(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 December 2016 (29.12.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/210395 Al

(51) International Patent Classification:
C12P 21/06 (2006.01) *C12N 15/80* (2006.01)
C12N 9/48 (2006.01)

(21) International Application Number:
PCT/US20 16/039494

(22) International Filing Date:
27 June 2016 (27.06.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/185,503 26 June 2015 (26.06.2015) US
62/235,937 1 October 201 5 (01. 10.2015) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: AMINOPEPTIDASES FOR PROTEIN HYDRLYZATES

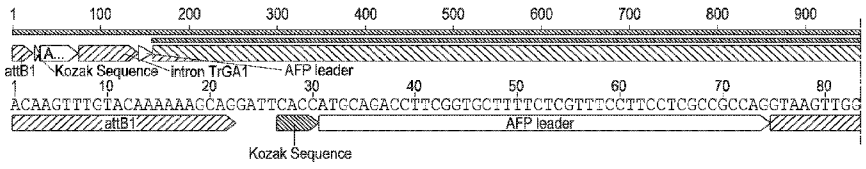

FIG. 1

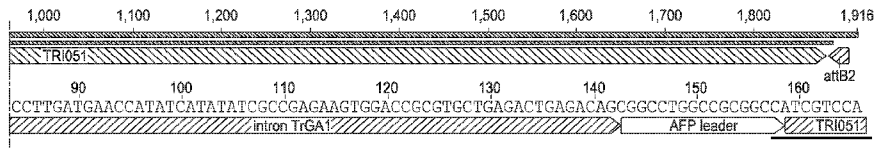

FIG. 1 Continued

(57) Abstract: The present disclosure provides polypeptides having aminopeptidase activity and isolated nucleic acid sequences encoding the polypeptides. In some embodiments, the disclosure also provides to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing the polypeptides. In some embodiments, the present disclosure further provides to methods of obtaining protein hydrolysates useful as flavor improving agents.

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TITLE

AMINOPEPTIDASES FOR PROTEIN HYDRLYZATES

CROSS-REFERENCE TO RELATED APPLICATIONS

This applications claims priority to and the benefit of United States provisional patent application numbers 62/185503, filed June 26, 2015; and 62/235937, filed October 1, 2015; each provisional application titled "NOVEL AMINOPEPTIDASES FOR PROTEIN HYDROLYSATES".

INCORPORATION BY REFERENCE OF THE SEQUENCE LISTING

The sequence listing provided in the file named "20160610_NB40989-PCT sequence listing prj_ST25.txt" with a size of 192,505 bytes which was created on June 10, 2016 and which is filed herewith, is incorporated by reference herein in its entirety.

BACKGROUND

[0001] Various food and feed products contain protein hydrolysates. This hydrolysis was conventionally accomplished using chemical hydrolysis. However, such chemical hydrolysis led to severe degradation of the amino acids obtained during the hydrolysis, and also to hazardous byproducts formed in the course of the chemical reaction. Increasing concern over the use of protein hydrolysates obtained by chemical hydrolysis has led to the development of enzymatic hydrolysis processes.

[0002] Enzymatic hydrolysis processes of proteinaceous materials aim at obtaining a high degree of hydrolysis. Polypeptides having aminopeptidase activity catalyze the removal of one or more amino acid residues from the N-terminus of peptides, polypeptides, and proteins. The production of protein hydrolysates with desirable properties and high degrees of hydrolysis generally requires the use of a mixture of peptidase activities. It would be desirable to provide a single component peptidase enzyme which has activity useful for improving the properties and degree of hydrolysis of protein hydrolysates used in food and or feed products either alone or in combination with other enzymes.

[0003] The present disclosure provides novel polypeptides having improved aminopeptidase activity as well as methods for obtaining protein hydrolysates with desirable qualities and high degrees of hydrolysis.

BRIEF DESCRIPTION OF THE DRAWINGS

[0004] A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0005] Figure 1 shows on the top an example of the overall design of the synthetic genes encoding the pepN_2's enzymes, and at the bottom a close-up of the leader sequence that was used instead of the endogenous secretion signal sequences.

[0006] Figure 2 shows pH dependent glutamic acid liberation of the PepN 2 from *Neosartorya fischeri* in gluten hydrolyses (no pH-control during hydrolyses).

[0007] Figure 3 shows Temperature dependent glutamic acid liberation of the PepN 2 from *Neosartoryafischeri* in gluten hydrolyses (no pH-control during hydrolyses).

[0008] Figure 4 shows glutamic acid liberation of different PepN 2.

[0009] Figure 5 shows hydrolysis of WHWLQLKPGQPMY (SEQ ID NO: 26)

[0010] Figure 6 shows hydrolysis of KPGQPMY. (SEQ ID NO: 27)

[0011] Figure 7 shows hydrolysis of QPMY. (SEQ ID NO: 28)

[0012] Figure 8 shows peptide sequences of the substrate as well as cleavage products were typed into Skyline and intensities were calculated in each sample.

[0013] Figure 9 shows the spatial arrangement of six panels Fig. 9A-9F which detail a sequence alignment of several PepN 2 enzymes.

[0014] Figure 10 Hydrolysis of TPAAAR (SEQ ID NO: 29) by TRI032, TRI035, TRI063 (A. oryzae) as well as COROLASE® LAP (fungal exo-peptidase;

[0015] DETAILED DESCRIPTION OF THE INVENTION

[0016] The present disclosure provides polypeptides having aminopeptidase activity. The present invention also relates to nucleic acid sequences encoding the polypeptides and to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing the polypeptides. The present disclosure also relates to methods for obtaining

hydrolysates from proteinaceous substrates which comprise subjecting the proteinaceous material to a polypeptide with aminopeptidase activity alone or in combination with a protease, e.g. an endopeptidase. The present disclosure also relates to methods for obtaining from a proteinaceous substrate a hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues, which methods comprise subjecting the substrate to a polypeptide having aminopeptidase activity. The present disclosure further relates to flavor improving compositions comprising a polypeptide with aminopeptidase activity. The compositions may further comprise additional enzymatic activities.

[0017] In another aspect, the methods described herein may be used in food related applications to improve flavor, such as baking. Alternatively, flavor improvement in foods may be achieved by the addition of hydrolysates obtained by the methods of the invention. In some embodiments, the hydrolysate produced using the aminopeptidases described herein may also have reduced bitterness when compared to an untreated hydrolysate.

[0018] In some embodiments, the invention provides new fungal aminopeptidases (PepNs), their high yield production in a production host (e.g. *Trichoderma reesei)*, and their use for the generation of protein hydrolysates, e.g., for debittering and for glutamate production. As demonstrated in Example 9, aminopeptidases type 2 according to the invention showed improved glutamate release compared with aminopeptidases type 1. Initial results suggest that these PepNs are tolerant to Proline in PΓ which is surprising compared to other known aminopeptidases. Thus, the proline tolerant aminopeptidases taught for use in the present invention are capable of acting on a wide range of peptide and/or protein substrates and due to having such a broad substrate-specificity are not readily inhibited from cleaving substrates enriched in certain amino acids (e.g. proline and/or lysine and/or arginine and/or glycine).

[0019] The present inventors surprisingly found that aminopeptidases type 2 have better performance that aminopeptidases type 1. Furthermore, the present inventors surprisingly found a group of fungal aminopeptidases type 2 with improved performance. This group of fungal aminopeptidases type 2 has a longer N-terminal in their mature amino acid sequences. Figure 9 shows the amino acid sequence alignment of several aminopeptidases type 2.

[0020] The term "aminopeptidase activity" is defined herein as a peptidase activity which catalyzes the removal of amino acids from the N-terminal end of peptides, oligopeptides or proteins. Defined in a general manner, the aminopeptidase activity is capable of cleaving the

amino acid X from the N-terminus of a peptide, polypeptide, or protein, wherein X may represent any amino acid residue selected from the group consisting of Ala, Arg, Asn, Asp, Cys, Gin, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val, but at least Leu, Glu, Gly, Ala, and/or Pro. It will be understood that the polypeptides having aminopeptidase activity of the present invention may be unspecific as to the amino acid to be cleaved of the N-terminus of the peptide, polypeptide substrate.

[0021] In some embodiments, the present invention relates to an isolated polypeptide having aminopeptidase activity which has a predicted mature sequence with more than 13 residues on the N-terminal side of the conserved residue I/V in position 67 as shown in the sequence alignment of Figure 9, or a fragment thereof, wherein the fragment has aminopeptidase activity.

[0022] In some embodiments, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to the mature amino acid sequence of SEQ ID NO: 1 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have aminopeptidase activity (hereinafter "homologous polypeptides"). In some embodiments, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 1. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 1, the mature amino sequence or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 1. In another embodiment, the polypeptide of the present invention has the amino acid sequence of SEQ ID NO: 1 or a fragment thereof, wherein the fragment has aminopeptidase activity. A fragment of SEQ ID NO: 1 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. In some embodiments the fragment comprises the sequence of SEQ ID NO: 18. In some embodiments, the polypeptide has the amino acid sequence of SEQ ID NO: 1.

[0023] In some embodiments, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to the mature amino acid sequence of SEQ ID NO: 2 of at least about 50%, preferably at least about 60%, preferably at least about 70%,

more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have aminopeptidase activity (hereinafter "homologous polypeptides"). In some embodiments, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 2. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 2, the mature amino sequence or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 2. In another embodiment, the polypeptide of the present invention has the amino acid sequence of SEQ ID NO: 2 or a fragment thereof, wherein the fragment has aminopeptidase activity. A fragment of SEQ ID NO: 2 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. In some embodiments the fragment comprises the sequence of SEQ ID NO: 19. In some embodiments, the polypeptide has the amino acid sequence of SEQ ID NO: 2.

[0024] In some embodiments, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to the mature amino acid sequence of SEQ ID NO: 3 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have aminopeptidase activity (hereinafter "homologous polypeptides"). In some embodiments, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 3. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 3, the mature amino sequence or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 3. In another embodiment, the polypeptide of the present invention has the amino acid sequence of SEQ ID NO: 3 or a fragment thereof, wherein the fragment has aminopeptidase activity. A fragment of SEQ ID NO: 3 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of

this amino acid sequence. In some embodiments the fragment comprises the sequence of SEQ ID NO:20. In some embodiments, the polypeptide has the amino acid sequence of SEQ ID NO: 3.

[0025] In some embodiments, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to the mature amino acid sequence of SEQ ID NO: 4 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have aminopeptidase activity (hereinafter "homologous polypeptides"). In some embodiments, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 4. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 4, the mature amino sequence or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 4. In another embodiment, the polypeptide of the present invention has the amino acid sequence of SEQ ID NO: 4 or a fragment thereof, wherein the fragment has aminopeptidase activity. A fragment of SEQ ID NO: 4 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. In some embodiments the fragment comprises the sequence of SEQ ID NO:21. In some embodiments, the polypeptide has the amino acid sequence of SEQ ID NO: 4.

[0026] In some embodiments, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to the mature amino acid sequence of SEQ ID NO: 5 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have aminopeptidase activity (hereinafter "homologous polypeptides"). In some embodiments, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 5. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 5, the mature amino sequence or an allelic variant; and a fragment thereof, wherein

the fragment has aminopeptidase activity. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 5. In another embodiment, the polypeptide of the present invention has the amino acid sequence of SEQ ID NO: 5 or a fragment thereof, wherein the fragment has aminopeptidase activity. A fragment of SEQ ID NO: 5 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. In some embodiments the fragment comprises the sequence of SEQ ID NO:22. In some embodiments, the polypeptide has the amino acid sequence of SEQ ID NO: 5.

[0027] In some embodiments, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to the mature amino acid sequence of SEQ ID NO: 6 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have aminopeptidase activity (hereinafter "homologous polypeptides"). In some embodiments, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 6. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 6, the mature amino sequence or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 6. In another embodiment, the polypeptide of the present invention has the amino acid sequence of SEQ ID NO: 6 or a fragment thereof, wherein the fragment has aminopeptidase activity. A fragment of SEQ ID NO: 6 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. In some embodiments the fragment comprises the sequence of SEQ ID NO:23. In some embodiments, the polypeptide has the amino acid sequence of SEQ ID NO: 6.

