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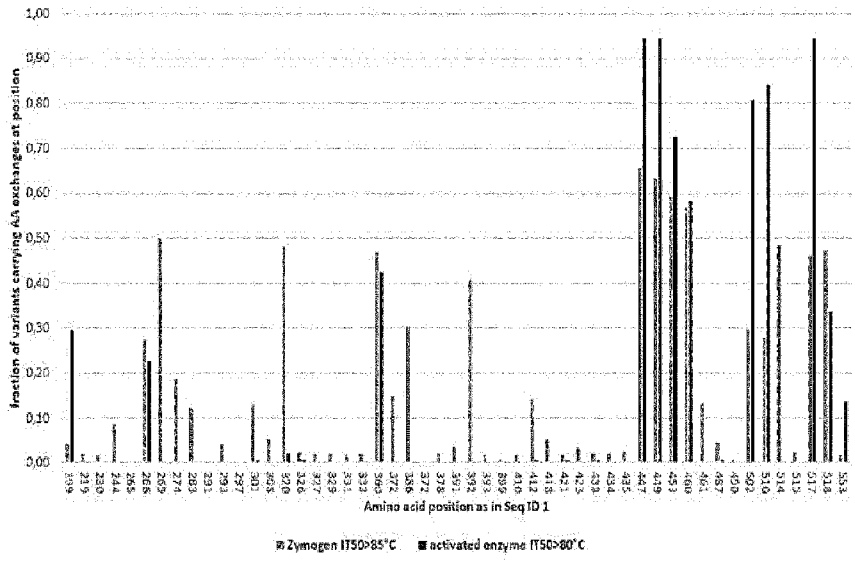

Fig. 1

(57) Abstract: The present invention relates to a protease variant which is at least 90% identical to the full length amino acid sequence of a Kumamolisin AS backbone as set forth in any of SEQ ID NOs 1 - 3, while maintaining proteolytic activity, or a fragment, fraction or shuffled variant thereof maintaining proteolytic activity, which protease variant demonstrates altered or improved stability compared to the Kumamolisin AS wildtype as set forth in SEQ ID NO 4, or the Kumamolisin AS backbone as set forth in any of SEQ ID NOs 1 - 3.

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Stable protease variants

Field of the invention

The present invention relates to the field of proteases.

Background

Proteases are today used in large array of industrial applications, including animal feed, detergents, fruit and beverage processing, leather processing, production of protein hydrolysates, hard surface cleaning or biofilm cleaning, treatment of necrotic or burned tissue to promote wound healing and/or food preparation including baking dough preparation.

In many of these applications, improved stability of the enzyme is a significant advantage. Improved thermostability helps to increase the processability of the respective protease, because the latter oftentimes undergoes thermal treatment during the manufacturing process.

This applies, *inter alia*, for the use of proteases in animal feed where they help to improve the digestibility and nutrient exploitation of the feed.

During feed processing, the feed is often subjected to heat, e.g., by application of steam, to reduce or eliminate pathogens, increase storage life of the feed and optimized utilization of the ingredients leading to improved feed conversion. The conditioning time can vary from a few seconds up to several minutes depending on the type and formulation of the feed. The temperature during conditioning typically ranges from 70°C to 100°C. After conditioning, the

feed is sometimes extruded through a pelleting die, which for a short time raises the temperature of the feed incrementally due to heat dissipation caused by friction.

Yet in other applications, protease enzymes are exposed to heat as well. This includes the use in detergents (e.g. exposure to hot water during laundry washing), fruit and beverage processing (heat exposure during the squeezing process or due to pasteurization or sterilization), leather processing, production of protein hydrolysates, hard surface cleaning or biofilm cleaning, treatment of necrotic or burned tissue to promote wound healing, processing aid in tissue engineering (sterilization, and denaturation of prion proteins) and/or food preparation including baking dough preparation.

Because proteases are proteins, they are susceptible to denaturation by heat and pressure. Denaturing essentially alters the structure of the enzyme, resulting in decreased activity levels and decreased efficacy of the enzyme.

There are different ways to improve protease stability or protect proteases from thermal impact. In animal feed applications, one option is Post-pellet liquid application, which is relatively complex and expensive because it requires the purchase and installation of specialized equipment, space in which to store the liquid enzyme and careful calculation of the amount of enzyme to apply.

Another option is the application of a protective coating before pelleting of the protease with other ingredients (e.g., in feed or detergents). This approach may reduce the efficacy of the enzyme because the coating may not fully dissolve, e.g., in the washing medium, or in the digestive tract of the animal. It is furthermore difficult to achieve a coating design that can withstand the high heat and moisture content of the pelleting process, but subsequently dissolve in the lower temperature and higher moisture conditions, e.g., in the animal's gut or the washing machine.

Another option is to use intrinsically thermostable proteases. These proteases are derived from thermophilic and hyper-thermophilic organisms and have unique structure and function properties of high thermostability. However, these proteases may suffer from other limitations, like suboptimal activity, specificity, bioavailability, pH-range or processability.

It is hence one object of the present invention to provide stable protease variants which do not suffer from the above discussed limitations.

Summary of the invention

These and further objects are met with methods and means according to the independent claims of the present invention. The dependent claims are related to specific embodiments.

Embodiments of the invention

Before the invention is described in detail, it is to be understood that this invention is not limited to the particular component parts or structural features of the devices or compositions described or process steps of the methods described as such devices and methods may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting. The mere fact that certain measures are recited in mutually different dependent claims does not indicate that a combination of these measures cannot be used to advantage. Any reference signs in the claims should not be construed as limiting the scope. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include singular and/or plural referents unless the context clearly dictates otherwise. Further, in the claims, the word “comprising” does not exclude other elements or steps.

It is moreover to be understood that, in case parameter ranges are given which are delimited by numeric values, the ranges are deemed to include these limitation values.

It is further to be understood that embodiments disclosed herein are not meant to be understood as individual embodiments which would not relate to one another. Features discussed with one embodiment are meant to be disclosed also in connection with other embodiments shown herein. If, in one case, a specific feature is not disclosed with one embodiment, but with another, the skilled person would understand that does not necessarily mean that said feature is not meant to be disclosed with said other embodiment. The skilled person would understand that it is the gist of this application to disclose said feature also for the other embodiment, but that just for purposes of clarity and to keep the specification in a manageable volume this has not been done.

According to one embodiment of the invention, a protease variant is provided which is at least 90% identical to the full length amino acid sequence of a Kumamolisin AS backbone as set forth in any of SEQ ID NOs 1 – 3, or a fragment, fraction or shuffled variant thereof maintaining proteolytic activity. The protease variant demonstrates altered or improved stability compared to

(i) the Kumamolisin AS wildtype as set forth in SEQ ID NO 4, or
(ii) the Kumamolisin AS backbone as set forth in any of SEQ ID NOs 1 – 3.

The term "shuffled variant" relates to a combination of such fragment or fraction with one or more fragments from other homologous enzymes, as long as such combination maintains proteolytic activity.

The term "homologous enzyme" describes enzymes belonging to the same structural fold as Kumamolisin and at least 40% sequence identity. This category encompasses Sedolisins as discussed below herein.

Some mutants of Kumamolisin AS have been described. The discovery of N291D mutant strain of Kumamolisin AS has been discussed to providing a useful treatment against celiac disease. There are many proposals that suggest creating a genetically modified organism that could produce N291D Kumamolisin AS protein in human's gastrointestinal tracts. See US application US 20140178355 A1.

Preferably, the Kumamolisin AS variant according to the invention has 93 % identity, more preferably 95 % identity, more preferably 98 % identity, most preferably 99 % identity.

The term "Kumamolisin" refers to acid proteases from the Sedolisin family of peptidases, also called S53 (MEROPS Accession MER000995, see also Wlodawer et al, 2003), comprising acid-acting endopeptidases and a tripeptidyl-peptidase. Sedolisins are endopeptidases with acidic pH optima that differ from the majority of endopetidases in being resistant to inhibition by pepstatin (Terashita et al., 1981; Oda et al., 1998).

The activation of sedolisins involves autocatalytic cleavage at pH below pH 6.5, better below pH 3.5 (see also patent application EP16176044 and Okubo et al., 2016), which releases one or more peptides to deliver the maturated and active form. Said autocatalytic cleavage is inhibited under alkaline, neutral and lightly acidic conditions.

Sedolisins comprise a catalytic triad with Glu, Asp and Ser, which in Kumamolisin AS according to SEQ ID NO 1 reside in positions Glu267, Asp271 and Ser278. The Ser residue is the nucleophile equivalent to Ser in the catalytic triad Asp, His, Ser triad of subtilisin proteases (MEROPS family S8), and the Glu of the triad is a functional substitution for the His general base in subtilisin though not in structural equivalent positions.

The protein folds of sedolisins are clearly related to that of subtilisin, and both groups are sometimes called serine proteases. However, sedolisins have additional loops. The amino acid sequences are not closely similar to subtilisins, and this, taken together with the quite different active site residues and the resulting lower pH for maximal activity, justifies the separate families.

In one embodiment, a protease variant is provided which comprises an amino acid sequence derived from a Kumamolisin AS as set forth in SEQ ID NO. 1, or a fragment, fraction or shuffled variant thereof maintaining proteolytic activity, which protease variant has one or more amino acid substitutions at one or more residue positions in SEQ ID NO. 1 selected from the group consisting of D447, A449, A517, N510, V502, E453, E360, A514, A460, A392, A386, T301, D199, Q518, G266, P553, E269, R412, S435, G320, T326, T461, Q244, D293, A487, V274, A372, K283, T308, A418, I391, A423, A331, S327, I219, M333, A329, N515, A378, S434, E421, A433, S230, Q393, D399, Y490, G281, Y287, R516, A475, S354, S315P, W325, L442, A470, S324, Q361, A190, T196, Q202, E228, A229, A242, D251, S262, N291, L297, H305, D306, V314, A328, I330, L338, A342, A351, D358, G388, D402, V455, E459, A478, K483, Q497, T507, L540, Q542, A548, P551, R166 and/or D265.

Note that, while the numbering set forth above refers to SEQ ID NO 1 or 4 (which are almost identical, with 4 being the wildtype and 1 being the actual backbone used for mutagenesis, the difference between the two being the N terminal AA residue), the claimed protease can be a fragment, fraction or shuffled variant thereof maintaining proteolytic activity. In such case, the resulting amino acid sequence is shorter than that of SEQ ID NO 1 or 4, while the numbering

of the mutant residues still refers to the full length SEQ ID NO 1 or 4, and has to be translated respectively to the numbering of the shorter form.

In one embodiment, the protease variant demonstrates altered or improved stability compared to

(i) the Kumamolisin AS wildtype as set forth in SEQ ID NO 4, or
(ii) the Kumamolisin AS backbone as set forth in any of SEQ ID NOs 1 – 3,

In one embodiment, the protease variant has at least one amino acid substitution selected from the group consisting of D447S, A449Y, A517T, N510H, E360L, E360V, E360C, V502C, E453W, A514T, A514Y, A514D, A514S, A460W, A386I, A392V, A392L, A392I, A392M, T301S, D199E, Q518G, P553K, E269M, E269T, E269C, E269H, E269Q, G266A, D293Y, G320A, R412Q, E421R, A487Q, T461V, T461C, A331F, A331Y, A329Q, A329H, A329T, S435I, S435R, S435T, S435V, V274I, A372S, K283L, Q244C, Q244G, T308C, A418W, I391W, A423V, T326R, T326W, T326L, T326K, I219L, S327F, S327L, S327W, M333I, N515G, A378G, S434G, A433G, S230D, Q393S, D399S, Y490W, A190D, T196S, Q202D, E228Q, A229W, A242S, D251S, S262C, G281R, Y287K, N291T, N291S, D293F, L297T, T301C, T301M, H305F, H305W, D306S, V314M, V314L, S315P, G320Q, G320S, S324L, S324R, W325K, A328W, A328D, A328R, A328Y, I330L, M333Y, M333L, L338R, A342R, A351S, S354E, S354Q, D358G, Q361C, Q361L, A386L, A386V, A386M, G388C, D402E, R412M, R412E, R412D, L442W, L442W, D447C, D447A, A449L, A449M, A449E, A449N, E453Y, E453F, V455I, V455L, E459W, A460R, A470V, A475V, A478L, K483A, Q497Y, Q497M, Q497D, Q497R, V502T, T507L, R516L, R516E, R516I, A517S, L540V, Q542H, Q542D, Q542S, A548S, P551N, P551R, P553L, R166I, D265T, compared to the Kumamolisin as as set forth in SEQ ID NO 1 or 4.

These individual amino acid substitutions are shown in Table 1. Note that, while the numbering set forth above refers to SEQ ID NO 1 or 4, the claimed protease can be a fragment, fraction or shuffled variant thereof maintaining proteolytic activity. In such case, the resulting amino acid sequence is shorter, or longer, than that of SEQ ID NO 1 or 4, while the numbering of the mutant residues still refers to the full length SEQ ID NO 1 or 4.

