activity (EC 3.4). Proteases of the invention are endopeptidases (EC 3.4.21). For purposes of the present invention, protease activity is determined according to the procedure described in “Materials and Methods” below.

[0011] The term “parent”, “protease parent” or “precursor protease” means a protease in which an alteration is made to produce protease variant. The parent may be a naturally occurring (wild-type) polypeptide. In a particular embodiment the parent is a protease with at least 60%, at least 62%, at least 63%, at least 64%, at least 65%, at least 70%, at least 72%, at least 73%, at least 74%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identity to a polypeptide with the amino acid sequence shown in SEQ ID NO 1, 2, 3, 4, 5 or 6.

[0012] The term “protease variant” means a protease having protease activity comprising an alteration, i.e., a substitution, insertion, and/or deletion, preferably substitution, at one or more (or one or several) positions compared to its parent which is a protease having the identical amino acid sequence of said variant but not having the alterations at one or more of said specified positions. A substitution means a replacement of an amino acid occupying a position with a different amino acid; a deletion means removal of an amino acid occupying a position; and an insertion means adding amino acids e.g. 1 to 10 amino acids, preferably 1-3 amino acids adjacent to an amino acid occupying a position. Preferably, the variant is modified by the hand of man.

[0013] The term “wild-type protease” means a protease expressed by a naturally occurring organism, such as a bacterium, archaea, yeast, fungus, plant or animal found in nature.

[0014] The term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc.

[0015] The term “mature polypeptide coding sequence” means a polynucleotide that encodes a mature polypeptide having protease activity.

[0016] The term “cDNA” means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a prokaryotic or eukaryotic cell. A cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

[0017] The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”. For purposes of the present invention, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular

[0018] Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The

output of Needle labeled “longest identity” (obtained using the -nbrief option) is used as the percent identity and is calculated as follows:

$$\frac{(\text{Identical Residues} \times 100)}{(\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})}$$

[0019] The term “improved property” means a characteristic associated with an enzyme that is improved compared to a reference enzyme one example is a protease variants which is improved compared to the parent protease. Such improved properties include, but are not limited to, wash performance, enzyme activity, thermal activity profile, thermostability, pH activity profile, pH stability, substrate/co-factor specificity, surface properties, substrate specificity, product specificity, increased stability and/or inhibitor binding.

[0020] The term “improved protease activity” is defined herein as an altered protease activity e.g. by increased protein conversion of a protease displaying an alteration of the activity relative (or compared) to the activity of a reference protease, e.g. altered activity of a protease variant compared to the parent protease, by increased protein conversion.

[0021] The term “stability” includes storage stability and stability during use, e.g. during a wash process and reflects the stability of an enzyme, such as a protease as a function of time e.g. how much activity is retained when the protease variant is kept in solution in particular in a detergent solution or in a wash solution. The stability is influenced by many factors e.g. pH, temperature, detergent composition e.g. amount of builder, surfactants, other stabilizers etc.

[0022] The terms “improved stability” and “increased stability” includes but are not limited to: improved stability under storage conditions, improved performance after storage conditions, improved tolerance to ingredients present in the detergent composition, increased binding to a specific inhibitor, improved detergent stability, improved in-wash stability, improved auto-proteolytic stability (including reduced auto-proteolysis) and/or improved chemical stability “improved chemical stability”, “detergent stability”.

[0023] The term “improved chemical stability” is defined herein as an enzyme, e.g. a protease variant displaying retention of enzymatic activity after a period of incubation in the presence of a chemical or chemicals, either naturally occurring or synthetic, which reduces the enzymatic activity of the reference enzyme, e.g. protease parent. Likewise, the term “improved detergent stability” is defined herein as an enzyme e.g. protease variant displaying retention of enzymatic activity after a period of incubation in the presence of a detergent component such as bleach, surfactants, builder and chelators, which reduces the enzymatic activity of the reference enzyme e.g. parent protease.

[0024] The term “improved wash performance” or improved “cleaning performance” is defined herein as e.g. a protease variant displaying an improved wash performance relative to the wash performance of the parent protease, or relative to a reference protease when measured in a relevant assay such as AMSA. The term “wash performance” includes wash performance in laundry but also hand wash and dish wash.

[0025] The terms “cleaning composition” or “detergent composition”, includes unless otherwise indicated, granular or powder-form all-purpose or heavy-duty washing agents, especially cleaning detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-

duty liquid (HDL) types; unit dose/liquid capsules formats, liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type;

[0026] machine dishwashing agents, including the various tablet, granular, liquid, unit dose/liquid or solid capsules and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, including antibacterial hand-wash types, cleaning bars, soap bars, mouthwashes, denture cleaners, car or carpet shampoos, bathroom cleaners; hair shampoos and hair-rinses; shower gels, foam baths; metal cleaners; as well as cleaning auxiliaries such as bleach additives and “stain-stick” or pre-treat types. The terms “detergent composition” and “detergent formulation” are used in reference to mixtures which are intended for use in a wash medium for the cleaning of soiled objects. In some embodiments, the term is used in reference to laundering fabrics and/or garments (e.g., “laundry detergents”). In alternative embodiments, the term refers to other detergents, such as those used to clean dishes, cutlery, etc. (e.g., “dishwashing detergents”). The terms “cleaning composition” and “detergent composition” are not intended to be limited to compositions that contain surfactants. It is intended that the term encompasses detergents that may contain, surfactants, builders, chelators or chelating agents, bleach system or bleach components, polymers, fabric conditioners, foam boosters, suds suppressors, dyes, perfume, tannish inhibitors, optical brighteners, bactericides, fungicides, soil suspending agents, anti corrosion agents, dye transfer inhibitors, acids or bases; buffering agents, salts, enzymes and enzyme inhibitors or stabilizers, enzyme activators, bluing agents and fluorescent dyes, antioxidants, or solubilizers and other functional or visually impacting ingredients (ex opacifiers, pearlescences).

[0027] The term “effective amount of enzyme” refers to the quantity of enzyme necessary to achieve the enzymatic activity required in the specific application, e.g., in a defined detergent composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and are based on many factors, such as the particular enzyme used, the cleaning application, the specific composition of the detergent composition, and whether a liquid or dry (e.g., granular, bar) composition is required, and the like.

[0028] The term “water hardness” or “degree of hardness” or “dH” or “° dH” as used herein refers to German degrees of hardness. One degree is defined as 10 milligrams of calcium oxide per liter of water.

[0029] The term “stain removing enzyme” as used herein, describes an enzyme that aids the removal of a stain or soil from a fabric or a hard surface. Stain removing enzymes act on specific substrates, e.g., protease on protein, amylase on starch, lipase and cutinase on lipids (fats and oils), pectinase on pectin; mannanases on mannan/processed foods and hemicellulases on hemicellulose. Stains are often depositions of complex mixtures of different components which either results in a local discoloration of the material by itself or which leaves a sticky surface on the object which may attract soils dissolved in the washing liquor thereby resulting in discoloration of the stained area. When an enzyme acts on its specific substrate present in a stain the enzyme degrades or partially degrades its substrate thereby aiding the removal of soils and stain components associated with the substrate

during the washing process. For example, when a protease acts on a blood stain it degrades the protein components in the blood.

[0030] The term “reduced amount” means in this context that the amount of the component is smaller than the amount which would be used in a reference process under otherwise the same conditions.

DETAILED DESCRIPTION OF THE INVENTION

[0031] The invention relates to a method of modifying enzymes in order to optimize their binding to a suitable inhibitor. Enzymes have long been applied in cleaning compositions. However, a generally encountered problem using proteases with or without other enzymes in particular liquid detergent compositions, is that its needed to stabilize the protease in order to minimize loss due to proteolysis or auto-proteolysis during storage in the detergent in order not to get reduced enzyme wash performance when finally washed. This may lead to reduced stability of the proteases and other enzymes in the composition and an overall reduced performance of the enzymes in the cleaning compositions. The storage stability of enzymes in cleaning compositions may be improved by adding protease inhibitors or stabilizers. The interaction between an enzyme and an inhibitor suitable for the enzyme is preferably reversible thus the effect of the inhibitor is reversed by removing the inhibitor. The inhibitor constant, K_i , is an indication of the potency of an inhibitor to a specific protease and it is the inhibitor concentration required to decrease the maximal rate of the reaction to half of the uninhibited value. The K_i is a measure of the effectiveness of the inhibition of a specific function, preferably the effectiveness of inhibition or stabilizing of a protease. The present invention relates to a method of producing enzyme variants, in particular protease variants which are improved compared to the parent enzyme or proteases by being more efficiently inhibited or stabilized by a specific inhibitor than the corresponding parent protease. Therefore, K_i is lower for the enzyme or protease variants than K_i for the corresponding parent. With a lower K_i , either higher stability (more inhibition during storage) or less inhibitor (more economically feasible) is needed to obtain the same degree of inhibition. Preferably, the inhibitor is a reversible inhibitor of enzyme/protease activity, e.g., serine protease activity and the dilution into the wash liqueur leads to dissociation. It is advantageous to stabilize proteases by protease inhibitors, preferably, by a reversible protease inhibitor. Preferred inhibitors include peptide aldehydes or hydrosulfite derivatives inhibitors as described in WO 2009/118375; and WO13004636 and WO11036153 (Novozymes NS), peptide aldehyde inhibitors as described in WO2007/141736, WO2007/145963 and WO2007/145964 (Procter and Gamble Co.) and boric acid and boronic acids inhibitors as described e.g. in WO1996041859 (Novozymes A/S).

[0032] As mentioned the interaction between an enzyme such as a protease with its inhibitor is specific and depends on the inhibitor but also of the enzyme e.g. the protease. The inhibitors described above are designed to fit to specific enzymes to obtained optimal inhibition. However, an optimal inhibitor interaction also depend on the structure of the enzyme as such e.g. the protease. Preferably, the enzymes to be inhibited are proteases in particular serine proteases, preferably subtilases which may be of the subtilisin-type.

Many commercially available proteases are performance optimized by introducing specific mutations into the back bone. The mutations may influence the way the protease interacts with the inhibitor. Preferably, the protease variant produced by the method of the invention has improved binding to a specific inhibitor compared to the binding of the protease parent to the same inhibitor. Even more preferably the protease is a protease which has improved binding to a specific inhibitor compared to the binding of the protease parent to the same inhibitor and has improved or retained cleaning performance such as wash performance in laundry compared to the parent protease. Several commercially available detergent proteases have been developed and many of these are formulated in cleaning compositions with various inhibitors. To obtain an optimal cleaning composition with good cleaning properties, also after storage, the proteases comprised in the compositions usually needs to be stabilized by an inhibitor.

[0033] One embodiment of the invention is providing a method for modifying enzymes such as proteases for optimal binding to an inhibitor. There are numerous ways of modifying a protease one way of modifying a protease is by protein engineering i.e. introducing alterations in the primary sequence. The method of the invention describes how the enzymes such as protease could be modified to increase the binding to an inhibitor. Thus one embodiment of the invention relates to a method for improve binding of an enzyme to an inhibitor of the enzyme, comprising the steps of

[0034] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the enzyme,

[0035] b) modify the enzyme by modifying the amino acid residues identified in step a)

[0036] One embodiment of the invention relates to a method for improve binding of an enzyme to an inhibitor of the enzyme, comprising the steps of

[0037] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the enzyme,

[0038] b) modify the enzyme by modifying the amino acid residues identified in step a)

[0039] c) optionally determine the binding constant K_i

[0040] d) select modified enzyme generated in step b) optionally with improvement factor >1.0

[0041] Calculation of Improvement Factor (IF)

[0042] Having the K_i for each enzyme, it is possible to calculate the improvement factor e.g. the effect of a single mutation, as described in Materials and Methods. The relative K_i for the mutation (Improvement Factor, or IF) is calculated:

$$\text{Improvement Factor (IF)} = \frac{K_{i_{\text{enzyme without mutation}}}}{K_{i_{\text{enzyme variant with mutation}}}}$$

[0043] A lower K_i is desirable, as a satisfactory inhibition can be obtained at a lower inhibitor concentration, so beneficial mutations have an IF >1.0.

[0044] Preferably the improvement factor is above 1.0 and below 50, preferably above 2.0 and below 40, preferably above 2.0 and below 30, preferably above 5.0 and below 20, preferably above 5.0 and below 10. Preferably the improvement factor is above 1.0, preferably above 2.0, preferably above 5.0, preferably above 10.0, preferably above 15.0, preferably above 20.

[0045] In one embodiment the enzyme is a protease, preferred proteases includes those of bacterial, fungal, plant, viral or animal origin e.g. vegetable or microbial origin, preferably microbial origin. In one aspect of the invention the enzyme is an alkaline protease, preferably a serine protease and preferably of the S1 family, such as trypsin, or the S8 family such as subtilisin. In a preferred aspect the protease is a serine protease such as a subtilase and/or a subtilisin. The term "subtilases" refers to a sub-group of serine protease according to Siezen et al., Protein Engng. 4 (1991) 719-737 and Siezen et al. Protein Science 6 (1997) 501-523. Examples of subtilases are those derived from *Bacillus* such as *Bacillus lentus*, *B. alkalophilus*, *B. subtilis*, *B. amyloliquefaciens*, *Bacillus pumilus* and *Bacillus gibsonii* described in; U.S. Pat. No. 7,262,042 and WO99/021867, and subtilisin *lentos*, subtilisin Novo, subtilisin Carlsberg, *Bacillus licheniformis*, subtilisin BPN', subtilisin 309, subtilisin 147 and subtilisin 168 described in WO89/06279 and protease PD138 described in (WO93/18140). Other useful proteases may be those described in WO92/175177, WO01/016285, WO02/026024 and WO02/016547. A further preferred protease is the alkaline protease from *Bacillus lentus* DSM 5483, as described for example in WO95/23221, and variants thereof which are described in WO92/21760, WO95/23221, EP1921147 and EP1921148. In a preferred embodiment the protease is a protease having an amino acid sequence which has at least 60%, at least 62%, at least 63%, at least 64%, at least 65%, at least 70%, at least 72%, at least 73%, at least 74%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to a polypeptide having the amino acid sequence of SEQ ID NO 1. In a preferred embodiment the protease is a protease having an amino acid sequence which has at least 60%, at least 62%, at least 63%, at least 64%, at least 65%, at least 70%, at least 72%, at least 73%, at least 74%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to a polypeptide having the amino acid sequence of SEQ ID NO 2. In a preferred embodiment the protease is a protease having an amino acid sequence which has at least 60%, at least 62%, at least 63%, at least 64%, at least 65%, at least 70%, at least 72%, at least 73%, at least 74%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to a polypeptide having the amino acid sequence of SEQ ID NO 3.

[0046] In a preferred embodiment the protease is a protease having an amino acid sequence which has at least 60%, at least 62%, at least 63%, at least 64%, at least 65%, at least 70%, at least 72%, at least 73%, at least 74%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to a polypeptide having the amino acid sequence of SEQ ID NO 4.

[0047] In a preferred embodiment the protease is a protease having an amino acid sequence which has at least 60%,

at least 62%, at least 63%, at least 64%, at least 65%, at least 70%, at least 72%, at least 73%, at least 74%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to a polypeptide having the amino acid sequence of SEQ ID NO 5.

