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(71) Applicant: **CHR. HANSEN A/S** [DK/DK]; Boege Alle 10-12, 2970 Hoersholm (DK).

(72) Inventors: **JAECKEL, Christian**; Ny Hjortespringvej 3, 3500 Vaerloese (DK). **LUND, Martin**; Holsteinsgade 29D, 2., 2100 Copenhagen Ø (DK). **VAN DEN BRINK, Johannes Maarten**; Skelhoejen 1, 2730 Herlev (DK).

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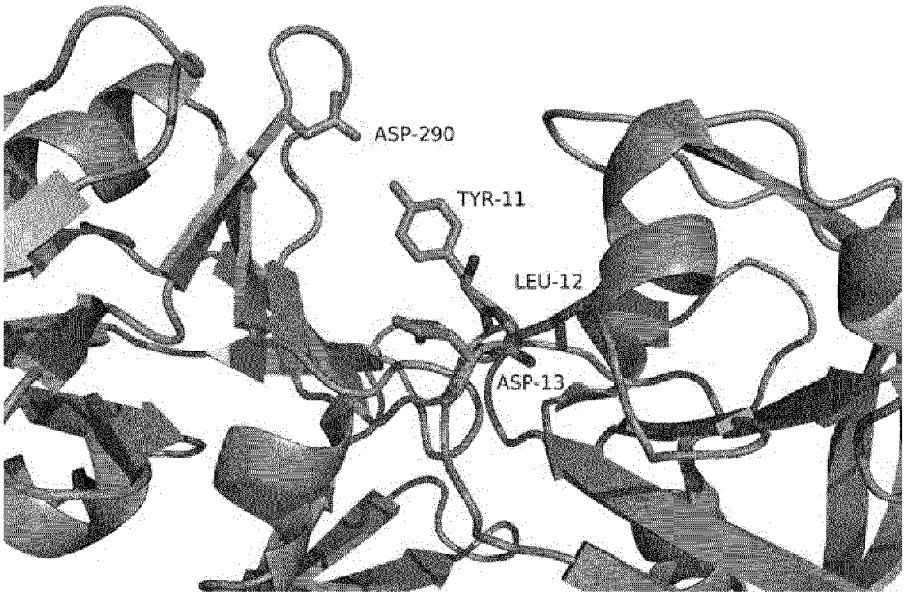
(54) Title: VARIANTS OF CHYMOSIN WITH IMPROVED MILK-CLOTTING PROPERTIES

Figure 4



(57) Abstract: Variants of chymosin with improved milk clotting properties.

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TITLE: Variants of chymosin with improved milk-clotting properties

FIELD OF THE INVENTION

The present invention relates to variants of chymosin with improved milk-clotting properties.

BACKGROUND ART

Chymosin (EC 3.4.23.4) and pepsin (EC 3.4.23.1), the milk clotting enzymes of the mammalian stomach, are aspartic proteases belonging to a broad class of peptidases.

When produced in the gastric mucosal cells, chymosin and pepsin occur as enzymatically inactive pre-prochymosin and pre-pepsinogen, respectively. When chymosin is excreted, an N-terminal peptide fragment, the pre-fragment (signal peptide) is cleaved off to give prochymosin including a pro-fragment. Prochymosin is a substantially inactive form of the enzyme which, however, becomes activated under acidic conditions to the active chymosin by autocatalytic removal of the pro-fragment. This activation occurs *in vivo* in the gastric lumen under appropriate pH conditions or *in vitro* under acidic conditions.

The structural and functional characteristics of bovine, i.e. *Bos taurus*, pre-prochymosin, prochymosin and chymosin have been studied extensively. The pre-part of the bovine pre-prochymosin molecule comprises 16 aa residues and the pro-part of the corresponding prochymosin has a length of 42 aa residues. The active bovine chymosin comprises 323 aa.

Chymosin is produced naturally in mammalian species such as bovines, camels, caprines, buffaloes, sheep, pigs, humans, monkeys and rats.

Bovine and camel chymosin has for a number of years been commercially available to the dairy industry.

Enzymatic coagulation of milk by milk-clotting enzymes, such as chymosin and pepsin, is one of the most important processes in the manufacture of cheeses. Enzymatic milk coagulation is a two-phase process: a first phase where a proteolytic enzyme, chymosin or pepsin, attacks κ-casein, resulting in a metastable

state of the casein micelle structure and a second phase, where the milk subsequently coagulates and forms a coagulum (reference 1).

WO02/36752A2 (Chr. Hansen) describes recombinant production of camel chymosin.

WO2013/174840A1 (Chr. Hansen) describes mutants/variants of bovine and camel chymosin.

WO2013/164479A2 (DSM) describes mutants of bovine chymosin.

The references listed immediately below may in the present context be seen as references describing mutants of chymosin:

- Suzuki et al: Site directed mutagenesis reveals functional contribution of Thr218, Lys220 and Asp304 in chymosin, Protein Engineering, vol. 4, January 1990, pages 69-71;
- Suzuki et al: Alteration of catalytic properties of chymosin by site-directed mutagenesis, Protein Engineering, vol. 2, May 1989, pages 563-569;
- van den Brink et al: Increased production of chymosin by glycosylation, Journal of biotechnology, vol. 125, September 2006, pages 304-310;
- Pitts et al: Expression and characterisation of chymosin pH optima mutants produced in Tricoderma reesei, Journal of biotechnology, vol. 28, March 1993, pages 69-83;
- M.G. Williams et al: Mutagenesis, biochemical characterization and X-ray structural analysis of point mutants of bovine chymosin, Protein engineering design and selection, vol. 10, September 1997, pages 991-997;
- Strop et al: Engineering enzyme subsite specificity: preparation, kinetic characterization, and x-ray analysis at 2.0 ANG resolution of Val111phe site mutated calf chymosin, Biochemistry, vol. 29, October 1990, pages 9863-9871;
- Chitpinityol et al: Site-specific mutations of calf chymosin B which influence milk-clotting activity, Food Chemistry, vol. 62, June 1998, pages 133-139;
- Zhang et al: Functional implications of disulfide bond, Cys45-Cys50, in recombinant prochymosin, Biochimica et biophysica acta, vol. 1343, December 1997, pages 278-286.

None of the prior art references mentioned above describe directly and unambiguously any of the chymosin variants with improved specific clotting activity or increased C/P ratios compared to the parent from which the variant is derived, as described below.

SUMMARY OF THE INVENTION

The problem to be solved by the present invention is to provide variants of chymosin which when compared to the parent polypeptide, have either increased specific clotting activity or increased C/P ratio or both.

Based on intelligent design and comparative analyses of different variants, the present inventors identified a number of amino acid positions that are herein important in the sense that by making a variant in one or more of these positions in a parent peptide one may get an improved chymosin variant with either increased specific clotting activity or increased C/P ratios or both.

The amino acid numbering as used herein to specify the variant is based on the mature peptide. As known in the art – different natural wildtype chymosin polypeptide sequences obtained from different mammalian species (such as e.g. bovines, camels, sheep, pigs, or rats) are having a relatively high sequence similarity/identity. In figure 1 this is exemplified by an alignment of herein relevant different chymosin sequences.
In view of this relatively close sequence relationship – it is believed that the 3D structures of different natural wildtype chymosins are also relatively similar.

In the present context – a naturally obtained wildtype chymosin (such as bovine chymosin or camel chymosin) may herein be an example of a parent polypeptide – i.e. a parent polypeptide to which an alteration is made to produce a variant chymosin polypeptide of the present invention.

Without being limited to theory – it is believed that the herein discussed chymosin related amino acid positions are of general importance in any herein relevant chymosin enzyme of interest (e.g. chymosins of e.g. bovines, camels, sheep, pigs, rats etc.) – in the sense that by making a variant in one or more of these positions one may get an improved chymosin variant in general (e.g. an improved bovine, camel, sheep, pig or rat chymosin variant).

As discussed herein - as a reference sequence for determining the amino acid position of a parent chymosin polypeptide of interest (e.g. camel, sheep, bovine etc.) is herein used the public known *Camelius dromedarius* mature chymosin

sequence of SEQ ID NO:2 herein. It may herein alternatively be termed camel chymosin. The sequence is also shown in Figure 1 herein.

In the present context it is believed that a parent chymosin polypeptide (e.g. from sheep or rat) that has at least 80% sequence identity with the mature polypeptide of SEQ ID NO:2 (camel chymosin) may herein be seen as sufficient structural related to e.g. bovine or camel chymosin in order to be improved by making a variant in any of the amino acid positions as described herein.

Embodiments of the present invention are described below.

DEFINITIONS

All definitions of herein relevant terms are in accordance of what would be understood by the skilled person in relation to the herein relevant technical context.

The term "chymosin" relates to an enzyme of the EC 3.4.23.4 class. Chymosin has a high specificity and predominantly clots milk by cleavage of a single 104-Ser-Phe-|-Met-Ala-107 bond in κ-chain of casein. As a side-activity, chymosin also cleaves α-casein primarily between Phe23 and Phe24 and β-casein primarily between Leu192 and Tyr193 (references 2, 3). The resulting peptides αS1(1-23) and β(193-209) will be further degraded by proteases from microbial cultures added to the ripening cheese (reference 4). An alternative name of chymosin used in the art is rennin.

The term "chymosin activity" relates to chymosin activity of a chymosin enzyme as understood by the skilled person in the present context.

The skilled person knows how to determine herein relevant chymosin activity.

As known in the art – the herein relevant so-called C/P ratio is determined by dividing the specific clotting activity (C) with the proteolytic activity (P).
As known in the art - a higher C/P ratio implies generally that the loss of protein during e.g. cheese manufacturing due to non-specific protein degradation is reduced which may lead to cheese yield improvements.

The term "isolated variant" means a variant that is modified by the act of man. In one aspect, the variant is at least 1% pure, e.g., at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, and at least 90% pure, as determined by SDS PAGE.

The term "mature polypeptide" means a peptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In the present context may a herein relevant mature chymosin polypeptide be seen as the active chymosin polypeptide sequence – i.e. without the pre-part and/or pro-part sequences. Herein relevant examples of a mature polypeptide are e.g. the mature polypeptide of SEQ ID NO:1 (bovine chymosin), which is from amino acid position 59 to amino acid position 381 of SEQ ID NO:1 or the mature polypeptide of SEQ ID NO:2 (camel chymosin), which is from amino acid position 59 to amino acid position 381 of SEQ ID NO:2.

The term "parent" or "parent polypeptide having chymosin activity" means a polypeptide to which an alteration is made to produce the enzyme variants of the present invention. The parent may be a naturally occurring (wild-type) polypeptide or a variant thereof.

The term "Sequence Identity" relates to the relatedness between two amino acid sequences or between two nucleotide sequences.

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 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 nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

For purposes of the present invention, the degree of sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), 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 EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:
(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment).

The term "variant" means a peptide having chymosin activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion, at one or more (several) 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 1-3 amino acids adjacent to an amino acid occupying a position.
The amino acid may be natural or unnatural amino acids – for instance, substitution with e.g. a particularly D-isomers (or D-forms) of e.g. D-alanine could theoretically be possible.

The term "wild-type" peptide refers to a nucleotide sequence or peptide sequence as it occurs in nature, i.e. nucleotide sequence or peptide sequence which hasn't been subject to targeted mutations by the act of man.

DRAWINGS

Figure 1: An alignment of herein relevant different chymosin sequences.
As understood by the skilled person in the present context – herein relevant sequence identity percentages of mature polypeptide sequences of e.g. sheep, C. bactrianus, camel, pig or rat chymosin with the mature polypeptide of SEQ ID NO:3 (bovine chymosin – i.e. amino acid positions 59 to 381 of SEQ ID NO:3) are relatively similar to above mentioned sequence identity percentages.

Figure 2:

3D structure of camel chymosin (detail, PDB: 4AA9) with a model of bound κ-casein shown in green. κ-casein is placed in the chymosin substrate binding cleft with the scissile bond between residues 105 and 106. Mutations R242E, Y243E, N249D, G251D, N252D, R254E, S273D, Q280E, F282E are highlighted in blue.

Figure 3:

3D structure of bovine chymosin (PDB: 4AA8) with a model of bound κ-casein shown in green. κ-casein is placed in the chymosin substrate binding cleft with the scissile bond between residues 105 and 106. Positions H292 and Q294 are highlighted in yellow.

Figure 4:

3D structure of camel chymosin (detail, PDB: 4AA9). Residues Y11, L12, and D13 of the protein N-terminus as well as the potential Y11 interaction partner D290 are highlighted in purple.

DETAILED DESCRIPTION OF THE INVENTION

Determining the amino acid position of a chymosin of interest

As discussed above - as a reference sequence for determining the amino acid position of a herein relevant chymosin polypeptide of interest (e.g. camel, sheep, bovine etc.) is herein used the public known camel chymosin sequence disclosed as SEQ ID NO:2 herein.

The amino acid sequence of another chymosin polypeptide is aligned with the polypeptide disclosed in SEQ ID NO:2, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the polypeptide disclosed in SEQ ID NO:2 is determined using the ClustalW algorithm as described in working Example 1 herein.

Based on above well-known computer programs – it is routine work for the skilled person to determine the amino acid position of a herein relevant chymosin polypeptide of interest (e.g. camel, sheep, bovine etc.).

In figure 1 herein is shown an example of an alignment.
Just as an example – in figure 1 can e.g. be seen that herein used bovine reference SEQ ID NO:3 has a "G" in position 50 and "*Camelus_dromedarius*" (SEQ ID NO:2 herein) has an "A" in this position 50.

Nomenclature of variants

In describing the variants of the present invention, the nomenclature described below is adapted for ease of reference. The accepted IUPAC single letter or three letter amino acid abbreviations are employed.
The specific variants discussed in this "nomenclature" section below may not be herein relevant variants of the present invention – i.e. this "nomenclature" section is just to describe the herein relevant used nomenclature as such.

Substitutions. For an amino acid substitution, the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, a theoretical substitution of threonine with alanine at position 226 is designated as "Thr226Ala" or "T226A". Multiple mutations are separated by addition marks ("+"), *e.g.*, "Gly205Arg + Ser411Phe" or "G205R + S411F", representing substitutions at positions 205 and 411 of glycine (G) with arginine (R) and serine (S) with phenylalanine (F), respectively. A substitution e.g. designated "226A" refers to a substitution of a parent amino acid (e.g. T, Q, S or another parent amino acid) with alanine at position 226.