In one embodiment of the invention, the protease variant has at least one amino acid substitution compared to the Kumamolisin AS as set forth in SEQ ID NO 1 or 4, which substitution is selected from the group consisting of:

- A517T or A517S
- A514S, A514T or A514D
- N510H
- V502C
- A449Y, A449N or less preferred A449E
- D447S or D447C
- A392I, A392L, A392V or A392M
- E360L, E360V or E360C
- E269H, E269T , E269M, E269C or E269Q
- Q518G
- G320Q, G320A or less preferred G320S
- A386I, A386L, A386V or A386M
- G266A
- A372S
- E453Y, E453W or less preferred E453F
- A460W
- A329Q, A329H or A329T
- D293Y
- R412E, R412D, R412Q or R412M
- T301S
- D199E
- A331F or A331Y
- S435T, S435R or S435I
- V274I
- D399S
- S230D
- S434G
- M333I or M333L
- N515G

- A418W
- I391W
- E421R
- A487Q
- A378G
- A423V
- T326K, T326L, T326 R or T326W
- A433G
- D399S
- Y490W
- R516E or R516I
- P553K
- V314L
- S327W, S327L or S327FA475V
- A342R
- S354E or S354Q
- S315P

Some of these substitutions cause a high ΔIT50 when introduced individually into the Kumamolisin AS as set forth in SEQ ID NO 1 or 4, and are therefore preferred, while others have a high occurrence in the combinatorial and distinct clones of Tables 2a, 2b and 4 and some combinations, which have a combination of individual substitutions with a high overall ΔIT50.

Some can interchangeably be used to stabilize the enzyme and some combinations results in other traits that are relevant for the production or performance in feed, like fermentation titers, the hydrolysis of anti-nutritive factors as protease inhibitors (soy bean Bowman-Birk and Kunitz-type trypsin and/or chymotrypsin inhibitors), pH profile, pH and pepsin stability, or stability against and performance under higher ionic strength.

Note that, while the numbering set forth above refers to SEQ ID NO 1 or 4, the claimed protease can be a fragment, fraction or shuffled variant thereof maintaining proteolytic activity. In such case, the resulting amino acid sequence is shorter than that of SEQ ID NO 1 or 4, while the numbering of the mutant residues still refers to the full length SEQ ID NO 1 or 4.

In one embodiment of the invention, the protease variant has at least two amino acid substitutions compared to the Kumamolisin AS backbone as set forth in SEQ ID NO 1 or 4. Preferably the protease variant has at least three, more preferably at least four, more preferably at least five and most preferably at least six amino acid substitutions selected from said group. Preferably, these amino acid substitutions are combinations of the individual substitutions discussed above.

In one embodiment of the invention, the protease variant has at least two amino acid substitutions compared to the Kumamolisin AS backbone as set forth in SEQ ID NO 1 or 4, the at least 2 amino acid substitutions being at two or more residue positions in SEQ ID NO 1 or 4 selected from the group consisting of 447 and 449, 453, 502, 510, 517, 360, 460, 199, 266, 301, 386 and 514. Preferably the protease variant has at least three, more preferably at least four, more preferably at least five and most preferably at least six amino acid substitutions selected from said group.

In one preferred embodiment, the protease variant has at least one, preferably at least two, more preferably at least three, more preferably at least four, more preferably at least five, most preferably at least six amino acid substitutions selected from the group consisting of D447S, A449Y, A517T, N510H, E360L, E360V, E360C, V502C, E453W, A514T, A514Y, A460W, A386I, D199E, G266A, T301S.

Tables 2a, 2b and 4 show sets of such so-called “distinct clones” or “combinatorial clones” which have combinations of the individual mutations set forth above.

As used herein, the term “combinatorial clone or variant” means a clone or variant screened from a recombination library. Such a recombination library contains a population carrying different amounts and mutations selected from the group of table 1.

As used herein, the term “distinct clone or variant” means A clone constructed containing a defined set of mutations selected from the group of table 1 in a rational approach.

Preferably, said improved stability which the protease variant according to the invention has is improved thermostability (IT50). The thermostability of an enzyme is usually determined by

measuring the inactivation temperature (IT 50). The "inactivation temperature" is defined as the temperature at which the residual activity of the enzyme after incubation for a certain duration and subsequent cooling to room temperature is 50% of the residual activity of the same enzyme incubated for the same duration under the same conditions at room temperature.

According to one embodiment, the protease variant has a set of substitutions at selected residues in the Kumamolisin AS backbone as set forth in SEQ ID NO 1 or 4, which set is at least one of the following

a) 360, 447, 449 and 510
b) 447, 449 and 514, and/or
c) 447, 449, 453, and 517.

These three sets of simultaneously substituted residues occur in three sets of specific distinct or combinatorial clones which are particularly preferred (consensus mutations). See Table 2a/Fig 3, Table 2b/Fig. 4 and Table 4/Fig. 5. For these reasons, these sets of simultaneously substituted residues seem to be particularly synergistic when it comes to improvement of stability.

According to one embodiment said improved stability is improved thermostability (IT50) of either the activated enzyme or the zymogen. In one embodiment of, the protease variant has an IT50 of between $\geq$ 75 and $\leq$ 105 °C.

In some embodiments, for the activated enzyme an IT50 of between $\geq$ 70 and $\leq$ 90°C is provided, while a for the zymogen an IT50 of between $\geq$ 80 and $\leq$ 105°C is provided.

The Kumamolisin AS wildtype enzyme has an IT50 of 79,6 °C +/- 0,4°C (n = 46) as the zymogen, i.e., the inactive zymogen, and an IT50 of 59°C +/- 1°C (n= 10) as the activated enzyme. In the course of this specification, the different variants are either characterized by their IT50, or by ΔIT50 (i.e., the difference compared to the wildtype IT50).

According to another embodiment of the invention, a nucleic acid molecule encoding a protease variant according the above description is provided. Furthermore, a plasmid or vector system comprising said nucleic acid molecule is provided, as well as a host cell being transformed with said plasmid or vector and/or comprising said nucleic acid molecule is provided.

Further, a method for producing a protease or protease variant is provided, said method encompassing:

a) cultivating said host cell, and
b) isolating the protease or protease variant from said host cell, or harvesting the protease or protease variant from the medium.

According to another embodiment of the invention, a composition comprising a protease variant according to the above description is provided, which composition has a pH of ≥ 5.

Such composition is generally discussed – yet not with the specific protease variants disclosed herein – in EP application No 16176044.2-1375 and later applications claiming the priority thereof, the content of which is incorporated by reference herein.

According to another embodiment of the invention, a feed additive, feed ingredient, feed supplement, and/or feedstuff comprising a protease variant or a composition according to the above description is provided.

Further, the use of a protease variant according to the above description for the manufacture of a feedstuff is provided.

Such feed additive, feed ingredient, feed supplement, and/or feedstuff is preferably meant for monogastric poultry, pig, fish and aquaculture, where it helps to increase protein digestion and absorbance from the feedstuff, plus degrade proteinogenic compounds which are detrimental for animal health or digestion.

Furthermore, the use of a protease according to the above description is provided for at least one purpose or agent selected from the group consisting of:

- detergent
- fruit and beverage processing
- leather processing
- production of protein hydrolysates

- hard surface cleaning or biofilm cleaning
- treatment of necrotic or burned tissue to promote wound healing,
- processing aid in tissue engineering and/or
- food preparation including baking dough preparation.

Likewise, an additive, ingredient or agent for one purpose or agent selected from the group consisting of:

- detergent
- fruit and beverage processing
- leather processing
- production of protein hydrolysates
- hard surface cleaning or biofilm cleaning
- treatment of necrotic or burned tissue to promote wound healing
- processing aid in tissue engineering and/or
- food preparation including baking dough preparation.

is provided which additive, ingredient or agent comprises a composition according to the above description.

Furthermore, a process of generating a protease variant according to the above description is provided, which process comprises:

i) mutagenizing a DNA, cDNA or mRNA encoding a Kumamolisin AS amino acid sequence as set forth in any of SEQ ID NOs 1 - 4
ii) expressing one or more mutants of Kumamolisin AS thus obtained , and
iii) testing the one or more mutants of Kumamolisin AS for at least stability, preferably thermostability.

Preferably, in said method, the encoding nucleic acid sequence and/or the amino acid sequence of one or variants of Kumamolisin AS is determined. For this purpose, routine methods from the prior art can be used.

Experiments and Figures

While the invention has been illustrated and described in detail in the drawings and foregoing description, such illustration and description are to be considered illustrative or exemplary and not restrictive; the invention is not limited to the disclosed embodiments. Other variations to the disclosed embodiments can be understood and effected by those skilled in the art in practicing the claimed invention, from a study of the drawings, the disclosure, and the appended claims. Any reference signs should not be construed as limiting the scope.

All amino acid sequences disclosed herein are shown from N-terminus to C-terminus; all nucleic acid sequences disclosed herein are shown 5'->3'.

1. Amino acid Sequences of the Kumamolisin AS backbone

SEQ ID NO 1 shows the proenzyme (propeptide plus enzyme, also called zymogen herein) sequence of the Kumamolisin AS backbone used herein. It is important to understand that, while the wildtype sequence of Kumamolisin AS has an N-terminal M residue, the Kumamolisin AS backbone used herein lacks said M, because the latter was replaced by a signal sequence that was later cleaved off. Such signal sequence is for example. The sacB signal peptide MNIKKFAKQATVLTFTTA LLAGGATQAFA.

In SEQ ID NO 1, the propeptide hence comprises AAs 2 – 189 (former N-terminal M which is lacking is yet considered as AA NO 1 in the numbering of SEQ ID NO), and the enzyme comprises AAs 190 – 553:

```
 SDMEKPWKE GEEARAVLQG HARAQAPQAV DKGPVAGDER MAVTVVLRRQ RAGELAAHVE      60
RQAAIAPHAR EHLKREAFAA SHGASLDDFA ELRRFADAHG LALDRANVAA GTAVLSGPVD     120
AINRAFGVEL RHFDHPDGSY RSYLGEVTVP ASIAPMIEAV LGLDTRPVAR PHFRMQRRAE     180
GGFEARSQAA APTAYTPLDV AQAYQFPEGL DGQGQCIAII ELGGGYDEAS LAQYFASLGV      240
PAPQVVSVSV DGASNQPTGD PSGPDGEVEL DIEVAGALAP GAKFAVYFAP NTDAGFLDAI      300
TTAIHDPTLK PSVVSISWGG PEDSWTSAAI AAMNRAFLDA AALGVTVLAA AGDSGSTDGE      360
QDGLYHVDFP AASPYVLACG GTRLVASGGR IAQETVWNDG PDGGATGGGV SRIFPLPAWQ      420
EHANVPPSAN PGASSGRGVP DLAGNADPAT GYEVVIDGEA TVIGGTSAVA PLFAALVARI      480
NQKLGKAVGY LNPTLYQLPA DVFHDITEGN NDIANRAQIY QAGPGWDPCT GLGSPIGVRL      540
LQALLPSASQ PQP     553
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The propeptide is shaded in grey. The catalytic triad SED (=Ser/Glu/Asp) consists of E267, D271 and S467, shown in italics. The positions where the inventors have found mutations that result in altered/improved properties are underlined.

2. Amino acid Sequences of the Kumamolisin AS backbone plus leader sequence and HisTag

In SEQ ID NO 2, the sacB leader sequence comprises AAs 1 – 29 (wavy underline) and replaces the original N-terminal M of the propeptide. The propeptide (shaded in grey) comprises AA 30 – 217, the activated enzyme comprises AA 218 – 581 and the His-tag comprises AAs 582 – 587 (double underline).

```
MNIKKFAKQA TVLTFTTALL AGGATQAFAS DMEKPWKEGE EARAVLQGHA RAQAPQAVDK      60
GPVAGDERMA VTVVLRRQRA GELAAHVERQ AAIAPHAREH LKREAFAASH GASLDDFAEL     120
RRFADAHGLA LDRANVAAGT AVLSGPVDAI NRAFGVELRH FDHPDGSYRS YLGEVTVPAS     180
IAPMIEAVLG LDTRPVARPH FRMQRRAEGG FEARSQAAAP TAYTPLDVAQ AYQFPEGLDG      240
QGQCIAIIEL GGGYDEASLA QYFASLGVPA PQVVSVSVDG ASNQPTGDPS GPDGEVELDI      300
EVAGALAPGA KFAVYFAPNT DAGFLDAITT AIHDPTLKPS VVSISWGGPE DSWTSAAIAA      360
MNRAFLDAAA LGVTVLAAAG DSGSTDGEQD GLYHVDFPAA SPYVLACGGT RLVASGGRIA      420
QETVWNDGPD GGATGGGVSR IFPLPAWQEH ANVPPSANPG ASSGRGVPDL AGNADPATGY      480
EVVIDGEATV IGGTSAVAPL FAALVARINQ KLGKAVGYLN PTLYQLPADV FHDITEGNND      540
IANRAQIYQA GPGWDPCTGL GSPIGVRLLQ ALLPSASQPQ PHHHHHH   587
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3. Amino acid Sequences of the activated Kumamolisin AS backbone devoid of propeptide

In SEQ ID NO 3, the activated Kumamolisin AS backbone enzyme is shown with AAs 1 – 364:

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AAPTAYTPLD VAQAYQFPEG LDGQGQCIAI IELGGGYDEA SLAQYFASLG VPAPQVVSVS      60
VDGASNQPTG DPSGPDGEVE LDIEVAGALA PGAKFAVYFA PNTDAGFLDA ITTAIHDPTL     120
KPSVVSISWG GPEDSWTSAA IAAMNRAFLD AAALGVTVLA AAGDSGSTDG EQDGLYHVDF     180
PAASPYVLAC GGTRLVASGG RIAQETVWND GPDGGATGGG VSRIFPLPAW QEHANVPPSA     240
NPGASSGRGV PDLAGNADPA TGYEVVIDGE ATVIGGTSAV APLFAALVAR INQKLGKAVG     300
YLNPTLYQLP ADVFHDITEG NNDIANRAQI YQAGPGWDPC TGLGSPIGVRL LQALLPSAS     360
QPQP  364
```

4. Amino acid Sequence of the Kumamolisin AS Wildtype

SEQ ID NO 4 shows the proenzyme (propeptide plus enzyme) sequence of the Kumamolisin AS wildtype, as obtained from *Alicyclobacillus sendaiensis* (GenBank: AB085855.1). SEQ ID NO 4 differs from SEQ ID NO 1, which shows the sequence of the Kumamolisin AS backbone used herein in that the latter lacks the N-terminal M still present in the Wildtype SEQ ID No 4. This is because the N-terminal M was replaced, in SEQ ID No 1, by the sacB signal sequence, which was later cleaved off. In SEQ ID NO 4, the propeptide comprises AAs 1 – 189, and the enzyme comprises AAs 190 – 553:

```
MSDMEKPWKE GEEARAVLQG HARAQAPQAV DKGPVAGDER MAVTVVLRRQ RAGELAAHVE      60
RQAAIAPHAR EHLKREAFAA SHGASLDDFA ELRRFADAHG LALDRANVAA GTAVLSGPVD      120
AINRAFGVEL RHFDHPDGSY RSYLGEVTVP ASIAPMIEAV LGLDTRPVAR PHFRMQRRAE      180
GGFEARSQAA APTAYTPLDV AQAYQFPEGL DGQGQCIAII ELGGGYDEAS LAQYFASLGV      240
PAPQVVSVSV DGASNQPTGD PSGPDGEVEL DIEVAGALAP GAKFAVYFAP NTDAGFLDAI      300
TTAIHDPTLK PSVVSISWGG PEDSWTSAAI AAMNRAFLDA AALGVTVLAA AGDSGSTDGE      360
QDGLYHVDFP AASPYVLACG GTRLVASGGR IAQETVWNDG PDGGATGGGV SRIFPLPAWQ      420
EHANVPPSAN PGASSGRGVP DLAGNADPAT GYEVVIDGEA TVIGGTSAVA PLFAALVARI      480
NQKLGKAVGY LNPTLYQLPA DVFHDITEGN NDIANRAQIY QAGPGWDPCT GLGSPIGVRL      540
LQALLPSASQ PQP     553
```

Again, the propeptide is shaded in grey. The catalytic triad SED (=Ser/Glu/Asp) consists of E267, D271 and S467, shown in italics.

Short description of the Figures

Fig. 1 shows the distribution of mutations in variants optimized for thermal stability of the zymogen and the activated enzyme.

Fig. 2 shows the effects of the ionic strength on stability and performance for the WT and top variants #1 to #7 from table 4.

Fig. 3 – 5 show the occurrence of substitutions at AA position in different sets of distinct clones and combinatorial clones.

Example 1: Protease activity assay

Protease activity assays were carried out in microtiter plates

a) AAPF assay 96 well formate

Assay buffer:	200 mM Sodium Acetate, 1 mM $CaCl_2$, 0,01% Triton X-100 at pH 3 depending on the experiment
Substrate stock solution:	100 mM in water free DMSO
Substrate working solution:	Substrate Stock solution diluted 1:50 in assay buffer,

Execution: Load 50 µL of the diluted sample into the wells of a Nunc 96 clear flat bottom plate. Dilution is made in water containing 0.01% Triton-X100 corresponding to the volumetric activity of the sample. Start reaction by adding 50 µL of substrate working solution. Measure kinetics at 37°C by monitoring the increase in adsorption at 410 nm as a measure for enzymatic activity. The activity was calculated by building a calibration curve with a reference enzyme preparation of the backbone with known proteolytic activity measured by a reference method.

For assaying the protease activity at different pH values the following buffers were used, each 200 mM: glycine/HCL between pH 2.0 – 3.0, trisodium citrate/citric acid between 3.0 and 6.0 and Tris/maleic acid between 6.0 and 7.5.

b) IT_{50}

IT_{50} defines the temperature where 50% of the activity is inactivated under the conditions described above. Although not equivalent to, it is a measure for the thermal stability in the application, e.g. pelleting conditions or conditions in a detergent application, either dish washing or the cleaning of a fabric or hard surface and other technical applications.

The screening of enzyme variants under predictive conditions is essential. For proteases like those described herein, screening for thermally more stable variants by methods as also described herein can be affected by the self-hydrolysis of the protease. As already described in

patent application EP16176044 Example 9, screening for variants with higher thermal stability under conditions where the protease is active results in a large number of false positives, as the result of a mixed effect of thermal inactivation and self-hydrolysis. The same applications teaches to circumvent this problem in the absence of small molecule reversible enzyme inhibitors, as is the case for the class of acid protease described herein, by executing the test for thermal stability of the enzyme and enzyme variants in the form of the inactive enzyme zymogen in the way described below.

Assay buffers: 50 mM sodium phosphate, 0,25mM $CaCl_2$ pH6.5
800 mM glycine/HCl pH2.8

Thermal inactivation execution: Samples were diluted corresponding to the volumetric activity in potassium phosphate buffer. The pH of the final solution was checked to be above pH 6.3. The samples were transferred in replicates, 20 µL per well, into a 384 well PCR plate according to the direction of the temperature gradient of the PCR machine. The plates were sealed with an adhesive or hot melting cover foil and incubated on a thermal gradient cycler with a temperature gradient of +/- 12 °C around the expected IT50 value for 10 minutes. The samples were cooled to 8°C before measuring the residual activity of the samples with AAPF-pNA as followed. Samples, 15 µL each from the temperature incubation plate were transferred into a 384 well greiner clear flat bottom PS-microplate and 9 µL of glycine buffer was added to activate the protease during an incubation of 1 hour at 37°C. After the activation of the protease the assay was started by adding 24 µL of an AAPF-pNA solution (2 mM AAPF-pNA in water with 0.01% Triton-X100) and activity was measured by following the kinetics at 37°C. The normalized experimental data for residual activity at the inactivation temperatures were fitted to a four parameter logistics function to evaluate the IT50.

c) IT50 without propeptide – activated enzyme protein:

Enzyme activation prior to thermal inactivation execution. Samples were diluted corresponding to the volumetric activity in glycine buffer pH 2.8 as described in 2b) and pH was checked to be equal or lower than pH 4.0. Samples were activated by an incubation for 1 hour at 37°C. After the incubation pH was set to above 7.0 by diluting the samples 1:3 in 50 mM sodium phosphate buffer pH 8.0. Thermal inactivation of activated enzyme protein execution. Aliquots of the activated enzyme protein were transferred in replicates, 20 µL per well, into a 384 well

PCR plate according to the direction of the temperature gradient of the PCR machine. The plates were sealed with an adhesive or hot melting cover foil and incubated on a thermal gradient cycler with a temperature gradient of +/- 12 °C around the expected IT50 value for 10 minutes. The samples were cooled to 8°C before measuring the residual activity of the samples with AAPF-pNA as followed. Samples, 15 µL each from the temperature incubation plate were transferred into a 384 well greiner clear flat bottom PS-microplate and 9 µL of glycine/HCl buffer was added to adjust the pH to 3.0. The assay was started by adding 24 µL of an AAPF-pNA solution (2 mM AAPF-pNA in water with 0.01% Triton-X100) and activity was measured by following the kinetics at 37°C. The normalized experimental data for residual activity at the inactivation temperatures were fitted to a four parameter logistics function to evaluate the IT50.

d) pH-profile – activated enzyme protein

Undiluted bacterial supernatant containing enzyme protein was titrated with 1 M HCl to pH 4 and enzyme was activated at 37°C for 60 min. 20 µL of sample were added to 200 µL Britton Robinson buffer with pH 1.8-7.0 (adjusted to conductivity of 15 mS/cm with NaCl). 20 µL were then transferred into a 384-well Greiner flat bottom PS-microplate plus 20 µl substrate solution (2 mM AAPF-pNA in water with 0.01% Triton-X100) and activity was measured by monitoring the kinetics at 410 nm and 37°C as described in example 1a). Each kinetic experiment was run in quadruplet.

e) **pH/Pepsin-Resistance**

Undiluted bacterial supernatant containing enzyme protein was titrated with 1 M HCl to pH 2.5. 90 µl were then transferred to a Nunc 96-well clear flat bottom microtiter plate. 10 µl of a 250 µg/mL Pepsin stock solution in pH 2.5 buffer (final concentration in assay 25 µg/mL) or pH 2.5 buffer were added to each well and then incubated at 37°C for 30 min. Finally, 5 µl of a 100 µM Pepstatin A solution (final concentration 5 µM) was added to each well to stop the pepsin reaction. 25 µl of the sample were transferred in 175 µl glycine/HCl buffer pH 3.0 in a new Nunc 96-well clear flat bottom microtiter plate. 20 µl were then transferred into a 384-well Greiner flat bottom PS-microplate plus 20 µl substrate solution (2 mM AAPF-pNA in water with 0.01% Triton-X100) and activity was measured by monitoring the kinetics at 410 nm and 37°C as described in example 1 a). Each kinetic experiment was run in quadruplet.

f) **Conductivity dependency**