[0048] In a preferred embodiment the protease is a protease having an amino acid sequence which has at least 60%, at least 62%, at least 63%, at least 64%, at least 65%, at least 70%, at least 72%, at least 73%, at least 74%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to a polypeptide having the amino acid sequence of SEQ ID NO 6.

[0049] The invention further relates to a method of generating modified protease having improved binding to an inhibitor. In one embodiment the amino acid identified in a) binds the inhibitor through hydrogen binding or e.g. through hydrophobic attraction, which allows modifying the binding pockets to better accommodate the inhibitor and/or modifying side chains filling up the binding pockets.

[0050] In one aspect of the invention the protease to be modified by the method of the invention is a protease which is optimized for a specific purpose such as wash performance or stability. Thus the protease may be a variant (i.e. comprising one or more alterations) of protease e.g. any of the proteases listed above. In one aspect the protease to be modified according to the method of the invention is a protease having a polypeptide sequence which has at least 60% sequence identity to SEQ ID NO 1, 2, 3, 4, 5 or 6. In one aspect the protease to be modified according to the method of the invention is a protease comprising one or more of the following mutations compared to SEQ ID NO 1: S9E, S9R, A15T, S24G, S24R, K27R, N42R, G52S, S55P, T56P, G59D, G59E, N60D, N60E, V66A, N74D, S76N, S85N, S85R, S97A, S97E, S97D, S99E, S99D, S99G, S99N, S99H, S99M, S101A, V102I, V102N, G116V, G116R, S126A, S126L, S154D, A156E, G157S, G157D, G157P, S158E, Y161A, R164S, Q176E, N179E, S182E, Q185N, A188P, N198D, V199I, Q200L, Y203W, S206G, S210V, L211D, L211Q, L211E, N212D, N212E, N212S, M216S, M216A, N232H, Q239R, N242D, S250D, S253D, N255W, N255D, N255E, S256E, S256D or R269H, wherein the protease has at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 1. In another aspect of the invention the protease to be modified in the method according to the invention comprises the following mutations compared to SEQ ID NO 1: Y161A+R164S+A188P, S97AD, S97SE, V66A+S104A, S9R+A15T+V66A+N212D+Q239R, S9R+A15T+G59E+V66A+A188P+V199I+Q239R+N255D, S9E+N42R+N74D, N74D+Q200L, N74D+N198D, Q200L+Y203W, S85N+S99G+V102N, K27R+V102Y+N115S+T268A, N74D+S101A+V102I, S99G+S101A+V102I+G157D+A226V+Q230H+Q239R+N242D+N246K, S99G+S101A+V102I+G157D+A226V+Q230H+Q239R+N242D+N246K, S24G+S52G+S76N+S99N+G126A+Y211Q, S24G+S52G+S76N+S99N+G126S+Y211Q,

S85N+G116V+S126L+P127Q+S129A, S85N+G116V+S99M+S126L+P127Q+S129A, N74D+S85R+G116R+S126L+P127Q+S129A, S97D+S99R+S101A+V102I+G157S, S3T+V4I+S97D+S99R+S101A+V102I+G157S+A188P+V193M+V199I+L211D, S3T+V4I+S97D+S99R+S101A+V102I+G157S+V193M+V205I+L217D, S3T+V4I+S97D+S99R+S101A+V102I+G157S+V199I, S97D+S99E+S101A+V102I+G157S, S3T+V4I+S97D+S99E+S101A+V102I+G157S+V199I, S9E+N42R+N74D+H118V+Q176E+A188P+V199I+Q200L+Y203W+S250D+S253D+N255W+L256E, S9E+N42R+N74D+A188P+V199I+Q200L+Y203W+S253D+N255W+L256E, S9E+N42R+N74D+Q176E+A188P+V199I+Q200L+Y203W+S250D+S253D+N255W+L256E, S9E+N42R+N74D+H118V+Q176E+A188P+V199I+Q200L+Y203W+S250D+N255W+L256E+*269a H+*269bH, or S3V+N74D+H118V+Q176E+N179E+S182E+V199I+Q200L+Y203W+S210V+S250D+N255W+L256E, wherein the protease has at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 1.

[0051] In one aspect the protease to be modified according to the method of the invention is a protease comprising one or more of the following mutations compared to SEQ ID NO 4: S9E, S9R, A15T, S24G, S24R, K27R, N42R, G52S, S55P, T56P, G59D, G59E, N60D, N60E, V66A, N74D, S76N, S85N, S85R, S97A, S97E, S99E, S99D, S99G, S99N, S99H, S99M, V102N, G116V, G116R, S126A, S126L, S154D, A156E, G157D, G157P, S158E, Y161A, R164S, Q176E, N179E, S182E, Q185N, A188P, N198D, V199I, Q200L, Y203W, S206G, S210V, L211D, L211Q, L211E, N212D, N212E, N212S, M216S, M216A, N232H, Q239R, N242D, S250D, S253D, N255W, N255D, N255E, S256E, S256D or R269H, wherein the protease has at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 4. In another aspect of the invention the protease to be modified in the method according to the invention comprises the following mutations compared to SEQ ID NO 4: S3T+V4I, S3T+V4I+A188P+V193M+V199I+L211D, S3T+V4I+V193M+V199I+L211D, S3T+V4I+V193M+V199I+L211D, S3T+V4I+S99E+V199I, S99E, wherein the protease has at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 4.

[0052] In one aspect of the invention the protease to be modified in the method according to the invention comprises one or more of the following mutations compared to SEQ ID NO 2: S9E, S9R, A15T, S24G, S24R, K27R, K43R, S53G, F58P, N61D, N61E, N62D, N62E, V68A, N76D, S78N, S87N, S87R, D99A, D99E, S101E, S101D, S101G, S101N, S101H, S101M, Q103A, Y104I, Y104N, N118V, N118R, G128A, G128L, E156D, E156S, G160S, G160D, G160P, S161E, Y167A, K170S, S182E, Q185E, S188E, S191N, S204D, Q206L, L209W, N212G, A216V, Y217D, L217Q, L217E, N218D, N218E, N218S, M222S, M222A, Q245R, S248D, K256D, F261W, F261D, F261E, Y262E, Y262D or Q275R. In another aspect of the invention the protease comprises one or more following mutations compared to

SEQ ID NO 2: S9E+K43R+N76D, N76D+Q206L, N76D+S204D, Q206L+L209W, S87N+S101G+Y104N, K27R+N117S, N76D+Q103A+Y104I, S101G+Q103A+Y104I+G160D+A232V+S236H+Q245R+S248D+N252K, S101G+Q103A+Y104I+G160D+A232V+S236H+Q239R+S248D+N252K, S24G+S53G+S78N+S101N+G128A+Y217Q, S24G+S53G+S78N+S101N+G128S+Y217Q, S87N+G118V+G128L+P129Q+S130A, S87N+G118V+S101M+G128L+P129Q+S130A, N76D+S87R+G118R+G128L+P129Q+S130A, S101R+Q103A+Y104I+G160S, S3T+V4I+S101R+Q103A+Y104I+G160S, Y217L, Y217D, Y217E or S101E wherein the protease has at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 2.

[0053] In one aspect the protease to be modified according to the method of the invention is a protease which comprise a substitution at one or more positions corresponding to positions 171, 173, 175, 179, or 180 of SEQ ID NO: 3. In one aspect the protease to be modified according to the method of the invention is a protease comprise one or more of the following mutations compared to SEQ ID NO 3, S173P, S175P, F180Y, S175P+F180Y, S173P+F180Y, S173P+S175P, S173P+S175P+F180Y, wherein the protease has at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 3.

[0054] In one aspect of the invention the protease to be modified is a commercially available protease. Examples of suitable commercially available protease include those sold under the trade names Alcalase®, Duralase™, Durazym™, Relase®, Savinase®, Primase®, Polarzyme®, Kannase®, Liquanase®, Blaze®, Ovozyme®, Coronase®, Neutrase®, Everlase® and Esperase® (Novozymes NS), those sold under the tradename Maxatase®, Maxacal®, Maxapem®, Purafect®, Purafect Prime®, Purafect MA®, Purafect Ox®, Purafect OxP®, Puramax®, Purafast®, Properase®, FN2®, FN3®, FN4®, Excellase®, Eraser®, Ultimase®, Opticlean®, Optimase®, ExcellenzP®, Preferenz® and Effectenz® (Danisco/DuPont), Axapem™ (Gist-Brocades N.V.), BLAP (sequence shown in FIG. 29 of U.S. Pat. No. 5,352, 604) and variants hereof (Henkel AG) and KAP (*Bacillus alkalophilus subtilisin*) from Kao.

[0055] The invention relates to a method for improve binding of a protease to an inhibitor of the protease, comprising the steps of:

- [0056]** a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,
- [0057]** b) modify the protease by modifying the amino acid residues identified in step a)
- [0058]** c) optionally determine the binding constant K_i
- [0059]** d) select modified protease generated in step b) which has improved binding to the protease inhibitor.

[0060] In step a) amino acid residues exposed to the inhibitor when the inhibitor binds to the protease are identified. To identify the amino acids binding the inhibitor the three dimensional structure of the protease may be consulted.

Protease TY145 (SEQ ID NO 3)

[0061] In one embodiment the amino acids binding the inhibitor is determined in the protease TY145, having the polypeptide sequence shown in SEQ ID NO 3.

[0062] The structure of TY145 (SEQ ID NO: 3) in complex with the CI2 inhibitor is disclosed in WO2004067737A2 (Novozymes NS). CI2 is Chymotrypsin Inhibitor 2 from Barley, which is known to inhibit chymotrypsin and subtilisin family proteases (Svendsen, I., Jonassen, I., Hejgaard, J., & Boisen, S. (1980) Carlsberg Res. Commun. 45, 389-395).

[0063] From this structure, where TY145 (SEQ ID NO:3) is chain A and the CI2 inhibitor is chain B, the CI2 inhibitor residues occupying the binding sub-sites S1-S4 in TY145 (SEQ ID NO 3) can be identified as:

[0064] P4—Chain B, residue Ile 353

[0065] P3—Chain B, residue Val 354

[0066] P2—Chain B, residue Thr 355

[0067] P1—Chain B, residue Met 356

An initial selection of residues to test for altered inhibitor binding is identified by selecting all residues in chain A having their alpha-carbon within 16 angstroms of the alpha-carbon of Ile 353 of chain B. From this set, all residues having their alpha-carbon within 10 angstroms of Met 356 are removed, leaving residues with alpha carbon closer than 16 Å to Ile 353 but at least 10 Å away from Met 356. The residues selected in this way are: 33, 34, 35, 36, 37, 54, 55, 56, 57, 70, 73, 103, 104, 105, 106, 107, 108, 109, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 138, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 177, 178, 180, 181, 182, 183 and 184.

[0068] In one embodiment of the invention the protease selected in step d) is a protease having at least 60% sequence identity to SEQ ID NO 3; and the amino acid residues identified in step a) is selected from the group consisting of 33, 34, 35, 36, 37, 54, 55, 56, 57, 70, 73, 103, 104, 105, 106, 107, 108, 109, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 138, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 177, 178, 180, 181, 182, 183 and 184. One embodiment relates to a method for improve binding of a protease comprising the amino acid sequence of SEQ ID NO 3 or a polypeptide having at least 60% sequence identity hereto to an inhibitor of the protease, comprising the steps of:

[0069] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0070] b) modify the protease by modifying the amino acid residues identified in step a),

wherein the amino acids identified are selected from the group consisting of 33, 34, 35, 36, 37, 54, 55, 56, 57, 70, 73, 103, 104, 105, 106, 107, 108, 109, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 138, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 177, 178, 180, 181, 182, 183 and 184 corresponding to the positions of SEQ ID NO 3 and/or c) determine the binding constant K_i and/or d) selecting the modified protease generated in step b) with improved inhibitor binding.

[0071] Example 5 shows variants of SEQ ID NO 3 generated by the method according to the invention, which have improved inhibitor binding. Using the above method it will be possible to identify specific mutations which improve the inhibitor binding of the targeted protease. One embodiment relates to a method for improving binding of a protease comprising the amino acid sequence of SEQ ID NO 3 or a

polypeptide having at least 60% sequence identity hereto to an inhibitor of the protease, comprising the steps of:

[0072] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0073] b) modify the protease by modifying the amino acid residues identified in step a),

wherein the modifications are selected from the group consisting of: Q70F, Q70A, Q70N, Q70H, Q70Y, S111R, S111E, S111D, S111I, S114A, S114Q, S114V, S114F, S144R, A145E, K146T, K146S, K146W, K146N, K146F, K146A, K146R, I150N, I150S, I150I, I150T, I150A, I150V, N176 I178Y, I178F, I178P, G182A, G182S, L184F, L184Y, L184W, L184D and L184H.

Protease P (SEQ ID NO 5)

[0074] The structure of the protease P with the amino acid sequence shown in SEQ ID NO 5 may be obtained by standard X-ray crystallographic methods or by standard homology modelling known in the art, see e.g. Emilio Xavier Esposito, Dror Tobi and Jeffry D. Madura: Comparative Protein Modeling, in Reviews in Computational Chemistry, Vol. 22, edited by Kenny B. Lipkowitz, Thomas R. Cundari, and Valerie J. Gillet. 2006 Wiley-VCH, John Wiley & Sons, Inc. A particular implementation of a homology modelling tool is described in Z. Xiang, 2006, Curr. Protein Pept. Sci. 7(3): 217-227.

[0075] The structure of the protease with SEQ ID NO 5 can be aligned to the TY145 structure (SEQ ID NO 3) of the TY145/CI2 inhibitor complex described above using standard superposition methods, see eg. Marc A. Marti-Renom, Emidio Capriotti, Ilya N. Shindyalov, and Philip E. Bourne, in Structural Bioinformatics, 2nd. Ed, by Jenny Gu and Philip E. Bourne, (2009) John Wiley & Sons, Inc. A particular implementation of molecular superposition is found in The PyMOL Molecular Graphics System, Version 1.6, Schrödinger, LLC. In this way, a model of the Protease P/CI2 inhibitor complex is obtained and used for selection of Protease P residues to test for altered inhibitor binding. The residues are identified by selecting all residues in Protease P having their alpha-carbon within 16 angstroms of the alpha-carbon of Ile353 of the CI2 inhibitor. From this set, all residues having their alpha-carbon within 10 angstroms of Met356 are removed, leaving residues with alpha carbon closer than 16 Å to Ile353 but at least 10Å away from Met356. The residues selected in this way are: 31, 32, 33, 34, 35, 54, 55, 56, 64, 67, 92, 93, 94, 95, 96, 97, 98, 100, 101, 102, 103, 103, 104, 105, 106, 107, 108, 109, 110, 122, 128, 129, 130, 131, 132, 133, 134, 135, 137, 138, 149, 160, 160, 161, 161, 162, 163, 164, 165 and 166. In one embodiment of the invention the protease selected in step d) is a protease having at least 60% sequence identity to SEQ ID NO 5; and the amino acid residues identified in step a) is selected from the group consisting of: 31, 32, 33, 34, 35, 54, 55, 56, 64, 67, 92, 93, 94, 95, 96, 97, 98, 100, 101, 102, 103, 103, 104, 105, 106, 107, 108, 109, 110, 122, 128, 129, 130, 131, 132, 133, 134, 135, 137, 138, 149, 160, 160, 161, 161, 162, 163, 164, 165 and 166. One embodiment relates to a method for improve binding of a protease comprising the amino acid sequence of SEQ ID NO 5 or a polypeptide having at least 60% sequence identity hereto to an inhibitor of the protease, comprising the steps of:

[0076] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0077] b) modify the protease by modifying the amino acid residues identified in step a),

wherein the amino acids identified are selected from the group consisting of 31, 32, 33, 34, 35, 54, 55, 56, 64, 67, 92, 93, 94, 95, 96, 97, 98, 100, 101, 102, 103, 103, 104, 105, 106, 107, 108, 109, 110, 122, 128, 129, 130, 131, 132, 133, 134, 135, 137, 138, 149, 160, 160, 161, 161, 162, 163, 164, 165 and 166 corresponding to the positions of SEQ ID NO 5 and/or c) determine the binding constant K_i and/or d) selecting the modified protease generated in step b) with improved inhibitor binding.

