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# DESCRIPTION

Description

## FIELD OF THE INVENTION

**[0001]** The present invention relates to variants of chymosin with improved milk-clotting properties.

## BACKGROUND ART

**[0002]** 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 x-casein, resulting in a metastable state of the casein micelle structure and a second phase, where the milk subsequently coagulates and forms a coagulum.

**[0003]** 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.

**[0004]** 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.

**[0005]** 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 is a mixture of two forms, A and B, both of which are active.

**[0006]** Chymosin is produced naturally in mammalian species such as bovines, camels, caprines, buffaloes, sheep, pigs, humans, monkeys and rats.

**[0007]** Bovine chymosin has for a number of years been commercially available to the dairy industry.

**[0008]** WO02/36752A2 (Chr. Hansen) describes recombinant production of camel chymosin.

**[0009]** WO2013/174840A1 (Chr. Hansen) describes mutants/variants of bovine and camel chymosin.

**[0010]** WO2013/164479A2 (DSM) describes mutants of bovine chymosin.

**[0011]** 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;
- Supannee 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.

## SUMMARY OF THE INVENTION

**[0012]** Embodiments falling outside of the claims are provided as disclosure not forming part of the invention.

**[0013]** The problem to be solved by the present invention is to provide variants of chymosin with improved milk-clotting properties.

**[0014]** As discussed in working examples herein - the present inventors have identified a number of improved camel (see Example 6 herein) and bovine/camel (see Example 7 herein) chymosin variants.

**[0015]** Based on a comparative analysis of the camel and bovine variants - the present inventors identified a number of further amino acid positions that are herein important in the sense that by making a variant in one or more of these positions one may get an improved chymosin variant.

**[0016]** 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.

**[0017]** In figure 1 herein this is exemplified by an alignment of herein relevant different chymosin sequences.

**[0018]** In view of this relatively close sequence relationship - it is believed that the 3D structures of different natural wildtype chymosins are also relatively similar.

**[0019]** In the present context - a natural obtained wildtype chymosin (such as 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.

**[0020]** 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).

**[0021]** 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 bovine chymosin B preprochymosin sequence (Genbank accession number P00794 - disclosed as SEQ ID NO: 1 herein).

**[0022]** The bovine chymosin B preprochymosin of SEQ ID NO: 1 may herein alternatively be termed Bovine (Bos bovis) chymosin B or simply bovine chymosin. The sequence is also shown in Figure 1 herein.

**[0023]** Another herein relevant chymosin sequence is publically known *Camelius drome-darius* 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.

**[0024]** Accordingly, a first aspect of the invention relates to a method for making an isolated chymosin polypeptide variant of claim 1.

**[0025]** As known in the art - the skilled person may, based on his common general knowledge, routinely produce and purify chymosin and chymosin variants.

**[0026]** 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.

**[0027]** As discussed herein - in working examples herein were made variants using the polypeptide of SEQ ID NO: 2 (camel chymosin) as parent polypeptide - such variant may herein be termed camel chymosin variant.

**[0028]** Accordingly, a second aspect of the invention relates to an isolated chymosin polypeptide variant of claim 8.

**[0029]** An isolated chymosin polypeptide variant as described herein may be used according to the art - e.g. to make a food or feed product of interest (such as e.g. a milk based product of interest that e.g. could be a cheese product).

**[0030]** Accordingly, a third 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.

**[0031]** Embodiment of the present invention is described below, by way of examples only.

**DEFINITIONS**

**[0032]** 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.

**[0033]** The term "chymosin" relates to an enzyme of the EC 3.4.23.4 class. Chymosin has a high specificity and it clots milk by cleavage of a single 105-Ser-Phe-|-Met-Ala-108 bond in kappa-chain of casein. An alternative name used in the art is rennin.

**[0034]** The term "chymosin activity" relates to chymosin activity of a chymosin enzyme as understood by the skilled person in the present context.

**[0035]** The skilled person knows how to determine herein relevant chymosin activity.

**[0036]** In working Example 4 herein is provided an example of a standard method to determine specific chymosin activity - alternatively termed clotting activity or milk clotting activity.

**[0037]** In working Example 5 herein is provided an example of a standard method to determine proteolytical activity.

**[0038]** As known in the art - the herein relevant so-called C/P ratio is determined by dividing the specific clotting activity (C) with the proteolytical activity (P).

**[0039]** 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, and that the development of bitter taste in the cheese during maturation is reduced.

**[0040]** The term "isolated variant" means a variant that is modified by the hand 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.

**[0041]** 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.

**[0042]** 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.

**[0043]** The term "Sequence Identity" relates to the relatedness between two amino acid sequences or between two nucleotide sequences.

**[0044]** 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)

**[0045]** 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).

**[0046]** 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.

**[0047]** 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.

**[0048]** The term "wild-type" chymosin peptide means a chymosin expressed by a naturally occurring organism, such as a mammalian (e.g. camel or bovine) found in nature.

**DRAWINGS**

**[0049]**

Figure 1: An alignment of herein relevant different chymosin sequences. The shown "Bos_bovis_chymosin_B" is bovine chymosin of SEQ ID NO: 1 herein and the shown "Camelus_dromedarius" is camel chymosin of SEQ ID NO: 2 herein. Using bovine chymosin of SEQ ID NO: 1 as reference sequence as described herein is can e.g. be seen that bovine chymosin has "V" in position 10 and camel chymosin has "A" in the same position 10. It may e.g. also be seen that bovine/Rat have "Q" in position 352 and Camel/C._bactrianus have "E" in the same position 352.

In relation to the chymosin sequences shown in figure 1 - sheep has 94.5% sequence identity with bovine SEQ ID NO: 1; C._bactrianus has 83.2% sequence identity with bovine SEQ ID NO: 1; Camelus_dromedarius (camel chymosin of SEQ ID NO: 2) has 84% sequence identity with bovine SEQ ID NO: 1; pig has 80.3% sequence identity with bovine SEQ ID NO: 1 and rat has 71.9% sequence with bovine identity SEQ ID NO: 1.

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: 1 (bovine chymosin - i.e. amino acid positions 59 to 381 of SEQ ID NO: 1) are relatively similar to above mentioned sequence identity percentages.

Figure 2: The 3D structure of bovine chymosin - the 3D structure is public available. As an example are shown where the amino acid positions 296 and 294 are present in bovine Chymosin.

Figure 3: Show a graphical representation the REMCAT and Proteol values of a number of chymosin variants.

Figure 4: PCA plot of effect of individual substitutions. All position numbers are 15 lower than numbers used in text.

**DETAILED DESCRIPTION OF THE INVENTION**

**[0050]** Embodiments falling outside of the claims are provided as disclosure not forming part of the invention.

**Determining the amino acid position of a chymosin of interest**

**[0051]** 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 bovine chymosin sequence disclosed as SEQ ID NO: 1 herein.

**[0052]** For purposes of the present invention, the polypeptide disclosed in SEQ ID NO: 1 (bovine chymosin) is used to determine the corresponding amino acid residue in another chymosin polypeptide. The amino acid sequence of another chymosin polypeptide is aligned with the polypeptide disclosed in SEQ ID NO: 1, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the polypeptide disclosed in SEQ ID NO: 1 is determined using the ClustalW algorithm as described in working Example 1 herein.

**[0053]** Identification of the corresponding amino acid residue in another chymosin polypeptide can be confirmed by 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.

**[0054]** 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.).

**[0055]** In figure 1 herein is shown an example of an alignment.

**[0056]** Just as an example - in figure 1 can e.g. be seen that herein used bovine reference SEQ ID NO: 1 has a "G" in position 50 and "Camelus_dromedarius" (SEQ ID NO: 2 herein) has an "A" in this position 50.

**Nomenclature of variants**

**[0057]** 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.

**[0058]** 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.

**[0059]** *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.

**[0060]** *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*".

**[0061]** *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".

**[0062]** 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 |

**[0063]** *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.

**[0064]** *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".

**A method for making an isolated chymosin polypeptide variant**

**[0065]** 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.

**[0066]** 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.

**[0067]** 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).

**[0068]** 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.

**[0069]** As known to the skilled person - this may e.g. be done by so-called site directed mutagenesis and recombinant expression/production based technology.

**[0070]** 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.

**[0071]** As known in the art - chymosin activity may be determined by the so-called C/P ratio, which is determined by dividing the specific clotting activity (C) with the proteolytical activity (P).

**[0072]** 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, and that the development of bitter taste in the cheese during maturation is reduced.

**[0073]** In working example 4 herein is described a suitable method to determine the specific clotting activity (C) and in working example 5 herein is described a suitable method to determine proteolytical activity (P).

**[0074]** Preferably, an isolated chymosin polypeptide variant as described herein is a variant, wherein the variant has a chymosin activity giving a higher C/P ratio as compared to the C/P ratio of bovine chymosin comprising the mature polypeptide of SEQ ID NO: 1 herein.

**[0075]** Preferably, an isolated chymosin polypeptide variant as described herein is a variant, wherein the variant has a chymosin activity giving a higher C/P ratio as compared to the C/P ratio of camel chymosin comprising the mature polypeptide of SEQ ID NO: 2 herein.

**[0076]** More preferably, an isolated chymosin polypeptide variant as described herein is a variant, wherein the variant has

- a chymosin activity giving a higher C/P ratio as compared to the C/P ratio of bovine chymosin comprising the mature polypeptide of SEQ ID NO: 1 herein; and
- a chymosin activity giving a higher C/P ratio as compared to the C/P ratio of camel chymosin comprising the mature polypeptide of SEQ ID NO: 2 herein.

**[0077]** 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 bovine chymosin sequence disclosed as SEQ ID NO: 1 herein.

**[0078]** As discussed above - based on e.g. the computer sequence alignment programs discussed herein - it is routine work for the skilled person to determine the herein relevant amino acid position of a herein relevant chymosin polypeptide of interest (e.g. camel, sheep, bovine etc).

**[0079]** The camel chymosin polypeptide of SEQ ID NO: 2 has 84% sequence identity with the bovine polypeptide of SEQ ID NO: 1 (i.e. the complete SEQ ID NO: 1 from position 1 to 381, which includes pre and pro sequence).

**[0080]** 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.

**[0081]** Said in other words, a herein relevant isolated chymosin polypeptide variant may comprise alterations (e.g. substitutions) in other position than the positions of e.g. the first aspect herein.

**[0082]** In relation to the chymosin sequences shown in figure 1 herein - sheep has 94.5% sequence identity with bovine SEQ ID NO: 1; C._bactrianus has 83.2% sequence identity with bovine SEQ ID NO: 1; pig has 80.3% sequence identity with bovine SEQ ID NO: 1 and rat has 71.9% sequence with bovine identity SEQ ID NO: 1.