20 µl undiluted bacterial supernatant was diluted in 180 µL glycine/HCl buffer pH 3.0 adjusted with NaCl to conductivity of 2, 4, 6, 10, 20, 30, 40, 50 mS/cm in a Nunc 96-well clear flat bottom microtiter plate. The samples were incubated at 37°C for 20 min and then 20 µL sample were then transferred into a 384-well Greiner flat bottom PS-microplate plus 20 µl substrate solution (2 mM AAPF-pNA in water with 0.01% Triton-X100) and activity was measured by monitoring the kinetics at 410 nm and 37°C as described in example 1 a). Each kinetic experiment was run in quadruplet.

g) **BBI/KTI Hydrolysis - Functional Trypsin Assay**

Bowman-Birk and Kunitz-type inhibitors (BBI/KTI) are strong inhibitors of serine proteases which are widely spread in seed of legumes and cereal grains. The assay principle is that a proteolytic degradation of the BBI/KTI by protease activity recovers the natural trypsin activity on Benzyl-Arginine-pNA (Bz-R-pNA) substrate without inhibitors. 90 µL of bacterial supernatant containing enzyme protein was diluted in glycine/HCl buffer to pH 3.0 and then incubated at 37°C for 30 min. 20 µl of the sample was then mixed with 20 µl inhibitor solution (KTI: 8 µg/mL; BBI: 16 µg/mL; KTI/BBI: 4/8 µg/mL diluted in glycine buffer pH 3.0) and further incubated at 37°C for 60 min. 15 µl of the sample were transferred into a 384-well Greiner flat bottom PS-microplate and then 15 µl trypsin solution in pH 8.0 (final trypsin concentration 1 µg/mL; final pH 7.0 or pH 7.5) was added to each well and the plate was incubated at 37°C for 10 min. Finally, 30 µL substrate solution (2 mM Bz-R-pNA in water with 0.01% Triton-X100) was added to each well and activity was measured by monitoring the kinetics at 410 nm and 37°C as described in example 1 a). Each kinetic experiment was run in quadruplet.

Example 2: Generation of genetic diversity

Initial genetic diversity was introduced by randomizing each position of the active enzyme core sequence of SEQ ID NO 1. Mutant enzyme single site saturation libraries were introduced in the gene carried on an E.coli / Bacillus shuttle vector using mutagenesis methods as described in Green & Sambrook (eds), Molecular Cloning, 4th edition, CSHL and suitable mutagenic PCR methods as disclosed in Cadwell and Joyce (PCR Methods Appl. 3 [194], 136-140. Protease

enzyme variants were characterized after heterologous expression in Bacillus subtilis and phenotypically optimized variants selected by the screening procedure outlined in Example 3.

In general, methods to mutagenize a protein, like an enzyme, to obtain a library of mutated proteins members of which may have altered characteristics, are well established. Methods to mutagenize a protein encompass site directed mutagenesis and others, as described e.g. in Hsieh & Vaisvila (2013), content of which incorporated herein by reference for enablement purposes.

Such methods are sometimes called "directed evolution", namely when the established library is then screened for particular features. Packer & Liu (2015) provide an overview of the respective methodology, content of which incorporated herein by reference for enablement purposes.

Example 3: Phenotypically screening for enzyme variants with increased thermal stability

The generated genetic diversity either in the initial stage in form of single site saturation libraries or in the subsequent stage in the form of recombination libraries or distinct clones was screened for variants with an optimized phenotype, i.e. increased thermal stability using the method as described in example 1b) with adaptations required to run them in a fully automated robotic workstation at high throughput. These were mainly adaptation in incubation times, volumes, substrate and the main adaptation was to select optimized variants not by the thermal inactivation profile on a temperature gradient but by the residual activity after incubation at a single temperature, the temperature which was set to discriminate optimized variants from the average of the genetic diversity. Protease variants were derived which differed in one or more amino acid positions from SEQ ID NO 2, including two positions, three positions, n positions. Appropriate iterative rounds of the procedures described herein were performed to satisfy the demands of the application

Example 4:

The following individual mutations which increase the IT50 compared to the used backbone were identified. The IT50 was analyzed as described above and compared to the IT50 of the used backbone (=wildtype with missing N-terminal methionine) characterizing the variant by

the corresponding ΔIT50. The backbone has an IT50 of 79,6 °C +/- 0,4°C (n = 46) as zymogen and an IT50 of 59°C +/- 1°C (n= 10) as activated enzyme.

Position	Mutation	ΔIT50 Zymogen	ΔIT50 activated Enzyme
A190	D	1,5	0,8
T196	S	0,7	0,3
D199	E	0,5	1,0
Q202	D	0,4	-0,3
I219	L	1,1	0,8
E228	Q	0,7	0,1
A229	W	0,2	n.d.
S230	D	2,8	-0,8
A242	S	0,3	-0,4
Q244	C	0,5	-3,6
Q244	G	0,7	1,5
D251	S	0,8	-0,3
S262	C	0,9	-0,3
G266	A	1,7	0,0
E269	M	2,4	-0,1
E269	T	2,6	-0,1
E269	C	2,1	-1,1
E269	H	4,0	-0,5
E269	Q	2,0	-1,4
V274	I	1,8	1,3
G281	R	2,0	5,4
K283	L	0,6	-0,2
Y287	K	0,2	5,2
N291	T	0,7	0,5
N291	S	-0,2	1,0
D293	Y	0,8	1,0
D293	F	1,1	1,3
L297	T	1,2	0,2
T301	S	0,6	7,6
T301	C	0,8	1,0
T301	M	0,7	0,5
H305	F	0,4	-0,4
H305	W	0,1	-2,7
D306	S	0,3	-0,5
T308	C	0,5	-0,8
V314	M	0,6	0,3
V314	L	2,5	0,7
S315P	P	0,8	3,0

G320	A	3,0	-0,2
G320	Q	3,6	1,5
G320	S	1,0	0,6
S324	L	0,1	1,3
S324	R	0,7	2,0
W325	K	-0,3	2,7
T326	R	1,7	1,2
T326	W	0,9	0,2
T326	L	1,7	1,6
T326	K	1,9	1,2
S327	F	1,2	0,6
S327	L	1,5	1,1
S327	W	2,0	1,0
A328	W	0,6	0,5
A328	D	1,3	1,1
A328	R	1,1	0,1
A328	Y	1,5	0,8
A329	Q	2,8	0,2
A329	H	2,1	0,3
A329	T	1,0	0,9
I330	L	1,1	0,8
A331	F	2,0	0,6
A331	Y	1,3	0,6
M333	I	2,5	-0,7
M333	Y	0,3	1,0
M333	L	2,4	-1,0
L338	R	-0,5	1,5
A342	R	-0,6	3,9
A351	S	1,3	-0,9
S354	E	1,6	3,3
S354	Q	2,0	0,3
D358	G	-2,0	0,7
E360	L	1,4	3,1
E360	V	2,4	2,9
E360	C	2,3	2,3
Q361	C	0,9	1,5
Q361	L	0,2	0,1
A372	S	2,4	-0,7
A378	G	1,5	1,5
A386	I	3,6	0,5
A386	L	2,7	1,3
A386	V	2,1	1,2
A386	M	1,7	0,0

G388	C	0,6	-3,5
I391	W	1,7	0,6
A392	V	2,8	0,7
A392	L	3,0	0,9
A392	I	3,7	2,4
A392	M	2,3	2,0
Q393	S	0,9	0,2
D399	S	2,3	2,1
D402	E	0,6	1,7
R412	Q	0,5	2,4
R412	M	1,5	2,9
R412	E	1,8	4,4
R412	D	0,4	3,5
A418	W	2,8	0,2
E421	R	1,0	0,5
A423	V	1,1	0,8
A433	G	1,4	1,9
S434	G	1,9	0,7
S435	I	1,7	1,6
S435	R	1,8	0,5
S435	T	2,5	4,7
S435	V	1,6	2,1
L442	W	1,4	0,3
L442	W	-0,7	2,4
D447	S	4,0	3,2
D447	C	3,0	1,4
D447	A	1,6	1,3
A449	Y	1,7	0,7
A449	L	0,8	0,3
A449	M	1,9	-0,9
A449	E	1,6	0,4
A449	N	1,6	3,3
E453	W	2,4	0,0
E453	Y	2,6	0,7
E453	F	1,1	-0,5
V455	I	1,2	0,3
V455	L	1,8	0,7
E459	W	0,9	-0,3
A460	W	2,6	0,5
A460	R	2,0	-0,6
T461	V	1,2	0,0
T461	C	1,2	0,6
A470	V	0,6	2,3
A475	V	-0,3	3,7

A478	L	1,2	0,2
K483	A	1,5	0,7
A487	Q	0,0	1,6
Y490	W	1,5	0,3
Q497	Y	1,8	1,2
Q497	M	0,8	0,8
Q497	D	0,3	1,0
Q497	R	0,6	0,2
V502	C	2,3	1,9
V502	T	1,5	1,6
T507	L	0,2	1,0
N510	H	2,4	7,9
A514	T	2,2	1,3
A514	Y	1,3	-1,2
A514	D	1,5	1,2
A514	S	2,4	0,5
N515	G	2,0	-0,2
R516	L	0,5	1,2
R516	E	1,1	3,5
R516	I	1,2	4,3
A517	T	1,3	3,9
A517	S	0,3	7,7
Q518	G	1,6	4,1
L540	V	0,7	0,5
Q542	H	0,9	-0,2
Q542	D	1,1	0,4
Q542	S	0,4	0,5
A548	S	0,2	n.d.
P551	N	0,9	-0,4
P551	R	0,6	0,3
P553	K	0,5	0,3
P553	L	0,8	0,2
R166	I	1,0	0,7
D265	T	1,7	n.d.

Table 1: Kumamolisin AS single amino acid substitutions relative to SEQ ID NO 1, and their ΔIT50 compared to the backbone for the zymogen and the activated enzyme

Quite a few distinct clones and combinatorial clones as shown in Table 3 have substitutions in these positions, leading to synergistic effects in thermal stabilization, when two or more residues thereof are mutated simultaneously.

Example 5

Distinct variants were generated by introducing selected distinct mutations into the Kumamolisin AS wild-type sequence via site-directed mutagenesis. Suitable mutagenic PCR methods known in the art and standard cloning techniques as described in Green & Sambrook (eds), Molecular Cloning, 4th edition, CSHL were used. Protease enzyme variants were characterized after heterologous expression in Bacillus subtilis and phenotypically analysis using the methods described above.

Combinatorial libraries, combining mutations identified in the examples provided above and outlined in Table 1 were generated by well-known PCR methods as described in Yolov and Shabarova (1990) and standard cloning techniques as described in Green & Sambrook (eds), Molecular Cloning, 4th edition, CSHL were used. Combinatorial libraries were screened for optimized variants as described in example 3.

Example 6

