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Description

FIELD OF THE INVENTION

- 5 **[0001]** This invention relates to polypeptides, specifically amylase polypeptides and nucleic acids encoding these, and their uses e.g. as non-maltogenic exoamylases in producing food or feed products.

BACKGROUND OF THE INVENTION

- 10 **[0002]** Improved amylases can ameliorate problems inherent in certain processes, such as in conversion of vegetable starches or baking.
- [0003]** Crystallisation of amylopectin takes place in starch granules days after baking, which leads to increased firmness of bread and causes bread staling. When bread stales, bread loses crumb softness and crumb moisture. As a result, crumbs become less elastic, and bread develops a leathery crust.
- 15 **[0004]** Enzymatic hydrolysis (by amylases, for example) of amylopectin side chains can reduce crystallization and increase anti-staling. Crystallization depends upon the length of amylopectin side chains: the longer the side chains, the greater the crystallization. Most starch granules are composed of a mixture of two polymers: amylopectin and amylose, of which about 75% is amylopectin. Amylopectin is a very large, branched molecule consisting of chains of α -D-glucopyranosyl units joined by (1-4) linkages, where the chains are attached by α -D-(1-6) linkages to form branches. Amylose is a linear chain of (1-4) linked α -D-glucopyranosyl units having few α -D-(1-6) branches.
- 20 **[0005]** Baking of farinaceous bread products such as white bread, bread made from bolted rye flour and wheat flour and rolls is accomplished by baking the bread dough at oven temperatures in the range of from 180 to 250°C for about 15 to 60 minutes. During the baking process a steep temperature gradient (200 to 120°C) prevails over the outer dough layers where the crust of the baked product is developed. However, due to steam, the temperature in the crumb is only
- 25 about 100°C at the end of the baking process. Above temperatures of about 85°C, enzyme inactivation can take place and the enzyme will have no anti-staling properties. Only thermostable amylases, thus, are able to modify starch efficiently during baking.
- [0006]** Endoamylase activity can negatively affect the quality of the final bread product by producing a sticky or gummy crumb due to the accumulation of branched dextrans. Exo-amylase activity is preferred, because it accomplishes the
- 30 desired modification of starch that leads to retardation of staling, with fewer of the negative effects associated with endo-amylase activity. Reduction of endoamylase activity can lead to greater exospecificity, which can reduce branched dextrans and produce a higher quality bread.
- [0007]** The conversion of vegetable starches, especially corn starch, to ethanol is a rapidly expanding industry. Maltotetraose (G4 or DP4) syrup is one of many commercially important products derived from enzymatic treatment of
- 35 starch. The conversion of vegetable starches, especially cornstarch, to maltotetraose and lower sugars, such as glucose or maltose, is a rapidly expanding industry.
- [0008]** The current process consists of two sequential enzyme-catalyzed steps that result in the production of glucose or maltose. Yeast can then be used to ferment the glucose to ethanol.
- [0009]** The first enzyme-catalyzed step is starch liquefaction. Typically, a starch suspension is gelatinized by rapid
- 40 heating to 85°C or more. α -Amylases (EC 3.2.1.1) are used to degrade the viscous liquefact to maltodextrins. α -amylases are endohydrolases that catalyze the random cleavage of internal α -1,4-D-glucosidic bonds. As α -amylases break down the starch, the viscosity decreases. Because liquefaction typically is conducted at high temperatures, thermostable α -amylases, such as an α -amylase from *Bacillus* sp., are preferred for this step.
- [0010]** The maltodextrins produced in this manner generally cannot be fermented by yeast to form alcohol. A second
- 45 enzyme-catalyzed saccharification step thus is required to break down the maltodextrins. Glucoamylases and/or maltogenic α -amylases commonly are used to catalyze the hydrolysis of non-reducing ends of the maltodextrins formed after liquefaction, releasing D-glucose, maltose and isomaltose. Debranching enzymes, such as pullulanases, can be used to aid saccharification. Saccharification typically takes place under acidic conditions at elevated temperatures, e.g., 60°C, pH 4.3.
- 50 **[0011]** G4 (also referred to as DP4) syrup has a number of advantageous properties compared to sucrose syrups. For example, partially replacing sucrose with G4 syrup in a food reduces the food's sweetness without affecting its taste or flavor. G4 syrup has high moisture retention in foods and exhibits less deleterious Maillard reaction products because of its lower glucose and maltose content. G4 syrup also has higher viscosity than sucrose, thus improving food texture. G4 syrup depresses the freezing point of water less than sucrose or high fructose syrup, so G4 syrup can better control
- 55 the freezing points of frozen foods. After ingestion, G4 syrup also affects osmotic pressure less than sucrose. Together, these qualities make G4 syrup ideally suited as an ingredient in foods and medical products. G4 syrup is useful in other industries, as well. For example, G4 syrup imparts gloss and can be used advantageously as a paper sizer. See, e.g., Kimura et al., "Maltotetraose, a new saccharide of tertiary property," Starch 42: 151-57 (1990).

[0012] One of the yeasts used to produce ethanol is *Saccharomyces cerevisiae*. *S. cerevisiae* contains α -glucosidase that has been shown to utilize mono-, di-, and tri-saccharides as substrates. Yoon et al., Carbohydrate Res. 338: 1127-32 (2003). The ability of *S. cerevisiae* to utilize tri-saccharides can be improved by Mg^{2+} supplementation and over-expression of AGT1 permease (Stambuck et al., Lett. Appl. Microbiol. 43: 370-76 (2006)), over-expression of MTT1 and MTT1alt to increase maltotriose uptake (Dietvorst et al., Yeast 22: 775-88 (2005)), or expression of the maltase MAL32 on the cell surface (Dietvorst et al., Yeast 24: 27-38 (2007)). The saccharification step could be omitted altogether, if the liquefaction step produced sufficient levels of mono-, di-, or tri-saccharides and *S. cerevisiae* or its genetically modified variants were used for the fermentation step.

[0013] *Pseudomonas saccharophila* expresses a maltotetraose-forming maltotetraohydrolase (EC 3.2.1.60; G4-forming amylase; G4-amylase). The nucleotide sequence of the *P. saccharophila* gene encoding PS4 has been determined. Zhou et al., "Nucleotide sequence of the maltotetraohydrolase gene from *Pseudomonas saccharophila*," FEBS Lett. 255: 37-41 (1989); GenBank Acc. No. X16732. PS4 is expressed as a precursor protein with an N-terminal 21-residue signal peptide. The mature form of PS4, as set forth in SEQ ID NO: 10, contains 530 amino acid residues with a catalytic domain at the N-terminus and a starch binding domain at the C-terminus. PS4 displays both endo- and exo- α -amylase activity. Endo- α -amylase activity is useful for decreasing the viscosity of gelatinized starch, and exo- α -amylase activity is useful for breaking down maltodextrins to smaller saccharides.

[0014] WO 2007/148224 relates to a PS4 variant polypeptide derivable from a parent polypeptide having non-maltogenic exoamylase activity.

[0015] WO 02/068589 relates to alpha amylases and to polynucleotides encoding the alpha amylases, which have increased activity and stability at acidic and alkaline pH and increased temperature.

SUMMARY OF THE INVENTION

[0016] The present invention provides a polypeptide having amylase activity comprising an amino acid sequence having at least 78% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises an amino acid substitution at position 240, with reference to the position numbering of the sequence shown as SEQ ID NO: 1; wherein the polypeptide has improved thermostability compared to the amylase of SEQ ID NO: 1. The present invention also provides a nucleic acid capable of encoding a polypeptide of the invention.

[0017] The present invention further provides an expression vector comprising a nucleic acid of the invention, or capable of expressing a polypeptide of the invention.

[0018] The present invention also provides a plasmid comprising a nucleic acid of the invention. The present invention further provides a host cell comprising an expression vector of the invention or a plasmid of the invention.

[0019] The present invention also provides a host cell transformed with an expression vector of the invention or a plasmid of the invention.

[0020] The present invention further provides the use of a polypeptide of the invention as a food or feed additive.

[0021] The present invention also provides a method of making a saccharide syrup, comprising adding a polypeptide of the invention to a granular starch liquefact to form the saccharide syrup.

[0022] The present invention further provides a food product comprising a polypeptide of the invention.

[0023] This invention relates to the above described amylase polypeptides and nucleic acids encoding these, and their uses e.g. as non-maltogenic exoamylases in producing food products. The amylases of the present invention have been engineered to have more beneficial qualities. The amylases described herein show an altered exospecificity and/or altered thermostability. In particular, the polypeptides are derived from polypeptides having non-maltogenic exoamylase activity, in particular, glucan 1,4- α -maltotetrahydrolase (EC 3.2.1.60) activity. In one aspect, the polypeptides as defined herein have an improved antistaling effect compared to the amylase of SEQ ID NO: 1.

[0024] In another aspect, the polypeptides as defined herein have an improved thermostability compared to the amylase of SEQ ID NO: 1.

[0025] A variant polypeptide as set out in the claims is provided. In a further aspect, the use of such a variant polypeptide, including in and as food additives, food products, bakery products, improver compositions, feed products including animal feeds, etc as set out in the claims, is provided. In yet a further aspect, nucleic acids which encode and which relate to variant polypeptides, as set out in the claims, are provided. Methods for producing such variant polypeptides, as well as other aspects, are also set out in the claims.

LEGENDS TO THE FIGURE

[0026] The accompanying drawings are incorporated in and constitute a part of this specification and illustrate various embodiments.

Fig. 1 shows firmness development of bread baked with pMS1776 (SEQ ID NO: 12), pMS2020 (SEQ ID NO: 14),

pMS2022 (SEQ ID NO: 15) and pMS2062 (SEQ ID NO: 16) compared to bread baked with a composition comprising SEQ ID NO: 1.

Fig. 2 shows firmness development of bread baked with pMS1776 (SEQ ID NO: 12), pMS1934 (SEQ ID NO: 13), pMS2022 (SEQ ID NO: 15) and pMS2062 (SEQ ID NO: 16) compared to bread baked with a composition comprising SEQ ID NO: 1 and Novamyl 1500.

DETAILED DISCLOSURE OF THE INVENTION

[0027] In accordance with this detailed description, the following abbreviations and definitions apply. It should be noted that as used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an enzyme" includes a plurality of such enzymes, and reference to "the formulation" includes reference to one or more formulations and equivalents thereof known to those skilled in the art, and so forth.

[0028] Described herein is a polypeptide having amylase activity comprising an amino acid sequence having

a) at least 65 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88 or 205, and/or

b) at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 16, 48, 97, 105, 235, 240, 248, 266, 311, 347, 350, 362, 364, 369, 393, 395, 396, 400, 401, 403, 412 or 409, and/or

c) at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D, 392 K/D/E/Y/N/Q/R/T/G or 399C/H, and/or

d) at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 44, 96, 204, 354 or 377, and/or

e) at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises the following amino acid substitution: 392S

with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0029] Described herein is a polypeptide having amylase activity comprising an amino acid sequence having at least 65% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88, or 205 and/or one or more of the following amino acid substitutions: 42K, 235H/K/R, 240E, 392 K/D/E/Y or 409E with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0030] Described herein is the use of polypeptides having a degree of sequence identity or sequence homology with amino acid sequence(s) defined herein or with a polypeptide having the specific properties defined herein. Also described herein are peptides having a degree of sequence identity with SEQ ID NO: 1, defined below, or homologues thereof. Here, the term "homologue" means an entity having sequence identity with the subject amino acid sequences or the subject nucleotide sequences, where the subject amino acid sequence preferably is SEQ ID NO: 1 and the subject nucleotide sequence preferably is SEQ ID NO: 52. In one aspect, the homologous amino acid sequence and/or nucleotide sequence should provide and/or encode a polypeptide which retains the functional activity and/or enhances the activity of a polypeptide of SEQ ID NO: 1.

[0031] In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 65%, 70%, 75%, 78%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%, identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

[0032] Sequence identity comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs use complex comparison algorithms to align two or more sequences that best reflect the evolutionary events that might have led to the difference(s) between the two or more sequences. Therefore, these algorithms operate with a scoring system rewarding alignment of identical or similar amino acids and penalising the insertion of gaps, gap extensions and alignment of non-similar amino acids.

The scoring system of the comparison algorithms include:

- i) assignment of a penalty score each time a gap is inserted (gap penalty score),
- ii) assignment of a penalty score each time an existing gap is extended with an extra position (extension penalty score),
- iii) assignment of high scores upon alignment of identical amino acids, and
- iv) assignment of variable scores upon alignment of non-identical amino acids.

[0033] Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons.

[0034] The scores given for alignment of non-identical amino acids are assigned according to a scoring matrix also called a substitution matrix. The scores provided in such substitution matrices are reflecting the fact that the likelihood of one amino acid being substituted with another during evolution varies and depends on the physical/chemical nature of the amino acid to be substituted. For example, the likelihood of a polar amino acid being substituted with another polar amino acid is higher compared to being substituted with a hydrophobic amino acid. Therefore, the scoring matrix will assign the highest score for identical amino acids, lower score for non-identical but similar amino acids and even lower score for non-identical non-similar amino acids. The most frequently used scoring matrices are the PAM matrices (Dayhoff et al. (1978), Jones et al. (1992)), the BLOSUM matrices (Henikoff and Henikoff (1992)) and the Gonnet matrix (Gonnet et al. (1992)).

[0035] Suitable computer programs for carrying out such an alignment include, but are not limited to, Vector NTI (Invitrogen Corp.) and the ClustalV, ClustalW and ClustalW2 programs (Higgins DG & Sharp PM (1988), Higgins et al. (1992), Thompson et al. (1994), Larkin et al. (2007)). A selection of different alignment tools are available from the ExPASy Proteomics server at www.expasy.org. Another example of software that can perform sequence alignment is BLAST (Basic Local Alignment Search Tool), which is available from the webpage of National Center for Biotechnology Information which can currently be found at <http://www.ncbi.nlm.nih.gov/> and which was firstly described in Altschul et al. (1990) J. Mol. Biol. 215; 403-410.

[0036] Once the software has produced an alignment, it is possible to calculate % similarity and % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[0037] In one embodiment, it is preferred to use the ClustalW software for performing sequence alignments. Preferably, alignment with ClustalW is performed with the following parameters for pairwise alignment:

Substitution matrix:	Gonnet 250
Gap open penalty:	20
Gap extension penalty:	0.2
Gap end penalty:	None

[0038] ClustalW2 is for example made available on the internet by the European Bioinformatics Institute at the EMBL-EBI webpage www.ebi.ac.uk under tools - sequence analysis - ClustalW2. Currently, the exact address of the ClustalW2 tool is www.ebi.ac.uk/Tools/clustalw2.

[0039] In another embodiment, it is preferred to use the program Align X in Vector NTI (Invitrogen) for performing sequence alignments. In one embodiment, Exp10 has is used with the default settings:

Gap opening penalty: 10

Gap extension penalty: 0.05

Gapseparation penalty range: 8

Score matrix: blosum62mt2

[0040] Described herein is the use of variants, homologues and derivatives of any amino acid sequence of a protein or polypeptide as defined herein, particularly those of SEQ ID NO: 1 or those of SEQ ID NO: 2, 4, 6, 8 or 10 defined below.

[0041] The sequences, particularly those of variants, homologues and derivatives of SEQ ID NO: 1, may also have

deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

[0042] The present invention also encompasses conservative substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-conservative substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyrrolidine, thienylalanine, naphthylalanine and phenylglycine.

[0043] Conservative substitutions that may be made are, for example within the groups of basic amino acids (Arginine, Lysine and Histidine), acidic amino acids (glutamic acid and aspartic acid), aliphatic amino acids (Alanine, Valine, Leucine, Isoleucine), polar amino acids (Glutamine, Asparagine, Serine, Threonine), aromatic amino acids (Phenylalanine, Tryptophan and Tyrosine), hydroxyl amino acids (Serine, Threonine), large amino acids (Phenylalanine and Tryptophan) and small amino acids (Glycine, Alanine).

[0044] Replacements may also be made by unnatural amino acids include; α * and α -disubstituted* amino acids, N-alkyl amino acids*, lactic acid*, halide derivatives of natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*, p-Br-phenylalanine*, p-I-phenylalanine*, L-allyl-glycine*, β -alanine*, L- α -amino butyric acid*, L- γ -amino butyric acid*, L- α -amino isobutyric acid*, L- ϵ -amino caproic acid#, 7-amino heptanoic acid*, L-methionine sulfone#, L-norleucine*, L-norvaline*, p-nitro-L-phenylalanine*, L-hydroxyproline#, L-thiopropine*, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe*, pentamethyl-Phe*, L-Phe (4-amino)#, L-Tyr (methyl)*, L-Phe (4-isopropyl)*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)*, L-diaminopropionic acid# and L-Phe (4-benzyl)*. The notation * has been utilised for the purpose of the discussion above (relating to homologous or non-conservative substitution), to indicate the hydrophobic nature of the derivative whereas # has been utilised to indicate the hydrophilic nature of the derivative, #* indicates amphipathic characteristics.

[0045] Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β -alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α -carbon substituent group is on the residue's nitrogen atom rather than the α -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ *et al.* (1992), Horwell DC. (1995).

[0046] Described herein, the variant polypeptide is a *Pseudomonas saccharophila* amylase (PS4) variant having the sequence shown in SEQ ID NO: 1 and having at least at least 65%, at least 70%, at least 75%, at least 78%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% amino acid sequence identity therewith.

[0047] In one aspect, preferably the sequence used in the present invention is in a purified form. The term "purified" means that a given component is present at a high level. The component is desirably the predominant active component present in a composition.

Definitions

[0048] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. The following terms are provided below.

[0049] "Amylase" means an enzyme that is, among other things, capable of catalyzing the degradation of starch. An endo-acting amylase activity cleaves α -D-(1 $\rightarrow$ 4) O-glycosidic linkages within the starch molecule in a random fashion. In contrast, an exo-acting amylolytic activity cleaves a starch molecule from the non-reducing end of the substrate. "Endo-acting amylase activity," "endo-activity," "endo-specific activity," and "endo-specificity" are synonymous, when the terms refer to the variant polypeptides as defined in the claims. The same is true for the corresponding terms for exo-activity.

[0050] "maltotetraose-forming maltotetrahydrolase; EC 3.2.1.60; G4-forming amylase; G4-amylase and glucan 1,4-alpha-maltotetrahydrolase" may be used interchangeably.

[0051] In the present context, "PS4" is used to designate the maltotetrahydrolase from *Pseudomonas saccharophila*.

[0052] A "variant" or "variants" refers to either polypeptides or nucleic acids. The term "variant" may be used interchangeably with the term "mutant." Variants include insertions, substitutions, transversions, truncations, and/or inversions

at one or more locations in the amino acid or nucleotide sequence, respectively. The phrases "variant polypeptide", "polypeptide", "variant" and "variant enzyme" mean a polypeptide/protein that has an amino acid sequence that has been modified from the amino acid sequence of SEQ ID NO: 1. The variant polypeptides include a polypeptide having a certain percent, e.g., 65%, 66%, 68%, 70%, 72%, 74%, 76%, 78%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, of sequence identity with SEQ ID NO: 1. As used herein, "parent enzymes," "parent sequence," "parent polypeptide" mean enzymes and polypeptides from which the variant polypeptides are based, e.g., SEQ ID NO: 1. A "parent nucleic acid" means a nucleic acid sequence encoding the parent polypeptide. The signal sequence of a "variant" may be the same (such as SEQ ID NO: 11) or may differ from the signal sequence of the wild-type PS4. A variant may be expressed as a fusion protein containing a heterologous polypeptide.

[0053] For example, the variant can comprise a signal peptide of another protein or a sequence designed to aid identification or purification of the expressed fusion protein, such as a His-Tag sequence.

[0054] To describe the various variants that are contemplated to be encompassed by the present disclosure, the following nomenclature will be adopted for ease of reference. Where the substitution includes a number and a letter, e.g., 141P, then this refers to {position according to the numbering system/substituted amino acid}. Accordingly, for example, the substitution of an amino acid to proline in position 141 is designated as 141P. Where the substitution includes a letter, a number, and a letter, e.g., A141P, then this refers to {original amino acid/position according to the numbering system/substituted amino acid}. Accordingly, for example, the substitution of alanine with proline in position 141 is designated as A141P.

[0055] Where two or more substitutions are possible at a particular position, this will be designated by contiguous letters, which may optionally be separated by slash marks "/", e.g., G303ED or G303E/D.

[0056] Position(s) and amino acid substitutions referred to herein are listed with reference to the corresponding position and the corresponding amino acid of SEQ ID NO: 1. Equivalent positions in another sequence may be found by aligning this sequence with SEQ ID NO: 1 to find an alignment with the highest percent identity and thereafter determining which amino acid aligns to correspond with an amino acid of a specific position of SEQ ID NO: 1. Such alignment and use of one sequence as a first reference is simply a matter of routine for one of ordinary skill in the art.

[0057] "Variant nucleic acids" can include sequences that are complementary to sequences that are capable of hybridizing to the nucleotide sequences presented herein, in particular to SEQ ID NO: 52. For example, a variant sequence is complementary to sequences capable of hybridizing under stringent conditions, e.g., 50°C and 0.2X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), to the nucleotide sequences presented herein, in particular to SEQ ID NO: 52 (pMS 382 DNA). More particularly, the term variant encompasses sequences that are complementary to sequences that are capable of hybridizing under highly stringent conditions, e.g., 65°C and 0.1X SSC, to the nucleotide sequences presented herein, in particular to SEQ ID NO: 52 (pMS 382 DNA). The melting point (T_m) of a variant nucleic acid may be about 1, 2, or 3°C lower than the T_m of the wild-type nucleic acid. The variant nucleic acids include a polynucleotide having a certain percent, e.g., 80%, 85%, 90%, 95%, or 99%, of sequence identity with the nucleic acid encoding SEQ ID NO: 1.

[0058] As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

[0059] "Isolated" means that the sequence is at least substantially free from at least one other component that the sequence is naturally associated and found in nature, e.g., genomic sequences.

[0060] "Purified" means that the material is in a relatively pure state, e.g., at least about 90% pure, at least about 95% pure, or at least about 98% pure.

[0061] "Thermostable" means the enzyme retains activity after exposure to elevated temperatures. The thermostability of an enzyme may be measured by its half-life ($t_{1/2}$), where half of the enzyme activity is lost by the half-life. The half-life value is calculated under defined conditions by measuring the residual amylase activity. To determine the half-life of the enzyme, the sample is heated to the test temperature for 1-10 min, and activity is measured using a standard assay for amylase activity, such as the Betamyl® assay (Megazyme, Ireland).

[0062] As used herein, "optimum pH" means the pH at which the variant polypeptides disclosed herein displays the activity in a standard assay for amylase activity, measured over a range of pH's.

[0063] As used herein, "polypeptide" is used interchangeably with the terms "amino acid sequence", "enzyme", "peptide" and/or "protein." As used herein, "nucleotide sequence" or "nucleic acid sequence" refers to an oligonucleotide sequence or polynucleotide sequence and variants, homologues, fragments and derivatives thereof. The nucleotide sequence may be of genomic, synthetic or recombinant origin and may be double-stranded or single-stranded, whether representing the sense or anti-sense strand. As used herein, the term "nucleotide sequence" includes genomic DNA, cDNA, synthetic DNA, and RNA.

[0064] "Homologue" means an entity having a certain degree of identity or "homology" with the subject amino acid sequences and the subject nucleotide sequences. A "homologous sequence" includes a polynucleotide or a polypeptide having a certain percent, e.g., 80%, 85%, 90%, 95%, or 99%, of sequence identity with another sequence. Percent identity means that, when aligned, that percentage of bases or amino acid residues are the same when comparing the

two sequences. Amino acid sequences are not identical, where an amino acid is substituted, deleted, or added compared to the subject sequence. The percent sequence identity typically is measured with respect to the mature sequence of the subject protein, i.e., following removal of a signal sequence, for example. Typically, homologues will comprise the same active site residues as the subject amino acid sequence. Homologues also retain amylase activity, although the

homologue may have different enzymatic properties than the wild-type PS4.

[0065] As used herein, "hybridization" includes the process by which a strand of nucleic acid joins with a complementary strand through base pairing, as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies. The variant nucleic acid may exist as single- or double-stranded DNA or RNA, an RNA/DNA heteroduplex or an RNA/DNA copolymer. As used herein, "copolymer" refers to a single nucleic acid strand that comprises both ribonucleotides and deoxyribonucleotides. The variant nucleic acid may be codon-optimized to further increase expression.

[0066] As used herein, a "synthetic" compound is produced by *in vitro* chemical or enzymatic synthesis. It includes, but is not limited to, variant nucleic acids made with optimal codon usage for host organisms, such as a yeast cell host or other expression hosts of choice.

[0067] As used herein, "transformed cell" includes cells, including both bacterial and fungal cells, which have been transformed by use of recombinant DNA techniques. Transformation typically occurs by insertion of one or more nucleotide sequences into a cell. The inserted nucleotide sequence may be a heterologous nucleotide sequence, i.e., is a sequence that is not natural to the cell that is to be transformed, such as a fusion protein.

[0068] As used herein, "operably linked" means that the described components are in a relationship permitting them to function in their intended manner. For example, a regulatory sequence operably linked to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

[0069] As used herein the term "starch" refers to any material comprised of the complex polysaccharide carbohydrates of plants, such as corn, comprised of amylose and amylopectin with the formula $(C_6H_{10}O_5)_x$, where X can be any number. The term "granular starch" refers to raw, i.e., uncooked starch, e.g., starch that has not been subject to gelatinization.

[0070] The term "liquefaction" refers to the stage in starch conversion in which gelatinized starch is hydrolyzed to give low molecular weight soluble dextrans. As used herein the term "saccharification" refers to enzymatic conversion of starch to glucose. The term "degree of polymerization" (DP) refers to the number (n) of anhydroglucopyranose units in a given saccharide. Examples of DP1 are the monosaccharides glucose and fructose. Examples of DP2 are the disaccharides maltose and sucrose.

[0071] As used herein the term "dry solids content" (ds) refers to the total solids of a slurry in a dry weight percent basis. The term "slurry" refers to an aqueous mixture containing insoluble solids.

[0072] The phrase "simultaneous saccharification and fermentation (SSF)" refers to a process in the production of biochemicals in which a microbial organism, such as an ethanol producing microorganism and at least one enzyme, such as PS4 or a variant thereof, are present during the same process step. SSF refers to the contemporaneous hydrolysis of granular starch substrates to saccharides and the fermentation of the saccharides into alcohol, for example, in the same reactor vessel.

[0073] As used herein "ethanologenic microorganism" refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol.

1.2. Abbreviations

[0074] The following abbreviations apply unless indicated otherwise:

ADA	azodicarbonamide
cDNA	complementary DNA
CGTase	cyclodextrin glucanotransferase
DEAE	diethylamino ethanol
dH ₂ O	deionized water
DNA	deoxyribonucleic acid
ds-DNA	double-stranded DNA
EC	enzyme commission for enzyme classification
FGSC	Fungal Genetics Stock Center
G121F	glycine (G) residue at position 121 of SEQ ID NO: 2 is replaced with a phenylalanine (F) residue, where amino acids are designated by single letter abbreviations commonly known in the art
HPLC	High Performance Liquid Chromatography
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PDB	Protein Database Base

PEG	polyethyleneglycol
ppm	parts per million
PS4	<i>P. saccharophila</i> G4-forming amylase
RT-PCR	reverse transcriptase polymerase chain reaction
5 SAS	<i>P. saccharophila</i> G4-forming amylase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
1X SSC	0.15 M NaCl, 0.015 M sodium citrate, pH 7.0
SSF	simultaneous saccharification and fermentation
$t_{1/2}$	half life
10 T _m	melting temperature (°C) at which 50% of the subject protein is melted
ΔT _m	°C increase in the T _m
w/v	weight/volume
w/w	weight/weight

2. Polypeptide variants of SEQ ID NO: 1

[0075] In one aspect, a polypeptide having a substitution at one or more positions which effect an altered property, which may be any combination of altered exospecificity, endospecificity or altered thermostability, or an altered handling property, relative to SEQ ID NO: 1, is provided. Such variant polypeptides are also referred to in this document for convenience as "variant polypeptide", "polypeptide variant" or "variant". In one aspect, the polypeptides as defined herein have an improved antistaling effect compared to the amylase of SEQ ID NO: 1. In another aspect, the polypeptides as defined herein have an improved thermostability compared to the amylase of SEQ ID NO: 1. In highly preferred embodiments, they have the added advantage of higher thermostability, or higher exoamylase activity or higher pH stability, or any combination.

[0076] In one aspect, the variants as defined herein exhibit enzyme activity. In one aspect, the variant polypeptides comprise amylase activity. In a further aspect, the variant polypeptides comprise exoamylase activity. In a further aspect, the variant polypeptides exhibit non-maltogenic exoamylase activity. In one aspect, the variants as defined herein are derivable from *Pseudomonas saccharophila* α-amylase (PS4). In one aspect, the variants as defined herein are a maltotetraose-forming maltotetrahydrolase, also called EC 3.2.1.60; G4-forming amylase; G4-amylase or glucan 1,4-alpha-maltotetrahydrolase.

[0077] Compositions, including food additives, food products, bakery products, improver compositions, feed products including animal feeds, etc comprising such variant polypeptides as defined herein, such as those which have non-maltogenic exoamylase activity, as well as methods of making and using such polypeptides and the compositions, are provided herein.