[0078] In example 6 is shown variants of SEQ ID NO 5 generated by the method according to the invention which have improved inhibitor binding. Using the above method it will be possible to identify specific mutations which improve the inhibitor binding of the targeted protease. One embodiment relates to a method for improve binding of a protease comprising the amino acid sequence of SEQ ID NO 5 or a polypeptide having at least 60% sequence identity hereto to an inhibitor of the protease, comprising the steps of:

[0079] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0080] b) modify the protease by modifying the amino acid residues identified in step a),

wherein the modifications are selected from the group consisting of: Q64N, Q64F, M103S, M103A, G128P and G127S+G128P+G129S+Y130P+D131S+M134L.

Protease C (SEQ ID NO 6)

[0081] The structure of the protease with the amino acid sequence shown in SEQ ID NO 6 may be obtained by standard X-ray crystallographic methods or by standard homology modelling known in the art, see e.g. Emilio Xavier Esposito, Dror Tobi and Jeffry D. Madura: Comparative Protein Modeling, in Reviews in Computational Chemistry, Vol. 22, edited by Kenny B. Lipkowitz, Thomas R. Cundari, and Valerie J. Gillet. 2006 Wiley-VCH, John Wiley & Sons, Inc. A particular implementation of a homology modelling tool is described in Z. Xiang, 2006, Curr. Protein Pept. Sci. 7(3): 217-227.

[0082] The structure of the protease with SEQ ID NO 6 can be aligned to the TY145 structure (SEQ ID NO 3) of the TY145/CI2 inhibitor complex described above using standard superposition methods, see e.g. Marc A. Marti-Renom, Emidio Capriotti, Ilya N. Shindyalov, and Philip E. Bourne, in Structural Bioinformatics, 2nd. Ed, by Jenny Gu and Philip E. Bourne, (2009) John Wiley & Sons, Inc. A particular implementation of molecular superposition is found in The PyMOL Molecular Graphics System, Version 1.6, Schrödinger, LLC. In this way, a model of the SEQ ID NO 6/CI2 inhibitor complex is obtained and used for selection of residues in the protease with the amino acid sequence shown in SEQ ID NO 6 to test for altered inhibitor binding. The residues are identified by selecting all residues in the protease with SEQ ID NO 6 having their alpha-carbon within 16 angstroms of the alpha-carbon of Ile353 of the inhibitor. From this set, all residues having their alpha-carbon within 10 angstroms of Met356 are removed, leaving residues with alpha carbon closer than 16 Å to Ile353 but at least 10Å away from Met356. The residues selected in this way are: 30, 31, 32, 33, 34, 48, 49, 50, 51, 60, 63, 91, 92, 93, 94, 95, 96, 97, 98, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110,

122, 128, 129, 130, 131, 132, 133, 134, 135, 137, 138, 149, 160, 162, 163, 164, 165, 166 and 169.

[0083] In one embodiment of the invention the protease by selected in step d) is a protease having at least 60% sequence identity to SEQ ID NO 1 and comprise, compared to SEQ ID NO 1, one insertion of the amino acid Glutamic acid (Glu) in a position corresponding to position 97 of SEQ ID

[0084] NO 6, in short S97SE; and the amino acid residues identified in step a) is selected from the group consisting of 30, 31, 32, 33, 34, 48, 49, 50, 51, 60, 63, 91, 92, 93, 94, 95, 96, 97, 98, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 122, 128, 129, 130, 131, 132, 133, 134, 135, 137, 138, 149, 160, 162, 163, 164, 165, 166 and 169 and 166. One embodiment relates to a method for improve binding of a protease comprising the amino acid sequence of SEQ ID NO 6 or a polypeptide having at least 60% sequence identity hereto to an inhibitor of the protease, comprising the steps of:

[0085] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0086] b) modify the protease by modifying the amino acid residues identified in step a),

wherein the amino acids identified are selected from the group consisting of 30, 31, 32, 33, 34, 48, 49, 50, 51, 60, 63, 91, 92, 93, 94, 95, 96, 97, 98, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 122, 128, 129, 130, 131, 132, 133, 134, 135, 137, 138, 149, 160, 162, 163, 164, 165, 166 and 169 and 166 corresponding to the positions of SEQ ID NO 6 and/or c) determine the binding constant K_i and/or d) selecting the modified protease generated in step b) with improved inhibitor binding.

[0087] Example 7 shows variants of SEQ ID NO 6 generated by the method according to the invention, which have improved inhibitor binding. Using the above method it will be possible to identify specific mutations which improve the inhibitor binding of the targeted protease. One embodiment relates to a method for improve binding of a protease comprising the amino acid sequence of SEQ ID NO 6 or a polypeptide comprising, compared to SEQ ID NO 1, one insertion of the amino acid Glutamic acid (Glu) in a position corresponding to position 97 of SEQ ID NO 1, in short S97SE and having at least 60% sequence identity to SEQ ID NO 6, to an inhibitor of the protease, comprising the steps of:

[0088] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0089] b) modify the protease by modifying the amino acid residues identified in step a), wherein the modifications are selected from the group consisting of: N60A, N60F, N60H, S100I, V103A, L134V, A164S, Y166F, N60H+S100I, N60F+S100I, S100I+L134V and S100I+Y166F.

Savinase (SEQ ID NO 1)

[0090] The structure of Savinase is known from several published x-ray crystallography structures, e.g. C. Betzel, S. Klupsch, G. Papendorf, S. Hastrup, S. Branner, K. S. Wilson, 1992, J. Mol. Biol. (223), p 427. The structure of Savinase can be aligned to the TY-145 (SEQ ID NO 3) structure of the TY145/CI2 inhibitor complex described above using standard superposition methods, see e.g. Marc A. Marti-Renom, Emidio Capriotti, Ilya N. Shindyalov, and Philip E. Bourne, in Structural Bioinformatics, 2nd. Ed. by Jenny Gu and Philip E. Bourne, (2009) John Wiley & Sons,

Inc. A particular implementation of molecular superposition is found in The PyMOL Molecular Graphics System, Version 1.6, Schrödinger, LLC. In this way, a model of the Savinase/CI2 inhibitor complex is obtained and used for selection of Savinase residues to test for altered inhibitor binding. The residues are identified by selecting all residues in Savinase having their alpha-carbon within 16 angstroms of the alpha-carbon of Ile353 of the CI2 inhibitor. From this set, all residues having their alpha-carbon within 10 angstroms of Met356 are removed, leaving residues with alpha carbon closer than 16 Å to Ile353 but at least 10Å away from Met356. The residues selected in this way are: 30, 31, 32, 33, 34, 48, 49, 50, 51, 60, 63, 91, 92, 93, 94, 95, 96, 97, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 121, 127, 128, 129, 130, 131, 132, 133, 134, 136, 165 and 168. In one embodiment of the invention the protease selected in step d) is a protease having at least 60% sequence identity to SEQ ID NO 1; and the amino acid residues identified in step a) is selected from the group consisting of: 30, 31, 32, 33, 34, 48, 49, 50, 51, 60, 63, 91, 92, 93, 94, 95, 96, 97, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 121, 127, 128, 129, 130, 131, 132, 133, 134, 136, 137, 148, 159, 161, 162, 163, 164, 165 and 168. One embodiment relates to a method for improve binding of a protease comprising the amino acid sequence of SEQ ID NO 1 or a polypeptide having at least 60% sequence identity hereto to an inhibitor of the protease, comprising the steps of:

[0091] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0092] b) modify the protease by modifying the amino acid residues identified in step a),

wherein the amino acids identified are selected from the group consisting of 30, 31, 32, 33, 34, 48, 49, 50, 51, 60, 63, 91, 92, 93, 94, 95, 96, 97, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 121, 127, 128, 129, 130, 131, 132, 133, 134, 136, 137, 148, 159, 161, 162, 163, 164, 165 and 168 corresponding to the positions of SEQ ID NO 1 and/or c) determine the binding constant K_i and/or d) selecting the modified protease generated in step b) with improved inhibitor binding.

[0093] BPN' (SEQ ID NO 2)

[0094] The structure of BPN' is known from several published x-ray crystallography structures, e.g. R. Bott, M. Ullsch, A. Kossiakoff, T. Graycar, B. Katz, S. Power (1988) J. Biol. Chem. (263), p. 7895. The structure of BPN' can be aligned to the TY145 structure of the TY145/CI2 inhibitor complex described above using standard superposition methods, see eg. Marc A. Marti-Renom, Emidio Capriotti, Ilya N. Shindyalov, and Philip E. Bourne, in Structural Bioinformatics, 2nd. Ed. by Jenny Gu and Philip E. Bourne, (2009) John Wiley & Sons, Inc. A particular implementation of molecular superposition is found in The PyMOL Molecular Graphics System, Version 1.6, Schrödinger, LLC. In this way, a model of the BPN'/inhibitor complex is obtained and used for selection of BPN' residues to test for altered inhibitor binding. The residues are identified by selecting all residues in BPN' having their alpha-carbon within 16 angstroms of the alpha-carbon of Ile353 of the CI2 inhibitor. From this set, all residues having their alpha-carbon within 10 angstroms of Met356 are removed, leaving residues with alpha carbon closer than 16 Å to Ile353 but at least 10Å away from Met356. The residues selected in this way are: 30, 31, 32, 33, 34, 49, 50, 51, 52, 62, 65, 93, 94, 95, 96, 97,

98, 99, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 123, 129, 130, 131, 132, 133, 134, 135, 136, 138, 139, 150, 164, 165, 167, 168, 169, 170 and 171. In one embodiment of the invention the protease selected in step d) is a protease having at least 60% sequence identity to SEQ ID NO 2; and the amino acid residues identified in step a) is selected from the group consisting of: 30, 31, 32, 33, 34, 49, 50, 51, 52, 62, 65, 93, 94, 95, 96, 97, 98, 99, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 123, 129, 130, 131, 132, 133, 134, 135, 136, 138, 139, 150, 164, 165, 167, 168, 169, 170. One embodiment relates to a method for improve binding of a protease comprising the amino acid sequence of SEQ ID NO 2 or a polypeptide having at least 60% sequence identity hereto to an inhibitor of the protease, comprising the steps of:

[0095] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0096] b) modify the protease by modifying the amino acid residues identified in step a),

wherein the amino acids identified are selected from the group consisting of 30, 31, 32, 33, 34, 49, 50, 51, 52, 62, 65, 93, 94, 95, 96, 97, 98, 99, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 123, 129, 130, 131, 132, 133, 134, 135, 136, 138, 139, 150, 164, 165, 167, 168, 169, 170 corresponding to the positions of SEQ ID NO 2 and/or c) determine the binding constant K_i and/or d) selecting the modified protease generated in step b) with improved inhibitor binding.

Bacillus Lentus protease BLAP (SEQ ID NO 4)

[0097] The structure of BLAP is known from several published x-ray crystallography structures, e.g. D. W. Goddette, C. Paech, S. S. Yang, J. R. Mielenz, C. Bystroff, M. E. Wilke, R. J. Fletterick (1992), J. Mol. Biol. (228), p 580. The structure of BLAP can be aligned to the TY145 (SEQ ID NO 3) structure of the TY145/inhibitor complex described above using standard superposition methods, see eg. Marc A. Marti-Renom, Emidio Capriotti, Ilya N. Shindyalov, and Philip E. Bourne, in Structural Bioinformatics, 2nd. Ed, by Jenny Gu and Philip E. Bourne, (2009) John Wiley & Sons, Inc. A particular implementation of molecular superposition is found in The PyMOL Molecular Graphics System, Version 1.6, Schrödinger, LLC. In this way, a model of the BLAP/CI2 inhibitor complex is obtained and used for selection of BLAP residues to test for altered inhibitor binding. The residues are identified by selecting all residues in BLAP having their alpha-carbon within 16 angstroms of the alpha-carbon of Ile353 of the CI2 inhibitor. From this set, all residues having their alpha-carbon within 10 angstroms of Met356 are removed, leaving residues with alpha carbon closer than 16 Å to Ile353 but at least 10Å away from Met356. The residues selected in this way are: 30, 31, 32, 33, 34, 48, 49, 50, 51, 60, 63, 91, 92, 93, 94, 95, 96, 97, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 121, 127, 128, 129, 130, 131, 132, 133, 134, 136, 137, 148, 159, 160, 161, 162, 163, 164 and 165. In one embodiment of the invention the protease selected in step d) is a protease having at least 60% sequence identity to SEQ ID NO 4; and the amino acid residues identified in step a) is selected from the group consisting of: 30, 31, 32, 33, 34, 48, 49, 50, 51, 60, 63, 91, 92, 93, 94, 95, 96, 97, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 121, 127, 128, 129, 130, 131, 132, 133, 134, 136, 137, 148, 159, 160, 161, 162, 163, 164 and 165. One embodiment relates to a method for improve binding of a protease comprising the amino acid sequence of SEQ ID NO

4 or a polypeptide having at least 60% sequence identity hereto to an inhibitor of the protease, comprising the steps of:

[0098] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0099] b) modify the protease by modifying the amino acid residues identified in step a),

wherein the amino acids identified are selected from the group consisting of 30, 31, 32, 33, 34, 48, 49, 50, 51, 60, 63, 91, 92, 93, 94, 95, 96, 97, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 121, 127, 128, 129, 130, 131, 132, 133, 134, 136, 137, 148, 159, 160, 161, 162, 163, 164 and 165 corresponding to the positions of SEQ ID NO 4 and/or c) determine the binding constant K_i and/or d) selecting the modified protease generated in step b) with improved inhibitor binding.

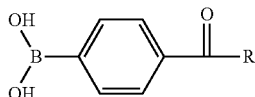
[0100] The protease variants obtained by the method of the invention are tested as described in examples 5 to 8. In one embodiment the modified enzymes e.g. protease variants generated by the method according to the invention have improved inhibitor binding compared to the non-modified enzyme e.g. the reference or parent protease. In one particular embodiment the protease variants generated by the method of the invention have improved inhibition by inhibitor compared to the parent protease or compared to a reference protease. In another particular embodiment the protease variants generated by the method of the present invention have increased inhibition by a protease inhibitor compared to the inhibition of a protease with SEQ ID NO 1, 2, 3, 4, 5 or 6 by the same inhibitor.