[0028] In some embodiments, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to the mature amino acid sequence of SEQ ID NO: 7 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have aminopeptidase activity (hereinafter "homologous polypeptides"). In some embodiments, the homologous

polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 7. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 7, the mature amino sequence or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 7. In another embodiment, the polypeptide of the present invention has the amino acid sequence of SEQ ID NO: 7 or a fragment thereof, wherein the fragment has aminopeptidase activity. A fragment of SEQ ID NO: 7 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. In some embodiments the fragment comprises the sequence of SEQ ID NO:24. In some embodiments, the polypeptide has the amino acid sequence of SEQ ID NO: 7.

[0029] In some embodiments, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to the mature amino acid sequence of SEQ ID NO: 8 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have aminopeptidase activity (hereinafter "homologous polypeptides"). In some embodiments, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 8. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 8, the mature amino sequence or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 8. In another embodiment, the polypeptide of the present invention has the amino acid sequence of SEQ ID NO: 8 or a fragment thereof, wherein the fragment has aminopeptidase activity. A fragment of SEQ ID NO: 8 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. In some embodiments the fragment comprises the sequence of SEQ ID NO:25. In some embodiments, the polypeptide has the amino acid sequence of SEQ ID NO: 8.

[0030] Preferably, a fragment contains at least 330 amino acid residues, more preferably at least 380 amino acid residues, and most preferably at least 430 amino acid residues.

[0031] An allelic variant denotes any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations.

[0032] The "parent enzyme" as used herein is an aminopeptidase enzyme which has all of the amino acid residues of the polypeptide as shown in SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8. In this respect, and for example, the parent will have all of the modifications (which may be zero, one or more than one depending on the variant) of the variant polypeptide.

[0033] In some embodiments, the present invention relates to isolated polypeptides having aminopeptidase activity which are encoded by nucleic acid sequences which hybridize under low stringency conditions, more preferably medium stringency conditions, and most preferably high stringency conditions, with an oligonucleotide probe which hybridizes under the same conditions with the polypeptide encoding part of nucleic acid sequence of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16 or its complementary strand (J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, New York); or allelic variants and fragments of the polypeptides, wherein the fragments have aminopeptidase activity.

[0034] Hybridization indicates that the nucleic acid sequence hybridizes to the oligonucleotide probe corresponding to the polypeptide encoding part of the nucleic acid sequence shown in SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16, under low to high stringency conditions (i.e., prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 Pg/ml sheared and denatured salmon sperm DNA, and either 25, 35 or 50% formamide for low, medium and high stringencies, respectively), following standard Southern blotting procedures.

[0035] The amino acid sequence of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8, or a partial sequence thereof may be used to design an oligonucleotide probe, or a nucleic acid sequence encoding a polypeptide of the present invention, such as the polypeptide encoding part

of nucleic acid sequence of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16, or a subsequence thereof, may be used to identify and clone DNA encoding polypeptides having aminopeptidase activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, preferably at least 25, and more preferably at least 40 nucleotides in length. Longer probes can also be used. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with 32P, 3H, 35S, biotin, or avidin).

[0036] Thus, a genomic, cDNA or combinatorial chemical library prepared from such other organisms may be screened for DNA which hybridizes with the probes described above and which encodes a polypeptide having aminopeptidase activity. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA which is homologous with the polypeptide encoding part of nucleic acid sequence of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16, the carrier material is used in a Southern blot in which the carrier material is finally washed three times for 30 minutes each using 2 x SSC, 0.2% SDS preferably at least 50°C, more preferably at least 55°C, more preferably at least 60°C, more preferably at least 65°C, even more preferably at least 70°C, and most preferably at least 75°C. Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using X-ray film.

[0037] The terms "modifying" and "modification" as used herein refers to a substitution when compared with the wild-type aminopeptidase polypeptide sequence of the closest identity. The comparison is made by aligning both the variant aminopeptidase polypeptide and the wild-type aminopeptidase with the reference sequence shown as SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8.

[0038] Likewise, "equivalent modification" as used herein refers to carrying out the same modification (usually a substitution) to an amino acid which is at an equivalent position in another aminopeptidase.

[0039] In one aspect, the aminopeptidase sequence according to the present invention is in an isolated form. The term "isolated" means that the aminopeptidase sequence is at least substantially free from at least one other component with which the aminopeptidase sequence is naturally associated in nature and as found in nature. The aminopeptidase sequence of the present invention may be provided in a form that is substantially free of one or more contaminants with which the substance might otherwise be associated. Thus, for example it may be substantially free of one or more potentially contaminating polypeptides and/or nucleic acid molecules.

[0040] In one aspect, the aminopeptidase sequence according to 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 component present in a composition. Preferably, the aminopeptidase is present at a level of at least about 90%, or at least about 95% or at least about 98%, said level being determined on a dry weight/dry weight basis with respect to the total composition under consideration.

[0041] As used herein, the singular "a," "an" and "the" includes the plural unless the context clearly indicates otherwise. Unless otherwise indicated, nucleic acid sequences are written left to right in 5' to 3' orientation; and amino acid sequences are written left to right in amino to carboxy orientation. It is to be understood that this disclosure is not limited to the particular methodology, protocols, and reagents described herein, absent an indication to the contrary.

[0042] Terms and abbreviations not defined should be accorded their ordinary meaning as used in the art. Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. Unless otherwise indicated, the practice of the present disclosure involves conventional techniques commonly used in molecular biology, protein engineering, and microbiology. Although any methods and materials similar or equivalent to those described herein find use in the practice of the present disclosure, some suitable methods and materials are described herein. The terms defined immediately below are more fully described by reference to the Specification as a whole.

Nucleotide sequence

[0043] The scope of the present invention encompasses nucleotide sequences encoding aminopeptidases having the specific properties as defined herein.

[0044] In some embodiments, the nucleic acid sequence encodes a polypeptide obtained from *Aspergillus,* e.g., *Aspergillus clavatus.* In some embodiments, the nucleic acid sequence encodes a polypeptide obtained from *Neosartorya,* e.g. *Neosartorya fischeri.*

[0045] In some embodiments, the present invention relates to a nucleic acid encoding a polypeptide having aminopeptidase activity which has a predicted mature sequence with more than 13 residues on the N-terminal side of the conserved residue I/V in position 67 as shown in the sequence alignment of Figure 9, or a fragment thereof, wherein the fragment has aminopeptidase activity.

[0046] In some embodiments, the polynucleotide of the present invention is a polynucleotide having a specified degree of nucleic acid homology to the exemplified polynucleotide. In some embodiments, the polynucleotide comprises a nucleic acid sequence having at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to the polypeptide encoding part of nucleic acid sequence of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16. In some embodiments, the polynucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16. In other embodiments, the polynucleotide of the present invention may also have a complementary nucleic acid sequence to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16. In some embodiments, the polynucleotide comprises a nucleic acid sequence encoding a recombinant polypeptide or an active fragment thereof, comprising an amino acid sequence having at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% identity to the amino acid sequence selected from the group consisting of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8. In some embodiments, the polynucleotide comprises a nucleic acid sequence encoding a recombinant polypeptide or an active fragment thereof, comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ

ID NO: 7, or SEQ ID NO: 8. Homology can be determined by amino acid sequence alignment, e.g., using a program such as BLAST, ALIGN, or CLUSTAL, as described herein.

[0047] The present invention also encompasses nucleic acid sequences which encode a polypeptide having the amino acid sequence of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8, which differ from SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8 by virtue of the degeneracy of the genetic code. The present invention also relates to subsequences of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16 which encode fragments of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8 which have aminopeptidase activity. A subsequence of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16 is a nucleic acid sequence encompassed by SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16 except that one or more nucleotides from the 5' end and/or 3' end have been deleted. Preferably, a subsequence contains at least 990 nucleotides, more preferably at least 1140 nucleotides, and most preferably at least 1290 nucleotides.

[0048] The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-stranded or single-stranded whether representing the sense or anti-sense strand.

[0049] The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA sequence coding for the present invention.

[0050] In a preferred embodiment, the nucleotide sequence when relating to and when encompassed by the *per se* scope of the present invention does not include the native nucleotide sequence according to the present invention when in its natural environment and when it is linked to its naturally associated sequence(s) that is/are also in its/their natural environment. For ease of reference, we shall call this preferred embodiment the "non-native nucleotide sequence". In this

regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally associated, which promoter is also in its native environment. However, the amino acid sequence encompassed by scope the present invention can be isolated and/or purified post expression of a nucleotide sequence in its native organism. Preferably, however, the amino acid sequence encompassed by scope of the present invention may be expressed by a nucleotide sequence in its native organism but wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

[0051] Typically, the nucleotide sequence encompassed by the scope of the present invention is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an alternative embodiment of the invention, the nucleotide sequence could be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al,* (1980) *Nuc Acids Res Symp Ser* 215-23 and Horn T *et al,* (1980) *Nuc Acids Res Symp Ser* 225-232).

[0052] The present invention also relates to nucleic acid sequences which have a degree of homology to the nucleic acid sequence of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16of at least about 50%, preferably about 60%, preferably about 70%, preferably about 80%, more preferably about 90%, even more preferably about 95%, and most preferably about 97% homology, which encode an active polypeptide. For purposes of the present invention, the degree of homology between two nucleic acid sequences is determined by the CLUSTAL method (Higgins, 1989, supra) with an identity table, a gap penalty of 10, and a gap length penalty of 10.

[0053] Modification of a nucleic acid sequence encoding a polypeptide of the present invention may be necessary for the synthesis of polypeptides substantially similar to the polypeptide. The term "substantially similar" to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source. For example, it may be of interest to synthesize variants of the polypeptide where the variants differ in specific activity, thermostability, pH optimum, or the like using, e.g., site-directed mutagenesis. The analogous sequence may be constructed on the basis of the nucleic acid sequence presented as the polypeptide encoding part of nucleic acid sequence of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16, e.g., a subsequence thereof,

and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the polypeptide encoded by the nucleic acid sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, e.g., Ford *et al.,* 1991, *Protein Expression and Purification* 2: 95-107.

[0054] It will be apparent to those skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the polypeptide encoded by the isolated nucleic acid sequence of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine scanning mutagenesis (see, e.g., Cunningham and Wells, 1989, Science 244: 1081-1085). In the latter technique, mutations are introduced at every positively charged residue in the molecule, and the resultant mutant molecules are tested for aminopeptidase activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of the three-dimensional structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (see, e.g., de Vos *et al.,* 1992, *Science* 255: 306-312; Smith *et al.,* 1992, *Journal of Molecular Biology* 224: 899-904; Wlodaver *et al.,* 1992, *FEBS Letters* 309: 59-64).

Compositions

[0055] In one aspect, the present invention also relates to compositions comprising aminopeptidases and amino acid sequences and/or nucleotide sequences as described herein.

[0056] In some embodiments, the present invention provides compositions comprising a polypeptide having aminopeptidase activity which has a predicted mature sequence with more than 13 residues on the N-terminal side of the conserved residue I/V in position 67 as shown in the sequence alignment of Figure 9, or a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise polypeptides having at least about 50%, preferably at least about 60%>, preferably at least about 70%, more preferably at least about 80%>, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97% homology to a polypeptide having aminopeptidase

activity which has a predicted mature sequence with more than 13 residues on the N-terminal side of the conserved residue I/V in position 67 as shown in the sequence alignment of Figure 9 .

[0057] In some embodiments, the present invention provides a general composition comprising at least one aminopeptidase having an amino acid sequence which has a degree of identity to the amino acid sequence of SEQ ID NO: 1 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%. In some embodiments, the compositions comprise homologous polypeptides having an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 1. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 1 or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 1. In another embodiment, the compositions comprise an aminopeptidase having the amino acid sequence of SEQ ID NO: lor a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise a polypeptide having the amino acid sequence of SEQ ID NO: 1

[0058] In some embodiments, the present invention provides a general composition comprising at least one aminopeptidase having an amino acid sequence which has a degree of identity to the amino acid sequence of SEQ ID NO: 2 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%. In some embodiments, the compositions comprise homologous polypeptides having an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 2. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 2 or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 2. In another embodiment, the compositions comprise an aminopeptidase having the amino acid sequence of SEQ ID NO: 2 or a fragment

thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise a polypeptide having the amino acid sequence of SEQ ID NO: 2.