**[0083]** As understood by the skilled person in the present context - herein relevant sequence identity percentages of e.g. mature sheep, C._bactrianus, camel, pig or rat chymosin with the mature polypeptide of SEQ ID NO: 1 (bovine chymosin - i.e. amino acid positions 59 to 381 of SEQ ID NO: 1) are relatively similar to above mentioned sequence identity percentages.

**Preferred variants:**

**[0084]** As discussed above - e.g. the first aspect relates to an isolated chymosin polypeptide variant, wherein the alteration is comprising a substitution, a deletion or an insertion in at least one amino acid position corresponding to position 70.Preferably, an isolated chymosin polypeptide variant, wherein the alteration is comprising a substitution in at least one amino acid position corresponding to any of positions L70M.

**[0085]** As understood by the skilled person in the present context - if the parent chymosin polypeptide already has e.g. "V" in position 156 then is does not make sense to talk about making the substitution 156V for this specific parent chymosin polypeptide. As can be seen in figure 1 herein - rat wildtype chymosin has "V" in position 156 - the substitution 156V may be seen as herein irrelevant for the specific rat chymosin polypeptide sequence of figure 1.

**[0086]** As understood by the skilled person in the present context - if the parent chymosin polypeptide does not have e.g. "D" in position 156 then is does not make sense to talk about making the substitution D156V for this specific parent chymosin polypeptide. As can be seen in figure 1 herein - rat wildtype chymosin has "V" in position 156 - the substitution D156V may therefore be seen as herein irrelevant for the specific rat chymosin polypeptide sequence of figure 1.

**[0087]** In a preferred embodiment, the substitution is wherein the substitution is: L280I + G309D + H134Q + M223E + L70M.

**[0088]** In a preferred embodiment, the parent polypeptide is 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 and wherein the substitution is:

L70M + Y79S + D117N + H134Q + M223E + V256I + L280I + G309D + Q346E;

L70M + Y79S + D117N + H134Q + M223E + L280I + G309W + S331Y;

L70M + D117N + H134Q + M223E + V256I + L280I + G309D + S331Y + K379P;

L70M + D117N + H134Q + S212A + M223E + V261A + L280I + G309D + V367I;

L70M + D117N + H134Q + D156V + L280I;

L70M + K77T + V90L + D117N + H134Q + D202Q + M223E + L280I + G309D;

L70M + Y79S + D117N + H134Q + M223E + V261A + L280I + G309D + E320T;

L70M + D117N + F124Y + H134Q + M223E + L238I + L280I + G309D + V367I;

L70M + D117N + H134Q + S212A + M223E + L280I + G309W + Q346E;

L70M + D117N + H134Q + D156V + M223E + L280I + G309D + E320T + Q346E;

L70M + V109L + D117N + H134Q + L224V + L280I + G309D;

L70M + D117N + H134Q + D202Q + M223E + V261A + L280I;

L70M + D117N + D202Q + M223E + L224V + L280I + G309D;

L70M + K77T + D117N + H134Q + S212A + M223E + V256I + L280I + G309D;

L70M + H134Q + D156V + M223E + L280I + G309W;

L70M + D117N + H134Q + S212A + M223E + S331Y;

L70M + V109L + D117N + F124Y + H134Q + M223E + V261A + L280I + G309W;

L70M + N108D + D117N + H134Q + M223E + G309W + E320T;

L70M + D117N + H134Q + M223E + G309D + Q346E + V367I + K379P;

L70M + N108D + D117N + V261A + L280I + G309D;

L70M + D117N + H134Q + L238I + L280I + G309W + K379P;

L70M + Y79S + D117N + M223E + L280I + K379P;

L70M + K77T + N108D + D117N + H134Q + M223E + L280I + Q346E; or

L70M + Y79S + N108D + D117N + F124Y + H134Q + D202Q + M223E + L280I + G309D.

**Preferred parent polypeptide having chymosin activity:**

**[0089]** In general - a herein relevant isolated chymosin polypeptide variant may comprise alterations (e.g. substitutions) in other positions than the positions of e.g. the first aspect herein.

**[0090]** As discussed above - in working examples herein were made variants using the polypeptide of SEQ ID NO: 2 (Camel) as parent polypeptide - such variant may herein be termed camel chymosin variant.

**[0091]** Accordingly, in a preferred embodiment - the parent polypeptide has at least 95% sequence identity with the mature polypeptide of SEQ ID NO: 2 (Camel chymosin) and even more preferably the parent polypeptide has at least 97% sequence identity with the mature polypeptide of SEQ ID NO: 2 (Camel chymosin). It may be preferred that the parent polypeptide is the mature polypeptide of SEQ ID NO: 2 (Camel chymosin).

**An isolated variant of camel chymosin:**

**[0092]** As discussed above - in working examples herein were made variants using the polypeptide of SEQ ID NO: 2 (camel chymosin) as parent polypeptide - such variant may herein be termed camel chymosin variant.

**[0093]** As discussed above - the second aspect accordingly relates to an isolated chymosin polypeptide variant according to claim 8.

**[0094]** The above described definitions and preferred embodiments are also relevant for this aspect.

**[0095]** Preferably, an isolated camel chymosin polypeptide variant as described herein is a variant, wherein the variant has a chymosin activity giving a higher C/P ratio as compared to the C/P ratio of camel chymosin comprising the mature polypeptide of SEQ ID NO: 2.

**[0096]** In a preferred embodiment - the parent polypeptide has at least 92% sequence identity with the mature polypeptide of SEQ ID NO: 2 (camel chymosin), more preferably the parent polypeptide has at least 95% sequence identity with the mature polypeptide of SEQ ID NO: 2 (camel chymosin) and even more preferably the parent polypeptide has at least 97% sequence identity with the mature polypeptide of SEQ ID NO: 2 (camel chymosin). It may be preferred that the parent polypeptide is the mature polypeptide of SEQ ID NO: 2 (Camel chymosin).

**[0097]** 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.

**[0098]** For instance, a camel 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 (camel chymosin).

**[0099]** It may be preferred that the isolated camel 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 (camel chymosin) or it may be preferred that the isolated camel chymosin variant comprises less than 10 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 5 amino acid alterations (e.g. substitutions) as compared to the mature polypeptide of SEQ ID NO: 2 (camel chymosin).

**[0100]** As understood by the skilled person in the present context - the term "the isolated variant polypeptide has less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 2 (camel chymosin)" of point (iii) above relates to that the herein described isolated camel chymosin variant shall of course not have a polypeptide sequence that is 100% identical to the public known wildtype camel chymosin sequence of SEQ ID NO: 2.

**[0101]** Preferably, the substitution is L70M.

**[0102]** In a preferred embodiment, the substitution is:
L280I + G309D + H134Q + M223E + L70M.

**[0103]** In a preferred embodiment, the parent polypeptide is 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 and wherein the substitution is:

L70M + Y79S + D117N + H134Q + M223E + V256I + L280I + G309D + Q346E;

L70M + Y79S + D117N + H134Q + M223E + L280I + G309W + S331Y;

L70M + D117N + H134Q + M223E + V256I + L280I + G309D + S331Y + K379P;

L70M + D117N + H134Q + S212A + M223E + V261A + L280I + G309D + V367I;

L70M + D117N + H134Q + D156V + L280I;

L70M + K77T + V90L + D117N + H134Q + D202Q + M223E + L280I + G309D;

L70M + Y79S + D117N + H134Q + M223E + V261A + L280I + G309D + E320T;

L70M + D117N + F124Y + H134Q + M223E + L238I + L280I + G309D + V367I;

L70M + D117N + H134Q + S212A + M223E + L280I + G309W + Q346E;

L70M + D117N + H134Q + D156V + M223E + L280I + G309D + E320T + Q346E;

L70M + V109L + D117N + H134Q + L224V + L280I + G309D;

L70M + D117N + H134Q + D202Q + M223E + V261A + L280I;

L70M + D117N + D202Q + M223E + L224V + L280I + G309D;

L70M + K77T + D117N + H134Q + S212A + M223E + V256I + L280I + G309D;

L70M + H134Q + D156V + M223E + L280I + G309W;

L70M + D117N + H134Q + S212A + M223E + S331Y;

L70M + V109L + D117N + F124Y + H134Q + M223E + V261A + L280I + G309W;

L70M + N108D + D117N + H134Q + M223E + G309W + E320T;

L70M + D117N + H134Q + M223E + G309D + Q346E + V367I + K379P;

L70M + N108D + D117N + V261A + L280I + G309D;

L70M + D117N + H134Q + L238I + L280I + G309W + K379P;

L70M + Y79S + D117N + M223E + L280I + K379P;

L70M + K77T + N108D + D117N + H134Q + M223E + L280I + Q346E; or

L70M + Y79S + N108D + D117N + F124Y + H134Q + D202Q + M223E + L280I + G309D.

**A method for making a milk based product**

**[0104]** 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).

**[0105]** 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.

**[0106]** 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.

**[0107]** The milk may e.g. be soy milk, sheep milk, goat milk, buffalo milk, yak milk, lama milk, camel milk or cow milk.

**[0108]** The milk based product may e.g. be a fermented milk product, a quark or a cheese.

**EXAMPLES**

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

**[0109]** 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). Bioin-formatics 23(21), 2947-2948.

**[0110]** ClustalW2 settings for multiple sequence alignments were Protein weight Matrix = BLOSUM, GAP open = 10, GAP EXTENSION= 0,05, 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**

**[0111]** Chymosin variants were designed using different strategies.

**[0112]** When there is referred to camel chymosin there is referred to camel chymosin comprising the polypeptide of SEQ ID NO: 2 herein.

**[0113]** 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.

**[0114]** When there is referred to bovine chymosin there is referred to bovine chymosin comprising the polypeptide of SEQ ID NO: 1 herein.

**[0115]** Bovine chymosin of SEQ ID NO: 1 may be seen as a herein relevant parent polypeptide having chymosin activity used to make bovine chymosin variants thereof.

**[0116]** Variants 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.

**[0117]** Variations were generally introduced in hypervariable regions, while conserved regions were not changed. Multiple variations were introduced in each variant construct, ensuring that each single mutation was present in multiple variant constructs (for discussion of results - see example 6 below).

**[0118]** Variants of bovine chymosin were designed based on a comparison of bovine and camel chymosin. Bovine residues were e.g. changed to the camel counterpart (for discussion of results - see example 7 below).

**EXAMPLE 3: Preparation of chymosin variant enzyme material**

**[0119]** All chymosin variants were synthesized as synthetic genes and cloned into a fungal expression vector corresponding essentially to pGAMpR-C (described in WO02/36752A2)

**[0120]** 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.

**[0121]** 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.

**[0122]** 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 clotting activity**

**[0123]** 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 pH 5.5 buffer. Hereafter, 200 µl enzyme was added to 10 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.