[0101] The protease variants obtained by a method according to the invention may be formulated into cleaning compositions such as detergent compositions comprising a protease variant produced by the method of the invention. In one embodiment the cleaning composition is a liquid or powder laundry detergent, suitable for e.g. washing at high temperature and/or pH, such as at or above 40° C. and/or at or above pH 8. In one embodiment the cleaning composition is a liquid or powder laundry detergent, suitable for e.g. washing at low temperature and/or pH, such as at or below 20° C. and/or pH 6. The detergent may also be formulated as a unit dose detergent and/or compact detergent optionally with minimum or no water. The detergent may also be a dish wash detergent which is preferably phosphate-free. The cleaning composition may further comprise at least one additional enzyme, such as carbohydrate-active enzymes like carbohydrase, pectinase, mannanase, amylase, cellulase, arabinase, galactanase, xylanase, or additional proteases such as metalloproteases, lipase, a, cutinase, oxidase, e.g., a laccase, and/or peroxidase.

[0102] Inhibitors

[0103] Several protease inhibitors have been described in the art and include protease inhibitors such as peptide aldehyde, hydrosulfite adducts of peptide aldehydes; boric acid, or a boronic acid; or a derivative of any of these. Preferably, the protease inhibitor is a (reversible) subtilisin protease inhibitor. In particular, the protease inhibitor may be a peptide aldehyde, boric acid, or a boronic acid; or a derivative of any of these.

[0104] The protease inhibitors may be a phenyl boronic acid, or a derivative thereof, of the following formula:



wherein R is selected from the group consisting of hydrogen, hydroxy, C1-C6 alkyl, substituted C1-C6 alkyl, C1-C6 alkenyl and substituted C1-C6 alkenyl. Preferably, R is hydrogen, CH₃, CH₃CH₂ or CH₃CH₂CH₂. Preferably the protease inhibitor (phenyl boronic acid derivative) is 4-formylphenyl-boronic acid (4-FPBA). The protease inhibitor is selected from the group consisting of:

[0105] thiophene-2 boronic acid, thiophene-3 boronic acid, acetamidophenyl boronic acid, benzofuran-2 boronic acid, naphthalene-1 boronic acid, naphthalene-2 boronic acid, 2-FPBA, 3-FBPA, 4-FPBA, 1-thianthrene boronic acid, 4-dibenzofuran boronic acid, 5-methylthiophene-2 boronic acid, thionaphthene boronic acid, furan-2 boronic acid, furan-3 boronic acid, 4,4 biphenyl-diboronic acid, 6-hydroxy-2-naphthalene, 4-(methylthio) phenyl boronic acid, 4 (trimethyl-silyl)phenyl boronic acid, 3-bromothiophene boronic acid, 4-methylthiophene boronic acid, 2-naphtyl boronic acid, 5-bromothiophene boronic acid, 5-chlorothiophene boronic acid, dimethylthiophene boronic acid, 2-bromophenyl boronic acid, 3-chlorophenyl boronic acid, 3-methoxy-2-thiophene, p-methyl-phenylethyl boronic acid, 2-thianthrene boronic acid, di-benzothiophene boronic acid, 4-carboxyphenyl boronic acid, 9-anthryl boronic acid, 3,5 dichlorophenyl boronic acid, diphenyl boronic acid, anhydride, o-chlorophenyl boronic acid, p-chlorophenyl boronic acid, m-bromophenyl boronic acid, p-bromophenyl boronic acid, p-fluorophenyl boronic acid, p-tolyl boronic acid, o-tolyl boronic acid, octyl boronic acid, 1,3,5 trimethylphenyl boronic acid, 3-chloro-4-fluorophenyl boronic acid, 3-aminophenyl boronic acid, 3,5-bis-(trifluoromethyl) phenyl boronic acid, 2,4 dichlorophenyl boronic acid, 4-methoxyphenyl boronic acid.

[0106] Further boronic acid derivatives suitable as protease inhibitors in the cleaning composition are described in U.S. Pat. No. 4,963,655, U.S. Pat. No. 5,159,060, WO 95/12655, WO 95/29223, WO 92/19707, WO 94/04653, WO 94/04654, U.S. Pat. No. 5,442,100, U.S. Pat. No. 5,488,157 and U.S. Pat. No. 5,472,628.

[0107] The protease inhibitor may also be a peptide aldehyde having the formula $X-B^1-B^0-H$, wherein the groups have the following meaning:

[0108] a) H is hydrogen;

[0109] b) B^0 is a single amino acid residue with L- or D-configuration and with the formula: $NH-CHR'-CO$;

[0110] c) B^1 is a single amino acid residue; and

[0111] d) X consists of one or more amino acid residues (preferably one or two), optionally comprising an N-terminal protection group.

[0112] $NH-CHR'-CO (B^0)$ is an L or D-amino acid residue, where R' may be an aliphatic or aromatic side chain, e.g., alkylaryl, such as benzyl, where R' may be optionally substituted. More particularly, the B^0 residue may be bulky, neutral, polar, hydrophobic and/or aromatic. Examples are

the D- or L-form of Tyr (p-tyrosine), m-tyrosine, 3,4-dihydroxyphenylalanine, Phe, Val, Met, norvaline (Nva), Leu, Ile or norleucine (Nle).

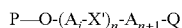
[0113] In the above formula, $X-B^1-B^0-H$, the B^1 residue may particularly be small, aliphatic, hydrophobic and/or neutral. Examples are alanine (Ala), cysteine (Cys), glycine (Gly), proline (Pro), serine (Ser), threonine (Thr), valine (Val), norvaline (Nva) and norleucine (Nle), particularly alanine, glycine, or valine.

X may in particular be one or two amino acid residues with an optional N-terminal protection group (i.e. the compound is a tri- or tetrapeptide aldehyde with or without a protection group). Thus, X may be B^2 , B^3-B^2 , $Z-B^2$, or $Z-B^3-B^2$ where B^3 and B^2 each represents one amino acid residue, and Z is an N-terminal protection group. The B^2 residue may in particular be small, aliphatic and/or neutral, e.g., Ala, Gly, Thr, Arg, Leu, Phe or Val. The B^3 residue may in particular be bulky, hydrophobic, neutral and/or aromatic, e.g., Phe, Tyr, Trp, Phenylglycine, Leu, Val, Nva, Nle or Ile. The N-terminal protection group Z (if present) may be selected from formyl, acetyl, benzoyl, trifluoroacetyl, fluoromethoxy carbonyl, methoxysuccinyl, aromatic and aliphatic urethane protecting groups, benzyloxycarbonyl (Cbz), t-butyloxycarbonyl, adamantyloxycarbonyl, p-methoxybenzyl carbonyl (MOZ), benzyl (Bn), p-methoxybenzyl (PMB) or p-methoxyphenyl (PMP), methoxycarbonyl (Moc); methoxyacetyl (Mac); methyl carbamate or a methylamino carbonyl/methyl urea group. In the case of a tetrapeptide aldehyde with a protection group (i.e. $X=Z-B^3-B^2$), Z is preferably a small aliphatic group, e.g., formyl, acetyl, fluoromethoxy carbonyl, t-butyloxycarbonyl, methoxycarbonyl (Moc); methoxyacetyl (Mac); methyl carbamate or a Methylamino carbonyl/methyl urea group. In the case of a tripeptide aldehyde with a protection group (i.e. $X=Z-B^2$), Z is preferably a bulky aromatic group such as benzoyl, benzyloxycarbonyl, p-methoxybenzyl carbonyl (MOZ), benzyl (Bn), p-methoxybenzyl (PMB) or p-methoxyphenyl (PMP).

[0114] In the case of a tripeptide aldehyde with a protection group (i.e. $X=Z-B^2$), Z is preferably a bulky aromatic group such as benzoyl, benzyloxycarbonyl, p-methoxybenzyl carbonyl (MOZ), benzyl (Bn), p-methoxybenzyl (PMB) or p-methoxyphenyl (PMP) or Z is preferably a small aliphatic group, e.g., formyl, acetyl, fluoromethoxy carbonyl, t-butyloxycarbonyl, methoxycarbonyl (Moc); methoxyacetyl (Mac); methyl carbamate or a Methylamino carbonyl/methyl urea group. In the case of a tetrapeptide aldehyde with a protection group (i.e. $X=Z-B^3-B^2$), Z is preferably a small aliphatic group, e.g., formyl, acetyl, fluoromethoxy carbonyl, t-butyloxycarbonyl, methoxycarbonyl (Moc); methoxyacetyl (Mac); methyl carbamate or a Methylamino carbonyl/methyl urea group.

[0115] Suitable peptide aldehydes are described in WO 94/04651, WO 95/25791, WO 98/13458, WO 98/13459, WO 98/13460, WO 98/13461, WO 98/13461, WO 98/13462, WO 2007/141736, 2007/145963, WO 2009/118375, WO 2010/055052 and WO 2011/036153. More particularly, the peptide aldehyde may be Cbz-RAY-H, Ac-GAY-H, Cbz-GAY-H, Cbz-GAL-H, Cbz-VAL-H, Cbz-GAF-H, Cbz-GAV-H, Cbz-GGY-H, Cbz-GGF-H, Cbz-RVY-H, Cbz-LVY-H, Ac-LGAY-H, Ac-FGAY-H, Ac-YGAY-H, Ac-FGAL-H, Ac-FGAF-H, Ac-FGVY-H, Ac-FGAM-H, Ac-WLVY-H, MeO-CO-VAL-H, MeNCO-VAL-H, MeO-CO-FGAL-H, MeO-CO-FGAF-H,

MeSO₂-FGAL-H, MeSO₂-VAL-H, PhCH₂O(OH)(O)P-VAL-H, EtSO₂-FGAL-H, PhCH₂SO₂-VAL-H, PhCH₂O(OH)(O)P-LAL-H, PhCH₂O(OH)(O)P-FAL-H, or MeO(OH)(O)P-LGAL-H. Here, Cbz is benzyloxycarbonyl, Me is methyl, Et is ethyl, Ac is acetyl, H is hydrogen, and the other letters represent amino acid residues denoted by standard single letter notation (e.g., F=Phe, Y=Tyr, L=Leu). Alternatively, the peptide aldehyde may have the formula as described in WO 2011/036153:



[0116] wherein Q is hydrogen, CH₃, CX''₃, CHX''₂, or CH₂X'', wherein X'' is a halogen atom;

[0117] wherein one X is the "double N-capping group" CO, CO—CO, CS, CS—CS or CS—CO, most preferred urido (CO), and the other X' are nothing,

[0118] wherein n=1-10, preferably 2-5, most preferably 2,

[0119] wherein each of A_i and A_{n+1} is an amino acid residue having the structure:

[0120] —NH—CR"—CO— for a residue to the right of X'=CO—, or

[0121] —CO—CR"—NH— for a residue to the left of X'=CO—

[0122] wherein R" is H— or an optionally substituted alkyl or alkylaryl group which may optionally include a hetero atom and may optionally be linked to the N atom, and wherein P is hydrogen or any C-terminal protection group.

[0123] Examples of such peptide aldehydes include α-MAPI, β-MAPI, F-urea-RVY-H, F-urea-GGY-H, F-urea-GAF-H, F-urea-GAY-H, F-urea-GAL-H, F-urea-GA-Nva-H, F-urea-GA-Nle-H, Y-urea-RVY-H, Y-urea-GAY-H, F—CS-RVF-H, F—CS-RVY-H, F—CS-GAY-H, Antipain, GE20372A, GE20372B, Chymostatin A, Chymostatin B, and Chymostatin C. Further examples of peptide aldehydes are disclosed in WO 2010/055052 and WO 2009/118375, WO 94/04651, WO 98/13459, WO 98/13461, WO 98/13462, WO 2007/145963, hereby incorporated by reference.

[0124] Alternatively to a peptide aldehyde, the protease inhibitor may be a hydrosulfite adduct having the formula X—B¹—NH—CHR'—CHOH—SO₃M, wherein X, B¹ and R' are defined as above, and M is H or an alkali metal, preferably Na or K. Peptide aldehyde hydrosulfite adducts are described in WO 2013/004636.

[0125] The peptide aldehyde may be converted into a water-soluble hydrosulfite adduct by reaction with sodium bisulfite, as described in textbooks, e.g., March, J. Advanced Organic Chemistry, fourth edition, Wiley-Interscience, US 1992, p 895.

[0126] An aqueous solution of the bisulfite adduct may be prepared by reacting the corresponding peptide aldehyde with an aqueous solution of sodium bisulfite (sodium hydrogen sulfite, NaHSO₃); potassium bisulfite (KHSO₃) by known methods, e.g., as described in WO 98/47523; U.S. Pat. No. 6,500,802; U.S. Pat. No. 5,436,229; J. Am. Chem. Soc. (1978) 100, 1228; Org. Synth., Coll. vol. 7: 361.

[0127] All the mentioned peptide aldehydes described here could be either a peptide aldehyde or a hydrosulfite adduct thereof.

[0128] The molar ratio of the above-mentioned peptide aldehydes (or hydrosulfite adducts) to the protease may be at least 1:1 or 1.5:1 and it may be less than 1000:1, more

preferred less than 500:1, even more preferred from 100:1 to 2:1 or from 20:1 to 2:1, or most preferred, the molar ratio is from 10:1 to 2:1.

[0129] Formate salts (e.g., sodium formate) and formic acid have also shown good effects as inhibitor of protease activity. Formate can be used synergistically with the above-mentioned protease inhibitors, as shown in WO2013/004635 (Novozymes NS). The formate salts may be present in the detergent composition in an amount of at least 0.1% w/w or 0.5% w/w, e.g., at least 1.0%, at least 1.2% or at least 1.5%. The amount of the salt is typically below 5% w/w, below 4% or below 3%.

[0130] WO2007/141736, WO2007/145963 and WO2007/145964 (Proctor and Gamble Co.)

[0131] disclose the use of a reversible peptide protease inhibitor to stabilize liquid detergent compositions. US2003/157088 (Proctor and Gamble Co.) describes compositions containing enzymes stabilized with inhibitors.

The invention is further described in the following embodiments.

Embodiment 1

[0132] a method for improve binding of a protease to an inhibitor of the protease, comprising the steps of:

[0133] a) identify the amino acid residues exposed to the inhibitor when the inhibitor binds to the protease,

[0134] b) modify the protease by modifying the amino acid residues identified in step a)

[0135] c) determine the binding constant K_i

[0136] d) select modified protease generated in step b) optionally with improvement factor>1.0.

Embodiment 2

[0137] The method according to embodiment 1, wherein the improvement factor of the protease selected in step d) is above 1.0 and below 50, preferably above 2.0 and below 40, preferably above 2.0 and below 30, preferably above 5.0 and below 20, preferably above 5.0 and below 10. Preferably the improvement factor is above 1.0, preferably above 2.0, preferably above 5.0, preferably above 10.0, preferably above 15.0, preferably above 20.