[0059] In some embodiments, the present invention provides a general composition comprising at least one aminopeptidase having an amino acid sequence which has a degree of identity to the amino acid sequence of SEQ ID NO: 3 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%. In some embodiments, the compositions comprise homologous polypeptides having an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 3. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 3 or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 3. In another embodiment, the compositions comprise an aminopeptidase having the amino acid sequence of SEQ ID NO: 3 or a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise a polypeptide having the amino acid sequence of SEQ ID NO: 3.

[0060] In some embodiments, the present invention provides a general composition comprising at least one aminopeptidase having an amino acid sequence which has a degree of identity to the amino acid sequence of SEQ ID NO: 4 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%. In some embodiments, the compositions comprise homologous polypeptides having an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 4. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 4 or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 4. In another embodiment, the compositions comprise an aminopeptidase having the amino acid sequence of SEQ ID NO: 4 or a fragment

thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise a polypeptide having the amino acid sequence of SEQ ID NO: 4.

[0061] In some embodiments, the present invention provides a general composition comprising at least one aminopeptidase having an amino acid sequence which has a degree of identity to the amino acid sequence of SEQ ID NO: 5 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%. In some embodiments, the compositions comprise homologous polypeptides having an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 5. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 5 or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 5. In another embodiment, the compositions comprise an aminopeptidase having the amino acid sequence of SEQ ID NO: 5 or a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise a polypeptide having the amino acid sequence of SEQ ID NO: 5.

[0062] In some embodiments, the present invention provides a general composition comprising at least one aminopeptidase having an amino acid sequence which has a degree of identity to the amino acid sequence of SEQ ID NO: 6 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%. In some embodiments, the compositions comprise homologous polypeptides having an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 6. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 6 or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 6. In another embodiment, the compositions comprise an aminopeptidase having the amino acid sequence of SEQ ID NO: 6 or a fragment

thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise a polypeptide having the amino acid sequence of SEQ ID NO: 6.

[0063] In some embodiments, the present invention provides a general composition comprising at least one aminopeptidase having an amino acid sequence which has a degree of identity to the amino acid sequence of SEQ ID NO: 7 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%. In some embodiments, the compositions comprise homologous polypeptides having an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 7. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 7 or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 7. In another embodiment, the compositions comprise an aminopeptidase having the amino acid sequence of SEQ ID NO: 7 or a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise a polypeptide having the amino acid sequence of SEQ ID NO: 7.

[0064] In some embodiments, the present invention provides a general composition comprising at least one aminopeptidase having an amino acid sequence which has a degree of identity to the amino acid sequence of SEQ ID NO: 8 of at least about 50%, preferably at least about 60%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%. In some embodiments, the compositions comprise homologous polypeptides having an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence of SEQ ID NO: 8. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 8 or an allelic variant; and a fragment thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise the amino acid sequence of SEQ ID NO: 8. In another embodiment, the compositions comprise an aminopeptidase having the amino acid sequence of SEQ ID NO: 8 or a fragment

thereof, wherein the fragment has aminopeptidase activity. In some embodiments, the compositions comprise a polypeptide having the amino acid sequence of SEQ ID NO: 8.

[0065] In some embodiments, the compositions comprise a polynucleotide having a specified degree of nucleic acid homology to the exemplified polynucleotide. In some embodiments, the compositions comprise a polynucleotide comprising a nucleic acid sequence having at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to the nucleic acid sequence of the polypeptide encoding part of nucleic acid sequence of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16. In some embodiments, the polynucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16 In other embodiments, the compositions comprise a polynucleotide with a complementary nucleic acid sequence to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 9, or SEQ ID NO: 10, or SEQ ID NO: 11, or SEQ ID NO: 12, or SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 16. In some embodiments, the compositions comprise a polynucleotide comprising a nucleic acid sequence encoding a recombinant polypeptide or an active fragment thereof, comprising an amino acid sequence having at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% identity to the amino acid sequence selected from the group consisting of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8. In some embodiments, the compositions comprise a polynucleotide comprising a nucleic acid sequence encoding a recombinant polypeptide or an active fragment thereof, comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8.

[0066] In some embodiments, the composition may comprise multiple enzymatic activities, such as an aminopeptidase, an amylase, a carbohydrase, a carboxypeptidase, a catalase, a cellulase, a chitinase, a cutinase, a cyclodextrin glycosyltransferase, a deoxyribonuclease, an esterase, an alpha-galactosidase, a beta-galactosidase, a glucoamylase, an alpha-glucosidase, a beta-glucosidase, a haloperoxidase, an invertase, a laccase, a lipase, a mannosidase, an oxidase, a pectinolytic enzyme, a peptidoglutaminase, a peroxidase, a phytase, a polyphenoloxidase, a

proteolytic enzyme, a ribonuclease, a transglutaminase, or a xylanase. The additional enzyme(s) may be producible by means of a microorganism belonging to the genus *Aspergillus,* preferably *Aspergillus aculeatus, Aspergillus awamori, Aspergillus niger,* or *Aspergillus oryzae,* or *Trichoderma, Humicola,* preferably *Humicola insolens,* or *Fusarium,* preferably *Fusarium bactridioides, Fusarium cerealis, Fusarium crookwellense, Fusarium culmorum, Fusarium graminearum, Fusarium graminum, Fusarium heterosporum, Fusarium negundi, Fusarium oxysporum, Fusarium reticulatum, Fusarium roseum, Fusarium sambucinum, Fusarium sarcochroum, Fusarium sporotrichioides, Fusarium sulphureum, Fusarium torulosum, Fusarium trichothecioides,* or *Fusarium venenaium.*

[0067] In some embodiments, the invention relates to compositions comprising a polypeptide with aminopeptidase activity and a suitable carrier. Any suitable carrier known in the art may be used including those described herein. In another embodiment, the compositions further comprise an endopeptidase. In some embodiments, the compositions further comprise one or more unspecific-acting endo- and/or exo-peptidase enzymes. In some embodiments, the compositions further comprise one or more specific-acting endo- and/or exo-peptidase enzymes.

[0068] In some embodiments, the specific acting proteolytic enzyme is an endopeptidase such as a glutamylendopeptidase (EC 3.4.21.19); a lysyl endopeptidase (EC 3.4.21.50); a leucyl endopeptidase (EC 3.4.21.57); a glycyl endopeptidase (EC 3.4.22.25); a prolyl endopeptidase (EC 3.4.21.26); trypsin (EC 3.4.21.4) or a trypsin-like (lysine/arginine specific) endopeptidase; or a peptidyl-Asp metalloendopeptidase (EC 3.4.24.33).

[0069] In some embodiments, the exopeptidase enzyme is selected from the group consisting of tripeptidyl aminopeptidase, dipeptidyl aminopeptidase, carboxypeptidase and other aminopeptidases.

[0070] In some embodiments, the one or more endo- and/or exo-peptidase enzymes are selected from the group consisting of acid fungal endopeptidase, metallo neutral endopeptidase, alkaline serine endopeptidase, subtilisin, bromelain, thermostable bacterial neutral endopeptidase, alkaline serine endopeptidase.

[0071] In some embodiments, the endo- and/or exo-peptidase enzymes for use in the present invention may be one or more of the proteases in one or more of the commercial products below:

Commercial product	Company	Protease type	Protease source

ALPHALASE® AFP	Genencor/DuPont	Acid fungal endopeptidase	*Trichoderma reesei*
FOODPRO® PAL	Genencor/DuPont	Acid fungal endopeptidase	*Aspergillus niger*
FOODPRO® PNL	Genencor/DuPont	Metallo neutral endopeptidase	*Bacillus amyloliquefaciens*
FOODPRO® Alkaline Protease	Genencor/DuPont	Alkaline Serine Endopeptidase	*Bacillus licheniformis*
FOODPRO® PBR	Genencor/DuPont	Bromelain	*Ananas comosus*
FOODPRO® PHT	Genencor/DuPont	Thermostable bacterial neutral endopeptidase	*Geobacillus sp.*
FOODPRO® 30L	Genencor/DuPont	Alkaline Serine Endopeptidase	
FOODPRO® 51FP	Genencor/DuPont	Endo-/Exopeptidase	
FOODPRO® PXT	Genencor/DuPont	subtilisin	*B. lentus*
ESPERASE® 8.0L	Novozymes	protease	*Bacillus* sp.
EVERLASE® 16.0		subtilisin	*Bacillus* sp.
ALCALASE® 2.4	Novozymes	subtilisin	*Bacillus* sp.
NEUTRASE® 0.8L	Novozymes	protease	*B. amyloliquefaciens*
Allzyme FD	Alltech	Serine protease*	*Aspergillus niger*
Arazyme One-Q	Insect Biotech Co.	metalloprotease	*Serratia proteamaculans* HY-3
SAVINASE®	Novozymes	subtilisin	*Bacillus* sp.
RONOZYME® ProAct	DSM/Novozymes	Alkaline serine protease	*Nocardiopsis prasina gene* expressed in *Bacillus licheniformis*
VALKERASE®/ CIBENZA® IND900	Novus	Keratinase	*Bacillus licheniformis*

[0072] Additionally, or in the alternative endo- and/or exo-peptidase enzymes may be comprised in one or more of the following commercially available products: KANNASE™ , NOVOCARNE™ Tender and Novozym 37020, NOVO-PRO™ D (all available from Novozymes); BioSorb-ACDP (Noor Creations, India); ANGEL® Acid Protease (Angel Yeast Co, Ltd., China) or COROLASE® LAP (from AB Enzymes).

[0073] In some embodiments, the invention also provides a feed and/or food additive composition comprising at least one of the aminopeptidases described herein.

[0074] In another embodiment there is provided a composition and/or food additive and/or feed additive composition comprising a hydrolysate of the invention. Suitably, such a food and/or feed additive composition may further comprise an aminopeptidase (optionally in combination with an endoprotease).

[0075] Materials may be added to an enzyme-containing liquid to improve the properties of the liquid composition. Non-limiting examples of such additives include: salts (e.g., alkali salts, earth metal salts, additional chloride salts, sulfate salts, nitrate salts, carbonate salts, where exemplary counter ions are calcium, potassium, and sodium), inorganic minerals or clays (e.g., zeolites, kaolin, bentonite, talc's and/or silicates), carbohydrates (e.g., sucrose and/or starch), coloring pigments (e.g., titanium dioxide), biocides (e.g., RODALON®, PROXEL®), dispersants, anti-foaming agents, reducing agents, acid agents, alkaline agents, enzyme stabilizers (e.g. polyol such as glycerol, propylene glycol, sorbitol, inorganic salts, sugars, sugar or a sugar alcohol, lactic acid, boric acid, or a boric acid derivative and combinations thereof), enzyme inhibitors, preservative (e.g. methyl paraben, propyl paraben, benzoate, sorbate or other food approved preservatives) and combinations thereof. Excipients which may be used in the preparation/composition include maltose, sucrose, glucose including glucose syrup or dried glucose syrup, pre-cooked starch, gelatinised starch, L-lactic, ascorbyl palmitate, tocopherols, lecithins, citric acid, citrates, phosphoric, phosphates, sodium alginate, carrageenan, locust bean gum, guar gum, xanthan gum, pectins, sodium carboxymethylcellulose, mono- and diglycerides, citric acid esters of mono- and diglycerides, sucrose esters, carbon dioxide, argon, helium, nitrogen, nitrous oxide, oxygen, hydrogen, and starch sodium octenylsuccinate. As demonstrated in Example 7 herein, an aminopeptidase according to the present invention may retain its enzymatic activity in a composition comprising sodium chloride.