**[0124]** 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:

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

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

**[0125]** For clotting activity determination of camel variant evaluation in Example 9, 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**

**[0126]** 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**

**[0127]** 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**

**[0128]** General proteolytic activity was measured using fluoresecently labelled Bodipy-FL casein as a substrate (EnzChek; Molecular Bioprobes, E6638). Casein derivatives heavily labeled with pH-insensitive green-fluorescent Bodipy-FL result in almost complete 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.

**[0129]** The assay was conducted in a 0.2 M phosphate buffer adjusted to the desired pH at a final substrate concentration of 0.04 mg/ml. Prior to mixing 1 part of substrate with 1 part of enzyme, both prepared in the phosphate buffer, all enzyme variants where normalized to 50 IMCU/ml (according to Example 4). The substrate and enzyme were mixed in a 96-well Nunc Fluoro microtitter plates, sealed and incubated at 32°C for 60 min. After incubation the sealing was removed and the fluorescence recorded in a fluorimeter. For variants evaluated in Examples 9 and 10, 1 part of substrate was mixed with 1 part of non-normalized enzyme samples in 386-well Nunc Fluoro microtitter plates and the fluorescence was continuously recorded in a fluorimeter at 32C for 10 hours. Slopes of the linear part of fluorescence increase were used to determine general proteolytic activity.

**EXAMPLE 6: Evaluation of camel chymosin variants**

**[0130]** For all variants the specific clotting activity (IMCU/mg of total protein) was determined at pH 6.5 according to Example 4 and the proteolytical activity was determined according to example 5 at pH 6.5 The C/P ratio was determined for all variants at pH 6.5 by dividing the specific clotting activity (IMCU/mg) with the proteolytical activity.

**[0131]** As a reference the camel wildtype gene was included.

**Variants with multiple substitutions**

**[0132]** It can be concluded that there are clear combinatorial effects, where different substitutions have an effect on the respective effects.

| | | IMCU/mg | Proteol | C/P |
|---|---|---|---|---|
| 1 | H134Q,Q246E,Y326F | 104% | 211% | 49% |
| 2 | D117N, L280I, G309D | 122% | 66% | 185% |
| 3 | H134Q, D156V, G309D | 117% | 179% | 66% |
| 4 | D156V, Q246E, L280I | 105% | 199% | 53% |
| 5 | D117N, H134Q, L280I | 67% | 683% | 10% |
| 6 | D156V, G309D, Y326F | 100% | 115% | 87% |
| 8 | D117N, D156V, D325M | 127% | 457% | 28% |
| 9 | L280I, D325M, Y326F | 113% | 94% | 121% |
| 10 | D117N, Q246E, Y326F | 127% | 121% | 105% |
| 11 | D117N, H134Q, D325M | 134% | 192% | 69% |
| Ref | camel | 100% | 100% | 100% |

**[0133]** It can be concluded that variants 1, 2, 3, 4, 8 ,9, 10 and 11 have a higher specific milk clotting activity, with variants 2, 8, 10 and 11 having the strongest improvement

**[0134]** It can be concluded that variants 2 and 9 have a reduced proteolytical activity.

**[0135]** It can be concluded that variants 2, 9 and 10 have an increased C/P ratio.

**[0136]** Based on this variant 2 is the most preferred variant, while variants 9 and 10 also show preferred characteristics.

**Individual mutations**

**[0137]** As all variants included multiple mutations, the data of the ranked variants were investigated in more details using statistical methods and 3D structure analysis, to determine the individual amino acid changes that have a positive or negative effect.

**[0138]** The effects of the individual amino acid changes can be summarized as follows but depend much upon the other amino acid changes in the different variants. Based on these the preferred mutations are D117N, Q246E, G 309D, Y326F and L280I.

| | C | P | C/P | |
|---|---|---|---|---|
| H134Q | + | -- | -- | Exposed lobe |
| Q246E | + | -- | - | Backbone |
| Y326F | + | - | +/- | Backbone |
| D117N | ++ | -- | - | Backbone lobe |
| L280I | + | +/- | +/- | In cleft |
| G309D | + | -- | +/- | Outside small lobe |
| D156V | + | -- | -- | Backbone |
| D325M | ++ | -- | -- | Backbone |

**[0139]** The term "+" refers to a positive amino acid exchange - i.e. "++" is more positive than "+".

**[0140]** The term "-" refers to a negative amino acid exchange - i.e. "- -" is more negative than "-".

**[0141]** The term "positive" refers to a positive effect on the cheese making properties of the variants, i.e. improved clotting activity ("C") and increased C/P ratio are considered to be positive ("+" or "++") while increased general proteolytical activity ("P") is considered to be a negative property ("-" or "--"). The qualification "+/-" indicates a relatively neutral effect

**[0142]** The descriptions of the right column of the table relates to where the individual mutations are situated in the 3D structure of camel chymosin. The 3D structure of camel chymosin is publicly available.

**Conclusions:**

**[0143]** The results above demonstrate that following individual mutations in camel chymosin were preferred (i.e. with improved C/P ratio as compared to camel wildtype chymosin): D117N, Q246E, G309D, Y326F, L280I.

**[0144]** The results above demonstrate that following multiple substitutions/mutations in camel chymosin were preferred (i.e. with improved C/P ratio as compared to camel wildtype chymosin):

D117N + L280I + G309D;

L280I + D325M + Y326F;

D117N + Q246E + Y326F.

**EXAMPLE 7: Evaluation of camel and bovine chymosin variants**

**[0145]** For all variants the specific clotting activity (IMCU/mg of total protein) was determined at pH 6.5 according to Example 4, while the general or aspecific proteolytical activity was determined as described in example 5.

**[0146]** The C/P ratio was determined for all variants at pH 6.5 by dividing the specific clotting activity (IMCU/mg) with the proteolytical activity.

**[0147]** As a reference a camel wildtype gene was included.

**[0148]** For better comparison all variants were made in a background that did not have active N-glycosylation sites, the so called Ugly variants. These were made by changing the N in the two potential N-glycosylation sites into a Q.

**[0149]** For further results, see Figure 3.

**Description of the variants**

**[0150]** In variant J2, K279 was replaced by V in bovine non-glycosylated chymosin

**[0151]** In variant J32, the flap region from bovine non-glycosylated chymosin was replaced by the flap region from Pepsin.

**[0152]** In variant J72, the negative patch from bovine chymosin was used to replace the corresponding regions in camel chymosin. In variant J44, R300 was replaced in camel chymosin by Q, the corresponding amino acid in bovine chymosin. This mutation is also found in variant J72.

| | | | Relative to camel | | |
|---|---|---|---|---|---|
| | | | RemCat | Prot | C/P |
| J2 | BovUgly | N310Q, N349Q, K279V | 54% | 227% | 24% |
| J22 | BovUgly | Pepsin positive patch | 15% | 115% | 13% |
| J32 | BovUgly | K279V, L80I, K129E, P130T, H134T, Q141T, V171F, E191S, Y192G, N310Q, N349Q | 6% | 63% | 10% |
| J44 | CamUGly | R300Q | 123% | 114% | 108% |
| J72 | CamUgly | N158Q, N349Q, R300Q, N307D, G309D, R312S, K336Q | 125% | 58% | 215% |
| CamUgly | Camel | N158Q, N349Q | 100% | 98% | 102% |
| BovUgly | Bovine | N310Q, N349Q | 40% | 208% | 19% |
| | | | | | |
| Camel | | N/A | 100% | 100% | 100% |

**Conclusions:**

**[0153]** Mutation of the Lysin at position 279 of bovine chymosin resulted in a variant that showed comparable proteolytical activity and an increased specific clotting activity as compared to bovine chymosin (variant J2). Accordingly, it can be concluded that Valine at position 279 is the preferred amino acid.

**[0154]** The effect of glycosylation of Camel chymosin on the cheese making properties is neglectible. Comparison of the unglycosylated camel variant with the wildtype camel chymosin indicates no significant changes. However, introduction of the negative patch reason from bovine chymosin in camel chymosin (variant J72) shows a positive effect on the specific clotting activity, while the general proteolytical activity is approximately 2 fold reduced, resulting in a doubling of the C/P ratio. Introduction of the single mutation R300Q from this patch (variant J44) shows a similar positive effect on clotting activity as seen for variant J72. Q is concluded to be the preferred amino acid in position 300.

**[0155]** The negative patch region in bovine chymosin is expected to have an important effect for positioning of the enzyme outward the correct cleavage site, thus improving the enzymes specificity. The effect is expected to be mostly charge related, i.e. any change that increases the negative charge in this reason will result in increased specificity.

**[0156]** Below is shown an alignment of the negative charged region of bovine and camel chymosin. Only charged residues are indicated.

| | |
|---|---|
| **R**xxxxxxNxGxx**R**xxxxxxxxxxxxxxxxxxxxxxxx**K** | Camel |
| Qxxxxxx**D**x**D**xxSxxxxxxxxxxxxxxxxxxxxxxxQ | Bovine |

**[0157]** With respect to position numbers and using the Camel as reference the numbering is starting from the right:

R300

N307

G309

R312

K336

**EXAMPLE 8: Evaluation of camel variants**

**[0158]** A number of different variants, each having multiple substitutions as compared to the wild type camel chymosin, was analyzed.

**[0159]** For all variants the specific clotting activity (IMCU/mg of total protein) was determined at pH 6.5 according to Example 4, while the aspecific proteolytical activity was determined as described in Example 5 by measuring proteolytical activity per 100 IMCU.

**[0160]** As a reference a camel wildtype gene was included.

**Analysis of variants**

**[0161]** The variants indicated in the table have an amino acid sequence identical to the camel chymosin gene (indicated by camel wt), except for the variations mentioned for each variant.

**[0162]** Clotting activity is mentioned as IMCU per mg of total protein. Improved clotting activities are indicated with one or more "+" symbols. Proteolytical activity is expressed in artificial units per 100 IMCU. Improved variants, i.e. variants with reduced proteolytical activities, are indicated with one or more "+" symbols. More "+" symbols indicate a stronger improvement. In the "Overall" column "+" symbols indicate variants that have generally improved properties, i.e. a low proteolytical activity with a high clotting activity.