Embodiment 3

[0138] Method of embodiment 1 wherein at least one of the amino acids identified in step a) bind to the inhibitor via hydrogen binding.

Embodiment 4

[0139] The method according to any of embodiment 1 to 3 wherein the structure of a targeted protease is aligned to the TY-145 structure (SEQ ID NO 3) of the TY145/CI2 inhibitor complex as disclosed in WO2004067737A2.

Embodiment 5

[0140] The method according to any of embodiment 4, wherein the at least one binding amino acids are identified by selecting all residues in the protease chain A having their alpha-carbon within 16 angstroms of the alpha-carbon of Ile 353 of the CI2 inhibitor chain B.

Embodiment 6

[0141] The method of embodiment 5, wherein all residues having their alpha-carbon within 10 angstroms of Met 356 of the CI2 inhibitor chain B are removed, Embodiment 7: The method according to any of the proceeding embodiments wherein the amino acids identified in step a) have alpha carbon closer than 16 Å to the isoleucine amino acid (Ile) at position 353 of chain B of the CI2 inhibitor but at least 10Å away from methionine amino acid (Met) at position 356 of chain B of the CI2 inhibitor.

Embodiment 8

[0142] The method of any one of embodiments 1-7, wherein the subtilisin inhibitor is a peptide aldehyde, or a hydrosulfite adduct thereof; or a phenyl boronic acid, or a derivative thereof, such as 4-FPBA.

Embodiment 9

[0143] The method of any one of embodiments 1-8, wherein the inhibitor is a peptide aldehyde or ketone having the formula $P-(A)_x-L-(B)_x-B^0-R^*$ or a hydrosulfite adduct of such aldehyde, wherein:

- [0144] a) R^* is H (hydrogen), CH_3 , CX_3 , CHX_2 , or CH_2X ;
- [0145] b) X is a halogen atom;
- [0146] c) B^0 is a single amino acid residue with L- or D-configuration of the formula $-NH-CH(R)-C(=O)-$;
- [0147] d) x is 1, 2 or 3;
- [0148] e) B_x is independently a single amino acid residue, each connected to the next B or to B^0 via its C-terminal;
- [0149] f) L is absent or independently a linker group of the formula $-C(=O)-$, $-C(=O)-C(=O)-$, $-C(=S)-C(=S)-C(=S)-$ or $-C(=S)-C(=O)-$;
- [0150] g) A is absent if L is absent or is independently a single amino acid residue connected to L via the N-terminal of the amino acid;
- [0151] h) P is selected from the group consisting of hydrogen or if L is absent an N-terminal protection group;
- [0152] i) y is 0, 1, or 2,
- [0153] j) R is independently selected from the group consisting of C_{1-6} alkyl, C_{6-10} aryl or C_{7-10} arylalkyl optionally substituted with one or more, identical or different, substituent's R' ;
- [0154] k) R' is independently selected from the group consisting of halogen, $-OH$, $-OR''$, $-SH$, $-SR''$, $-NH_2$, $-NHR''$, $-NR''_2$, $-CO_2H$, $-CONH_2$, $-CONHR''$, $-CONR''_2$, $-NHC(=N)NH_2$; and
- [0155] l) R'' is a C_{1-6} alkyl group.
- [0156] m) x may be 1, 2 or 3.

Embodiment 10

[0157] The method of embodiment 9, wherein the inhibitor is an aldehyde having the formula $P-B^2-B^1-B^0-H$ or an adduct having the formula $P-B^2-B^1-N(H)-CHR-CHOH-SO_3M$, wherein

- [0158] a) H is hydrogen;
- [0159] b) B^0 is a single amino acid residue with L- or D-configuration of the formula $-NH-CH(R)-C(=O)-$;
- [0160] c) B^1 and B^2 are independently single amino acid residues;
- [0161] d) R is independently selected from the group consisting of C_{1-6} alkyl, C_{6-10} aryl or C_{7-10} arylalkyl optionally substituted with one or more, identical or different, substituent's R' ;
- [0162] e) R' is independently selected from the group consisting of halogen, $-OH$, $-OR''$, $-SH$, $-SR''$, $-NH_2$, $-NHR''$, $-NR''_2$, $-CO_2H$, $-CONH_2$, $-CONHR''$, $-CONR''_2$, $-NHC(=N)NH_2$;
- [0163] f) R'' is a C_{1-6} alkyl group; and
- [0164] g) P is an N-terminal protection group.

Embodiment 11

[0165] The method of embodiment 9 or 10, wherein R is such that $B^0=-NH-CH(R)-C(=O)-$ is Phe, Tyr or Leu; and B^1 is Ala, Gly or Val; and B^2 is Arg, Phe, Tyr or Trp.

Embodiment 12

[0166] The method of any one of embodiments 9-11, wherein x=2, L is absent, A is absent, and P is p-methoxycarbonyl (Moc) or benzyloxycarbonyl (Cbz).

Embodiment 13

[0167] The method of any one of embodiments 9-12, wherein the inhibitor is Cbz-Arg-Ala-Tyr-H, Ac-Gly-Ala-Tyr-H, Cbz-Gly-Ala-Tyr-H, Cbz-Gly-Ala-Tyr- CF_3 , Cbz-Gly-Ala-Leu-H, Cbz-Val-Ala-Leu-H, Cbz-Val-Ala-Leu- CF_3 , Moc-Val-Ala-Leu- CF_3 , Cbz-Gly-Ala-Phe-H, Cbz-Gly-Ala-Phe- CF_3 , Cbz-Gly-Ala-Val-H, Cbz-Gly-Gly-Tyr-H, Cbz-Gly-Gly-Phe-H, Cbz-Arg-Val-Tyr-H, Cbz-Leu-Val-Tyr-H, Ac-Leu-Gly-Ala-Tyr-H, Ac-Phe-Gly-Ala-Tyr-H, Ac-Tyr-Gly-Ala-Tyr-H, Ac-Phe-Gly-Ala-Leu-H, Ac-Phe-Gly-Ala-Phe-H, Ac-Phe-Gly-Val-Tyr-H, Ac-Phe-Gly-Ala-Met-H, Ac-Trp-Leu-Val-Tyr-H, MeO-CO-Val-Ala-Leu-H, MeNCO-Val-Ala-Leu-H, MeO-CO-Phe-Gly-Ala-Leu-H, MeO-CO-Phe-Gly-Ala-Phe-H, MeSO₂-Phe-Gly-Ala-Leu-H, MeSO₂-Val-Ala-Leu-H, PhCH₂O-P(OH)(O)-Val-Ala-Leu-H, EtSO₂-Phe-Gly-Ala-Leu-H, PhCH₂SO₂-Val-Ala-Leu-H, PhCH₂O-P(OH)(O)-Leu-Ala-Leu-H, PhCH₂O-P(OH)(O)-Phe-Ala-Leu-H, or MeO-P(OH)(O)-Leu-Gly-Ala-Leu-H or a hydrosulfite adduct of any of these, wherein Cbz is benzyloxycarbonyl and Moc is methoxycarbonyl.

Embodiment 14

[0168] The method of any one of embodiments 9-13, wherein the inhibitor is Cbz-Gly-Ala-Tyr-H or Moc-Val-Ala-Leu-H, or a hydrosulfite adduct thereof, wherein Cbz is benzyloxycarbonyl and Moc is methoxycarbonyl.

Embodiment 15

[0169] The method according to any of the proceeding embodiments wherein the protease is selected from a protease having the amino acid sequence shown in SEQ ID NO: 1 or a protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto.

Embodiment 16

[0170] The method according to embodiment 15 wherein the protease is a variant of SEQ ID NO 1 comprising compared to SEQ ID NO 1 one or more mutation selected from the list consisting of: S9E, S9R, A15T, S24G, S24R, K27R, N42R, G52S, S55P, T56P, G59D, G59E, N60D, N60E, V66A, N74D, S76N, S85N, S85R, S97A, S97E, S97D, S99E, S99D, S99G, S99N, S99H, S99M, S101A, V102I, V102N, G116V, G116R, S126A, S126L, S154D, A156E, G157S, G157D, G157P, S158E, Y161A, R164S, Q176E, N179E, S182E, Q185N, A188P, N198D, V199I, Q200L, Y203W, S206G, S210V, L211D, L211Q, L211E, N212D, N212E, N212S, M216S, M216A, N232H, Q239R, N242D, S250D, S253D, N255W, N255D, N255E, S256E, S256D and R269H, wherein the protease variant has at least 60%, at least 70%, at least 80%, at least 90% or at least 95%, sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 1.

Embodiment 17

[0171] The method according to embodiment 15 or 16, wherein the protease is a variant of SEQ ID NO 1 comprising compared to SEQ ID NO 1 the mutations selected from the list consisting of: Y161A+R164S+A188P, S97AD, S97SE, V66A+S104A, S9R+A15T+V66A+N212D+Q239R, S9R+A15T+G59E+V66A+A188P+V199I+Q239R+N255D, S9E+N42R+N74D, N74D+Q200L, N74D+N198D, Q200L+Y203W, S85N+S99G+V102N, K27R+V102Y+N115S+T268A, N74D+S101A+V102I, S99G+S101A+V102I+G157D+A226V+Q230H+Q239R+N242D+N246K, S99G+S101A+V102I+G157D+A226V+Q230H+Q239R+N242D+N246K, S24G+S52G+S76N+S99N+G126A+Y211Q, S24G+S52G+S76N+S99N+G126S+Y211Q, S85N+G116V+S126L+P127Q+S129A, S85N+G116V+S99M+S126L+P127Q+S129A, N74D+S85R+G116R+S126L+P127Q+S129A, S97D+S99R+S101A+V102I+G157S, S3T+V4I+S97D+S99R+S101A+V102I+G157S+A188P+V193M+V199I+L211D, S3T+V4I+S97D+S99R+S101A+V102I+G157S+V193M+V205I+L217D, S3T+V4I+S97D+S99R+S101A+V102I+G157S+V199I, S97D+S99E+S101A+V102I+G157S, S3T+V4I+S97D+S99E+S101A+V102I+G157S+V199I, S9E+N42R+N74D+H118V+Q176E+A188P+V199I+Q200L+Y203W+S250D+S253D+N255W+

[0172] L256E, S9E+N42R+N74D+A188P+V199I+Q200L+Y203W+S253D+N255W+L256E, S9E+N42R+N74D+Q176E+A188P+V199I+Q200L+Y203W+S250D+S253D+N255W+L256E, S9E+N42R+N74D+H118V+Q176E+A188P+V199I+Q200L+Y203W+S250D+N255W+L256E+*269aH+*269bH and S3V+N74D+H118V+Q176E+N179E+S182E+V199I+Q200L+Y203W+S210V+S250D+N255W+

[0173] L256E, wherein the protease variant has at least 60%, at least 80%, at least 90%, at least 95% sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 1.

Embodiment 18

[0174] The method according to any of embodiments 1 to 14, wherein the protease is selected from a protease having the amino acid sequence shown in SEQ ID NO: 2 or a

protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto.

Embodiment 19

[0175] The method according to embodiment 18 wherein the protease is a variant of SEQ ID NO 2 comprising compared to SEQ ID NO 2 one or more mutation selected from the list consisting of: S9E, S9R, A15T, S24G, S24R, K27R, K43R, S53G, F58P, N61D, N61E, N62D, N62E, V68A, N76D, S78N, S87N, S87R, D99A, D99E, S101E, S101D, S101G, S101N, S101H, S101M, Q103A, Y104I, Y104N, N118V, N118R, G128A, G128L, E156D, E156S, G160S, G160D, G160P, S161E, Y167A, K170S, S182E, Q185E, S188E, S191N, S204D, Q206L, L209W, N212G, A216V, Y217D, L217Q, L217E, N218D, N218E, N218S, M222S, M222A, Q245R, S248D, K256D, F261W, F261D, F261E, Y262E, Y262D and Q275R, wherein the protease variant has at least 60%, at least 70%, at least 80%, at least 90% or at least 95%, sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 2.

Embodiment 20

[0176] The method according to embodiment 18 or 19, wherein the protease is a variant of SEQ ID NO 2 comprising compared to SEQ ID NO 2 the mutations selected from the list consisting of: S9E+K43R+N76D, N76D+Q206L, N76D+S204D, Q206L+L209W, S87N+S101G+Y104N, K27R+N117S, N76D+Q103A+Y104I, S101G+Q103A+Y104I+G160D+A232V+S236H+Q245R+S248D+N252K, S101G+Q103A+Y104I+G160D+A232V+S236H+Q239R+S248D+N252K, S24G+S53G+S78N+S101N+G128A+Y217Q, S24G+S53G+S78N+S101N+G128S+Y217Q, S87N+G118V+G128L+P129Q+S130A, S87N+G118V+S101M+G128L+P129Q+S130A, N76D+S87R+G118R+G128L+P129Q+S130A, S101R+Q103A+Y104I+G160S, S3T+V4I+S101R+Q103A+Y104I+G160S, Y217L, Y217D, Y217E and S101E, wherein the protease variant has at least 60%, at least 80%, at least 90%, at least 95% sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 2.

Embodiment 21

[0177] The method according to any of embodiments 1 to 14, wherein the protease is selected from a protease having the amino acid sequence shown in SEQ ID NO: 3 or a protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto.

Embodiment 22

[0178] The method according to embodiment 21, wherein the protease is a variant of SEQ ID NO 3 comprising compared to SEQ ID NO 3 one or more mutation selected from the list consisting of: S173P, S175P, F180Y, S175P+ F180Y, S173P+F180Y, S173P+S175P, S173P+S175P+ F180Y, wherein the protease variant has at least 60%, at least 70%, at least 80%, at least 90% or at least 95%, sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 3.

Embodiment 23

[0179] The method according to any of embodiments 1 to 14, wherein the protease is selected from a protease having the amino acid sequence shown in SEQ ID NO: 4 or a protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto.

Embodiment 24

[0180] The method according to embodiment 23 wherein the protease is a variant of SEQ ID NO 4 comprising compared to SEQ ID NO 4 one or more mutation selected from the list consisting of: S9E, S9R, A15T, S24G, S24R, K27R, N42R, G52S, S55P, T56P, G59D, G59E, N60D, N60E, V66A, N74D, S76N, S85N, S85R, S97A, S97E, S99E, S99D, S99G, S99N, S99H, S99M, V102N, G116V, G116R, S126A, S126L, S154D, A156E, G157D, G157P, S158E, Y161A, R164S, Q176E, N179E, S182E, Q185N, A188P, N198D, V199I, Q200L, Y203W, S206G, S210V, L211D, L211Q, L211E, N212D, N212E, N212S, M216S, M216A, N232H, Q239R, N242D, S250D, S253D, N255W, N255D, N255E, S256E, S256D and R269H, wherein the protease variant has at least 60%, at least 70%, at least 80%, at least 90% or at least 95%, sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 4.

Embodiment 25

[0181] The method according to embodiment 23 or 24, wherein the protease is a variant of SEQ ID NO 4 comprising compared to SEQ ID NO 4 the mutations selected from the list consisting of: S3T+V4I, S3T+V4I+A188P+V193M+V199I+L211D, S3T+V4I+V193M+V199I+L211D, S3T+V4I+V193M+V199I+L211D, S3T+V4I+V199I, S3T+V4I+S99E+V199I and S99E, wherein the protease variant has at least 60%, at least 80%, at least 90%, at least 95% sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 4.