Forms

[0076] The product and/or the composition of the present invention may be used in any suitable form - whether when alone or when present in a composition. Likewise, aminopeptidase of the present invention when combined with, e.g. exo- and/or endo- proteases may be used in any suitable form for use in the food industry as a food processing aid or foodstuff additive (i.e. ingredients - such as food ingredients, functional food ingredients or pharmaceutical ingredients).

[0077] Suitable examples of forms include one or more of: tablets, pills, capsules, ovules, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed-, modified-, sustained-, pulsed- or controlled-release applications.

[0078] By way of example, if the product and/or the composition are used in a tablet form - such as for use as a functional ingredient - the tablets may also contain one or more of: excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine; disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates; granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxypropylcellulose (HPC), sucrose, gelatin and acacia; lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

[0079] Examples of nutritionally acceptable carriers for use in preparing the forms include, for example, water, salt solutions, alcohol, silicone, waxes, petroleum jelly, vegetable oils, polyethylene glycols, propylene glycol, liposomes, sugars, gelatin, lactose, amylose, magnesium stearate, talc, surfactants, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, and the like.

[0080] Preferred excipients for the forms include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols.

[0081] For aqueous suspensions and/or elixirs, aminopeptidase and/or the composition of the present invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerine, and combinations thereof.

[0082] The forms may also include gelatin capsules; fibre capsules, fibre tablets etc.

Methods

[0083] In some embodiments, the polypeptides of the present invention may be used in the production of protein hydrolysates, e.g., for enhancing the degree of hydrolysis, general debittering of protein hydrolysates and enhancing flavor development, production of glutamate and/or other uses like FAN generation during malting or brewing.

[0084] The present invention further relates to methods for using a polypeptide of the present invention in combination with a protease (e.g., an endopeptidase) to produce a high degree of hydrolysis of a protein-rich material. The method comprises treating of a proteinaceous substrate with the polypeptide and an endopeptidase. The substrate may be treated with the enzymes concurrently or consecutively.

[0085] A polypeptide of the present invention is added to the proteinaceous substrate in an effective amount conventionally employed in protein hydrolysis processes. In some embodiments, a polypeptide of the present invention is added to the proteinaceous substrate in the range of from about 0.1 to about 100,000 aminopeptidase units per 100 g of protein, or in the range of from about 1 to about 10,000 aminopeptidase units per 100 g of protein. As defined herein, one aminopeptidase unit (APU) is the amount of enzyme needed to release 1 micromole of p-nitroanilide per minute from Ala-p-nitroanilide (Sigma Chemical Co., St. Louis MO) under the specified conditions. In the aminopeptidase assay the hydrolysis of the peptide substrate H-Ala-nitroanilide is measured by the release of p-nitroanilid (pNA). The absorbance of pNA is determined at a wavelength of 405 nm using an ELISA reader. The reaction is run with 180 µï 20 mM CPB buffer, 15 µï diluted enzyme and 20 µï substrate at 30°C. The CPB-Buffer is made up of 20mM Citric acid, 20mM Phosphate, 20mM Boric acid and adjusted to pH 9.0. The substrate is 20 mg H-Ala-pNA from BACHEM (L-1070) in 1 ml DMSO (Dimethyl Sulphoxide from SIGMA (catalog # D2650).

[0086] The endopeptidase may be obtained from a strain of *Bacillus*, preferably *Bacillus licheniformis* or *Bacillus subtilis*, a strain of *Staphylococcus*, preferably *Staphylococcus aureus*, a strain of *Streptomyces*, preferably *Streptomyces thermovularis* or *Streptomyces griseus*, a strain of *Actinomyces species*, a strain of *Aspergillus*, preferably *Aspergillus aculeatus, Aspergillus awamori, Aspergillus foetidus, Aspergillus nidulans, Aspergillus niger, ox Aspergillus oryzae*, or a strain of Trichoderma, preferably Trichoderma reesei, or *Fusarium*, preferably *Fusarium venenatum*. In some embodiments, the endopeptidase is selected from the group consisting of

ALPHALASE® AFP, FOODPRO® PAL, FOODPRO® PNL, FOODPRO® Alkaline Protease, FOODPRO® PXT, FOODPRO® PBR, FOODPRO® PHT, FOODPRO® 30L, and FOODPRO® 51FP.

[0087] The endopeptidase is added to the proteinaceous substrate in an effective amount conventionally employed in protein hydrolysis processes, preferably in the range of from about 0.05 to about 15 AU/100 g of protein, and more preferably from about 0.1 to about 8 AU/100 g of protein. One AU (Anson Unit) is defined as the amount of enzyme which under standard conditions (i.e., 25°C, pH 7.5 and 10 min. reaction time) digests hemoglobin at an initial rate such that there is liberated per minute an amount of TCA soluble product which gives the same color with phenol reagent as one milliequivalent of tyrosine. In some embodiments, the endopeptidase may be dosed in an amount of about 10 to about 3000 mg of enzyme per kg of protein substrate, e.g. 0.01 to 3 g of enzyme per metric ton (MT) of protein substrate.

[0088] The enzymatic treatment, i.e., the incubation of the substrate with the enzyme preparations, may take place at any convenient temperature at which the enzyme preparation does not become inactivated, preferably in the range of from about 20°C to about 70°C. In accordance with established practice, the enzyme preparations may be suitably inactivated by increasing the temperature of the incubation mixture to a temperature where the enzymes become inactivated, e.g., to above about 70°C, or similarly by decreasing the pH of the incubation mixture to a point where the enzymes become inactivated, e.g., below about 4.0.

[0089] Furthermore, the methods of the present invention result in enhancement of the degree of hydrolysis of a proteinaceous substrate. As used herein, the degree of hydrolysis (DH) is the percentage of the total number of amino bonds in a protein that has been hydrolyzed by a proteolytic enzyme. In one aspect, an enzyme according to the present invention may facilitate an increase or enhancement of at least about 5, 7, 10, 12, 15, 17, 20, 22, 25, 27, 30, 35, 40, 45 or 50% of the DH of a proteinaceous substrate. In one example the increase may be relative to a type 1 aminopeptidase, although any suitable comparison may be made. In a preferred embodiment, the protein hydrolysates have an increased content of Leu, Gly, Glu, Ser, Asp, Asn, Pro, Cys, Ala, and/or Gin, e.g., at least 1.1 times greater.

[0090] In some embodiments, the protein hydrolysates have an increased content of Glu. In some embodiments, the protein hydrolysates have an increased content of Leu. In some embodiments, the protein hydrolysates have an increased content of Gly. In some embodiments,

the protein hydrolysates have an increased content of Ser. In some embodiments, the protein hydrolysates have an increased content of Asp. In some embodiments, the protein hydrolysates have an increased content of Asn. In some embodiments, the protein hydrolysates have an increased content of Pro. In some embodiments, the protein hydrolysates have an increased content of Cys. In some embodiments, the protein hydrolysates have an increased content of Ala. In another more preferred embodiment, the protein hydrolysates have an increased content of Gin.

[0091] Aminopeptidase enzymes according to the present invention may exhibit decreased inhibition from the product of the enzymatic reaction. As demonstrated in Example 8 herein, two PepN 2 enzymes according to the present invention exhibited less inhibition by product than the enzyme leucine aminopeptidase 2 from *Aspergillus oryzae* RIB40 (NCBI Reference Sequence: XP_001819545.1), which is set out below as SEQ ID NO: 17:

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  1 mrsllwasll sgvlagralv spdefpediq ledllegsqq ledfayaype rnrvfggkah
 61 ddtvnylyee lkktgyydvy kqpqvhlwsn adqtlkvgde eieaktmtys psvevtadva
121 vvknlgcsea dypsdvegkv alikrgecpf gdksvlaaka kaaasivynn vagsmagtlg
181 aaqsdkgpys aivgisledg qkliklaeag svsvdlwvds kqenrttynv vaqtkggdpn
241 nvvalgghtd sveagpgind dgsgiisnlv iakaltqysv knavrflfwt aeefgllgsn
301 yyvshlnate lnkirlylnf dmiaspnyal miydgdgsaf nqsgpagsaq ieklfedyyd
361 sidlphiptq fdgrsdyeaf ilngipsggl ftgaegimse enasrwggqa gvaydanyha
421 agdnmtnlnh eaflinskat afavatyand lssipkrntt sslhrrartm rpfgkrapkt
481 hahvsgsgcw hsqvea
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[0092] As such, in one embodiment the invention provides an isolated polypeptide that has aminopeptidase activity that has decreased product inhibition compared to the aminopeptidase of SEQ ID NO: 17 (which is also referred to as TRI063 herein - see e.g. the Examples). In particular, an isolated polypeptide according to the present invention that has aminopeptidase activity may have a product inhibition constant (Ki) value of greater than 2, 3, 4 or 5 mM. In a preferred embodiment the isolated polypeptide that has aminopeptidase activity is an aminopeptidase type 2 enzyme. In a further preferred embodiment the aminopeptidase type 2 enzyme is as set out in any of SEQ ID NOs: 1-8 as defined herein, particularly SEQ ID NO: 1 or 5.

[0093] Aminopeptidase enzymes according to the present invention may be capable of hydrolyzing a polypeptide with a proline residue in position 2, as numbered from the N-terminus. In particular, aminopeptidase enzymes according to the present invention may be capable of hydrolyzing a polypeptide with a proline residue in position 2, as numbered from the N-terminus, to over half the starting concentration within 2 hours of incubation. The degree of hydrolysis can be measured by any suitable protocol known in the art, for example, the method presented in Example 10. As demonstrated in Example 10, two PepN 2 enzymes according to the present invention (TRI032 and TRI035) are able to hydrolyze the peptide TPAAAR over time to less than half the concentration within 2 hr incubation, whereas TRI063 *(A. oryzae)* and COROLASE® LAP show no hydrolysis of TPAAAR within 12 h.

[0094] The present invention also relates to methods for obtaining a protein hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues, which method comprises: subjecting the substrate to the action of a polypeptide having aminopeptidase activity. The present invention also relates to methods for obtaining a protein hydrolysate enriched in free glutamine or glutamic acid and/or oligopeptide bound glutamine or glutamic acid residues, which method comprises: subjecting the substrate to the action of a polypeptide having aminopeptidase activity.

[0095] The present invention also relates to methods for debittering a protein hydrolysate, which method comprises: subjecting the substrate to the action of a polypeptide having aminopeptidase activity.

[0096] In some embodiments, the methods further comprise subjecting the substrate to a deamidation process. The deamidation process may be performed simultaneously, prior or subsequently to the subjecting the substrate to the action of a polypeptide having aminopeptidase activity.

[0097] In some embodiments, the methods of the present invention produce protein hydrolysates with enhanced flavor because glutamic acid (Glu), whether free or oligopeptide bound, plays an important role in the flavor and palatability of protein hydrolysates. In some embodiments, the method also produces protein hydrolysates having improved functionality, in particular, improved solubility, improved emulsifying properties, increased degree of hydrolysis, and improved foaming properties.

[0098] The conversion of amides (glutamine or asparagine) into charged acids (glutamic acid or aspartic acid) via the liberation of ammonia is known as deamidation. Deamidation may take place as a non-enzymatic or as an enzymatic deamidation process.

[0099] In some embodiments, the deamidation is carried out as an enzymatic deamidation process, e.g., by subjecting the substrate to a glutaminase, transglutaminase and/or peptidoglutaminase.

[00100] In some embodiments the glutaminase is GLUTAMINASE SD-C100S™ (Amano, Japan).

[00101] In some embodiments, the glutaminase may be dosed in an amount of about 1 mg to 20 mg per g of substrate protein. In some embodiments, the glutaminase may be dosed in an amount of about 5 mg to 15 mg per g of substrate protein. In some embodiments, the glutaminase may be dosed in an amount of about 10 mg per g of substrate protein.

[00102] The present invention also relates to methods for the production of glutamate, which method comprises: subjecting a protein substrate to the action of a polypeptide having aminopeptidase activity.