[0078] As noted above, the variant polypeptides may comprise one or more improved handling properties, preferably improved baking properties. Thus, the variant polypeptides are such that the food products so treated have one or more of (preferably all of) a lower firmness, a higher resilience, a higher cohesiveness, a lower crumbliness or a higher foldability. Such improved handling or baking properties exhibited by the variant polypeptides are described in further detail below.

[0079] Furthermore a treatment of food products, particularly doughs and bakery products with such polypeptides, and such that the food products exhibit the desired qualities set out above, is provided.

[0080] Further is provided other uses of the variants described herein and compositions comprising these variants, such as in the preparation of detergents, as sweeteners, syrups, etc. The compositions include the polypeptide together with at least one other component. In particular, food or feed additives comprising the polypeptides, are provided.

[0081] An isolated and/or purified polypeptide comprising a variant polypeptide as defined herein is provided. In one embodiment, the variant polypeptide is a mature form of the polypeptide (SEQ ID NO: 1), wherein the 21 amino acid leader sequence is cleaved, so that the N-terminus of the polypeptide begins at the aspartic acid (D) residue. In one aspect, the variants include a C-terminal starch binding domain. A representative amino acid sequence of a starch binding domain comprises or consist of an amino acid sequence of SEQ ID NO: 36. Other variants include variants wherein between one and about 25 amino acid residues have been added or deleted with respect to SEQ ID NO: 1. In one aspect, the variant has the amino acid sequence of SEQ ID NO: 1, wherein any number between one and about 25 amino acids have been substituted. In a further aspect, the variant has the amino acid sequence of SEQ ID NO: 1, wherein any number between three and twelve amino acids has been substituted. In a further aspect, the variant has the amino acid sequence of SEQ ID NO: 1, wherein any number between five and nine amino acids has been substituted.

[0082] In one aspect, at least two, in another aspect at least three, and yet in another aspect at least five amino acids of SEQ ID NO: 1 has been substituted.

[0083] In a further aspect, the length of the polypeptide variant is 390 to 540 amino acids. In a further aspect, the length of the polypeptide variant is 410 to 440 amino acids. In a further aspect, the length of the polypeptide variant is

420 to 435 amino acids. In a further aspect, the length of the polypeptide variant is 429 to 430 amino acids.

[0084] Described herein is a polypeptide having amylase activity comprising an amino acid sequence having:

a) at least 65 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88 or 205, and/or

b) at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 16, 48, 97, 105, 235, 240, 248, 266, 311, 347, 350, 362, 364, 369, 393, 395, 396, 400, 401, 403, 412 or 409, and/or

c) at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D, 392 K/D/E/Y/N/Q/R/T/G or 399C/H, and/or

d) at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 44, 96, 204, 354 or 377, and/or

e) at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises the following amino acid substitution: 392S

wherein an amino acid substitution is relative to the corresponding amino acid of SEQ ID NO: 1 and the positions are with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0085] In a further aspect, the polypeptide as defined herein comprises one or more amino acid substitutions at the following positions: 88, 205, 235, 240, 248, 266, 311, 377 or 409 and/or one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D or 392K/D/E/Y/N/Q/R/S/T/G. In a further aspect, the polypeptide as defined herein comprises one or more amino acid substitutions at the following positions: 88, 205, 235, 240, 311 or 409 and/or one or more of the following amino acid substitutions: 42K/N/I/H/F, 272D, or 392 K/D/E/Y/N/Q/R/S/T/G. In a further aspect, the polypeptide as defined herein comprises amino acid substitutions at least in four, five or in all of the following positions: 88, 205, 235, 240, 311 or 409 and/or has at least one, or two the following amino acid substitutions: 42K/N/I/H/F, 272D or 392 K/D/E/Y/N/Q/R/S/T/G. In a further aspect, the polypeptide as defined herein further comprises one or more of the following amino acids 33Y, 34N, 70D, 121F, 134R, 141P, 146G, 157L, 161A, 178F, 179T, 223E/S/K/A, 229P, 307K, 309P and 334P. In a further aspect, the polypeptide as defined herein further has the following amino acids 33Y, 34N, 70D, 121F, 134R, 141P, 146G, 157L, 161A, 178F, 179T, 229P, 307K, 309P and 334P. In a further aspect, the polypeptide as defined herein has at least one of the amino acid substitutions in a) of claim 1 and further comprises one or more amino acid substitutions at the following positions: 44, 96, 204, 354 or 377. In a further aspect, the polypeptide as defined herein has at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 1. In a further aspect, the polypeptide as defined herein has the amino acid 34Q. In a further aspect, the polypeptide as defined herein has the amino acid 42K/F/H/I/N/A/V. In a further aspect, the polypeptide as defined herein has the amino acid 42K/F/H/I/N. In a further aspect, the polypeptide as defined herein has the amino acid 42K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 88. In a further aspect, the polypeptide as defined herein has the amino acid 88L/Y/K. In a further aspect, the polypeptide as defined herein has the amino acid 88L. In a further aspect, the polypeptide as defined herein has the amino acid 88Y. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 205. In a further aspect, the polypeptide as defined herein has the amino acid 205L/K/M/N/Q/R/V/Y. In a further aspect, the polypeptide as defined herein has the amino acid 205L/K/M/N/Q/R/V. In a further aspect, the polypeptide as defined herein has the amino acid 205L. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 235. In a further aspect, the polypeptide as defined herein has the amino acid 235H/K/R/Q/S. In a further aspect, the polypeptide as defined herein has the amino acid 235H/K/R/Q. In a further aspect, the polypeptide as defined herein has the amino acid 235H/K/R. In a further aspect, the polypeptide as defined herein has the amino acid 235R. In a further aspect, the polypeptide as defined herein has the amino acid 235H. In a further aspect, the polypeptide as defined herein has the amino acid 235K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 240. In a further aspect, the polypeptide as defined herein has the amino acid 240E/H/M/D/S. In a further aspect, the polypeptide as defined herein has the amino acid 240E/H/M/D. In a further aspect, the polypeptide as defined herein has the amino acid 240E. In a further aspect, the polypeptide as defined herein has the amino acid 272D. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 311. In a further aspect, the polypeptide as defined herein has the amino acid 311P. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 409. In a further aspect, the polypeptide as defined herein has the amino acid 409H/Q/T/E. In a further aspect, the polypeptide as defined herein has the amino acid 409E. In a further aspect, the

polypeptide as defined herein has the amino acid 100Q/K/N/R. In a further aspect, the polypeptide as defined herein has the amino acid 100Q. In a further aspect, the polypeptide as defined herein has the amino acid 392D/E/K/Y/N/Q/R/S/T/G. In a further aspect, the polypeptide as defined herein has the amino acid 392D/E/K/Y/N/Q/R/S/T. In a further aspect, the polypeptide as defined herein has the amino acid 392D/E/K/Y. In a further aspect, the polypeptide as defined herein has the amino acid 392D. In a further aspect, the polypeptide as defined herein has the amino acid 392E. In a further aspect, the polypeptide as defined herein has the amino acid 392K. In a further aspect, the polypeptide as defined herein has the amino acid 392Y. In a further aspect, the polypeptide as defined herein has both of the following amino acids 235R and 311P. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 16. In a further aspect, the polypeptide as defined herein has the amino acid 16/A/E/K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 48. In a further aspect, the polypeptide as defined herein has the amino acid 48/C/L. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 97. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 105. In a further aspect, the polypeptide as defined herein has the amino acid 105/N/R. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 248. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 266. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 347. In a further aspect, the polypeptide as defined herein has the amino acid 347/C/D/H/K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 350. In a further aspect, the polypeptide as defined herein has the amino acid 350E/H/N. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 354. In a further aspect, the polypeptide as defined herein has the amino acid 354D/E. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 362. In a further aspect, the polypeptide as defined herein has the amino acid 362E/H/P. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 364. In a further aspect, the polypeptide as defined herein has the amino acid 364E/K/NQ. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 369. In a further aspect, the polypeptide as defined herein has the amino acid 369I/N. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 377. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 393. In a further aspect, the polypeptide as defined herein has the amino acid 393D/E/K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 395. In a further aspect, the polypeptide as defined herein has the amino acid 395C/E/K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 396. In a further aspect, the polypeptide as defined herein has the amino acid 396D/E. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 399. In a further aspect, the polypeptide as defined herein has the amino acid 399C/H. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 400. In a further aspect, the polypeptide as defined herein has the amino acid 400S/W. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 401. In a further aspect, the polypeptide as defined herein has the amino acid 401D/K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 403. In a further aspect, the polypeptide as defined herein has the amino acid 403E/T/V. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 412. In a further aspect, the polypeptide as defined herein has the amino acid 412D/N. In a further aspect, the polypeptide as defined herein further comprises one or more of the following amino acids 121F, 134R, 141P, 229P, or 307K. In a further aspect, the polypeptide as defined herein has one or more of the following amino acids 42K, 88L, 205L, 235R, 240E, 272D, 311P, 392D, or 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acids 42K, 88L, 235R, 311P, 392D and either 223S, 223K or 223A. In a further aspect, the polypeptide as defined herein further comprises one or more selected from the group consisting of 272D, 409E, 205L and 240E. In a further aspect, the polypeptide as defined herein has at least four, five, six, seven or eight of the following amino acids 42K, 88L, 205L, 235R, 240E, 272D, 311P, 392D, or 409E. In a further aspect, the polypeptide as defined herein has the amino acid 223A/K/S. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 223S, 235R, 311P and 392D. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 223K, 235R, 272D, 311P and 392D. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 223S, 235R, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 205L, 223S, 235R, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 205L, 223K, 240E, 235R, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 205L, 223A, 235R, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein has one or more amino acids in the N-terminus. In one aspect, the polypeptide as defined herein has the amino acid M at the N-terminus. In a further aspect, the polypeptide as defined herein comprises one or more amino acid substitutions at the following positions: 88, 205, 235 or 409 and/or one or more of the following amino acid substitutions: 42K, 34Q, 100Q, 223A/S, 240E, 311P, 392D, or 409E with reference to the position numbering of the sequence shown as SEQ ID NO: 1. In a further aspect, the polypeptide as defined herein comprises one or more of the following amino acid substitutions: 42K,

34Q, 88K/L/Y, 100Q, 205L, 223A/S/K, 235K/H/Q/R, 240E/D, 248H, 266T, 311P, 377D/E/P, 392K/D/Y or 409E. In a further aspect, the polypeptide as defined herein comprises one or more of the following amino acid substitutions: 34Q, 42K, 88L, 100Q, 205L, 223A/S/K, 235K/R, 240E, 311P, 392D or 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 235R and 311P. In a further aspect, the polypeptide as defined herein comprises the amino acid substitution 42K. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K and 223S. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 223S and 392D. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 223A, 235R, 311P and 392D. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 205L, 223S, 235R, 240E, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 205L, 223K, 235R, 240E, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 205L, 223S, 235R, 311P and 392D. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 34Q, 42K, 88L, 205L, 223K, 235R, 240E, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 100Q, 205L, 223K, 235R, 240E, 311P, 392D and 409E.

[0086] Representative embodiments of the polypeptides as defined herein comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34. Further representative embodiments of the polypeptides as defined herein comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17. Further representative embodiments of the polypeptides as defined herein has an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34. Further representative embodiments of the polypeptides as defined herein has an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17, and optionally one or more amino acids at the N-terminus.

[0087] In one aspect, the polypeptides as defined herein comprises the sequence HGGDEIILQFHWN (SEQ ID NO: 37) at the positions corresponding to positions 13-26 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0088] In one aspect, the polypeptides as defined herein comprises the sequence DGF-X1-AIW-X2-P-X3-PWRD-X4-SSW (SEQ ID NO: 38), wherein X1 is S or T, X2 is M or L, X3 is V or P and X4 is any natural occurring amino acid residue, preferably an L-amino acid, at the positions corresponding to positions 49-66 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0089] In one aspect, the polypeptides as defined herein comprises the sequence GGEGYFW (SEQ ID NO: 39) at the positions corresponding to positions 79-85 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0090] In one aspect, the polypeptides as defined herein comprises the sequence VPNH (SEQ ID NO: 40) at the positions corresponding to positions 114-117 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0091] In one aspect, the polypeptides as defined herein comprises the sequence CDDGD (SEQ ID NO: 41) at the positions corresponding to positions 150-154 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0092] In one aspect, the polypeptides as defined herein comprises the sequence AGGFRFDFVRG (SEQ ID NO: 42) at the positions corresponding to positions 187-197 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0093] In one aspect, the polypeptides as defined herein comprises the sequence FALK (SEQ ID NO: 43) at the positions corresponding to positions 256-259 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0094] In one aspect, the polypeptides as defined herein comprises the sequence WREVAVTFVDNHD (SEQ ID NO: 44) at the positions corresponding to positions 282-294 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0095] In one aspect, the polypeptides as defined herein comprises the sequence GYSPG (SEQ ID NO: 45) at the positions corresponding to positions 296-300 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0096] In one aspect, the polypeptides as defined herein comprises the sequence GQH (SEQ ID NO: 46) at the

positions corresponding to positions 304-306 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0097] In one aspect, the polypeptides as defined herein comprises the sequence AYAYI (SEQ ID NO: 47) at the positions corresponding to positions 318-322 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0098] In one aspect, the polypeptides as defined herein comprises the sequence SPGTP (SEQ ID NO: 48) at the positions corresponding to positions 325-329 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0099] In one aspect, the polypeptides as defined herein comprises the sequence VYW (SEQ ID NO: 49) at the positions corresponding to positions 331-333 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0100] In one aspect, the polypeptides as defined herein comprises the sequence HMYDWG (SEQ ID NO: 50) at the positions corresponding to positions 335-340 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

[0101] In another aspect, the polypeptide variant as defined herein has the sequence of SEQ ID NO:1, wherein any number between one and about 25 amino acids have been substituted. Representative examples of polypeptide variants as defined herein having single or several amino acid substitutions are shown in below TABLE A:

pMS	Mutations compared to pMS382 having SEQ ID NO:1
pms1104 (SEQ ID NO: 20)	Q42K
pms1105 (SEQ ID NO: 27)	Q42K, E223S
pms1723 (SEQ ID NO: 28)	Q42K, E223S, A392D
pms2104 (SEQ ID NO: 29)	Q42K, R88L, E223A, T235R, Q311P, A392D
pms2118 (SEQ ID NO: 34)	Q42K, R88L, S205L, E223S, T235R, Q240E, Q311P, A392D, S409E
pms2124 (SEQ ID NO: 31)	Q42K, R88L, S205L, E223K, T235R, Q240E, Q311P, A392D, S409E
pms1284 (SEQ ID NO: 23)	T235R, Q311P
pms1286 (SEQ ID NO: 22)	T235R
pms1290 (SEQ ID NO: 24)	T235K
pms465 (SEQ ID NO: 18)	E223S
pms1042 (SEQ ID NO: 19)	Q311P
pms1153 (SEQ ID NO: 21)	R88L
pms1484 (SEQ ID NO: 25)	S205L
pms1579 (SEQ ID NO: 26)	S409E
pms2138 (SEQ ID NO: 30)	Q42K, R88L, S205L, E223S, T235R, Q311P, A392D
pms2177 (SEQ ID NO: 32)	N34Q, Q42K, R88L, S205L, E223K, T235R, Q240E, Q311P, A392D, S409E
pms2178 (SEQ ID NO: 33)	Q42K, R88L, G100Q, S205L, E223K, T235R, Q240E, Q311P, A392D, S409E
pms1776 (SEQ ID NO: 12)	Q42K, R88L, E223S, T235R, Q311P, A392D
pms1934 (SEQ ID NO: 13)	Q42K, R88L, E223K, T235R, H272D, Q311P, A392D
pms2020 (SEQ ID NO: 14)	Q42K, R88L, E223S, T235R, Q311P, A392D, S409E
pms2022 (SEQ ID NO: 15)	Q42K, R88L, S205L, E223S, T235R, Q311P, A392D, S409E
pms2062 (SEQ ID NO: 16)	Q42K, R88L, S205L, E223K, Q240E, T235R, Q311P, A392D, S409E
pms2171 (SEQ ID NO: 17)	Q42K, R88L, S205L, E223A, T235R, Q311P, A392D, S409E

[0102] The polypeptide variants as defined herein may have an altered thermostability, an altered endo-amylase activity, an altered exo-amylase activity, and/or an altered ratio of exo- to endo amylase activity compared to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 10. In one aspect, the polypeptides as defined herein have an improved antistaling effect compared to the amylase of SEQ ID NO: 1. In another aspect, the polypeptides as defined herein have

an improved thermostability compared to the amylase of SEQ ID NO: 1.

[0103] The polypeptide variants as defined herein may have up to 25, 23, 21, 19, 17, 15, 13, 11, 9, 8, 7, 6, 5 amino acid deletions, additions, insertions, or substitutions compared to the amino acid sequence of SEQ ID NO: 1.

[0104] The present disclosure also relates to each and every G4-amylase backbone having SEQ ID NO: 2, 4, 6, or 8 comprising the substitutions as further defined herein.

[0105] Nucleic acids encoding the polypeptide variants above also are provided. In one embodiment, a nucleic acid encoding a polypeptide variant as defined herein is a nucleotide sequence encoding the protein comprising an amino acid sequence of residues 1-429 of SEQ ID NO: 1 as set forth in SEQ ID NO: 52. For example nucleotide sequence SEQ ID NO: 3 encodes the G4 amylase backbone with SEQ ID NO: 2. As is well understood by one skilled in the art, the genetic code is degenerate, meaning that multiple codons in some cases may encode the same amino acid. Nucleic acids can include genomic DNA, mRNA, and cDNA that encodes a polypeptide variant

2.1. Polypeptide variant characterization

[0106] Enzyme variants can be characterized by their nucleic acid and primary polypeptide sequences, by three dimensional structural modeling, and/or by their specific activity. Additional characteristics of the polypeptide variants as defined herein include stability, pH range, oxidation stability, and thermostability, for example. Levels of expression and enzyme activity can be assessed using standard assays known to the artisan skilled in this field. In another aspect, variants demonstrate improved performance characteristics relative to the polypeptide with SEQ ID NO: 1, such as improved stability at high temperatures, e.g., 65-85°C. In one aspect, the polypeptide variants as defined herein are advantageous for use in liquefaction or other processes that require elevated temperatures, such as baking. For example, a thermostable polypeptide variant as defined herein can degrade starch at temperatures of about 55°C to about 85°C or more.

[0107] An expression characteristic means an altered level of expression of the variant, when the variant is produced in a particular host cell. Expression generally relates to the amount of active variant that is recoverable from a fermentation broth using standard techniques known in this art over a given amount of time. Expression also can relate to the amount or rate of variant produced within the host cell or secreted by the host cell. Expression also can relate to the rate of translation of the mRNA encoding the variant enzyme.

[0108] A nucleic acid complementary to a nucleic acid encoding any of the polypeptide variants as defined herein set forth herein is provided. Additionally, a nucleic acid capable of hybridizing to the complement is provided. In another embodiment, the sequence for use in the methods and compositions described here is a synthetic sequence. It includes, but is not limited to, sequences made with optimal codon usage for expression in host organisms, such as yeast.

3. Production of the polypeptide variants as defined herein

[0109] The polypeptide variants as provided herein may be produced synthetically or through recombinant expression in a host cell, according to procedures well known in the art. The expressed polypeptide variant as defined herein optionally is isolated prior to use. In another embodiment, the polypeptide variant as defined herein is purified following expression. Methods of genetic modification and recombinant production of polypeptide variants are described, for example, in U.S. Patent Nos. 7,371,552, 7,166,453; 6,890,572; and 6,667,065; and U.S. Published Application Nos. 2007/0141693; 2007/0072270; 2007/0020731; 2007/0020727; 2006/0073583; 2006/0019347; 2006/0018997; 2006/0008890; 2006/0008888; and 2005/0137111. The relevant teachings of these disclosures, include polypeptide-encoding polynucleotide sequences, primers, vectors, selection methods, host cells, purification and reconstitution of expressed polypeptide variants, and characterization of polypeptide variants as defined herein, including useful buffers, pH ranges, Ca²⁺ concentrations, substrate concentrations and enzyme concentrations for enzymatic assays.

[0110] In another embodiment, suitable host cells include a Gram positive bacterium selected from the group consisting of *Bacillus subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. thuringiensis*, *Streptomyces lividans*, or *S. murinus*; or a Gram negative bacterium, wherein said Gram negative bacterium is *Escherichia coli* or a *Pseudomonas* species. In one aspect, the host cell is a *B. subtilis* or *B. licheniformis*. In one embodiment, the host cell is *B. subtilis*, and the expressed protein is engineered to comprise a *B. subtilis* signal sequence, as set forth in further detail below. In one aspect, the host cell expresses the polynucleotide as set out in the claims.

[0111] In some embodiments, a host cell is genetically engineered to express a polypeptide variant as defined herein with an amino acid sequence having at least about 78%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NO: 1. In some embodiments, the polynucleotide encoding a polypeptide variant as defined herein will have a nucleic acid sequence encoding the protein of SEQ ID NO: 1 or a nucleic acid sequence having at least about 78%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleic acid encoding the protein of SEQ ID NO: 1. In one embodiment, the nucleic acid sequence has at least about 78%, 80%,

85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the nucleic acid of SEQ ID NO: 52.

3.1. Vectors

[0112] In one aspect, the invention relates to a vector comprising a polynucleotide. In one aspect, of the invention a bacterial cell comprises the vector. In some embodiments, a DNA construct comprising a nucleic acid encoding a variant is transferred to a host cell in an expression vector that comprises regulatory sequences operably linked to an encoding sequence. The vector may be any vector that can be integrated into a fungal host cell genome and replicated when introduced into the host cell. The FGSC Catalogue of Strains, University of Missouri, lists suitable vectors. Additional examples of suitable expression and/or integration vectors are provided in Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001); Bennett et al., *MORE GENE MANIPULATIONS IN FUNGI*, Academic Press, San Diego (1991), pp. 396-428; and U.S. Patent No. 5,874,276. Exemplary vectors include pFB6, pBR322, PUC18, pUC100 and pENTR/D, pDONTM201, pDONRTM221, pENTRTM, pGEM@3Z and pGEM@4Z. Exemplary for use in bacterial cells include pBR322 and pUC19, which permit replication in *E. coli*, and pE194, for example, which permits replication in *Bacillus*.

[0113] In some embodiments, a nucleic acid encoding a variant is operably linked to a suitable promoter, which allows transcription in the host cell. The promoter may be derived from genes encoding proteins either homologous or heterologous to the host cell. Suitable non-limiting examples of promoters include *cbh1*, *cbh2*, *egl1*, and *egl2* promoters. In one embodiment, the promoter is one that is native to the host cell. For example, when *P. saccharophila* is the host, the promoter is a native *P. saccharophila* promoter. An "inducible promoter" is a promoter that is active under environmental or developmental regulation. In another embodiment, the promoter is one that is heterologous to the host cell.

[0114] In some embodiments, the coding sequence is operably linked to a DNA sequence encoding a signal sequence. A representative signal peptide is SEQ ID NO: 11, which is the native signal sequence of the PS4 precursor. In other embodiments, the DNA encoding the signal sequence is replaced with a nucleotide sequence encoding a signal sequence from a species other than *P. saccharophila*. In this embodiment, the polynucleotide that encodes the signal sequence is immediately upstream and in-frame of the polynucleotide that encodes the polypeptide. The signal sequence may be selected from the same species as the host cell. In one non-limiting example, the signal sequence is a cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) signal sequence from *Bacillus* sp., and the variant polypeptides disclosed herein is expressed in a *B. subtilis* host cell. A methionine residue may be added to the N-terminus of the signal sequence.

[0115] In additional embodiments, a signal sequence and a promoter sequence comprising a DNA construct or vector to be introduced into a fungal host cell are derived from the same source. In some embodiments, the expression vector also includes a termination sequence. In one embodiment, the termination sequence and the promoter sequence are derived from the same source. In another embodiment, the termination sequence is homologous to the host cell.

[0116] In some embodiments, an expression vector includes a selectable marker. Examples of suitable selectable markers include those that confer resistance to antimicrobial agents, e.g., hygromycin or phleomycin. Nutritional selective markers also are suitable and include *amdS*, *argB*, and *pyr4*. In one embodiment, the selective marker is the *amdS* gene, which encodes the enzyme acetamidase; it allows transformed cells to grow on acetamide as a nitrogen source. The use of an *A. nidulans amdS* gene as a selective marker is described in Kelley et al., *EMBO J.* 4: 475-479 (1985) and Penttila et al., *Gene* 61: 155-164 (1987).

[0117] A suitable expression vector comprising a DNA construct with a polynucleotide encoding a variant may be any vector that is capable of replicating autonomously in a given host organism or integrating into the DNA of the host. In some embodiments, the expression vector is a plasmid. In some embodiments, two types of expression vectors for obtaining expression of genes are contemplated. The first expression vector comprises DNA sequences in which the promoter, coding region, and terminator all originate from the gene to be expressed. In some embodiments, gene truncation is obtained by deleting undesired DNA sequences, e.g., DNA encoding the C-terminal starch-binding domain, to leave the domain to be expressed under control of its own transcriptional and translational regulatory sequences. The second type of expression vector is preassembled and contains sequences required for high-level transcription and a selectable marker. In some embodiments, the coding region for a gene or part thereof is inserted into this general-purpose expression vector, such that it is under the transcriptional control of the expression construct promoter and terminator sequences. In some embodiments, genes or part thereof are inserted downstream of the strong *cbh1* promoter.

3.2. Transformation, expression and culture of host cells

[0118] Introduction of a DNA construct or vector into a host cell includes techniques such as transformation; electroporation; nuclear microinjection; transduction; transfection, e.g., lipofection mediated and DEAE-Dextrin mediated transfection; incubation with calcium phosphate DNA precipitate; high velocity bombardment with DNA-coated microprojectiles; and protoplast fusion. General transformation techniques are known in the art. See, e.g., Ausubel et al. (1987), *supra*, chapter 9; Sambrook et al. (2001), *supra*; and Campbell et al., *Curr. Genet.* 16: 53-56 (1989). The expression of

heterologous protein in *Trichoderma* is described, for example, in U.S. Patent No. 6,022,725; U.S. Patent No. 6,268,328; Harkki et al., Enzyme Microb. Technol. 13: 227-233 (1991); Harkki et al., BioTechnol. 7: 596-603 (1989); EP 244,234; and EP 215,594. In one embodiment, genetically stable transformants are constructed with vector systems whereby the nucleic acid encoding a variant is stably integrated into a host cell chromosome. Transformants are then purified by known techniques.

[0119] In one non-limiting example, stable transformants including an *amdS* marker are distinguished from unstable transformants by their faster growth rate and the formation of circular colonies with a smooth, rather than ragged outline on solid culture medium containing acetamide. Additionally, in some cases a further test of stability is conducted by growing the transformants on solid non-selective medium, e.g., a medium that lacks acetamide, harvesting spores from this culture medium and determining the percentage of these spores that subsequently germinate and grow on selective medium containing acetamide. Other methods known in the art may be used to select transformants.

3.3. Identification of activity

[0120] To evaluate the expression of a variant in a host cell, assays can measure the expressed protein, corresponding mRNA, or α -amylase activity. For example, suitable assays include Northern and Southern blotting, RT-PCR (reverse transcriptase polymerase chain reaction), and *in situ* hybridization, using an appropriately labeled hybridizing probe. Suitable assays also include measuring activity in a sample. Suitable assays of the exo-activity of the variant include, but are not limited to, the Betamyl® assay (Megazyme, Ireland). Suitable assays of the endo-activity of the variant include, but are not limited to, the Phadebas blue assay (Pharmacia and Upjohn Diagnostics AB). Assays also include HPLC analysis of liquefact prepared in the presence of the variant. HPLC can be used to measure amylase activity by separating DP-3 and DP-4 saccharides from other components of the assay.

Improved Handling Properties

[0121] The variant polypeptides described here preferably have improved properties when compared to SEQ ID NO:1, such as any one or more of improved thermostability, improved pH stability, or improved exo-specificity. The variant polypeptides described here preferably also have improved handling properties, such that a food product treated with a variant polypeptide has any one or all of lower firmness, higher resilience, higher cohesiveness, lower crumbliness, or higher foldability compared to a food product which has been treated with SEQ ID NO: 1.

[0122] Without wishing to be bound by any particular theory, we believe that the mutations at the particular positions have individual and cumulative effects on the properties of a polypeptide comprising such mutations.

Thermostability and pH Stability

[0123] Preferably, the variant polypeptide is thermostable; preferably, it has higher thermostability than SEQ ID NO: 1.

[0124] In wheat and other cereals the external side chains in amylopectin are in the range of DP 12-19. Thus, enzymatic hydrolysis of the amylopectin side chains, for example, by variant polypeptides as described having non-maltogenic exoamylase activity, can markedly reduce their crystallisation tendencies.

[0125] Starch in wheat and other cereals used for baking purposes is present in the form of starch granules which generally are resistant to enzymatic attack by amylases. Thus starch modification is mainly limited to damaged starch and is progressing very slowly during dough processing and initial baking until gelatinisation starts at about 60°C. As a consequence hereof only amylases with a high degree of thermostability are able to modify starch efficiently during baking. And generally the efficiency of amylases is increased with increasing thermostability. That is because the more thermostable the enzyme is the longer time it can be active during baking and thus the more antistaling effect it will provide.