Deletions. For an amino acid deletion, the following nomenclature is used: Original amino acid, position, *. Accordingly, the deletion of glycine at position 195 is designated as "Gly195*" or "G195*". Multiple deletions are separated by addition marks ("+"), *e.g.*, "Gly195* + Ser411*" or "G195* + S411*".

Insertions. For an amino acid insertion, the following nomenclature is used: Original amino acid, position, original amino acid, inserted amino acid. Accordingly the insertion of lysine after glycine at position 195 is designated "Gly195GlyLys" or "G195GK". An insertion of multiple amino acids is designated [Original amino acid, position, original amino acid, inserted amino acid #1, inserted amino acid #2; etc.]. For example, the insertion of lysine and alanine after glycine at position 195 is indicated as "Gly195GlyLysAla" or "G195GKA".

In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s). In the above example, the sequence would thus be:

Parent:	Variant:
195	195 195a 195b
G	G - K - A

Multiple alterations. Variants comprising multiple alterations are separated by addition marks ("+"), *e.g.*, "Arg170Tyr+Gly195Glu" or "R170Y+G195E" representing a substitution of tyrosine and glutamic acid for arginine and glycine at positions 170 and 195, respectively.

Different substitutions. Where different substitutions can be introduced at a position, the different substitutions are separated by a comma, *e.g.*, "Arg170Tyr,Glu" or "R170Y,E" represents a substitution of arginine with tyrosine or glutamic acid at position 170. Thus, "Tyr167Gly,Ala + Arg170Gly,Ala" or "Y167G,A + R170G,A" designates the following variants:
"Tyr167Gly+Arg170Gly", "Tyr167Gly+Arg170Ala", "Tyr167Ala+Arg170Gly", and "Tyr167Ala+Arg170Ala".

Preferred parent polypeptide having chymosin activity
Preferably, the parent polypeptide has at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% sequence identity with the mature polypeptide of SEQ ID NO:3 (bovine chymosin) and/or SEQ ID NO:2 (camel chymosin).

Just as an example – a herein suitable relevant parent polypeptide could e.g. be bovine chymosin A – as known in the art bovine chymosin A may only have one amino acid difference as compared to bovine chymosin B of SEQ ID NO:3 herein.

In a preferred embodiment - the parent polypeptide has at least 90% sequence identity with the mature polypeptide of SEQ ID NO:3 (bovine chymosin), more preferably the parent polypeptide has at least 95% sequence identity with the mature polypeptide of SEQ ID NO:3 (bovine chymosin) and even more preferably the parent polypeptide has at least 97% sequence identity with the mature poly-

peptide of SEQ ID NO:3 (bovine chymosin). It may be preferred that the parent polypeptide is the mature polypeptide of SEQ ID NO:3 (bovine chymosin).

As understood by the skilled person in the present context – a herein relevant parent polypeptide having chymosin activity may already e.g. be a variant of e.g. a corresponding wildtype chymosin.

For instance, a bovine chymosin variant with e.g. 5-10 alterations (e.g. substitutions) as compared to mature wildtype bovine chymosin polypeptide of SEQ ID NO:3 may still be a parent polypeptide that has at least 95% sequence identity with the mature polypeptide of SEQ ID NO:3 (Bovine chymosin).

Said in other words and in general - a herein relevant isolated chymosin polypeptide variant may comprise alterations (e.g. substitutions) in other positions than the positions claimed herein.

As understood by the skilled person in the present context – a parent polypeptide may be a polypeptide that has at least 80% sequence identity with the mature polypeptide of SEQ ID NO:2 (Camel). In a preferred embodiment - the parent polypeptide has at least 92% sequence identity with the mature polypeptide of SEQ ID NO:2 and/or SEQ ID NO:3, more preferably the parent polypeptide has at least 95% sequence identity with the mature polypeptide of SEQ ID NO:2 and/or SEQ ID NO:3 and even more preferably the parent polypeptide has at least 97% sequence identity with the mature polypeptide of SEQ ID NO:2 or SEQ ID NO:3. It may be preferred that the parent polypeptide is the mature polypeptide of SEQ ID NO:2 (camel chymosin).

As understood by the skilled person in the present context – an isolated chymosin variant may comprise alterations (e.g. substitutions) in other amino acid positions than given above.
For instance, a bovine chymosin variant with e.g. 5-10 alterations (e.g. substitutions) as compared to wildtype camel chymosin polypeptide of SEQ ID NO:2 will still be a parent polypeptide that has at least 95% sequence identity with the mature polypeptide of SEQ ID NO:2.

It may be preferred that the isolated bovine chymosin variant comprises less

than 30 amino acid alterations (e.g. substitutions) as compared to the mature polypeptide of SEQ ID NO:2 (camel chymosin) or it may be preferred that the isolated camel chymosin variant comprises less than 20 amino acid alterations (e.g. substitutions) as compared to the mature polypeptide of SEQ ID NO:2 or it may be preferred that the isolated bovine chymosin variant comprises less than 10 amino acid alterations (e.g. substitutions) as compared to the mature polypeptide of SEQ ID NO:2 or it may be preferred that the isolated camel chymosin variant comprises less than 5 amino acid alterations (e.g. substitutions) as compared to the mature polypeptide of SEQ ID NO:2 (camel chymosin).

Method for making isolated chymosin polypeptide variants

As discussed above - as known in the art, the skilled person may, based on his common general knowledge, routinely produce and purify chymosin and chymosin variants.
Said in other words, once the skilled person is in possession of a herein relevant parent polypeptide having chymosin activity of interest (e.g. from bovines, camels, sheep, pigs, or rats) it is routine work for the skilled person to make a variant of such a parent chymosin of interest when guided by present disclosure.

An example of a suitable method to produce and isolate a chymosin (variant or parent) may be by well-known e.g. fungal recombinant expression/production based technology as e.g. described in WO02/36752A2 (Chr. Hansen).

It is also routine work for the skilled person to make alteration at one or more positions in a parent polypeptide having chymosin activity, wherein the alteration is comprising a substitution, a deletion or an insertion in at least one amino acid position as disclosed herein.

As known to the skilled person – this may e.g. be done by so-called site directed mutagenesis and recombinant expression/production based technology.

It is also routine work for the skilled person to determine if a herein relevant parent polypeptide (e.g. camel or bovine wildtype chymosin) and/or a herein relevant variant has chymosin activity or not.

As known in the art – chymosin specificity may be determined by the so-called C/P ratio, which is determined by dividing the specific clotting activity (C) with the proteolytic activity (P). As known in the art - a higher C/P ratio implies generally that the loss of protein during e.g. cheese manufacturing due to non-specific protein degradation is reduced, i.e. the yield of cheese is improved.

Determination of milk clotting activity

Milk clotting activity may be determined using the REMCAT method, which is the standard method developed by the International Dairy Federation (IDF method). Milk clotting activity is determined from the time needed for a visible flocculation of a standard milk substrate prepared from a low-heat, low fat milk powder with a calcium chloride solution of 0.5 g per liter (pH ≈ 6.5). The clotting time of a rennet sample is compared to that of a reference standard having known milk-clotting activity and having the same enzyme composition by IDF Standard 110B as the sample. Samples and reference standards are measured under identical chemical and physical conditions. Variant samples are adjusted to approximately 3 IMCU/ml using an 84 mM acetic acid buffer pH 5.5. Hereafter, 20 µl enzyme preparation is added to 1 ml preheated milk (32°C) in a glass test tube placed in a water bath, capable of maintaining a constant temperature of 32°C ± 1°C under constant stirring.

The total milk-clotting activity (strength) of a rennet is calculated in International Milk-Clotting Units (IMCU) per ml relative to a standard having the same enzyme composition as the sample according to the formula:

$$\text{Strength in IMCU/ml} = \frac{\text{Sstandard} \times \text{Tstandard} \times \text{Dsample}}{\text{Dstandard} \times \text{Tsample}}$$

Sstandard:	The milk-clotting activity of the international reference standard for rennet.
Tstandard:	Clotting time in seconds obtained for the standard dilution.
Dsample:	Dilution factor for the sample
Dstandard:	Dilution factor for the standard
Tsample:	Clotting time in seconds obtained for the diluted rennet sample from addition of enzyme to time of flocculation.

For clotting activity determination the µIMCU method may be used instead of the REMCAT method. As compared to REMCAT, flocculation time of chymosin vari-

ants in the μIMCU assay is determined by OD measurements in 96-well microtiter plates at 800 nm in a UV/VIS plate reader. A standard curve of various dilutions of a reference standard with known clotting strength is recorded on each plate. Samples are prepared by diluting enzyme in 84 mM acetate buffer, 0.1% triton X-100, pH 5.5. Reaction at 32°C is started by adding 250 uL of a standard milk substrate containing 4% (w/w) low-heat, low fat milk powder and 7.5% (w/w) calcium chloride (pH ≈ 6.5) to 25 uL enzyme sample. Milk clotting activity of chymosin variants in International Milk-Clotting Units (IMCU) per ml is determined based on sample flocculation time relative to the standard curve.

Determination of total protein content

Total protein content may preferably be determined using the Pierce BCA Protein Assay Kit from Thermo Scientific following the instructions of the providers.

Calculation of specific clotting activity

Specific clotting activity (IMCU/mg total protein) was determined by dividing the clotting activity (IMCU/ml) by the total protein content (mg total protein per ml).

Determination of proteolytic activity

General proteolytic activity may preferably be measured using fluorescently labelled Bodipy-FL casein as a substrate (EnzChek; Molecular Bioprobes, E6638). Casein derivatives heavily labeled with pH-insensitive green-fluorescent Bodipy-FL result in quenching of the conjugate's fluorescence. Protease catalyzed hydrolysis releases fluorescent Bodipy-FL. This method is very sensitive which was essential for this experiment as the reference has the lowest general proteolytical activity of all coagulants known to date. A 0.04 mg/ml substrate solution is prepared in 0.2M phosphate buffer pH 6.5, containing 100mM NaCl, 5% glycerol, and 0.1% Brij. Chymosin variants are dissolved in 20mM malonate buffer, containing 100mM NaCl, 5% glycerol, and 0.1% Brij. Of both reference and chymosin variant solutions, 20μL are mixed in a black 384-well Corning flat bottom polystyrene microtitter plate and fluorescence was continuously recorded in a fluorometer at 32C for 10 hours. Slopes of the linear part of fluorescence change are used to determine general proteolytic activity.

Determination of the C/P ratio

The C/P ratio is calculated by dividing the clotting activity (C) with the proteolytic activity (P).

Statistical analysis of the positional and mutational effects on specific clotting activity and C/P ratio

A statistical machine-learning approach and PCA-based analysis may preferably be used to determine the effects of single mutations present in the multi-substitution variants, i.e. specific milk clotting activity, as well as on the ratio of clotting and general proteolytic activity (C/P).

Preferred embodiments of the invention

As outlined above and illustrated in the examples below, the inventors of present disclosure have made a number of preferred chymosin polypeptide variants with improved clotting activity and/or C/P ratio when compared to the corresponding parent polypeptide under comparable conditions.

In a preferred aspect, the present invention relates to an isolated chymosin polypeptide variant comprising an alteration in one or more positions compared to a parent polypeptide having chymosin activity, wherein the alteration comprise a substitution, a deletion or an insertion in at least one amino acid position corresponding to any of positions: D59, V309, S132, N249, L166, N249, Q56, Y134, K295, M157, M256, R242, I96, H76, S164, S273, G251, Y11, L166, K19, Y21, S74, Y243, N249, S273, Q280, F282, L295, N252, R254, G70, V136, L222, K231, N291.

wherein

(i): the amino acid position of the parent polypeptide is determined by an alignment of the parent polypeptide with the polypeptide of SEQ ID NO:2 and

(ii): the parent polypeptide has at least 80% sequence identity with the mature polypeptide of SEQ ID NO:2 (camel chymosin);

wherein the isolated chymosin polypeptide variant has a higher specific clotting activity and/or C/P ratio than its corresponding parent polypeptide.

More specifically, the isolated chymosin polypeptide variant of present invention has a specific clotting activity (IMCU/mg total protein) that is at least 85% such as e.g. at least 90%, 100%, 110%, 120%, 130%, 160% or 200% of the specific clotting activity of its parent polypeptide. Alternatively or additionally, the isolated chymosin polypeptide variant of present invention has a C/P ratio that is at least 200%, such as e.g. at least 400%, at least 500%, at least 750% or at least 1000% of the C/P ratio of its parent polypeptide.

As specified above, the parent polypeptide may have at least 80%, such as at least e.g. 82%, 85%, 95%, 97%, 98%, 99% or 100% sequence identity with the mature polypeptide of SEQ ID NO:2 (camel chymosin).

The alteration may comprise a substitution selected from a list consisting of: D59N, V309I, S132A, N249E, L166V, N249D, Q56H, Y134G, K295L, M157L, M256L, R242E, I96L, H76Q, S164G, S273Y, G251D, Y11I, R242D, L222V, Y11V, L166I, K19T, Y21S, S74D, Y243E, N249D, S273D, Q280E, F282E, L295K, N252D, R254E, G70D, V136I, L222I, K231N, N291Q.