[00103] The transglutaminase may be of any convenient source including mammals, see e.g., JP 1050382 and JP 5023182, including activated Factor XIII, see e.g., WO 93/15234; those derived from fish, see e.g., EP 555,649; and those obtained from microorganisms, see e.g., EP 379,606, WO 96/06931 and WO 96/22366. In some embodiments, the transglutaminase is obtained from an Oomycete, including a strain of *Phytophthora,* preferably *Phytophthora cactorum,* or a strain of *Pythium,* preferably *Pythium irregulare, Pythium sp., Pythium intermedium, Pythium Itimum,* or *Pythium periilum* (or *Pythium periplocum).* In some embodiments, the transglutaminase is of bacterial origin and is obtained from a strain of *Bacillus,* preferably *Bacillus subtilis,* a strain of *Streptoverticillium,* preferably *Streptoverticillium mobaraensis, Streptoverticillium griseocarneum,* or *Streptoverticillium cinnamoneum,* and a strain of *Streptomyces,* preferably *Streptomyces lydicus.*

[00104] The peptidoglutaminase may be a peptidoglutaminase I (peptidyl-glutaminase; EC 3.5.1.43), or a peptidoglutaminase II (protein-glutamine glutaminase; EC 3.5.1.44), or any mixture thereof. The peptidoglutaminase may be obtained from a strain of *Aspergillus,* preferably *Aspergillus japonicus,* a strain of *Bacillus,* preferably *Bacillus circulans,* a strain of

Cryptococcus, preferably *Cryptococcus albidus,* or a strain of *Debaryomyces,* preferably *Debaryomyces kloecheri.*

[00105] The transglutaminase is added to the proteinaceous substrate in an effective amount conventionally employed in deamidation processes, preferably in the range of from about 0.01 to about 5% (w/w), and more preferably in the range of from about 0.1 to about 1 % (w/w) of enzyme preparation relating to the amount of substrate.

[00106] The peptidoglutaminase is added to the proteinaceous substrate in an effective amount conventionally employed in deamidation processes, preferably in the range of from about 0.01 to about 100,000 PGase Units per 100 g of substrate, and more preferably in the range of from about 0.1 to about 10,000 PGase Units per 100 g of substrate.

[00107] The peptidoglutaminase activity may be determined according to the procedure of Cedrangoro *et al.* (1965, *Enzymologia* Vol. 29 pagel43). According to this procedure, 0.5 ml of an enzyme sample, adjusted to pH 6.5 with 1 N NaOH, is charged into a small vessel. Then 1 ml of a borate pH 10.8 buffer solution is added to the vessel. The discharged ammonia is absorbed by 5 N sulphuric acid, and by use of Nessler's reagent the mixture is allowed to form color which is measured at 420 nm. One PGase unit is the amount of enzyme capable of producing 1 micromole of ammonia per minute under these conditions.

[00108] The present invention also relates to methods for the production of free amino nitrogen (FAN) during malting and/or brewing, which method comprises: subjecting a substrate during a malting and/or brewing process to the action of a polypeptide having aminopeptidase activity.

[00109] In some embodiments of the methods of the present invention, a protein substrate is subjected to a polypeptide of the present invention. A polypeptide of the present invention is added to the proteinaceous substrate in an effective amount conventionally employed in protein hydrolysis processes, preferably in the range of from about 0.001 to about 0.5 AU/100 g of substrate, more preferably in the range of from about 0.01 to about 0.1 AU/100 g of substrate.

[00110] In another embodiment, the methods of the present invention for producing a hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues further comprise: subjecting the substrate to one or more unspecific acting endo- and/or exo-peptidase enzymes. This step may take place simultaneously, or may follow the step of subjecting a protein substrate with a polypeptide of the present invention.

[00111] In a preferred embodiment, the unspecific acting endo- and/or exo-peptidase enzyme is obtained from a strain of *Aspergillus*, or a strain of *Bacillus.*

[00112] The unspecific acting endo- and/or exo-peptidase enzyme is added to the substrate in an effective amount conventionally employed in protein hydrolysis processes, preferably in the range of from about.

[00113] In some embodiments, the endo- and/or exo-peptidase may be dosed in an amount of about 50 to about 3000 mg of enzyme per kg of protein substrate, e.g. 0.05 to 3 g of enzyme per metric ton (MT) of protein substrate.

[00114] Suitably, the endo- and/or exo-peptidase may be dosed in an amount of less than about 4.0 g of enzyme per MT of protein substrate.

[00115] In another embodiment, the endo- and/or exo-peptidase may be dosed at between about 0.5 g and about 5.0 g of enzyme per MT of protein substrate. Suitably the endo- and/or exo-peptidase may be dosed at between about 0.5 g and about 3.0 g of enzyme per MT of protein substrate. More suitably, the endoprotease may be dosed at about 1.0 g to about 2.0 g of enzyme per MT of protein substrate.

[00116] In some embodiments, a polypeptide of the present invention may be dosed in an amount of between about 0.5 mg to about 2 g of enzyme per kg of protein substrate and/or food and/or feed additive composition. Suitably a polypeptide of the present invention may be dosed in an amount of between about 1 mg to about 2 g of enzyme per kg of protein substrate and/or food and/or feed additive composition. More suitably in an amount of between about 5 mg to about 1.5 g of enzyme per kg of protein substrate and/or food and/or feed additive composition.

[00117] In the preparation of a hydrolysate a polypeptide of the present invention may be dosed in an amount of between about 0.5 mg to about 2 g of enzyme per kg of protein substrate. Suitably a polypeptide of the present invention may be dosed in an amount of between about 1 mg to about 2 g of enzyme per kg of protein substrate. More suitably in an amount of between about 5 mg to about 1.5 g of enzyme per kg of protein substrate.

[00118] In one embodiment, a polypeptide of the present invention may be dosed in an amount of between about 5 mg to about 500 mg of enzyme per kg of protein substrate. Suitably a polypeptide of the present invention may be dosed in an amount of between about 50 mg to about 500 mg of enzyme per kg of protein substrate. Suitably a polypeptide of the present

invention may be dosed in an amount of between about 100 mg to about 450 mg of enzyme per kg of protein substrate.

[00119] Each enzymatic treatment may take place at any temperature at which the enzyme preparation does not become inactivated, preferably in the range of from about 20°C to about 70°C. The enzyme preparation may then be inactivated by increasing the temperature, e.g., to above about 70°C, or by decreasing the pH, e.g., below about 4.0.

[00120] The proteinaceous substrate used in the methods of the present invention may consist of intact proteins, prehydrolyzed proteins (i.e., peptides), or a mixture thereof. The proteinaceous substrate may be of vegetable or animal origin. In some embodiments, the proteinaceous substrate is of vegetable origin, e.g., soy protein, grain protein, e.g., wheat gluten, corn gluten, barley, rye, oat, rice, zein, lupine, cotton seed protein, rape seed protein, peanut, alfalfa protein, pea protein, fabaceous bean protein, sesame seed protein, or sunflower. A proteinaceous substrate of animal origin may be whey protein, casein, meat proteins, fish protein, red blood cells, egg white, gelatin, lactoalbumin, hair proteins or feather proteins.

[00121] The present invention also relates to protein hydrolysates produced by these methods.

Preparation of the nucleotide sequence

[00122] A nucleotide sequence encoding either a protein which has the specific properties as defined herein or a protein which is suitable for modification may be identified and/or isolated and/or purified from any cell or organism producing said protein. Various methods are well known within the art for the identification and/or isolation and/or purification of nucleotide sequences. By way of example, PCR amplification techniques to prepare more of a sequence may be used once a suitable sequence has been identified and/or isolated and/or purified.

[00123] By way of further example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the enzyme. If the amino acid sequence of the enzyme is known, labelled oligonucleotide probes may be synthesized and used to identify enzyme-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known enzyme gene could be used to identify enzyme-encoding clones. In the latter case, hybridization and washing conditions of lower stringency are used.

[00124] Alternatively, enzyme-encoding clones could be identified by inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar plates containing a substrate for enzyme (i.e., maltose), thereby allowing clones expressing the enzyme to be identified.

[00125] In a yet further alternative, the nucleotide sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beucage S.L. *et al,* (1981) *Tetrahedron Letters* 22, p 1859-1869, or the method described by Matthes *et al,* (1984) *EMBO J*. 3, p 801-805. In the phosphoramidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

[00126] The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in Saiki R K *et al, {Science* (1988) 239, pp 487-491).

Amino acid sequences

[00127] The scope of the present invention also encompasses amino acid sequences of enzymes having the specific properties as defined herein.

[00128] As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". In some instances, the term "amino acid sequence" is synonymous with the term "enzyme".

[00129] The amino acid sequence may be prepared/isolated from a suitable source, or it may be made synthetically or it may be prepared by use of recombinant DNA techniques.

[00130] The protein encompassed in the present invention may be used in conjunction with other proteins, particularly proline endoprotease, tripeptidyl exopeptidases, and other forms of endo or exoproteases. Thus the present invention also covers a combination of proteins wherein the combination comprises the aminopeptidase of the present invention and another enzyme, which

may be another aminopeptidase according to the present invention. This aspect is discussed in a later section.

[00131] Preferably the amino acid sequence when relating to and when encompassed by the *per se* scope of the present invention is not a native enzyme. In this regard, the term "native enzyme" means an entire enzyme that is in its native environment and when it has been expressed by its native nucleotide sequence.

Sequence identity or sequence homology

[00132] The present invention also encompasses the use of sequences having a degree of sequence identity or sequence homology with amino acid sequence(s) of a polypeptide having the specific properties defined herein or of any nucleotide sequence encoding such a polypeptide (hereinafter referred to as a "homologous sequence(s)"). Here, the term "homologue" means an entity having a certain homology with the subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can be equated with "identity".

[00133] 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 the aminopeptidase.

[00134] In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% 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.

[00135] In the present context, a homologous sequence is taken to include a nucleotide sequence which may be at least 75, 80, 85 or 90% identical, preferably at least 95 or 98% identical to a nucleotide sequence encoding a polypeptide of the present invention (the subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject 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.

[00136] Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate percentage homology between two or more sequences.

[00137] Percentage homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

[00138] Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in percentage homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalizing unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximize local homology.

[00139] However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimized alignments with fewer gaps. 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.

[00140] Calculation of maximum percentage homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the Vector NTI (Invitrogen Corp.). Examples of software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *etal.* 1999, Short Protocols in Molecular Biology, 4th Ed - Chapter 18), BLAST 2 (see *FEMS Microbiol Lett* 1999 174(2): 247-50; *FEMS Microbiol Lett* 1999 177(1):

187-8 and tatiana@ncbi.nlm.nih.gov), FASTA (Altschul *etal*, 1990 *J. Mol. Biol.* 403-410) and AlignX for example. At least BLAST, BLAST 2 and FASTA are available for offline and online searching (see Ausubel *et al.* 1999, *supra*, pages 7-58 to 7-60).

[00141] Although the final percentage homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. Vector NTI programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the default values for the Vector NTI package.

[00142] Alternatively, percentage homologies may be calculated using the multiple alignment feature in Vector NTI (Invitrogen Corp.), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244).

[00143] Once the software has produced an optimal alignment, it is possible to calculate percentage homology, preferably percentage sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[00144] Should Gap Penalties be used when determining sequence identity, then preferably the following parameters are used for pairwise alignment:

FOR BLAST	
GAP OPEN	0
GAP EXTENSION	0

FOR CLUSTAL	DNA	PROTEIN	
WORD SIZE	2	1	K triple
GAP PENALTY	15	10	
GAP EXTENSION	6.66	0.1	

[00145] In one embodiment, CLUSTAL may be used with the gap penalty and gap extension set as defined above.

[00146] Suitably, the degree of identity with regard to a nucleotide sequence is determined over at least 20 contiguous nucleotides, preferably over at least 30 contiguous nucleotides, preferably over at least 40 contiguous nucleotides, preferably over at least 50 contiguous

nucleotides, preferably over at least 60 contiguous nucleotides, preferably over at least 100 contiguous nucleotides.

[00147] Suitably, the degree of identity with regard to a nucleotide sequence may be determined over the whole sequence.