[0126] Accordingly, the use of variant polypeptides as described here when added to the starch at any stage of its processing into a food product, e.g., before during or after baking into bread can retard or impede or slow down the retrogradation. Such use is described in further detail below.

[0127] As used herein the term "thermostable" relates to the ability of the enzyme to retain activity after exposure to elevated temperatures. Preferably, the variant polypeptide is capable of degrading starch at temperatures of from about 55°C to about 80°C or more. Suitably, the enzyme retains its activity after exposure to temperatures of up to about 95°C.

[0128] The thermostability of an enzyme such as a non-maltogenic exoamylase is measured by its half life. Thus, the variant polypeptides described here have half lives extended relative to the parent enzyme by preferably 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more, preferably at elevated temperatures of from 55°C to about 95°C or more, preferably at about 80°C.

[0129] As used here, the half life ($t_{1/2}$) is the time (in minutes) during which half the enzyme activity is inactivated under defined heat conditions. In preferred embodiments, the half life is assayed at 80 degrees C. Preferably; the sample is heated for 1-10 minutes at 80°C or higher. The half life value is then calculated by measuring the residual amylase

activity, by any of the methods described here. Preferably, a half life assay is conducted as described in more detail in the Examples.

[0130] Preferably, the variant polypeptides described here are active during baking and hydrolyse starch during and after the gelatinization of the starch granules which starts at temperatures of about 55 degrees C. The more thermostable the non-maltogenic exoamylase is the longer time it can be active and thus the more antistaling effect it will provide. However, during baking above temperatures of about 85 degrees C, enzyme inactivation can take place. If this happens, the non-maltogenic exoamylase may be gradually inactivated so that there is substantially no activity after the baking process in the final bread. Therefore preferentially the non-maltogenic exoamylases suitable for use as described have an optimum temperature above 50 degrees C and below 98 degrees C.

[0131] The thermostability of the variant polypeptides described here can be improved by using protein engineering to become more thermostable and thus better suited for the uses described here; we therefore encompass the use of variant polypeptides modified to become more thermostable by protein engineering.

[0132] Preferably, the variant polypeptide is pH stable; more preferably, it has a higher pH stability than SEQ ID NO: 1. As used herein the term "pH stable" relates to the ability of the enzyme to retain activity over a wide range of pHs. Preferably, the variant polypeptide is capable of degrading starch at a pH of from about 5 to about 10.5. In one embodiment, the degree of pH stability may be assayed by measuring the half life of the enzyme in specific pH conditions. In another embodiment, the degree of pH stability may be assayed by measuring the activity or specific activity of the enzyme in specific pH conditions. The specific pH conditions may be any pH from pH5 to pH10.5.

[0133] Thus, the variant polypeptide may have a longer half life, or a higher activity (depending on the assay) when compared to SEQ ID NO: 1 under identical conditions. The variant polypeptides may have 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or longer half life when compared to SEQ ID NO:1 under identical pH conditions. Alternatively, or in addition, they may have such higher activity when compared to SEQ ID NO: 1 under identical pH conditions.

Exo-Specificity

[0134] It is known that some non-maltogenic exoamylases can have some degree of endoamylase activity. In some cases, this type of activity may need to be reduced or eliminated since endoamylase activity can possibly negatively effect the quality of the final bread product by producing a sticky or gummy crumb due to the accumulation of branched dextrins.

[0135] Exo-specificity can usefully be measured by determining the ratio of total amylase activity to the total endoamylase activity. This ratio is referred to in this document as a "Exo-specificity index". In preferred embodiments, an enzyme is considered an exoamylase if it has a exo-specificity index of 20 or more, i.e., its total amylase activity (including exoamylase activity) is 20 times or more greater than its endoamylase activity. In highly preferred embodiments, the exo-specificity index of exoamylases is 30 or more, 40 or more, 50 or more, 60 or more, 70 or more, 80 or more, 90 or more, or 100 or more. In highly preferred embodiments, the exo-specificity index is 150 or more, 200 or more, 300 or more, 400 or more, 500 or more or 600 or more.

[0136] The total amylase activity and the endoamylase activity may be measured by any means known in the art. For example, the total amylase activity may be measured by assaying the total number of reducing ends released from a starch substrate. Alternatively, the use of a Betamyl assay is described in further detail in the Examples, and for convenience, amylase activity as assayed in the Examples is described in terms of "Betamyl Units" in the Tables.

[0137] Endoamylase activity may be assayed by use of a Phadebas Kit (Pharmacia and Upjohn). This makes use of a blue labelled crosslinked starch (labelled with an azo dye); only internal cuts in the starch molecule release label, while external cuts do not do so. Release of dye may be measured by spectrophotometry. Accordingly, the Phadebas Kit measures endoamylase activity, and for convenience, the results of such an assay are referred to in this document as "Phadebas units".

[0138] In a highly preferred embodiment, therefore, the exo-specificity index is expressed in terms of Betamyl Units / Phadebas Units, also referred to as "B/Phad".

[0139] Exo-specificity may also be assayed according to the methods described in the prior art, for example, in our International Patent Publication Number WO99/50399. This measures exo-specificity by way of a ratio between the endoamylase activity to the exoamylase activity. Thus, in a preferred aspect, the variant polypeptide described here will have less than 0.5 endoamylase units (EAU) per unit of exoamylase activity. Preferably the non-maltogenic exoamylases which are suitable for use according to the present invention have less than 0.05 EAU per unit of exoamylase activity and more preferably less than 0.01 EAU per unit of exoamylase activity.

[0140] The variant polypeptide described here will preferably have exospecificity, for example measured by exo-specificity indices, as described above, consistent with their being exoamylases. Moreover, they preferably have higher or increased exospecificity when compared to SEQ ID NO: 1. Thus, for example, the variant polypeptide may have 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or higher exo-specificity index when compared to SEQ ID

NO:1, preferably under identical conditions. They may have 1.5x or higher, 2x or higher, 5 x or higher, 10 x or higher, 50 x or higher, 100 x or higher, when compared to SEQ ID NO:1, preferably under identical conditions.

3.4. Methods for purifying variants

[0141] In general, a variant produced in cell culture is secreted into the medium and may be purified or isolated, e.g., by removing unwanted components from the cell culture medium. In some cases, a variant may be recovered from a cell lysate. In such cases, the enzyme is purified from the cells in which it was produced using techniques routinely employed by those of skill in the art. Examples include, but are not limited to, affinity chromatography, ion-exchange chromatographic methods, including high resolution ion-exchange, hydrophobic interaction chromatography, two-phase partitioning, ethanol precipitation, reverse phase HPLC, chromatography on silica or on a cation-exchange resin, such as DEAE, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, and gel filtration using Sephadex G-75, for example.

4. Com positions and uses of variants

[0142] A polypeptide variant produced and purified by the methods described above is useful for a variety of industrial applications. In one embodiment, the polypeptide variant as defined herein is useful in a starch conversion process, particularly in a liquefaction process of a starch, e.g., corn starch, wheat starch, or barley starch. The desired end-product may be any product that may be produced by the enzymatic conversion of the starch substrate. For example, the desired product may be a syrup rich in saccharides useful for fermentation, particularly maltotriose, glucose, and/or maltose. The end product then can be used directly in a fermentation process to produce alcohol for fuel or drinking (i.e., potable alcohol). The skilled artisan is aware of various fermentation conditions that may be used in the production of ethanol or other fermentation end-products. A microbial organism capable of fermenting maltotrioses and/or less complex sugars, such as *S. cerevisiae* or a genetically modified variant thereof, is particularly useful. Suitable genetically altered variants of *S. cerevisiae* particularly useful for fermenting maltotrioses include variants that express AGT1 permease (Stambuck et al., Lett. Appl. Microbiol. 43: 370-76 (2006)), MTT1 and MTT1alt (Dietvorst et al., Yeast 22: 775-88 (2005)), or MAL32 (Dietvorst et al., Yeast 24: 27-38 (2007)). The present polypeptide variants also are useful in compositions and methods of food preparation, where enzymes that express amylase activity at high temperatures are desired.

[0143] The desirability of using a particular polypeptide variant will depend on the overall properties displayed by the polypeptide variant relative to the requirements of a particular application. As a general matter, polypeptide variants useful for a starch conversion process have substantial endo-amylase activity compared to wild-type PS4 or SEQ ID NO:1, and/or have a lower exo- to endo-amylase activity compared to wild-type PS4 or SEQ ID NO: 1. Such polypeptide variants may be particularly useful in a liquefaction process, when used alone or combination with other amylase variants, where internal cleavage of complex branching saccharides lowers the viscosity of the substrate. Some polypeptide variants useful for liquefaction, however, are expected to have an endo-amylase activity comparable or even lower than wild-type PS4. Useful polypeptide variants include those with more or less exo-amylase activity than the wild-type PS4 or the SEQ ID NO: 1 polypeptide, depending on the application. Compositions may include one or a combination of amylase variants, each of which may display a different set of properties.

4.1. Preparation of starch substrates

[0144] Those of skill in the art are well aware of available methods that may be used to prepare starch substrates for use in the processes disclosed herein. For example, a useful starch substrate may be obtained from tubers, roots, stems, legumes, cereals or whole grain. More specifically, the granular starch comes from plants that produce high amounts of starch. For example, granular starch may be obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes. Corn contains about 60-68% starch; barley contains about 55-65% starch; millet contains about 75-80% starch; wheat contains about 60-65% starch; and polished rice contains 70-72% starch. Specifically contemplated starch substrates are cornstarch, wheat starch, and barley starch. The starch from a grain may be ground or whole and includes corn solids, such as kernels, bran and/or cobs. The starch may be highly refined raw starch or feedstock from starch refinery processes. Various starches also are commercially available. For example, cornstarch is available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starch is available from Sigma; sweet potato starch is available from Wako Pure Chemical Industry Co. (Japan); and potato starch is available from Nakaari Chemical Pharmaceutical Co. (Japan).

[0145] Maltodextrins are useful as starch substrates in embodiments of the present invention. Maltodextrins comprise starch hydrolysis products having about 20 or fewer dextrose (glucose) units. Typical commercial maltodextrins contain mixtures of polysaccharides including from about three to about nineteen linked dextrose units. Maltodextrins are defined by the FDA as products having a dextrose equivalence (DE) of less than 20. They are generally recognized as safe

(GRAS) food ingredients for human consumption. Dextrose equivalence (DE) is a measure of reducing power compared to a dextrose (glucose) standard of 100. The higher the DE, the greater the extent of starch depolymerization, resulting in a smaller average polymer (polysaccharide) size, and the greater the sweetness. A particularly useful maltodextrin is MALTRIN® M040 obtained from cornstarch, available from Grain Processing Corp. (Muscatine, Iowa): DE 4.0-7.0; bulk density 0.51 g/cc; measured water content 6.38% by weight.

[0146] The starch substrate can be a crude starch from milled whole grain, which contains non-starch fractions, e.g., germ residues and fibers. Milling may comprise either wet milling or dry milling. In wet milling, whole grain is soaked in water or dilute acid to separate the grain into its component parts, e.g., starch, protein, germ, oil, kernel fibers. Wet milling efficiently separates the germ and meal (i.e., starch granules and protein) and is especially suitable for production of syrups. In dry milling, whole kernels are ground into a fine powder and processed without fractionating the grain into its component parts. Dry milled grain thus will comprise significant amounts of non-starch carbohydrate compounds, in addition to starch. Most ethanol comes from dry milling. Alternatively, the starch to be processed may be a highly refined starch quality, for example, at least about 90%, at least 95%, at least 97%, or at least 99.5% pure.

4.2. Saccharification, gelatinization and liquefaction of starch

[0147] As used herein, the term "liquefaction" or "liquefy" means a process by which starch is converted to less viscous and shorter chain dextrans. This process involves gelatinization of starch simultaneously with or followed by the addition of a polypeptide variant as described herein. A thermostable polypeptide variant as described herein is preferably used for this application. Additional liquefaction-inducing enzymes optionally may be added.

[0148] In some embodiments, the starch or maltodextrin substrate prepared as described above is slurried with water. The starch or maltodextrin slurry may contain starch as a weight percent of dry solids of about 10-55%, about 20-45%, about 30-45%, about 30-40%, or optionally about 30-35%. The α -amylases, e.g., bacterial α -amylases, including *Bacillus* α -amylases, is usually supplied, for example, at about 1500 units per kg dry matter of starch, for example. To optimize α -amylase stability and activity, the pH of the slurry may be adjusted to the optimal pH for the used polypeptide variant. Other α -amylases may be added and may require different optimal conditions. Bacterial α -amylase remaining in the slurry following liquefaction may be deactivated by lowering pH in a subsequent reaction step or by removing calcium from the slurry.

[0149] The slurry of starch plus the polypeptide variant may be pumped continuously through a jet cooker, which is steam heated from about 85°C to up to 105°C. Gelatinization occurs very rapidly under these conditions, and the enzymatic activity, combined with the significant shear forces, begins the hydrolysis of the starch substrate. The residence time in the jet cooker is very brief. The partly gelatinized starch may be passed into a series of holding tubes maintained at about 85-105°C and held for about 5 min. to complete the gelatinization process. These tanks may contain baffles to discourage back mixing. As used herein, the term "secondary liquefaction" refers the liquefaction step subsequent to primary liquefaction, when the slurry is allowed to cool to room temperature. This cooling step can be about 30 minutes to about 180 minutes, e.g. about 90 minutes to 120 minutes.

[0150] A polypeptide variant as defined herein can be added to the liquefied starch obtained by the process above or to a maltodextrin slurry at about 0.01 to about 1.0 kg/MTDS. 1 kg/MTDS = 0.1 % by weight dissolved solids. In one embodiment, a polypeptide as defined herein can be added to a liquefied starch or maltodextrin slurry at a treatment level in a range from about 0.001% by weight to about 0.01% by weight based on dissolved solids. In a typical embodiment, a polypeptide as defined herein can be added to a liquefied starch or maltodextrin slurry at a treatment level in a range from about 0.0025% by weight to about 0.01% by weight based on dissolved solids. In one embodiment, the polypeptide as defined herein is immobilized, and the liquefied starch or maltodextrin substrate is passed over the immobilized polypeptide as defined herein and converted to product in a continuous reaction. In this embodiment, the polypeptide as defined herein may be immobilized with additional enzymes, such as a pullulanase.

[0151] The production of maltotetraose may further comprise contacting the liquefied starch or other source of maltodextrins with an isoamylase, a protease, a cellulase, a hemicellulase, a lipase, a cutinase, or any combination thereof.

[0152] Also provided is a method of making a saccharide (e.g., maltotetraose) syrup, comprising adding a polypeptide variant as defined herein or a composition comprising the variant to a starch liquefact and saccharifying the starch liquefact to form the saccharide syrup. The polypeptide variant as defined herein may be added to the starch liquefact in a range from about 0.001% by weight to about 0.1% by weight based on dissolved solids. In one embodiment, the variant is added to the starch liquefact in a range from about 0.0025% by weight to about 0.01% by weight based on dissolved solids. The units of concentration also are expressed herein as kg of polypeptide variant per metric ton of dry solids (MTDS), where 1 kg/MTDS = 0.1 % by weight dissolved solids. The liquefied starch solution may be a slurry of liquefied starch at about 20-35% w/w dry solids. The starch may be obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes. The starch liquefact may be saccharified at about 55°C to about 65°C such as at about 60°C to about 65°C. The starch liquefact may be saccharified at about pH 5.0 to about pH 7.0. A pullulanase, isoamylase, pullulanase, protease, cellulase, hemicellulase, lipase, cutinase, or any

combination thereof, may be added with the polypeptide variant to the starch liquefact. In one embodiment, the saccharide syrup may be fermented to produce ethanol. The saccharide syrup produced by the method may comprise at least about 40%, about 45%, about 50%, about 55%, or about 60% by weight maltotetraose based on total saccharide content.

[0153] In another aspect a method of making a saccharide syrup, including adding a polypeptide variant as defined herein and an alpha-amylase to granular starch and hydrolyzing the granular starch to form the saccharide syrup is provided. In one embodiment, the granular starch liquefact is produced by an alpha-amylase. In one embodiment, the granular starch liquefact is an acid produced liquefact. In one embodiment the polypeptide variant as defined herein is added to the granular starch in a range from about 0.001% by weight to about 0.1% by weight based on dissolved solids. In another embodiment the polypeptide variant as defined herein is added to the granular starch in a range from about 0.0025% by weight to about 0.01% by weight based on dissolved solids. The granular starch can be obtained from starch from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes.

[0154] In a particular embodiment the granular starch is saccharified at 55°C to 65°C such as 60°C to 65°C. In another embodiment the granular starch is saccharified at pH 5.0 to pH 7.0. It is envisioned that the method can also include fermenting the saccharide syrup to produce ethanol.

[0155] In one embodiment the method includes a step of adding an enzyme having debranching activity to the granular starch. The enzyme having debranching activity can include but is not limited to an isoamylase, a pullulanase, an isopullulanase, a neopullulanase or any combination thereof. It is also envisioned that the method can optionally include a further step of adding a protease, a cellulase, a hemicellulase, a lipase, a cutinase, a pectate liase or any combination thereof to the granular starch.

[0156] In one embodiment the saccharide syrup includes at least 35% by weight maltotetraose based on total saccharide content. Alternatively, the saccharide syrup includes at least 45% by weight maltotetraose based on total saccharide content. In another embodiment the saccharide syrup includes at least 50% by weight maltotetraose based on total saccharide content. In a further embodiment the saccharide syrup includes from 45% by weight to 60% by weight maltotetraose based on total saccharide content.

[0157] It is envisioned that the polypeptide variant as defined herein of the method can be immobilized.

[0158] In another aspect a method is provided for making IMO, including adding a) a polypeptide variant as defined herein, b) an alpha-amylase, and c) a transglucosidase to starch in the form of a starch liquefact or granular starch and saccharifying the starch to form IMO. It is envisioned that the IMO can be formed at an IMO number of at least 30, at least 40 and/or at least 45. In one embodiment the starch is obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes.

[0159] In another aspect a method is provided for making IMO, including adding a) a polypeptide variant, b) an alpha-amylase, and c) a transglucosidase to starch in the form of a starch liquefact or granular starch and saccharifying the starch to form IMO. Any of a number of transglucosidase enzymes (TG) can be use, for example, TRANSGLUCOSIDASE L-500® (Danisco US Inc., Genencor Division).

[0160] It is envisioned that the IMO can be formed at an IMO number of at least 30, at least 40 and/or at least 45. In one embodiment the starch is obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes.

4.3. Processes of fermentation

[0161] Yeast typically from *Saccharomyces spp.* is added to the mash and the fermentation is ongoing for 24-96 hours, such as typically 35-60 hours. The temperature is between about 26-34°C, typically at about 32°C, and the pH is from about pH 3-6, typically around about pH 4-5.

[0162] In one embodiment, a batch fermentation process is used in a closed system, where the composition of the medium is set at the beginning of the fermentation and is not altered during the fermentation. At the beginning of the fermentation, the medium is inoculated with the desired microbial organism(s). In this method, fermentation is permitted to occur without the addition of any components to the system. Typically, a batch fermentation qualifies as a "batch" with respect to the addition of the carbon source, and attempts are often made to control factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures, cells progress through a static lag phase to a high growth log phase and finally to a stationary phase, where growth rate is diminished or halted. If untreated, cells in the stationary phase eventually die. In general, cells in log phase are responsible for the bulk of production of product.

[0163] A suitable variation on the standard batch system is the "fed-batch fermentation" system. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression likely inhibits the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors, such as pH, dissolved oxygen and the partial pressure of waste gases, such as CO₂. Batch and fed-batch fermentations are common and well known in the art.

[0164] Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor, and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density, where cells are primarily in log phase growth. Continuous fermentation allows for the modulation of one or more factors that affect cell growth and/or product concentration. For example, in one embodiment, a limiting nutrient, such as the carbon source or nitrogen source, is maintained at a fixed rate and all other parameters are allowed to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, cell loss due to medium being drawn off should be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes, as well as techniques for maximizing the rate of product formation, are well known in the art of industrial microbiology.

[0165] Following the fermentation, the mash is distilled to extract the ethanol. The ethanol obtained according to the process of the disclosure may be used as, e.g., fuel ethanol; drinking ethanol, *i.e.*, potable neutral spirits or industrial ethanol. Left over from the fermentation is the grain, which is typically used for animal feed, either in liquid form or dried. Further details on how to carry out liquefaction, saccharification, fermentation, distillation, and recovery of ethanol are well known to the skilled person. According to the process of the disclosure, the saccharification and fermentation may be carried out simultaneously or separately.

5. Compositions and methods for baking and food preparation

[0166] In one aspect, compositions, including food additives, food products, bakery products, improver compositions, feed products including animal feeds, etc comprising such altered variants as described herein, such as those which have non-maltogenic exoamylase activity, as well as methods of making and using such polypeptides and the compositions are provided.

[0167] As noted above, the variant polypeptides as described herein may comprise one or more improved handling properties, preferably improved baking properties. Thus, the variant polypeptides as described herein may provide that the food products so treated have one or more of (preferably all of) a lower firmness, a higher resilience, a higher cohesiveness, a lower crumbliness or a higher foldability. Such improved handling or baking properties exhibited by the variant polypeptides as described herein are described in further detail below. In one aspect, a variant, in which the half life ($t_{1/2}$), preferably at 60 degrees C, is increased by 15% or more, preferably 50% or more, most preferably 100% or more, compared to the amino acid sequence of SEQ ID NO: 1, is provided.

[0168] In one aspect, a variant as described herein, in which a food product treated with the variant has any one or more, preferably all of the following properties: (a) lower firmness; (b) higher resilience; (c) higher cohesiveness; (d) lower crumbliness; and (e) higher foldability compared to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

[0169] In one aspect, a variant as described herein, in which the resilience, cohesiveness or foldability of the food product is independently increased by 15% or more, preferably 50% or more, most preferably 100% or more, compared to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

[0170] In one aspect, a variant as described herein, in which each of resilience cohesiveness and foldability of a food product treated with the variant is increased compared to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

[0171] In one aspect, a variant as described herein, in which the firmness or the crumbliness of the food product is independently decreased by 15% or more, preferably 50% or more, most preferably 100% or more, relative to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

[0172] In one aspect, a variant as described herein, in which each of the firmness and crumbliness of a food product treated with the polypeptide is decreased compared to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

[0173] In one aspect, a variant as described herein comprising a fragment of at least 20 residues of a polypeptide as set out in the claims, in which the polypeptide has non-maltogenic exoamylase activity, is provided.

[0174] In one aspect, a variant as described herein has non-maltogenic exoamylase activity. Such activity may be determined by the methods described in US 6,667,065.

[0175] In one aspect, the treatment of food products, particularly doughs and bakery products with such polypeptides, and such that the food products exhibit the desired qualities set out above are provided.

[0176] In one aspect, a process for treating a starch comprising contacting the starch with a variant as described herein and allowing the polypeptide to generate from the starch one or more linear products, is provided. In one aspect, the use of a variant as described herein in preparing a food or feed product, is provided. In one aspect, a process of preparing a food or feed product comprising admixing a variant as described herein with a food or feed ingredient, is provided. In one aspect, the use, or a process, in which the food product comprises a dough or a dough product, preferably

a processed dough product, is provided. In one aspect, the use or process, in which the food product is a bakery product, is provided. In one aspect, a process for making a bakery product comprising: (a) providing a starch medium; (b) adding to the starch medium a variant as described herein; and (c) applying heat to the starch medium during or after step (b) to produce a bakery product, is provided. In one aspect, a food product, feed product, dough product or a bakery product obtained by a process as defined in the claims, is provided.

[0177] In one aspect, the use of a variant as described herein, in a dough product to retard or reduce staling, preferably detrimental retrogradation, of the dough product, is provided.

[0178] In one aspect, the use of a variant as described herein, in a dough product to improve any one or more of firmness, resilience, cohesiveness, crumbliness or foldability of the dough product, is provided.

[0179] In one aspect, a combination of a variant as described herein, together with any one or more of the following:

(a) maltogenic alpha-amylase also called glucan 1,4- α -maltohydrolase (EC 3.2.1.133) from *Bacillus stearothermophilus*, or a variant, homologue, or mutants thereof which have maltogenic alpha-amylase activity;

(b) a bakery xylanase (EC 3.2.1.8) from e.g. *Bacillus* sp., *Aspergillus* sp., *Thermomyces* sp. or *Trichoderma* sp.;

(c) α -amylase (EC 3.2.1.1) from *Bacillus amyloliquefaciens* or from *Aspergillus* sp. or a variant, homologue, or mutants thereof which have alpha-amylase activity; and

(d) a lipase such as glycolipase from *Fusarium heterosporum*, is provided.

[0180] The variants as described herein preferably comprise one or more improved handling properties compared to a parent polypeptide or a wild type polypeptide, such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1. The improved handling properties may in preferred embodiments comprise improved baking properties.

[0181] Thus, the variant as described herein are in one aspect such that a food product treated with the variant polypeptide has an improved handling or preferably baking property compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1. The handling or baking property may be selected from the group consisting of: firmness, resilience, cohesiveness, crumbliness and foldability.

[0182] These handling properties may be tested by any means known in the art. For example, firmness, resilience and cohesiveness may be determined by analysing bread slices by Texture Profile Analysis using for example a Texture Analyser, as e.g. described in 11.

Firmness

[0183] The variants described here are in one aspect such that a food product treated with the variant polypeptide has lower firmness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

[0184] The firmness is in preferred embodiments inversely correlated with the softness of the food product; thus, a higher softness may reflect a lower firmness, and vice versa.

[0185] Firmness may be measured by the "Firmness Evaluation Protocol" set out in example 11.

[0186] In one aspect, the variants described herein are such that a food product treated with the variant polypeptide has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more lower firmness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1. A food product treated with the variant polypeptides disclosed herein may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more lower firmness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

Resilience

[0187] In one aspect, the variants described herein are such that a food product treated with the variant polypeptide has a higher resilience compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

[0188] Resilience may be measured by the "Resilience Evaluation Protocol" set out in 11.

[0189] Thus in one aspect, the variants described herein are such that a food product treated with the variant polypeptide has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more higher resilience compared to a food

product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1. A food product treated with the variant polypeptide may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more higher resilience compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

Cohesiveness

[0190] In one aspect, the variants described herein are such that a food product treated with the variant polypeptide has higher cohesiveness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

[0191] Cohesiveness may be measured by the "Cohesiveness Evaluation Protocol" set out 11.

[0192] Thus in one aspect, the variants described here are such that a food product treated with the variant polypeptide as disclosed herein has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more higher cohesiveness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1. A food product treated with the variant polypeptide may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more higher cohesiveness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

Crumbliness

[0193] In one aspect, the variants described here are such that a food product treated with the variant polypeptide has lower crumbliness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

[0194] Crumbliness may be measured by the "Crumbliness Evaluation Protocol" set out in Example 13.

[0195] Thus in one aspect, the variants described here are such that a food product treated with the variant polypeptide has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more lower crumbliness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1. A food product treated with the variant polypeptide may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more lower crumbliness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

Foldability

[0196] In one aspect, the variants described here are such that a food product treated with the variant polypeptide has higher foldability compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

[0197] Foldability is preferably measured by the "Foldability Evaluation Protocol" set out in Example 14.

[0198] Thus, the variants described here are such that a food product treated with the variant polypeptide has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more higher foldability compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1. A food product treated with the variant polypeptide may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more higher foldability compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO: 1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1.

[0199] In one aspect, the use of the variant polypeptides described here in combination with a xylanase for improving foldability are provided.

[0200] Further, a method for preparing a food product, the method comprising: (a) obtaining a non-maltogenic exoamylase; (b) introducing a mutation at any one or more of the positions of the non-maltogenic exoamylase as set out in this document; (c) admixing the resulting polypeptide with a food ingredient is provided.

[0201] The variant polypeptides may be used to enhance the volume of bakery products such as bread. While not wishing to be bound by any particular theory, we believe that this results from the reduction in viscosity of the dough during heating (such as baking) as a result of the amylase shortening amylose molecules. This enables the carbon dioxide generated by fermentation to increase the size of the bread with less hindrance.

[0202] Thus, food products comprising or treated with variant polypeptides are expanded in volume when compared to products which have not been so treated, or treated with parent polypeptides such as a polypeptide of SEQ ID NO:

1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO: 1. In other words, the food products have a larger volume of air per volume of food product. Alternatively, or in addition, the food products treated with variant polypeptides as described herein have a lower density, or weight (or mass) per volume ratio. In particularly preferred embodiments, the variant polypeptides are used to enhance the volume of bread. Volume enhancement or expansion is beneficial because it reduces the gumminess or starchiness of foods. Light foods are preferred by consumers, and the customer experience is enhanced. In preferred embodiments, the use of variant polypeptides enhances the volume by 10%, 20%, 30% 40%, 50% or more.

[0203] The variant polypeptides and nucleic acids described here may be used as - or in the preparation of - a food. In particular, they may be added to a food, i.e., as a food additive. The term "food" is intended to include both prepared food, as well as an ingredient for a food, such as a flour. In a preferred aspect, the food is for human consumption. The food may be in the form of a solution or as a solid - depending on the use and/or the mode of application and/or the mode of administration.