In a related embodiment, the present invention relates to an isolated chymosin polypeptide variant wherein the alteration comprises one or more combinations of substitutions comprising:
Y11V, K19T, D59N, I96L, S164G, L166V, L222V, R242E, N249E, L253I;
Y11I, D59N, I96L, S164G, L166V, L222V, R242E, G251D, L253I;
Y11I, I96L, S164G, L222I, R242E;
Y11I, K19T, D59N, I96L, S164G, L222I, R242E, N249E, G251D;
H76Q, I96L, S164G, L222I, R242E, G251D, S273Y;
K19T, D59N, H76Q, S164G, L222I, N249D, S273Y;
K19T, D59N, H76Q, L166V, L222I, R242E, G251D, S273Y;
K19T, D59N, H76Q, S132A, L222I, G251D, S273Y, V309I;
Y21S, H76Q, S164G, L222I, R242E, G251D, S273Y;
D59N, S132A, S164G, L222I, R242E, N249D, G251D, S273Y;
D59N, H76Q, I96L, S132A, S164G, L166V, L222I, G251D, S273Y;
H76Q, S164G, L166V, L222I, R242E, G251D, S273Y;
D59N, H76Q, S132A, S164G, L166V, S273Y;
K19T, D59N, H76Q, S164G, R242E, N249D, G251D, S273Y;
Y21S, D59N, H76Q, I96L, S164G, L222I, N249D, G251D, S273Y;

K19T, D59N, I96L, S164G, L222I, G251D;
D59N, H76Q, S164G, L222I, S226T, R242E;
H76Q, L130I, L222I, S226T, G251D, S273Y;
Y21S, D59N, H76Q, I96L, L222I, S273Y;
H76Q, S164G, L222I, N249D, G251D, S273Y, V309I;
D59N, I96L, L166V, L222I, R242E, G251D;
Y11V, K19T, D59N, I96L, S164G, L166V, L222I, R242E, G251D, L253I;
K19S, D59N, I96L, S164G, L222I, R242E, N249E, G251D;
K19T, D59N, I96L, S164G, L166I, L222I, R242E, N249D;
H76Q, I96L, S164G, L222I, R242E, G251D, S273Y;
K19T, I96L, L222I, R242E, L253I;
K19T, D59N, I96L, S164G, L222V, R242E, N249D, L253I;
I96L, S164G, L222I, R242E, G251D, S274Y;
R242E, N252D, N100Q, N291Q;
R242E, R254E, Q280E, N100Q, N291Q;
R242E, Q280E, N100Q, N291Q;
R242E, R254E, S273D, Q280E, N100Q, N291Q;
R67Q, S132A, L222I, K231N, R242E, V248I;
R67Q, I96L, L130I, M157L, K231N, R242E;
R67Q, M157L, L222I, K231N, V248I;
R67Q, I96L, M157L, L222I, K231N or
R67Q, G70D, M157L, L222I, N291Q;

Optionally, the isolated chymosin polypeptide variant may further comprise substitutions that alter the glycosylation pattern, such as e.g. substitutions in one or more of positions N100, N252 and/or N291, more specifically N100Q, N252Q and/or N291Q.

Preferred methods for making isolated chymosin polypeptide variants

The present invention further relates to methods for producing an isolated polypeptide according to present disclosure.

As a related embodiment, the present invention relates to a method for making an isolated chymosin polypeptide variant wherein the method comprises the steps:

(a): making an alteration at one or more positions in a parent polypeptide, wherein the alteration is comprising a substitution, a deletion or an insertion in at least one amino acid position corresponding to any of positions: D59, V309, S132, N249, L166, N249, Q56, Y134, K295, M157, M256, R242, I96, H76, S164, S273, G251, Y11, L166, K19, Y21, S74, Y243, N249, S273, Q280, F282, L295, N252, R254, G70, V136, L222, K231, N291,

(b): producing and isolating the altered polypeptide of step (a),

and wherein:

(i): the amino acid position of the parent polypeptide is determined by an alignment of the parent polypeptide with the polypeptide of SEQ ID NO:2 (camel chymosin); and

(ii): the parent polypeptide has at least 80% sequence identity with the mature polypeptide of SEQ ID NO:3 (bovine chymosin) and/or at least 80% sequence identity with the mature polypeptide of SEQ ID NO:2 (camel chymosin).

More specifically, the alteration may be one or more of the substitutions: D59N, V309I, S132A, N249E, L166V, N249D, Q56H, Y134G, K295L, M157L, M256L, R242E, I96L, H76Q, S164G, S273Y, G251D, Y11I, R242D, L222V, Y11V, L166I, K19T, Y21S, S74D, Y243E, N249D, S273D, Q280E, F282E, L295K, N252D, R254E, G70D, V136I, L222I, K231N, N291Q.

In yet a related aspect, the present invention relates to a method for making an isolated chymosin polypeptide variant as specified above, wherein the isolated chymosin polypeptide variant comprises substitution in one or more of the combinations of positions comprising the positions corresponding to:

Y11V, K19T, D59N, I96L, S164G, L166V, L222V, R242E, N249E, L253I;

Y11I, D59N, I96L, S164G, L166V, L222V, R242E, G251D, L253I;

Y11I, I96L, S164G, L222I, R242E;

Y11I, K19T, D59N, I96L, S164G, L222I, R242E, N249E, G251D;

H76Q, I96L, S164G, L222I, R242E, G251D, S273Y;

K19T, D59N, H76Q, S164G, L222I, N249D, S273Y;

K19T, D59N, H76Q, L166V, L222I, R242E, G251D, S273Y;

K19T, D59N, H76Q, S132A, L222I, G251D, S273Y, V309I;

Y21S, H76Q, S164G, L222I, R242E, G251D, S273Y;

D59N, S132A, S164G, L222I, R242E, N249D, G251D, S273Y;

D59N, H76Q, I96L, S132A, S164G, L166V, L222I, G251D, S273Y;

H76Q, S164G, L166V, L222I, R242E, G251D, S273Y;

D59N, H76Q, S132A, S164G, L166V, S273Y;
K19T, D59N, H76Q, S164G, R242E, N249D, G251D, S273Y;
Y21S, D59N, H76Q, I96L, S164G, L222I, N249D, G251D, S273Y;
K19T, D59N, I96L, S164G, L222I, G251D;
D59N, H76Q, S164G, L222I, S226T, R242E;
H76Q, L130I, L222I, S226T, G251D, S273Y;
Y21S, D59N, H76Q, I96L, L222I, S273Y;
H76Q, S164G, L222I, N249D, G251D, S273Y, V309I;
D59N, I96L, L166V, L222I, R242E, G251D;
Y11V, K19T, D59N, I96L, S164G, L166V, L222I, R242E, G251D, L253I;
K19S, D59N, I96L, S164G, L222I, R242E, N249E, G251D;
K19T, D59N, I96L, S164G, L166I, L222I, R242E, N249D;
H76Q, I96L, S164G, L222I, R242E, G251D, S273Y;
K19T, I96L, L222I, R242E, L253I;
K19T, D59N, I96L, S164G, L222V, R242E, N249D, L253I;
I96L, S164G, L222I, R242E, G251D, S274Y;
R242E, N252D, N100Q, N291Q;
R242E, R254E, Q280E, N100Q, N291Q;
R242E, Q280E, N100Q, N291Q;
R242E, R254E, S273D, Q280E, N100Q, N291Q;
R67Q, S132A, L222I, K231N, R242E, V248I;
R67Q, I96L, L130I, M157L, K231N, R242E;
R67Q, M157L, L222I, K231N, V248I;
R67Q, I96L, M157L, L222I, K231N or
R67Q, G70D, M157L, L222I, N291Q.

The parent polypeptide may as a preferred embodiment have at least 95% sequence identity with the mature polypeptide of SEQ ID NO:2 (Camel chymosin).

Further the present invention relates to method for making a food or feed product comprising adding an effective amount of the isolated chymosin polypeptide variant as described herein to the food or feed ingredient(s) and carrying our further manufacturing steps to obtain the food or feed product.

A further related aspect of present invention concerns a method for making a food or feed product comprising adding an effective amount of the isolated chy-

mosin polypeptide variant as described herein to the food or feed ingredient(s) and carrying our further manufacturing steps to obtain the food or feed product, in particular wherein the food or feed product is a milk-based product or a food or feed product comprising a chymosin polypetide of present invention.

A further related aspect of present invention relates to a chymosin polypetide variant according to present invention in a process for making a milk based product such as e.g. cheese, such as e.g. pasta filata, cheddar, continental type cheeses, soft cheese or white brine cheese.

As discussed above - an isolated chymosin polypeptide variant as described herein may be used according to the art – e.g. to make a milk based product of interest (such as e.g. a cheese product).

As discussed above – an aspect of the invention relates to a method for making a food or feed product comprising adding an effective amount of the isolated chymosin polypeptide variant as described herein to the food or feed ingredient(s) and carrying our further manufacturing steps to obtain the food or feed product.

Preferably, the food or feed product is a milk-based product and wherein the method comprises adding an effective amount of the isolated chymosin polypeptide variant as described herein to milk and carrying our further manufacturing steps to obtain the milk based product.

The milk may e.g. be soy milk, sheep milk, goat milk, buffalo milk, yak milk, lama milk, camel milk or cow milk.

The milk based product may e.g. be a fermented milk product such as a quark or a cheese.

As known in the art, the growth, purification, testing and general handling may influence the performance of enzymes and hence also the enzyme of present invention. Hence the present invention relates to chymosin polypeptide variants, methods for making these and products containing these, wherein the chymosin polypeptide variant has an improved clotting activity and/or C/P ratio when compared to the corresponding parent polypeptide under comparable conditions and

preferably after being produced and otherwise handled under comparable conditions.

EXAMPLES

EXAMPLE 1: alignment and numbering of chymosin protein sequences and variant sequences

Chymosin protein sequences were aligned using the ClustalW algorithm as provided by the EBI (EBI, tools, multiple sequence alignment, CLUSTALW", http://www.ebi.ac.uk/Tools/msa/clustalw2/) and as described in Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). Bioinformatics 23(21), 2947-2948.

ClustalW2 settings for multiple sequence alignments were Protein weight Matrix = BLOSUM, GAP open = 10, GAP EXTENSION = 0.5, GAP DISTANCES = 8, No End Gaps, ITERATION = none, NUMITER = 1, CLUSTERING = NJ

As a reference sequence the bovine chymosin B preprochymosin was used (Genbank accession number P00794 – disclosed herein as SEQ ID NO:1), where the N-terminal Methionin has number 1 (MRCL……) and the C-terminal Isoleucin (in the protein sequence …LAKAI) has number 381. Variants were aligned against the bovine B pre-pro-chymosin and residues were numbered according to the corresponding bovine chymosin residue.

EXAMPLE 2: Design of chymosin variants

Chymosin variants were designed using different strategies.

When there is referred to camel chymosin there is referred to camel chymosin comprising the polypeptide of SEQ ID NO:2 herein.
Camel chymosin of SEQ ID NO:2 may be seen as a herein relevant parent polypeptide having chymosin activity used to make camel chymosin variants thereof.

When there is referred to bovine chymosin there is referred to bovine chymosin comprising the polypeptide of SEQ ID NO:1 herein. Bovine chymosin of SEQ ID

NO:1 may be seen as a relevant parent polypeptide having chymosin activity used to make bovine chymosin variants thereof.

Variants 180 to 269 and 367 to 461 of camel chymosin were designed based on an alignment of a large set of public known aspartic protease sequences having an identity of 25% or more compared to bovine chymosin B.
Variations were generally introduced in regions with a high level of amino acid variation between species, while conserved regions were not changed. Amino acid substitutions were chosen based on phylogenetic, structural and experimental information to identify changes with high probability to show beneficial effects on specific clotting activity and the C/P ratio. Multiple variations were introduced in each variant construct, ensuring that each single mutation was present in multiple variant constructs to minimize the effect of covariation between various substitutions. Machine learning and statistical analysis of experimental data were used to determine the relative contributions of the amino acid substitutions to measured coagulant performance of the chymosin variants (references 14, 15).

Variants 271 to 366 were designed based on detailed structural analysis of bovine chymosin (PDB code: 4AA8) and camel chymosin (PDB code: 4AA9). Variations were chosen based on the chemical nature of the respective amino acid side chains and their expected impact on either casein substrate binding or general enzyme properties. Most of the amino acid substitutions in variants 271 to 346 were made in sequence positions either within or in close structural proximity to the substrate binding cleft, or in secondary structural elements that get into contact with the bound casein substrate. Furthermore, changes were made in positions on the protein surface that alter the charge profile of these regions (reference 5) and are therefore expected to have an impact on enzyme performance. Variants 347 to 366 were made based on the different structural conformation of the N-terminal sequence in bovine and camel chymosin. Amino acid substitutions were made in positions within the substrate binding cleft that interact with the N-terminus in camel chymosin.

EXAMPLE 3: Preparation of chymosin variant enzyme material

All chymosin variants were synthesized as synthetic genes and cloned into a fungal expression vector such as e.g. pGAMpR-C (described in WO02/36752A2)

The vectors were transformed into *E. coli* and plasmid DNA was purified using standard molecular biology protocols, known to the person skilled in the art.
The variant plasmids were individually transformed into an *Aspergillus niger* or *Aspergillus nidulans* strain and protein was produced essentially as described in WO02/36752A2 and purified using standard chromatography techniques. For enzyme library screening, all chymosin variants were produced in 20-60mL fermentations. For more detailed characterization of variants 433, 436, 453, and 457, the respective enzymes were fermented again in 70L scale.

As known in the art - the skilled person may, based on his common general knowledge, produce and purify chymosin and chymosin variants – such as herein described bovine and camel chymosin variants.

EXAMPLE 4: Determination of specific chymosin activity

4.1 Determination of milk clotting activity

Milk clotting activity was determined using the REMCAT method, which is the standard method developed by the International Dairy Federation (IDF method).
Milk clotting activity is determined from the time needed for a visible flocculation of a standard milk substrate prepared from a low-heat, low fat milk powder with a calcium chloride solution of 0.5 g per liter (pH ≈ 6.5). The clotting time of a rennet sample is compared to that of a reference standard having known milk-clotting activity and having the same enzyme composition by IDF Standard 110B as the sample. Samples and reference standards were measured under identical chemical and physical conditions. Variant samples were adjusted to approximately 3 IMCU/ml using an 84 mM acetic acid buffer pH 5.5. Hereafter, 20 µl enzyme preparation was added to 1 ml preheated milk (32°C) in a glass test tube placed in a water bath, capable of maintaining a constant temperature of 32°C ± 1°C under constant stirring.
The total milk-clotting activity (strength) of a rennet was calculated in International Milk-Clotting Units (IMCU) per ml relative to a standard having the same enzyme composition as the sample according to the formula:

$$\text{Strength in IMCU/ml} = \frac{\text{Sstandard} \times \text{Tstandard} \times \text{Dsample}}{\text{Dstandard} \times \text{Tsample}}$$

Sstandard: The milk-clotting activity of the international reference standard for rennet.

Tstandard:	Clotting time in seconds obtained for the standard dilution.
Dsample:	Dilution factor for the sample
Dstandard:	Dilution factor for the standard
Tsample:	Clotting time in seconds obtained for the diluted rennet sample from addition of enzyme to time of flocculation.