**Table 1, analysis of camel chymosin variants**

| | | | | | | Clotting | | Proteolytical | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | IMCU/mg | | AU/100 IMCU | | Overall |
| 1 | L280I | G309D | E141S | Q220S | R324I | 196 | | 161852 | | |
| 2 | L280I | G309W | F75Y | Y79S | | 119 | ++ | 43355 | | |
| 3 | L280I | G309D | H134Q | S222G | S331Y | 299 | | 36409 | | |
| 4 | L280I | G309D | K120Q | M223E | H239N | 250 | | 13642 | +++ | |

| | | | | | | Clotting | | Proteolytical | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | IMCU/mg | | AU/100 IMCU | | Overall |
| 5 | L280I | G309D | Q220S | V213F | T342S | 231 | | 139775 | | |
| 6 | L280I | G309D | H134Q | V213F | F281A | 376 | + | 23575 | + | |
| 7 | L280I | G309D | S331Y | L224V | Y326F | 318 | | 12257 | ++++ | + |
| 8 | L280I | G309D | Y326F | V241I | E305T | 353 | + | 33177 | | |
| 9 | L280I | G309D | S331Y | F124Y | I346L | 338 | | 37156 | | |
| 10 | L280I | G309D | M223E | L224V | L273V | 324 | | 36425 | | |
| 11 | L280I | G309D | H134Q | M223E | L70M | 386 | + | 10664 | ++++ | ++ |
| 12 | L280I | G309D | F75Y | S331Y | Q346E | 418 | ++ | 40393 | | |
| 13 | L280I | G309D | L224V | I103V | L238I | 412 | ++ | 50010 | | |
| 14 | L280I | G309W | L238I | T342S | | 420 | +++ | 21087 | + | ++ |
| 15 | L280I | G309D | | L70M | T342S | 395 | ++ | 22743 | + | |
| 16 | L280I | G309D | Y79S | L224V | S212A | 403 | ++ | 23684 | + | |
| 17 | L280I | G309D | V213F | E320T | V90L | 426 | +++ | 21956 | + | ++ |
| 18 | L280I | G309D | L163E | S222G | V261A | 246 | | 97468 | | |
| 19 | L280I | G309W | S212A | V261A | | 344 | | 10865 | ++++ | + |
| 20 | L280I | G309D | 0220S | L224V | H134Q | 425 | +++ | 35156 | | |
| 21 | L280I | G309W | K77T | R324I | | 434 | +++ | 45616 | | |
| 22 | L280I | G309W | I361L | I103V | | 324 | | 32966 | | |
| 23 | L280I | G309D | E141S | R324V | V367I | 360 | + | 77215 | | |
| 24 | L280I | G309D | Y79S | L273V | L163E | 317 | | 62132 | | |
| 25 | L280I | G309D | I154L | T235S | K379P | 333 | | 93587 | | |
| 26 | L280I | G309D | F75Y | T342S | V261A | 361 | + | 108877 | | |
| 27 | L280I | G309D | V90L | K379P | V318T | 317 | | 52280 | | |
| 28 | L280I | G309D | V256I | V90L | E141S | 289 | | 81720 | | |
| 29 | L280I | G309D | I154L | V261A | V367I | 405 | ++ | 59055 | | |
| 30 | L280I | G309D | Y326F | L273V | V90L | 312 | | 54833 | | |
| 31 | L280I | G309D | H134Q | L163E | V318T | 344 | | 43594 | | |
| 32 | L280I | G309D | Y79S | H134Q | Y326F | 337 | | 30815 | | |
| 33 | L280I | G309D | Y79S | I103V | F281A | 379 | + | 104307 | | |
| 31 | L280I | G309D | V256I | V261A | K379P | 378 | + | 39517 | | |
| 35 | L280I | G309D | S331Y | L238I | I154L | 293 | | 60312 | | |
| 36 | L280I | G309D | S222G | R324V | I154L | 223 | | 62784 | | |
| 37 | L280I | G309D | H239N | F124Y | V90L | 312 | | 55432 | | |
| 38 | L280I | G309D | H239N | R324I | D325Q | 377 | + | 17261 | ++ | |
| 39 | L280I | G309W | K120Q | V367I | | 354 | | 75440 | | |
| 40 | L280I | G309D | Y326F | L70M | D325Q | 373 | + | 72792 | | |
| 41 | L280I | G309D | L224V | E320T | T235S | 446 | +++ | 32453 | | |
| 42 | L280I | G309D | S331Y | T342S | D325Q | 175 | ++++ | 70103 | | |
| 43 | L280I | G309D | F124Y | Q346E | I154L | 410 | ++ | 33586 | | |
| 44 | L280I | G309D | V261A | R324V | F281A | 198 | | 34974 | | |
| 45 | L280I | G309D | I361L | S212A | V318T | 343 | | 64876 | | |
| 46 | L280I | G309D | Y79S | T342S | I154L | 382 | + | 122413 | | |
| 47 | L280I | G309D | Q316E | K77T | T235S | 377 | + | 34716 | | |
| 48 | L280I | G309D | K120Q | Y326F | K77T | 264 | | 46463 | | |
| Camel wt | | | | | | 366 | + | 15.664 | +++ | |
| Bovine wt | | | | | | 208 | | 62.662 | | |

**[0163]** High specific clotting activity is essential for a good milk clotting enzymes. In total 21 variants with an increased specific clotting activity, relative to the camel chymosin, were identified and included in Table 2 below.

**Table 2, Camel chymosin variants with increased Clotting activity**

| | | | | | | Clotting | | Proteolytical | | Overall |
|---|---|---|---|---|---|---|---|---|---|---|
| 42 | L280I | G309D | S331Y | T342S | D325Q | 475 | ++++ | 70.103 | | |
| 41 | L280I | G309D | L224V | E320T | T235S | 446 | +++ | 30.953 | | |
| 21 | L280I | G309W | K77T | R324I | | 434 | +++ | 45.616 | | |
| 17 | L280I | G309D | V213F | E320T | V90L | 426 | +++ | 21.956 | + | ++ |
| 20 | L280I | G309D | Q220S | L224V | H134Q | 425 | +++ | 35.156 | | |
| 14 | L280I | G309W | L238I | T342S | | 420 | +++ | 21.087 | + | ++ |
| 2 | L280I | G309W | F75Y | Y79S | | 419 | ++ | 43.355 | | |
| 12 | L280I | G309D | F75Y | S331Y | Q346E | 418 | ++ | 40.393 | | |
| 13 | L280I | G309D | L224V | I103V | L238I | 412 | ++ | 50.010 | | |
| 43 | L280I | G309D | F124Y | Q346E | I154L | 410 | ++ | 33.586 | | |
| 29 | L280I | G309D | I154L | V261A | V367I | 405 | ++ | 59.055 | | |
| 16 | L280I | G309D | Y79S | L224V | S212A | 403 | ++ | 23.684 | + | |
| 15 | L280I | G309D | | L70M | T342S | 395 | ++ | 22.743 | + | |
| 11 | L280I | G309D | H134Q | M223E | L70M | 386 | + | 10.664 | ++++ | ++ |
| 46 | L280I | G309D | Y79S | T342S | I154L | 382 | + | 122.413 | | |
| 33 | L280I | G309D | Y79S | I103V | F281A | 379 | + | 104.307 | | |
| 34 | L280I | G309D | V2561 | V261A | K379P | 378 | + | 39.517 | | |
| 47 | L280I | G309D | Q346E | K77T | T235S | 377 | + | 34.716 | | |
| 38 | L280I | G309D | H239N | R324I | D325Q | 377 | + | 17.261 | ++ | |
| 6 | L280I | G309D | H134Q | V213F | F281A | 376 | + | 23.575 | + | |
| 40 | L280I | G309D | Y326F | L70M | D325Q | 373 | + | 72.792 | | |
| Camel | | | | | | | | | | |
| wt | | | | | | 366 | + | 15.664 | +++ | |

**[0164]** Reduced proteolytical activity is a perquisite for a good milk clotting enzymes. In total 10 variants with a reduced proteolytical activity, relative to the camel chymosin, were identified (see Table 3 below).

**Table 3, Camel chymosin variants with reduced proteolytical activity**

| | | | | | | Clotting | | Proteolytical | | Overall |
|---|---|---|---|---|---|---|---|---|---|---|
| 11 | L280I | G309D | H134Q | M223E | L70M | 386 | + | 10.664 | ++++ | ++ |
| 19 | L280I | G309W | S212A | V261A | | 344 | | 10.865 | ++++ | + |
| 7 | L280I | G309D | S331Y | L224V | Y326F | 318 | | 12.257 | ++++ | + |
| 4 | L280I | G309D | K120Q | M223E | H239N | 250 | | 13.642 | +++ | |
| 38 | L280I | G309D | H239N | R324I | D325Q | 377 | + | 17.261 | ++ | |
| 14 | L280I | G309W | L238I | T342S | | 420 | +++ | 21.087 | + | ++ |
| 17 | L280I | G309D | V213F | E320T | V90L | 426 | +++ | 21.956 | + | ++ |
| 15 | L280I | G309D | | L70M | T342S | 395 | ++ | 22.743 | + | |
| 6 | L280I | G309D | H134Q | V213F | F281A | 376 | + | 23.575 | + | |
| 16 | L280I | G309D | Y79S | L224V | S212A | 403 | ++ | 23.684 | + | |
| Camel | | | | | | | | | | |
| wt | | | | | | 366 | + | 15.664 | +++ | |

**[0165]** Based on an overall analysis five variants were identified that had improved properties for both milk clotting and proteolytical activities. These five variants are indicated in table 4 below.

**Table 4, Camel chymosin variants with increased clotting activity and decreased proteolytical activity**

| | | | | | | Clotting | | Proteolytical | | Overall |
|---|---|---|---|---|---|---|---|---|---|---|
| 7 | L280I | G309D | S331Y | L224V | Y326F | 318 | | 12.257 | ++++ | + |
| 11 | L280I | G309D | H134Q | M223E | L70M | 386 | + | 10.664 | ++++ | ++ |
| 14 | L280I | G309W | L238I | T342S | | 420 | +++ | 21.087 | + | ++ |
| 17 | L280I | G309D | V213F | E320T | V90L | 426 | +++ | 21.956 | + | ++ |

| | | | | | | Clotting | | Proteolytical | | Overall |
|---|---|---|---|---|---|---|---|---|---|---|
| 19 | L280I | G309W | S212A | V261A | | 344 | | 10.865 | ++++ | + |
| Camel wt | | | | | | 366 | + | 15.664 | +++ | |

**Statistical analysis of the effects of individual mutations**

**[0166]** A statistical, PCA based, analysis was used to identify single mutations with positive effects on either proteolytical activity, milk clotting activity, or both. In the table below, mutations resulting in increased clotting activity, decreased proteolytical activity or both increased clotting and decreased proteolytical activity are summarized. The PCA plot is indicated in the Figure 4.

**Table 5, single substitutions having positive effects on clotting, proteolytical activity or on both**

| Clotting + proteolytical | Clotting | Proteolytical |
|---|---|---|
| H134Q | I103V | R324V |
| L224V | F75Y | K120Q |
| Q346E | D325Q | M223E |
| L70M | I154L | S331Y |
| G309W | I361L | K379P |
| E320T | Y79S | L163E |
| L238I | D117N | |
| V90L | L280I | |
| V367I | V261A | |
| V256I | | |
| K77T | | |
| S212A | | |
| F124Y | | |

**Positional effects**

**[0167]** It was expected that most mutations that would have an effect on clotting activity or on general proteolytical activity (i.e. specificity) would be located in or close to the catalytical cleft. The substrate is entering the catalytical cleft and it is also here that cleavage takes place.