Embodiment 26

[0182] The method according to any of embodiments 1 to 14, wherein the protease is selected from a protease having the amino acid sequence shown in SEQ ID NO: 5 or a protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto.

Embodiment 27

[0183] The method according to any of embodiments 1 to 14, wherein the protease is a variant of SEQ ID NO 5 comprising compared to SEQ ID NO 5 one or more mutation selected independently from insertions, deletions and substitutions, wherein the protease variant has at least 60%, at least 70%, at least 80%, at least 90% or at least 95%, sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 5.

Embodiment 28

[0184] The method according to any of embodiments 1 to 14, wherein the protease is selected from a protease having the amino acid sequence shown in SEQ ID NO: 6 or a protease comprising compared to SEQ ID NO 1 an insertion

of glutamic acid at the position corresponding to position 97 of SEQ ID NO 1 and having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity to SEQ ID NO 1.

Embodiment 29

[0185] The method according to claim 28, wherein the protease is a variant of SEQ ID

[0186] NO 6 comprising compared to SEQ ID NO 6 one or more mutation selected independently from insertions, deletions and substitutions, wherein the protease variant has at least 60%, at least 70%, at least 80%, at least 90% or at least 95%, sequence identity to the polypeptide having the amino acid sequence of SEQ ID NO 6.

Embodiment 30

[0187] A protease obtained by the method of any of embodiments 1-29

Embodiment 31

[0188] The method according to any of the proceeding embodiments wherein the modified protease variant has improved wash performance.

Materials and Methods

Protease Activity Assay

1) Suc-AAPF-pNA Activity Assay:

[0189] The proteolytic activity can be determined by a method employing the Suc-AAPF-pNA substrate. Suc-AAPF-pNA is an abbreviation for N-Succinyl-Alanine-Alanine-Proline-Phenylalanine-p-Nitroanilide, and it is a blocked peptide which can be cleaved by endo-proteases. Following cleavage a free pNA molecule is liberated and it has a yellow colour and thus can be measured by visible spectrophotometry at wavelength 405 nm. The Suc-AAPF-PNA substrate is manufactured by Bachem (cat. no. L1400, dissolved in DMSO).

[0190] The protease sample to be analyzed was diluted in residual activity buffer (100 mM Tris pH 8.6). The assay was performed by transferring 30 µl of diluted enzyme samples to 96 well microtiter plate and adding 70 µl substrate working solution (0.72 mg/ml in 100 mM Tris pH8.6). The solution was mixed at room temperature and absorption is measured every 20 sec. over 5 minutes at OD 405 nm. The slope (absorbance per minute) of the time dependent absorption-curve is directly proportional to the activity of the protease in question under the given set of conditions. The protease sample should be diluted to a level where the slope is linear.

Calculation of Ki

[0191] It is assumed that protease and inhibitor react according to the equation:



[0192] Where E is the protease, I is the inhibitor and EI is the protease-inhibitor complex. It is furthermore assumed that equilibrium between protease, inhibitor and protease-inhibitor complex is reached during the 1 hour incubation in Model B, and that addition of substrate solution does not result in significant alteration of this equilibrium during the

measuring time used for linear regression. The concentration of enzyme-inhibitor in the detergent solution is then given by:

$$[EI] = \frac{([E_{tot}] + [I_{tot}] + K_i - \sqrt{([E_{tot}] + [I_{tot}] + K_i)^2 - 4 * [E_{tot}] * [I_{tot}]})}{2}$$

[0193] where E_{tot} is the total protease concentration ($[E_{tot}] = [E] + [EI]$), I_{tot} is the total inhibitor concentration ($[I_{tot}] = [I] + [EI]$), and K_i is the equilibrium binding constant for the reaction. The measured slopes V are given by:

$$V = V_0 * (1 - [EI] / [E_{tot}]) = V_0 * (1 - \frac{([E_{tot}] + [I_{tot}] + K_i - \sqrt{([E_{tot}] + [I_{tot}] + K_i)^2 - 4 * [E_{tot}] * [I_{tot}]})}{2 * [E_{tot}]})$$

[0194] where V_0 is the slope without inhibitor added. To calculate apparent binding constant K_i least square fitting of measured slopes at various inhibitor concentrations to this equation is performed with K_i and V_0 as variables.

[0195] The K_i for the parent protease and for each variant was calculated as described above

[0196] Having the K_i for each variant, it is possible to calculate the effect of a single mutation, if data are available for at least two variants that differ only by that mutation. In this case, the relative K_i for the mutation (Improvement Factor IF) is calculated:

$$\text{Improvement Factor (IF)} = \frac{K_{i \text{ variant without mutation}}}{K_{i \text{ variant with mutation}}}$$

A lower K_i is desirable, as a satisfactory inhibition can be obtained at a lower inhibitor concentration, so beneficial mutations have an IF > 1.0.

Detergent

[0197]

TABLE 1

Model B composition		
	Ingredient	wt %
Laundry liquid detergent	(C10-C13)alkylbenzene sulfonic acid	7.2
	sodium lauryl ether sulfate	10.6
	cocoa fatty acid	2.75
	soy fatty acid	2.75
	alcohol ethoxylate with 8 mol EO	6.6
	sodium hydroxide	1.1
	Ethanol	3
	propane-1,2-diol	6

TABLE 1-continued

Model B composition	
Ingredient	wt %
Glycerol	1.7
Triethanolamine	3.3
sodium formiate	1
sodium citrate	2
diethylenetriaminepentakis(methylene)-pentakis(phosphonic acid), heptasodium salt	0.5
copoly(acrylic acid/maleic acid), sodium salt	0.5
deionized water	ad 100

Example 1: Construction of Variants by Site-Directed Mutagenesis

[0198] Site-directed variants were constructed of the relevant back bone e.g. SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 6, comprising specific substitutions according to the invention. The variants were made by traditional cloning of 30 DNA fragments (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989) using PCR together with properly designed mutagenic oligonucleotides that introduced the desired mutations in the resulting sequence.

[0199] Mutagenic oligos were designed corresponding to the DNA sequence flanking the desired site(s) of mutation, separated by the DNA base pairs defining the insertions/deletions/substitutions, and purchased from an oligo vendor such as Life Technologies. In this manner, the variants listed in Table 2, Table 3 and Table 4 below were constructed and produced.

[0200] In order to test the protease variants of the invention, the mutated DNA comprising a variant of the invention were transformed into a competent *B. subtilis* strain and fermented using standard protocols (liquid media, 3-4 days, 30° C.). The culture broth was centrifuged (26000×g, 20 5 min) and the supernatant was carefully decanted from the precipitate. The supernatant was filtered through a Nalgene 0.2 µm filtration unit in order to remove the rest of the *Bacillus* host cells.

TABLE 2

Variants of SEQ ID NO 3 Mutations compared to SEQ ID NO 3	
	Q70F
	Q70A
	Q70N
	S111R

TABLE 2-continued

Variants of SEQ ID NO 3 Mutations compared to SEQ ID NO 3
S111E
S111D
S114A
S144R
A145E
I150N
I150S
G182A
L184F
L184Y
Y240R
S173P + S175P
Q70N + S173P + S175P
S114V + S173P + S175P
I150L + S173P + S175P
S114V + I150L + S173P + S175P
S173P + S175P + F180Y
Q70H + S173P + S175P + F180Y
Q70Y + S173P + S175P + F180Y
Q70F + S173P + S175P + F180Y
I150T + S173P + S175P + F180Y
I150A + S173P + S175P + F180Y
I150S + S173P + S175P + F180Y
I150N + S173P + S175P + F180Y
I150V + S173P + S175P + F180Y
K146T + S173P + S175P + F180Y
K146S + S173P + S175P + F180Y
K146N + S173P + S175P + F180Y
K146W + S173P + S175P + F180Y
K146F + S173P + S175P + F180Y
K146A + S173P + S175P + F180Y
S114A + S173P + S175P + F180Y
S114Q + S173P + S175P + F180Y
S114F + S173P + S175P + F180Y
Q70A + S173P + S175P + F180Y
S173P + S175P + F180Y + L184F
S173P + S175P + F180Y + G182A
S173P + S175P + I178Y + F180Y
S173P + S175P + I178F + F180Y
S173P + S175P + I178P + F180Y
K146R + S173P + S175P + F180Y
S173P + S175P + F180Y + L184F
S173P + S175P + F180Y + L184Y
S173P + S175P + F180Y + G182A
S173P + S175P + I178F + F180Y
S173P + S175P + F180Y + L184W
S111I + S173P + S175P + F180Y
S173P + S175P + F180Y + G182S
S173P + S175P + F180Y + L184D
S173P + S175P + F180Y + L184H

TABLE 3

Variants of SEQ ID NO 5 Mutations compared to SEQ ID NO 5
Q64N
Q64F
M103S
M103A
G128P
G127S + G128P + G129S + Y130P + D131S + M134L

TABLE 4

Variants of SEQ ID NO 6 Mutations compared to SEQ ID NO 6
N60A
N60F
N60H
S100I
V103A
L134V
A164S
Y166F
N60H + S100I
N60F + S100I
S100I + L134V
S100I + Y166F

Example 2: Purification Protease Variants of SEQ ID NO 3

[0201] The 0.2 μ m filtrate was mixed 1:1 with 3.0M (NH₄)₂SO₄ and the mixture was applied to a Phenyl-sepharose FF (high sub) column (from GE Healthcare) equilibrated in 100 mM H₃BO₃, 10 mM MES/NaOH, 2 mM CaCl₂, 1.5M (NH₄)₂SO₄, pH 6.0. After washing the column with the equilibration buffer, the protease was step-eluted with 100 mM H₃BO₃, 10 mM MES, 2 mM CaCl₂, pH 6.0. The eluted peak (containing the protease activity) was collected and applied to a Bacitracin agarose column (from Upfront chromatography) equilibrated in 100 mM H₃BO₃, 10 mM MES, 2 mM CaCl₂, pH 6.0. After washing the column extensively with the equilibration buffer, the protease was eluted with 100 mM H₃BO₃, 10 mM MES, 2 mM CaCl₂, 1M NaCl, pH 6.0 with 25% (v/v) 2-propanol. The elution peak (containing the protease activity) was transferred to 20 mM MES, 2 mM CaCl₂, pH 6.0 on a G25 sephadex column (from GE Healthcare). The G25 transferred peak was the purified preparation and was used for further experiments.

Example 3: Purification of Protease Variants of SEQ ID NO 5

[0202] Solid ammonium sulphate was added to the 0.2 μ m filtrate to a final ammonium sulfate concentration of 1.5M (NH₄)₂SO₄. The solution became slightly turbid and was again filtered through a Nalgene 0.2 μ m filtration unit. The clear filtrate was applied to a Phenyl-sepharose FF (high sub) column (from GE Healthcare) equilibrated in 20 mM HEPES, 2 mM CaCl₂, 1.5M (NH₄)₂SO₄, pH 7.0. After washing the column extensively with the equilibration buffer, the protease was eluted with a linear gradient over three column volumes between the equilibration buffer and 20 mM HEPES, 2 mM CaCl₂, pH 7.0 with 25% (v/v) 2-propanol. Fractions from the column were analysed for protease activity (using the Suc-AAPF-pNA assay at pH 9). The protease peak was pooled and the pool was transferred to 100 mM H₃BO₃, 10 mM MES, 2 mM CaCl₂, pH 6.0 on a G25 Sephadex column (from GE Healthcare). The G25 sephadex transferred enzyme was applied to a SP-sepharose FF column (from GE Healthcare) equilibrated in 100 mM H₃BO₃, 10 mM MES, 2 mM CaCl₂, pH 6.0. After washing the column extensively with the equilibration buffer, the protease was eluted with a linear NaCl gradient (0-->0.5M) in the same buffer over five column volumes. Fractions from the column were analysed for protease activity (using the

Suc-AAPF-pNA assay at pH 9) and active fractions were further analysed by SDS-PAGE. Fractions, where only one band was seen on the coomassie stained SDS-PAGE gel, were pooled as the purified preparation and was used for further characterization.

Example 4: Purification of Protease Variants of
SEQ ID NO: 6

[0203] The pH in the 0.2 µm filtrate was adjusted to pH 8 with 3 M Tris base and the pH adjusted filtrate was applied to a MEP Hypercel column (Pall Corporation) equilibrated in 20 mM Tris/HCl, 1 mM CaCl₂, pH 8.0. After washing the column with the equilibration buffer, the column was step-eluted with 20 mM CH₃COOH/NaOH, 1 mM CaCl₂, pH 4.5. Fractions from the column were analyzed for protease activity using the Suc-AAPF-pNA assay at pH 9 and peak-fractions were pooled. The pH of the pool from the MEP Hypercel column was adjusted to pH 6 with 20% (v/v) CH₃COOH or 3 M Tris base and the pH adjusted pool was diluted with deionized water to the same conductivity as 20 mM MES/NaOH, 2 mM CaCl₂, pH 6.0. The diluted pool was applied to a SP-Sepharose® Fast Flow column (GE Healthcare) equilibrated in 20 mM MES/NaOH, 2 mM CaCl₂, pH 6.0. After washing the column with the equilibration buffer, the protease variant was eluted with a linear NaCl gradient (0-0.5 M) in the same buffer over five column volumes. Fractions from the column were analyzed for protease activity using the Suc-AAPF-pNA assay at pH 9 and active fractions were analyzed by SDS-PAGE. Fractions, where only one band was observed on the Coomassie stained SDS-PAGE gel, were pooled as the purified preparation and was used for further experiments.