For clotting activity determination of libraries 1 and 3 variants as well as variants by structural design, the µIMCU method was used instead of the REMCAT method. As compared to REMCAT, flocculation time of chymosin variants in the µIMCU assay was determined by OD measurements in 96-well microtiter plates at 800 nm in a UV/VIS plate reader. A standard curve of various dilutions of a reference standard with known clotting strength was recorded on each plate. Samples were prepared by diluting enzyme in 84 mM acetate buffer, 0.1% triton X-100, pH 5.5. Reaction at 32°C was started by adding 250 uL of a standard milk substrate containing 4% (w/w) low-heat, low fat milk powder and 7.5% (w/w) calcium chloride (pH ≈ 6.5) to 25 uL enzyme sample. Milk clotting activity of chymosin variants in International Milk-Clotting Units (IMCU) per ml was determined based on sample flocculation time relative to the standard curve.

4.2 Determination of total protein content

Total protein content was determined using the Pierce BCA Protein Assay Kit from Thermo Scientific following the instructions of the providers.

4.3 Calculation of specific clotting activity

Specific clotting activity (IMCU/mg total protein) was determined by dividing the clotting activity (IMCU/ml) by the total protein content (mg total protein per ml).

EXAMPLE 5 Determination of proteolytic activity

General proteolytic activity was measured using fluorescently labelled Bodipy-FL casein as a substrate (EnzChek; Molecular Bioprobes, E6638). Casein derivatives heavily labeled with pH-insensitive green-fluorescent Bodipy-FL result in quenching of the conjugate's fluorescence. Protease catalyzed hydrolysis releases fluorescent Bodipy-FL. This method is very sensitive which was essential for this experiment as CHYMAX M has the lowest general proteolytical activity of all coagulants known to date.

A 0.04 mg/ml substrate solution was prepared in 0.2M phosphate buffer pH 6.5, containing 100mM NaCl, 5% glycerol, and 0.1% Brij. Chymosin variants were solved in 20mM malonate buffer, containing 100mM NaCl, 5% glycerol, and 0.1% Brij. Of both substrate and chymosin variant solutions, 20µL were mixed in a black 384-well Corning flat bottom polystyrene microtitter plate and fluorescence was continuously recorded in a fluorometer at 32°C for 10 hours. Slopes of the linear part of fluorescence change were used to determine general proteolytic activity.

EXAMPLE 6 Statistical analysis of the positional and mutational effects on specific clotting activity and C/P ratio

A statistical machine-learning approach and PCA-based analysis was used to determine the effects of all single mutations present in the variants of multi-substitution libraries 1 to 3 on cleavage of κ-casein between positions Phe105 and Met106, i.e. specific milk clotting activity, as well as on the ratio of clotting and general proteolytic activity (C/P).

Results

Multi-substitution library 1

Variants of camel chymosin, each having multiple substitutions compared to wild type, were generated and analyzed as described above. All variants have an amino acid sequence identical to camel chymosin (SEQ ID NO:2), except for the variations mentioned in the table. Camel chymosin (CHY-MAX M) is included as reference.

Clotting activities were determined using the µIMCU method.

Table 1: *Enzymatic activities of camel chymosin variants 180-222. Numbers are given in % cleavage of wild type camel chymosin (CHY-MAX M).*

variant	mutations									Clotting (C)	Proteolytic (P)	C/P
CHY-MAX M										100	100	100
180	H76Q	S132A	S164G	L222I	N249D	G251D				72	37	194
181	Y21S	D59N	H76Q	S164G	L166V	N249D	G251D	S273Y		77	37	210
182	D59N	H76Q	S164G	L222I	R242E	S273Y	V309I			96	21	449
183	D59N	H76Q	L130I	L166V	L222I	N249D	G251D	S273Y		84	55	152
184	Y21S	D59N	S164G	L222I	R242E	G251D	S273Y	V309I		102	35	287
185	K19T	Y21S	D59N	H76Q	S132A	S164G	L222I	G251D	S273Y	97	29	334
186	D59N	H76Q	I96L	L130I	S164G	L222I	R242E	G251D		85	16	524
187	H76Q	S164G	L166V	L222I	S226T	S273Y				103	21	504
188	K19T	D59N	I96L	S164G	L222I	G251D				126	31	403
189	Y21S	H76Q	S164G	L222I	R242E	G251D	S273Y			138	14	975
190	H76Q	I96L	S164G	L222I	R242E	G251D	S273Y			153	10	1479
191	H76Q	S164G	L222I	N249D	G251D	S273Y	V309I			112	19	606
192	K19T	D59N	H76Q	S164G	L222I	N249D	S273Y			152	42	363
193	Y21S	D59N	H76Q	S164G	L222I	S226T	G251D	S273Y	V309I	107	32	340
194	H76Q	S164G	L166V	L222I	R242E	G251D	S273Y			132	14	949
195	D59N	H76Q	I96L	S164G	L222I	S226T	N249D	G251D	S273Y	96	19	498
196	D59N	H76Q	L130I	S164G	L166V	L222I	G251D	S273Y	V309I	76	24	316
197	D59N	S132A	S164G	L222I	R242E	N249D	G251D	S273Y		138	38	365
198	H76Q	I96L	S164G	G251D	S273Y	V309I				71	16	443
199	D59N	H76Q	L130I	S164G	G251D	V309I				54	18	309
200	K19T	D59N	S164G	L166V	L222I	S226T	G251D	S273Y		107	31	342
201	D59N	H76Q	I96L	S132A	S164G	L222I	S226T	G251D	S273Y	96	23	426
202	K19T	D59N	H76Q	I96L	S164G	L166V	L222I	G251D	S273Y	90	41	218
203	K19T	D59N	H76Q	L130I	S164G	L222I	S226T	G251D	S273Y	64	21	309
204	K19T	D59N	H76Q	S132A	L222I	G251D	S273Y	V309I		141	48	294
205	H76Q	L130I	L222I	S226T	G251D	S273Y				124	38	322
206	K19T	Y21S	D59N	H76Q	L130I	S164G	L222I	S273Y		75	25	295
207	Y21S	D59N	H76Q	I96L	S164G	L222I	N249D	G251D	S273Y	129	17	762
208	K19T	D59N	H76Q	S164G	R242E	N249D	G251D	S273Y		129	15	879
209	D59N	H76Q	S164G	L222I	S226T	R242E				124	30	417
210	D59N	H76Q	I96L	S132A	S164G	L166V	L222I	G251D	S273Y	136	21	657
211	D59N	H76Q	S132A	S164G	L166V	S273Y				131	31	423
212	Y21S	D59N	S164G	L222I	S226T	N249D	G251D	S273Y		92	48	190
213	D59N	H76Q	L130I	S132A	S164G	L222I	R242E	G251D	S273Y	108	24	441
214	D59N	H76Q	S164G	L166V	L222I	N249D	G251D	S273Y	V309I	111	65	171
215	D59N	H76Q	I96L	S164G	L222I	S226T	G251D	S273Y	V309I	87	24	369
216	K19T	D59N	H76Q	L166V	L222I	R242E	G251D	S273Y		146	30	494
217	Y21S	D59N	H76Q	I96L	L222I	S273Y				118	52	228
218	D59N	H76Q	I96L	L130I	S164G	L222I	N249D	G251D	S273Y	75	23	323
219	L130I	S164G	L222I	S273Y						46	38	121
220	K19T	Y21S	H76Q	S164G	L222I	G251D	S273Y			65	28	228
221	Y21S	D59N	H76Q	L130I	S132A	S164G	L222I	G251D	S273Y	65	31	213
222	D59N	H76Q	S226T	R242E	G251D	S273Y				102	37	273

In table 1 are shown camel chymosin variants with data on specific clotting activity (C), unspecific proteolytic activity (P) as well as the C/P ratio. Out of 43 variants 17 reveal between 10% and 50% increased specific clotting activity compared to wild type camel chymosin (CHY-MAX M). All variants have significantly increased C/P ratios, with the best one, 190, showing a ca. 15x improvement compared to wild type camel chymosin.

Mutational analysis of multi-substitution library 1

A statistical analysis of the positional and mutational effects on specific clotting activity (C) and the C/P ratio was performed based on the proteolytic data of library 1. The most beneficial mutations for increased specific clotting and C/P are shown in tables 2 and 3, respectively.

Table 2: *Mutational contributions (mean) to increased specific clotting activity and standard deviations (sd) based on statistical analysis.*

mutation	mean	sd
R242E	1.98E-01	2.47E-02
L222I	1.09E-01	3.35E-02
D59N	6.06E-02	3.12E-02
S273Y	6.06E-02	3.47E-02
K19T	5.13E-02	2.65E-02
V309I	4.37E-02	2.92E-02
S132A	4.18E-02	2.46E-02
N249D	3.85E-02	2.54E-02
I96L	3.38E-02	2.59E-02

Based on the results shown in table 2 it is concluded that mutations **K19T**, **D59N**, **I96L**, **S132A**, **L222I**, **R242E**, **N249D**, **S273Y**, and **V309I** increase the specific clotting activity of chymosin. It can consequently be expected that these mutations enable a lower dosing of chymosin in cheese manufacturing.

Table 3: *Mutational contributions (mean) to increased C/P ratio and standard deviations (sd) based on statistical analysis.*

mutation	mean	sd
R242E	2.12E-01	2.82E-02
I96L	1.20E-01	2.81E-02
H76Q	9.10E-02	2.16E-02
S164G	8.59E-02	2.19E-02
S273Y	7.77E-02	2.01E-02
G251D	3.59E-02	1.99E-02

Based on the results shown in table 3 it is concluded that mutations **H76Q**, **I96L**, **S164G**, **R242E**, **G251D**, and **S273Y** increase the C/P ratio of chymosin. It can consequently be expected that these mutations result in increased yields during cheese manufacturing using the respective chymosin variants.

Multi-substitution library 2

Another set of camel chymosin variants, each having multiple substitutions compared to wild type, were generated and analyzed as described above. All variants have an amino acid sequence identical to camel chymosin (SEQ ID NO:2), except for the variations mentioned in the table. Camel chymosin (CHY-MAX M) is included as reference.

Clotting activities were determined using the REMCAT method.

Table 4: *Enzymatic activities of camel chymosin variants 223-269. Numbers are given in % cleavage of wild type camel chymosin (CHY-MAX M).*

variant	mutations										Clotting (C)	Proteolytic (P)	C/P
CHY-MAX M											100	100	100
223	K19T	D59N	I96L	S164G	L222I	G251D					89	37	242
224	Y11I	K19T	D59N	I96V	L222I	R242D	G251D				82	31	262
225	K19S	D59N	I96V	S164G	G251D						72	40	182
226	K19S	I96L	S164G	L166V	L222I	R242E					91	38	242
227	K19T	D59N	I96L	S164G	L166V	L222I	R242D	G251D	L253I		92	24	378
228	D59N	I96L	S164G	L222I	R242E	L253I	I263L				108	23	467
229	K19T	D59N	E83T	I96L	L222I	G251D	I263L				99	106	93
230	Y11I	K19T	D59N	S164G	L222I	G251D	I263V				54	16	343
231	K19T	D59N	I96L	S164G	L166I	G251D	L253V				63	30	206
232	K19T	I96V	S164G	L222I	N249D	G251D	L253I				56	29	193
233	K19T	I96L	L222I	R242E	L253I						125	57	220
234	K19T	E83S	I96L	S164G	L222I	R242E	G251D	L253I			83	35	235
235	D59N	E83T	I96L	S164N	L222V	G251D					42	53	80
236	K19S	D59N	I96L	S164G	L222I	R242E	N249E	G251D			130	28	459
237	K19T	I96L	S164G	L166V	L222I	N249D	I263L				65	30	217
238	D59N	I96L	L166V	L222I	R242E	G251D					178	51	347
239	K19T	D59N	E83T	S164G	L166V	L222I	R242D	G251D			101	43	235
240	Y11I	K19T	D59N	E83S	I96L	S164G	L222I	N249D			53	60	87
241	K19T	E83T	I96L	S164G	L222I	R242E	L253V				97	37	261
242	K19T	D59N	I96L	S164G	L166I	L222I	R242E	N249D			129	21	623
243	Y11V	K19T	D59N	I96L	S164G	L166V	L222I	R242E	G251D	L253I	130	17	759
244	K19T	I96L	S164N	L222I	R242E	I263L					51	22	236
245	Y11V	D59N	I96L	S164G	L222I	G251D	L253V				63	24	265
246	K19T	D59N	I96V	S164G	L166V	L222I	R242E	I263L			98	28	347
247	Y11V	K19T	D59N	I96L	S164N	L166I	L222I	G251D			32	16	202
248	K19T	I96L	S164G	L166V	L222I	R242E	N249D	G251D	I263V		105	19	566
249	K19T	I96L	S164G	R242E	L253I						73	14	516
250	K19S	D59N	E83S	I96L	S164N	L222I	G251D				47	64	74
251	K19T	D59N	I96L	S164G	L222V	N249E	G251D	I263V			79	27	293
252	K19T	D59N	I96L	S164G	L222I	N249E	G251D	L253V	I263L		69	21	332
253	Y11I	K19T	I96L	S164G	L222V	R242E	G251D				58	2	3265
254	I96L	S164G	L222I	R242E	N249D	G251D	I263L				82	14	601
255	K19T	D59N	I96L	S164G	L166I	L222I	R242D	G251D	I263V		108	25	427
256	K19T	D59N	I96L	S164G	L222V	R242E	N249D	L253I			111	19	574
257	H76Q	I96L	S164G	L222I	R242E	G251D	S273Y				128	8	1597
258	K19T	E83S	I96L	S164G	L222I	R242E	N249D	G251D	L253I		95	30	315
259	I96L	S164G	L166V	L222I	R242E	N249D	I263L				104	26	405
260	Y11V	K19T	E83S	I96L	S164G	L166V	L222I	R242E	G251D		97	14	676
261	Y11V	K19T	I96L	S164G	L166V	L222I	R242E				94	19	491
262	Y11V	E83S	I96L	S164G	L222I	R242E	G251D	L253I	I263L		61	18	332
263	Y11V	I96L	S164G	L222I	R242E	N249D	L253I	I263L			67	7	961
264	K19T	I96L	S164G	L166V	L222I	R242E	N249D	I263L			75	50	149
265	Y11V	E83S	I96L	S164G	L222I	R242E	L253I	I263L			62	28	222
266	K19T	E83S	I96L	S164G	L166V	L222I	R242E	N249D	G251D	L253I	97	32	302
267	I96L	S164G	L222I	R242E	G251D	S274Y					110	19	569
268	H76Q	I96L	S164G	L222I	R242E	G251D					102	10	1054
269	I96L	S164G	L222I	R242E	G251D						101	22	465

In table 4 are shown camel chymosin variants with data on specific clotting activity (C), unspecific proteolytic activity (P) as well as the C/P ratio. Out of 47 variants, 8 reveal between 10% and 78% increased specific clotting activity compared to wild type camel chymosin (CHY-MAX M). While 43 variants have

significantly increased C/P ratios, the best one, 253, shows a ca. 33x improvement compared to wild type camel chymosin.