**[0168]** Suprisingly, only few of the substitutions that were shown to have a positive effect on clotting activity and/or specificity were located in this region (for example L280I L70M and F75Y). Many mutations that had a positive effect were found on other parts of the molecule

**Substitutions resulting in improved clotting activity**

**[0169]** Most of the substitutions resulting in improved clotting activity were located in the body of the enzyme and are likely to have caused conformational changes in the molecule. Substitution F75Y is located at the entrance of the cleft and is rather subtle, resulting in increased polarity.

**Table6,substitutions giving improved clotting**

| | |
|---|---|
| I103V | Lobe, back |
| F75Y | Cleft entrance |
| D325Q | Backbone |
| I154L | Backbone |
| I361L | Body |
| Y79S | Backbone |
| D117N | Side |
| L280I | Close to cleft |
| V261A | Side |

**Substitutions resulting in reduced proteolytical activity**

**[0170]** Most of the substitutions are located in the body of the molecule. The resulting conformational changes might result in increased accessibility for the substrate. Two mutations were found at the lobes that mark the entrance of the catalytical cleft. The L163E substitution increases the negative charge. This strengthens the results from example 7, showing the importance of charge in these positions.

Table 6 Mutations resulting in reduced proteolytical activity

| | |
|---|---|
| R324V | Backbone |
| K120Q | Side |
| M223E | Body |
| S331Y | Lobe |
| K379P | Backbone |
| L163E | Lobe |

**Substitutions resulting in improved clotting and reduced proteolytical activity**

**[0171]** Some of the substitutions that result in an overall improvement of the milk clotting capabilities result in charge changes that are likely to be involved in substrate recognition. These include H134Q resulting in higher positive, as well as the Q346E substitution resulting in more negative charge. Other substitutions with positive effects on both clotting and specificity are most likely resulting in more general conformational changes of the chymosin molecule.

Table 7 Mutations giving improved clotting and reduced proteolytical activity

| | |
|---|---|
| H134Q | Outside flap |
| L224V | Backbone |
| Q346E | Entrance cleft |
| L70M | Cleft |
| G309W | Side lobe |
| E320T | Backbone |
| L238I | Backbone |
| V90L | Close to cleft |
| V367I | Backbone |
| V256I | Backbone |
| K77T | Side protruding |
| S212A | Backbone |
| F124Y | Backbone |

**EXAMPLE 9: Evaluation of camel variants**

**Variant characterization**

**[0172]** Camel chymosin variants evaluated in Example 7 regarding their milk clotting (C) and general proteolytic (P) activities were produced again and evaluated regarding their casein cleavage specificity C/P (Table 1 below). The C/P ratio is a measure for a coagulant's efficiency in cheese making, i.e., the yield of cheese curd obtained from a certain volume of milk. Milk clotting and general proteolytic activities were determined as described in Examples 4 and 5, respectively. In this example, however, proteolytic activity was measured without normalization for clotting activity.

**[0173]** Camel chymosin was analyzed as reference. C/P values of all variants are shown as relative values to wild type camel chymosin. An impact of total protein concentration in the enzyme samples on C/P was detected, and C/P values were corrected for this correlation

accordingly.

**Table 1, Analysis of camel chymosin variants**

| variant | mutations | | | | | Clotting (C) | Proteolytical (P) | C/P |
|---|---|---|---|---|---|---|---|---|
| 1 | L280I | G309D | E141S | Q220S | R324I | 92% | 125% | 25% |
| 2 | L280I | G309W | F75Y | Y79S | | 108% | 129% | 78% |
| 3 | L280I | G309D | H 134Q | S222G | S331Y | 103% | 34% | 271% |
| 4 | L280I | G309D | K120Q | M223E | H239N | 96% | 81% | 85% |
| 5 | L280I | G309D | Q220S | V213F | T3425 | 75% | 113% | 42% |
| 6 | L280I | G309D | H 134Q | V213F | F281A | 62% | 31% | 339% |
| 7 | L280I | G309D | S331Y | L224V | Y326F | 91% | 110% | 143% |
| 8 | L280I | G309D | Y326F | V241I | E305T | 135% | 114% | 94% |
| 9 | L280I | G309D | S331Y | F124Y | I346L | 98% | 123% | 81% |
| 10 | L280I | G309D | M223E | L224V | L273V | 93% | 78% | 105% |
| 11 | L280I | G309D | H 134Q | M223E | L70M | 116% | 68% | 246% |
| 12 | L280I | G309D | F75Y | S331Y | Q346E | 155% | 83% | 172% |
| 13 | L280I | G309D | L224V | I103V | L238I | 136% | 89% | 128% |
| 14 | L280I | G309W | L238I | T3425 | | 124% | 159% | 89% |
| 15 | L280I | G309D | | L70M | T3425 | 93% | 152% | 35% |
| 16 | L280I | G309D | Y79S | L224V | S212A | 137% | 91% | 100% |
| 17 | L280I | G309D | V213F | E320T | V90L | 133% | 163% | 46% |
| 18 | L280I | G309D | L163E | S222G | V261A | 72% | 49% | 182% |
| 19 | L280I | G309W | S212A | V261A | | 104% | 122% | 138% |
| 20 | L280I | G309D | Q220S | L224V | H 134Q | 201% | 52% | 315% |
| 21 | L280I | G309W | K77T | R324I | | 160% | 102% | 139% |
| 22 | L280I | G309W | I361L | I103V | | 108% | 132% | 79% |
| 24 | L280I | G309D | Y79S | L273V | L163E | 91% | 76% | 112% |
| 25 | L280I | G309D | I154L | T2355 | K379P | 112% | 118% | 112% |
| 26 | L280I | G309D | F75Y | T3425 | V261A | 108% | 90% | 141% |
| 27 | L280I | G309D | V90L | K379P | V318T | 95% | 135% | 55% |
| 28 | L280I | G309D | V256I | V90L | E141S | 109% | 146% | 139% |
| 29 | L280I | G309D | I154L | V261A | V367I | 157% | 95% | 156% |
| 30 | L280I | G309D | Y326F | L273V | V90L | 99% | 119% | 58% |
| 31 | L280I | G309D | H 134Q | L163E | V318T | 95% | 59% | 247% |
| 32 | L280I | G309D | Y79S | H134Q | Y326F | 105% | 66% | 219% |
| 33 | L280I | G309D | Y79S | I103V | F281A | 124% | 66% | 342% |
| 34 | L280I | G309D | V256I | V261A | K379P | 146% | 102% | 134% |
| 36 | L280I | G309D | S222G | R324V | I154L | 76% | 68% | 161% |
| 37 | L280I | G309D | H239N | F124Y | V90L | 102% | 125% | 67% |
| 38 | L280I | G309D | H239N | R324I | D325Q | 90% | 143% | 127% |
| 39 | L280I | G309W | K120Q | V367I | | 103% | 94% | 139% |
| 40 | L280I | G309D | Y326F | L70M | D325Q | 96% | 207% | 10% |
| 41 | L280I | G309D | L224V | E320T | T2355 | 116% | 102% | 134% |
| 42 | L280I | G309D | S331Y | T3425 | D325Q | 145% | 102% | 158% |
| 43 | L280I | G309D | F124Y | Q346E | I154L | 135% | 94% | 176% |
| 44 | L280I | G309D | V261A | R324V | F281A | 71% | 63% | 137% |
| 45 | L280I | G309D | I361L | S212A | V318T | 116% | 122% | 100% |
| 46 | L280I | G309D | Y79S | T3425 | I154L | 137% | 102% | 115% |
| 47 | L280I | G309D | Q346E | K77T | T235S | 124% | 107% | 123% |
| 48 | L280I | G309D | K120Q | Y326F | K77T | 90% | 86% | 113% |
| Camel wt | | | | | | 100% | 100% | 100% |

**[0174]** A total of 30 out of 46 characterized variants show improved C/P compared to wild type camel chymosin (Table 2 below). A more than 3-fold improvement was observed for the three top variants 33, 6 and 20.

**Table 2, Camel chymosin variants with improved C/P**

| variant | | mutations | | | | Clotting (C) | Proteolytical (P) | C/P |
|---|---|---|---|---|---|---|---|---|
| 33 | L280I | G309D | Y79S | I103V | F281A | 124% | 66% | 342% |
| 6 | L280I | G309D | H 134Q | V213F | F281A | 62% | 31% | 339% |
| 20 | L280I | G309D | Q220S | L224V | H134Q | 201% | 52% | 315% |
| 3 | L280I | G309D | H 134Q | S222G | S331Y | 103% | 34% | 271% |
| 31 | L280I | G309D | H 134Q | L163E | V318T | 95% | 59% | 247% |
| 11 | L280I | G309D | H 134Q | M223E | L70M | 116% | 68% | 246% |
| 32 | L280I | G309D | Y79S | H134Q | Y326F | 105% | 66% | 219% |
| 18 | L280I | G309D | L163E | 5222G | V261A | 72% | 49% | 182% |
| 43 | L280I | G309D | F124Y | Q346E | I154L | 135% | 94% | 176% |
| 12 | L280I | G309D | F75Y | S331Y | Q346E | 155% | 83% | 172% |
| 36 | L280I | G309D | S222G | R324V | I154L | 76% | 68% | 161% |
| 42 | L280I | G309D | S331Y | T342S | D325Q | 145% | 102% | 158% |
| 29 | L280I | G309D | I154L | V261A | V367I | 157% | 95% | 156% |
| 7 | L280I | G309D | S331Y | L224V | Y326F | 91% | 110% | 143% |
| 26 | L280I | G309D | F75Y | T342S | V261A | 108% | 90% | 141% |
| 21 | L280I | G309W | K77T | R324I | | 160% | 102% | 139% |
| 28 | L280I | G309D | V256I | V90L | E141S | 109% | 146% | 139% |
| 39 | L280I | G309W | K120Q | V367I | | 103% | 94% | 139% |
| 19 | L280I | G309W | S212A | V261A | | 104% | 122% | 138% |
| 44 | L280I | G309D | V261A | R324V | F281A | 71% | 63% | 137% |
| 34 | L280I | G309D | V256I | V261A | K379P | 146% | 102% | 134% |
| 41 | L280I | G309D | L224V | E320T | T235S | 116% | 102% | 134% |
| 13 | L280I | G309D | L224V | I103V | L238I | 136% | 89% | 128% |
| 38 | L280I | G309D | H239N | R324I | D325Q | 90% | 143% | 127% |
| 47 | L280I | G309D | Q346E | K77T | T235S | 124% | 107% | 123% |
| 46 | L280I | G309D | Y79S | T342S | I154L | 137% | 102% | 115% |
| 48 | L280I | G309D | K120Q | Y326F | K77T | 90% | 86% | 113% |
| 24 | L280I | G309D | Y79S | L273V | L163E | 91% | 76% | 112% |
| 25 | L280I | G309D | I154L | T235S | K379P | 112% | 118% | 112% |
| 10 | L280I | G309D | M223E | L224V | L273V | 93% | 78% | 105% |
| Camel wt | | | | | | 100% | 100% | 100% |

**Statistical analysis of the positional and mutational effects on C/P**

**[0175]** A statistical, PCA based, analysis was used to identify single mutations with positive effects on the specificity of milk clotting over general casein proteolysis (C/P) of camel chymosin. The following mutations were found to be beneficial for high C/P ratios: H134Q, F281A, I103V, V256I, I154L, S222G, L224V, Q346E, S331Y, K77T, V367I, G309D, V261A, D325Q, L280I, D117N, L163E, S212A

**EXAMPLE 10: Evaluation of camel variants**

**Variant characterization**

**[0176]** Based on the positional and mutational effects determined in Example 7, another set of camel chymosin variants was generated with multiple substitutions as compared to wild type camel chymosin and evaluated regarding their casein substrate specificity (C/P) as described in Example 9 (Table 1 below).