Distinct clones and combinatorial clones comprising two or more mutations from Table 1 were identified, the IT50 analyzed as described above and compared to the IT50 of the used backbone (=wildtype with missing N-terminal methionine) characterizing the variant by the corresponding ΔIT50. As the IT50 of the backbone was determined in the same experiment as the variant the measured IT50 of the backbone can be slightly different from the average value. Results are shown in the following Table 2a (Fig. 3 shows results in graphic form):

#	Mutations in distinct clones and selected combinatorial clones							
1						E360L		A392V
2				T301S		E360V	A386I	
3						E360L	A386I	A392V
4						E360L		A392I
5						E360V	A386I	A392I
6				T301S	G320A	E360L		
7				T301S		E360L	A386I	A392I
8				T301S		E360V		A392I
9						E360V		A392V
10						E360L	A386I	
11				T301S		E360L		A392I
12				T301S		E360L	A386I	
13						E360L	A386I	A392I
14						E360V		A392V
15						E360L	A386I	
16				T301S		E360L		
17				T301S		E360L		A392V
18				T301S		E360V	A386I	
19						E360V	A386I	
20				T301S		E360V		A392I
21	D199E					E360V		
22						E360L	A386I	
23						E360L	A386I	A392I
24						E360V		A392V
25			E269T			E360V	A386I	
26				T301S		E360L		A392V
27						E360L		A392V
28						E360V		A392I
29				T301S		E360L		
30						E360L	A386I	A392I
31				T301S		E360L	A386I	A392V
32						E360V		A392I
33				T301S		E360L		A392I
34						E360V		
35						E360L	A386I	A392V
36				T301S		E360V	A386I	
37						E360L	A386I	
38				T301S		E360L	A386I	A392V
39				T301S		E360V		A392V
40				T301S		E360L	A386I	A392V

Table 2a: Distinct clones comprising selected combinations of mutations from table 1, and their ΔIT50 compared to the wildtype

#	Mutations in distinct clones and selected combinatorial clones						
1		D447S	A449Y		A460W	V502C	N510H
2		D447S	A449Y	E453W	A460W	V502C	N510H
3		D447S	A449Y	E453W	A460W	V502C	N510H
4		D447S	A449Y		A460W	V502C	N510H
5		D447S	A449Y	E453W	A460W	V502C	N510H
6		D447S	A449Y	E453W	A460W	V502C	N510H
7		D447S	A449Y	E453W		V502C	N510H
8		D447S	A449Y	E453W	A460W	V502C	N510H
9		D447S	A449Y	E453W	A460W		N510H
10		D447S	A449Y	E453W	A460W	V502C	N510H
11		D447S	A449Y	E453W	A460W	V502C	N510H
12		D447S	A449Y	E453W	A460W	V502C	N510H
13		D447S	A449Y	E453W	A460W	V502C	N510H
14		D447S	A449Y		A460W	V502C	N510H
15		D447S	A449Y	E453W	A460W	V502C	N510H
16		D447S	A449Y		A460W	V502C	N510H
17		D447S	A449Y	E453W	A460W	V502C	N510H
18		D447S	A449Y	E453W	A460W	V502C	N510H
19		D447S	A449Y	E453W	A460W	V502C	N510H
20		D447S	A449Y	E453W	A460W	V502C	N510H
21		D447S	A449Y		A460W	V502C	N510H
22		D447S	A449Y		A460W	V502C	N510H
23		D447S	A449Y	E453W	A460W	V502C	N510H
24		D447S	A449Y	E453W	A460W	V502C	N510H
25		D447S	A449Y			V502C	N510H
26		D447S	A449Y	E453W	A460W	V502C	N510H
27		D447S	A449Y	E453W	A460W	V502C	N510H
28		D447S	A449Y		A460W	V502C	N510H
29		D447S	A449Y	E453W	A460W	V502C	N510H
30		D447S	A449Y	E453W	A460W	V502C	N510H
31		D447S	A449Y	E453W	A460W	V502C	N510H
32		D447S	A449Y	E453W	A460W	V502C	N510H
33		D447S	A449Y	E453W	A460W	V502C	N510H
34		D447S	A449Y	E453W	A460W	V502C	N510H
35		D447S	A449Y	E453W		V502C	N510H
36		D447S	A449Y	E453W	A460W	V502C	N510H
37		D447S	A449Y	E453W	A460W	V502C	N510H
38		D447S	A449Y	E453W	A460W	V502C	N510H
39		D447S	A449Y	E453W	A460W	V502C	N510H
40		D447S	A449Y	E453W	A460W	V502C	N510H

Table 2a ctd': Distinct clones comprising selected combinations of mutations from table 1, and their ΔIT50 compared to the wildtype

#	Mutations in distinct clones and selected combinatorial clones				IT50 Zymogen	ΔIT50 Zymogen	IT50 active enzyme	ΔIT50 active enzyme
1		A517T			95,5	17,0	90,1	30,6
2		A517T			>95	>17	90,1	30,6
3		A517T			99,5	21,0	89,2	29,7
4		A517T			97,3	18,8	89,1	29,6
5		A517T			99,4	20,9	88,8	29,3
6		A517T			96,4	17,9	88,6	29,1
7		A517T			96,4	17,9	88,5	29,0
8		A517T			99,1	20,6	88,5	29,0
9		A517T	Q518G		97,8	19,3	88,5	29,0
10		A517T			98,4	19,9	88,4	28,9
11		A517T			97,7	19,2	88,4	28,9
12		A517T			98,6	20,1	88,3	28,8
13		A517T			99,5	21,0	88,2	28,7
14		A517T			>95	>17	88,2	28,7
15		A517T			98,3	19,8	88,1	28,6
16		A517T			95,8	17,3	88,0	28,5
17		A517T			97,2	18,7	88,0	28,5
18		A517T			97,6	19,1	87,8	28,3
19		A517T			98,5	20,0	87,8	28,3
20		A517T	Q518G		97,0	18,5	87,8	28,3
21		A517T			>95	>17	87,8	28,3
22		A517T			>95	>17	87,8	28,3
23		A517T			97,1	18,6	87,8	28,3
24		A517T			99,0	20,5	87,8	28,3
25		A517T			94,0	16,0	87,7	27,0
26		A517T			97,4	18,9	87,7	28,2
27		A517T	Q518G		98,0	19,5	87,7	28,2
28		A517T			>95	>17	87,6	28,1
29		A517T			96,5	18,0	87,6	28,1
30		A517T			99,0	20,2	87,5	28,0
31		A517T			98,1	19,6	87,5	28,0
32		A517T	Q518G		97,9	19,4	87,4	27,9
33		A517T	Q518G		97,1	18,6	87,4	27,9
34		A517T			95,6	17,1	87,4	27,9
35		A517T			98,2	19,7	87,4	27,9
36		A517T			98,5	20,0	87,4	27,9
37		A517T	Q518G		97,9	19,4	87,4	27,9
38		A517T			>95	>17	87,3	27,8
39		A517T			96,2	17,7	87,2	27,7
40		A517T	Q518G		>95	>17	87,1	27,6

Table 2a ctd': Distinct clones comprising selected combinations of mutations from table 1, and their ΔIT50 compared to the wildtype

#	Mutations in distinct clones and selected combinatorial clones							
41				T301S		E360L	A386I	
42						E360V		A392I
43						E360V	A386I	A392I
44						E360L		A392V
45	D199E					E360V		
46				T301S		E360L	A386I	A392I
47	D199E	G266A				E360V		A392V
48		G266A				E360V		A392V
49						E360L		A392I
50				T301S		E360V	A386I	
51						E360L	A386I	
52						E360L	A386I	
53	D199E	G266A				E360V		A392V
54						E360V	A386I	A392I
55	D199E	G266A	E269H			E360V		A392L
56						E360V	A386I	
57						E360V		A392V
58				T301S		E360L	A386I	A392I
59	D199E					E360V	A386I	
60						E360V	A386I	A392I
61	D199E					E360V	A386I	
62						E360V	A386I	
63	D199E	G266A		T301S		E360L		
64	D199E	G266A	E269T		G320A	E360V		A392L
65						E360L	A386I	
66		G266A				E360V		A392V
67						E360L	A386I	
68	D199E					E360V		
69	D199E					E360V	A386I	
70	D199E					E360L		
71	D199E	G266A	E269H	T301S		E360L		
72	D199E					E360L		
73	D199E	G266A				E360V		
74	D199E	G266A	E269H			E360V		A392L
75	D199E					E360V		
76				T301S		E360L		A392I
77	D199E					E360V		
78	D199E					E360L		
79						E360V	A386I	

Table 2a ctd': Distinct clones comprising selected combinations of mutations from table 1, and their ΔIT50 compared to the wildtype

#	Mutations in distinct clones and selected combinatorial clones						
41		D447S	A449Y	E453W	A460W	V502C	N510H
42		D447S	A449Y		A460W		N510H
43		D447S	A449Y	E453W		V502C	N510H
44		D447S	A449Y	E453W	A460W		N510H
45		D447S	A449Y	E453W	A460W	V502C	N510H
46		D447S	A449Y	E453W	A460W	V502C	N510H
47		D447S	A449Y	E453W	A460W	V502C	N510H
48	R412E	D447S	A449Y	E453W	A460W		
49		D447S	A449Y	E453W		V502C	N510H
50		D447S	A449Y		A460W	V502C	N510H
51		D447S	A449Y	E453W		V502C	N510H
52		D447S	A449Y		A460W	V502C	N510H
53		D447S	A449Y	E453W	A460W	V502C	N510H
54		D447S	A449Y	E453W	A460W	V502C	N510H
55		D447S	A449Y	E453W	A460W	V502C	N510H
56		D447S	A449Y	E453W		V502C	N510H
57		D447S	A449Y	E453W	A460W	V502C	N510H
58		D447S	A449Y		A460W	V502C	N510H
59		D447S	A449Y			V502C	N510H
60		D447S	A449Y	E453W	A460W	V502C	N510H
61		D447S	A449Y	E453W		V502C	N510H
62		D447S	A449Y		A460W	V502C	N510H
63		D447S	A449Y	E453W		V502C	N510H
64		D447S	A449Y	E453W	A460W	V502C	N510H
65		D447S	A449Y			V502C	N510H
66		D447S	A449Y	E453W	A460W		
67		D447S	A449Y	E453W		V502C	N510H
68		D447S	A449Y	E453W		V502C	N510H
69		D447S	A449Y	E453W	A460W	V502C	N510H
70		D447S	A449Y		A460W	V502C	N510H
71		D447S	A449Y	E453W		V502C	N510H
72		D447S	A449Y	E453W		V502C	N510H
73		D447S	A449Y	E453W		V502C	N510H
74		D447S	A449Y	E453W	A460W	V502C	N510H
75		D447S	A449Y		A460W	V502C	N510H
76		D447S	A449Y	E453W	A460W		N510H
77		D447S	A449Y	E453W		V502C	N510H
78		D447S	A449Y	E453W		V502C	N510H
79		D447S	A449Y			V502C	N510H

Table 2a ctd': Distinct clones comprising selected combinations of mutations from table 1, and their ΔIT50 compared to the wildtype

#	Mutations in distinct clones and selected combinatorial clones				IT50 Zymogen	ΔIT50 Zymogen	IT50 active enzyme	ΔIT50 active enzyme
41		A517T	Q518G		98,4	19,9	87,0	27,5
42		A517T			92,7	14,2	86,9	27,4
43		A517T			>95	>17	86,9	27,4
44		A517T			96,9	18,4	86,8	27,3
45		A517T			93,5	15,1	86,7	26,0
46		A517T	Q518G		97,4	18,9	86,7	27,2
47		A517T	Q518G		101,5	23,0	86,6	27,1
48		A517T	Q518G		100,3	21,8	86,6	27,1
49		A517T	Q518G		>95	>17	86,6	27,1
50		A517T	Q518G		94,7	16,2	86,5	27,0
51		A517T			>95	>17	86,5	27,0
52		A517T			>95	>17	86,4	26,9
53		A517T	Q518G	P553K	102,2	23,7	86,4	26,9
54		A517T	Q518G		>95	>17	86,4	26,9
55		A517T	Q518G	P553K	101,7	23,2	86,3	26,8
56		A517T			93,1	14,6	86,2	27,0
57		A517T	Q518G		>95	>17	86,2	26,7