Example 5: Inhibitor Binding Assay of SEQ ID NO
3 Variants

[0204] Purified protease variants are pre-diluted to approximately 0.2 mg/ml in dilution buffer. The experimental conditions are described in Table 5. 40 µl diluted protease is then mixed with 40 µl inhibitor solution Inhibitor 1: (Cbz-Gly-Ala-NHCH(CH₂C₆H₄pOH)C(OH)(SO₃Na)—H, wherein Cbz is benzyloxycarbonyl) or 40 µl Inhibitor 2: (4-FPBA) solution in the well of a 96 well microtiter plate (Nunc F 96-MTP). For each protease variant 8 concentrations of Inhibitor 1 solutions (600 µM, 200 µM, 67 µM, 22 µM, 7.4 µM, 2.47 µM, 0.82 µM and 0 µM) and 8 concentrations of inhibitor 2 (120 mM, 40 mM, 13.3 mM, 4.44 mM, 1.48 mM, 0.494 mM, 0.164 mM and 0 mM) are tested. After 10 min mixing of protease and inhibitor at room temperature, 30 µl of the mixture is transferred to a microtiter plate (Nunc U96 PP 0.5 ml) with 270 µl Model B detergent (described in Table 1) in the wells resulting in inhibitor 1 concentrations of 0-30 µM and inhibitor 2 concentrations of 0-6000 µM. Protease, inhibitor and detergent is mixed for 1 hour using magnetic bars for 1 hour at room temperature to reach equilibrium. Then 20 µl is transferred to a microtiter plate (Nunc F 96-MTP). 100 µl substrate solution is added and after 5 sec mixing absorbance at 405 nm is measured every 20 sec for 5 min on a SpectraMax Plus reader. Slope from linear regression of initial increase in absorbance at 405 nm is used for calculation of apparent binding constants. K_i values were calculated according to the “Calculation of K_i” method described in “Materials and Methods”

TABLE 5

Experimental Conditions	
Dilution buffer	0.01% Triton X-100
Detergent	Laundry liquid Model B detergent (Table 1)
Substrate buffer	100 mM Tris, pH 8.6
Substrate stock	100 mg/ml Suc-Ala-Ala-Pro-Phe-pNA (Bachem L-1400) in DMSO
Substrate solution	7 µl/ml substrate stock solution in substrate buffer
Inhibitor 1 stock	Inhibitor (414 mM)
Inhibitor 1 working solutions	Inhibitor 1 stock diluted to 600 µM, 200 µM, 67 µM, 22 µM, 7.4 µM, 2.47 µM, 0.82 µM and 0 µM with dilution buffer
Inhibitor 2 stock	4-FPBA (1801 mM)
Inhibitor 2 working solutions	inhibitor 2 stock solution diluted to 120 mM, 40 mM, 13.3 mM, 4.44 mM, 1.48 mM, 0.494 mM, 0.164 mM and 0 mM with dilution buffer

[0205] Calculation of Improvement Factor (IF)

[0206] Having the K_i for each variant, it is possible to calculate the effect of a single mutation, if data are available for at least two variants that differ only by that mutation. In this case, the relative K_i for the mutation (Improvement Factor, or IF) is calculated:

$$\text{Improvement Factor (IF)} = \frac{K_{i \text{ variant without mutation}}}{K_{i \text{ variant with mutation}}}$$

[0207] A lower K_i is desirable, as a satisfactory inhibition can be obtained at a lower inhibitor concentration, so beneficial mutations have an IF>1.0

TABLE 6

Inhibitor 1 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 3	
Mutations compared to SEQ ID NO 3	IF (inhibitor 1)
SEQ ID NO 3	1.00
Y240R	1.19
S111R	1.11
Q70F	8.53
Q70A	2.54
Q70N	3.33
S114A	2.14
I150N	3.68
I150S	4.94
G182A	1.86
L184F	2.27
L184Y	4.47

TABLE 7

inhibitor 2 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 3	
Mutations compared to SEQ ID NO 3	IF Inhibitor 2
SEQ ID NO 3	1.00
S111E	1.23
S111D	1.53
A145E	1.03
S144R	2.03
Q70N	3.59

TABLE 7-continued

inhibitor 2 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 3	
Mutations compared to SEQ ID NO 3	IF Inhibitor 2
S114A	1.40
L184F	1.39

TABLE 8

Inhibitor 1 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 3 comprising S173P + S175P.	
Mutations compared to SEQ ID NO 3	IF (inhibitor 1)
S173P + S175P	1.00
Q70N + S173P + S175P	4.48
S114V + S173P + S175P	1.83
I150L + S173P + S175P	2.05
S114V + I150L + S173P + S175P	6.14

TABLE 9

inhibitor 1 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 3 comprising S173P + S175P + F180Y	
Mutations compared to SEQ ID NO 3	IF (inhibitor 1)
S173P + S175P + F180Y	1.00
Q70H + S173P + S175P + F180Y	3.78
Q70Y + S173P + S175P + F180Y	7.44
Q70F + S173P + S175P + F180Y	11.76
I150T + S173P + S175P + F180Y	4.15
I150A + S173P + S175P + F180Y	2.52
I150S + S173P + S175P + F180Y	5.70
I150N + S173P + S175P + F180Y	3.58
I150V + S173P + S175P + F180Y	1.80
K146T + S173P + S175P + F180Y	15.91
K146S + S173P + S175P + F180Y	1.13
K146N + S173P + S175P + F180Y	1.36
K146W + S173P + S175P + F180Y	5.47
K146F + S173P + S175P + F180Y	5.17
K146A + S173P + S175P + F180Y	3.72
S114A + S173P + S175P + F180Y	3.33
S114Q + S173P + S175P + F180Y	2.03
S114F + S173P + S175P + F180Y	1.41
Q70A + S173P + S175P + F180Y	4.13
S173P + S175P + F180Y + L184F	4.28
S173P + S175P + F180Y + G182A	2.49
S173P + S175P + I178Y + F180Y	2.15
S173P + S175P + I178F + F180Y	2.34
S173P + S175P + I178P + F180Y	2.22
K146R + S173P + S175P + F180Y	1.31
S173P + S175P + F180Y + L184F	3.02
S173P + S175P + F180Y + L184Y	3.91
S173P + S175P + F180Y + G182A	2.01
S173P + S175P + I178F + F180Y	1.83
S173P + S175P + F180Y + L184W	2.23
S111I + S173P + S175P + F180Y	1.12
S173P + S175P + F180Y + G182S	1.69
S173P + S175P + F180Y + L184D	3.28
S173P + S175P + F180Y + L184H	2.50

Example 6: Inhibitor Binding Assay of SEQ ID NO 5 Variants

[0208] Purified protease variants are pre-diluted to approximately 0.2 mg/ml in dilution buffer. The experimen-

tal conditions are described in Table 5. 40 μ l diluted protease is then mixed with 40 μ l inhibitor solution Inhibitor 1 or 40 μ l Inhibitor 2 solution in the well of a 96 well microtiter plate (Nunc F 96-MTP). For each protease variant 8 concentrations of inhibitor 1 solutions (600 μ M, 200 μ M, 67 μ M, 22 μ M, 7.4 μ M, 2.47 μ M, 0.82 μ M and 0 μ M) and 8 concentrations of Inhibitor 2 (120 mM, 40 mM, 13.3 mM, 4.44 mM, 1.48 mM, 0.494 mM, 0.164 mM and 0 mM) are tested. After 10 min mixing of protease and inhibitor at room temperature, 30 μ l of the mixture is transferred to a microtiter plate (Nunc U96 PP 0.5 ml) with 270 μ l Model B detergent (described in Table 1) in the wells resulting in inhibitor 1 concentrations of 0-30 μ M and inhibitor 2 concentrations of 0-6000 μ M. Protease, inhibitor and detergent is mixed for 1 hour using magnetic bars for 1 hour at room temperature to reach equilibrium. Then 20 μ l is transferred to a microtiter plate (Nunc F 96-MTP). 100 μ l substrate solution is added and after 5 sec mixing absorbance at 405 nm is measured every 20 sec for 5 min on a SpectraMax Plus reader. Slope from linear regression of initial increase in absorbance at 405 nm is used for calculation of apparent binding constants. K_i values were calculated according to the "Calculation of K_i " method described in "Materials and Methods"

[0209] Calculation of Improvement Factor (IF)

[0210] Having the K_i for each variant, it is possible to calculate the effect of a single mutation, if data are available for at least two variants that differ only by that mutation. In this case, the relative K_i for the mutation (Improvement Factor, or IF) is calculated:

$$\text{Improvement Factor (IF)} = \frac{K_{i \text{ variant without mutation}}}{K_{i \text{ variant with mutation}}}$$

[0211] A lower K_i is desirable, as a satisfactory inhibition can be obtained at a lower inhibitor concentration, so beneficial mutations have an IF>1.0

TABLE 10

inhibitor 1 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 5	
Mutations compared to SEQ ID NO 5	IF (inhibitor 1)
SEQ ID NO 5	1.00
Q64N	1.38
Q64F	3.44
M103S	1.73
M103A	2.31
G128P	1.19
G127S + G128P + G129S + Y130P + D131S + M134L	1.05

TABLE 11

inhibitor 2 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 5	
Mutations compared to SEQ ID NO 5	IF (Inhibitor 2)
SEQ ID NO 5	1.00
Q64N	1.24

TABLE 11-continued

inhibitor 2 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 5	
Mutations compared to SEQ ID NO 5	IF (Inhibitor 2)
G128P	1.54
G127S + G128P + G129S + Y130P + D131S + M134L	1.37

Example 7: Inhibitor Binding Assay of SEQ ID NO
6 Variants

[0212] Purified protease variants are pre-diluted to approximately 0.2 mg/ml in dilution buffer. The experimental conditions are described in Table 5 40 μ l diluted protease is then mixed with 40 μ l inhibitor solution Inhibitor 1 or 40 μ l inhibitor 2 solution in the well of a 96 well microtiter plate (Nunc F 96-MTP). For each protease variant 8 concentrations of inhibitor 1 solutions (600 μ M, 200 μ M, 67 μ M, 22 μ M, 7.4 μ M, 2.47 μ M, 0.82 μ M and 0 μ M) and 8 concentrations of inhibitor 2 (120 mM, 40 mM, 13.3 mM, 4.44 mM, 1.48 mM, 0.494 mM, 0.164 mM and 0 mM) are tested. After 10 min mixing of protease and inhibitor at room temperature, 30 μ l of the mixture is transferred to a microtiter plate (Nunc U96 PP 0.5 ml) with 270 μ l Model B detergent (described in Table 1) in the wells resulting in inhibitor 1 concentrations of 0-30 μ M and inhibitor 2 concentrations of 0-6000 μ M. Protease, inhibitor and detergent is mixed for 1 hour using magnetic bars for 1 hour at room temperature to reach equilibrium. Then 20 μ l is transferred to a microtiter plate (Nunc F 96-MTP). 100 μ l substrate solution is added and after 5 sec mixing absorbance at 405 nm is measured every 20 sec for 5 min on a SpectraMax Plus reader. Slope from linear regression of initial increase in absorbance at 405 nm is used for calculation of apparent binding constants. K_i values were calculated according to the "Calculation of K_i " method described in "Materials and Methods"

[0213] Calculation of Improvement Factor (IF)

[0214] Having the K_i for each variant, it is possible to calculate the effect of a single mutation, if data are available for at least two variants that differ only by that mutation. In this case, the relative K_i for the mutation (Improvement Factor, or IF) is calculated:

$$\text{Improvement Factor (IF)} = \frac{K_{i \text{ variant without mutation}}}{K_{i \text{ variant with mutation}}}$$

[0215] A lower K_i is desirable, as a satisfactory inhibition can be obtained at a lower inhibitor concentration, so beneficial mutations have an IF>1.0

TABLE 12

inhibitor 1 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 6	
Mutations compared to SEQ ID NO 6	IF (inhibitor 1)
SEQ ID NO 6	1.00
N60A	1.22
N60F	3.25
N60H	1.24
S100I	6.46
V103A	1.49
L134V	2.89
Y166F	1.31
N60H + S100I	6.65
N60F + S100I	11.26
S100I + L134V	15.14
S100I + Y166F	11.26

TABLE 14

inhibitor 2 binding constant improvement factors (IF) in Model B relative to SEQ ID NO 6	
Mutations compared to SEQ ID NO 6	IF (inhibitor 2)
SEQ ID NO 6	1.00
N60A	1.74
V103A	1.86
A164S	1.84
Y166F	1.62
S100I + L134V	1.1
S100I + Y166F	2.31

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 6

<210> SEQ ID NO 1

<211> LENGTH: 269

<212> TYPE: PRT

<213> ORGANISM: Bacillus lentus

<400> SEQUENCE: 1

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His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp
20 25 30

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Thr	Gly	Ile	Ser	Thr	His	Pro	Asp	Leu	Asn	Ile	Arg	Gly	Gly	Ala	Ser
		35					40					45			
Phe	Val	Pro	Gly	Glu	Pro	Ser	Thr	Gln	Asp	Gly	Asn	Gly	His	Gly	Thr
	50					55					60				
His	Val	Ala	Gly	Thr	Ile	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu
65					70					75					80
Gly	Val	Ala	Pro	Ser	Ala	Glu	Leu	Tyr	Ala	Val	Lys	Val	Leu	Gly	Ala
				85					90					95	
Ser	Gly	Ser	Gly	Ser	Val	Ser	Ser	Ile	Ala	Gln	Gly	Leu	Glu	Trp	Ala
			100					105					110		
Gly	Asn	Asn	Gly	Met	His	Val	Ala	Asn	Leu	Ser	Leu	Gly	Ser	Pro	Ser
		115						120				125			
Pro	Ser	Ala	Thr	Leu	Glu	Gln	Ala	Val	Asn	Ser	Ala	Thr	Ser	Arg	Gly
	130					135					140				
Val	Leu	Val	Val	Ala	Ala	Ser	Gly	Asn	Ser	Gly	Ala	Gly	Ser	Ile	Ser
145					150					155					160
Tyr	Pro	Ala	Arg	Tyr	Ala	Asn	Ala	Met	Ala	Val	Gly	Ala	Thr	Asp	Gln
				165					170					175	
Asn	Asn	Asn	Arg	Ala	Ser	Phe	Ser	Gln	Tyr	Gly	Ala	Gly	Leu	Asp	Ile
			180					185					190		
Val	Ala	Pro	Gly	Val	Asn	Val	Gln	Ser	Thr	Tyr	Pro	Gly	Ser	Thr	Tyr
		195					200					205			
Ala	Ser	Leu	Asn	Gly	Thr	Ser	Met	Ala	Thr	Pro	His	Val	Ala	Gly	Ala
	210					215					220				
Ala	Ala	Leu	Val	Lys	Gln	Lys	Asn	Pro	Ser	Trp	Ser	Asn	Val	Gln	Ile
225					230					235					240
Arg	Asn	His	Leu	Lys	Asn	Thr	Ala	Thr	Ser	Leu	Gly	Ser	Thr	Asn	Leu
			245						250					255	
Tyr	Gly	Ser	Gly	Leu	Val	Asn	Ala	Glu	Ala	Ala	Thr	Arg			
		260						265							

<210> SEQ ID NO 2

<211> LENGTH: 275

<212> TYPE: PRT

<213> ORGANISM: Bacillus amyloliquefaciens

<400> SEQUENCE: 2

Ala	Gln	Ser	Val	Pro	Tyr	Gly	Val	Ser	Gln	Ile	Lys	Ala	Pro	Ala	Leu
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His	Ser	Gln	Gly	Tyr	Thr	Gly	Ser	Asn	Val	Lys	Val	Ala	Val	Ile	Asp
		20					25					30			
Ser	Gly	Ile	Asp	Ser	Ser	His	Pro	Asp	Leu	Lys	Val	Ala	Gly	Gly	Ala
	35					40					45				
Ser	Met	Val	Pro	Ser	Glu	Thr	Asn	Pro	Phe	Gln	Asp	Asn	Asn	Ser	His
	50					55					60				
Gly	Thr	His	Val	Ala	Gly	Thr	Val	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly
65					70					75					80
Val	Leu	Gly	Val	Ala	Pro	Ser	Ala	Ser	Leu	Tyr	Ala	Val	Lys	Val	Leu
			85						90					95	
Gly	Ala	Asp	Gly	Ser	Gly	Gln	Tyr	Ser	Trp	Ile	Ile	Asn	Gly	Ile	Glu
		100					105						110		
Trp	Ala	Ile	Ala	Asn	Asn	Met	Asp	Val	Ile	Asn	Met	Ser	Leu	Gly	Gly
		115				120						125			

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Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
 130                135                140

Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
145                150                155                160

Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
                165                170                175

Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
                180                185                190

Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
195                200                205

Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
210                215                220

Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
225                230                235                240

Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
                245                250                255

Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
260                265                270

Ala Ala Gln
275

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<210> SEQ ID NO 3
<211> LENGTH: 311
<212> TYPE: PRT
<213> ORGANISM: Bacillus sp.