Table 1, Analysis of camel chymosin variants

| variant | mutations | | | | | | | | | | Clotting (C) | Proteolytical (P) | C/P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | L70M | Y79S | D117N | H134Q | M223E | V256I | L280I | G309D | Q346E | | 132% | 116% | 117% |
| 2 | L70M | Y79S | D117N | H134Q | M223E | L280I | G309W | S331Y | | | 131% | 56% | 194% |
| 3 | L70M | D117N | H134Q | M223E | V256I | L280I | G309D | S331Y | K379P | | 109% | 75% | 135% |
| 4 | L70M | D117N | H134Q | S212A | M223E | V261A | L280I | G309D | V367I | | 83% | 115% | 108% |
| 5 | L70M | D117N | H134Q | D156V | L280I | | | | | | 135% | 108% | 137% |
| 6 | L70M | K77T | V90L | D117N | H134Q | D202Q | M223E | L280I | G309D | | 135% | 113% | 124% |
| 7 | L70M | Y79S | D117N | H134Q | M223E | V261A | L280I | G309D | E320T | | 141% | 124% | 143% |
| 8 | L70M | V109L | H134Q | M223E | G309D | | | | | | 82% | 86% | 87% |
| 9 | L70M | D117N | F124Y | H134Q | M223E | L238I | L280I | G309D | V367I | | 105% | 97% | 115% |
| 10 | L70M | D117N | H134Q | S212A | M223E | L280I | G309W | Q346E | | | 101% | 79% | 133% |
| 11 | L70M | D117N | H134Q | D156V | M223E | L280I | G309D | E320T | Q346E | | 153% | 101% | 119% |
| 12 | L70M | V109L | D117N | H134Q | L224V | L280I | G309D | | | | 98% | 71% | 128% |
| 13 | L70M | D117N | H134Q | D202Q | M223E | V261A | L280I | | | | 116% | 144% | 126% |
| 14 | L70M | D117N | D202Q | M223E | L224V | L280I | G309D | | | | 85% | 126% | 111% |
| 15 | L70M | K77T | D117N | H134Q | S212A | M223E | V256I | L280I | G309D | | 154% | 130% | 129% |
| 16 | L70M | H134Q | D156V | M223E | L280I | G309W | | | | | 136% | 131% | 137% |
| 17 | L70M | V90L | D117N | H134Q | M223E | L238I | V256I | L280I | G309D | | 121% | 101% | 97% |
| 18 | L70M | D117N | H134Q | S212A | M223E | S331Y | | | | | 124% | 76% | 151% |
| 19 | L70M | V109L | D117N | F124Y | H134Q | M223E | V261A | L280I | G309W | | 96% | 98% | 128% |
| 20 | L70M | V90L | H134Q | M223E | L280I | E320T | | | | | 138% | 110% | 98% |
| 21 | L70M | N108D | D117N | H134Q | M223E | G309W | E320T | | | | 187% | 151% | 138% |
| 22 | V109L | D117N | H134Q | M223E | L238I | L280I | G309D | E320T | | | 110% | 93% | 106% |
| 23 | L70M | D117N | H134Q | M223E | G309D | Q346E | V367I | K379P | | | 67% | 102% | 118% |
| 24 | L70M | N108D | D117N | V261A | L280I | G309D | | | | | 95% | 117% | 102% |
| 25 | L70M | D117N | H134Q | L238I | L280I | G309W | K379P | | | | 97% | 92% | 113% |
| 26 | L70M | Y79S | D117N | M223E | L280I | K379P | | | | | 137% | 123% | 129% |
| 27 | D117N | H134Q | M223E | L224V | V256I | L280I | | | | | 132% | 102% | 127% |
| 28 | L70M | K77T | N108D | D117N | H134Q | M223E | L280I | Q346E | | | 167% | 106% | 166% |
| 29 | L70M | Y79S | N108D | D117N | F124Y | H134Q | D202Q | M223E | L280I | G309D | 183% | 57% | 151% |
| Bovine wt | | | | | | | | | | | 100% | 100% | 100% |

**[0177]** A total of 26 out of 29 variants show improved C/P ratios, as compared to wild type camel chymosin. A 2-fold improvement was observed for the best variant (Table 2, below).

Table 2, Camel chymosin variants with improved C/P

| variant | mut ations | | | | | | | | | | Clotting (C) | Proteolytical (P) | C/P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | L70M | Y79S | D117N | H134Q | M223E | L280I | G309W | S331Y | | | 131% | 56% | 194% |
| 28 | L70M | K77T | N108D | D117N | H134Q | M223E | L280I | Q346E | | | 167% | 106% | 166% |
| 18 | L70M | D117N | H134Q | S212A | M223E | S331Y | | | | | 124% | 76% | 151% |
| 29 | L70M | Y79S | N108D | D117N | F124Y | H134Q | D202Q | M223E | L280I | G309D | 183% | 57% | 151% |
| 7 | L70M | Y79S | D117N | H134Q | M223E | V261A | L280I | G309D | E320T | | 141% | 124% | 143% |
| 21 | L70M | N108D | D117N | H134Q | M223E | G309W | E320T | | | | 187% | 151% | 138% |
| 5 | L70M | D117N | H134Q | D156V | L280I | | | | | | 135% | 108% | 137% |
| 16 | L70M | H134Q | D156V | M223E | L280I | G309W | | | | | 136% | 131% | 137% |
| 3 | L70M | D117N | H134Q | M223E | V256I | L280I | G309D | S331Y | K379P | | 109% | 75% | 135% |
| 10 | L70M | D117N | H134Q | S212A | M223E | L280I | G309W | Q346E | | | 101% | 79% | 133% |
| 15 | L70M | K77T | D117N | H134Q | S212A | M223E | V256I | L280I | G309D | | 154% | 130% | 129% |
| 26 | L70M | Y79S | D117N | M223E | L280I | K379P | | | | | 137% | 123% | 129% |
| 12 | L70M | V109L | D117N | H134Q | L224V | L280I | G309D | | | | 98% | 71% | 128% |

| variant | mut ations | | | | | | | | | | Clotting (C) | Proteolytical (P) | C/P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | L70M | V109L | D117N | F124Y | H134Q | M223E | V261A | L280I | G309W | | 96% | 98% | 128% |
| 27 | D117N | H134Q | M223E | L224V | V256I | L280I | | | | | 132% | 102% | 127% |
| 13 | L70M | D117N | H134Q | D202Q | M223E | V261A | L280I | | | | 116% | 144% | 126% |
| 6 | L70M | K77T | V90L | D117N | H134Q | D202Q | M223E | L280I | G309D | | 135% | 113% | 124% |
| 11 | L70M | D117N | H134Q | D156V | M223E | L280I | G309D | E320T | Q346E | | 153% | 101% | 119% |
| 23 | L70M | D117N | H134Q | M223E | G309D | Q346E | V367I | K379P | | | 67% | 102% | 118% |
| 1 | L70M | Y79S | D117N | H134Q | M223E | V256I | L280I | G309D | Q346E | | 132% | 116% | 117% |
| 9 | L70M | D117N | F124Y | H134Q | M223E | L238I | L280I | G309D | V367I | | 105% | 97% | 115% |
| 25 | L70M | D117N | H134Q | L238I | L280I | G309W | K379P | | | | 97% | 92% | 113% |
| 14 | L70M | D117N | D202Q | M223E | L224V | L280I | G309D | | | | 85% | 126% | 111% |
| 4 | L70M | D117N | H134Q | S212A | M223E | V261A | L280I | G309D | V367I | | 83% | 115% | 108% |
| 22 | V109L | D117N | H134Q | M223E | L238I | L280I | G309D | E320T | | | 110% | 93% | 106% |
| 24 | L70M | N108D | D117N | V261A | L280I | G309D | | | | | 95% | 117% | 102% |
| Camel wt | | | | | | | | | | | 100% | 100% | 100% |

**Statistical analysis of the positional and mutational effects on C/P**

**[0178]** A statistical, PCA based, analysis was used to identify single mutations with positive effects on the specificity of milk clotting over general casein proteolysis (C/P) of camel chymosin. The following mutations were found to be beneficial for high C/P ratios:
S331Y, Y79S, K77T, D117N, H134Q, N108D, G309W, L224V, D156V, L280I, M223E, V367I, F114Y

**EXAMPLE 11: Evaluation of camel variants**

**[0179]** A statistical, PCA based, analysis was performed on the combined set of variants from Examples 9 and 10, and single mutations were identified with positive effects on the specificity of milk clotting over general casein proteolysis (C/P) of camel chymosin. The following mutations were found to be beneficial for high C/P ratios:
F281A, H134Q, I103V, S331Y, S222G, I154L, L280I, G309D, D117N, L224V, N108D, L163E, G309W, K77T, Y79S

**[0180]** These mutations agree well with the beneficial mutations determined in Examples 9 and 10.

**Structural evaluation of positional and mutational effects on C/P**

**[0181]** As seen in Example 8, the majority of beneficial mutations are again located distant from the substrate binding cleft. Only L280I and F281A are located directly in the cleft (Gilliland et al. 1990). I280 points into the hydrophobic core of the C-terminal lobe. This mutation might therefore lead to subtle conformational changes of the binding cleft and, thus, influence substrate specificity. Position 281 is part of the S2 binding site and interacts with the P2 position in the casein substrate. A mutation in this position is very likely to have an impact on casein binding and, thus, proteolysis. Mutations G309W and S331Y are positioned on the surface of the C-terminal lobe in a region that has been described to interact with κ-casein to aid substrate binding in the catalytic cleft (Gilliland et al. 1990). These mutations might therefore have a positive impact on substrate binding. I154L and D156V, and L163E represent changes to the core of the N-terminal lobe, possibly leading to subtle structural rearrangements of the enzyme with impact on catalytic activity. Mutations S222G and L224V introduce changes into the beta sheet that might interact with the protein N-terminus in its activated form (Langholm Jensen et al.). Potential effects on the activation state of the enzyme could result in shifted casein substrate specificity. The remaining hit mutations K77T, Y79S, I103V, N108D, D117N, and H134Q are located on the surface of the N-terminal lobe and, with exception of I103V, represent exchanges of polar amino acids. These changes on the surface of the enzyme most probably influence interactions with casein molecules leading to improved specificity in favor of κ-casein.