[0204] The variant polypeptides and nucleic acids may be used as a food ingredient. As used herein the term "food ingredient" includes a formulation, which is or can be added to functional foods or foodstuffs and includes formulations which can be used at low levels in a wide variety of products that require, for example, acidifying or emulsifying. The food ingredient may be in the form of a solution or as a solid - depending on the use and/or the mode of application and/or the mode of administration.

[0205] The variant polypeptides and nucleic acids disclosed here may be - or may be added to - food supplements. The variant polypeptides and nucleic acids disclosed here may be - or may be added to - functional foods. As used herein, the term "functional food" means food which is capable of providing not only a nutritional effect and/or a taste satisfaction, but is also capable of delivering a further beneficial effect to consumer. Although there is no legal definition of a functional food, most of the parties with an interest in this area agree that they are foods marketed as having specific health effects.

[0206] The variant polypeptides may also be used in the manufacture of a food product or a foodstuff. Typical foodstuffs include dairy products, meat products, poultry products, fish products and dough products. The dough product may be any processed dough product, including fried, deep fried, roasted, baked, steamed and boiled doughs, such as steamed bread and rice cakes. In highly preferred embodiments, the food product is a bakery product.

[0207] Preferably, the foodstuff is a bakery product. Typical bakery (baked) products include bread - such as loaves, rolls, buns, bagels, pizza bases etc. pastry, pretzels, tortillas, cakes, cookies, biscuits, crackers etc.

[0208] The food products preferably benefit from one or more of the improved handling or baking properties of the variant polypeptides described here. The improved handling or baking property may be selected from the group consisting of: improved firmness, improved resilience, improved cohesiveness, improved crumbliness and improved foldability.

[0209] Further in one aspect, a method of modifying a food additive comprising a non-maltogenic exoamylase, the method comprising introducing a mutation at any one or more of the positions of the non-maltogenic exoamylase as set out in this document is provided. The same method can be used to modify a food ingredient, or a food supplement, a food product, or a foodstuff.

[0210] In one aspect, the use of variant polypeptides that are capable of retarding the staling of starch media, such as starch gels are provided. The variant polypeptides are especially capable of retarding the detrimental retrogradation of starch.

[0211] Most starch granules are composed of a mixture of two polymers: an essentially linear amylose and a highly branched amylopectin. Amylopectin is a very large, branched molecule consisting of chains of α -D-glucopyranosyl units joined by (1-4) linkages, wherein said chains are attached by α -D-(1-6) linkages to form branches. Amylopectin is present in all natural starches, constituting about 75% of most common starches. Amylose is essentially a linear chain of (1-4) linked α -D-glucopyranosyl units having few α -D-(1-6) branches. Most starches contain about 25% amylose.

[0212] Starch granules heated in the presence of water undergo an order-disorder phase transition called gelatinization, where liquid is taken up by the swelling granules. Gelatinization temperatures vary for different starches. Upon cooling of freshly baked bread the amylose fraction, within hours, retrogrades to develop a network. This process is beneficial in that it creates a desirable crumb structure with a low degree of firmness and improved slicing properties. More gradually crystallisation of amylopectin takes place within the gelatinised starch granules during the days after baking. In this process amylopectin is believed to reinforce the amylose network in which the starch granules are embedded. This reinforcement leads to increased firmness of the bread crumb. This reinforcement is one of the main causes of bread staling.

[0213] It is known that the quality of baked products gradually deteriorates during storage. As a consequence of starch recrystallisation (also called retrogradation), the water-holding capacity of the crumb is changed with important implications on the organoleptic and dietary properties. The crumb loses softness and elasticity and becomes firm and crumbly. The increase in crumb firmness is often used as a measure of the staling process of bread.

[0214] The rate of detrimental retrogradation of amylopectin depends on the length of the side chains of amylopectin. Thus, enzymatic hydrolysis of the amylopectin side chains, for example, by variant polypeptides as described herein

having non-maltogenic exoamylase activity, can markedly reduce their crystallisation tendencies.

[0215] Accordingly, in one aspect the use of variant polypeptides as described here when added to the starch at any stage of its processing into a food product e.g., before during or after baking into bread can retard or impede or slow down the retrogradation. Such use is described in further detail below.

[0216] In one aspect, a method of improving the ability of a non-maltogenic exoamylase to prevent staling, preferably detrimental retrogradation, of a dough product, the method comprising introducing a mutation at any one or more of the positions of the non-maltogenic exoamylase as set out in this document are provided.

[0217] For evaluation of the antistaling effect of the variant polypeptides such a variant having non-maltogenic exoamylase activity described here, the crumb firmness can be measured 1, 3 and 7 days after baking by means of an Instron 4301 Universal Food Texture Analyzer or similar equipment known in the art.

[0218] Another method used traditionally in the art and which is used to evaluate the effect on starch retrogradation of a variant polypeptide such as a variant having exoamylase activity is based on DSC (differential scanning calorimetry). Here, the melting enthalpy of retrograded amylopectin in bread crumb or crumb from a model system dough baked with or without enzymes (control) is measured. The DSC equipment applied may be a Mettler-Toledo DSC 820 run with a temperature gradient of 10°C per min. from 20 to 95°C. For preparation of the samples 10-20 mg of crumb are weighed and transferred into Mettler-Toledo aluminium pans which then are hermetically sealed.

[0219] The model system doughs may contain standard wheat flour and optimal amounts of water or buffer with or without the polypeptide variant. They are mixed in a 10 or 50 g Brabender Farinograph for 6 or 7 min., respectively. Samples of the doughs are placed in glass test tubes (15*0.8 cm) with a lid. These test tubes are subjected to a baking process in a water bath starting with 30 min. incubation at 33°C followed by heating from 33 to 95°C with a gradient of 1.1°C per min. and finally a 5 min. incubation at 95°C. Subsequently, the tubes are stored in a thermostat at 20°C prior to DSC analysis.

[0220] In one embodiment, the variants described here have a reduced melting enthalpy, compared to the control. In a further embodiment, the variants have a 10% or more reduced melting enthalpy. In yet a further aspect, they have a 20% or more, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more reduced melting enthalpy when compared to the control.

[0221] In one aspect, the use of variant polypeptides in the preparation of food products, in particular, starch products is provided. The method comprises forming the starch product by adding a non-maltogenic exoamylase enzyme such as a variant polypeptide, to a starch medium. If the starch medium is a dough, then the dough is prepared by mixing together flour, water, the non-maltogenic exoamylase which is a variant polypeptide as described herein and optionally other possible ingredients and additives.

[0222] The term "starch" should be taken to mean starch per se or a component thereof, especially amylopectin. The term "starch medium" means any suitable medium comprising starch. The term "starch product" means any product that contains or is based on or is derived from starch. Preferably, the starch product contains or is based on or is derived from starch obtained from wheat flour. The term "flour" as used herein is a synonym for the finely-ground meal of wheat or other grain. Preferably, however, the term means flour obtained from wheat per se and not from another grain. Thus, and unless otherwise expressed, references to "wheat flour" as used herein preferably mean references to wheat flour per se as well as to wheat flour when present in a medium, such as a dough.

[0223] A preferred flour is wheat flour or rye flour or mixtures of wheat and rye flour. However, dough comprising flour derived from other types of cereals such as for example from rice, maize, barley, and durra are also contemplated. Preferably, the starch product is a bakery product. More preferably, the starch product is a bread product. Even more preferably, the starch product is a baked farinaceous bread product. The term "baked farinaceous bread product" refers to any baked product based on a dough obtainable by mixing flour, water, and a leavening agent under dough forming conditions. Further components can of course be added to the dough mixture.

[0224] Thus, if the starch product is a baked farinaceous bread product, then the process comprises mixing - in any suitable order - flour, water, and a leavening agent under dough forming conditions and further adding a variant polypeptide as described herein, optionally in the form of a premix. The leavening agent may be a chemical leavening agent such as sodium bicarbonate or any strain of *Saccharomyces cerevisiae* (Baker's Yeast).

[0225] The variant polypeptide as described herein can be added together with any dough ingredient including the water or dough ingredient mixture or with any additive or additive mixture. The dough can be prepared by any conventional dough preparation method common in the baking industry or in any other industry making flour dough based products.

[0226] Baking of farinaceous bread products such as for example white bread, bread made from bolted rye flour and wheat flour, rolls and the like is typically accomplished by baking the bread dough at oven temperatures in the range of from 180 to 250°C for about 15 to 60 minutes. During the baking process a steep temperature gradient (200 to 120°C) is prevailing in the outer dough layers where the characteristic crust of the baked product is developed. However, owing to heat consumption due to steam generation, the temperature in the crumb is only close to 100°C at the end of the baking process.

[0227] A process for making a bread product comprising: (a) providing a starch medium; (b) adding to the starch medium a variant polypeptide as described in this document; and (c) applying heat to the starch medium during or after

step (b) to produce a bread product is provided. A process for making a bread product comprising adding to a starch medium a variant polypeptide as described is also provided.

[0228] In one aspect, the variant polypeptide can be added as a liquid preparation or as a dry pulverulent composition either comprising the enzyme as the sole active component or in admixture with one or more additional dough ingredient or dough additive.

[0229] In a further aspect, the improver compositions, which include bread improving compositions and dough improving compositions are provided. These comprise a variant polypeptide, optionally together with a further ingredient, or a further enzyme, or both.

[0230] In one aspect, an improver composition for a dough, in which the improver composition comprises a variant as set out in any of the claims, and at least one further dough ingredient or dough additive, is provided.

[0231] In one aspect, a composition comprising a flour and a variant as set out in any of the claims, is provided.

[0232] In a further aspect, the use of such a bread and dough improving compositions in baking is provided. In a further aspect, a baked product or dough obtained from the bread improving composition or dough improving composition is provided. In another aspect, a baked product or dough obtained from the use of a bread improving composition or a dough improving composition is provided.

[0233] A dough may be prepared by admixing flour, water, a dough improving composition comprising variant polypeptide (as described above) and optionally other ingredients and additives.

[0234] The dough improving composition can be added together with any dough ingredient including the flour, water or optional other ingredients or additives. The dough improving composition can be added before the flour or water or optional other ingredients and additives. The dough improving composition can be added after the flour or water, or optional other ingredients and additives. The dough can be prepared by any conventional dough preparation method common in the baking industry or in any other industry making flour dough based products.

[0235] The dough improving composition can be added as a liquid preparation or in the form of a dry powder composition either comprising the composition as the sole active component or in admixture with one or more other dough ingredients or additive.

[0236] The amount of the variant polypeptide that is added is normally in an amount which results in the presence in the finished dough of 50 to 100,000 units per kg of flour, preferably 100 to 50,000 units per kg of flour. Preferably, the amount is in the range of 200 to 20,000 units per kg of flour. Alternatively, the variant polypeptide is added in an amount which results in the presence in the finished dough of 0.02 - 50 ppm of enzyme based on flour (0.02 - 50 mg enzyme per kg of flour), preferably 0.2 - 10 ppm.

[0237] In the present context, 1 unit of the non-maltogenic exoamylase is defined as the amount of enzyme which releases hydrolysis products equivalent to 1 μ mol of reducing sugar per min. when incubated at 50 degrees C in a test tube with 4 ml of 10 mg/ml waxy maize starch in 50 mM MES, 2 mM calcium chloride, pH 6.0 as described hereinafter.

[0238] The dough as described here generally comprises wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, corn starch, maize flour, rice flour, rye meal, rye flour, oat flour, oat meal, soy flour, sorghum meal, sorghum flour, potato meal, potato flour or potato starch. The dough may be fresh, frozen, or part-baked.

[0239] The dough may be a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways, such as by adding chemical leavening agents, e.g., sodium bicarbonate or by adding a leaven (fermenting dough), but it is preferred to leaven the dough by adding a suitable yeast culture, such as a culture of *Saccharomyces cerevisiae* (baker's yeast), e.g. a commercially available strain of *S. cerevisiae*.

[0240] The dough may comprise fat such as granulated fat or shortening. The dough may further comprise a further emulsifier such as mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxethylene stearates, or lysolecithin.

[0241] A pre-mix comprising flour together with the combination as described herein are furthermore provided. The pre-mix may contain other dough-improving and/or bread-improving additives, e.g. any of the additives, including enzymes, mentioned herein.

[0242] In order to improve further the properties of the baked product and impart distinctive qualities to the baked product further dough ingredients and/or dough additives may be incorporated into the dough. Typically, such further added components may include dough ingredients such as salt, grains, fats and oils, sugar or sweetener, dietary fibres, protein sources such as milk powder, gluten soy or eggs and dough additives such as emulsifiers, other enzymes, hydrocolloids, flavouring agents, oxidising agents, minerals and vitamins

[0243] The emulsifiers are useful as dough strengtheners and crumb softeners. As dough strengtheners, the emulsifiers can provide tolerance with regard to resting time and tolerance to shock during the proofing. Furthermore, dough strengtheners will improve the tolerance of a given dough to variations in the fermentation time. Most dough strengtheners also improve on the oven spring which means the increase in volume from the proofed to the baked goods. Lastly, dough strengtheners will emulsify any fats present in the recipe mixture.

[0244] Suitable emulsifiers include lecithin, polyoxyethylene stearat, mono- and diglycerides of edible fatty acids, acetic acid esters of mono- and diglycerides of edible fatty acids, lactic acid esters of mono- and diglycerides of edible fatty

acids, citric acid esters of mono- and diglycerides of edible fatty acids, diacetyl tartaric acid esters of mono- and diglycerides of edible fatty acids, sucrose esters of edible fatty acids, sodium stearyl-2-lactylate, and calcium stearyl-2-lactylate.

[02445] The further dough additive or ingredient can be added together with any dough ingredient including the flour, water or optional other ingredients or additives, or the dough improving composition. The further dough additive or ingredient can be added before the flour, water, optional other ingredients and additives or the dough improving composition. The further dough additive or ingredient can be added after the flour, water, optional other ingredients and additives or the dough improving composition.

[02446] The further dough additive or ingredient may conveniently be a liquid preparation. However, the further dough additive or ingredient may be conveniently in the form of a dry composition.

[02447] In one aspect the further dough additive or ingredient is at least 1% the weight of the flour component of dough. In a further aspect, the further dough additive or ingredient is at least 2%, preferably at least 3%, preferably at least 4%, preferably at least 5%, preferably at least 6%. If the additive is a fat, then typically the fat may be present in an amount of from 1 to 5%, typically 1 to 3%, more typically about 2%.

[02448] For the commercial and home use of flour for baking and food production, it is important to maintain an appropriate level of α -amylase activity in the flour. A level of activity that is too high may result in a product that is sticky and/or doughy and therefore unmarketable. Flour with insufficient α -amylase activity may not contain enough sugar for proper yeast function, resulting in dry, crumbly bread, or baked products. Accordingly, a variant as described herein, by itself or in combination with another α -amylase(s) or another variant, may be added to the flour to augment the level of endogenous α -amylase activity in flour. The variant in this embodiment can have a temperature optimum in the presence of starch in the ranges of about 30-90°C, 40-80°C, 40-50°C, 45-65°C, or 50-60°C, for example. The pH optimum in a 1% solution of soluble starch may be between pH 4.5 to 6.0, for example.

[02449] Grains, such as barley, oats, wheat, as well as plant components, such as corn, hops, and rice, also are used for brewing, both in industry and for home brewing. The components used in brewing may be unmalted or may be malted, i.e., partially germinated, resulting in an increase in the levels of enzymes, including α -amylase. For successful brewing, adequate levels of α -amylase enzyme activity are necessary to ensure the appropriate levels of sugars for fermentation. A variant, by itself or in combination with another α -amylase(s), accordingly may be added to the components used for brewing.

[02500] As used herein, the term "flour" means milled or ground cereal grain. The term "flour" also may mean Sago or tuber products that have been ground or mashed. In some embodiments, flour may also contain components in addition to the milled or mashed cereal or plant matter. An example of an additional component, although not intended to be limiting, is a leavening agent. Cereal grains include wheat, oat, rye, and barley. Tuber products include tapioca flour, cassava flour, and custard powder. The term "flour" also includes ground corn flour, maize-meal, rice flour, whole-meal flour, self-rising flour, tapioca flour, cassava flour, ground rice, enriched flower, and custard powder.

[02511] As used herein, the term "stock" means grains and plant components that are crushed or broken. For example, barley used in beer production is a grain that has been coarsely ground or crushed to yield a consistency appropriate for producing a mash for fermentation. As used herein, the term "stock" includes any of the aforementioned types of plants and grains in crushed or coarsely ground forms. The methods described herein may be used to determine α -amylase activity levels in both flours and stock.

[02521] One or more further enzymes may be used in combination with the variant polypeptides as described herein. Such combinations may for example be added to the food, dough preparation, foodstuff or starch composition.

[02531] Baking amylases may be from a fungus, bacterium or plant. It may be an α -amylase (EC 3.2.1.1). The α -amylase may be a fungal α -amylase from *Aspergillus* or *Trichoderma*, e.g. from *Aspergillus oryzae*. Or it may be an α -amylase from *Bacillus*, e.g. *B. amyloliquefaciens* or *B. licheniformis*. Or it may be a plant β -amylase (EC 3.2.1.2), e.g. β -amylase from barley malt or soy bean. Or it may be an α -amylase used for antistaling, e.g. non-maltogenic α -amylase (EC 3.2.1.60) developed from *Pseudomonas saccharophila* maltotetrahydrolase (SEQ ID NO: 10) or maltogenic α -amylase (EC 3.2.1.133) developed from *Bacillus stearothermophilus* as further described below.

[02541] In one aspect, the further amylase is a maltogenic α -amylase. A "maltogenic α -amylase" (glucan 1,4- α -maltotetrahydrolase, E.C. 3.2.1.133) is able to hydrolyze amylose and amylopectin to maltose in the α -configuration. A maltogenic α -amylase from *Bacillus* (EP 120 693) is commercially available under the trade name Novamyl (Novozymes, Denmark) and is widely used in the baking industry as an anti-staling agent due to its ability to reduce retrogradation of starch. Novamyl is described in detail in International Patent Publication WO 91/04669. The maltogenic α -amylase Novamyl shares several characteristics with cyclodextrin glucanotransferases (CGTases), including sequence homology (Henrissat B, Bairoch A; *Biochem. J.*, 316, 695-696 (1996)) and formation of transglycosylation products (Christophersen, C., et al., 1997, *Starch*, vol. 50, No. 1, 39-45). The Novamyl may in particular comprise Novamyl 1500 MG. Other documents describing Novamyl and its uses include Christophersen, C., Pedersen, S., and Christensen, T., (1993) Method for production of maltose and a limit dextrin, the limit dextrin, and use of the limit dextrin. Denmark, and WO 95/10627. It is further described in U.S. Pat. No. 4,598,048 and U.S. Pat. No. 4,604,355. Preferred examples

of antistaling amylases are exo-amylases, e.g. non-maltogenic exo-amylase or G4-amylase (EC 3.2.1.60) developed from *Pseudomonas saccharophila* maltotetraohydrolase having SEQ ID NO: 1 or maltogenic amylase (EC 3.2.1.133) developed from *Bacillus stearothermophilus* having SEQ ID NO: 51.

[0255] An antistaling amylase reduces staling after baking and leads to a reduction in firmness increase and a reduction in resilience decrease from day 1 to day 7 after baking relative to a control without enzyme. An antistaling amylase can be assayed by doing a baking trial and using texture analysis to determine firmness and resilience development over time. Texture analysis is described in example 11.

[0256] The further enzymes may be selected from, for example, any combination of the following: (a) Novamyl, or a variant, homologue, or mutants thereof which have maltogenic α -amylase activity; (b) a xylanase such as GRIN-DAMYL™ POWERBake 900 (Danisco A/S); (c) a bacterial α -amylase such as Max-Life U4 (Danisco A/S); and (d) a lipase such as GRINDAMYL™ POWERBake 4050 (Danisco A/S).

[0257] In one embodiment a variant polypeptide as described herein is used in combination with at least one enzyme selected from the list consisting of oxidoreductases, hydrolases, lipases, esterases, glycosidases, amylases, pullulanases, xylanases, cellulases, hemicellulases, starch degrading enzymes, proteases and lipoxygenases. In one embodiment, the composition comprises at least one variant polypeptide as described herein and a maltogenic amylase from *Bacillus*, as disclosed in WO91/04669. One embodiment comprises a variant polypeptide as described herein and flour.

[0258] Further enzymes that may be added to the dough include oxidoreductases, hydrolases, such as lipases and esterases as well as glycosidases like α -amylase, pullulanase, and xylanase. Oxidoreductases, such as for example glucose oxidase and hexose oxidase, can be used for dough strengthening and control of volume of the baked products and xylanases and other hemicellulases may be added to improve dough handling properties, crumb firmness and bread volume. Lipases are useful as dough strengtheners and crumb softeners and α -amylases and other amylolytic enzymes may be incorporated into the dough to control bread volume and further reduce crumb firmness.

[0259] Further enzymes that may be used may be selected from the group consisting of a cellulase, a hemicellulase, a starch degrading enzyme, a protease, a lipoxygenase.

[0260] A variant as defined herein further can be added alone or in a combination with other amylases, including other amylase variants, to prevent or retard staling, i.e., crumb firming of baked products. The amount of anti-staling amylase will typically be in the range of 0.01-10 mg of enzyme protein per kg of flour, e.g., 0.5 mg/kg ds. Additional anti-staling amylases that can be used in combination with a variant polypeptide as described herein include an endo-amylase, e.g., a bacterial endo-amylase from *Bacillus*. The additional amylase can be a maltogenic α -amylase (EC 3.2.1.133), e.g., from *Bacillus*. Novamyl® is an exemplary maltogenic α -amylase from *B. stearothermophilus* strain NCIB 11837 and is described in Christophersen et al., Starch 50: 39-45 (1997). Other examples of anti-staling endo-amylases include bacterial α -amylases derived from *Bacillus*, such as *B. licheniformis* or *B. amyloliquefaciens*. The anti-staling amylase may be an exo-amylase, such as β -amylase, e.g., from plant sources, such as soybean, or from microbial sources, such as *Bacillus*.

[0261] The baking composition comprising a variant polypeptide as described herein further can comprise a phospholipase. The phospholipase may have A₁ or A₂ activity to remove fatty acid from the phospholipids, forming a lyso-phospholipid. It may or may not have lipase activity, i.e., activity on triglyceride substrates. The phospholipase typically has a temperature optimum in the range of 30-90°C, e.g., 30-70°C. The added phospholipases can be of animal origin, for example, from pancreas, e.g., bovine or porcine pancreas, snake venom or bee venom. Alternatively, the phospholipase may be of microbial origin, e.g., from filamentous fungi, yeast or bacteria, such as the genus or species. Exemplary sources of phospholipases include *Aspergillus*, *A. niger*; *Dictyostelium*, *D. discoideum*; *Mucor*, *M. javanicus*, *M. mucedo*, *M. subtilissimus*; *Neurospora*, *N. crassa*; *Rhizomucor*, *R. pusillus*; *Rhizopus*, *R. arrizus*, *R. japonicus*, *R. stolonifer*; *Sclerotinia*, *S. libertiana*; *Trichophyton*, *T. rubrum*; *Whetzelinia*, *W. sclerotiorum*; *Bacillus*, *B. megaterium*, *B. subtilis*; *Citrobacter*, *C. freundii*; *Enterobacter*, *E. aerogenes*, *E. cloacae*; *Edwardsiella*, *E. tarda*; *Ectwinia*, *E. herbicola*; *Escherichia*, *E. coli*; *Klebsiella*, *K. pneumoniae*; *Proteus*, *P. vulgaris*; *Providencia*, *P. stuartii*; *Salmonella*, *S. typhimurium*; *Serratia*, *S. liquefaciens*, *S. marcescens*; *Shigella*, *S. flexneri*; *Streptomyces*, *S. violeceoruber*; *Yersinia*, *Y. enterocolitica*; *Fusarium*, and *F. oxysporum* (strain DSM 2672, for example).

[0262] The phospholipase is added in an amount that improves the softness of the bread during the initial period after baking, particularly the first 24 hours. The amount of phospholipase will typically be in the range of about 0.01-10 mg of enzyme protein per kg of flour, e.g., 0.1-5 mg/kg. Phospholipase activity generally will be in the range of about 20-1000 Lipase Unit (LU)/kg of flour, where a Lipase Unit is defined as the amount of enzyme required to release 1 μ mol butyric acid per minute at 30°C, pH 7.0, with gum arabic as emulsifier and tributyrin as substrate.

[0263] Compositions of dough generally comprise wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, cornstarch, rye meal, rye flour, oat flour, oatmeal, soy flour, sorghum meal, sorghum flour, potato meal, potato flour or potato starch. The dough may be fresh, frozen or par-baked. The dough can be a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways, such as by adding chemical leavening agents, e.g., sodium bicarbonate or by adding a leaven, i.e., fermenting dough. Dough also may be leavened by adding a suitable yeast culture, such as a culture of *Saccharomyces cerevisiae* (baker's yeast), e.g., a commercially

available strain of *S. cerevisiae*.

[0264] The dough may also comprise other conventional dough ingredients, e.g., proteins, such as milk powder, gluten, and soy; eggs (e.g., whole eggs, egg yolks or egg whites); an oxidant, such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or ammonium persulfate; an amino acid such as L-cysteine; a sugar; or a salt, such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate. The dough further may comprise fat, e.g., triglyceride, such as granulated fat or shortening. The dough further may comprise an emulsifier such as mono- or diglycerides, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxyethylene stearates, or lysolecithin. In particular, the dough can be made without addition of emulsifiers.

[0265] Optionally, an additional enzyme may be used together with the anti-staling amylase and the phospholipase. The additional enzyme may be a second amylase, such as an amyloglucosidase, a β -amylase, a cyclodextrin glucanotransferase, or the additional enzyme may be a peptidase, in particular an exopeptidase, a transglutaminase, a lipase, a cellulase, a hemicellulase, in particular a pentosanase, such as xylanase, a protease, a protein disulfide isomerase, e.g., a protein disulfide isomerase as disclosed in WO 95/00636, for example, a glycosyltransferase, a branching enzyme (1,4- α -glucan branching enzyme), a 4- α -glucanotransferase (dextrin glycosyltransferase) or an oxidoreductase, e.g., a peroxidase, a laccase, a glucose oxidase, a pyranose oxidase, a lipoxygenase, an L-amino acid oxidase or a carbohydrate oxidase. The additional enzyme(s) may be of any origin, including mammalian and plant, and particularly of microbial (bacterial, yeast or fungal) origin and may be obtained by techniques conventionally used in the art.

[0266] The xylanase is typically of microbial origin, e.g., derived from a bacterium or fungus, such as a strain of *Aspergillus*, in particular of *A. aculeatus*, *A. niger* (cf. WO 91/19782), *A. awamori* (e.g., WO 91/18977), or *A. tubigensis* (e.g., WO 92/01793); from a strain of *Trichoderma*, e.g., *T. reesei*, or from a strain of *Humicola*, e.g., *H. insolens* (e.g., WO 92/17573). Pentopan® and Novozym 384® are commercially available xylanase preparations produced from *Trichoderma reesei*. The amyloglucosidase may be an *A. niger* amyloglucosidase (such as AMG®). Other useful amylase products include Grindamyl® A 1000 or A 5000 (available from Danisco, Denmark) and Amylase® H or Amylase® P (available from Gist-Brocades, The Netherlands). The glucose oxidase may be a fungal glucose oxidase, in particular an *Aspergillus niger* glucose oxidase (such as Gluzyme®). An exemplary protease is Neutrase®. An exemplary lipase can be derived from strains of *Thermomyces* (*Humicola*), *Rhizomucor*, *Candida*, *Aspergillus*, *Rhizopus*, or *Pseudomonas*, in particular from *Thermomyces lanuginosus* (*Humicola lanuginosa*), *Rhizomucor miehei*, *Candida antarctica*, *Aspergillus niger*, *Rhizopus delemar* or *Rhizopus arrhizus*, or *Pseudomonas cepacia*. In specific embodiments, the lipase may be Lipase A or Lipase B derived from *Candida antarctica* as described in WO 88/02775, for example, or the lipase may be derived from *Rhizomucor miehei* as described in EP 238,023, for example, or *Humicola lanuginosa*, described in EP 305,216, for example, or *Pseudomonas cepacia* as described in EP 214,761 and WO 89/01032, for example.

[0267] The process may be used for any kind of baked product prepared from dough, either of a soft or a crisp character, either of a white, light or dark type. Examples are bread, particularly white, whole-meal or rye bread, typically in the form of loaves or rolls, such as, but not limited to, French baguette-type bread, pita bread, tortillas, cakes, pancakes, biscuits, cookies, pie crusts, crisp bread, steamed bread, pizza and the like.

[0268] In another embodiment, a variant polypeptide as described herein may be used in a pre-mix, comprising flour together with an anti-staling amylase, a phospholipase and a phospholipid. The pre-mix may contain other dough-improving and/or bread-improving additives, e.g., any of the additives, including enzymes, mentioned above. In one aspect, the variant polypeptide as described herein is a component of an enzyme preparation comprising an anti-staling amylase and a phospholipase, for use as a baking additive.

[0269] The enzyme preparation is optionally in the form of a granulate or agglomerated powder. The preparation can have a narrow particle size distribution with more than 95% (by weight) of the particles in the range from 25 to 500 μ m. Granulates and agglomerated powders may be prepared by conventional methods, e.g., by spraying the variant polypeptide as described herein onto a carrier in a fluid-bed granulator. The carrier may consist of particulate cores having a suitable particle size. The carrier may be soluble or insoluble, e.g., a salt (such as NaCl or sodium sulfate), a sugar (such as sucrose or lactose), a sugar alcohol (such as sorbitol), starch, rice, corn grits, or soy.