58		A517T	Q518G		>95	>17	86,2	26,7
59		A517T			92,2	14,1	86,1	25,4
60		A517T	Q518G		97,2	18,7	86,0	26,5
61		A517T			93,2	14,9	85,9	26,2
62		A517T			92,4	13,9	85,9	26,6
63		A517T			95,7	17,2	85,8	26,3
64		A517T	Q518G	P553K	100,1	21,6	85,8	26,8
65		A517T			94,0	15,9	85,8	25,1
66	R516I	A517T	Q518G		100,1	21,6	85,7	26,2
67		A517T			>95	>17	85,7	26,2
68		A517T	Q518G		>95	>17	85,7	26,2
69		A517T			94,9	16,6	85,4	25,7
70		A517T			-10,0	-10,0	85,4	25,9
71		A517T			95,8	17,3	85,4	25,9
72		A517T	Q518G		>95	>17	85,4	25,9
73		A517T	Q518G		>95	>17	85,4	25,9
74		A517T	Q518G		100,4	21,9	85,3	25,8
75		A517T			>95	>17	85,3	25,8
76		A517T	Q518G		95,1	16,6	85,2	25,7
77		A517T			94,9	16,4	85,1	25,5
78		A517T			>95	>17	85,1	25,6
79		A517T			93,0	15,0	85,0	25,7

Table 2a ctd': Distinct clones comprising selected combinations of mutations from table 1, and their ΔIT50 compared to the wildtype

G320	A	3,0	-0,2	2	186
	Q	3,6	1,5		46
	S	1,0	0,6		35
T326	R	1,7	1,2		11
	W	0,9	0,2		
	L	1,7	1,6	1	6
	K	1,9	1,2	1	1
T461	V	1,2	0,0	1	26
	C	1,2	0,6		48
Q244	C	0,5	-3,6		46
	G	0,7	1,5	1	1
D293	Y	0,8	1,0	1	24
	F	1,1	1,3		
A487	Q	0,0	1,6	1	24
V274	I	1,8	1,3		104
A372	S	2,4	-0,7		82
K283	L	0,6	-0,2		68
T308	C	0,5	-0,8		30
A418	W	2,8	0,2		12
	H	1,1	1,3		16
I391	W	1,7	0,6		21
A423	V	1,1	0,8		18
A331	F	2,0	0,6		7
	Y	1,3	0,6		9
S327	F	1,2	0,6		
	L	1,5	1,1		16
	W	2,0	1,0		
I219	L	1,1	0,8		16
M333	I	2,5	-0,7		16
A329	Q	2,8	0,2		5
	H	2,1	0,3		3
	T	1,0	0,9		7
N515	G	2,0	-0,2		13
A378	G	1,5	1,5		12
S434	G	1,9	0,7		12
E421	R	1,0	0,5	1	11
A433	G	1,4	1,9		11
S230	D	2,8	-0,8		9
Q393	S	0,9	0,2		3
D399	S	2,3	2,1		4
Y490	W	1,5	0,3		2
G281	R	2,0	5,4		

Y287	K	0,2	5,2	
R516	I	1,2	4,3	
	E	1,1	3,5	
	L	0,5	1,2	
A475	V	-0,3	3,7	
S354	E	1,6	3,3	
S315P	P	0,8	3,0	
W325	K	-0,3	2,7	
L442	W	-0,7	2,4	
	W	1,4	0,3	
A470	V	0,6	2,3	
S324	R	0,7	2,0	
S324	L	0,1	1,3	
Q361	C	0,9	1,5	
Q361	L	0,2	0,1	
A190	D	1,5	0,8	
T196	S	0,7	0,3	
Q202	D	0,4	-0,3	
E228	Q	0,7	0,1	
A229	W	0,2	n.d.	
A242	S	0,3	-0,4	
D251	S	0,8	-0,4	
S262	C	0,9	-0,3	
N291	T	0,7	0,5	
N291	S	-0,2	1,0	
L297	T	1,2	0,2	
H305	F	0,4	-0,4	
H305	W	0,1	-2,7	
D306	S	0,3	-0,5	
V314	M	0,6	0,3	
V314	L	2,5	0,7	
A328	W	0,6	0,5	
A328	D	1,3	1,1	
A328	R	1,1	0,1	
A328	Y	1,5	0,8	
I330	L	1,1	0,8	
M333	Y	0,3	1,0	
M333	L	2,4	-1,0	
L338	R	-0,5	1,5	
A342	R	-0,6	3,9	
A351	S	1,3	-0,9	
S354	Q	2,0	0,3	
D358	G	-2,0	0,7	

G388	C	0,6	-3,5	
D402	E	0,6	1,7	
V455	I	1,2	0,3	
V455	L	1,8	0,7	
E459	W	0,9	-0,3	
A478	L	1,2	0,2	
K483	A	1,5	0,7	
Q497	Y	1,8	1,2	
Q497	M	0,8	0,8	
Q497	D	0,3	1,0	
Q497	R	0,6	0,2	
V502	T	1,5	1,6	
T507	L	0,2	1,0	
L540	V	0,7	0,5	
Q542	H	0,9	-0,2	
Q542	D	1,1	0,4	
Q542	S	0,4	0,5	
A548	S	0,2	n.d.	
P551	N	0,9	-0,4	
P551	R	0,6	0,3	
P553	L	0,8	0,2	
R166	I	1,0	0,7	
D265	T	1,7	n.d.	

Table 3: Some preferred substitutions and their key characteristics

It is further to be understood that the mutations can have positive or negative effects on other enzyme parameters, as the producibility in fermentative microbial production systems or the stability against pH- conditions or endogenous proteases of the animal, like pepsin. Testing the stability of feed enzymes at low pH and in the presence of pepsin is a standard for feed enzymes and was performed in this study as outlined in example 1e. The stability against higher ionic strength is not a standard test for feed enzymes though high ion concentrations can interfere with the enzyme stability and with the enzyme performance under such conditions and can be found for example in the gut. The secretion of acid in the gut and the feed ingredients translate to an increased ionic strength.

Fig. 2 shows that the wildtype suffers from combined effects of stability and performance reduction in the presence of higher ionic strength. Fig. 2 also shows the effect of ionic strength on the top variants also shown in table 4, variants #1 to #7.

The performance and stability in high ionic strength was tested as described in example 1d. The pH profile was a control parameter and tested as described in example 1f. The digestion of proteinaceous antinutritive factors like the Trypsin/chymotrypsin inhibitors BBI and KTI (Bowman-Birk inhibitors and Kunitz-type inhibitors) is a potential beneficial performance characteristic of a protease which was tested as described in example 1g.

From the 651 individual combinatorial and distinct variants tested in detail, Table 4 describes the variants consolidating a multitude of performance and stability parameters (Fig. 5 shows results in graphic form).

All variants shown in table 4 are better or equally well produced in a microbial production system than the wildtype and have no relevant changes in their pH activity profile tested as described in example 1d. Table 4 ranks these variants based on the thermal stability of the activated enzyme, the pH/pepsin stability and the stability against and the performance under higher ionic strength.

It was further found that the best variants can hydrolyze BBI and KTI (Bowman-Birk inhibitors and Kunitz-type inhibitors) as tested in a functional trypsin inhibition assay, which differentiates these variants from the parent enzyme, beside the high thermal stability engineered into these variants.

#	mutant code	Mutations in distinct clones and selected combinatorial clones																				IT50 [°C] Zymogen	IT50 [°C] activated	pH/pepsin Stability % residual activity	Stability / performance at 25 mS cm-1	Inhibitor hydrolysis	
																										BBI	KTI
1	GIN_3B2_08.42a				T301S		E360V		A392I		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T			99,10	88,48	93	62	48%	41%
2	Gin_48a5xa	D199E	G266A				E360V		A392V		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G	P553K	102,15	86,37	96	87	58%	46%
3	Gin_85b5xa	D199E	G266A	E269H			E360V		A392L		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G	P553K	101,73	86,34	92	62	60%	47%
4	Gin_24b5xa	D199E	G266A	E269T		G320A	E360V		A392L		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G	P553K	100,13	85,82	95	77	68%	65%
5	GIN_3B2_08.34a						E360V	A386I			D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T			98,48	87,82	99	68	50%	48%
6	GIN_3B2_08.58a						E360L		A392V		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G		98,00	87,65	91	48	45%	40%
7	Gin_48a4xa	D199E	G266A				E360V		A392V		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G		101,45	86,59	92	81	58%	52%
8	GIN_48a1xbxe		G266A				E360V		A392V		D447S	A449Y	E453W	A460W			A514Y	R516I	A517T	Q518G		100,10	85,73	88	98	75%	56%
9	GIN_3B2_08.85c						E360V		A392V		D447S	A449Y	E453W	A460W		N510H	A514T		A517T	Q518G		97,78	88,46	93	33	41%	40%
10	GIN_3B2_08.47a						E360L	A386I			D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T			98,33	88,10	92	38	pos.	pos.
11	GIN_3B3_05.84c				T301S		E360L		A392V		D447S	A449Y	E453W	A460W	V502C	N510H	A514T		A517T			97,18	87,96	90	32	pos.	pos.
12	GIN_3B2_10.87a				T301S		E360V		A392I		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T			96,99	87,81	81	29	pos.	pos.
13	GIN_3B3_03.04c						E360L	A386I	A392I		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T			97,06	87,79	68	24	pos.	pos.
14	GIN_3B3_01.11a						E360V		A392V		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T			99,01	87,75	102	49	pos.	40%
15	GIN_3B3_02.55a						E360V		A392I		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G		97,88	87,42	92	32	pos.	pos.
16	GIN_3B2_09.09c						E360L	A386I	A392V		D447S	A449Y	E453W		V502C	N510H	A514Y		A517T			98,23	87,38	80	18	pos.	pos.
17	GIN_3B3_05.09c						E360L	A386I			D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G		97,85	87,35	68	32	pos.	pos.
18	GIN_3B2_09.31a				T301S		E360L	A386I			D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G		98,41	86,97	79	29	pos.	pos.
19	GIN_3B3_01.52a						E360L		A392V		D447S	A449Y	E453W	A460W		N510H	A514Y		A517T			96,85	86,75	61	39	pos.	pos.
20	GIN_48a1xbxd		G266A				E360V		A392V	R412E	D447S	A449Y	E453W	A460W			A514Y		A517T	Q518G		100,30	86,59	90	89	58%	41%
21	GIN_3B3_01.21b				T301S		E360L	A386I	A392I		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G		98,50	86,59	94	34	pos.	pos.
22	Gin_28a5x2xf	D199E	G266A		T301S		E360L				D447S	A449Y	E453W		V502C	N510H	A514Y		A517T			95,73	85,83	74	51	pos.	pos.
23	Gin_28a5x3xa	D199E	G266A	E269H	T301S		E360L				D447S	A449Y	E453W		V502C	N510H	A514Y		A517T			95,81	85,38	75	34	pos.	pos.
24	Gin_85b4xa	D199E	G266A	E269H			E360V		A392L		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G		100,40	85,34	89	57	pos.	pos.