Variants/homologues/derivatives

[00148] The present invention also encompasses the use of variants, homologues and derivatives of any amino acid sequence of a protein or of any nucleotide sequence encoding such a protein

[00149] Here, the term "homologue" means an entity having a certain homology with the subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can be equated with "identity".

[00150] In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 80, 85 or 90% identical, preferably at least 95, 96, 97, 98 or 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.

[00151] In the present context, a homologous sequence is taken to include a nucleotide sequence which may be at least 75, 80, 85 or 90% identical, preferably at least 95, 96, 97, 98 or 99% identical to a nucleotide sequence encoding an enzyme of the present invention (the subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject 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.

[00152] Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate percentage homology between two or more sequences. Percentage homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment.

Typically, such ungapped alignments are performed only over a relatively short number of residues.

[00153] Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in percentage homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalizing unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximize local homology.

[00154] However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimized alignments with fewer gaps. 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. For example, when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

[00155] Calculation of maximum percentage homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux *et al* 1984 Nuc. Acids Research 12 p387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al,* 1999, *supra),* FASTA (Altschul *et al,* 1990 *J. Mol Biol.* 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al,* 1999, *supra).* However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence.

[00156] Although the final percentage homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

[00157] Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *supra).*

[00158] Once the software has produced an optimal alignment, it is possible to calculate percentage homology, preferably percentage sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[00159] The sequences 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.

[00160] Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q

	Polar - charged	D E
		K R
AROMATIC		H F W Y

[00161] The present invention also encompasses homologous 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-homologous 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), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

[00162] Replacements may also be made by unnatural amino acids include; alpha* and alpha-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*, B-alanine*, L-a-amino butyric acid*, L-y-amino butyric acid*, L-a-amino isobutyric acid*, L-s-amino caproic acid#, 7-amino heptanoic acid*, L-methionine sulfone#*, L-norleucine*, L-norvaline*, p-nitro-L-phenylalanine*, L-hydroxyproline #, L-thioproline*, 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 utilized for the purpose of the discussion above (relating to homologous or non-homologous substitution), to indicate the hydrophobic nature of the derivative whereas # has been utilized to indicate the hydrophilic nature of the derivative, #* indicates amphipathic characteristics.

[00163] 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 a-carbon

substituent group is on the residue's nitrogen atom rather than the a-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ *et al., PNAS* (1992) 89 (20), 9367-9371 and Horwell DC, *Trends Biotechnol.* (1995) 13 (4), 132-134.

[00164] The nucleotide sequences for use in the present invention may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences of the present invention.

[00165] The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

[00166] Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other homologues may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridizing to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

[00167] Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from

several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example, the GCG Wisconsin PileUp program is widely used.

[00168] The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

[00169] Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterized sequences. This may be useful where for example silent codon sequence changes are required to optimize codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

[00170] Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

[00171] Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

[00172] In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing these using automated techniques are readily available in the art.

[00173] Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Hybridization

[00174] The present invention also encompasses sequences that are complementary to the nucleic acid sequences of the present invention or sequences that are capable of hybridizing either to the sequences of the present invention or to sequences that are complementary thereto.

[00175] The term "hybridization" as used herein shall include "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.

[00176] The present invention also encompasses the use of nucleotide sequences that are capable of hybridizing to the sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof.

[00177] The term "variant" also encompasses sequences that are complementary to sequences that are capable of hybridizing to the nucleotide sequences presented herein.

[00178] Preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridizing under stringent conditions (e.g. 50°C and 0.2xSSC {lxSSC = 0.15 M NaCl, 0.015 M Na_3citrate pH 7.0}) to the nucleotide sequences presented herein.

[00179] More preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridizing under high stringent conditions (e.g. 65°C and O.lxSSC {lxSSC = 0.15 M NaCl, 0.015 M Na_3citrate pH 7.0}) to the nucleotide sequences presented herein.

[00180] The present invention also relates to nucleotide sequences that can hybridize to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

[00181] The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridize to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

[00182] Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridizing to the nucleotide sequences presented herein under conditions of intermediate to maximal stringency.

[00183] In a preferred aspect, the present invention covers nucleotide sequences that can hybridize to the nucleotide sequence of the present invention, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC).

[00184] In a more preferred aspect, the present invention covers nucleotide sequences that can hybridize to the nucleotide sequence of the present invention, or the complement thereof, under high stringent conditions (e.g. 65°C and O.lxSSC).

Molecular evolution

[00185] As a non-limiting example, it is possible to produce numerous site directed or random mutations into a nucleotide sequence, either in vivo or in vitro, and to subsequently screen for improved functionality of the encoded polypeptide by various means.

[00186] In addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wildtype or other mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide. The production of new preferred variants can be achieved by various methods well established in the art, for example the Error Threshold Mutagenesis (WO 92/18645), oligonucleotide mediated random mutagenesis (US 5,723, 323), DNA shuffling (US 5,605,793), exo-mediated gene assembly WO00/58517. The application of these and similar random directed molecular evolution methods allows the identification and selection of variants of the enzymes of the present invention which have preferred characteristics without any prior knowledge of protein structure or function, and allows the production of non-predictable but beneficial mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimization or alteration of enzyme activity, such examples include, but are not limited to one or more of the following:

optimized expression and/or activity in a host cell or in vitro,
increased enzymatic activity, altered substrate and/or product specificity,
increased or decreased enzymatic or structural stability, altered enzymatic activity/specificity in preferred environmental conditions, e.g.
temperature, pH, substrate

Site-directed mutagenesis

[00187] Once a protein-encoding nucleotide sequence has been isolated, or a putative protein-encoding nucleotide sequence has been identified, it may be desirable to mutate the sequence in order to prepare a protein of the present invention.

[00188] Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites.

[00189] A suitable method is disclosed in Morinaga *et al.*, *{Biotechnology* (1984) 2, p646-649). Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long *{Analytical Biochemistry* (1989), 180, p 147-151).

Recombinant

[00190] In one aspect the sequence for use in the present invention is a recombinant sequence - i.e. a sequence that has been prepared using recombinant DNA techniques.

[00191] These recombinant DNA techniques are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, Molecular Cloning: A Laboratory Manual Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press.

Synthetic

[00192] In one aspect the sequence for use in the present invention is a synthetic sequence - i.e. a sequence that has been prepared by *in vitro* chemical or enzymatic synthesis. It includes, but is not limited to, sequences made with optimal codon usage for host organisms - such as the methylotrophic yeasts *Pichia* and *Hansenula.*

Expression

Expression of enzymes

[00193] The nucleotide sequence for use in the present invention may be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence, in protein/enzyme form, in and/or from a compatible host cell.

[00194] Expression may be controlled using control sequences e.g. regulatory sequences.

[00195] The protein produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences may be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

Expression vector

[00196] The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

[00197] Preferably, the expression vector is incorporated into the genome of a suitable host organism. The term "incorporated" preferably covers stable incorporation into the genome.

[00198] The nucleotide sequence of the present invention may be present in a vector in which the nucleotide sequence is operably linked to regulatory sequences capable of providing for the expression of the nucleotide sequence by a suitable host organism.

[00199] The vectors for use in the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide of the present invention.

[00200] The choice of vector e.g. a plasmid, cosmid, or phage vector will often depend on the host cell into which it is to be introduced.

[00201] The vectors for use in the present invention may contain one or more selectable marker genes- such as a gene, which confers antibiotic resistance e.g. ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Alternatively, the selection may be accomplished by co-transformation (as described in W091/17243).

[00202] Vectors may be used *in vitro,* for example for the production of RNA or used to transfect, transform, transduce or infect a host cell.

[00203] Thus, in a further embodiment, the invention provides a method of making nucleotide sequences of the present invention by introducing a nucleotide sequence of the present invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector.

[00204] The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUBl lO, pE194, pAMBl and pIJ702.

Regulatory sequences

[00205] In some applications, the nucleotide sequence for use in the present invention is operably linked to a regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the nucleotide sequence of the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

[00206] The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. 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.

[00207] The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

[00208] The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

[00209] Enhanced expression of the nucleotide sequence encoding the enzyme of the present invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions.

[00210] Preferably, the nucleotide sequence according to the present invention is operably linked to at least a promoter.

[00211] Other promoters may even be used to direct expression of the polypeptide of the present invention.

[00212] Examples of suitable promoters for directing the transcription of the nucleotide sequence in a bacterial, fungal or yeast host are well known in the art.

[00213] The promoter can additionally include features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box.

Constructs

[00214] The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence for use according to the present invention directly or indirectly attached to a promoter.

[00215] An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Shl-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

[00216] The construct may even contain or express a marker, which allows for the selection of the genetic construct.

[00217] For some applications, preferably the construct of the present invention comprises at least the nucleotide sequence of the present invention operably linked to a promoter.

Host cells

[00218] The term "host cell" - in relation to the present invention includes any cell that comprises either the nucleotide sequence or an expression vector as described above and which is used in the recombinant production of a protein having the specific properties as defined herein.

[00219] Thus, a further embodiment of the present invention provides host cells transformed or transfected with a nucleotide sequence that expresses the protein of the present invention. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells.

[00220] Examples of suitable bacterial host organisms are gram positive or gram negative bacterial species.

[00221] Depending on the nature of the nucleotide sequence encoding the polypeptide of the present invention, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a different fungal host organism should be selected.

[00222] The use of suitable host cells - such as yeast, fungal and plant host cells - may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

[00223] The host cell may be an aminopeptidase deficient or aminopeptidase minus strain. The present invention also relates to methods for producing a mutant cell of a parent cell, which comprises disrupting or deleting a nucleic acid sequence encoding the polypeptide or a control sequence thereof, which results in the mutant cell producing less of the polypeptide than the parent cell.

[00224] The construction of strains which have reduced aminopeptidase activity may be conveniently accomplished by modification or inactivation of a nucleic acid sequence necessary for expression of the polypeptide having aminopeptidase activity in the cell.

Organism

[00225] The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the polypeptide according to the present invention and/or products obtained therefrom, and/or wherein a promoter can allow expression of the nucleotide sequence according to the present invention when present in the organism.

[00226] Suitable organisms may include a prokaryote, fungus, yeast or a plant.

[00227] The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the polypeptide according to the present invention and/or the products obtained therefrom, and/or wherein a promoter can allow expression of the nucleotide sequence according to the present invention within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

[00228] The term "transgenic organism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

[00229] Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the polypeptide according to the present invention, constructs according to the present invention, vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention, or the products thereof.

[00230] For example, the transgenic organism may also comprise the nucleotide sequence coding for the polypeptide of the present invention under the control of a heterologous promoter.

Transformation of host cells/organisms

[00231] As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis.*

[00232] Teachings on the transformation of prokaryotic hosts are well documented in the art, for example see Sambrook *et al {supra).* If a prokaryotic host is used, then the nucleotide

sequence may need to be suitably modified before transformation - such as by removal of introns.

[00233] Filamentous fungi cells may be transformed using various methods known in the art - such as a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known. The use of *Aspergillus* as a host microorganism is described in EP 0 238 023.

[00234] Another host organism can be a plant. A review of the general techniques used for transforming plants may be found in articles by Potrykus *(Annu Rev Plant Physiol Plant Mol Biol*, 1991, 42:205-225) and Christou *{Agro-Food-IndustryHi-Tech* March/ April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

[00235] General teachings on the transformation of fungi, yeasts and plants are presented in following sections.

Transformed fungus

[00236] A host organism may be a fungus - such as a mold. Examples of suitable such hosts include any member belonging to the genera Thermomyces, Acremonium, Aspergillus, Penicillium, Mucor, Neurospora, Trichoderma and the like.

[00237] In one embodiment, the host organism may be a filamentous fungus.

[00238] Transforming filamentous fungi is discussed in US-A-5741665 which states that standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N. crassa* is found, for example in Davis and de Serres, *Methods Enzymol* (1971) 17A: 79-143.

[00239] Further teachings which may also be utilized in transforming filamentous fungi are reviewed in US-A-5674707.

[00240] In addition, gene expression in filamentous fungi is taught in in Punt *et al.* (2002) *Trends Biotechnol* 2002 May;20(5):200-6, Archer & Peberdy, *Crit Rev Biotechnol* (1997) 17(4):273-306.