Mutational analysis of multi-substitution library 2

A statistical analysis of the positional and mutational effects on specific clotting activity (C) and the C/P ratio was performed based on the proteolytic data of library 2. The most beneficial mutations for increased specific clotting and C/P are shown in tables 5 and 6, respectively.

Table 5: *Mutational contributions (mean) to increased specific clotting activity and standard deviations (sd) based on statistical analysis.*

mutation	mean	sd
R242E	4.00E-01	3.19E-02
D59N	2.94E-01	2.26E-02
N249E	1.47E-01	3.22E-02
L166V	1.27E-01	2.70E-02
S273Y	1.23E-01	2.94E-02
L222I	1.07E-01	3.53E-02
H76Q	5.93E-02	2.94E-02
N249D	4.26E-02	2.38E-02

Based on the results shown in table 5 it is concluded that mutations **D59N**, **H76Q**, **L166V**, **L222I**, **R242E**, **N249D**, **N249E**, and **S273Y** increase the specific clotting activity of chymosin. It can consequently be expected that these mutations enable a lower dosing of chymosin in cheese manufacturing.

Table 6: *Mutational contributions (mean) to increased C/P ratio and standard deviations (sd) based on statistical analysis.*

mutation	mean	sd
R242E	4.13E-01	2.20E-02
H76Q	2.50E-01	3.24E-02
Y11I	2.49E-01	6.43E-02
S164G	2.27E-01	2.07E-02
G251D	2.10E-01	2.65E-02
R242D	1.85E-01	2.69E-02
L222V	1.75E-01	4.53E-02
Y11V	1.75E-01	2.83E-02
S273Y	8.29E-02	3.35E-02
L166I	7.64E-02	2.91E-02
I96L	3.85E-02	2.59E-02
K19T	3.85E-02	2.43E-02

Based on the results shown in table 6 it is concluded that mutations **Y11I**, **Y11V**, **K19T**, **H76Q**, **I96L**, **S164G**, **L166I**, **L222V**, **R242D**, **R242E**, **G251D**, and **S273Y** increase the C/P ratio of chymosin. It can consequently be expected that these mutations result in increased yields during cheese manufacturing using the respective chymosin variants.

Structure-based variations in camel chymosin

Variants of camel chymosin (SEQ ID NO:2) were made with amino acid changes in positions determined by protein structural analysis (Tab. 7). Mutations N100Q and N291Q were introduced into both N-glycosylation sites of these variants and the reference camel chymosin (CamUGly) to yield non-glycosylated, homogeneous protein samples.

Clotting activities were determined using the μIMCU method.

Table 7: *Enzymatic activities of camel chymosin variants 271-308. Numbers are given in % cleavage of non-glycosylated camel chymosin (CamUGly).*

Table 7	CamBov					
variant	**mutations**			**Clotting (C)**	**Proteolytic (P)**	**C/P**
CamUGly		N100Q	N291Q	100	100	100
271	V221K	N100Q	N291Q	47	61	77
272	D290E	N100Q	N291Q	92	100	92
273	V136I	N100Q	N291Q	80	90	89
274	E240Q	N100Q	N291Q	84	144	58
276	G289S	N100Q	N291Q	93	107	86
277	N292H	N100Q	N291Q	95	93	100
278	L295K	N100Q	N291Q	102	70	146
279	V136E	N100Q	N291Q	102	102	100
280	D290L	N100Q	N291Q	44	198	22
281	F119Y	N100Q	N291Q	8	45	18
282	Q280E	N100Q	N291Q	79	72	110
283	F282E	N100Q	N291Q	93	80	116
284	N249D	N100Q	N291Q	118	84	140
285	R254S	N100Q	N291Q	47	94	50
286	R242E	N100Q	N291Q	114	67	170
288	V203R	N100Q	N291Q	99	113	88
289	N249R	N100Q	N291Q	76	108	70
290	H56K	N100Q	N291Q	99	133	74
291	S74D	N100Q	N291Q	94	87	108
292	A131D	N100Q	N291Q	17	39	44
293	Y190A	N100Q	N291Q	3	33	9
294	I297A	N100Q	N291Q	26	37	70
302	Y21S	N100Q	N291Q	97	87	111
303	L130I	N100Q	N291Q	77	82	95
306	G251D	N100Q	N291Q	100	81	123
307	Y243E	N100Q	N291Q	86	58	149
308	S273D	N100Q	N291Q	102	98	104

Based on the results shown in table 7 it is concluded that mutations **Y21S**, **S74D**, **R242E**, **Y243E**, **N249D**, **G251D**, **S273D**, **Q280E**, **F282E**, and **L295K** increase the C/P ratio of chymosin. Mutations **R242E** and **N249D** also result in **increased specific clotting activity**. Seven out of ten variants with increased C/P ratios shown in table 7 bear mutations (R242E, N249D, G251D, Y243E, S273D, Q280E, F282E) in a distinct region on the protein surface that is located in proximity to the binding cleft as seen in figure 2. This region has been suggested to support binding of the κ-casein substrate by interacting with its positively charged sequence Arg96 to His102 (references 5, 16-18) in positions P10

to P4 (reference 10). The negative charges introduced with the mutations may strengthen these interactions, resulting in increased specificity for κ-casein (C/P). **The results show that single amino acid substitutions in this region can increase C/P significantly.**

Negative charge combinations in camel chymosin

More variants of camel chymosin (SEQ ID NO:2) were made with combinations of mutations that introduce negative charges into the surface region described above (R242E, Y243E, G251D, N252D, R254E, S273D, Q280E). Mutations N100Q and N291Q were introduced into both N-glycosylation sites of these variants and the reference camel chymosin (CamUGly) to yield non-glycosylated, homogeneous protein samples (Tab. 8).

Clotting activities were determined using the μIMCU method.

Table 8: *Enzymatic activities of camel chymosin variants 309-323. Numbers are given in % cleavage of non-glycosylated camel chymosin (CamUGly).*

variant	mutations						Clotting (C)	Proteolytic (P)	C/P
CamUGly					N100Q	N291Q	100	100	100
309	R242E	Q280E			N100Q	N291Q	133	59	225
310	R242E	N252D			N100Q	N291Q	136	63	216
311	N252D	Q280E			N100Q	N291Q	108	96	112
312	Y243E	Q280E			N100Q	N291Q	112	71	158
313	Y243E	N252D			N100Q	N291Q	91	77	117
314	R254E	Q280E			N100Q	N291Q	106	84	127
315	S273D	Q280E			N100Q	N291Q	77	51	150
316	R242E	G251D			N100Q	N291Q	107	72	148
317	R242E	G251D	Q280E		N100Q	N291Q	138	84	164
318	R242E	S273D	Q280E		N100Q	N291Q	136	98	139
319	N252D	S273D	Q280E		N100Q	N291Q	115	67	171
320	G251D	S273D	Q280E		N100Q	N291Q	114	64	176
321	R242E	R254E	Q280E		N100Q	N291Q	134	66	202
322	R242E	R254E	S273D	Q280E	N100Q	N291Q	126	60	211
323	Y243E	R254E	S273D		N100Q	N291Q	103	71	144

All variants shown in table 8 reveal increased C/P ratios compared to non-glycosylated camel chymosin. Several of these variants (309, 310, 321, 322, 323) had even higher C/P than the best variant with single negative charge mutation (286). **It is concluded that the C/P-increasing effect, caused by introducing negative charges into the P10-P4 interacting region on the chymosin structure, can be further enhanced by combinations of the respective mutations.**

Structure-based variations in bovine chymosin

Variants of bovine chymosin (SEQ ID NO:1) were made with amino acid changes in positions determined by protein structural analysis (Table 9). Mutations N252Q and N291Q were introduced into both N-glycosylation sites of these variants and the reference bovine chymosin (BovUGly) to yield non-glycosylated, homogeneous protein samples.

Clotting activities were determined using the µIMCU method.

Table 9: *Enzymatic activities of bovine chymosin variants 325-346. Numbers are given in % cleavage of non-glycosylated bovine chymosin (BovUGly).*

variant	mutations			Clotting (C)	Proteolytic (P)	C/P
BovUGly		N252Q	N291Q	100	100	100
325	V223F	N252Q	N291Q	54	130	41
327	A117S	N252Q	N291Q	75	76	96
329	Q242R	N252Q	N291Q	76	166	45
330	Q278K	N252Q	N291Q	94	112	83
332	H292N	N252Q	N291Q	96	71	133
333	Q294E	N252Q	N291Q	99	79	123
334	K295L	N252Q	N291Q	106	182	58
335	D249N	N252Q	N291Q	89	129	68
337	G244D	N252Q	N291Q	100	106	93
338	Q56H	N252Q	N291Q	110	140	77
339	L32I	N252Q	N291Q	86	124	69
340	K71E	N252Q	N291Q	44	50	86
341	P72T	N252Q	N291Q	103	172	59
342	Q83T	N252Q	N291Q	92	103	88
343	V113F	N252Q	N291Q	42	44	95
344	E133S	N252Q	N291Q	93	199	46
345	Y134G	N252Q	N291Q	106	115	91
346	K71A	N252Q	N291Q	79	131	60

The data in table 9 demonstrate that variations **Q56H**, **Y134G**, and **K295L** lead to **increased specific clotting activity** and variations **H292N** and **Q294E** result in **enhanced C/P ratios**. Both H292 and Q294 are located in a loop partially covering the substrate binding cleft (Fig. 3), which explains the observed impact of the respective mutations in these positions on casein substrate specificity (C/P). Notably, while substitutions H292N increased C/P and D249N as well as

K295L decreased C/P of bovine chymosin, inverse effects on C/P were observed by the respective reverse mutations N292H, N249D, and L295K in camel chymosin (Tab. 7). This demonstrates that these amino acid changes exert similar effects on chymosin specificity across species.

Variations of the camel chymosin N-terminus

Variants of camel chymosin (SEQ ID NO:2) were made with amino acid changes in positions determined by protein structural analysis of the molecular interactions of the N-terminal sequence Y11-D13 within the substrate binding cleft (Tab. 10). Mutations N100Q and N291Q were introduced into both N-glycosylation sites of these variants and the reference camel chymosin (CamUGly) to yield non-glycosylated, homogeneous protein samples.

Clotting activities were determined using the µIMCU method.

Table 10: *Enzymatic activities of camel chymosin variants 347-366. Numbers are given in % cleavage of non-glycosylated camel chymosin (CamUGly).*

variant	mutations				Clotting (C)	Proteolytic (P)	C/P
CamUGly			N100Q	N291Q	100	100	100
347	Y11H		N100Q	N291Q	76	131	58
348	Y11K		N100Q	N291Q	63	82	76
349	Y11R		N100Q	N291Q	55	277	20
350	Y11H	D290E	N100Q	N291Q	74	105	71
351	Y11R	D290E	N100Q	N291Q	62	101	62
352	Y11F		N100Q	N291Q	91	146	62
353	Y11I		N100Q	N291Q	96	83	116
354	Y11L		N100Q	N291Q	79	108	74
355	Y11V		N100Q	N291Q	101	64	157
356	L12F		N100Q	N291Q	96	147	66
357	L12I		N100Q	N291Q	83	91	91
359	D13N		N100Q	N291Q	88	131	67
360	D13Q		N100Q	N291Q	100	169	59
361	D13S		N100Q	N291Q	88	164	54
362	D13T		N100Q	N291Q	62	89	70
363	D13F		N100Q	N291Q	73	155	48
364	D13L		N100Q	N291Q	82	196	42
365	D13V		N100Q	N291Q	49	86	57
366	D13Y		N100Q	N291Q	74	99	75

Analysis of the camel chymosin structure guided variations in the N-terminal sequence Y11-D13 as well as in position D290, a potential interaction partner of Y11 (fig. 4). Since casein substrates compete with the N-terminal chymosin sequence for binding within the binding cleft, amino acid substitutions that change interactions between binding cleft and the motif Y11-D13 are expected to impact enzymatic activity toward various casein substrates and, thus, the C/P ratio. The results of the respective variants 347-366 show strong variation of both specific clotting activity and C/P. Notably, variants 353 and 355 reveal increased C/P ratios. It is therefore concluded that amino acid substitutions **Y11I** and **Y11V** result in **increased C/P ratios**. Since the chymosin binding cleft consists mainly of hydrophobic amino acids (reference 9), both mutations might enhance binding of the N-term in the binding cleft by improved hydrophobic interactions and, thus, inhibit non-specific binding and hydrolysis of caseins (P).

Multi-substitution library 3

Another set of camel chymosin variants, each having multiple substitutions compared to wild type, were generated and analyzed as described above. All variants have an amino acid sequence identical to camel chymosin (SEQ ID NO:2), except for the variations mentioned in the table. Camel chymosin (CHY-MAX M) is included as reference.

Clotting activities were determined using the µIMCU method.