25	Gin_28a5x1xf	D199E			T301S		E360L				D447S	A449Y	E453W	A460W	V502C	N510H			A517T			94,77	84,81	95	35	55%	40%
26	Gin_28a4xa5x	D199E					E360V		A392V		D447S	A449Y	E453W		V502C	N510H	A514Y		A517T	Q518G	P553K	98,90	84,73	95	52	55%	pos.
27	GIN_48a1xbxg		G266A				E360V		A392V	R412D	D447S	A449Y	E453W	A460W			A514Y		A517T	Q518G		96,46	84,30	94	70	50%	pos.
28	Gin_28a4xa6x	D199E					E360L		A392V		D447S	A449Y	E453W		V502C	N510H	A514Y		A517T	Q518G	P553K	99,08	84,08	112	33	43%	pos.
29	Gin_28a5x1xg	D199E			T301S		E360L				D447S	A449Y	E453W		V502C	N510H	A514Y		A517T			>97	83,84	n.d.	32	pos.	pos.
30	GIN_3B3_05.20b						E360L		A392V		D447S	A449Y	E453W	A460W		N510H	A514Y		A517T			>97	83,75	80	30	54%	48%
31	GIN_3B2_08.81c				T301S		E360L	A386I			D447S	A449Y	E453W	A460W	V502C	N510H	A514T		A517T			96,61	83,73	73	28	46%	46%
32	Gin_28a5x2xd	D199E	G266A	E269T	T301S		E360L				D447S	A449Y	E453W		V502C	N510H			A517T			94,68	83,66	81	44	pos.	pos.
33	Gin_48a3xa	D199E	G266A						A392V		D447S	A449Y	E453W	A460W	V502C	N510H	A514Y		A517T	Q518G		97,99	82,38	101	42	50%	pos.

Table 4: Some distinct and combinatorial clones with particularly good performance

The following Table 5 shows the frequency of occurrence of given mutations preferred combinatorial and distinct variants. The frequency of occurrence is a measure for the role and importance of a given mutation.

Mutation	Frequency of occurence in preferred combinatorial and distinct variants (activated enzyme (IT50>80°C))
D447S	127
A449Y	127
A517T	127
N510H	125
E360L/V	125
V502C	120
E453W	92
A514Y/T	84
A460W	72
A386I	56
A392V/I	50
T301S	44
D199E	43
Q518G	36
P553K	20
E269T/H	12
G266A	19
D293Y	1
G320A	1
R412Q	1
E421R	1
A487Q	1
T461V	1

Mutation	Frequency of occurence in preferred combinatorial and distinct variants zymogen (IT50>85°C)
D447S	365
A449Y	353
A517T	256
N510H	175
E360V/L	262
V502C	185
E453W	320
A514Y/T	265
A460W	308
A386I	178
A392V/I	392
T301S	79
D199E	30
Q518G	250
P553K	6
E269T/H	133
G266A	133
D293Y	24
G320A	265
R412Q	74
E421R	11
A487Q	24
T461V	26
T461C	48

Mutation	Frequency of occurence in preferred combinatorial and distinct variants zymogen (IT50>85°C)
A331F	7
A331Y	9
A329Q/H/T	15
S435R/I	11
V274I	104
A372S	82
K283L	66
Q244C	46
T380C	30
A418W	28
I391W	21
A423V	18
T326L	16
I219L	16
S327L	16
M333I	16
N515G	13
A378G	12
S434G	12
A433G	10
S230D	9
Q393S	3
D399S	3
Y490W	3

Table 5: Frequency of occurrence of given mutations in preferred combinatorial and distinct variants. Frequency of occurrence is a measure for the role and importance of a given mutation.

The following Table 6 shows the impact of single mutations on ΔIT50 of the zymogen or the activated form. Again, the amount of impact of a single mutation on ΔIT50 is a measure for the role and importance of a said mutation.

Mutation	ΔIT50 Zymogen	Mutation	ΔIT50 Zymogen	Mutation	ΔIT50 activated Enzyme	Mutation	ΔIT50 activated Enzyme
D447S	4,8	L297T	1,2	A517S	7,7	D358G	0,7
E269H	4,0	S327F	1,2	N510H	7,6	A331Y	0,6
A392I	3,7	V455I	1,2	T301S	7,6	S327F	0,6
G320Q	3,6	T461V	1,2	G281R	5,4	T461C	0,6
A386I	3,6	T461C	1,2	Y287K	5,2	G320S	0,6
G320A	3,0	A478L	1,2	S435T	4,7	A386I	0,5
A392L	3,0	R516I	1,2	R412E	4,4	A460W	0,5
D447C	3,0	I219L	1,1	R516I	4,3	A514S	0,5
S230D	2,8	D293F	1,1	Q518G	4,1	S435R	0,5
A329Q	2,8	A328R	1,1	A517T	3,9	A190D	0,5
A392V	2,8	I330L	1,1	A475V	3,7	E421R	0,5
A418W	2,8	A423V	1,1	R516E	3,5	N291T	0,5
A386L	2,7	E453F	1,1	R412D	3,5	T301M	0,5
E269T	2,6	R516E	1,1	A342R	3,4	L540V	0,5
E453Y	2,6	Q542D	1,1	D447S	3,3	A328W	0,5
A460W	2,6	G320S	1,0	S354E	3,3	Q542S	0,5
V314L	2,5	A329T	1,0	A449N	3,3	A449E	0,4
M333I	2,5	E421R	1,0	E360L	3,1	Q542D	0,4
S435T	2,5	R166I	1,0	S315PP	3,0	A329H	0,3
E269M	2,4	V410I	1,0	E360V	2,9	S354Q	0,3
M333L	2,4	S262C	0,9	R412M	2,9	Y490W	0,3
E360V	2,4	T326W	0,9	W325K	2,7	L442W	0,3
A372S	2,4	Q361C	0,9	A392I	2,4	V455I	0,3
E453W	2,4	Q393S	0,9	R412Q	2,4	A449L	0,3
N510H	2,4	E459W	0,9	L442W	2,4	T196S	0,3
A514S	2,4	Q542H	0,9	E360C	2,3	V314M	0,3
E360C	2,3	P551N	0,9	A470V	2,3	P551R	0,3
A392M	2,3	D251S	0,8	D399S	2,1	P553K	0,3
D399S	2,3	D293Y	0,8	S435V	2,1	A329Q	0,2
V502C	2,3	T301C	0,8	A392M	2,0	A418W	0,2

Table 6: Impact of single mutations on ΔIT50 of the zymogen (left) or the activated form (right). The amount of impact of a single mutation on ΔIT50 is a measure for the role and importance of a said mutation.

Mutation	ΔIT50 Zymogen	Mutation	ΔIT50 Zymogen	Mutation	ΔIT50 activated Enzyme	Mutation	ΔIT50 activated Enzyme
A514T	2,2	S315P	0,8	V502C	1,9	L297T	0,2
E269C	2,1	A449L	0,8	A433G	1,9	A478L	0,2
A329H	2,1	Q497M	0,8	S324R	1,9	T326W	0,2
A331F	2,1	P553L	0,8	D402E	1,7	Q393S	0,2
A386V	2,1	T196S	0,7	T326L	1,6	P553L	0,2
E269Q	2,0	E228Q	0,7	S435I	1,6	Q497R	0,2
G281R	2,0	Q244G	0,7	V502T	1,6	A331F	0,1
S327W	2,0	N291T	0,7	A487Q	1,6	A328R	0,1
S354Q	2,0	T301M	0,7	G320Q	1,5	E228Q	0,1
A460R	2,0	L540V	0,7	A378G	1,5	Q361L	0,1
N515G	2,0	K283L	0,6	Q361C	1,5	E453W	0
T326K	1,9	T301S	0,6	Q244G	1,5	G266A	0
S434G	1,9	V314M	0,6	L338R	1,5	A386M	0
A449M	1,9	S324R	0,6	D447C	1,4	I391W	0
V274I	1,8	A328W	0,6	A386L	1,3	T461V	0
R412E	1,8	G388C	0,6	A514T	1,3	E269T	-0,1
S435R	1,8	D402E	0,6	V274I	1,3	E269M	-0,1
V455L	1,8	A470V	0,6	D447A	1,3	Q542H	-0,1
Q497Y	1,8	Q497R	0,6	D293F	1,3	G320A	-0,2
G266A	1,7	P551R	0,6	S324L	1,3	N515G	-0,2
T326R	1,7	D199E	0,5	A386V	1,2	K283L	-0,2
T326L	1,7	Q244C	0,5	T326K	1,2	S262C	-0,3
A386M	1,7	T308C	0,5	Q497Y	1,2	E459W	-0,3
I391W	1,7	R412Q	0,5	T326R	1,2	D251S	-0,3
S435I	1,7	R516L	0,5	A514D	1,2	Q202D	-0,3
A449Y	1,7	P553K	0,5	R516L	1,2	P551N	-0,4
D265T	1,7	Q202D	0,4	S327L	1,1	H305F	-0,4
S354E	1,6	H305F	0,4	A328D	1,1	A242S	-0,4
S435V	1,6	R412D	0,4	S327W	1,0	E269H	-0,5
D447A	1,6	Q542S	0,4	D293Y	1,0	E453F	-0,5
A449E	1,6	A242S	0,3	T301C	1,0	D306S	-0,5
A449N	1,6	D306S	0,3	D199E	1,0	A460R	-0,6
Q518G	1,6	M333Y	0,3	M333Y	1,0	M333I	-0,7
A190D	1,5	Q497D	0,3	Q497D	1,0	A372S	-0,7
S327L	1,5	A517S	0,3	T507L	1,0	S230D	-0,8
A328Y	1,5	A229W	0,2	N291S	1,0	T308C	-0,8
A378G	1,5	Y287K	0,2	A392L	0,9	A449M	-0,9

Table 6 ctd': Impact of single mutations on ΔIT50 of the zymogen (left) or the activated form (right). The amount of impact of a single mutation on ΔIT50 is a measure for the role and importance of a said mutation.

Mutation	ΔIT50 Zymogen	Mutation	ΔIT50 Zymogen	Mutation	ΔIT50 activated Enzyme	Mutation	ΔIT50 activated Enzyme
R412M	1,5	Q361L	0,2	A329T	0,9	A351S	-0,9
K483A	1,5	T507L	0,2	V455L	0,8	M333L	-1,0
Y490W	1,5	A548S	0,2	A328Y	0,8	E269C	-1,1
A514D	1,5	S324L	0,1	I330L	0,8	E269Q	-1,4
E360L	1,4	A487Q	0	A423V	0,8	H305W	-2,7
A433G	1,4	N291S	-0,2	Q497M	0,8	G388C	-3,5
L442W	1,4	W325K	-0,3	A392V	0,7	Q244C	-3,6
A328D	1,3	A475V	-0,3	E453Y	0,7	D265T	n.d.
A331Y	1,3	L338R	-0,5	V314L	0,7	R166I	n.d.
A351S	1,3	A342R	-0,6	S434G	0,7	V410I	n.d.
A514Y	1,3	L442W	-0,7	A449Y	0,7	A229W	n.d.
A517T	1,3	D358G	-2	K483A	0,7	A548S	n.d.

Table 6 ctd': Impact of single mutations on ΔIT50 of the zymogen (left) or the activated form (right). The amount of impact of a single mutation on ΔIT50 is a measure for the role and importance of a said mutation.

It is further to be understood that some mutations of Table 1 and Table 6 can interchangeably be used to engineer thermostability in Kumamolisin As. Table 7 shows a set of variants based on variant #1 of Table 7. In the course of engineering the mutations at position 502 and 510 seemed to change the activity at extrem acidic pH, below pH 2.

Excluding mutations at 502 and 510 reduced the thermostability significantly below the targeted temperature stability for the activated enzyme, as for example in Table 7, clone #2 which has a 7,8°C reduction in thermal stability compared to clone #1. A set of distinct variants were constructed by a rational approach taking advantage of the mutations identified and shown in Tables 1 and 6 to compensate for the effect of 502 and 510. With the exception of D399S substitutions can gradually or fully compensate the effect of mutations at 502 and 510.

#	Mutations in distinct clones and selected combinatorial clones																IT50 [°C]	IT50 [°C]
																	Zymogen	activated
1	G266A		E360V	A392V			D447S	A449Y	E453W	A460W		V502C	N510H	A514Y	A517T	Q518G	101,5	86,6
2	G266A		E360V	A392V			D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	>95	78,8
3	G266A		E360V	A392V	R412E,		D447S,	A449Y,	E453W,	A460W,				A514Y,	A517T,	Q518G,	100,3	86,6
4	G266A		E360V	A392V	R412D		D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	96,5	84,3
5	G266A		E360V	A392V	R412Q		D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	97,7	81,8