[00241] The present invention encompasses the production of transgenic filamentous fungi according to the present invention prepared by use of these standard techniques.

[00242] In one aspect, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*.

[00243] A transgenic *Aspergillus* according to the present invention can also be prepared by following, for example, the teachings of Turner, G. 1994 *(Vectorsfor genetic manipulation.* In: Martinelli S.D., Kinghorn J.R. (Editors) *Aspergillus:* 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp. 641-666).

[00244] In one aspect, the host organism can be of the genus *Trichoderma,* such as *Trichoderma Reesei.*

Transformed yeast

[00245] In another embodiment, the transgenic organism can be a yeast.

[00246] A review of the principles of heterologous gene expression in yeast are provided in, for example, *Methods Mol Biol* (1995), 49:341-54, and *Curr Opin Biotechnol* (1997) Oct;8(5):554-60

[00247] In this regard, yeast - such as the species *Saccharomyces cerevisae or Pichia pastoris* (see *FEMS Microbiol Rev* (2000 24(l):45-66), may be used as a vehicle for heterologous gene expression.

[00248] A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

[00249] For the transformation of yeast, several transformation protocols have been developed. For example, a transgenic Saccharomyces according to the present invention can be prepared by following the teachings of Hinnen *et al.*, (1978, *PNAS USA* **75,** 1929); Beggs, J D (1978, *Nature*, 275, 104); and Ito, H *et al* (1983, *J Bacteriology* 153, 163-168).

[00250] The transformed yeast cells may be selected using various selective markers - such as auxotrophic markers dominant antibiotic resistance markers.

Culturing and production

[00251] Host cells transformed with the nucleotide sequence of the present invention may be cultured under conditions conducive to the production of the encoded polypeptide and which facilitate recovery of the polypeptide from the cells and/or culture medium.

[00252] The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in questions and obtaining expression of the polypeptide.

[00253] The protein produced by a recombinant cell may be displayed on the surface of the cell.

[00254] The protein may be secreted from the host cells and may conveniently be recovered from the culture medium using well-known procedures.

Secretion

[00255] Often, it is desirable for the protein to be secreted from the expression host into the culture medium from where the protein may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

[00256] Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene *(glaA* - both 18 and 24 amino acid versions e.g. from *Aspergillus),* the a-factor gene (yeasts e.g. *Saccharomyces, Kluyveromyces* and *Hansenula)* or the a-amylase gene *{Bacillus).*

[00257] By way of example, the secretion of heterologous proteins in *E. coli* is reviewed in *Methods Enzymol* (1990) 182: 132-43.

Detection

[00258] A variety of protocols for detecting and measuring the expression of the amino acid sequence are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS).

[00259] A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays.

[00260] A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures.

[00261] Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US 3,817,837; US ,850,752; US 3,939,350; US 3,996,345; US 4,277,437; US 4,275,149 and US 4,366,241.

[00262] Also, recombinant immunoglobulins may be produced as shown in US 4,816,567.

Fusion proteins

[00263] The amino acid sequence for use according to the present invention may be produced as a fusion protein, for example to aid in extraction and purification. Examples of fusion protein partners include Glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and (β-galactosidase). It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences.

[00264] Preferably, the fusion protein will not hinder the activity of the protein sequence.

[00265] Gene fusion expression systems in *E. coli* have been reviewed in *Curr Opin Biotechnol* (1995) 6(5):501-6.

[00266] In another embodiment of the invention, the amino acid sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognized by a commercially available antibody.

Additional Proteins of interest (POIs)

[00267] The sequences for use according to the present invention may also be used in conjunction with one or more additional proteins of interest (POIs) or nucleotide sequences of interest (NOIs).

[00268] Non-limiting examples of POIs include: proteins or enzymes involved in starch metabolism, proteins or enzymes involved in glycogen metabolism, acetyl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carboxypeptidases, catalases, cellulases, chitinases, chymosin, cutinase, deoxyribonucleases, epimerases, esterases, a-galactosidases, β-galactosidases, a-glucanases, glucan lysases, endo -P-glucanases, glucoamylases, glucose oxidases, a-glucosidases, β-glucosidases, glucuronidases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, tripeptidyl exopeptidases, laccases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes, peroxidases, phenoloxidases, phytases, polygalacturonases, proline endoproteases, rhamno-galacturonases, rib onucl eases, thaumatin, transferases, exoproteases, transport proteins, transglutaminases, aminopeptidases,

hexose oxidase (D-hexose: (Voxidoreductase, EC 1.1.3.5) or combinations thereof. TheNOI may even be an antisense sequence for any of those sequences.

[00269] The POI may even be a fusion protein, for example to aid in extraction and purification.

[00270] The POI may even be fused to a secretion sequence.

[00271] Other sequences can also facilitate secretion or increase the yield of secreted POI. Such sequences could code for chaperone proteins as for example the product of *Aspergillus niger cyp B* gene described in UK patent application 9821 198.0.

[00272] The NOI may be engineered in order to alter their activity for a number of reasons, including but not limited to, alterations which modify the processing and/or expression of the expression product thereof. By way of further example, the NOI may also be modified to optimize expression in a particular host cell. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites.

[00273] The NOI may include within it synthetic or modified nucleotides- such as methylphosphonate and phosphorothioate backbones.

[00274] The NOI may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule.

General recombinant DNA methodology techniques

[00275] The present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, Molecular Cloning: A Laboratory Manual Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. *etal.* (1995 and periodic supplements; Current Protocols in Molecular Biology, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, DNA Isolation and Sequencing: Essential Techniques, John Wiley & Sons; M. J. Gait (Editor), 1984, Oligonucleotide Synthesis: A Practical Approach, Irl Press; and, D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis*

and Physical Analysis ofDNA in Methods in Enzymology , Academic Press. Each of these general texts is herein incorporated by reference.

EXAMPLES

[00276] The present disclosure is described in further detail in the following examples, which are not in any way intended to limit the scope of the disclosure as claimed. The attached figures are meant to be considered as integral parts of the specification and description of the disclosure. The following examples are offered to illustrate, but not to limit the claimed disclosure.

Example 1 - Cloning and transformation of TRI031, TRI032, TRI033, TRI034, TRI035, TRI036, TRI037, TRI038

[00277] Synthetic genes (TRI03 1, TRI032, TRI033, TRI034, TRI035, TRI036, TRI037, TRI038) encoding fungal aminopeptidases type-2 (pepN_2, belonging to Merops family M28.008.; merops.sanger.ac.uk/) were ordered from Geneart (Life Technologies) as codon-optimized genes for expression in *Trichoderma reesei.* TRI03 1 corresponds to NCBI accession number: XP_001258675 from *Neosartorya fischeri* NRRL 181; TRI032 corresponds to NCBI accession number: XP_003667354 from *Myceliophthora thermophila* ATCC® 42464; TRI033 corresponds to NCBI accession number: EGU74500 from *Fusarium oxysporum* Fo5176; TRI034 corresponds to NCBI accession number: ENH69875from *Fusarium oxysporum* f. sp. cubense race 1; TRI035 corresponds to NCBI accession number: XP_001273779 from *Aspergillus clavatus* NRRL 1; TRI036 corresponds to NCBI accession number: EGS23402 *Chaetomium thermophilum* var. thermophilum DSM 1495; TRI037 corresponds to NCBI accession number: XP_001217759 from *Aspergillus terreus* NIH2624; and TRI038 corresponds to NCBI accession number: XP_681714 from *Aspergillus nidulans* FGSC A4.

[00278] The genes were ordered with Gateway-specific recombination sites (attBl and attB2) (Life Technologies) flanking the coding region and delivered as plasmid stocks in the pDonr221 Gateway vector (Life Technologies). The TRI031, TRI032, TRI033, TRI034, TRI035, TRI036, TRI037, TRI038 amino acid sequences have predicted secretion signal sequences (SignalP 4.0: discriminating signal peptides from transmembrane regions. Thomas Nordahl Petersen, S0ren Brunak, Gunnar von Heijne & Henrik Nielsen. *Nature Methods,* 8:785-

786, 201 1) and these endogenous signal sequences were replaced by a leader sequence containing a Kozak sequence, the secretion signal sequence from the *Trichoderma reesei* acidic fungal protease (AFP) and an intron from a Trichoderma reesei glucoamylase gene (TrGAl) (see Figure 1).

[00279] The synthetic genes in the pDonr221 vector was recombined into the destination vector pTrex8gM using LR CLONASE™ enzyme mix (Life Technologies) resulting in the expression vectors pTrex8gM_TRI03 1 (SEQ ID NO: 9), pTrex8gM_TRI032 (SEQ ID NO: 10), pTrex8gM_TRI033 (SEQ ID NO: 11), pTrex8gM_TRI034 (SEQ ID NO: 12), pTrex8gM_TRI035 (SEQ ID NO: 13), pTrex8gM_TRI036 (SEQ ID NO: 14), pTrex8gM_TRI037 (SEQ ID NO: 15), and pTrex8gM_TRI038 (SEQ ID NO: 16).

[00280] 1.5-17 μg of the expression vectors were transformed individually into a *Trichoderma reesei* strain Cellulight™ using PEG mediated protoplast transformation essentially as described in (US 8,592,194 B2). PEG-Protoplast method with slight modifications was used for transformation, as indicated. For protoplasts preparation, spores were grown for approximately 18 hours at 26°C in *Trichoderma* germination medium with 10 mM uridine to complement the pyr auxotrophy. {*Trichoderma* germination medium: 40 ml 50% glucose, 2 g/L peptone, 15g/L KH_2P0_4, 5g/L $(NH_4)_2S0_4$, 2.4 ml 1M $MgS0_4$, 4.1 ml 1M $CaCl_2$, 1 ml of 400X *T. reesei* Trace elements solution {200 g/L $FeS0_4x7H_20$, 16 g/L $ZnS0_4x7H_20$, 1.4 g/L $MnS0_4xH_20$, 3.2 g/L $CuS0_4x5H_20$, 0.8 g/L H_3B0_3, 175 g/L citric acid}) at shaking speed of 200 rpm. Germinating spores were harvested by centrifugation, washed and treated with 45 mg/ml of lysing enzyme solution *(Trichoderma harzianum,* Sigma cat# L1412) to lyse the fungal cell walls. Further preparation of protoplasts was performed by a standard method, as described by Penttila *et al. (Gene* 61(1987) 155-164).

[00281] In general, transformation mixtures containing 1.5-17 μg of DNA and ~5x 10^7 protoplasts in a total volume of 200 μL were treated with 2 mL of 25% PEG solution, diluted with 2 volumes of 1.2M sorbitol/ lOmM Tris, pH7.5/ lOmM $CaCl_2$ solution, mixed with 3% selective top agarose, 1 M sorbitol, 10 mM NH_4C1, lx MM solution (2x MM solution: 30 g/L KH_2P0_4, 20 mL 1M acetamide, 20 ml 1M CsCl, 6 ml 20% $MgS0_4x7H_20$, 6 ml 20% $CaCl_2x2H_20$, 2 mL *T. reesei* Trace elements solution (400x), 80 mL 50% glucose, make up to 1 liter, pH 4.5) and poured on to MM plates with 10 mM NH_4C1 (MM plates 2% agar, lx MM solution). Transformants were selected for prototrophic growth on MM plates (lacking uridine).

Plates were incubated for 5-7 days at 28 °C until sporulation occurred. Stable looking transformants were transferred to new MM plates with 10 mM NH_4Cl for better sporulation. When sporulated, spores were harvested using a solution of 0.85% NaCl, 0.01-5% TWEEN® 80. Spore suspensions were used to inoculate liquid cultures either in a 24-well MTP format (for screening) or shake flasks (for validation studies). Preculture in 3 mL YEG medium (5 g/L yeast extract, 22g/L glucose, H_20) Main culture in the following production medium (Production medium: 35 g/L 61% glucose/sophorose mix, 9 g/L casmino acids, 5 g/L $(NH_4)_2SO_4$, 4.5 g/L KH_2PO_4, 1 g/L $CaCl_2x2H_20$, 1 g/L $MgSO_4x7H_20$, 33 g/L PIPPS buffer, pH 5.5, 2.5 mL/L of 400X *T. reesei* trace elements (175 g/L citric acid, 200 g/L $FeSO_4x7H_20$, 16 g/L $ZnSO_4x7H_20$, 3.2 g/L $CuSO_4x5H_20$, 1.4 g/L $MnSO_4xH_20$, 0.8 g/L boric acid) pH 5.5. 3 mL of production medium was added to produce variants in 24-well MTPs. For shake flasks, volumes were scaled up.