Table 11: *Enzymatic activities of camel chymosin variants 367-416. Numbers are given in % cleavage of wild type camel chymosin (CHY-MAX M).*

variant	mutations						Clotting (C)	Proteolytic (P)	C/P
CHY-MAX M							100	100	100
367	R67Q	N100Q	L130I	M157L	V248I	N291Q	46	64	72
368	N100Q	L130I	S132A	M157L	K231N		87	104	83
369	R67Q	I96L	L130I	M157L	L222I	M256L	49	56	88
370	R67Q	L130I	S132A	M157L	R242E	V248I	23	32	70
371	R67Q	N100Q	M157L	R242E	M256L		100	62	162
372	R67Q	G70D	M157L	R242E	V248I		89	32	276
373	V32L	R67Q	M157L	L222I	R242E		64	63	102
374	Y11V	R67Q	M157L	V248I	M256L		71	45	158
375	R67Q	V136I	M157L	L222I	V248I		88	20	449
376	L130I	M157L	V248I	M256L	N291Q		90	80	112
377	R67Q	I96L	L130I	M157L	K231N	R242E	124	37	339
378	V32L	R67Q	L130I	M157L	L222I	K231N	52	103	51
379	L130I	V136I	M157L	L222I	N292H		55	47	118
380	R67Q	G70D	M157L	L222I	N291Q		117	34	339
381	V32L	R67Q	L130I	K231N	N292H		58	66	87
382	Y11V	R67Q	N100Q	L130I	V136I	M157L	60	55	109
383	R67Q	L130I	L222I	R242E	M256L		78	27	290
384	R67Q	M157L	L222I	V248I	N292H		83	97	86
385	V32L	R67Q	M157L	M256L	N291Q		85	143	60
386	R67Q	L130I	S132A	M157L	L222I	N292H	78	133	58
387	R67Q	N100Q	L130I	M157L	K231N	N291Q	59	70	84
388	R67Q	L130I	K231N	V248I	N291Q		91	87	105
389	Y11V	R67Q	L130I	M157L	L222I	K231N	63	47	134
390	I45V	L130I	M157L	K231N	R242E		108	43	253
391	V32L	R67Q	V136I	M157L	N291Q		104	84	124
392	R67Q	N100Q	L130I	D158S	V248I		70	67	105
393	I45V	R67Q	L130I	M157L	L222I	K231N	79	54	147
394	V32L	R67Q	L130I	S132A	M157L	V248I	74	130	57
395	Y11V	R67Q	L130I	M157L	N291Q	N292H	74	83	90
396	R67Q	N100Q	L130I	M157L	L222I	K231N	60	81	74
397	I45V	R67Q	G70D	L130I	S132A		68	75	90
398	I45V	R67Q	L130I	V248I	N292H		53	81	66
399	Y11V	R67Q	L130I	M157L	L222I	R242E	106	28	373
400	R67Q	N100Q	D158S	L130I	M157L	L222I	57	58	98
401	R67Q	L130I	V136I	M157L	K231N	V248I	71	79	89
402	I45V	R67Q	L130I	L222I	N291Q		91	89	103
403	R67Q	G70D	L130I	M157L	K231N	M256L	89	53	167
404	V32L	R67Q	L130I	M157L	D158S	V248I	58	82	71
405	R67Q	L130I	M157L	D158S	R242E	N291Q	92	16	556
406	R67Q	L130I	M157L	D158S	K231N	N292H	53	74	72
407	R67Q	L130I	V248I	M256L	N292H		58	107	55
408	V32L	R67Q	I96L	L130I	M157L	V248I	35	76	46
409	R67Q	I96L	N100Q	L130I	M157L	N292H	96	36	263
410	V32L	R67Q	G70D	N100Q	M157L		68	66	104
411	V32L	R67Q	L130I	M157L	K231N	M256L	102	18	574
412	R67Q	I96L	M157L	L222I	K231N		120	55	220
413	R67Q	M157L	L222I	K231N	V248I		124	46	268
414	R67Q	L130I	M157L	R242E	M256L	N292H	115	59	196
415	R67Q	L222I	K231N	V248I			82	67	123
416	R67Q	S132A	L222I	K231N	R242E	V248I	129	42	306

In table 11 are shown camel chymosin variants with data on specific clotting activity (C), unspecific proteolytic activity (P) as well as the C/P ratio. Out of 50 variants 6 reveal between 10% and 29% increased specific clotting activity compared to wild type camel chymosin (CHY-MAX M). While 23 variants have more than 10% increased C/P ratios, the best one, 411, shows a ca. 6x improvement compared to wild type camel chymosin (CHY-MAX M).

Mutational analysis of multi-substitution library 3

A statistical analysis of the positional and mutational effects on clotting activity (C) and the C/P ratio was performed based on the proteolytic data of library 3. The most beneficial mutations for increased clotting and C/P are shown in tables 12 and 13, respectively.

Table 12: *Mutational contributions (mean) to increased clotting activity and standard deviations (sd) based on statistical analysis.*

mutation	mean	sd
R242E	4.63E-01	4.21E-02
I96L	2.31E-01	4.82E-02
N291Q	1.67E-01	3.97E-02
K231N	1.34E-01	3.52E-02
M256L	1.28E-01	4.44E-02
S132A	1.04E-01	3.35E-02
M157L	7.99E-02	3.49E-02

Based on the results shown in table 12 it is concluded that mutations **I96L**, **S132A**, **M157L**, **K231N**, **R242E**, **M256L**, and **N291Q** increase the specific clotting activity of chymosin. It can consequently be expected that these mutations enable a lower dosing of chymosin in cheese manufacturing.

Table 13: *Mutational contributions (mean) to increased C/P ratio and standard deviations (sd) based on statistical analysis.*

muta-tion	mean	sd
R242E	6.66E-01	4.23E-02
G70D	3.32E-01	5.72E-02
Y11V	2.06E-01	3.61E-02
K231N	1.45E-01	2.92E-02
L222I	1.09E-01	3.71E-02
V136I	1.02E-01	4.53E-02
I96L	9.84E-02	6.02E-02
N291Q	4.78E-02	4.20E-02

Based on the results shown in table 13 it is concluded that mutations **Y11V**, **G70D**, **I96L**, **V136I**, **L222I**, **K231N**, **R242E**, and **N291Q** increase the C/P ratio of chymosin. It can consequently be expected that these mutations result in increased yields during cheese manufacturing using the respective chymosin variants.

Multi-substitution library 4

Another set of camel chymosin variants, each having multiple substitutions compared to wild type, were generated and analyzed as described above. All variants have an amino acid sequence identical to camel chymosin (SEQ ID NO:2), except for the variations mentioned in the table. Camel chymosin (CHY-MAX M) is included as reference.

Clotting activities were determined using the REMCAT method.

Table 14: *Enzymatic activities of camel chymosin variants 417-461. Numbers are given in % cleavage of wild type camel chymosin (CHY-MAX M).*

variant	mutations											Clotting (C)	Proteolytic (P)	C/P
CHY-MAX M												100	100	100
417	Y11V	K19T	D59N	S164G	L166V	L222I	R242E	N249E	G251D			132	20	651
418	Y11V	K19T	D59N	I96L	S164G	L166I	L222I	R242E	N249E	G251D		114	21	556
419	Y11I	K19T	D59N	I96L	S164G	L166V	L222I	R242E	N249E	G251D		108	20	554
420	Y11I	K19T	D59N	I96L	S164G	L166I	L222I	R242E	G251D			98	11	898
421	Y11V	K19T	D59N	I96L	L166V	L222V	R242E	N249E	G251D	L253I		132	84	156
422	Y11V	K19T	D59N	I96L	S164G	L166V	R242E					105	13	802
423	Y11V	K19T	D59N	I96L	S164G	L222V	R242E	G251D				89	8	1131
424	Y11V	K19T	D59N	I96L	S164G	L166I	R242E	N249E	G251D	L253I		93	8	1111
425	Y11V	K19T	D59N	I96L	S164G	L166V	L222V	R242E	N249E	G251D		105	18	572
426	Y11V	K19T	D59N	I96L	S164G	L166I	L222V	R242E	N249E	G251D	L253I	93	18	512
427	Y11V	K19T	D59N	L166V	L222I	R242E	N249E	G251D	L253I			137	42	323
428	Y11V	K19T	D59N	I96L	S164G	L166V	L222I	R242E	N249E			120	15	803
429	Y11V	K19T	D59N	S164G	L166I	L222I	R242E	G251D				107	17	630
430	Y11V	K19T	D59N	I96L	S164G	R242E	G251D					89	11	801
431	Y11V	D59N	I96L	S164G	L166I	L222V	R242E	G251D	L253I			79	28	283
432	Y11V	D59N	I96L	S164G	L166I	L222I	R242E	G251D				102	24	432
433	Y11I	D59N	I96L	S164G	L166V	L222V	R242E	G251D	L253I			97	25	392
434	Y11V	K19T	D59N	I96L	S164G	L222I	R242E	N249E	G251D			99	33	301
435	Y11V	K19T	D59N	I96L	S164G	L166I	L222V	R242E	G251D			88	17	514
436	Y11V	K19T	D59N	I96L	S164G	L166V	L222V	R242E	N249E	L253I		95	10	949
437	Y11V	K19T	D59N	I96L	S164G	L166I	L222V	R242E	N249E	G251D		114	22	520
438	Y11I	K19T	I96L	S164G	L166V	R242E	N249E	G251D				93	7	1262
439	Y11V	K19T	D59N	I96L	S164G	L166V	L222V	R242E	G251D			108	26	423
440	Y11V	K19T	D59N	I96L	S164G	L222V	R242E	N249E	G251D			105	9	1196
441	Y11I	K19T	L222V	R242E	N249E	G251D						122	26	469
442	Y11V	K19T	I96L	L222V	R242E	N249E	G251D					105	21	503
443	Y11I	K19T	D59N	I96L	S164G	L166V	L222V	R242E	N249E	G251D		105	18	595
444	Y11V	K19T	I96L	S164G	L166V	L222V	R242E	N249E	G251D			96	8	1242
445	Y11I	K19T	D59N	I96L	S164G	L166I	L222V	R242E	N249E	G251D		82	12	707
446	Y11I	I96L	S164G	L166V	L222V	R242E	N249E	G251D				95	16	579
447	Y11I	K19T	D59N	I96L	S164G	L222V	R242E	N249E				90	11	790
448	Y11I	K19T	D59N	I96L	L222V	R242E	N249E	G251D				153	40	381
449	Y11I	K19T	D59N	I96L	S164G	L222I	R242E					89	16	564
450	Y11I	K19T	D59N	I96L	S164G	L166V	R242E	G251D				88	5	1686
451	Y11I	K19T	D59N	S164G	L166I	L222V	R242E	G251D				93	21	440
452	Y11I	I96L	L222V	R242E	N249E	G251D						122	22	566
453	Y11I	I96L	S164G	L222I	R242E							74	5	1375
454	Y11V	K19T	I96L	L166V	L222V	R242E	G251D					119	52	228
455	Y11I	D59N	I96L	S164G	L222I	R242E	G251D					105	9	1139
456	Y11I	D59N	I96L	S164G	L222V	R242E	N249E	G251D				95	15	615
457	Y11I	K19T	D59N	I96L	S164G	L222I	R242E	N249E	G251D			101	7	1419
458	Y11I	D59N	I96L	S164G	L166V	L222V	R242E	G251D				89	16	572
459	Y11V	K19T	D59N	I96L	L222V	R242E	G251D					143	62	230
460	Y11I	K19T	S164G	L166I	L222V	R242E	N249E	G251D				80	13	625
461	Y11I	D59N	I96L	S164G	L166V	L222V	R242E	N249E	G251D			96	35	273

In table 14 are shown camel chymosin variants with data on specific clotting activity (C), unspecific proteolytic activity (P) as well as the C/P ratio. Out of 45 variants 11 reveal between 14% and 53% increased specific clotting activity compared to wild type camel chymosin (CHY-MAX M). While all 45 variants have more than 10% increased C/P ratios, the best one, 450, shows a ca. 17x improvement compared to wild type camel chymosin (CHY-MAX M).

Mutational analysis of multi-substitution library 4

A statistical analysis of the positional and mutational effects on clotting activity (C) and the C/P ratio was performed based on the proteolytic data of library 4. The most beneficial mutations for increased clotting and C/P are shown in tables 15 and 16, respectively.

Table 15: *Mutational contributions (mean) to increased clotting activity and standard deviations (sd) based on statistical analysis.*

mutation	mean	sd
D59N	3.99E-01	3.48E-02
L222I	2.05E-01	2.64E-02
L166V	1.92E-01	2.39E-02
N249E	1.45E-01	1.88E-02
G251D	9.79E-02	2.29E-02
Y11V	8.54E-02	1.56E-02
R242E	5.14E-02	2.06E-02

Based on the results shown in table 15 it is concluded that mutations **Y11V**, **D59N**, **L166V**, **L222I**, **R242E**, **N249E**, and **G251D** increase the specific clotting activity of chymosin. It can consequently be expected that these mutations enable a lower dosing of chymosin in cheese manufacturing.

Table 16: *Mutational contributions (mean) to increased C/P ratio and standard deviations (sd) based on statistical analysis.*

mutation	mean	sd
S164G	7.51E-01	4.50E-02
K19T	2.85E-01	4.93E-02
I96L	2.43E-01	4.16E-02
R242E	2.25E-01	7.12E-02
L253I	2.22E-01	4.61E-02
Y11I	1.30E-01	4.93E-02
N249E	9.52E-02	3.86E-02
Y11V	9.49E-02	3.55E-02

Based on the results shown in table 16 it is concluded that mutations **Y11I**, **Y11V**, **K19T**, **I96L**, **S164G**, **R242E**, **N249E**, and **L253I** increase the C/P ratio of chymosin. It can consequently be expected that these mutations result in in-

creased yields during cheese manufacturing using the respective chymosin variants.

Selected variants from multi-substitution library 4 were fermented again in 70L followed by purification and characterization regarding their proteolytic profile (table 17).

Table 17: *Enzymatic activities of selected camel chymosin variants from 70L fermentation. Numbers are given in % cleavage of wild type camel chymosin (CHY-MAX M).*

variant	mutations										Clotting (C)	Proteolytic (P)	C/P
CHY-MAX M											100	100	100
433	Y11I	D59N	I96L	S164G	L166V	L222V	R242E	G251D	L253I		151	11	1356
436	Y11V	K19T	D59N	I96L	S164G	L166V	L222V	R242E	N249E	L253I	188	9	2007
453	Y11I	I96L	S164G	L222I	R242E						153	8	1893
457	Y11I	K19T	D59N	I96L	S164G	L222I	R242E	N249E	G251D		217	7	3002

In table 17 are shown camel chymosin variants from 70L fermentation with data on specific clotting activity (C), unspecific proteolytic activity (P) as well as the C/P ratio. All 4 variants reveal between 51% and 117% increased specific clotting activity compared to wild type camel chymosin (CHY-MAX M). While all 4 variants have more than 13-fold increased C/P ratios, the best one, 457, shows a ca. 30x improvement compared to wild type camel chymosin (CHY-MAX M).