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## PATENTKRAV

1. Fremgangsmåde til fremstilling af en isoleret chymosin-polypeptidvariant omfattende følgende trin:

(a): udførelse af en ændring i én eller flere positioner i et moderpolypeptid med chymosinaktivitet, hvor ændringen omfatter en substitution, en deletion eller en insertion i mindst én aminosyreposition svarende til position 70; og

(b): fremstilling og isolering af det ændrede polypeptid fra trin (a) og derved opnåelse af den isolerede chymosin-polypeptidvariant, hvor varianten har chymosinaktivitet;

og hvor:

(i): aminosyrepositionen af moderpolypeptidet bestemmes ved en alignment af moderpolypeptidet med polypeptidet med SEQ ID NO: 1 (bovint chymosin) - dvs. polypeptidet med SEQ ID NO: 1 anvendes til at bestemme den tilsvarende aminosyresekvens i moderpolypeptidet; og

(ii): moderpolypeptidet har mindst 90% sekvensidentitet med det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin), som er fra aminosyreposition 59 til aminosyreposition 381 af SEQ ID NO: 2;

(iii): det isolerede variantpolypeptid har mindre end 100% sekvensidentitet med det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin);

hvor den isolerede chymosin-polypeptidvariant har:

- en chymosinaktivitet, der giver et højere C/P-forhold sammenlignet med C/P-forholdet for bovint chymosin omfattende det modne polypeptid med SEQ ID NO: 1; og
- en chymosinaktivitet, der giver et højere C/P-forhold sammenlignet med C/P-forholdet for kamel-chymosin omfattende det modne polypeptid med SEQ ID NO: 2;

den isolerede chymosinvariant omfatter færre end 30 aminosyreændringer sammenlignet med det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin);

en insertion betyder tilføjelse af 1-3 aminosyrer ved siden af en aminosyre, der indtager en position.

2. Fremgangsmåde til fremstilling af en isoleret chymosin-polypeptidvariant ifølge det foregående krav, hvor ændringen omfatter en substitution i mindst én aminosyreposition, og hvor substitutionen er L70M.

3. Fremgangsmåde til fremstilling af en isoleret chymosin-polypeptidvariant ifølge et hvilket som helst af de foregående krav, og hvor ændringen omfatter en substitution i

mindst én aminosyreposition, og hvor substitutionen er:
L280I + G309D + H134Q + M223E + L70M.

4. Fremgangsmåde til fremstilling af en isoleret chymosin-polypeptidvariant ifølge et hvilket som helst af kravene 1 til 3, hvor moderpolypeptidet har mindst 95% sekvensidentitet med det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin), som er fra aminosyreposition 59 til aminosyreposition 381 af SEQ ID NO: 2.

5. Fremgangsmåde til fremstilling af en isoleret chymosin-polypeptidvariant ifølge et hvilket som helst af kravene 1 til 4, hvor moderpolypeptidet har mindst 97% sekvensidentitet med det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin), som er fra aminosyreposition 59 til aminosyreposition 381 af SEQ ID NO: 2.

6. Fremgangsmåde til fremstilling af en isoleret chymosin-polypeptidvariant ifølge et hvilket som helst af kravene 1 til 5, hvor moderpolypeptidet er det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin), som er fra aminosyreposition 59 til aminosyreposition 381 af SEQ ID NO: 2.

7. Fremgangsmåde til fremstilling af en isoleret chymosin-polypeptidvariant ifølge krav 1, hvor moderpolypeptidet er det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin), som er fra aminosyreposition 59 til aminosyreposition 381 af SEQ ID NO: 2, og hvor substitutionen er:
L70M + Y79S + D117N + H134Q + M223E + V256I + L280I + G309D + Q346E;
L70M + Y79S + D117N + H134Q + M223E + L280I + G309W + S331Y;
L70M + D117N + H134Q + M223E + V256I + L280I + G309D + S331Y + K379P;
L70M + D117N + H134Q + S212A + M223E + V261A + L280I + G309D + V367I;
L70M + D117N + H134Q + D156V + L280I;
L70M + K77T + V90L + D117N + H134Q + D202Q + M223E + L280I + G309D;
L70M + Y79S + D117N + H134Q + M223E + V261A + L280I + G309D + E320T;
L70M + D117N + F124Y + H134Q + M223E + L238I + L280I + G309D + V367I;
L70M + D117N + H134Q + S212A + M223E + L280I + G309W + Q346E;
L70M + D117N + H134Q + D156V + M223E + L280I + G309D + E320T + Q346E;
L70M + V109L + D117N + H134Q + L224V + L280I + G309D;
L70M + D117N + H134Q + D202Q + M223E + V261A + L280I;
L70M + D117N + D202Q + M223E + L224V + L280I + G309D;

L70M + K77T + D117N + H134Q + S212A + M223E + V256I + L280I + G309D;
L70M + H134Q + D156V + M223E + L280I + G309W;
L70M + D117N + H134Q + S212A + M223E + S331Y;
L70M + V109L + D117N + F124Y + H134Q + M223E + V261A + L280I + G309W;
L70M + N108D + D117N + H134Q + M223E + G309W + E320T;
L70M + D117N + H134Q + M223E + G309D + Q346E + V367I + K379P;
L70M + N108D + D117N + V261A + L280I + G309D;
L70M + D117N + H134Q + L238I + L280I + G309W + K379P;
L70M + Y79S + D117N + M223E + L280I + K379P;
L70M + K77T + N108D + D117N + H134Q + M223E + L280I + Q346E; eller
L70M + Y79S + N108D + D117N + F124Y + H134Q + D202Q + M223E + L280I + G309D.

8. Isoleret chymosin-polypeptidvariant omfattende:
(a): en ændring i én eller flere positioner i et moderpolypeptid med chymosinaktivitet, hvor ændringen omfatter en substitution, en deletion eller en insertion i mindst én aminosyreposition svarende til position 70; og
(b): hvor varianten har chymosinaktivitet;
og hvor:
(i): aminosyrepositionen af moderpolypeptidet bestemmes ved en alignment af moderpolypeptidet med polypeptidet med SEQ ID NO: 1 (bovint chymosin) - dvs. polypeptidet med SEQ ID NO: 1 anvendes til at bestemme den tilsvarende aminosyresekvens i moderpolypeptidet; og
(ii): moderpolypeptidet har mindst 90% sekvensidentitet med det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin), som er fra aminosyreposition 59 til aminosyreposition 381 af SEQ ID NO: 2; og
(iii): det isolerede variantpolypeptid har mindre end 100% sekvensidentitet med det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin);
hvor den isolerede variant har en chymosinaktivitet, der giver et højere C/P-forhold sammenlignet med C/P-forholdet for kamel-chymosin omfattende det modne polypeptid med SEQ ID NO: 2;
den isolerede chymosinvariant omfatter færre end 30 aminosyreændringer sammenlignet med det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin);
en insertion betyder tilføjelse af 1-3 aminosyrer ved siden af en aminosyre, der indtager en position.

9. Isoleret chymosin-polypeptidvariant ifølge krav 8, hvor ændringen omfatter en substitution i mindst én aminosyreposition, og hvor substitutionen er:
L280I + G309D + H134Q + M223E + L70M.

10. Isoleret chymosin-polypeptidvariant ifølge krav 8, hvor moderpolypeptidet er det modne polypeptid med SEQ ID NO: 2 (kamel-chymosin), som er fra aminosyreposition 59 til aminosyreposition 381 af SEQ ID NO: 2, og hvor substitutionen er:
L70M + Y79S + D117N + H134Q + M223E + V256I + L280I + G309D + Q346E;
L70M + Y79S + D117N + H134Q + M223E + L280I + G309W + S331Y;
L70M + D117N + H134Q + M223E + V256I + L280I + G309D + S331Y + K379P;
L70M + D117N + H134Q + S212A + M223E + V261A + L280I + G309D + V367I;
L70M + D117N + H134Q + D156V + L280I;
L70M + K77T + V90L + D117N + H134Q + D202Q + M223E + L280I + G309D;
L70M + Y79S + D117N + H134Q + M223E + V261A + L280I + G309D + E320T;
L70M + D117N + F124Y + H134Q + M223E + L238I + L280I + G309D + V367I;
L70M + D117N + H134Q + S212A + M223E + L280I + G309W + Q346E;
L70M + D117N + H134Q + D156V + M223E + L280I + G309D + E320T + Q346E;
L70M + V109L + D117N + H134Q + L224V + L280I + G309D;
L70M + D117N + H134Q + D202Q + M223E + V261A + L280I;
L70M + D117N + D202Q + M223E + L224V + L280I + G309D;
L70M + K77T + D117N + H134Q + S212A + M223E + V256I + L280I + G309D;
L70M + H134Q + D156V + M223E + L280I + G309W;
L70M + D117N + H134Q + S212A + M223E + S331Y;
L70M + V109L + D117N + F124Y + H134Q + M223E + V261A + L280I + G309W;
L70M + N108D + D117N + H134Q + M223E + G309W + E320T;
L70M + D117N + H134Q + M223E + G309D + Q346E + V367I + K379P;
L70M + N108D + D117N + V261A + L280I + G309D;
L70M + D117N + H134Q + L238I + L280I + G309W + K379P;
L70M + Y79S + D117N + M223E + L280I + K379P;
L70M + K77T + N108D + D117N + H134Q + M223E + L280I + Q346E; eller
L70M + Y79S + N108D + D117N + F124Y + H134Q + D202Q + M223E + L280I + G309D.

11. Fremgangsmåde til fremstilling af et fødevare- eller foderprodukt, omfattende tilsætning af en effektiv mængde af den isolerede chymosin-polypeptidvariant ifølge krav 8 til 9 til fødevare- eller foderingrediensen/-ingredienserne og udførelse af yderligere fremstillingstrin for at opnå fødevare- eller foderproduktet.

12. Fremgangsmåde til fremstilling af et fødevare- eller foderprodukt ifølge krav 11, hvor produktet er et mælkebaseret produkt, og hvor fremgangsmåden omfatter tilsætning af en effektiv mængde af den isolerede chymosin-polypeptidvariant ifølge et hvilket som helst af kravene 8 til 9 til mælk og udførelse af yderligere fremstillingstrin for at opnå det mælkebaserede produkt.