[0270] Another aspect contemplates the enveloping of particles comprising a variant polypeptide as described herein, i.e., α -amylase particles. To prepare the enveloped α -amylase particles, the enzyme is contacted with a food grade lipid in sufficient quantity to suspend all of the α -amylase particles. Food grade lipids, as used herein, may be any naturally organic compound that is insoluble in water but is soluble in non-polar organic solvents such as hydrocarbon or diethyl ether. Suitable food grade lipids include, but are not limited to, triglycerides either in the form of fats or oils that are either saturated or unsaturated. Examples of fatty acids and combinations thereof which make up the saturated triglycerides include, but are not limited to, butyric (derived from milk fat), palmitic (derived from animal and plant fat), and/or stearic (derived from animal and plant fat). Examples of fatty acids and combinations thereof which make up the unsaturated triglycerides include, but are not limited to, palmitoleic (derived from animal and plant fat), oleic (derived from animal and plant fat), linoleic (derived from plant oils), and/or linolenic (derived from linseed oil). Other suitable food grade lipids include, but are not limited to, monoglycerides and diglycerides derived from the triglycerides discussed above, phos-

pholipids and glycolipids.

[0271] The food grade lipid, particularly in the liquid form, is contacted with a powdered form of the α -amylase particles in such a fashion that the lipid material covers at least a portion of the surface of at least a majority, e.g., 100% of the α -amylase particles. Thus, each α -amylase particle is individually enveloped in a lipid. For example, all or substantially all of the α -amylase particles are provided with a thin, continuous, enveloping film of lipid. This can be accomplished by first pouring a quantity of lipid into a container, and then slurring the α -amylase particles so that the lipid thoroughly wets the surface of each α -amylase particle. After a short period of stirring, the enveloped α -amylase particles, carrying a substantial amount of the lipids on their surfaces, are recovered. The thickness of the coating so applied to the particles of α -amylase can be controlled by selection of the type of lipid used and by repeating the operation in order to build up a thicker film, when desired.

[0272] The storing, handling and incorporation of the loaded delivery vehicle can be accomplished by means of a packaged mix. The packaged mix can comprise the enveloped α -amylase. However, the packaged mix may further contain additional ingredients as required by the manufacturer or baker. After the enveloped α -amylase has been incorporated into the dough, the baker continues through the normal production process for that product.

[0273] The advantages of enveloping the α -amylase particles are two-fold. First, the food grade lipid protects the enzyme from thermal denaturation during the baking process for those enzymes that are heat labile. Consequently, while the α -amylase is stabilized and protected during the proving and baking stages, it is released from the protective coating in the final baked good product, where it hydrolyzes the glucosidic linkages in polyglucans. The loaded delivery vehicle also provides a sustained release of the active enzyme into the baked good. That is, following the baking process, active α -amylase is continually released from the protective coating at a rate that counteracts, and therefore reduces the rate of, staling mechanisms.

[0274] In general, the amount of lipid applied to the α -amylase particles can vary from a few percent of the total weight of the α -amylase to many times that weight, depending upon the nature of the lipid, the manner in which it is applied to the α -amylase particles, the composition of the dough mixture to be treated, and the severity of the dough-mixing operation involved.

[0275] The loaded delivery vehicle, i.e., the lipid-enveloped enzyme, is added to the ingredients used to prepare a baked good in an effective amount to extend the shelf-life of the baked good. The baker computes the amount of enveloped α -amylase, prepared as discussed above, that will be required to achieve the desired anti-staling effect. The amount of the enveloped α -amylase required is calculated based on the concentration of enzyme enveloped and on the proportion of α -amylase to flour specified. A wide range of concentrations has been found to be effective, although, as has been discussed, observable improvements in anti-staling do not correspond linearly with the α -amylase concentration, but above certain minimal levels, large increases in α -amylase concentration produce little additional improvement. The α -amylase concentration actually used in a particular bakery production could be much higher than the minimum necessary in order to provide the baker with some insurance against inadvertent under-measurement errors by the baker. The lower limit of enzyme concentration is determined by the minimum anti-staling effect the baker wishes to achieve.

[0276] A method of preparing a baked good may comprise: (a) preparing lipid-coated α -amylase particles, where substantially all of the α -amylase particles are coated; (b) mixing a dough containing flour; (c) adding the lipid-coated α -amylase to the dough before the mixing is complete and terminating the mixing before the lipid coating is removed from the α -amylase; (d) proofing the dough; and (e) baking the dough to provide the baked good, where the α -amylase is inactive during the mixing, proofing and baking stages and is active in the baked good.

[0277] The enveloped α -amylase can be added to the dough during the mix cycle, e.g., near the end of the mix cycle. The enveloped α -amylase is added at a point in the mixing stage that allows sufficient distribution of the enveloped α -amylase throughout the dough; however, the mixing stage is terminated before the protective coating becomes stripped from the α -amylase particle(s). Depending on the type and volume of dough, and mixer action and speed, anywhere from one to six minutes or more might be required to mix the enveloped α -amylase into the dough, but two to four minutes is average. Thus, several variables may determine the precise procedure. First, the quantity of enveloped α -amylase should have a total volume sufficient to allow the enveloped α -amylase to be spread throughout the dough mix. If the preparation of enveloped α -amylase is highly concentrated, additional oil may need to be added to the pre-mix before the enveloped α -amylase is added to the dough. Recipes and production processes may require specific modifications; however, good results generally can be achieved when 25% of the oil specified in a bread dough formula is held out of the dough and is used as a carrier for a concentrated enveloped α -amylase when added near the end of the mix cycle. In bread or other baked goods, particularly those having a low fat content, e.g., French-style breads, an enveloped α -amylase mixture of approximately 1% of the dry flour weight is sufficient to admix the enveloped α -amylase properly with the dough. The range of suitable percentages is wide and depends on the formula, finished product, and production methodology requirements of the individual baker. Second, the enveloped α -amylase suspension should be added to the mix with sufficient time for complete mixture into the dough, but not for such a time that excessive mechanical action strips the protective lipid coating from the enveloped α -amylase particles.

6. Textile desizing compositions and use

[0278] Also contemplated are compositions and methods of treating fabrics (e.g., to desize a textile) using a variant polypeptide as described herein. In one aspect, a method of desizing textiles, comprising contacting the variant polypeptide as described herein with a textile for a time sufficient to desize the textile, is provided.

[0279] Fabric-treating methods are well known in the art (see, e.g., U.S. Patent No. 6,077,316). For example, in one aspect, the feel and appearance of a fabric is improved by a method comprising contacting the fabric with a variant polypeptide as described herein in a solution. In one aspect, the fabric is treated with the solution under pressure.

[0280] In one aspect, a variant polypeptide as described herein is applied during or after the weaving of a textile, or during the desizing stage, or one or more additional fabric processing steps. During the weaving of textiles, the threads are exposed to considerable mechanical strain. Prior to weaving on mechanical looms, warp yarns are often coated with sizing starch or starch derivatives to increase their tensile strength and to prevent breaking. A variant polypeptide as described herein can be applied during or after the weaving to remove these sizing starch or starch derivatives. After weaving, a variant polypeptide as described herein can be used to remove the size coating before further processing the fabric to ensure a homogeneous and wash-proof result.

[0281] A variant polypeptide as described herein can be used alone or with other desizing chemical reagents and/or desizing enzymes to desize fabrics, including cotton-containing fabrics, as detergent additives, e.g., in aqueous compositions. A variant polypeptide as described herein also can be used in compositions and methods for producing a stonewashed look on indigodyed denim fabric and garments. For the manufacture of clothes, the fabric can be cut and sewn into clothes or garments, which are afterwards finished. In particular, for the manufacture of denim jeans, different enzymatic finishing methods have been developed. The finishing of denim garment normally is initiated with an enzymatic desizing step, during which garments are subjected to the action of amylolytic enzymes to provide softness to the fabric and make the cotton more accessible to the subsequent enzymatic finishing steps. A variant polypeptide as disclosed herein can be used in methods of finishing denim garments (e.g., a "bio-stoning process"), enzymatic desizing and providing softness to fabrics, and/or finishing process.

Examples

Example 1. Cloning of PS4 and G4-amylases

[0282] *Pseudomonas sacharophila* is grown overnight on LB media and chromosomal DNA is isolated by standard methods (Sambrook J, 1989). A 2190 bp fragment containing the PS4 open reading frame (Zhou *et al.*, 1989) is amplified from *P. sacharophila* chromosomal DNA by PCR using the primers P1 and P2 (see Table 3). The resulting fragment is used as a template in a nested PCR with primers P3 and P4, amplifying the openreading frame of PS4 without its signal sequence and introducing a NcoI site at the 5' end of the gene and a BamHI site at the 3'end. Together with the NcoI site a codon for a N-terminal Methionine is introduced, allowing for intracellular expression of PS4. The 1605 bp fragment is cloned into pCRBLUNT TOPO (Invitrogen) and the integrity of the construct analysed by sequencing. The *E. coli* *Bacillus* shuttle vector pDP66K (Penninga *et al.*, 1996) is modified to allow for expression of the PS4 under control of the P32 promoter and the *ctgase* signal sequence. The resulting plasmid, pCSmta is transformed into *B. subtilis*.

[0283] A second expression construct is made in which the starch binding domain of PS4 is removed. In a PCR with primers P3 and P6 (Table 3) on pCSmta, a truncated version of the mta gene is generated. The full length mta gene in pCSmta is exchanged with the truncated version which resulted in the plasmid pCSmta-SBD.

[0284] G4 amylases of *Pseudomonas* sp. AM1 (2006), *Pseudomonas* sp. 7193, *Pseudomonas mendocina* (strain ymp) and *Hahella chejuensis* (strain KCTC 2396) were cloned into a *Bacillus* expression vector with signal sequence by gene synthesis (GeneScript; NJ, USA) of the sequences encoding the mature proteins with an M added at the N-terminus as shown in SEQ ID NO 3, 5, 7 and 9.

Example 2. Site-directed mutagenesis

[0285] Site-directed mutagenesis was used to produce variant polypeptide as disclosed herein. Mutations were introduced into a nucleic acid encoding pMS382 having SEQ ID: 1, using the Quick Change™ method (Stratagene, California), according to instructions supplied with the kit with some modifications. Briefly, a single colony was picked and inoculated in 3 ml LB (22 g/l Lennox L Broth Base, Sigma) supplemented with 50 µg/ml kanamycin (Sigma) in a 10 ml Falcon tube. After overnight incubation at 37°C at 200 rpm, the culture was spun down at 5000 rpm for 5 min. The medium was removed and the double-stranded DNA template was prepared using QIAGEN columns (QIAGEN). Primers were designed according to the manufacturers' protocol. For example, TABLE 1 lists primers that were used to generate new variants based on the nucleotide sequence of pMS382 as shown in SEQ ID NO: 52.

[0286] Next, PCR was performed to synthesize the mutant strand. The PCR reaction mix contained the following:

2.5 µl 10 X QuickChange Multi reaction buffer
 0.75 µl QuickSolution
 X µl primers (10 pmol for primers of 28-35 nt; 7 pmol for primers of 24-27 nt; or 5 pmol for primers of 20-23 nt)
 1 µl dNTP mix
 X µl ds-DNA template (200 ng)
 1 µl QuickChange Multi enzyme blend (2.5 U/µl) (PfuTurbo DNA polymerase)
 X µl dH₂O (to a final volume of 25 µl)

[0287] The PCR reaction was performed in an Eppendorf thermal cycler for 35 cycles of denaturation (96°C for 1 min), primer annealing (62.8°C for 1 min), and elongation (65°C for 15 min), and then hold at 4°C. For each amplification reaction, 2 µl of *DpnI* restriction enzyme (10 U/µl) was added, and the mixture was incubated at 37°C for ~3 hr.

[0288] The *DpnI*-treated DNA was then used to transform XL10-Gold® Ultracompetent cells (Stratagene). XL10-Gold® cells were thawed on ice. For each mutagenesis reaction, 45 µl cells were added to a pre-chilled Falcon tube. Subsequently, 2 µl of beta-mercaptoethanol mix was added to each tube. The mixture was incubated on ice for 10 min with swirling every 2 min. Then, 1.5 µl *DpnI*-treated DNA was added to each aliquot of cells, and the mixture was incubated on ice for 30 min. The sample was subject to a heat-pulse of 30 sec at 42°C, and was placed on ice for another 2 min. 0.5 ml of preheated NZY⁺ broth was added to each sample, and incubation was carried at 37°C for 1 hr with shaking at 225-250 rpm. 200 µl of each transformation reaction were plated on LB plates (33.6 g/l Lennox L Agar, Sigma) supplemented with 1% starch and 50 µg/ml kanamycin. The plates were incubated overnight at 37°C. Individual colonies harboring the desired mutations were identified by DNA sequencing and subjected to plasmid preps to harvest plasmids with the desired mutations.

Example 3. Transformation into *Bacillus subtilis*.

[0289] *Bacillus subtilis* (strain DB104A; Smith et al., Gene 70, 351-361 (1988)) is transformed with the mutated plasmid DNA according to the following protocol.

A. Media for protoplasting and transformation

[0290]

2 x SMM	per litre: 342 g sucrose (1 M); 4.72 g sodium maleate (0.04 M); 8.12 g MgCl ₂ ·6H ₂ O (0.04 M); pH 6.5 with concentrated NaOH. Distribute in 50-ml portions and autoclave for 10 min.
4 x YT (1/2 NaCl)	2 g Yeast extract + 3.2 g Tryptone + 0.5 g NaCl per 100 ml.
SMMP	mix equal volumes of 2 x SMM and 4 x YT.
PEG	10 g polyethyleneglycol 6000 (BDH) or 8000 (Sigma) in 25 ml 1 x SMM (autoclave for 10 min.).

B. Media for plating/regeneration

[0291]

agar	4% Difco minimal agar. Autoclave for 15 min.
sodium succinate	270 g/l (1 M), pH 7.3 with HCl. Autoclave for 15 min.
phosphate buffer	3.5 g K ₂ HPO ₄ + 1.5 g KH ₂ PO ₄ per 100ml. Autoclave for 15 min
MgCl ₂	20.3 g MgCl ₂ ·6H ₂ O per 100 ml (1 M).
casamino acids	5% (w/v) solution. Autoclave for 15 min.
yeast extract	10 g per 100 ml, autoclave for 15 min.
glucose	20% (w/v) solution. Autoclave for 10 min.
DM3 regeneration medium : mix at 60°C (water bath; 500-ml bottle):	
	250 ml sodium succinate
	50 ml casamino acids
	25 ml yeast extract
	50 ml phosphate buffer
	15 ml glucose
	10 ml MgCl ₂

(continued)

100 ml molten agar

- 5 **[0292]** Add appropriate antibiotics: chloramphenicol and tetracycline, 5 µg/ml; erythromycin, 1 µg/ml. Selection on kanamycin is problematic in DM3 medium: concentrations of 250 µg/ml may be required.

C. Preparation of protoplasts

- 10 **[0293]** Use detergent-free plastic or glassware throughout.
[0294] Inoculate 10 ml of 2 x YT medium in a 100-ml flask from a single colony. Grow an overnight culture at 25-30°C in a shaker (200 rev/min).
[0295] Dilute the overnight culture 20 fold into 100 ml of fresh 2 x YT medium (250-ml flask) and grow until OD₆₀₀ = 0.4-0.5 (approx. 2 h) at 37°C in a shaker (200-250 rev/min).
 15 **[0296]** Harvest the cells by centrifugation (9000 g, 20 min, 4°C).
[0297] Remove the supernatant with pipette and resuspend the cells in 5 ml of SMMP + 5 mg lysozyme, sterile filtered.
[0298] Incubate at 37°C in a waterbath shaker (100 rpm).
[0299] After 30 min and thereafter at 15 min intervals, examine 25 µl samples by microscopy. Continue incubation until 99% of the cells are protoplasted (globular appearance). Harvest the protoplasts by centrifugation (4000 g, 20 min, RT) and pipet off the supernatant. Resuspend the pellet gently in 1-2 ml of SMMP.
 20 **[0300]** The protoplasts are now ready for use. Portions (e.g. 0.15 ml) can be frozen at -80°C for future use (glycerol addition is not required). Although this may result in some reduction of transformability, 106 transformants per µg of DNA can be obtained with frozen protoplasts).

25 D. Transformation

- [0301]** Transfer 450 µl of PEG to a microtube.
[0302] Mix 1-10 µl of DNA (0.2 µg) with 150 µl of protoplasts and add the mixture to the microtube with PEG. Mix immediately, but gently.
 30 **[0303]** Leave for 2 min at room temperature, and then add 1.5 ml of SMMP and mix.
[0304] Harvest protoplasts by microfuging (10 min, 13,000 rpm (10,000-12,000 g)) and pour off the supernatant. Remove the remaining droplets with a tissue.
[0305] Add 300 µl of SMMP (do not vortex) and incubate for 60-90 min at 37°C in a waterbath shaker (100 rpm) to allow for expression of antibiotic resistance markers. (The protoplasts become sufficiently resuspended through the shaking action of the waterbath.) Make appropriate dilutions in 1 x SSM and plate 0.1 ml on DM3 plates.
 35

Example 4. Fermentation of Variants in Shake Flasks.

- [0306]** The shake flask substrate is prepared by dissolving the following in water:

40	Yeast extract	2% (w/v)
	Soy Flour	2% (w/v)
	NaCl	0.5% (w/v)
	Dipotassium phosphate	0.5% (w/v)
45	Antifoam agent	0.05% (w/v)

- [0307]** The substrate is adjusted to pH 6.8 with 4 N sulfuric acid or sodium hydroxide before autoclaving. 100 ml of substrate is placed in a 500 ml flask with one baffle and autoclaved for 30 minutes. Subsequently, 6 ml of sterile dextrose syrup is added. The dextrose syrup is prepared by mixing one volume of 50% w/v dextrose with one volume of water followed by autoclaving for 20 minutes.
 50 **[0308]** The shake flasks are inoculated with the variants and incubated for 24 hours at 35°C and 180 rpm in an incubator. After incubation cells are separated from broth by centrifugation (10,000 g in 10 minutes) and finally, the supernatant is made cell free by microfiltration at 0.2 µm. The cell free supernatant is used for assays and application tests.
 55

Table 1. Primers used to generate variants based on the nucleotide sequence of pMS382 as shown in SEQ ID NO: 52.

Mutation	5' Oligo Sequence 3'	modification	Strand	Purpose
Q42K	GTATAACATCCTGAGACAAaaaGCGAGCACAATTGC CG	5' phosphate	+	MSDM
Q42N	GTATAACATCCTGAGACAAaacGCGAGCACAATTGC C	5' phosphate	+	MSDM
R88L	GCATGACTTTAACAAAAACGGCctgTATGGAAGCGA TGCTC	5' phosphate	+	MSDM
R88Y	GGCATGACTTTAACAAAAACGGCtatTATGGAAGCG ATGCTCAAC	5' phosphate	+	MSDM
L88K	GGCATGACTTTAACAAAAACGGCaaaTATGGAAGC GATGCTCAAC	5' phosphate	+	MSDM
S205L	GCCCCGGAAAGAGTTGATctgTGGATGAGCGATTCA GCG	5' phosphate	+	MSDM
T235R	GTGGGATTGGAGAAATaqaGCGAGCTGGCAGC	5' phosphate	+	MSDM
T235K	GTGGGATTGGAGAAATaaaGCGAGCTGGCAGC	5' phosphate	+	MSDM
T235H	GCCGTGGGATTGGAGAAATcatGCGAGCTGGCAGC AG	5' phosphate	+	MSDM
T235Q	CCGTGGGATTGGAGAAATcagGCGAGCTGGCAGCA G	5' phosphate	+	MSDM
Q240E	CGCTCCAATCTTTGATGATTCCTGCCAGCTCGCTG	5' phosphate	-	MSDM
Q240D	CAGCGAGCTGGCAGgatATCATCAAAGATTGGAGC G	5' phosphate	+	MSDM
Q311P	CAACATAAATGGCCGCTTccgGATGGCCTTATCAGA CAG	5' phosphate	+	MSDM
A392D	GCCCTGAATAGCGATCTGgatAATCCGGGACAAGTT GC	5' phosphate	+	MSDM
A392Y	CGCCCTGAATAGCGATCTGtatAATCCGGGACAAGT TGCTAG	5' phosphate	+	MSDM
S409E	GCGAAGCAGTCAATGCCgaaAATGGCCAAGTCAGA GTCTG	5' phosphate	+	MSDM
Mutation	5' Oligo Sequence 3'	modification	Strand	Purpose
R248H	CATCAAAGATTGGAGCGATcatGCAAAATGCCCG GTCTTTGAC	5' phosphate	+	MSDM
S266T	CGCATGCAAAATGGAacgGTCGCCGATTGGAAAC ATG	5' phosphate	+	MSDM
S377D	CTACAGTTgatGGCAGCCAACAAAC	5' phosphate	+	MSDM

Example 5. Amylase Assays

Betamyl assay

[0309] One Betamyl unit is defined as activity degrading 0,0351 mmole per 1 min. of PNP-coupled maltopentaose so

that 0,0351 mmole PNP per 1 min. can be released by excess alpha-glucosidase in the assay mix. The assay mix contains 50 ul 50 mM Na-citrate, 5 mM CaCl₂, pH 6,5 with 25 ul enzyme sample and 25 ul Betamyl substrate (Glc5-PNP and alpha-glucosidase) from Megazyme, Ireland (1 vial dissolved in 10 ml water). The assay mix is incubated for 30 min. at 40°C and then stopped by adding 150 ul 4% Tris. Absorbance at 420 nm is measured using an ELISA-reader and the Betamyl activity is calculate based on Activity = A420 * d in Betamyl units/ml of enzyme sample assayed. For dosing in baking trials 1 BMK = 1000 Betamyl units are used.

Endo-amylase assay

[0310] The endo-amylase assay is identical to the Phadebas assay run according to manufacturer (Pharmacia & Upjohn Diagnostics AB).

Exo-specificity

[0311] The ratio of exo-amylase activity to Phadebas activity was used to evaluate exo-specificity.

Example 6. Procedure heat stability determinations based on residual activity

[0312] The enzyme samples in appropriate dilutions are incubated in 50 mM Na-acetate buffer, pH 5.0 with 0,5 M NaCl at room temperature (control) or for 4 minutes at 80°C (heated sample), and immediately after that the control and heated sample are analysed using the Betamyl assay. The residual activity is calculated as the Betamyl activity of the heated sample divided by the Betamyl activity of the control sample.

Example 7. Stabilising effects of mutations

[0313] Using the procedure described in Example 6 the stabilising effects of the mutations Q42K, R88L, S205L, E223S, Q311P, S409E and T235K are shown in Table 2 as an increase in residual activity after heating. Likewise the stabilising effect of A392D is shown in Table 3, of S205L, S223A and S205L combined with S409 in Table 4, of N34Q and G100Q in Table 5 and of Q240E in Table 6. In tables 2-6, the variants have the same sequence as the one mentioned in the first line except for the further mutation(s) made as described in the second column.

Table 2. Stabilising effect of Q42K, R88L, S205L, E223S, Q311P, S409E and T235K combined with Q311P

Variant	Further mutation in pMS382	Residual activity (%)
pMS382 (SEQ ID NO: 1)		8,4
pMS465 (SEQ ID NO: 18)	E223S	9,6
pMS1042 (SEQ ID NO: 19)	Q311P	13,1
pMS1104 (SEQ ID NO: 20)	Q42K	11,2
pMS1153 (SEQ ID NO: 21)	R88L	19,5
pMS1286 (SEQ ID NO: 22)	T235R	12,3
pMS1284 (SEQ ID NO: 23)	T235R, Q311P	17,2
pMS1290 (SEQ ID NO: 24)	T235K	14,1
pMS1484 (SEQ ID NO: 25)	S205L	8,8
pMS1579 (SEQ ID NO: 26)	S409E	16,4

Table 3. Stabilising effect of A392D

Variant	Further mutation in pMS1105	Residual activity (%)
pMS1105 (SEQ ID NO: 27)		11,0
pMS1723 (SEQ ID NO: 28)	A392D	12,3

Table 4. Stabilising effect of S205L, S223A and S205L combined with S409E

Variant	Further mutation in pMS1776	Residual activity (%)
pMS1776 (SEQ ID NO: 12)		18,9
pMS2104 (SEQ ID NO: 29)	S223A	22,1
pMS2138 (SEQ ID NO: 30)	S205L	22,4
pMS2022 (SEQ ID NO: 15)	S205L, S409E	23,3

Table 5. Stabilising effect of N34Q and G100Q

Variant	Further mutation in pMS2124	Residual activity (%)
pMS2124 (SEQ ID NO: 31)		24,7
pMS2177 (SEQ ID NO: 32)	N34Q.	28,8
pMS2178 (SEQ ID NO: 33)	G100Q	26,4

Table 6. Stabilising effect of Q240E

Variant	Further mutation in pMS2022	Residual activity (%)
pMS2022 (SEQ ID NO: 15)		23,3
pMS2118 (SEQ ID NO: 34)	Q240E	26,8

Example 10. Recipe for Baking Trials

[0314] Baking trials were carried out with a standard white bread sponge and dough recipe for US toast. The sponge dough is prepared from 1500 g of flour "Gold Medal" from General Mills, USA, 890 g of water, 40 g of soy bean oil, 7,5 g GRINDSTED™ SSL P55 Veg, 10 g emulsifier DIMODAN™ PH300, 26 g dry yeast and ascorbic acid (20ppm final concentration). The sponge is mixed for 1 min. at low speed and subsequently 3 min. at speed 2 on a Hobart spiral mixer. The sponge is subsequently fermented for 3 hours at 25°C, 85% RH.

[0315] Thereafter, 500 g of "Gold Medal" flour, 14 g dry yeast, 5 g of calcium propionate, 240 g High Fructose Corn Syrup (42%), 5 g of calcium propionate, 250 g of water and ascorbic acid (30 ppm final concentration) and 40 g of salt are added to the sponge. The resulting dough is mixed for 0.5 min. at low speed and then 10.5 min. on high speed on a Hobart spiral mixer.

[0316] The dough is rested for 5 min. at ambient temperature, and then 794 g dough pieces are scaled, moulded on a cross grain moulder and transferred to pans. After 60 min. proofing at 43°C at 80% RH the doughs are baked for 21 min. at 200°C in a Reed Rack Oven.

Example 11. Protocol for Evaluation of Firmness, Resilience and Cohesiveness*Texture Profile Analysis of Bread*

[0317] Firmness, resilience and cohesiveness are determined by analysing bread slices by Texture Profile Analysis using a Texture Analyser from Stable Micro Systems, UK. Calculation of firmness, resilience, and cohesiveness is done according to preset standard supplied by Stable Micro System, UK. The probe used is aluminium 50 mm round.

[0318] Firmness is determined at 40% compression during the first compression. The figure is the force needed to compress the slice to 40% of the total thickness. The lower the value, the softer the bread. The firmness is expressed for example in grams.

Example 12. Evaluation of antistaling effects of G4 amylase variant polypeptides

[0319] Bread was baked as described in Example 10 with pMS1776, pMS1934, pMS2020, pMS2022 and pMS2062 in dosages varying from 7.5 to 20 BMK/kg corresponding to 7,500 to 20,000 Betamyl units/kg.

[0320] The firmness of the bread is tested according to the protocol set out in Example 11 at various times after baking. As controls, bread baked with standard dosages of 600 ppm Novamyl 1500 and/or 416.66 ppm of a composition comprising SEQ ID NO: 1 was also baked.

[0321] Fig. 1 and 2 show the firmness results. Relative to the controls with standard dosages 600 ppm Novamyl 1500 and/or 416.66 ppm of a composition comprising SEQ ID NO: 1 a much stronger reduction in the firming rate from day 3 to day 10 after baking is obtained with pMS1776, pMS1934, pMS2020, pMS2022 and pMS2062. Also these breads were strongly improved in resilience and cohesiveness relative to the controls.

[0322] This indicates that the variant polypeptide(s) as described herein have highly improved antistaling effects compared to Novamyl 1500 and a composition comprising SEQ ID NO: 1.

Example 13. Protocol for Evaluation of Crumbliness (Resistance to Crumbling)

[0323] Two slices of bread are placed on a piece of paper. Each slice is divided into 4 squares by vertical and subsequent horizontal tears of the slice.

[0324] Tearing is done by pulling the crumb apart by the fingers. First the slice is torn from the middle of the top bread surface to the middle of the bottom bread surface. Thereafter, each half of the original slice is torn from the crust side to the inside of the slice. The small crumb pieces, which are separated from the 4 squares, are removed by shaking each piece after a tear at least 3 times by moving the hand up and down.

[0325] The weight of the separated small crumb pieces is determined as a measure of crumbliness. This assay may be referred to as the "Crumbliness Evaluation Protocol".

Example 14. Protocol for Evaluation of Foldability

[0326] The toast bread is sliced using an automatic bread slicer with set slice thickness of 15 mm. The slice is folded by hand from the top of the slice towards the bottom, so that the direction of the crease is from side to side.