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CLAIMS

1. An isolated chymosin polypeptide variant comprising an alteration in one or more positions compared to a parent polypeptide having chymosin activity, wherein the alteration comprise a substitution, a deletion or an insertion in at least one amino acid position corresponding to any of positions: S164, D59, V309, S132, N249, L166, N249, Q56, M157, M256, R242, I96, H76, S273, G251, Y11, L166, K19, Y21, S74, Y243, N249, S273, Q280, F282, L295, N252, R254, G70, V136, L222, K231, N291 wherein
(i): the amino acid position of the parent polypeptide is determined by an alignment of the parent polypeptide with the polypeptide of SEQ ID NO:2 and
(ii): the parent polypeptide has at least 80% sequence identity with the mature polypeptide of SEQ ID NO:2 (camel chymosin);
wherein the isolated chymosin polypeptide variant has a higher specific clotting activity and/or C/P ratio than its corresponding parent polypeptide.

2. The isolated chymosin polypeptide variant of claim 1, wherein the variant poly-peptide has a specific clotting activity (IMCU/mg total protein) that is at least 85% such as e.g. at least 90%, 100%, 110%, 120%, 130%, 160% or 200% of the specific clotting activity of its parent polypeptide.

3. The isolated chymosin polypeptide variant of claim 1 or 2, wherein the variant polypeptide has a C/P ratio that is at least 200%, such as e.g. at least 400%, at least 500%, at least 750% or at least 1000% of the C/P ratio of its parent polypeptide.

4. The isolated chymosin polypeptide variant of any of claims 1 to 3, wherein the parent polypeptide has at least 80%, such as at least e.g. 82%, 85%, 95%, 97%, 98%, 99% or 100% sequence identity with the mature polypeptide of SEQ ID NO:2 (camel chymosin).

5. The isolated chymosin polypeptide variant of any of claims 1 to 4, wherein the alteration is comprising a substitution selected from a list consisting of: D59N, V309I, S132A, N249E, L166V, N249D, Q56H, M157L, M256L, R242E, I96L, H76Q, S164G, S273Y, G251D, Y11I, R242D, L222V, Y11V, L166I, K19T, Y21S,

S74D, Y243E, N249D, S273D, Q280E, F282E, L295K, N252D, R254E, G70D, V136I, L222I, K231N, N291Q.

6. An isolated chymosin polypeptide variant according to any of claims 1 to 5, wherein the alteration comprise one or more combinations of substitutions comprising:

Y11V, K19T, D59N, I96L, S164G, L166V, L222V, R242E, N249E, L253I;
Y11I, D59N, I96L, S164G, L166V, L222V, R242E, G251D, L253I;
Y11I, I96L, S164G, L222I, R242E;
Y11I, K19T, D59N, I96L, S164G, L222I, R242E, N249E, G251D;
H76Q, I96L, S164G, L222I, R242E, G251D, S273Y;
K19T, D59N, H76Q, S164G, L222I, N249D, S273Y;
K19T, D59N, H76Q, L166V, L222I, R242E, G251D, S273Y;
K19T, D59N, H76Q, S132A, L222I, G251D, S273Y, V309I;
Y21S, H76Q, S164G, L222I, R242E, G251D, S273Y;
D59N, S132A, S164G, L222I, R242E, N249D, G251D, S273Y;
D59N, H76Q, I96L, S132A, S164G, L166V, L222I, G251D, S273Y;
H76Q, S164G, L166V, L222I, R242E, G251D, S273Y;
D59N, H76Q, S132A, S164G, L166V, S273Y;
K19T, D59N, H76Q, S164G, R242E, N249D, G251D, S273Y;
Y21S, D59N, H76Q, I96L, S164G, L222I, N249D, G251D, S273Y;
K19T, D59N, I96L, S164G, L222I, G251D;
D59N, H76Q, S164G, L222I, S226T, R242E;
H76Q, L130I, L222I, S226T, G251D, S273Y;
Y21S, D59N, H76Q, I96L, L222I, S273Y;
H76Q, S164G, L222I, N249D, G251D, S273Y, V309I;
D59N, I96L, L166V, L222I, R242E, G251D;
Y11V, K19T, D59N, I96L, S164G, L166V, L222I, R242E, G251D, L253I;
K19S, D59N, I96L, S164G, L222I, R242E, N249E, G251D;
K19T, D59N, I96L, S164G, L166I, L222I, R242E, N249D;
H76Q, I96L, S164G, L222I, R242E, G251D, S273Y;
K19T, I96L, L222I, R242E, L253I;
K19T, D59N, I96L, S164G, L222V, R242E, N249D, L253I;
I96L, S164G, L222I, R242E, G251D, S274Y;
R242E, N252D, N100Q, N291Q;
R242E, R254E, Q280E, N100Q, N291Q;

R242E, Q280E, N100Q, N291Q;
R242E, R254E, S273D, Q280E, N100Q, N291Q;
R67Q, S132A, L222I, K231N, R242E, V248I;
R67Q, I96L, L130I, M157L, K231N, R242E;
R67Q, M157L, L222I, K231N, V248I;
R67Q, I96L, M157L, L222I, K231N or
R67Q, G70D, M157L, L222I, N291Q;

7. A method for making an isolated chymosin polypeptide variant according to any of claims 1 to 6, the method comprising the steps:
(a): making an alteration at one or more positions in a parent polypeptide, wherein the alteration is comprising a substitution, a deletion or an insertion in at least one amino acid position corresponding to any of positions: Y11, S164, L253, D59, V309, S132, N249, L166, N249, Q56, M157, M256, R242, I96, H76, S164, S273, G251, Y11, L166, K19, Y21, S74, Y243, N249, S273, Q280, F282, L295, N252, R254, G70, V136, L222, K231, N291;
(b): producing and isolating the altered polypeptide of step (a),
and wherein:
(i): the amino acid position of the parent polypeptide is determined by an alignment of the parent polypeptide with the polypeptide of SEQ ID NO:2 (camel chymosin); and
(ii): the parent polypeptide has at least 80% sequence identity with the mature polypeptide of SEQ ID NO:3 (bovine chymosin) and/or at least 80% sequence identity with the mature polypeptide of SEQ ID NO:2 (camel chymosin).

8. The method for making an isolated chymosin polypeptide variant of claim 7, wherein the alteration is one or more of the substitutions: Y11I, Y11V, S164G, L253I, D59N, V309I, S132A, N249E, L166V, N249D, Q56H, M157L, M256L, R242E, I96L, H76Q, S164G, S273Y, G251D, Y11I, R242D, L222V, Y11V, L166I, K19T, Y21S, S74D, Y243E, N249D, S273D, Q280E, F282E, L295K, N252D, R254E, G70D, V136I, L222I, K231N, N291Q.

9. The method according to any of claims 7 and 8 wherein the isolated chymosin polypeptide variant comprise a substitution in one or more of the combinations of positions comprising the positions corresponding to:
Y11V, K19T, D59N, I96L, S164G, L166V, L222V, R242E, N249E, L253I;

Y11I, D59N, I96L, S164G, L166V, L222V, R242E, G251D, L253I;
Y11I, I96L, S164G, L222I, R242E;
Y11I, K19T, D59N, I96L, S164G, L222I, R242E, N249E, G251D;
H76Q, I96L, S164G, L222I, R242E, G251D, S273Y;
K19T, D59N, H76Q, S164G, L222I, N249D, S273Y;
K19T, D59N, H76Q, L166V, L222I, R242E, G251D, S273Y;
K19T, D59N, H76Q, S132A, L222I, G251D, S273Y, V309I;
Y21S, H76Q, S164G, L222I, R242E, G251D, S273Y;
D59N, S132A, S164G, L222I, R242E, N249D, G251D, S273Y;
D59N, H76Q, I96L, S132A, S164G, L166V, L222I, G251D, S273Y;
H76Q, S164G, L166V, L222I, R242E, G251D, S273Y;
D59N, H76Q, S132A, S164G, L166V, S273Y;
K19T, D59N, H76Q, S164G, R242E, N249D, G251D, S273Y;
Y21S, D59N, H76Q, I96L, S164G, L222I, N249D, G251D, S273Y;
K19T, D59N, I96L, S164G, L222I, G251D;
D59N, H76Q, S164G, L222I, S226T, R242E;
H76Q, L130I, L222I, S226T, G251D, S273Y;
Y21S, D59N, H76Q, I96L, L222I, S273Y;
H76Q, S164G, L222I, N249D, G251D, S273Y, V309I;
D59N, I96L, L166V, L222I, R242E, G251D;
Y11V, K19T, D59N, I96L, S164G, L166V, L222I, R242E, G251D, L253I;
K19S, D59N, I96L, S164G, L222I, R242E, N249E, G251D;
K19T, D59N, I96L, S164G, L166I, L222I, R242E, N249D;
H76Q, I96L, S164G, L222I, R242E, G251D, S273Y;
K19T, I96L, L222I, R242E, L253I;
K19T, D59N, I96L, S164G, L222V, R242E, N249D, L253I;
I96L, S164G, L222I, R242E, G251D, S274Y;
R242E, N252D, N100Q, N291Q;
R242E, R254E, Q280E, N100Q, N291Q;
R242E, Q280E, N100Q, N291Q;
R242E, R254E, S273D, Q280E, N100Q, N291Q;
R67Q, S132A, L222I, K231N, R242E, V248I;
R67Q, I96L, L130I, M157L, K231N, R242E;
R67Q, M157L, L222I, K231N, V248I;
R67Q, I96L, M157L, L222I, K231N or
R67Q, G70D, M157L, L222I, N291Q.

10. The method for making an isolated chymosin polypeptide variant of any of claims 7 to 9, wherein the parent polypeptide has at least 95% sequence identity with the mature polypeptide of SEQ ID NO:2 (Camel chymosin).

11. A method for making a food or feed product comprising adding an effective amount of the isolated chymosin polypeptide variant according to any of claims 1 to 6 to the food or feed ingredient(s) and carrying our further manufacturing steps to obtain the food or feed product.

12. A method according to claim 11, wherein the food or feed product is a milk-based product.

13. Use of a chymosin polypetide variant according to any of claims 1 to 6 in a process for making cheese.

14. Use of a chymosin polypetide variant according to any of claims 1 to 6 in a process for making Pasta filata, Cheddar, and Continental type cheeses.

15. Use of a chymosin polypetide variant according to any of claims 1 to 6 in a process for making Soft Cheese or White Brine Cheese.

Figure 1

```
                      1                                                  50
Bos_bovis_chymosin_B  MRCLVVLLAV FALSQGAEIT RIPLYKGKSL RKALKEHGLL EDFLQKQQYG
              Sheep   MRCLVVLLAV FALSQGAEIT RIPLYKGKPL RKALKERGLL EDFLQKQQYG
       C._bactrianus  MRCLVVLLAA LALSQASGIT RIPLHKGKTL RKALKERGLL EDFLQRQQYA
 Camelus_dromedarius  MRCLVVLLAA LALSQASGIT RIPLHKGKTL RKALKERGLL EDFLQRQQYA
                Pig   .IRGRVLLAV LALSQGSGIT RVPLRKGKSL RKELKERGLL EDFLQKQPYA
                Rat   MRCFVLLLAV LAIAQSHVVT RIPLHKGKSL RNTLKEQGLL EDFLRRHQYE

                      51                                                 100
Bos_bovis_chymosin_B  ISSKYSGFGE VASVPLTNYL DSQYFGKIYL GTPPQEFTVL FDTGSSDFWV
              Sheep   VSSEYSGFGE VASVPLTNYL DSQYFGKIYL GTPPQEFTVL FDTGSSDFWV
       C._bactrianus  VSSKYSSLGK VAREPLTSYL DSQYFGKIYI GTPPQEFTVV FDTGSSDLWV
 Camelus_dromedarius  VSSKYSSLGK VAREPLTSYL DSQYFGKIYI GTPPQEFTVV FDTGSSDLWV
                Pig   LSSKYSSFGE VASEPLTNYL DTQYFGKIYI GTPPQEFTVV FDTGSSELWV
                Rat   FSEKNSNIGM VASEPLTNYL DSEYFGLIYV GTPPQEFKVV FDTGSSELWV

                      101                                                150
Bos_bovis_chymosin_B  PSIYCKSNAC KNHQRFDPRK SSTFQNLGKP LSIHYGTGSM QGILGYDTVT
              Sheep   PSIYCKSNAC KNHQRFDPRK SSTFQNLGKP LSIRYGTGSM QGILGYDTVT
       C._bactrianus  PSIYCKSNAC KNHHRFDPRK SSTFRNLGKP LSIHYGTGSI EGFLGYDTVT
 Camelus_dromedarius  PSIYCKSNVC KNHHRFDPRK SSTFRNLGKP LSIHYGTGSM EGFLGYDTVT
                Pig   PSVYCKSDAC QNHHRFNPSK SSTFQNLDKP LSIQYGTGSI QGFLGYDTVM
                Rat   PSVYCSSKVC RNHNRFDPSK SFTFQNLSKP LFVQYGTGSV EGFLAYDTVT

                      151                                                200
Bos_bovis_chymosin_B  VSNIVDIQQT VGLSTQEPGD VFTYAEFDGI LGMAYPSLAS EYSIPVFDNM
              Sheep   VSNIVDIQQT VGLSTQEPGD VFTYAEFDGI LGMAYPSLAS EYSVPVFDNM
       C._bactrianus  VSNIVDPNQT VGLSTEQPGE VFTYSEFDGI LGLAYPSLAS EYSVPVFDNM
 Camelus_dromedarius  VSNIVDPNQT VGLSTEQPGE VFTYSEFDGI LGLAYPSLAS EYSVPVFDNM
                Pig   VAGIVDAHQT VGLSTQEPSD IFTYSEFDGI LGLGYPELAS EYTVPVFDNM
                Rat   VSDIVVPHQT VGLSTEEPGD IFTYSPFDGI LGLAYPTFAS KYSVPIFDNM

                      201                                                250
Bos_bovis_chymosin_B  MNRHLVAQDL FSVYMDRNGQ ESMLTLGAID PSYYTGSLHW VPVTVQQYWQ
              Sheep   MDRRLVAQDL FSVYMDRSGQ GSMLTLGAID PSYYTGSLHW VPVTLQKYWQ
       C._bactrianus  MDRHLVARDL FSVYMDRNGQ GSMLTLGATD PSYYTGSLHW VPVTVQQYWQ
 Camelus_dromedarius  MDRHLVARDL FSVYMDRNGQ GSMLTLGAID PSYYTGSLHW VPVTLQQYWQ
                Pig   MHRHLVAQDL FAVYMSRNDE GSMLTLGAID PSYYTGSLHW VPVTMQLYWQ
                Rat   MNRHLVAQDL FSVYMSRNDQ GSMLTLGAID QSYFIGSLHW VPVTVQGYWQ

                      251                                                300
Bos_bovis_chymosin_B  FTVDSVTISG VVVACEGGCQ AILDTGTSKL VGPSSDILNI QQAIGATQNQ
              Sheep   FTVDSVTISG AVVACEGGCQ AILDTGTSKL VGPSSDILNI QQAIGATQNQ
       C._bactrianus  VTVDSVTING VAVACVGGCQ AILDTGTSVL FGPSSDILKI QMAIGATENR
 Camelus_dromedarius  FTVDSVTING VAVACVGGCQ AILDTGTSVL FGPSSDILKI QMAIGATENR
                Pig   FTVDSVTING VVVACNGGCQ AILDTGTSML AGPSSDILNI QMAIGATESQ
                Rat   FTVDRITIND EVVACQGGCP AVLDTGTALL TGPGRDILNI QHAIGAVQGQ

                      301                                                350
Bos_bovis_chymosin_B  YGEFDIDCDN LSYMPTVVFE INGKMYPLTP SAYTSQDQGF CTSGFQSENH
              Sheep   YGEFDIDCDS LSSMPTVVFE INGKMYPLTP YAYTSQEEGF CTSGFQGENH
       C._bactrianus  YGEFDVNCGS LRSMPTVVFE INGRDFPLAP SAYTSKDQGF CTSGFQGDNN
 Camelus_dromedarius  YGEFDVNCGN LRSMPTVVFE INGRDYPLSP SAYTSKDQGF CTSGFQGDNN
                Pig   YGEFDIDCGS LSSMPTVVFE ISGRMYPLPP SAYTNQDQGF CTSGFQGDSK
                Rat   HDQFDIDCWR LNFMPTVVFE INGREFPLPP SAYTNQFQGS CSSGFR..HG

                      351                           381
Bos_bovis_chymosin_B  SQKWILGDVF IREYYSVFDR ANNLVGLAKA I
              Sheep   SHQWILGDVF IREYYSVFDR ANNLVGLAKA I
       C._bactrianus  SELWILGDVF IREYYSVFDR ANNRVGLAKA I
 Camelus_dromedarius  SELWILGDVF IREYYSVFDR ANNRVGLAKA I
                Pig   SQHWILGVVF IQEYYSVFDR ANNRVGLAKA I
                Rat   SQMWILGDVF IREFYSVFDR ANNRVGLAKA I
```