13. Fremgangsmåde til fremstilling af et fødevare- eller foderprodukt ifølge krav 11, hvor mælken er sojamælk, fåremælk, gedemælk, bøffelmælk, yakmælk, lamamælk, kamelmælk eller komælk.

14. Fremgangsmåde til fremstilling af et fødevare- eller foderprodukt ifølge et hvilket som helst af kravene 11 til 13, hvor det mælkebaserede produkt er et fermenteret mælkeprodukt, en kvark eller en ost.

# DRAWINGS

Drawing

## Figure 1

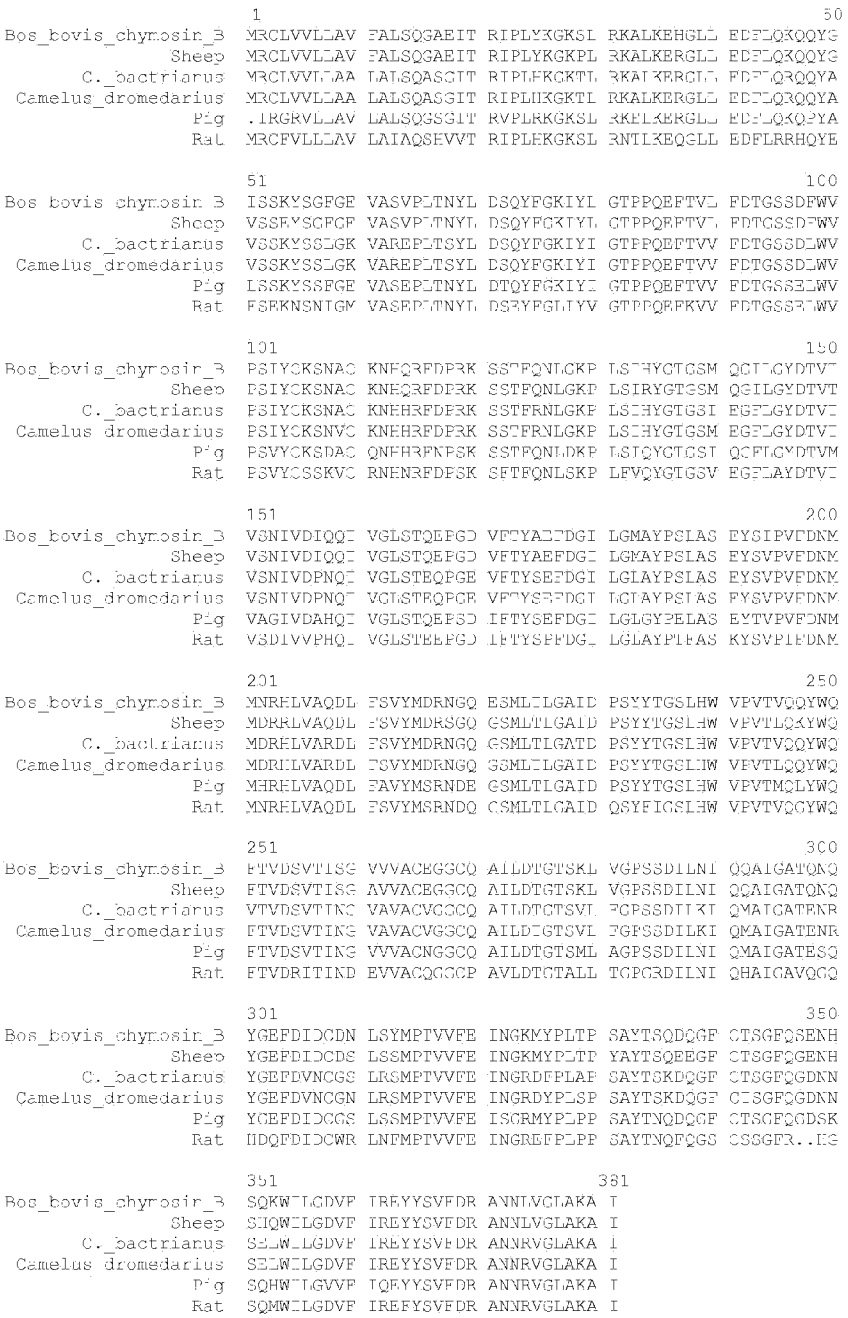
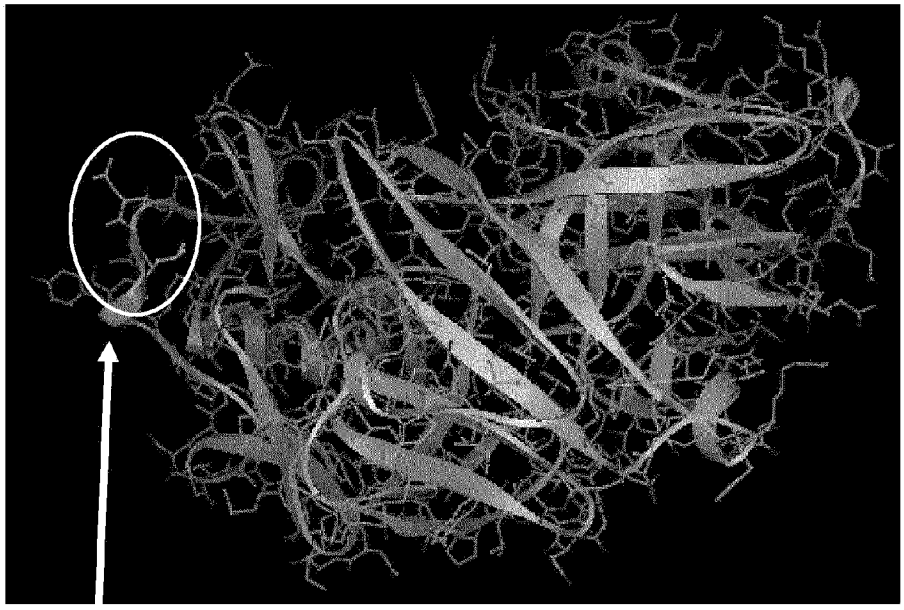
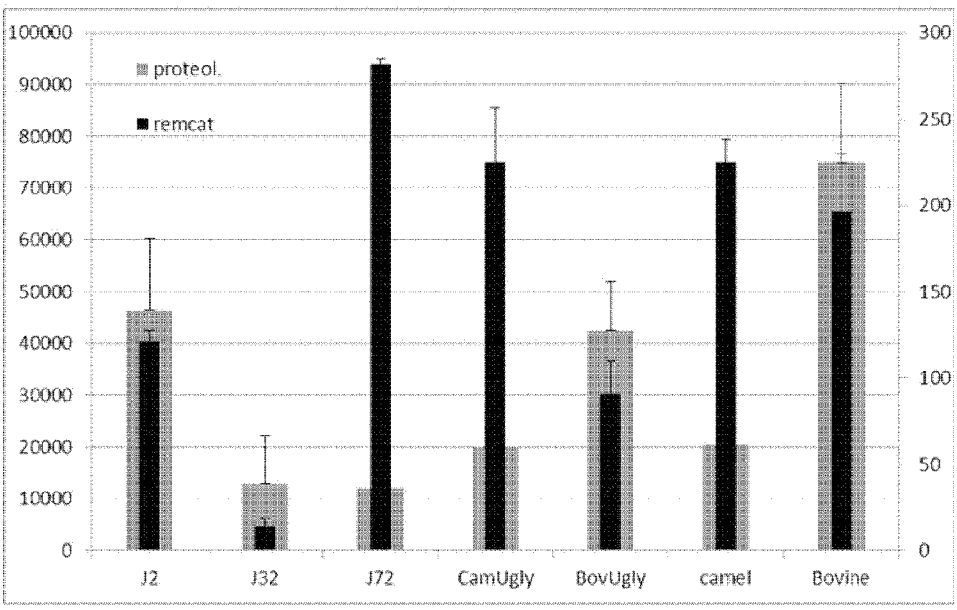


**Figure 2**



Positions 296 and 294 in
Bovine Chymosin

**Figure 3**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40000
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20000
10000
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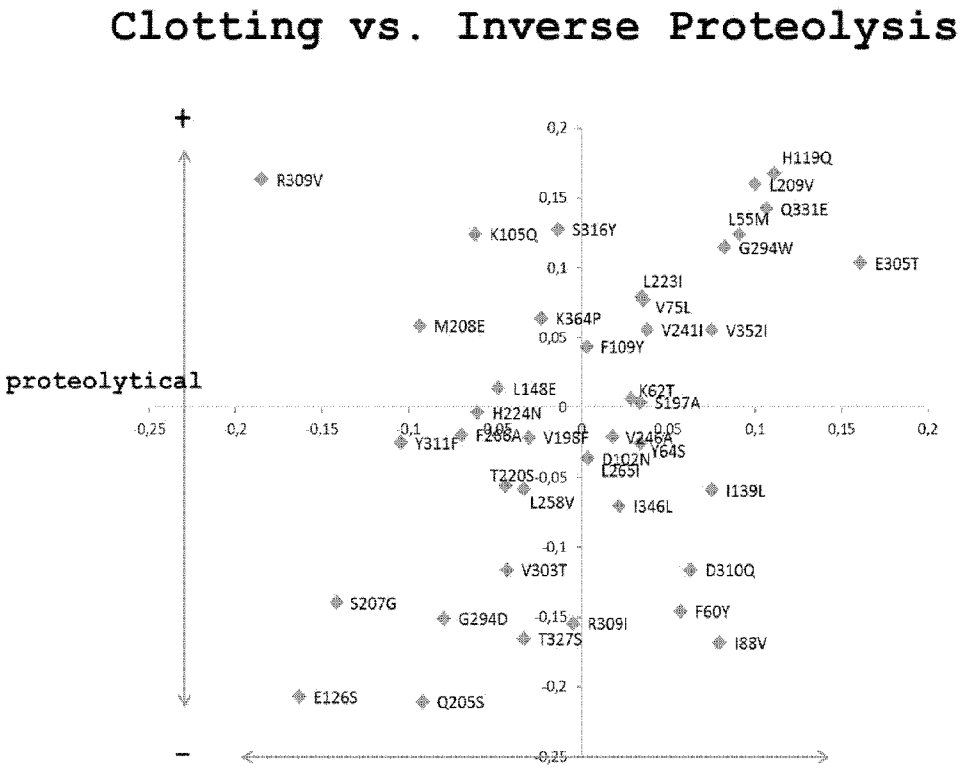
300
250
200
150
100
50
0
proteol.
remcat
J2
J32
J72
CamUgly
BovUgly
camel
Bovine

**Figure 4**


Clotting vs. Inverse Proteolysis
+
proteolytical
−
0,2
0,15
0,1
0,05
0
-0,05
-0,1
-0,15
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SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

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