[0327] The foldability is visually assessed using the following scoring system:

Score	Feature
1	Unfoldable, slice breaks upon folding
2	Foldable, whole slice breaks within 5 seconds after folding
3	Foldable, part of the slice breaks within 5 seconds after folding. Other parts break later.
4	Foldable, part of the slice breaks later than 5 seconds after folding. Other parts do not break.
5	Foldable, no part of the slice break after folding

[0328] This assay may be referred to as the "Foldability Evaluation Protocol".

Example 15. Preparation of DP4 syrup

Materials:

[0329] SPEZYME FRED® bacterial alpha amylase (Danisco US Inc., Genencor Division) treated granular corn starch liquefact (34%ds, 9.1DE).

[0330] Pullulanase (OPTIMAX L-1000, Danisco US Inc., Genencor Division)

Experimental:

[0331] Starch liquefact was pH adjusted to 5.2 with NaOH and then each of 100 g liquefact was incubated at 60 C for DP4 saccharification by dosing enzymes at 0.03 BMK/gds. Pullulanase when used was dosed at 0.5 ASPU/gds. Reaction was carried out up to 40 hours, stopped for periodical sampling to check peak DP4. When peak DP4 was observed, the reaction was stopped by heating in boiling water.

Results:

[0332]

Table 1.

		Sugar production (%)										
Enzymes	Hr	% DP1	% DP2	% DP3	% DP4	% DP5	% DP6	% DP7	% DP8	% DP9	% DP10	% DP11+
pMS2062	16	2.31	5.50	8.67	38.00	0.81	0.17	0.67	0.99	3.23	0.39	39.30
pMS2062+PU		2.42	5.70	8.99	38.12	1.06	0.19	0.85	1.29	4.10	1.06	36.20
pMS2062	19.5	2.49	5.73	9.03	39.86	0.63	0.00	0.51	0.83	2.62	0.38	37.90
pMS2062+PU		2.60	5.89	9.45	40.37	0.88	0.00	0.70	1.18	3.65	1.19	34.10
pMS2062	22	2.59	5.69	9.20	41.16	0.56	0.00	0.45	0.75	2.27	0.37	37.00
pMS2062+PU		2.81	5.95	9.91	41.96	0.78	0.00	0.58	1.02	3.30	1.26	32.40
pMS2062	40	3.41	6.32	10.47	43.76	0.24	0.12	0.16	0.87	0.30	0.20	34.10
pMS2062+PU		3.67	7.01	11.42	45.80	0.43	0.26	0.42	2.22	1.87	0.17	26.70

[0333] As shown in Table 1, the results indicated that PMS2062 produced DP4 as a major sugar product. As also indicated, pullulanase (PU) addition (PMS2062 + PU) resulted in faster reduction in higher sugars (see e.g., DP11+), while DP4 level was minimally affected.

5 SEQUENCES

[0334]

10 SEQ ID NO: 1. Mature protein sequence of pMS382
 DQAGKSPAGVRYHGGDEII LQGFHWNVVREAPYNWYNILRQQASTIAADGFS AIWMPVPWRDFSSWTDGDKSGGGEGYFWHDF
 NKNGRYGSDAQLRQAAGALGGAGVKVLYDVVPNHMNRFPDKEINLPAGQRFWRND CPDPGNPNDCDDGDRFLGGEADLNTG
 HPQIYGMFRDEF TNLRSYGAGGFRDFVVRGYAPERVD SWMSDSADSSFCV GELWKEPSEYPPWDWRNTASWQQIIKDWSRA
 KCPVFDFALKERMQNGSVADWKHGLNGNPDPRWREVAVTFVDNHD TGYSPGQNGGQHKWPLQDGLIRQAYAYILTSPGTPVVY
 WPHMYDWGYGDFIRQLIQVRRTAGVRADSAISFHSYGSGLVATVSGSQQTLVVALNSDLANPGQVASGSFSEAVNASNGQVRV
 15 WRSGSGDGGGNDGG

SEQ ID NO: 2. Mature protein of G4 amylase of *Pseudomonas* sp. AM1 (2006) with M added at the N-terminus (Q1EJP2 protein):

20 1 MDLAGKSPGG VRYHGGDEII LQGFHWNVIR ESPNNWYNTL RDMAPTIAAD
 51 GFS AIWMPVP WRDFSSWSDG ANSGGGEGYF WHDFNKNGRY GSDTQLKQAA
 101 GALNNAQVKV LYDVVPNHMN RGYDPKQINL PAGQGFWRND CADPGNYPND
 151 CDDGDRFMGG DADLNTANPQ VYGMFRDEFA NLRSNYGAGG FRFDFVRGYA
 201 GERVDSWMGA AHDNAFCVGE LWKAPAEYPS WDWRNTASWQ QVIKDWSRA
 251 KCPVFDFALK ERMQNGSIAD WKNGLNGNPD PRWREVAVTF VDNHDAGYSP
 301 GQNGGQHHWA LQDGLIRQAY AYILTSPGTP VVYWSHMYDW GYGDFIRQLI
 25 351 QVRRTAGVRA DSAISFHSYG SGLVATVSGS QQTLVVALNS DLANPGQVAS
 401 GSFSEAVNAS NGQVRVWRS GSGDGGGNDGG EGGLVNVNFR CDNGVTQMGD
 451 SVYAVGNVSQ LGNWSPASAV RLTDTSYPT WKGSIALPDG QNVEWKCLIR
 501 NEADATLVRQ WQSGGNNQVQ AAAGASTSGS F*

30 SEQ ID NO: 3. DNA sequence of nucleotide encoding G4 amylase of *Pseudomonas* sp. AM1 (2006) (Q1EJP2) with M added at the N-terminus (Q1EJP2 DNA):
 atggatctggcaggcaaatcaccggcgcgcttagatatcatggcgcgatgaaattattctgcaaggctttcattggaatgt
 tattagagaatcaccgaataatttggtataatacactgagagatatggcaccgacaattgcagcagatggcttttcagcaattt
 ggatgccggttccgtggagagatttttcatcatggtcagatggcgcaaatcaggcgcgcggaaggctatttttggcatgat
 35 ttttaataaaaaatggcagatatggctcagatacacaactgaaacaagcagcaggcgactgaataatgcacaagttaaagtctt
 gtatgatgttgttccgaatcatatgaatagaggctatccggataaacaattaatctgccggcaggccaaggcttttggagaa
 atgattgcgagatccgggcaattatccgaatgattgcatgatggcgatagatttatggcgcgcgatgctgatctgaataca
 gcaaatccgcaagtttatggcatgttagagatgaatttgcaaatctgagatcaaattatggcgaggcggttagatttga
 40 ttttgtagaggctatgcaggcgaaagagttgattcatggatggcgcgagcagcatgataatgcattttgcgttggcgaaactgt
 ggaaagcaccggcagaatccgtcatgggattggagaaatacagcatcatggcaacaagttatataagattggtcagataga
 gcaaaatgcccggtttttgattttgactgaaagaaagaatgcaaaatggctcaattgcagattggaaaaatggcctgaatgg
 caatccggatccgagatggagagaagttgcagttacatttgttgataatcatgatgcaggctattcaccgggccaatggcg
 gccacatcattgggcactgcaagatggcctgattagacaagcatatgcataatctctgacatcacccgggcacaccggttgtt
 tattggtcacatatgtatgattggggctatggcgattttattagacaactgattcaagttagaagaacagcaggcggttagagc
 45 agattcagcaatttcatttcattcaggctattcaggcctggttgcaacagtttcaggctcacaacaacactggttgtgcac
 tgaatagcgatctggcaaatccgggccaagttgcatcaggctcattttcagaagcagttaatgcatcaaatggccaagttaga
 gtttggagatcaggctcaggcgatggcgcggaatgatggcgcggaaggcggtggttaattgttaatttttagatgcgataa
 tggcggttacacaatggcgatcagtttatgcagttggcaatgtttcacaactgggcaattggtcaccggcatcagcagttta
 gactgacagatcatcatatccgacatggaaggctcaattgcactgccgatggccaaatgttgatggaatgcctg
 attagaaatgaagcagatgcaacactggttagacaatggcaatcaggcggaataatcaagttcaagcagcagcaggcgcatc
 aacatcaggctcatttttaa

50

55

SEQ ID NO: 4. Mature protein of G4 amylase of *Pseudomonas* sp. 7193 with M added at the N-terminus (A5CVD5 protein):

```

1      MDLAGKSPGG VRYHGGDEII LQGFHWNVIR ESPNNWYNTL RDMAPTIAAD
51     GFSAIWMPVP WRDSSWSWDG ANSGGGEGYF WHDFNKNRGY GSDTQLKQAA
101    GALNNAQVKV LYDAVPHNMN RGYDPKQINL PAGQGFWRND CADPGNYPND
151    CDDGDRFMGG DADLNTANPQ VYGMFRDEFA NLRSNYGAGG FRFDFVRGYA
201    GERVDSWMGD GACQRLLRGR ALEGTGRIPE LGLAQYQQLA AVIKDWSDBA
251    KVPGCSNFAL KGAHAERLPS PTGRTASTAT PMPRWREVAV TFVDNHDITGY
301    SPGQNGGQHH WALRDDLRQ AYAYILASPG TPVVYWSHMY DWGHGPLIRQ
351    LIQIRRAAGV RADSAIEFHS GYSGLVATVR GTAQTLVMAL GSNLSSPAEV
401    SSGSFSQALN QDSGQLRIWT TGSTGGDEGD GGGDGTMTSV NFRCDNGITQ
451    PGDSVYAVGS LAQLGSWSPA NAVRLTDVSN YPTWKGAISL PAGQAVEWKC
501    IVRSEADPTQ VRQWQAGDNN RVTAGAGATT IGRL*
```

SEQ ID NO: 5. DNA sequence of nucleotide encoding [G4 amylase of *Pseudomonas* sp. 7193 (A5CVD5) with M added at the N-terminus (A5CVD5 DNA):

```

atggatctggcaggcaaatcaccggcgccgcttagatatcatggcgcgatgaaattattctgcaaggctttcattggaatgt
tattagagaatcaccgaataattggtataatacactgagagatatggcaccgacaattgcagcagatggcttttcagcaattt
ggatgccgggttcggtggagagatttttcatcatggtcagatggcgcaaatcaggcgccggcgcaaggctatttttggcatgat
tttaataaaaaatggcagatatggctcagatacacaactgaaacaagcagcaggcgactgaataatgcacaagttaaagttct
gtatgatgcagttccgaatcatatgaatagaggtatccggataaacaattaatctgccggcaggccaaggcttttggagaa
atgattgcccagatccgggcaattatccgaatgattgcatgatggcgatagatttatggcgccgcatgctgatctgaataga
gcaaatccgcaagtttatggcatgttttagagatgaatttgcaaatctgagatcaaattatggcgaggcggttttagatttga
ttttgttagaggctatgcaggcgaaagagttgatcatggatggcgcatggcgcatgcaaagactgctgagaggcagagcac
tgaaggcacaggcagaattccggaactggcctggcacaatatggccaactggcagcagttatataagattggtcagataga
gcaaaagttccgggctgctcaaattttgcactgaaaggcgccatgcagaaagactgccgtcaccgacaggcagaacagcatc
aacagcaacaccgatgccgagatggagagaagttgcagttacatttgttgataatcatgatacaggctattcaccggggccaaa
atggcgcccaacatcattgggactgagagatgatctggttagacaagcatatgcatatattctggcatcaccgggacacccg
gttggtttattggtcacatattgattggggtcatggaccgctgattagacaactgattcaaatagaagagcagcaggcgt
tagagcagattcagcaattgaatttcattcaggctattcaggcctggttgcaacagtttagaggcacagcacaaactggtta
tggcactgggctcaaattctgcatcaccggcagaagtttcatcaggctcattttcacaagcactgaatcaagattcaggccaa
ctgagaatttgacaacaggctcaacaggcgccgatgaaggcgatggcgccggcgatggcacaatggtttcagttaatatttag
atgcgataatggcattacacaacccggcgattcagttatgcagttggctcactggcacaactgggctcatggtcaccggcaa
atgcagtttagactgacagatgtttcaaattatccgacatgaaaggcgcaatttactgccggcaggccaagcagttgaatgg
aatgcattggttagatcagaagcagatccgacacaagtttagacaatggcaagcaggcgataataatagagttacagcaggcgc
aggcgcaacaacaattggcagactgtaa
```

SEQ ID NO: 6. Mature protein of G4 amylase of *Pseudomonas mendocina* (strain ymp) with M added at the N-terminus (A4XX23 protein):

```

1      MDAPGKTASG VRYHGGDEII LQGFHWNTVR TSSNWYATLA SMAPTIAADG
51     FSAIWMPVPW RDFSSWSDPG NGTSGGGEGY FWHDFNKNRG YGSDSLLRQA
101    ASALNAAGVK PIYDVVPNHM NRGYPDKIN LPAGQGLWRH DCNDPGNYAN
151    DCDDGDRFMG GDADLNTGHP QNYAMFRDEF ARLRSQYAGG GFRFDFVRGY
201    AGERVASWMS DAHDNGFCLG ELWKAPGEYP SWDWRNGASW QQILKDWSDR
251    AKCTVFDFAL KERMQNGGIA DWRHGLNGNP DARWREVAVT FVDNHDITGYS
301    PGPHGGQHHW PLPDARLKQA YAYILSSPGT PVVYWPHMYD WGHGDFIRQL
351    IQIRRAAGVK AASAIQFHTG FSGLVATISG SQQQLLIALD SNLSSPGQVA
401    SGDFTQALNT DNGAIRIWSR GQGGGDGQGN LVSVNFRCDN GVTQWGDVSY
451    ALGNVTQLGN WSPAGAVRLT DTSAYPTWKG SIALPAGQQV QWKCIVRSES
501    NPTQVKTWQP GGNNSVTVAS GASTAGSF*
```

SEQ ID NO: 7. DNA sequence of nucleotide encoding G4 amylase of *Pseudomonas mendocina* (strain ymp) (A4XX23) with M added at the N-terminus: (A4XX23 DNA):

```

atggatgcaccgggcaaaacagcatcaggcgttagatatcatggcggcgatgaaattattctgcaaggctttcattggaatac
agttagaacatcatcaaattggtatgcaacactggcatcaatggcaccgacactggcagcagatggcttttcagcaatttggg
5  tgccgggttcggtggagagatttttcatcatggtcagatccgggcaatggcacatcaggcggcgcggaaggctattttggcat
gattttaataaaaaatggcagatatggctcagattcactgctgagacaagcagcatcagcactgaatgcagcaggcggttaaacc
gatttatgatgtgttccgaatcatatgaatagaggctatccggataaagaaataatctgccggcaggccaaggcctgtgga
gacatgattgcaatgatccgggcaattatgcaaatgattgcatgagatgagatgagatgagatgagatgagatgagatgagat
10 acaggccatccgcaaaattatgcaatgtttagagatgaatttgcaagactgagatcaccaatggcgcgaggcggtttagatt
tgattttgttagaggctatgcaggcgaaagagttgcatcatggatgtcagatgcacatgataatggcttttgctggcggaac
tgtggaaagcaccgggcaaatatccgtcatgggattggagaaatggcgcatcatggcaacaaattctgaaagattgggtcagat
agagcaaaatgcacagtttttgattttgactgaaagaaagaatgcaaaatggcgcgattgagatggagacatggcctgaa
tggaatccggatgcaagatggagagaagttgcagttacatttgttgataatcatgatacaggctattcaccgggcccgcatg
15 gcggccaacatcattggcgcgtgccggatgcaagactgaacaagcatatgcatatattctgtcatcaccgggcacaccgggt
gtttattggcgcatatgtatgattgggggtcatggagatttttagacaactgattcaaattagaagagcagcaggcggttaa
agcagcatcagcaattcaatttcatacaggctttcaggcctggttgcaacaatttcaggctcacacaacaactgctgattg
cactggattcaaatctgtcatcaccgggccaaagtgcacatcaggcgattttacacaagcactgaatacagataatggcgcaatt
agaatttgagatcaggccaaggcgcgcgatggccaaggcaatctggtttcagttaattttagatgagataatggcggttac
acaatggggcgattcagtttatgcactgggcaatgttacacaactgggcaattggtcaccggcgaggcgagttagactgacag
20 atacatcagcatatccgacatggaaaggctcaattgcactgccggcaggccaacaagttcaatggaaatgcattgttagatca
gaatcaaatccgacacaagttaaaacatggcaaccgggcggcaataattcagttacagttgcatcaggcgcatcaacagcagg
ctcatttttaa

```

SEQ ID NO: 8. Mature protein of G4 amylase of *Hahella chejuensis* (strain KCTC 2396) with M added at the N-terminus (Q2SEA8 protein):

```

1  MESSGKSGAG VRFHGGDEII LQGFHWNVVR TAERNWYNIL QSKAQQISED
25  51  GFTAIWMPVP WRDNSSWQAS SDTRFGGEGY FWADMDKNSR YGDDGQLKQA
101 ASALKNKGVK VIYDIVPNHH DRGHSNDSL NPSGQGYYS DCSSCDDGDP

```

```

151  FMDGGSDFST AHPDVYDLFK NELVNLKTN YSAGGFRFDFV RGYAPERISA
30  201  WMSASLDSGY CVGELWKGPS EYPSWDWRHS ASWQEILKDF TDASDCSVFD
251  FALKERMQNG SISDWRYGLN GNPSAQWREV AVTFVDNHD TGYSPGPLGGQ
301  HHWALPDWKR KMAYAYILSS PGTPVYVPH MYDWGMRDFI RNLIQLRKSA
351  GVKAYSGVQF HDGFSGLVGT TSGSNGKLLF AIDS NFSSPN QVAGGAWNLA
401  VNEDNGRIRI WRQ*

```

SEQ ID NO: 9. DNA sequence of nucleotide encoding of G4 amylase of *Hahella chejuensis* (strain KCTC 2396) with M added at the N-terminus (Q2SEA8 DNA):

```

atggaatcatcaggcaaatcaggcgaggcggttagatttcatggcggcgatgaaattattctgcaaggctttcattggaacgt
tgtagaacagcagaaagaaactggtacaacatcctgcaatcaaaagcacaacaaatttcagaagatggctttacagcaattt
ggatgccggttcggtggagagataattcatcatggcaagcatcatcagatacaagatttgccggcggaaggctattttgggca
40 gatatggataaaaattcaagatatggcgatgatggccaactgaacaagcagcatcagcactgaaaaataaaggcggttaaagt
tatttatgatattgttccgaatcatcatgatagaggccattcaaatgattcactgaatctgccgtcaggccaaggctattata
gatcagattgctcatcatgcgatgatggcgatccggtttatggatggcggtcagatttttcaacagcagcatccggtgtttac
gatctgttttaaaacgaactggttaacctgaaaacaaactactcagcaggcggttttagattttgattttgttagaggctatgc
accgaaagaatttcagcatggatgtcagcatcactggattcaggctattgcgttggcgaactgtggaaaggcccgtcagaat
45 atccgtcatgggattggagacattcagcatcatggcaagaaattctgaaagattttacagatgcatcagattgctcagttttt
gattttgactgaaagaaagaatgcaaaatggctcaatttcagattggagatggcctgaatggcaatccgtcagcacaatg
gagagaagttgcagttacatttgttgataatcatgatacaggctattcaccgggcccgctggcgcgccaacatcattgggcac
tgccgatttggaaaagaaaaatggcatatgcatatattctgtcatcaccgggcacaccggttgtttattggccgcatatgtat
50 gattggggcatgagagattttattagaaatctgattcaactgagaaaatcagcaggcggttaaagcatattcaggcggttcaatt
tcatgatggctttcaggcctggttggcacaacatcaggctcaaatggcaaaactgctgtttgcaattgattcaaattttcat
caccgaatcaagttgcaggcgcgcatggaatctggcagttaatgaagataatggcagaattagaatttggagacaataa

```

SEQ ID NO: 10. >gi|77787|pir||S05667 glucan 1, 4-alpha-maltotetraohydrolase (EC 3.2.1.60) mature protein without the signal sequence - *Pseudomonas saccharophila* DQAGKSPAGVRYHGGDEIILQGFHWNVREAPNDWYNILRQQASTIAADGFSAIWMPVPWRDFSSWTDGGKSGGGEGYFWHDF NKNGRYGSDAQLRQAAGALGGAGVKVLYDVVPNHMNRGYPDKIELNPAGQGFWRNDCADPGNYPNDCDDGDRFIGGESDLNTG HPQIYGMFRDELANLRSGYGAGGFRDFVRGYAPERVDSWMSDSADSSFCVGELWKGPSSEYPWDWRNTASWQQI IKDWS DRA KCPVDFDALKERMQNGSVADWKHGLNGNPDPRWREVAVTFVDNHD TGYSPGQNGGQHHWALQDGLIRQAYAYILTS PGTPV VY WSHMYDWGYGDFIRQLIQVRRTAGVRADS AISFSHSGYSLVATVSGSQTLVVALNSDLANPGQVASGSFSEAVNASNGQVRV WRS GSGDGGGNDGGEGGLVNVNFRCDNGVTQMGSVYAVGNVSQ LGNWSPASAVRLTDTSSYPTWKGSIALPDGQNV EWKCLI RNEADATLVRQWQSGGNNQVQAAGASTSGSF

SEQ ID NO: 11. Signal peptide of glucan 1, 4- α -maltotetraohydrolase (EC 3.2.1.60) - *Pseudomonas saccharophila*
MSHILRAAVLAAVLLPFPALA

SEQ ID NO: 12. Mature protein sequence of pMS1776

```

5      1   DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
      51   FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
     101   ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
     151   DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGGGF RFDFVRGYAP
     201   ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNRASWQQ IIKDWSDRAK
     251   CPVFDFALKE RMQNGSVADW KHGLNGNPD P RWREVAITFV DNHDTGYSPG
10    301   QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
     351   VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
     401   SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

```

SEQ ID NO: 13. Mature protein sequence of pMS1934

```

15      1   DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
      51   FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
     101   ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
     151   DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGGGF RFDFVRGYAP
     201   ERVDSWMSDS ADSSFCVGEL WKKPSEYPPW DWRNRASWQQ IIKDWSDRAK
     251   CPVFDFALKE RMQNGSVADW KDGLNGNPD P RWREVAITFV DNHDTGYSPG
20    301   QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
     351   VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
     401   SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

```

SEQ ID NO: 14 Mature protein sequence of pMS2020

```

25      1   DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
      51   FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
     101   ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
     151   DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGGGF RFDFVRGYAP
     201   ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNRASWQQ IIKDWSDRAK
     251   CPVFDFALKE RMQNGSVADW KHGLNGNPD P RWREVAITFV DNHDTGYSPG
30    301   QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
     351   VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
     401   SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*
35

```

SEQ ID NO: 15 Mature protein sequence of pMS2022

```

40      1   DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
      51   FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
     101   ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
     151   DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGGGF RFDFVRGYAP
     201   ERVDLWMSDS ADSSFCVGEL WKSPSEYPPW DWRNRASWQQ IIKDWSDRAK
     251   CPVFDFALKE RMQNGSVADW KHGLNGNPD P RWREVAITFV DNHDTGYSPG
     301   QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
     351   VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
45    401   SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

```

SEQ ID NO: 16 Mature protein sequence of pMS2062

```

50      1   DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
      51   FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
     101   ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
     151   DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGGGF RFDFVRGYAP
     201   ERVDSWMSDS ADSSFCVGEL WKKPSEYPPW DWRNRASWQQ IIKDWSDRAK
     251   CPVFDFALKE RMQNGSVADW KHGLNGNPD P RWREVAITFV DNHDTGYSPG
     301   QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
     351   VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
55    401   SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

```

SEQ ID NO: 17 Mature protein sequence of **pMS2171**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVRGYAP
 201 ERVDLWMSDS ADSSFCVGEL WKAPSEYPPW DWRNRASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 18 Mature protein sequence of **pMS465:**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 19 Mature protein sequence of **pMS1042:**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 20 Mature protein sequence of **pMS1104:**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 21 Mature protein sequence of **pMS1153:**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG
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 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
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 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 22 Mature protein sequence of pMS1286:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
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 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNRASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 23 Mature protein sequence of pMS1284:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNRASWQQ IIKDWSDRAK
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 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 24 Mature protein sequence of pMS1290:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
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 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 25 Mature protein sequence of pMS1484:

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 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
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 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
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 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 27 Mature protein sequence of pMS1105:

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 101 ALGGAGVKVL YDVVPNHMNR FYPDKEINLP AGQRFWRNDC PDPGNGPNDC
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 201 ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
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SEQ ID NO: 28 Mature protein sequence of pMS1723:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNRGYR SDAQLRQAAG
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 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
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SEQ ID NO: 29 Mature protein sequence of pMS2104:

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 201 ERVDSWMSDS ADSSFCVGEL WKAPSEYPPW DWRNRASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
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 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

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 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
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 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 31 Mature protein sequence of pMS2124:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
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 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 32 Mature protein sequence of pMS2177:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYQWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEYFW HDFNKNGLYG SDAQLRQAAG
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 10 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 33 Mature protein sequence of pMS2178:

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 20 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 34 Mature protein sequence of pMS2118:

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 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 30 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 35. Linker Sequence GSGDGGGNDGG

SEQ ID NO: 36: Sequence of PS4 SBD:

1 EGGLVNVNFR CDNGVTQMGD SVYAVGNVSQ LGNWSPASAV RLTDTSYPT
 35 51 WKGSIALPDG QNVEWKCLIR NEADATLVRQ WQSGGNNQVQ AAAGASTSGS
 101 F*

SEQ ID NO: 37
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SEQ ID NO: 51: Novamyl:

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SEQ ID NO: 52: Nucleotide sequence of pMS382:

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1261 ggagatggag gaggaaatga cggaggataa

Note: The derived N-terminus of this nucleotide sequence is DQA... However, the final construct may be extended at the 5' end with atg to encode M at the N-terminus.