Figure 2

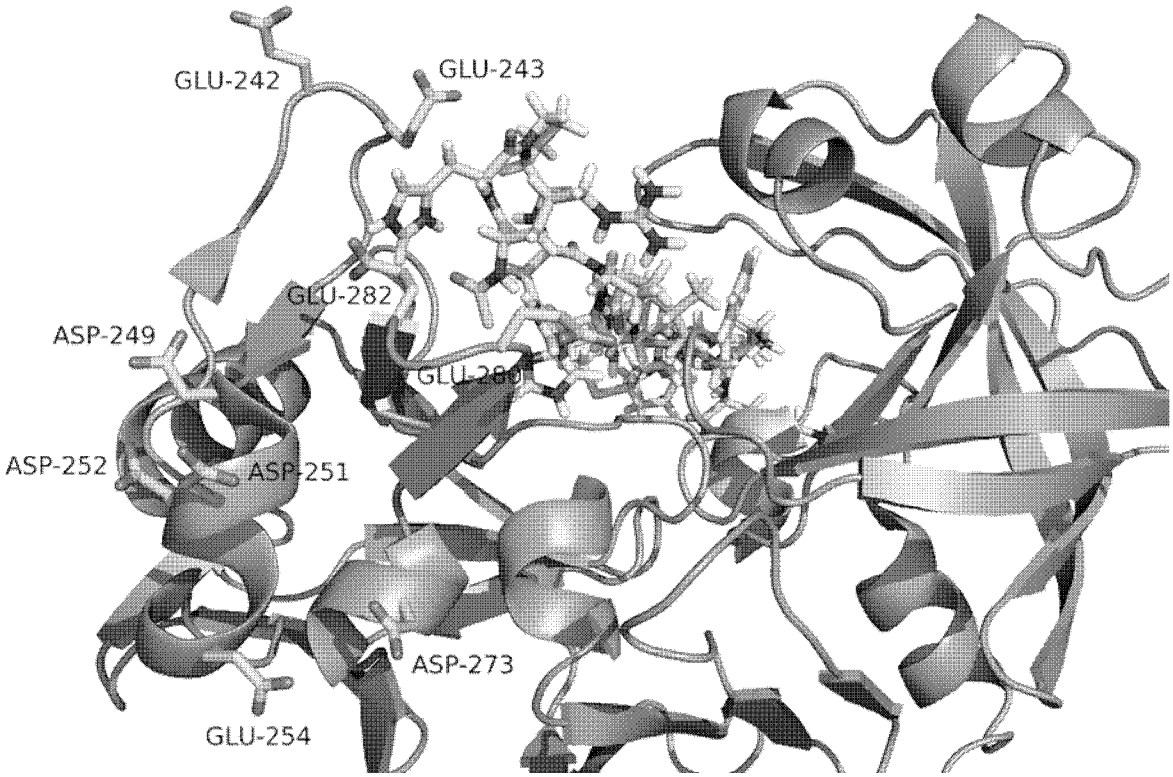

GLU-242
GLU-243
GLU-282
ASP-249
GLU-280
ASP-252
ASP-251
ASP-273
GLU-254

Figure 3

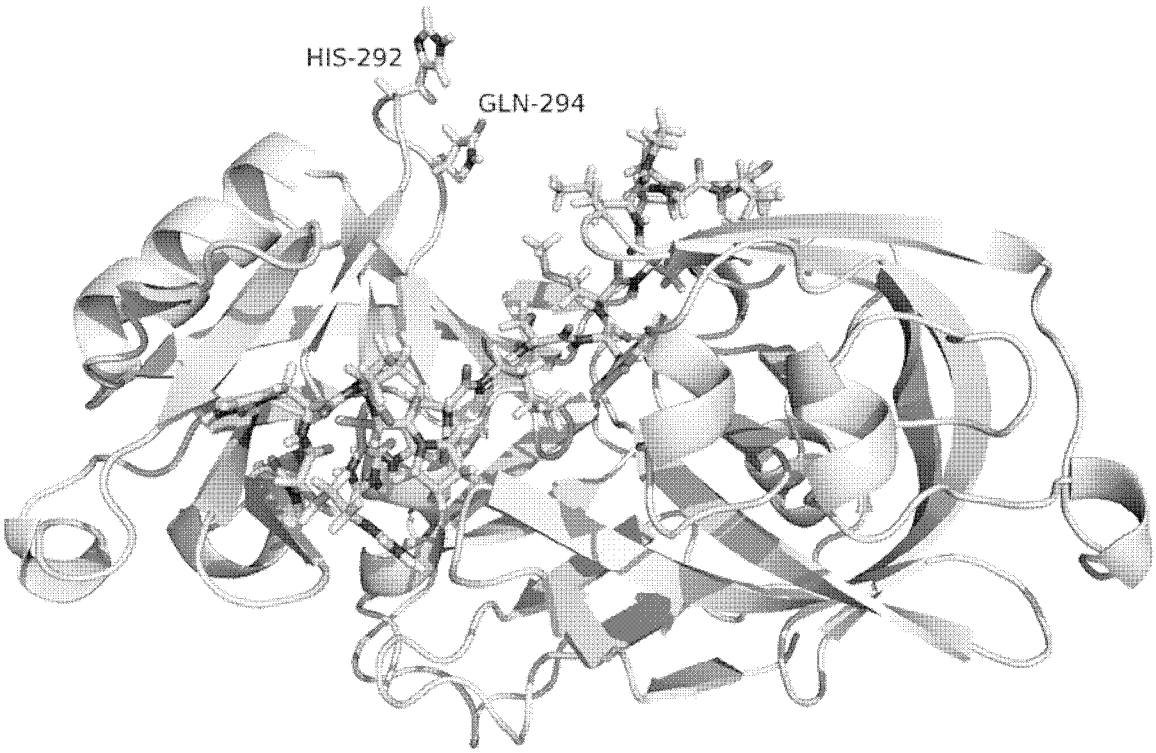
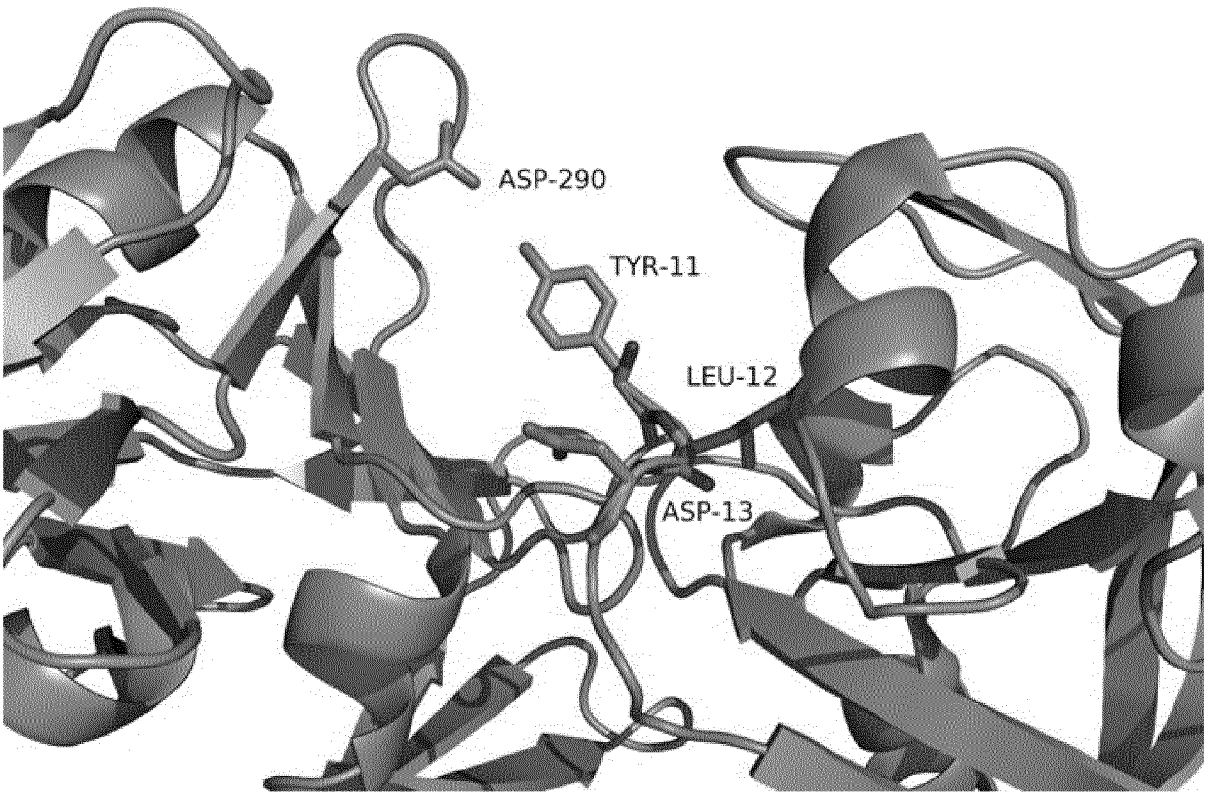


Figure 4



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/062086

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23C19/04 C12N9/64
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23C C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/128417 A1 (CHR HANSEN AS [DK]) 3 September 2015 (2015-09-03) claims 1-15; example 6; table 1	1-15
X	WO 2013/174840 A1 (CHR HANSEN AS [DK]) 28 November 2013 (2013-11-28) example 6	1-15
X,P	WO 2016/207214 A1 (CHR HANSEN AS [DK]) 29 December 2016 (2016-12-29) claims 1-17; tables 1-15	1-15
X,P	WO 2017/037092 A1 (CHR HANSEN AS [DK]) 9 March 2017 (2017-03-09) claims 1-17; tables 1-16	1-15

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 August 2017	06/10/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bulcao de Melo B., T

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/062086

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-15(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-15(partially)

An isolated chymosin polypeptide variant comprising an alteration in position S164 compared to a parent polypeptide having chymosin activity, wherein the alteration comprises a substitution, a deletion or an insertion in amino acid position corresponding to position S164.

2-29. claims: 1-15(partially)

An isolated chymosin polypeptide variant comprising an alteration in one or more positions compared to a parent polypeptide having chymosin activity, wherein the alteration comprise a substitution, a deletion or an insertion in at least one amino acid position corresponding to any of positions D59, V309, S132, N249, L166, Q56, M157, M256, R242, I96, H76, S273, G251, Y11, K19, Y21, S74, Y243, Q280, F282, L295, N252, R245, G70, V136, L222, K231, N291.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/062086

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015128417 A1	03-09-2015	AU 2015222149 A1	28-07-2016
		CA 2938015 A1	03-09-2015
		CN 106062187 A	26-10-2016
		EP 3110948 A1	04-01-2017
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		KR 20160125459 A	31-10-2016
		US 2017067041 A1	09-03-2017
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		CA 2874214 A1	28-11-2013
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		EP 2855674 A1	08-04-2015
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		NZ 702030 A	25-11-2016
		RU 2014152723 A	20-07-2016
		US 2015173383 A1	25-06-2015
		WO 2013174840 A1	28-11-2013
WO 2016207214 A1	29-12-2016	NONE	
WO 2017037092 A1	09-03-2017	NONE	