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<400> SEQUENCE: 3

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Asp Gln Ser Ile Thr Lys Thr Thr Gly Gly Ser Gly Ile Lys Val Ala
20                25                30

Val Leu Asp Thr Gly Val Tyr Thr Ser His Leu Asp Leu Ala Gly Ser
35                40                45

Ala Glu Gln Cys Lys Asp Phe Thr Gln Ser Asn Pro Leu Val Asp Gly
50                55                60

Ser Cys Thr Asp Arg Gln Gly His Gly Thr His Val Ala Gly Thr Val
65                70                75                80

Leu Ala His Gly Gly Ser Asn Gly Gln Gly Val Tyr Gly Val Ala Pro
85                90                95

Gln Ala Lys Leu Trp Ala Tyr Lys Val Leu Gly Asp Asn Gly Ser Gly
100               105               110

Tyr Ser Asp Asp Ile Ala Ala Ala Ile Arg His Val Ala Asp Glu Ala
115               120               125

Ser Arg Thr Gly Ser Lys Val Val Ile Asn Met Ser Leu Gly Ser Ser
130               135               140

Ala Lys Asp Ser Leu Ile Ala Ser Ala Val Asp Tyr Ala Tyr Gly Lys
145               150               155               160

Gly Val Leu Ile Val Ala Ala Ala Gly Asn Ser Gly Ser Gly Ser Asn
165               170               175

Thr Ile Gly Phe Pro Gly Gly Leu Val Asn Ala Val Ala Val Ala Ala
180               185               190

Leu Glu Asn Val Gln Gln Asn Gly Thr Tyr Arg Val Ala Asp Phe Ser

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195					200					205					
Ser	Arg	Gly	Asn	Pro	Ala	Thr	Ala	Gly	Asp	Tyr	Ile	Ile	Gln	Glu	Arg
210						215					220				
Asp	Ile	Glu	Val	Ser	Ala	Pro	Gly	Ala	Ser	Val	Glu	Ser	Thr	Trp	Tyr
225					230					235				240	
Thr	Gly	Gly	Tyr	Asn	Thr	Ile	Ser	Gly	Thr	Ser	Met	Ala	Thr	Pro	His
				245					250					255	
Val	Ala	Gly	Leu	Ala	Ala	Lys	Ile	Trp	Ser	Ala	Asn	Thr	Ser	Leu	Ser
			260					265					270		
His	Ser	Gln	Leu	Arg	Thr	Glu	Leu	Gln	Asn	Arg	Ala	Lys	Val	Tyr	Asp
			275				280					285			
Ile	Lys	Gly	Gly	Ile	Gly	Ala	Gly	Thr	Gly	Asp	Asp	Tyr	Ala	Ser	Gly
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Phe	Gly	Tyr	Pro	Arg	Val	Lys									
305					310										

<210> SEQ ID NO 4

<211> LENGTH: 269

<212> TYPE: PRT

<213> ORGANISM: bacillus lentus

<400> SEQUENCE: 4

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His	Asn	Arg	Gly	Leu	Thr	Gly	Ser	Gly	Val	Lys	Val	Ala	Val	Leu	Asp
			20					25					30		
Thr	Gly	Ile	Ser	Thr	His	Pro	Asp	Leu	Asn	Ile	Arg	Gly	Gly	Ala	Ser
		35					40					45			
Phe	Val	Pro	Gly	Glu	Pro	Ser	Thr	Gln	Asp	Gly	Asn	Gly	His	Gly	Thr
	50					55					60				
His	Val	Ala	Gly	Thr	Ile	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu
	65				70					75				80	
Gly	Val	Ala	Pro	Ser	Ala	Glu	Leu	Tyr	Ala	Val	Lys	Val	Leu	Gly	Ala
			85						90				95		
Asp	Gly	Arg	Gly	Ala	Ile	Ser	Ser	Ile	Ala	Gln	Gly	Leu	Glu	Trp	Ala
		100						105					110		
Gly	Asn	Asn	Gly	Met	His	Val	Ala	Asn	Leu	Ser	Leu	Gly	Ser	Pro	Ser
	115						120					125			
Pro	Ser	Ala	Thr	Leu	Glu	Gln	Ala	Val	Asn	Ser	Ala	Thr	Ser	Arg	Gly
	130					135					140				
Val	Leu	Val	Val	Ala	Ala	Ser	Gly	Asn	Ser	Gly	Ala	Ser	Ser	Ile	Ser
	145				150					155				160	
Tyr	Pro	Ala	Arg	Tyr	Ala	Asn	Ala	Met	Ala	Val	Gly	Ala	Thr	Asp	Gln
			165						170				175		
Asn	Asn	Asn	Arg	Ala	Ser	Phe	Ser	Gln	Tyr	Gly	Ala	Gly	Leu	Asp	Ile
			180					185					190		
Val	Ala	Pro	Gly	Val	Asn	Val	Gln	Ser	Thr	Tyr	Pro	Gly	Ser	Thr	Tyr
		195					200					205			
Ala	Ser	Leu	Asn	Gly	Thr	Ser	Met	Ala	Thr	Pro	His	Val	Ala	Gly	Ala
	210					215					220				
Ala	Ala	Leu	Val	Lys	Gln	Lys	Asn	Pro	Ser	Trp	Ser	Asn	Val	Gln	Ile
	225				230					235				240	

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Arg	Asn	His	Leu	Lys	Asn	Thr	Ala	Thr	Ser	Leu	Gly	Ser	Thr	Asn	Leu
			245						250					255	

Tyr	Gly	Ser	Gly	Leu	Val	Asn	Ala	Glu	Ala	Ala	Thr	Arg
	260						265					

<210> SEQ ID NO 5
 <211> LENGTH: 273
 <212> TYPE: PRT
 <213> ORGANISM: Bacillus sp.

<400> SEQUENCE: 5

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Ala	Trp	Gly	Ile	Thr	Thr	Gly	Ser	Ser	Asn	Val	Arg	Ile	Ala	Val	Leu
	20						25						30		
Asp	Thr	Gly	Ile	Asp	Ala	Asn	His	Pro	Asn	Leu	Arg	Asn	Leu	Val	Asp
	35					40					45				
Thr	Ser	Leu	Gly	Arg	Ser	Phe	Val	Gly	Gly	Gly	Thr	Gly	Asp	Val	Gln
	50					55					60				
Gly	His	Gly	Thr	His	Val	Ala	Gly	Thr	Ile	Ala	Ser	Tyr	Gly	Ser	Val
65				70					75					80	
Ser	Gly	Val	Met	Gln	Asn	Ala	Arg	Leu	Ile	Pro	Val	Lys	Val	Leu	Gly
		85						90						95	
Asp	Asn	Gly	Ser	Gly	Ser	Met	Tyr	Gly	Ile	Gln	Gln	Gly	Ile	Leu	Tyr
	100						105						110		
Ala	Ala	Ser	Ile	Asn	Ala	Asp	Val	Ile	Asn	Met	Ser	Leu	Gly	Gly	Gly
	115					120						125			
Gly	Tyr	Asp	Ser	Gly	Met	Asn	Asn	Ala	Ile	Asn	Thr	Ala	Val	Ser	Ser
	130					135					140				
Gly	Thr	Leu	Val	Ile	Ala	Ala	Ser	Gly	Asn	Asp	Gly	Arg	Gly	Ser	Ile
145			150						155					160	
Ser	Tyr	Pro	Ala	Ala	Tyr	Ser	Asn	Ala	Ile	Ala	Val	Gly	Ser	Val	Thr
		165						170						175	
Ser	Asn	Arg	Thr	Arg	Ser	Asn	Phe	Ser	Asn	Tyr	Gly	Ser	Gly	Leu	Glu
	180						185						190		
Leu	Met	Ala	Pro	Gly	Ser	Asn	Ile	Tyr	Ser	Thr	Tyr	Pro	Asn	Gly	Gln
	195					200						205			
Phe	Arg	Thr	Leu	Ser	Gly	Thr	Ser	Met	Ala	Thr	Pro	His	Val	Ala	Gly
	210					215					220				
Val	Ala	Gly	Leu	Ile	Lys	Ser	Ala	Asn	Pro	Asn	Leu	Ser	Val	Thr	Gln
225				230					235					240	
Val	Arg	Asn	Ile	Leu	Arg	Asp	Thr	Ala	Gln	Tyr	Ala	Gly	Ser	Ser	Asn
		245						250						255	
Gln	Tyr	Gly	Tyr	Gly	Ile	Val	Asn	Ala	Tyr	Ala	Ala	Val	Gln	Ala	Ala
		260					265						270		

Gly

<210> SEQ ID NO 6
 <211> LENGTH: 270
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: Protease C

<400> SEQUENCE: 6

- a) R* is H (hydrogen), CH₃, CX₃, CHX₂, or CH₂X;
- b) X is a halogen atom;
- c) B⁰ is a single amino acid residue with L- or D-configuration of the formula —NH—CH(R)—C(=O)—;
- d) x is 1, 2 or 3;
- e) B_x is independently a single amino acid residue, each connected to the next B or to B⁰ via its C-terminal;
- f) L is absent or independently a linker group of the formula —C(=O)—, —C(=O)—C(=O)—, —C(=S)—, —C(=S)—C(=S)— or —C(=S)—C(=O)—;
- g) A is absent if L is absent or is independently a single amino acid residue connected to L via the N-terminal of the amino acid;

- h) P is selected from the group consisting of hydrogen or if L is absent an N-terminal protection group;
- i) y is 0, 1, or 2;
- j) R is independently selected from the group consisting of C₁₋₆ alkyl, C₆₋₁₀ aryl or C₇₋₁₀ arylalkyl optionally substituted with one or more, identical or different, substituent's R';
- k) R' is independently selected from the group consisting of halogen, —OH, —OR", —SH, —SR", —NH₂, —NHR", —NR"₂, —CO₂H, —CONH₂, —CONHR", —CONR"₂, —NHC(=N)NH₂; and
- l) R" is a C₁₋₆ alkyl group.
- m) x may be 1, 2 or 3.
5. The method of claim 4, wherein the inhibitor is an aldehyde having the formula P—B²—B¹—B⁰—H or an adduct having the formula P—B²—B¹—N(H)—CHR—CHOH—SO₃M, wherein
- a) H is hydrogen;
- b) B⁰ is a single amino acid residue with L- or D-configuration of the formula —NH—CH(R)—C(=O)—;
- c) B¹ and B² are independently single amino acid residues;
- d) R is independently selected from the group consisting of C₁₋₆ alkyl, C₆₋₁₀ aryl or C₇₋₁₀ arylalkyl optionally substituted with one or more, identical or different, substituent's R';
- e) R' is independently selected from the group consisting of halogen, —OH, —OR", —SH, —SR", —NH₂, —NHR", —NR"₂, —CO₂H, —CONH₂, —CONHR", —CONR"₂, —NHC(=N)NH₂;
- f) R" is a C₁₋₆ alkyl group; and
- g) P is an N-terminal protection group.
6. The method of claim 4, wherein R is such that B⁰—NH—CH(R)—C(=O)— is Phe, Tyr or Leu; and B¹ is Ala, Gly or Val; and B² is Arg, Phe, Tyr or Trp.
7. The method of claim 4, wherein x=2, L is absent, A is absent, and P is p-methoxycarbonyl (Moc) or benzyloxycarbonyl (Cbz).
8. The method of claim 4, wherein the inhibitor is Cbz-Arg-Ala-Tyr-H, Ac-Gly-Ala-Tyr-H, Cbz-Gly-Ala-Tyr-H, Cbz-Gly-Ala-Tyr-CF₃, Cbz-Gly-Ala-Leu-H, Cbz-Val-Ala-Leu-H, Cbz-Val-Ala-Leu-CF₃, Moc-Val-Ala-Leu-CF₃, Cbz-Gly-Ala-Phe-H, Cbz-Gly-Ala-Phe-CF₃, Cbz-Gly-Ala-Val-H, Cbz-Gly-Gly-Tyr-H, Cbz-Gly-Gly-Phe-H, Cbz-Arg-Val-Tyr-H, Cbz-Leu-Val-Tyr-H, Ac-Leu-Gly-Ala-Tyr-H, Ac-Phe-Gly-Ala-Tyr-H, Ac-Tyr-Gly-Ala-Tyr-H, Ac-Phe-Gly-Ala-Leu-H, Ac-Phe-Gly-Ala-Phe-H, Ac-Phe-Gly-Val-

Tyr-H, Ac-Phe-Gly-Ala-Met-H, Ac-Trp-Leu-Val-Tyr-H, MeO—CO—Val-Ala-Leu-H, MeNCO—Val-Ala-Leu-H, MeO—CO—Phe-Gly-Ala-Leu-H, MeO—CO—Phe-Gly-Ala-Phe-H, MeSO₂-Phe-Gly-Ala-Leu-H, MeSO₂-Val-Ala-Leu-H, PhCH₂O—P(OH)(O)—Val-Ala-Leu-H, EtSO₂-Phe-Gly-Ala-Leu-H, PhCH₂SO₂-Val-Ala-Leu-H, PhCH₂O—P(OH)(O)—Leu-Ala-Leu-H, PhCH₂O—P(OH)(O)—Phe-Ala-Leu-H, or MeO—P(OH)(O)—Leu-Gly-Ala-Leu-H or a hydrosulfite adduct of any of these, wherein Cbz is benzyloxycarbonyl and Moc is methoxycarbonyl.

9. The method of claim 4, wherein the inhibitor is Cbz-Gly-Ala-Tyr-H or Moc-Val-Ala-Leu-H, or a hydrosulfite adduct thereof, wherein Cbz is benzyloxycarbonyl and Moc is methoxycarbonyl.

10. The method according to claim 1 wherein the protease is selected from a protease:

- a. having the amino acid sequence shown in SEQ ID NO: 1 or a protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto, or
- b. having the amino acid sequence shown in SEQ ID NO: 2 or a protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto, or
- c. having the amino acid sequence shown in SEQ ID NO: 3 or a protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto, or
- d. having the amino acid sequence shown in SEQ ID NO: 4 or a protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto, or
- e. having the amino acid sequence shown in SEQ ID NO: 5 or a protease having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity hereto, or
- f. protease having the amino acid sequence shown in SEQ ID NO: 6 or a protease comprising compared to SEQ ID NO 1 an insertion of glutamic acid at the position corresponding to position 97 of SEQ ID NO 1 and having at least 60%, preferably at least 70%, preferably at least 80% or at least 90% sequence identity to SEQ ID NO 1.

11. A protease obtained by the method of claim 1.

12. The method according to claim 1 wherein the modified protease variant has improved wash performance.

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