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5	Asp	Glu	Phe	Ala	Asn	Leu	Arg	Ser	Asn	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	
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	225					230					235					240	
	Gln	Val	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	
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25				260					265					270			
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		290					295					300					
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55	Gly	Ser	Phe	Ser	Glu	Ala	Val	Asn	Ala	Ser	Asn	Gly	Gln	Val	Arg	Val	
					405					410					415		

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 420 425 430

5

Gly Leu Val Asn Val Asn Phe Arg Cys Asp Asn Gly Val Thr Gln Met
 435 440 445

10

Gly Asp Ser Val Tyr Ala Val Gly Asn Val Ser Gln Leu Gly Asn Trp
 450 455 460

15

Ser Pro Ala Ser Ala Val Arg Leu Thr Asp Thr Ser Ser Tyr Pro Thr
 465 470 475 480

20

Trp Lys Gly Ser Ile Ala Leu Pro Asp Gly Gln Asn Val Glu Trp Lys
 485 490 495

25

Cys Leu Ile Arg Asn Glu Ala Asp Ala Thr Leu Val Arg Gln Trp Gln
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Ser Gly Gly Asn Asn Gln Val Gln Ala Ala Ala Gly Ala Ser Thr Ser
 515 520 525

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Gly Ser Phe
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<210> 3
 <211> 1596
 <212> DNA
 <213> Artificial Sequence

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<220>
 <223> DNA sequence of nucleotide encoding G4 amylase of Pseudomonas sp. AM1 (2006) (Q1EJP2) with M added
 at the N-terminus (Q1EJP2 DNA):

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<400> 3

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5	agagatatgg caccgacaat tgcagcagat ggcttttcag caatttggat gccggttccg	180
	tggagagatt tttcatcatg gtcagatggc gcaaattcag gcggcggcga aggctatatt	240
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10	ggcgactga ataatgcaca agttaaagt ctgtatgatg ttgttccgaa tcatatgaat	360
	agaggctatc cggataaaca aattaatctg ccggcaggcc aaggcttttg gagaaatgat	420
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15	gatgctgatc tgaatacagc aaatccgcaa gtttatggca tgtttagaga tgaatttgca	540
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20	ggcgaaagag ttgattcatg gatgggcgca gcacatgata atgcattttg cgttggcgaa	660
	ctgtggaaag caccggcaga atatccgtca tgggattgga gaaatacagc atcatggcaa	720
25	caagttatta aagattggtc agatagagca aaatgcccg tttttgattt tgcactgaaa	780
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	ccgagatgga gagaagttgc agttacattt gttgataatc atgatgcagg ctattcaccg	900
30	ggccaaaatg gcggccaaca tcattgggca ctgcaagatg gcctgattag acaagcatat	960
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35	gattcagcaa tttcatttca ttcaggctat tcaggcctgg ttgcaacagt ttcaggctca	1140
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	tcaggcgatg gcggcggcaa tgatggcggc gaaggcggcc tggttaatgt taattttaga	1320
	tgcgataatg gcgttacaca aatgggcgat tcagtttatg cagttggcaa tgtttcacia	1380
45	ctgggcaatt ggtcaccggc atcagcagtt agactgacag atacatcatc atatccgaca	1440
	tggaaaggct caattgcaact gccgatggc caaaatgttg aatggaaatg cctgattaga	1500
	aatgaagcag atgcaacact ggtagacaa tggcaatcag gcggcaataa tcaagttcaa	1560
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<210> 4

<211> 534

<212> PRT

<213> Artificial Sequence

<220>

<223> Mature protein of G4 amylase of Pseudomonas sp. 7193 with M added at the N-terminus (A5CVD5 protein)

<400> 4

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      35          40          45

20     Ala Asp Gly Phe Ser Ala Ile Trp Met Pro Val Pro Trp Arg Asp Phe
      50          55          60

25

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10	Lys	Gln	Ala	Ala	Gly	Ala	Leu	Asn	Asn	Ala	Gln	Val	Lys	Val	Leu	Tyr	
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5	Ser	His	Met	Tyr	Asp	Trp	Gly	His	Gly	Pro	Leu	Ile	Arg	Gln	Leu	Ile	
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25	Ser	Ser	Gly	Ser	Phe	Ser	Gln	Ala	Leu	Asn	Gln	Asp	Ser	Gly	Gln	Leu	
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	450						455					460					
45	Gly	Ser	Trp	Ser	Pro	Ala	Asn	Ala	Val	Arg	Leu	Thr	Asp	Val	Ser	Asn	
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50	Tyr	Pro	Thr	Trp	Lys	Gly	Ala	Ile	Ser	Leu	Pro	Ala	Gly	Gln	Ala	Val	
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55	Glu	Trp	Lys	Cys	Ile	Val	Arg	Ser	Glu	Ala	Asp	Pro	Thr	Gln	Val	Arg	
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60	Gln	Trp	Gln	Ala	Gly	Asp	Asn	Asn	Arg	Val	Thr	Ala	Gly	Ala	Gly	Ala	
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<211> 1605

<212> DNA

<213> Artificial Sequence

<220>

<223> DNA sequence of nucleotide encoding G4 amylase of Pseudomonas sp. 7193 (A5CVD5) with M added at the N-terminus (A5CVD5 DNA)

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	tggagagatt tttcatcatg gtcagatggc gcaaattcag gcggcgagca aggctatttt	240
	tggcatgatt ttaataaaaa tggcagatat ggctcagata cacaactgaa acaagcagca	300
15	ggcgagctga ataatgcaca agttaaaagt ctgtatgatg cagttccgaa tcatatgaat	360
	agaggctatc cggataaaca aattaatctg ccggcaggcc aaggcttttg gagaaatgat	420
	tgcgcagatc cgggcaatta tccgaatgat tgcgatgatg gcgatagatt tatgggaggc	480
20	gatgctgatc tgaatacagc aaatccgcaa gtttatggca tgtttagaga tgaatttgca	540
	aatctgagat caaattatgg cgcaggcggc tttagatttg attttgtag aggctatgca	600
	ggcgaaagag ttgattcatg gatgggagat ggcgatgcc aaagactgct gagaggcaga	660
25	gcaactggaag gcacaggcag aattccggaa ctgggcctgg cacaatatgg ccaactggca	720
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	gattggggtc atggaccgct gattagacaa ctgattcaaa ttagaagagc agcaggcggt	1080
40	agagcagatt cagcaattga atttcattca ggctattcag gcctgggtgc aacagttaga	1140
	ggcacagcac aaacactggt tatggcactg ggctcaaadc tgtcatcacc ggcagaagtt	1200
	tcatcaggct cattttcaca agcactgaat caagattcag gccaaactgag aatttgagca	1260
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50	ctggcacaac tgggctcatg gtcaccggca aatgcagtta gactgacaga tgtttcaaat	1440
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<210> 6

<211> 528

<212> PRT

<213> Artificial Sequence

<220>

5 <223> Mature protein of G4 amylase of *Pseudomonas mendocina* (strain ymp) with M added at the N-terminus (A4XX23 protein)

<400> 6

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10	Ser	Asn	Trp	Tyr	Ala	Thr	Leu	Ala	Ser	Met	Ala	Pro	Thr	Leu	Ala	Ala	
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20	Ser	Trp	Ser	Asp	Pro	Gly	Asn	Gly	Thr	Ser	Gly	Gly	Gly	Glu	Gly	Tyr	
	65					70					75					80	
25	Phe	Trp	His	Asp	Phe	Asn	Lys	Asn	Gly	Arg	Tyr	Gly	Ser	Asp	Ser	Leu	
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15	Arg	His	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Ala	Arg	Trp	Arg	Glu	Val	Ala	
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25	Gly	Gly	Gln	His	His	Trp	Pro	Leu	Pro	Asp	Ala	Arg	Leu	Lys	Gln	Ala	
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35	His	Met	Tyr	Asp	Trp	Gly	His	Gly	Asp	Phe	Ile	Arg	Gln	Leu	Ile	Gln	
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		370					375					380					
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					405					410					415		
60	Ile	Trp	Arg	Ser	Gly	Gln	Gly	Gly	Gly	Asp	Gly	Gln	Gly	Asn	Leu	Val	
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75	Gly	Ala	Val	Arg	Leu	Thr	Asp	Thr	Ser	Ala	Tyr	Pro	Thr	Trp	Lys	Gly	
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Ser Ile Ala Leu Pro Ala Gly Gln Gln Val Gln Trp Lys Cys Ile Val
485 490 495

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<210> 7

<211> 1587

<212> DNA

15 <213> Artificial Sequence

<220>

<223> DNA sequence of nucleotide encoding G4 amylase of Pseudomonas mendocina (strain ymp) (A4XX23) with M added at the N-terminus: (A4XX23 DNA)

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<400> 7

25

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35

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45

50

55

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<210> 8

<211> 413

<212> PRT

<213> Artificial Sequence

<220>

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15

Glu Arg Asn Trp Tyr Asn Ile Leu Gln Ser Lys Ala Gln Gln Ile Ser
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Glu Asp Gly Phe Thr Ala Ile Trp Met Pro Val Pro Trp Arg Asp Asn
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Ser Ser Trp Gln Ala Ser Ser Asp Thr Arg Phe Gly Gly Glu Gly Tyr
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Phe Trp Ala Asp Met Asp Lys Asn Ser Arg Tyr Gly Asp Asp Gly Gln
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Leu Lys Gln Ala Ala Ser Ala Leu Lys Asn Lys Gly Val Lys Val Ile
100 105 110

Tyr Asp Ile Val Pro Asn His His Asp Arg Gly His Ser Asn Asp Ser
115 120 125

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Leu Asn Leu Pro Ser Gly Gln Gly Tyr Tyr Arg Ser Asp Cys Ser Ser
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50	Leu	Pro	Asp	Trp	Lys	Arg	Lys	Met	Ala	Tyr	Ala	Tyr	Ile	Leu	Ser	Ser	305	310	315	320
55	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met	Tyr	Asp	Trp	Gly	Met	325	330	335	
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	Asn	Phe	Ser	Ser	Pro	Asn	Gln	Val	Ala	Gly	Gly	Ala	Trp	Asn	Leu	Ala	385	390	395	400

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50	Ala Leu Lys Glu Arg Met Gln Asn Gly Ser Val Ala Asp Trp Lys His 260 265 270		
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	Gln His Lys Trp Pro Leu Pro Asp Gly Leu Ile Arg Gln Ala Tyr Ala 305 310 315 320		
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	Arg	Thr	Ala	Gly	Val	Arg	Ala	Asp	Ser	Ala	Ile	Ser	Phe	His	Ser	Gly	
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 Tyr Asn Trp Tyr Asn Ile Leu Arg Gln Lys Ala Ser Thr Ile Ala Ala
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 Asp Gly Phe Ser Ala Ile Trp Met Pro Val Pro Trp Arg Asp Phe Ser
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 Ser Trp Thr Asp Gly Asp Lys Ser Gly Gly Gly Glu Gly Tyr Phe Trp
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 Leu Pro Ala Gly Gln Arg Phe Trp Arg Asn Asp Cys Pro Asp Pro Gly

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15	Ile Ile Lys Asp Trp Ser Asp Arg Ala Lys Cys Pro Val Phe Asp Phe 245 250 255		
20	Ala Leu Lys Glu Arg Met Gln Asn Gly Ser Val Ala Asp Trp Lys His 260 265 270		
25	Gly Leu Asn Gly Asn Pro Asp Pro Arg Trp Arg Glu Val Ala Val Thr 275 280 285		
30	Phe Val Asp Asn His Asp Thr Gly Tyr Ser Pro Gly Gln Asn Gly Gly 290 295 300		
35	Gln His Lys Trp Pro Leu Pro Asp Gly Leu Ile Arg Gln Ala Tyr Ala 305 310 315 320		
40	Tyr Ile Leu Thr Ser Pro Gly Thr Pro Val Val Tyr Trp Pro His Met 325 330 335		
45	Tyr Asp Trp Gly Tyr Gly Asp Phe Ile Arg Gln Leu Ile Gln Val Arg 340 345 350		
50	Arg Thr Ala Gly Val Arg Ala Asp Ser Ala Ile Ser Phe His Ser Gly 355 360 365		
55	Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val 370 375 380		
	Val Ala Leu Asn Ser Asp Leu Asp Asn Pro Gly Gln Val Ala Ser Gly 385 390 395 400		
	Ser Phe Ser Glu Ala Val Asn Ala Glu Asn Gly Gln Val Arg Val Trp 405 410 415		
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<223> Mature protein sequence of pMS2062

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

	225		230		235		240									
5	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe
				245						250					255	
10	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His
				260					265					270		
15	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Pro	Arg	Trp	Arg	Glu	Val	Ala	Val	Thr
			275					280					285			
20	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Tyr	Ser	Pro	Gly	Gln	Asn	Gly	Gly
		290					295					300				
25	Gln	His	Lys	Trp	Pro	Leu	Pro	Asp	Gly	Leu	Ile	Arg	Gln	Ala	Tyr	Ala
	305					310					315					320
30	Tyr	Ile	Leu	Thr	Ser	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met
					325					330					335	
35	Tyr	Asp	Trp	Gly	Tyr	Gly	Asp	Phe	Ile	Arg	Gln	Leu	Ile	Gln	Val	Arg
				340					345					350		
40	Arg	Thr	Ala	Gly	Val	Arg	Ala	Asp	Ser	Ala	Ile	Ser	Phe	His	Ser	Gly
			355					360					365			
45	Tyr	Ser	Gly	Leu	Val	Ala	Thr	Val	Ser	Gly	Ser	Gln	Gln	Thr	Leu	Val
		370					375					380				
50	Val	Ala	Leu	Asn	Ser	Asp	Leu	Asp	Asn	Pro	Gly	Gln	Val	Ala	Ser	Gly
	385					390					395					400
55	Ser	Phe	Ser	Glu	Ala	Val	Asn	Ala	Ser	Asn	Gly	Gln	Val	Arg	Val	Trp
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60	Arg	Ser	Gly	Ser	Gly	Asp	Gly	Gly	Gly	Asn	Asp	Gly	Gly			
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	Glu	Ile	Ile	Leu	Gln	Gly	Phe	His	Trp	Asn	Val	Val	Arg	Glu	Ala	Pro	
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5	Tyr	Asn	Trp	Tyr	Asn	Ile	Leu	Arg	Gln	Lys	Ala	Ser	Thr	Ile	Ala	Ala	
			35					40					45				
10	Asp	Gly	Phe	Ser	Ala	Ile	Trp	Met	Pro	Val	Pro	Trp	Arg	Asp	Phe	Ser	
		50					55					60					
15	Ser	Trp	Thr	Asp	Gly	Asp	Lys	Ser	Gly	Gly	Gly	Glu	Gly	Tyr	Phe	Trp	
	65					70					75					80	
20	His	Asp	Phe	Asn	Lys	Asn	Gly	Leu	Tyr	Gly	Ser	Asp	Ala	Gln	Leu	Arg	
				85						90					95		
25	Gln	Ala	Ala	Gly	Ala	Leu	Gly	Gly	Ala	Gly	Val	Lys	Val	Leu	Tyr	Asp	
				100					105					110			
30	Val	Val	Pro	Asn	His	Met	Asn	Arg	Phe	Tyr	Pro	Asp	Lys	Glu	Ile	Asn	
			115					120					125				
35	Leu	Pro	Ala	Gly	Gln	Arg	Phe	Trp	Arg	Asn	Asp	Cys	Pro	Asp	Pro	Gly	
		130					135					140					
40	Asn	Gly	Pro	Asn	Asp	Cys	Asp	Asp	Gly	Asp	Arg	Phe	Leu	Gly	Gly	Glu	
	145					150					155					160	
45	Ala	Asp	Leu	Asn	Thr	Gly	His	Pro	Gln	Ile	Tyr	Gly	Met	Phe	Arg	Asp	
				165						170					175		
50	Glu	Phe	Thr	Asn	Leu	Arg	Ser	Gly	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	Phe	
				180					185					190			
55	Asp	Phe	Val	Arg	Gly	Tyr	Ala	Pro	Glu	Arg	Val	Asp	Leu	Trp	Met	Ser	
			195					200					205				
60	Asp	Ser	Ala	Asp	Ser	Ser	Phe	Cys	Val	Gly	Glu	Leu	Trp	Lys	Ala	Pro	
		210					215					220					
65	Ser	Glu	Tyr	Pro	Pro	Trp	Asp	Trp	Arg	Asn	Arg	Ala	Ser	Trp	Gln	Gln	
	225					230					235					240	
70	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
				245						250					255		
75	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	

	260	265	270
5	Gly Leu Asn Gly Asn Pro Asp	Pro Arg Trp Arg Glu Val Ala Val Thr	
	275	280	285
10	Phe Val Asp Asn His Asp Thr Gly Tyr Ser Pro Gly Gln Asn Gly Gly		
	290	295	300
15	Gln His Lys Trp Pro Leu Pro Asp Gly Leu Ile Arg Gln Ala Tyr Ala		
	305	310	315
20	Tyr Ile Leu Thr Ser Pro Gly Thr Pro Val Val Tyr Trp Pro His Met		
	325	330	335
25	Tyr Asp Trp Gly Tyr Gly Asp Phe Ile Arg Gln Leu Ile Gln Val Arg		
	340	345	350
30	Arg Thr Ala Gly Val Arg Ala Asp Ser Ala Ile Ser Phe His Ser Gly		
	355	360	365
35	Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val		
	370	375	380
40	Val Ala Leu Asn Ser Asp Leu Asp Asn Pro Gly Gln Val Ala Ser Gly		
	385	390	395
45	Ser Phe Ser Glu Ala Val Asn Ala Glu Asn Gly Gln Val Arg Val Trp		
	405	410	415
50	Arg Ser Gly Ser Gly Asp Gly Gly Gly Asn Asp Gly Gly		
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<223> Mature protein sequence of pMS465

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 Tyr Asn Trp Tyr Asn Ile Leu Arg Gln Gln Ala Ser Thr Ile Ala Ala
 35 40 45

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	Asp	Gly	Phe	Ser	Ala	Ile	Trp	Met	Pro	Val	Pro	Trp	Arg	Asp	Phe	Ser	
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5	Ser	Trp	Thr	Asp	Gly	Asp	Lys	Ser	Gly	Gly	Gly	Glu	Gly	Tyr	Phe	Trp	
	65					70					75					80	
	His	Asp	Phe	Asn	Lys	Asn	Gly	Arg	Tyr	Gly	Ser	Asp	Ala	Gln	Leu	Arg	
10					85					90					95		
	Gln	Ala	Ala	Gly	Ala	Leu	Gly	Gly	Ala	Gly	Val	Lys	Val	Leu	Tyr	Asp	
				100					105					110			
15	Val	Val	Pro	Asn	His	Met	Asn	Arg	Phe	Tyr	Pro	Asp	Lys	Glu	Ile	Asn	
			115					120					125				
	Leu	Pro	Ala	Gly	Gln	Arg	Phe	Trp	Arg	Asn	Asp	Cys	Pro	Asp	Pro	Gly	
20		130					135					140					
	Asn	Gly	Pro	Asn	Asp	Cys	Asp	Asp	Gly	Asp	Arg	Phe	Leu	Gly	Gly	Glu	
25	145					150					155					160	
	Ala	Asp	Leu	Asn	Thr	Gly	His	Pro	Gln	Ile	Tyr	Gly	Met	Phe	Arg	Asp	
					165					170					175		
30	Glu	Phe	Thr	Asn	Leu	Arg	Ser	Gly	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	Phe	
				180					185					190			
	Asp	Phe	Val	Arg	Gly	Tyr	Ala	Pro	Glu	Arg	Val	Asp	Ser	Trp	Met	Ser	
35			195					200					205				
	Asp	Ser	Ala	Asp	Ser	Ser	Phe	Cys	Val	Gly	Glu	Leu	Trp	Lys	Ser	Pro	
40		210					215					220					
	Ser	Glu	Tyr	Pro	Pro	Trp	Asp	Trp	Arg	Asn	Thr	Ala	Ser	Trp	Gln	Gln	
	225					230					235				240		
45	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
					245					250					255		
	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	
50				260					265					270			
	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Pro	Arg	Trp	Arg	Glu	Val	Ala	Val	Thr	
			275					280					285				
55	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Tyr	Ser	Pro	Gly	Gln	Asn	Gly	Gly	

	290		295		300												
5	Gln	His	Lys	Trp	Pro	Leu	Gln	Asp	Gly	Leu	Ile	Arg	Gln	Ala	Tyr	Ala	
	305					310					315					320	
	Tyr	Ile	Leu	Thr	Ser	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met	
10					325					330					335		
	Tyr	Asp	Trp	Gly	Tyr	Gly	Asp	Phe	Ile	Arg	Gln	Leu	Ile	Gln	Val	Arg	
				340					345					350			
15	Arg	Thr	Ala	Gly	Val	Arg	Ala	Asp	Ser	Ala	Ile	Ser	Phe	His	Ser	Gly	
			355					360					365				
	Tyr	Ser	Gly	Leu	Val	Ala	Thr	Val	Ser	Gly	Ser	Gln	Gln	Thr	Leu	Val	
20		370					375					380					
	Val	Ala	Leu	Asn	Ser	Asp	Leu	Ala	Asn	Pro	Gly	Gln	Val	Ala	Ser	Gly	
25		385				390					395					400	
	Ser	Phe	Ser	Glu	Ala	Val	Asn	Ala	Ser	Asn	Gly	Gln	Val	Arg	Val	Trp	
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30	Arg	Ser	Gly	Ser	Gly	Asp	Gly	Gly	Gly	Asn	Asp	Gly	Gly				
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5 Glu Ile Ile Leu Gln Gly Phe His Trp Asn Val Val Arg Glu Ala Pro
 20 25 30

10 Tyr Asn Trp Tyr Asn Ile Leu Arg Gln Gln Ala Ser Thr Ile Ala Ala
 35 40 45

15 Asp Gly Phe Ser Ala Ile Trp Met Pro Val Pro Trp Arg Asp Phe Ser
 50 55 60

Ser Trp Thr Asp Gly Asp Lys Ser Gly Gly Gly Glu Gly Tyr Phe Trp
 65 70 75 80

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	His	Asp	Phe	Asn	Lys	Asn	Gly	Arg	Tyr	Gly	Ser	Asp	Ala	Gln	Leu	Arg	
					85					90					95		
5	Gln	Ala	Ala	Gly	Ala	Leu	Gly	Gly	Ala	Gly	Val	Lys	Val	Leu	Tyr	Asp	
				100					105					110			
10	Val	Val	Pro	Asn	His	Met	Asn	Arg	Phe	Tyr	Pro	Asp	Lys	Glu	Ile	Asn	
			115					120					125				
15	Leu	Pro	Ala	Gly	Gln	Arg	Phe	Trp	Arg	Asn	Asp	Cys	Pro	Asp	Pro	Gly	
		130					135					140					
20	Asn	Gly	Pro	Asn	Asp	Cys	Asp	Asp	Gly	Asp	Arg	Phe	Leu	Gly	Gly	Glu	
	145					150					155					160	
25	Ala	Asp	Leu	Asn	Thr	Gly	His	Pro	Gln	Ile	Tyr	Gly	Met	Phe	Arg	Asp	
					165					170					175		
30	Glu	Phe	Thr	Asn	Leu	Arg	Ser	Gly	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	Phe	
				180					185					190			
35	Asp	Phe	Val	Arg	Gly	Tyr	Ala	Pro	Glu	Arg	Val	Asp	Ser	Trp	Met	Ser	
			195					200					205				
40	Asp	Ser	Ala	Asp	Ser	Ser	Phe	Cys	Val	Gly	Glu	Leu	Trp	Lys	Glu	Pro	
		210					215					220					
45	Ser	Glu	Tyr	Pro	Pro	Trp	Asp	Trp	Arg	Asn	Thr	Ala	Ser	Trp	Gln	Gln	
	225					230					235					240	
50	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
				245						250					255		
55	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	
				260					265					270			
60	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Pro	Arg	Trp	Arg	Glu	Val	Ala	Val	Thr	
			275					280					285				
65	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Tyr	Ser	Pro	Gly	Gln	Asn	Gly	Gly	
		290					295					300					
70	Gln	His	Lys	Trp	Pro	Leu	Pro	Asp	Gly	Leu	Ile	Arg	Gln	Ala	Tyr	Ala	
	305					310					315					320	
75	Tyr	Ile	Leu	Thr	Ser	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met	

	325		330		335
5	Tyr Asp Trp Gly Tyr Gly Asp Phe Ile Arg Gln Leu Ile Gln Val Arg	340	345	350	
10	Arg Thr Ala Gly Val Arg Ala Asp Ser Ala Ile Ser Phe His Ser Gly	355	360	365	
15	Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val	370	375	380	
20	Val Ala Leu Asn Ser Asp Leu Ala Asn Pro Gly Gln Val Ala Ser Gly	385	390	395	400
25	Ser Phe Ser Glu Ala Val Asn Ala Ser Asn Gly Gln Val Arg Val Trp	405	410	415	
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				20					25					30			
	Tyr	Asn	Trp	Tyr	Asn	Ile	Leu	Arg	Gln	Lys	Ala	Ser	Thr	Ile	Ala	Ala	
10			35					40					45				
	Asp	Gly	Phe	Ser	Ala	Ile	Trp	Met	Pro	Val	Pro	Trp	Arg	Asp	Phe	Ser	
		50					55					60					
15	Ser	Trp	Thr	Asp	Gly	Asp	Lys	Ser	Gly	Gly	Gly	Glu	Gly	Tyr	Phe	Trp	
	65					70					75					80	
	His	Asp	Phe	Asn	Lys	Asn	Gly	Arg	Tyr	Gly	Ser	Asp	Ala	Gln	Leu	Arg	
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	Gln	Ala	Ala	Gly	Ala	Leu	Gly	Gly	Ala	Gly	Val	Lys	Val	Leu	Tyr	Asp	
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	Val	Val	Pro	Asn	His	Met	Asn	Arg	Phe	Tyr	Pro	Asp	Lys	Glu	Ile	Asn
			115					120					125			
5	Leu	Pro	Ala	Gly	Gln	Arg	Phe	Trp	Arg	Asn	Asp	Cys	Pro	Asp	Pro	Gly
		130					135					140				
10	Asn	Gly	Pro	Asn	Asp	Cys	Asp	Asp	Gly	Asp	Arg	Phe	Leu	Gly	Gly	Glu
	145					150					155					160
15	Ala	Asp	Leu	Asn	Thr	Gly	His	Pro	Gln	Ile	Tyr	Gly	Met	Phe	Arg	Asp
				165						170					175	
20	Glu	Phe	Thr	Asn	Leu	Arg	Ser	Gly	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	Phe
			180						185					190		
25	Asp	Phe	Val	Arg	Gly	Tyr	Ala	Pro	Glu	Arg	Val	Asp	Ser	Trp	Met	Ser
		195						200					205			
30	Asp	Ser	Ala	Asp	Ser	Ser	Phe	Cys	Val	Gly	Glu	Leu	Trp	Lys	Glu	Pro
	210						215					220				
35	Ser	Glu	Tyr	Pro	Pro	Trp	Asp	Trp	Arg	Asn	Thr	Ala	Ser	Trp	Gln	Gln
	225					230					235					240
40	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe
				245						250					255	
45	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His
			260						265					270		
50	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Pro	Arg	Trp	Arg	Glu	Val	Ala	Val	Thr
		275					280					285				
55	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Tyr	Ser	Pro	Gly	Gln	Asn	Gly	Gly
	290						295					300				
60	Gln	His	Lys	Trp	Pro	Leu	Gln	Asp	Gly	Leu	Ile	Arg	Gln	Ala	Tyr	Ala
	305					310					315					320
65	Tyr	Ile	Leu	Thr	Ser	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met
				325						330					335	
70	Tyr	Asp	Trp	Gly	Tyr	Gly	Asp	Phe	Ile	Arg	Gln	Leu	Ile	Gln	Val	Arg
			340						345					350		
75	Arg	Thr	Ala	Gly	Val	Arg	Ala	Asp	Ser	Ala	Ile	Ser	Phe	His	Ser	Gly

	355	360	365
5	Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val		
	370	375	380
10	Val Ala Leu Asn Ser Asp Leu Ala Asn Pro Gly Gln Val Ala Ser Gly		
	385	390	395 400
15	Ser Phe Ser Glu Ala Val Asn Ala Ser Asn Gly Gln Val Arg Val Trp		
	405	410	415
20	Arg Ser Gly Ser Gly Asp Gly Gly Gly Asn Asp Gly Gly		
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	1				5					10					15		
5	Glu	Ile	Ile	Leu	Gln	Gly	Phe	His	Trp	Asn	Val	Val	Arg	Glu	Ala	Pro	
				20					25					30			
	Tyr	Asn	Trp	Tyr	Asn	Ile	Leu	Arg	Gln	Gln	Ala	Ser	Thr	Ile	Ala	Ala	
10			35					40					45				
	Asp	Gly	Phe	Ser	Ala	Ile	Trp	Met	Pro	Val	Pro	Trp	Arg	Asp	Phe	Ser	
		50					55					60					
15	Ser	Trp	Thr	Asp	Gly	Asp	Lys	Ser	Gly	Gly	Gly	Glu	Gly	Tyr	Phe	Trp	
	65					70					75					80	
	His	Asp	Phe	Asn	Lys	Asn	Gly	Leu	Tyr	Gly	Ser	Asp	Ala	Gln	Leu	Arg	
20					85					90					95		
	Gln	Ala	Ala	Gly	Ala	Leu	Gly	Gly	Ala	Gly	Val	Lys	Val	Leu	Tyr	Asp	
25				100					105					110			
	Val	Val	Pro	Asn	His	Met	Asn	Arg	Phe	Tyr	Pro	Asp	Lys	Glu	Ile	Asn	
			115					120					125				
30	Leu	Pro	Ala	Gly	Gln	Arg	Phe	Trp	Arg	Asn	Asp	Cys	Pro	Asp	Pro	Gly	
		130					135					140					

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	Asn Gly Pro Asn Asp Cys Asp Asp Gly Asp Arg Phe Leu Gly Gly Glu	
	145	150 155 160
5	Ala Asp Leu Asn Thr Gly His Pro Gln Ile Tyr Gly Met Phe Arg Asp	
		165 170 175
10	Glu Phe Thr Asn Leu Arg Ser Gly Tyr Gly Ala Gly Gly Phe Arg Phe	
		180 185 190
15	Asp Phe Val Arg Gly Tyr Ala Pro Glu Arg Val Asp Ser Trp Met Ser	
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30	Ile Ile Lys Asp Trp Ser Asp Arg Ala Lys Cys Pro Val Phe Asp Phe	
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55	Tyr Ile Leu Thr Ser Pro Gly Thr Pro Val Val Tyr Trp Pro His Met	
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65	Arg Thr Ala Gly Val Arg Ala Asp Ser Ala Ile Ser Phe His Ser Gly	
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70	Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val	
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75	Val Ala Leu Asn Ser Asp Leu Ala Asn Pro Gly Gln Val Ala Ser Gly	

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30	Val	Val	Pro	Asn	His	Met	Asn	Arg	Phe	Tyr	Pro	Asp	Lys	Glu	Ile	Asn	
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		130					135					140					
40	Asn	Gly	Pro	Asn	Asp	Cys	Asp	Asp	Gly	Asp	Arg	Phe	Leu	Gly	Gly	Glu	
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45	Ala	Asp	Leu	Asn	Thr	Gly	His	Pro	Gln	Ile	Tyr	Gly	Met	Phe	Arg	Asp	
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50	Glu	Phe	Thr	Asn	Leu	Arg	Ser	Gly	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	Phe	
				180					185					190			
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70	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
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75	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	
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5 Phe Val Asp Asn His Asp Thr Gly Tyr Ser Pro Gly Gln Asn Gly Gly
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10 Gln His Lys Trp Pro Leu Gln Asp Gly Leu Ile Arg Gln Ala Tyr Ala
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20 Arg Thr Ala Gly Val Arg Ala Asp Ser Ala Ile Ser Phe His Ser Gly
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25 Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val
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Val Ala Leu Asn Ser Asp Leu Ala Asn Pro Gly Gln Val Ala Ser Gly
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55 Tyr Asn Trp Tyr Asn Ile Leu Arg Gln Gln Ala Ser Thr Ile Ala Ala
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	225					230					235					240	
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5 Tyr Ile Leu Thr Ser Pro Gly Thr Pro Val Val Tyr Trp Pro His Met
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10 Tyr Asp Trp Gly Tyr Gly Asp Phe Ile Arg Gln Leu Ile Gln Val Arg
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15 Arg Thr Ala Gly Val Arg Ala Asp Ser Ala Ile Ser Phe His Ser Gly
 355 360 365

20 Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val
 370 375 380

25 Val Ala Leu Asn Ser Asp Leu Ala Asn Pro Gly Gln Val Ala Ser Gly
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 35 40 45