6	G266A		E360V	A392V		S435I	D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	97,4	81,4
7	G266A		E360V	A392V		A433G	D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	98,1	81,3
8	G266A	T326L	E360V	A392V			D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	98,7	81,1
9	G266A		E360V	A392V	R412M		D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	98,3	81,0
10	G266A		E360V	A392V		S435T	D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	99,2	81,0
11	G266A		E360V	A392V			D447S	A449Y	E453W	A460W	A487Q			A514Y	A517T	Q518G	96,3	81,0
12	G266A	T326K	E360V	A392V			D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	97,2	80,5
13	G266A		E360V	A392V			D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	96,2	80,4
14	Q244G	G266A	E360V	A392V			D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	96,3	80,2
15	G266A		E360V	A392V		S435V	D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	98,6	80,1
16	G266A		E360V	A392V	D399S		D447S	A449Y	E453W	A460W				A514Y	A517T	Q518G	87,90	71,92

Table 7: A set of variants based on variant #1

References:

Wlodawer A1, Li M, Gustchina A, Oyama H, Dunn BM, Oda K., Acta Biochim Pol. 2003;50(1):81-102

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Packer & Liu, Methods for the directed evolution of proteins. Nature Reviews Genetics 16, 379–394 (2015)

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Cadwell and Joyce, Mutagenic PCR. PCR Methods Appl. 3, 1994, 136-140

Okubo et al, 2006 Jun;273(11):2563-76.

What is claimed is:

1. A protease variant which is at least 90% identical to the full length amino acid sequence of a Kumamolisin AS backbone as set forth in any of SEQ ID NOs 1 – 3 while maintaining proteolytic activity, or a fragment, fraction or shuffled variant thereof maintaining proteolytic activity, which protease variant demonstrates altered or improved stability compared to

(i) the Kumamolisin AS wildtype as set forth in SEQ ID NO 4, or
(ii) the Kumamolisin AS backbone as set forth in any of SEQ ID NOs 1 – 3

2. A protease variant comprising an amino acid sequence derived from a Kumamolisin AS as set forth in SEQ ID NO. 1, or a fragment, fraction or shuffled variant thereof maintaining proteolytic activity, which protease variant has one or more amino acid substitutions at one or more residue positions in SEQ ID NO. 1 selected from the group consisting of D447, A449, A517, N510, V502, E453, E360, A514, A460, A392, A386, T301, D199, Q518, G266, P553, E269, R412, S435, G320, T326, T461, Q244, D293, A487, V274, A372, K283, T308, A418, I391, A423, A331, S327, I219, M333, A329, N515, A378, S434, E421, A433, S230, Q393, D399, Y490, G281, Y287, R516, A475, S354, S315P, W325, L442, A470, S324, Q361, A190, T196, Q202, E228, A229, A242, D251, S262, N291, L297, H305, D306, V314, A328, I330, L338, A342, A351, D358, G388, D402, V455, E459, A478, K483, Q497, T507, L540, Q542, A548, P551, R166 and/or D265.

3. The protease variant according to claim 2, which protease variant demonstrates at least one altered or improved stability compared to

(iii) the Kumamolisin AS wildtype as set forth in SEQ ID NO 4, or
(iv) the Kumamolisin AS backbone as set forth in any of SEQ ID NOs 1 – 3.

4. The protease variant according to any of claims 2 - 3, which protease variant has at least one amino acid substitution selected from the group consisting of D447S, A449Y, A517T, N510H, E360L, E360V, E360C, V502C, E453W, A514T, A514Y, A514D, A514S, A460W, A386I, A392V, A392L, A392I, A392M, T301S, D199E, Q518G, P553K, E269M, E269T, E269C, E269H, E269Q, G266A, D293Y, G320A, R412Q, E421R, A487Q, T461V, T461C, A331F, A331Y, A329Q, A329H, A329T, S435I, S435R, S435T, S435V, V274I, A372S, K283L,

Q244C, Q244G, T308C, A418W, I391W, A423V, T326R, T326W, T326L, T326K, I219L, S327F, S327L, S327W, M333I, N515G, A378G, S434G, A433G, S230D, Q393S, D399S, Y490W, A190D, T196S, Q202D, E228Q, A229W, A242S, D251S, S262C, G281R, Y287K, N291T, N291S, D293F, L297T, T301C, T301M, H305F, H305W, D306S, V314M, V314L, S315P, G320Q, G320S, S324L, S324R, W325K, A328W, A328D, A328R, A328Y, I330L, M333Y, M333L, L338R, A342R, A351S, S354E, S354Q, D358G, Q361C, Q361L, A386L, A386V, A386M, G388C, D402E, R412M, R412E, R412D, L442W, L442W, D447C, D447A, A449L, A449M, A449E, A449N, E453Y, E453F, V455I, V455L, E459W, A460R, A470V, A475V, A478L, K483A, Q497Y, Q497M, Q497D, Q497R, V502T, T507L, R516L, R516E, R516I, A517S, L540V, Q542H, Q542D, Q542S, A548S, P551N, P551R, P553L, R166I, D265T compared to the Kumamolisin AS as set forth in SEQ ID NO 1.

5. The protease variant according to any one of the aforementioned claims, which protease variant has at least 2 amino acid substitutions compared to the Kumamolisin AS backbone as set forth in SEQ ID NO 1 or 4.

6. The protease variant according to any one of the aforementioned claims, which protease variant has at least one, preferably at least two, more preferably at least three, more preferably at least four, more preferably at least five, most preferably at least six amino acid substitutions selected from the group consisting of D447S, A449Y, A517T, N510H, E360L, E360V, E360C, V502C, E453W, A514T, A514Y, A514D, A514S, A460W, A386I

7. The protease variant according to any one of the aforementioned claims, which protease variant has a set of substitutions at selected residues in the Kumamolisin AS backbone as set forth in SEQ ID NO 1 or 4, which set is at least one of the following

a) 360, 447, 449 and 510
b) 447, 449 and 514, and/or
c) 447, 449, 453, and 517.

8. The protease variant according to any one of the aforementioned claims, wherein said improved stability is improved thermostability (IT50) of either the activated enzyme or the zymogen.

9. The protease variant according to any one of the aforementioned claims, which protease variant has an IT50 of between ≥ 75°C and ≤ 105°C.

10. A nucleic acid molecule encoding a protease variant according to any one of the aforementioned claims.

11. A plasmid or vector system comprising the nucleic acid molecule according claims 10

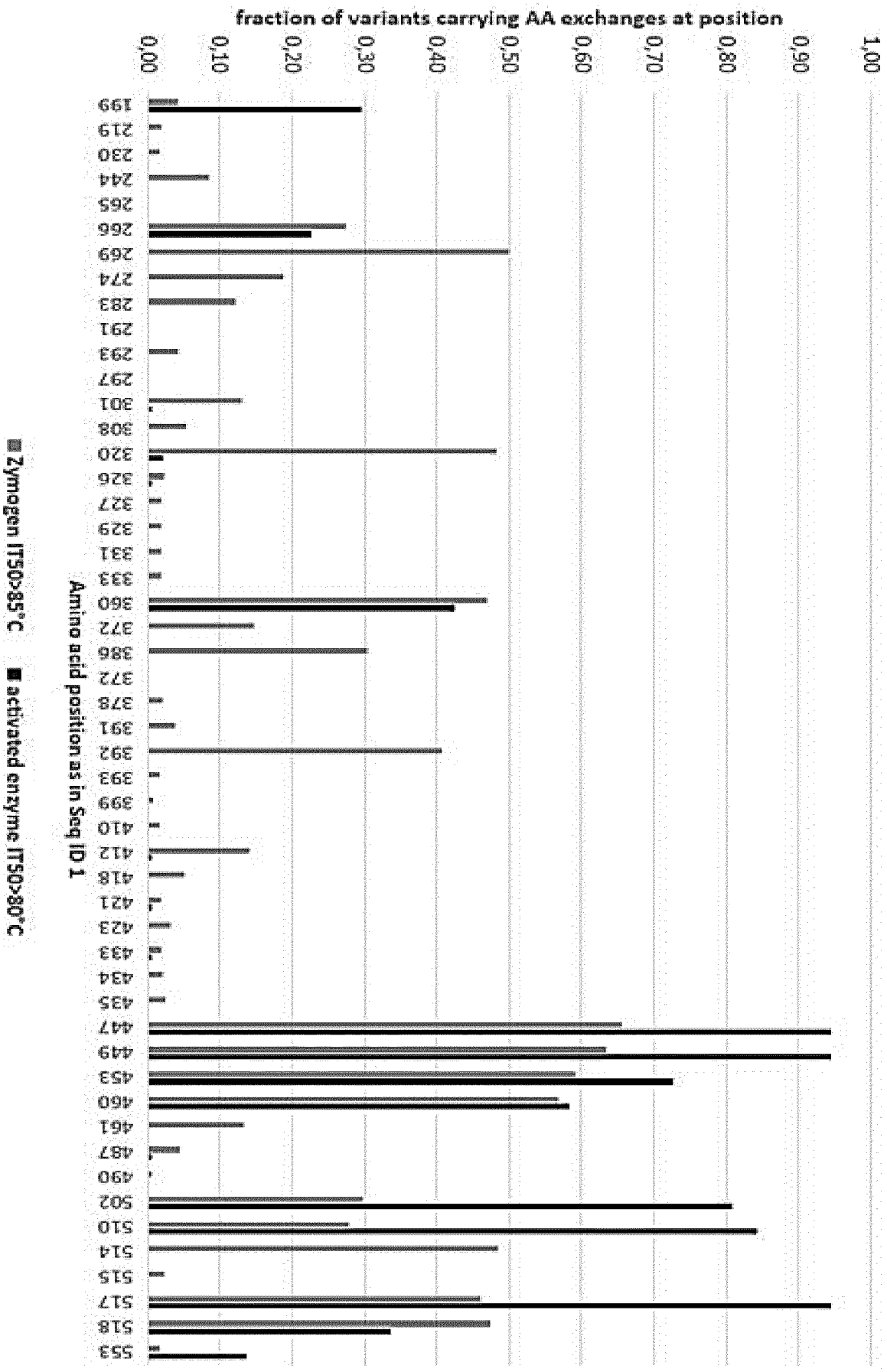
12. A composition comprising a protease variant or protease according to any one of the aforementioned claims, which composition has a pH of ≥ 5.

13. A feed additive, feed ingredient, feed supplement, and/or feedstuff comprising a protease variant or protease or a composition according to any one of the aforementioned claims.

14. Use of a protease variant according to any one of claims 1 – 9 or a composition according to claim 12 for the manufacture of a feedstuff.

15. A process of making a protease variant according to any one of the aforementioned claims, which process comprises:

i) mutagenizing a DNA, cDNA or mRNA encoding a Kumamolisin AS amino acid sequence as set forth in any of SEQ ID NOs 1 - 4
ii) expressing one or more mutants of Kumamolisin AS thus obtained, and
iii) testing the one or mutants of Kumamolisin AS for stability, preferably thermostability.


fraction of variants carrying AA exchanges at position
0,00
0,10
0,20
0,30
0,40
0,50
0,60
0,70
0,80
0,90
1,00
Amino acid position as in Seq ID 1
199
219
230
244
265
266
269
274
283
291
293
297
301
308
320
326
327
329
331
333
360
372
386
372
378
391
392
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410
412
418
421
423
433
434
435
447
449
453
460
461
487
490
502
510
514
515
517
518
553
Zymogen IT50>85°C
activated enzyme IT50>80°C

Fig. 1

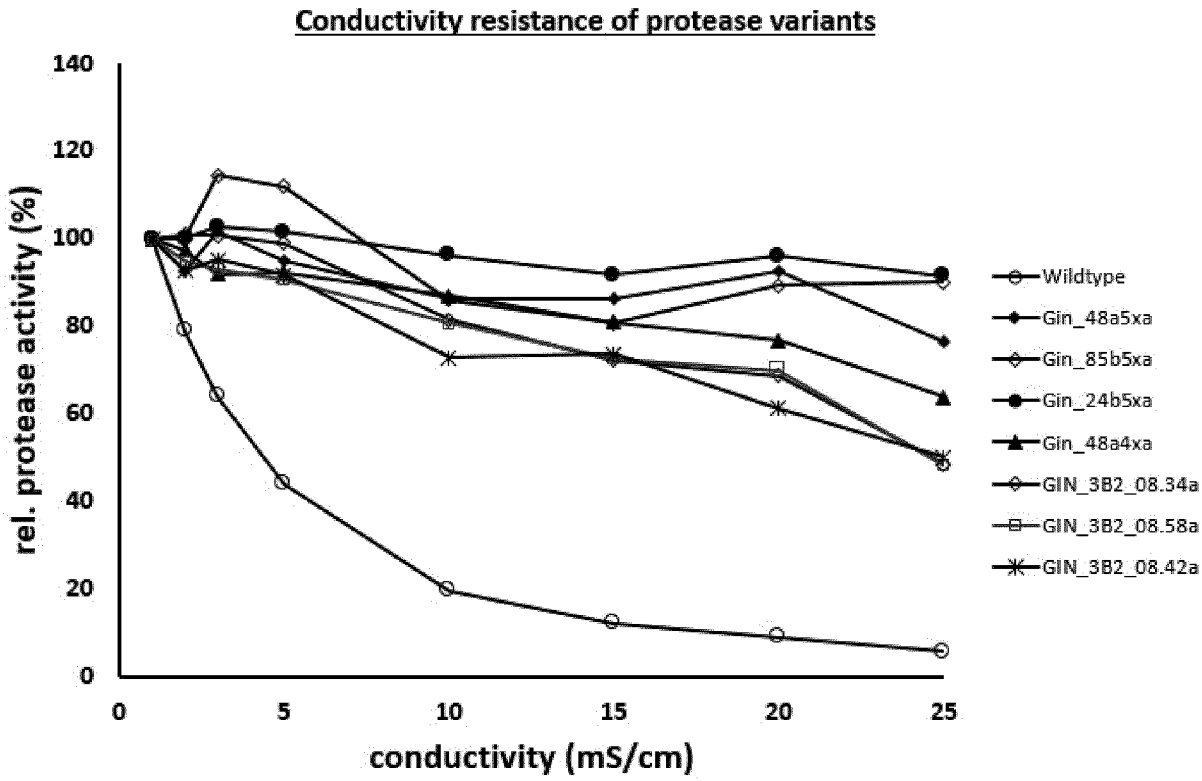
Conductivity resistance of protease variants
rel. protease activity (%)
140
120
100
80
60
40
20
0
0
5
10
15
20
25
conductivity (mS/cm)
Wildtype
Gin_48a5xa
Gin_85b5xa
Gin_24b5xa
Gin_48a4xa
GIN_3B2_08.34a
GIN_3B2_08.58a
GIN_3B2_08.42a

Fig. 2

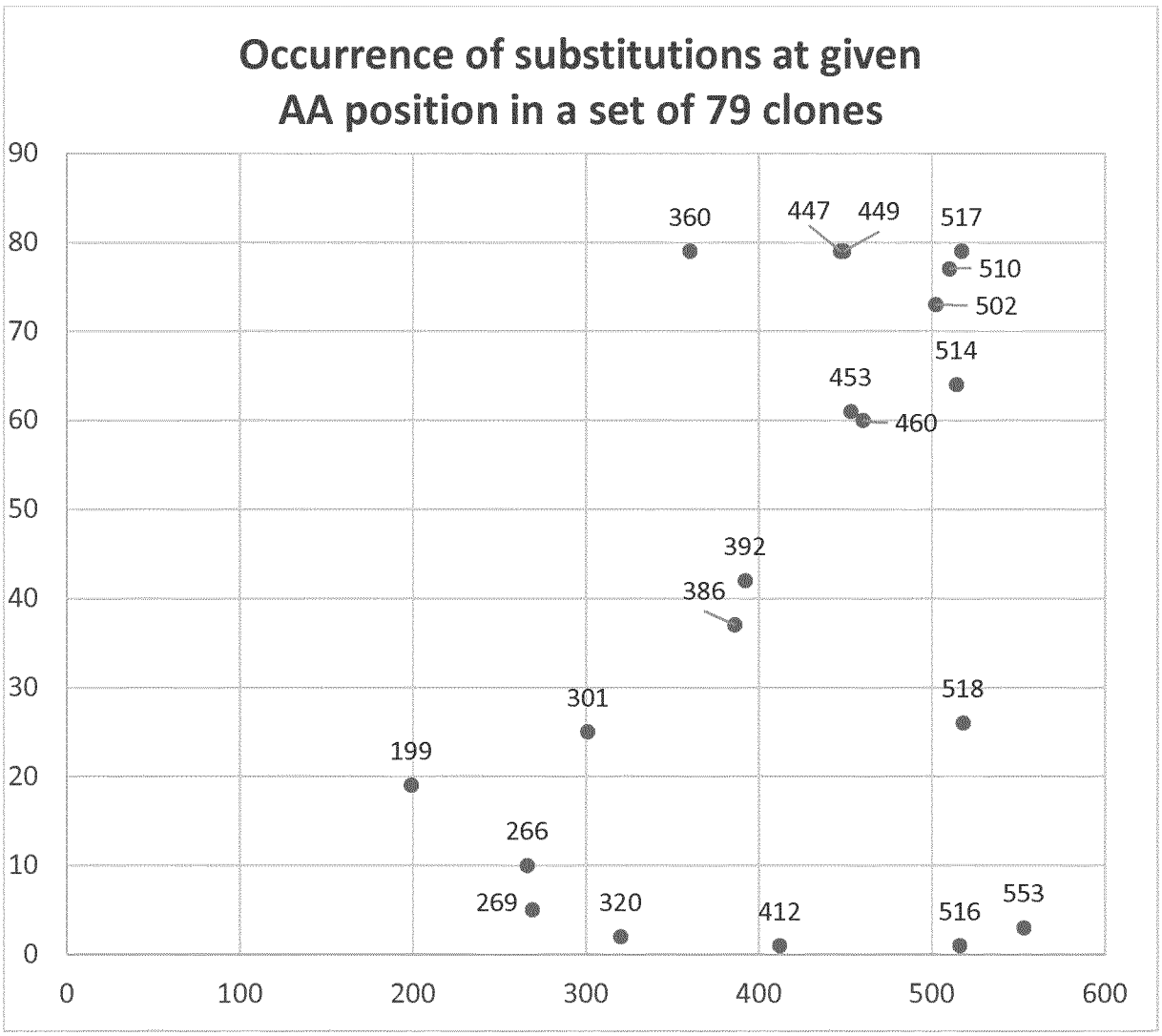
Occurrence of substitutions at given AA position in a set of 79 clones
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
360
447
449
517
510
502
514
453
460
392
386
301
518
199
266
269
320
412
516
553

Fig. 3

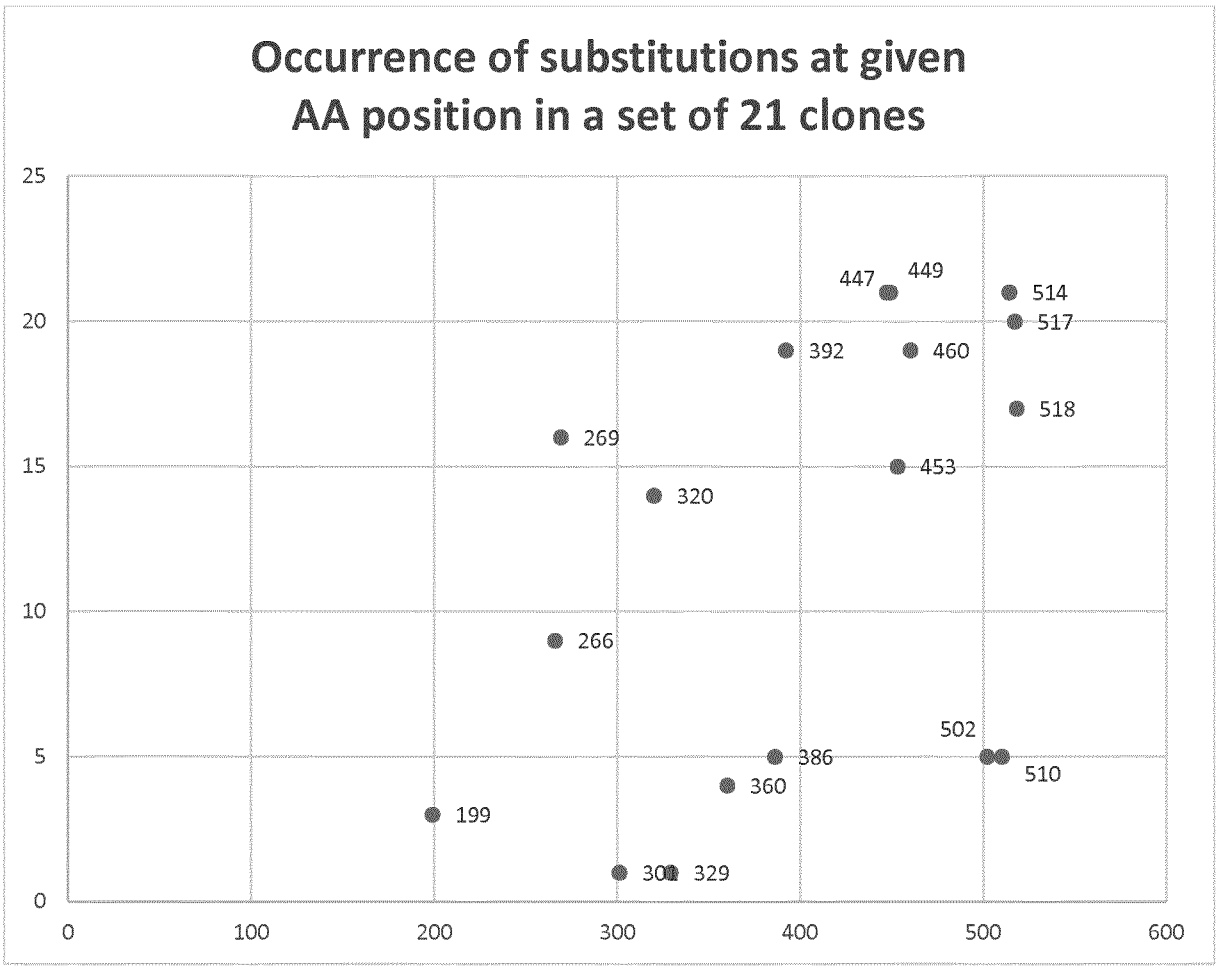
Occurrence of substitutions at given AA position in a set of 21 clones
25
20
15
10
5
0
0
100
200
300
400
500
600
447
449
514
517
392
460
518
269
453
320
266
502
386
510
360
199
329

Fig. 4

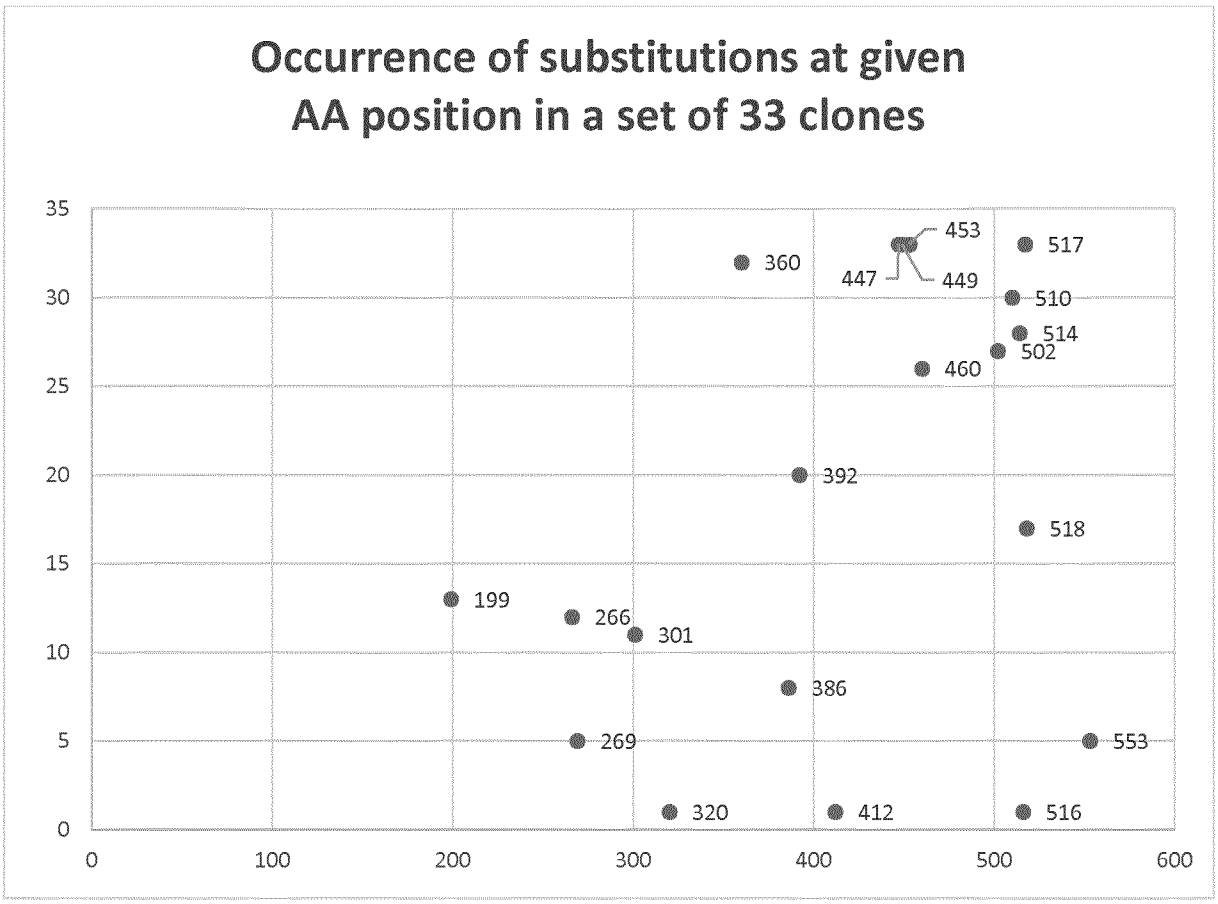
Occurrence of substitutions at given AA position in a set of 33 clones
35
30
25
20
15
10
5
0
0
100
200
300
400
500
600
360
453
447
449
517
510
514
502
460
392
518
199
266
301
386
269
553
320
412
516

Fig. 5