Asp Gly Phe Ser Ala Ile Trp Met Pro Val Pro Trp Arg Asp Phe Ser
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55 Ser Trp Thr Asp Gly Asp Lys Ser Gly Gly Gly Glu Gly Tyr Phe Trp
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10	Val	Val	Pro	Asn	His	Met	Asn	Arg	Phe	Tyr	Pro	Asp	Lys	Glu	Ile	Asn	
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15	Leu	Pro	Ala	Gly	Gln	Arg	Phe	Trp	Arg	Asn	Asp	Cys	Pro	Asp	Pro	Gly	
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20	Asn	Gly	Pro	Asn	Asp	Cys	Asp	Asp	Gly	Asp	Arg	Phe	Leu	Gly	Gly	Glu	
	145					150					155					160	
25	Ala	Asp	Leu	Asn	Thr	Gly	His	Pro	Gln	Ile	Tyr	Gly	Met	Phe	Arg	Asp	
					165					170					175		
30	Glu	Phe	Thr	Asn	Leu	Arg	Ser	Gly	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	Phe	
				180					185					190			
35	Asp	Phe	Val	Arg	Gly	Tyr	Ala	Pro	Glu	Arg	Val	Asp	Ser	Trp	Met	Ser	
			195					200					205				
40	Asp	Ser	Ala	Asp	Ser	Ser	Phe	Cys	Val	Gly	Glu	Leu	Trp	Lys	Ser	Pro	
		210					215					220					
45	Ser	Glu	Tyr	Pro	Pro	Trp	Asp	Trp	Arg	Asn	Thr	Ala	Ser	Trp	Gln	Gln	
	225					230					235					240	
50	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
					245					250					255		
55	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	
				260					265					270			
60	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Pro	Arg	Trp	Arg	Glu	Val	Ala	Val	Thr	
			275					280					285				
65	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Tyr	Ser	Pro	Gly	Gln	Asn	Gly	Gly	
		290					295					300					
70	Gln	His	Lys	Trp	Pro	Leu	Gln	Asp	Gly	Leu	Ile	Arg	Gln	Ala	Tyr	Ala	
	305					310					315					320	
75	Tyr	Ile	Leu	Thr	Ser	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met	
					325					330					335		

Tyr Asp Trp Gly Tyr Gly Asp Phe Ile Arg Gln Leu Ile Gln Val Arg
 340 345 350

5 Arg Thr Ala Gly Val Arg Ala Asp Ser Ala Ile Ser Phe His Ser Gly
 355 360 365

10 Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val
 370 375 380

15 Val Ala Leu Asn Ser Asp Leu Ala Asn Pro Gly Gln Val Ala Ser Gly
 385 390 395 400

Ser Phe Ser Glu Ala Val Asn Ala Ser Asn Gly Gln Val Arg Val Trp
 405 410 415

20 Arg Ser Gly Ser Gly Asp Gly Gly Gly Asn Asp Gly Gly
 420 425

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Glu Ile Ile Leu Gln Gly Phe His Trp Asn Val Val Arg Glu Ala Pro
 20 25 30

40 Tyr Asn Trp Tyr Asn Ile Leu Arg Gln Lys Ala Ser Thr Ile Ala Ala
 35 40 45

45 Asp Gly Phe Ser Ala Ile Trp Met Pro Val Pro Trp Arg Asp Phe Ser
 50 55 60

50 Ser Trp Thr Asp Gly Asp Lys Ser Gly Gly Gly Glu Gly Tyr Phe Trp
 65 70 75 80

His Asp Phe Asn Lys Asn Gly Arg Tyr Gly Ser Asp Ala Gln Leu Arg
 85 90 95

55 Gln Ala Ala Gly Ala Leu Gly Gly Ala Gly Val Lys Val Leu Tyr Asp
 100 105 110

	Val	Val	Pro	Asn	His	Met	Asn	Arg	Phe	Tyr	Pro	Asp	Lys	Glu	Ile	Asn	
			115					120					125				
5	Leu	Pro	Ala	Gly	Gln	Arg	Phe	Trp	Arg	Asn	Asp	Cys	Pro	Asp	Pro	Gly	
		130					135					140					
10	Asn	Gly	Pro	Asn	Asp	Cys	Asp	Asp	Gly	Asp	Arg	Phe	Leu	Gly	Gly	Glu	
	145					150					155					160	
15	Ala	Asp	Leu	Asn	Thr	Gly	His	Pro	Gln	Ile	Tyr	Gly	Met	Phe	Arg	Asp	
					165					170					175		
20	Glu	Phe	Thr	Asn	Leu	Arg	Ser	Gly	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	Phe	
				180					185					190			
25	Asp	Phe	Val	Arg	Gly	Tyr	Ala	Pro	Glu	Arg	Val	Asp	Ser	Trp	Met	Ser	
			195					200					205				
30	Asp	Ser	Ala	Asp	Ser	Ser	Phe	Cys	Val	Gly	Glu	Leu	Trp	Lys	Ser	Pro	
	210						215					220					
35	Ser	Glu	Tyr	Pro	Pro	Trp	Asp	Trp	Arg	Asn	Thr	Ala	Ser	Trp	Gln	Gln	
	225					230					235					240	
40	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
					245					250					255		
45	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	
				260					265					270			
50	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Pro	Arg	Trp	Arg	Glu	Val	Ala	Val	Thr	
			275					280					285				
55	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Tyr	Ser	Pro	Gly	Gln	Asn	Gly	Gly	
	290						295					300					
60	Gln	His	Lys	Trp	Pro	Leu	Gln	Asp	Gly	Leu	Ile	Arg	Gln	Ala	Tyr	Ala	
	305					310					315					320	
65	Tyr	Ile	Leu	Thr	Ser	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met	
					325					330					335		
70	Tyr	Asp	Trp	Gly	Tyr	Gly	Asp	Phe	Ile	Arg	Gln	Leu	Ile	Gln	Val	Arg	
				340					345					350			
75	Arg	Thr	Ala	Gly	Val	Arg	Ala	Asp	Ser	Ala	Ile	Ser	Phe	His	Ser	Gly	
			355					360					365				

Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val
 370 375 380

5 Val Ala Leu Asn Ser Asp Leu Asp Asn Pro Gly Gln Val Ala Ser Gly
 385 390 395 400

10 Ser Phe Ser Glu Ala Val Asn Ala Ser Asn Gly Gln Val Arg Val Trp
 405 410 415

Arg Ser Gly Ser Gly Asp Gly Gly Gly Asn Asp Gly Gly
 420 425

15 <210> 29
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 <212> PRT
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25 <400> 29

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30 Glu Ile Ile Leu Gln Gly Phe His Trp Asn Val Val Arg Glu Ala Pro
 20 25 30

35 Tyr Asn Trp Tyr Asn Ile Leu Arg Gln Lys Ala Ser Thr Ile Ala Ala
 35 40 45

Asp Gly Phe Ser Ala Ile Trp Met Pro Val Pro Trp Arg Asp Phe Ser
 50 55 60

40 Ser Trp Thr Asp Gly Asp Lys Ser Gly Gly Gly Glu Gly Tyr Phe Trp
 65 70 75 80

45 His Asp Phe Asn Lys Asn Gly Leu Tyr Gly Ser Asp Ala Gln Leu Arg
 85 90 95

50 Gln Ala Ala Gly Ala Leu Gly Gly Ala Gly Val Lys Val Leu Tyr Asp
 100 105 110

Val Val Pro Asn His Met Asn Arg Phe Tyr Pro Asp Lys Glu Ile Asn
 115 120 125

55 Leu Pro Ala Gly Gln Arg Phe Trp Arg Asn Asp Cys Pro Asp Pro Gly
 130 135 140

	Asn Gly Pro Asn Asp Cys Asp Asp Gly Asp Arg Phe Leu Gly Gly Glu	
	145	150 155 160
5	Ala Asp Leu Asn Thr Gly His Pro Gln Ile Tyr Gly Met Phe Arg Asp	
		165 170 175
10	Glu Phe Thr Asn Leu Arg Ser Gly Tyr Gly Ala Gly Gly Phe Arg Phe	
		180 185 190
15	Asp Phe Val Arg Gly Tyr Ala Pro Glu Arg Val Asp Ser Trp Met Ser	
		195 200 205
20	Asp Ser Ala Asp Ser Ser Phe Cys Val Gly Glu Leu Trp Lys Ala Pro	
		210 215 220
25	Ser Glu Tyr Pro Pro Trp Asp Trp Arg Asn Arg Ala Ser Trp Gln Gln	
		225 230 235 240
30	Ile Ile Lys Asp Trp Ser Asp Arg Ala Lys Cys Pro Val Phe Asp Phe	
		245 250 255
35	Ala Leu Lys Glu Arg Met Gln Asn Gly Ser Val Ala Asp Trp Lys His	
		260 265 270
40	Gly Leu Asn Gly Asn Pro Asp Pro Arg Trp Arg Glu Val Ala Val Thr	
		275 280 285
45	Phe Val Asp Asn His Asp Thr Gly Tyr Ser Pro Gly Gln Asn Gly Gly	
		290 295 300
50	Gln His Lys Trp Pro Leu Pro Asp Gly Leu Ile Arg Gln Ala Tyr Ala	
		305 310 315 320
55	Tyr Ile Leu Thr Ser Pro Gly Thr Pro Val Val Tyr Trp Pro His Met	
		325 330 335
60	Tyr Asp Trp Gly Tyr Gly Asp Phe Ile Arg Gln Leu Ile Gln Val Arg	
		340 345 350
65	Arg Thr Ala Gly Val Arg Ala Asp Ser Ala Ile Ser Phe His Ser Gly	
		355 360 365
70	Tyr Ser Gly Leu Val Ala Thr Val Ser Gly Ser Gln Gln Thr Leu Val	
		370 375 380
75	Val Ala Leu Asn Ser Asp Leu Asp Asn Pro Gly Gln Val Ala Ser Gly	
		385 390 395 400

Ser Phe Ser Glu Ala Val Asn Ala Ser Asn Gly Gln Val Arg Val Trp
 405 410 415

5 Arg Ser Gly Ser Gly Asp Gly Gly Gly Asn Asp Gly Gly
 420 425

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Glu Ile Ile Leu Gln Gly Phe His Trp Asn Val Val Arg Glu Ala Pro
 20 25 30

25 Tyr Asn Trp Tyr Asn Ile Leu Arg Gln Lys Ala Ser Thr Ile Ala Ala
 35 40 45

30 Asp Gly Phe Ser Ala Ile Trp Met Pro Val Pro Trp Arg Asp Phe Ser
 50 55 60

35 Ser Trp Thr Asp Gly Asp Lys Ser Gly Gly Gly Glu Gly Tyr Phe Trp
 65 70 75 80

His Asp Phe Asn Lys Asn Gly Leu Tyr Gly Ser Asp Ala Gln Leu Arg
 85 90 95

40 Gln Ala Ala Gly Ala Leu Gly Gly Ala Gly Val Lys Val Leu Tyr Asp
 100 105 110

45 Val Val Pro Asn His Met Asn Arg Phe Tyr Pro Asp Lys Glu Ile Asn
 115 120 125

50 Leu Pro Ala Gly Gln Arg Phe Trp Arg Asn Asp Cys Pro Asp Pro Gly
 130 135 140

Asn Gly Pro Asn Asp Cys Asp Asp Gly Asp Arg Phe Leu Gly Gly Glu
 145 150 155 160

55 Ala Asp Leu Asn Thr Gly His Pro Gln Ile Tyr Gly Met Phe Arg Asp
 165 170 175

	Glu	Phe	Thr	Asn	Leu	Arg	Ser	Gly	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	Phe	
				180					185					190			
5	Asp	Phe	Val	Arg	Gly	Tyr	Ala	Pro	Glu	Arg	Val	Asp	Leu	Trp	Met	Ser	
			195					200					205				
10	Asp	Ser	Ala	Asp	Ser	Ser	Phe	Cys	Val	Gly	Glu	Leu	Trp	Lys	Ser	Pro	
		210					215					220					
15	Ser	Glu	Tyr	Pro	Pro	Trp	Asp	Trp	Arg	Asn	Arg	Ala	Ser	Trp	Gln	Gln	
	225					230					235					240	
20	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
				245						250					255		
25	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	
				260					265					270			
30	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Pro	Arg	Trp	Arg	Glu	Val	Ala	Val	Thr	
			275				280						285				
35	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Tyr	Ser	Pro	Gly	Gln	Asn	Gly	Gly	
	290						295					300					
40	Gln	His	Lys	Trp	Pro	Leu	Pro	Asp	Gly	Leu	Ile	Arg	Gln	Ala	Tyr	Ala	
	305					310					315					320	
45	Tyr	Ile	Leu	Thr	Ser	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met	
				325						330					335		
50	Tyr	Asp	Trp	Gly	Tyr	Gly	Asp	Phe	Ile	Arg	Gln	Leu	Ile	Gln	Val	Arg	
			340					345						350			
55	Arg	Thr	Ala	Gly	Val	Arg	Ala	Asp	Ser	Ala	Ile	Ser	Phe	His	Ser	Gly	
			355					360					365				
60	Tyr	Ser	Gly	Leu	Val	Ala	Thr	Val	Ser	Gly	Ser	Gln	Gln	Thr	Leu	Val	
	370						375					380					
65	Val	Ala	Leu	Asn	Ser	Asp	Leu	Asp	Asn	Pro	Gly	Gln	Val	Ala	Ser	Gly	
	385					390					395					400	
70	Ser	Phe	Ser	Glu	Ala	Val	Asn	Ala	Ser	Asn	Gly	Gln	Val	Arg	Val	Trp	
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75	Arg	Ser	Gly	Ser	Gly	Asp	Gly	Gly	Gly	Asn	Asp	Gly	Gly				
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Glu Ile Ile Leu Gln Gly Phe His Trp Asn Val Val Arg Glu Ala Pro
 20 25 30

Tyr Asn Trp Tyr Asn Ile Leu Arg Gln Lys Ala Ser Thr Ile Ala Ala
 35 40 45

Asp Gly Phe Ser Ala Ile Trp Met Pro Val Pro Trp Arg Asp Phe Ser
 50 55 60

Ser Trp Thr Asp Gly Asp Lys Ser Gly Gly Gly Glu Gly Tyr Phe Trp
 65 70 75 80

His Asp Phe Asn Lys Asn Gly Leu Tyr Gly Ser Asp Ala Gln Leu Arg
 85 90 95

Gln Ala Ala Gly Ala Leu Gly Gly Ala Gly Val Lys Val Leu Tyr Asp
 100 105 110

Val Val Pro Asn His Met Asn Arg Phe Tyr Pro Asp Lys Glu Ile Asn
 115 120 125

Leu Pro Ala Gly Gln Arg Phe Trp Arg Asn Asp Cys Pro Asp Pro Gly
 130 135 140

Asn Gly Pro Asn Asp Cys Asp Asp Gly Asp Arg Phe Leu Gly Gly Glu
 145 150 155 160

Ala Asp Leu Asn Thr Gly His Pro Gln Ile Tyr Gly Met Phe Arg Asp
 165 170 175

Glu Phe Thr Asn Leu Arg Ser Gly Tyr Gly Ala Gly Gly Phe Arg Phe
 180 185 190

Asp Phe Val Arg Gly Tyr Ala Pro Glu Arg Val Asp Leu Trp Met Ser
 195 200 205

	Asp	Ser	Ala	Asp	Ser	Ser	Phe	Cys	Val	Gly	Glu	Leu	Trp	Lys	Lys	Pro	
	210						215					220					
5	Ser	Glu	Tyr	Pro	Pro	Trp	Asp	Trp	Arg	Asn	Arg	Ala	Ser	Trp	Gln	Glu	
	225					230					235					240	
10	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
					245					250					255		
15	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	
				260					265					270			
20	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Pro	Arg	Trp	Arg	Glu	Val	Ala	Val	Thr	
			275					280					285				
25	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Tyr	Ser	Pro	Gly	Gln	Asn	Gly	Gly	
	290						295					300					
30	Gln	His	Lys	Trp	Pro	Leu	Pro	Asp	Gly	Leu	Ile	Arg	Gln	Ala	Tyr	Ala	
	305					310					315					320	
35	Tyr	Ile	Leu	Thr	Ser	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met	
					325					330					335		
40	Tyr	Asp	Trp	Gly	Tyr	Gly	Asp	Phe	Ile	Arg	Gln	Leu	Ile	Gln	Val	Arg	
				340					345					350			
45	Arg	Thr	Ala	Gly	Val	Arg	Ala	Asp	Ser	Ala	Ile	Ser	Phe	His	Ser	Gly	
			355					360					365				
50	Tyr	Ser	Gly	Leu	Val	Ala	Thr	Val	Ser	Gly	Ser	Gln	Gln	Thr	Leu	Val	
	370						375					380					
55	Val	Ala	Leu	Asn	Ser	Asp	Leu	Asp	Asn	Pro	Gly	Gln	Val	Ala	Ser	Gly	
	385					390					395					400	
60	Ser	Phe	Ser	Glu	Ala	Val	Asn	Ala	Glu	Asn	Gly	Gln	Val	Arg	Val	Trp	
					405					410					415		
65	Arg	Ser	Gly	Ser	Gly	Asp	Gly	Gly	Gly	Asn	Asp	Gly	Gly				
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75	<211> 429																
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<223> Mature protein sequence of pMS2177

<400> 32

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

	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
					245					250					255		
5	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	
				260					265					270			
10	Gly	Leu	Asn	Gly	Asn	Pro	Asp	Pro	Arg	Trp	Arg	Glu	Val	Ala	Val	Thr	
			275					280					285				
15	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Tyr	Ser	Pro	Gly	Gln	Asn	Gly	Gly	
	290						295					300					
20	Gln	His	Lys	Trp	Pro	Leu	Pro	Asp	Gly	Leu	Ile	Arg	Gln	Ala	Tyr	Ala	
	305					310					315					320	
25	Tyr	Ile	Leu	Thr	Ser	Pro	Gly	Thr	Pro	Val	Val	Tyr	Trp	Pro	His	Met	
					325					330					335		
30	Tyr	Asp	Trp	Gly	Tyr	Gly	Asp	Phe	Ile	Arg	Gln	Leu	Ile	Gln	Val	Arg	
				340					345					350			
35	Arg	Thr	Ala	Gly	Val	Arg	Ala	Asp	Ser	Ala	Ile	Ser	Phe	His	Ser	Gly	
			355					360					365				
40	Tyr	Ser	Gly	Leu	Val	Ala	Thr	Val	Ser	Gly	Ser	Gln	Gln	Thr	Leu	Val	
		370					375					380					
45	Val	Ala	Leu	Asn	Ser	Asp	Leu	Asp	Asn	Pro	Gly	Gln	Val	Ala	Ser	Gly	
	385					390					395					400	
50	Ser	Phe	Ser	Glu	Ala	Val	Asn	Ala	Glu	Asn	Gly	Gln	Val	Arg	Val	Trp	
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				20					25					30			
5	Tyr	Asn	Trp	Tyr	Asn	Ile	Leu	Arg	Gln	Lys	Ala	Ser	Thr	Ile	Ala	Ala	
			35					40					45				
	Asp	Gly	Phe	Ser	Ala	Ile	Trp	Met	Pro	Val	Pro	Trp	Arg	Asp	Phe	Ser	
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	Ser	Trp	Thr	Asp	Gly	Asp	Lys	Ser	Gly	Gly	Gly	Glu	Gly	Tyr	Phe	Trp	
	65					70					75					80	
15																	
	His	Asp	Phe	Asn	Lys	Asn	Gly	Leu	Tyr	Gly	Ser	Asp	Ala	Gln	Leu	Arg	
					85					90					95		
	Gln	Ala	Ala	Gln	Ala	Leu	Gly	Gly	Ala	Gly	Val	Lys	Val	Leu	Tyr	Asp	
20				100					105					110			
	Val	Val	Pro	Asn	His	Met	Asn	Arg	Phe	Tyr	Pro	Asp	Lys	Glu	Ile	Asn	
25			115					120					125				
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	145					150					155					160	
	Ala	Asp	Leu	Asn	Thr	Gly	His	Pro	Gln	Ile	Tyr	Gly	Met	Phe	Arg	Asp	
35					165					170					175		
	Glu	Phe	Thr	Asn	Leu	Arg	Ser	Gly	Tyr	Gly	Ala	Gly	Gly	Phe	Arg	Phe	
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45	Asp	Ser	Ala	Asp	Ser	Ser	Phe	Cys	Val	Gly	Glu	Leu	Trp	Lys	Lys	Pro	
		210					215					220					
	Ser	Glu	Tyr	Pro	Pro	Trp	Asp	Trp	Arg	Asn	Arg	Ala	Ser	Trp	Gln	Glu	
50		225				230					235					240	
	Ile	Ile	Lys	Asp	Trp	Ser	Asp	Arg	Ala	Lys	Cys	Pro	Val	Phe	Asp	Phe	
					245					250					255		
55																	
	Ala	Leu	Lys	Glu	Arg	Met	Gln	Asn	Gly	Ser	Val	Ala	Asp	Trp	Lys	His	
				260					265					270			

Gly Leu Asn Gly Asn Pro Asp Pro Arg Trp Arg Glu Val Ala Val Thr
 275 280 285

5 Phe Val Asp Asn His Asp Thr Gly Tyr Ser Pro Gly Gln Asn Gly Gly
 290 295 300

10 Gln His Lys Trp Pro Leu Pro Asp Gly Leu Ile Arg Gln Ala Tyr Ala
 305 310 315 320

Tyr Ile Leu Thr Ser Pro Gly Thr Pro Val Val Tyr Trp Pro His Met
 325 330 335

15 Tyr Asp Trp Gly Tyr Gly Asp Phe Ile Arg Gln Leu Ile Gln Val Arg
 340 345 350

20 Arg Thr Ala Gly Val Arg Ala Asp Ser Ala Ile Ser Phe His Ser Gly
 355 360 365

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REFERENCES CITED IN THE DESCRIPTION

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AMYLASEPOLYPEPTID

PATENTKRAV

1. Polypeptid med amylaseaktivitet, der omfatter en aminosyresekvens med mindst 78 % sekvensidentitet med aminosyresekvensen ifølge SEQ ID NO: 1, og hvor polypeptidet omfatter en
5 aminosyresubstitution ved position: 240, med reference til positionsnummereringen fra sekvensen vist som SEQ ID NO: 1; hvor polypeptidet har forbedret termostabilitet sammenlignet med amylasen af SEQ ID NO: 1.
2. Polypeptid ifølge krav 1, hvor polypeptidet omfatter én eller flere aminosyresubstitutioner ved følgende positioner: 235, 88, 205, 248, 266, 311, 377 eller 409 og/eller én eller flere af følgende aminosyresubstitutioner: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D eller 392K/D/E/Y/N/Q/R/S/T/G
10 og/eller den følgende aminosyresubstitution: 240E/H/M/D/S.
3. Polypeptid ifølge krav 1 eller 2, hvor polypeptidet endvidere omfatter aminosyren 223S/K/A.
4. Polypeptid ifølge et hvilket som helst af kravene 1-3 med mindst 90, 91, 92, 93, 94, 95, 96, 97, 98, eller 99 % sekvensidentitet med aminosyresekvensen ifølge SEQ ID NO: 1.
5. Polypeptid ifølge et hvilket som helst af kravene 1-4 med en linker kondenseret ved C-terminus.
- 15 6. Polypeptid ifølge et hvilket som helst af kravene 1-5 med exoamylaseaktivitet, fortrinsvis ikke-maltogen exoamylaseaktivitet.
7. Nukleinsyre, der er i stand til at kode for et polypeptid ifølge et hvilket som helst af kravene 1-6.
8. Ekspressionsvektor, der omfatter en nukleinsyre ifølge krav 7, eller som er i stand til at udtrykke et polypeptid ifølge et hvilket som helst af kravene 1-6.
- 20 9. Plasmid, der omfatter en nukleinsyre ifølge krav 7.
10. Værtscelle, der omfatter en ekspressionsvektor ifølge krav 8 eller et plasmid ifølge krav 9.
11. Værtscelle transformeret med en ekspressionsvektor ifølge krav 8 eller et plasmid ifølge krav 9.
12. Anvendelse af et polypeptid ifølge et hvilket som helst af kravene 1-6 som et fødevare- eller foderadditiv.
- 25 13. Fremgangsmåde til fremstilling af en saccharidsirup, hvilken fremgangsmåde omfatter tilsætning af et polypeptid ifølge et hvilket som helst af kravene 1-6 til en granuløs stivelseslikvefaktion for at danne saccharidsiroppen.
14. Fødevareprodukt, der omfatter et polypeptid ifølge et hvilket som helst af kravene 1-6.

DRAWINGS

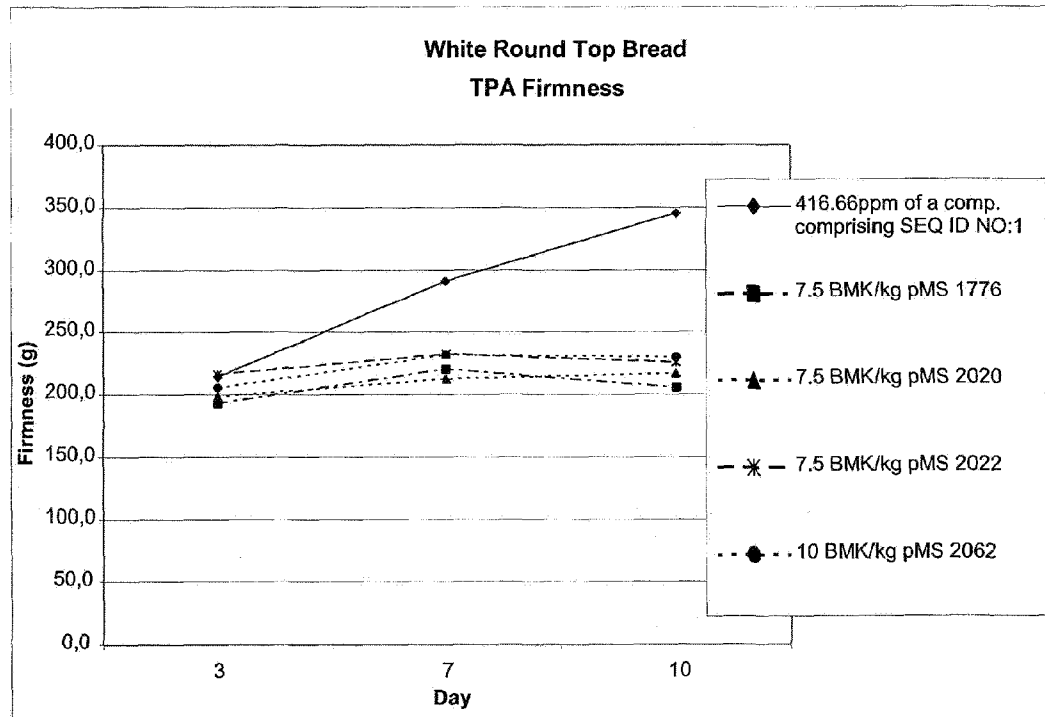


Fig. 1

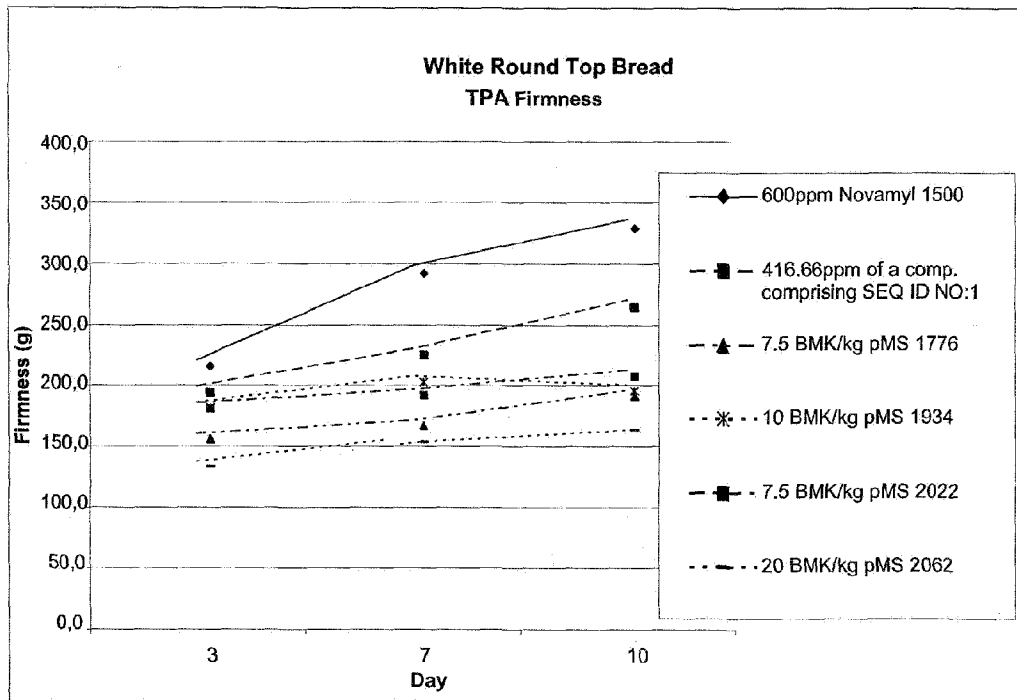


Fig. 2