[00282] Cultures were grown for 7 days at 28°C and 80% humidity with shaking at 180 rpm. Culture supernatants were harvested by vacuum filtration and used to assay their performance as well as expression level.

[00283] The amino acid and nucleotide sequences for the pepN_2 enzymes are depicted below:

[00284] SEQ ID NO: 1 = Amino acid sequence of TRI03 1. Underlined is the secretion signal encoded by the leader sequence described in figure 1:

MQTFGAFLVSFLAASGLAAANGPGWOWKPPVHPKVLPQMIHLWOLMHGAQKLEDFAYAYPERNRVFGG
PAHEDTVNYLYRELKKTGYYDVYKQPQVHQWTQADQALTVDGKSYVATTMTYSPSVNVTAPLAVVNNL
GCVESDYPADLKGKIALVSRGECPFATKSVLSAKAGAAAALVYNNIEGSMAGTLGGPTSELGPYAPIAGISL
ADGQALIQMIQAGTVTANLWIDSKVENRTTYNVIAQTKGGDPNNVVALGGHTDSVEAGPGINDDGSGIISN
LVVAKALTPJSVKNAVRFCFWTAEEFGLLGSSYYVNSLNATEKAKIRLYLNFDMIASPNYALMIYDGDGS
AFNLTGPAGSAQIERLFEDYYKSIRKPFVPTEFNGRSDYEAFILNGIPAGGIFTGAEAIKTEEQAKLFGGQAG
VALDANYHAKGDNMTNLNREAFLINSKATAIAVATYANSLDSIPSRNMSTVVKRSQLEQAKKSTPHTHTG
GTGCYKDRVEQ (Seq ID no 1)

The sequence absent the underlined leader sequence is denoted SEQ ID NO: 18.

[00285] SEQ ID NO: 2 = Amino acid sequence of TRI032. Underlined is the secretion signal encoded by the leader sequence described in figure 1.

MOTFGAFLVSFLAASGLAAAGGHGGSSGLGCDSQRPLVSSEKLQSLIKKEDLLAGSQELQDIATAHGGHRA
FGSSGHNATVDFLYYTLKALDYYNVmQPFKEIFSSGTGSLTVDGEDIEAETLTYTPSGSATDKPVVWAN
VGCDAADYPAEVAGNIALIKRGTCTFSQKSWAKAAGAVAAIIYNNAEGKLSGTLGQPFLDYAPVLGITLE
AGEALLAKLAGGPWATLQIDALVEERVTYNVIAETKEGDHSNVLVLGGHTDSWAGPGINDDGSGTIGML
TVAKALTKFRVKNAVRFAFWSAEEYGLLGSYAYIKSINSSAAELSKIRAYLNFDMIASPNYIYGIYDGDGNA
FNLTGPAGSDVIERNFENFFKRKHTPSVPTEFSGRSDYAAFIENGIPSGGLFTGAEVLKTEREAELFGGRAGV
AYDVNYHQAGDTVDNLALDAFLLNmAIADSVATYALSFDGLPRVDGKKRRWDAHRARMLKRSAGSHG
HAHLHSGPCGGGASI (Seq ID no 2)

The sequence absent the underlined leader sequence is denoted SEQ ID NO: 19.

[00286] SEQ ID NO: 3 = Amino acid sequence of TRI033. Underlined is the secretion signal encoded by the leader sequence described in figure 1.

MQTFGAFLVSFLAASGLAAATKKPLVNELKLQKDINIKDLMAGAQKLQDIAEANGNTRVFGGAGHNATV
DYLYKTLKATGYYNVKKQPFTELYSAGTASLKVDGDDITAAIMTYTPAGEATGPLWAENLGCEASDFPA
ESEGKVVLVLRGECPFSQKSTNGKTAGAAAVIVYNNWGELAGTLGEPFGEFAPIVGISQEDGQAILAKTKA
GEWVDLKVDATVENRWFNVIAETKEGDHDNVLVVGGHSDSVAAGPGINDDGSGIIGILKVAQALTKYR
VKNAVPJGFWSAEEFGLLGSYAYMKSINGSDAEVAKIRAYLNFDMIASPNYVYGIYDGDGSAFNLTGPAG
SDAIEKDFERFFKTK^GYVPSEFSGRSDYAAFIENGIPSGGLFTGAEQLKTEEEAKKFGGEAGVAYDINYH
KIGDDINM.NK£AFLWTQAIANSVARYAKTWKSLPKVTONTPJIWDAEVASVLKRSSGHSHAGGPCGSVS
V (Seq ID no 3)

The sequence absent the underlined leader sequence is denoted SEQ ID NO: 20.

[00287] SEQ ID NO: 4 = Amino acid sequence of TRI034. Underlined is the secretion signal encoded by the leader sequence described in figure 1.

MOTFGAFLVSFLAASGLAAALQIPLNLQWKLSWM,FGDDLPLVDTK£LQKSIKPENLEARAKDLYEIAKN
GEEEYGHPTRVIGSEGHLGTLSYIHAELAKLGGYYSVSNQQFPAVSGNVFESRLVIGDSVPKQASPMGLTPP
TKNKEPVHGTLVLVDNEGCDASDYPEAVKGNIALILRGTCPFGTKSGNAGKAGAVAAVVYNYEKDEVHG
TLGTPSPDHVATFGLGGEEGKAVAKKLKDGEKVDAIAYroAEVKTISTTNIIAQTRGGDPDNCVMLGGHSD
SVAEGPGINDDGSGSISVLEVAVQLmYRVNNCVRFAWWAAEEEGLLGSDHYVSVLPEDENRKIRLFMDY

DMMASPNFAYQIYNATNAENPKGSEELRDLYVNWYEEQGLNYTFIPFDGRSDYDGFIRGGIPAGGIATGAE
GVKTEDEVEMFGGEAGWYDKNYHQIGDDLTNVNYTAWEWmLIAHSVATYAKSFKGFPEP^IETSVQ
TYSDKTKYHGSKLFI (Seq ID no 4)

The sequence absent the underlined leader sequence is denoted SEQ ID NO: 21.

[00288] SEQ ID NO: 5 = Amino acid sequence of TRI035. Underlined is the secretion signal encoded by the leader sequence described in figure 1.

MQTFGAFLVSFLAASGLAAANAPGGPGGHGPJCLPVNPKTFPNEIPXKDLLHGSQKLEDFAYAYPEPJmVF
GGQAHLDTVNYLYRELKKTGYYDVYKQPQVHQWTRA^QSLTLGGDSIQASTMTYSPSVNVTAPLSLVSK
LGCAEGDYSADVKGKIALVSRGECSFAQKSVLSAKAGAVATIVYNNVDGSLAGTLGGATSELGPYSPIIGIT
LAAGQDLVAPXQAAPTEVSLWIDSKVENRTTYNVIAQmGGDPNNWALGGHTDSVENGPGINDDGSGVI
SNLWAKALTRYSVKNAVRFCFWTAEEFGLLGSNYYVDNLSPAELAKIRLYLNFDMIASPNYALMIYDGD
GSAFNLTGPPGSAQIESLFENYYKSIKQGFVPTAFDGRSDYEGFILKGIPAGGVFTGAESLKTEEQARLFGGQ
AGVALDANYHAKGDNMTNLNHKAFLINSRATAFAVATYANNLSSIPPPJSATVVKRESMKWTKREEPHTH
GADTGCFASRVKE (Seq ID no 5)

The sequence absent the underlined leader sequence is denoted SEQ ID NO: 22.

[00289] SEQ ID NO: 6 = Amino acid sequence of TRI036. Underlined is the secretion signal encoded by the leader sequence described in figure 1.

MQTFGAFLVSFLAASGLAAAGGPHGFGLPKIDLPJMVSSNRLQSMITLKDLMDGAKKLQDIATKNGGNRA
FGGAGHNATVDYLYKTLTSLGGYYTVKKQPFKEIFSSGSGSLIVDGQGIDAGIMTYTPGGSATANLVQVAN
LGCEDEDYPAEVAGNIALISRGSCTFSSKSLKAKAAGAVGAIVYNNVPGELSGTLGTPFLDYAPIVGISQED
GQVILEKLAAGPWATLNIDAIVEERTTYNVIAETKEGDHNNVLIVGGHSDSVAAGPGINDDGSGTIGILTV
AKALAKANWIKNAVPJAFWSAEEFGLLGSYAYMKSLNESEAEVAKIRAYLNFDMIASPNYIYGIYDGDG
NAFNLTGPAGSDIIEKDFEDFFKKKKTPSVPTEFSGRSDYAAFIENGIPSGGLFTGAEVLKTEEEAKLFGGKA
GVAYDWYHKAGDTVDNLAKDAFLLNmAIANSVAKYAASWAGFPKPSAVPJyiYDADMAQLLKRSGGV
HGHGPHTHSGPCGGGDLL (Seq ID no 6)

The sequence absent the underlined leader sequence is denoted SEQ ID NO: 23.

[00290] SEQ ID NO: 7 = Amino acid sequence of TRI037. Underlined is the secretion signal encoded by the leader sequence described in figure 1.

MOTFGAFLVSFLAASGLAAAEGLGNHGPJCLDPNKJmDIKLKDLLKGSQKLEDFAYAYPEPJvlRVFGGKAH
QDTWWIYNELKKTGYYDVYKQPQVHLWSNAEQSLTVDGEAIDATTMTYSPSLKETTAEVVVVPGLGCT
AADYPADVAGKIALIQRGSCTFGEKSVYAAAANAAAAIVYNNVDGSLSGTLGAATSELGPYAPIVGISLAD
GQNLVSLAQAGPLTVDLYINSQMENRTTHNVIAKSKGGDPNNVIVIGGHSDAVNQGPGVNDDGSGIISNLV
IAKALmYSLKNSWWAFWTAEEFGLLGSEFYWSLSAAEKDKIKLYLNFDMIASPNYALMIYDGDGSTFN
MTGPAGSAEIEHLFEDYYKSRGLSYIPTAFDGRSDYEAFILNGIPAGGLFTGAEQIKTEEQVAMFGGQAGVA
YDPNYHAAGDNMTNLSEEAFLINSKATAFAVATYANSLESIPPRNATMSIQTRSASRRAAAHRRAAKPHSH
SGGTGCWHTRVEL (Seq ID **no** 7)

The sequence absent the underlined leader sequence is denoted SEQ ID NO: 24.

[00291] SEQ ID NO: 8 = Amino acid sequence of TRI038. Underlined is the secretion signal encoded by the leader sequence described in figure 1.

MOTFGAFLVSFLAASGLAAAGKHKPLVTPEALQDLITLDDLLAGSQQLQDFAYAYPERNRVFGGRAHDDT
WWLYRELKRTGYYHVYKQPQVHLYSNAEESLTVNGEAIEATTMTYSPSANASAELAVISGLGCSPADFA
SDVAGKVVLVQRGNCTFGEKSVYAAAADAAATIVYNNVEGSLSGTLGAAQSEQGPYSGIVGISLADGEAL
LALAEEGPVHVDLWIDSVMENRTTYNVIAQT^GGDPDNVVTLGGHSDSVEAGPGINDDGSGIISNLVIARA
LTKFSTKHAVRFFFWTAEEFGLLGSDYYVSSLSPAELAKIRLYLNFDMIASPNYGLLLYDGDGSAFNLTGPA
GSDAIEKLFYDYFQSIGQATVETEFDGRSDYEAFILNGIPAGGVFTGAEEIKSEEEVALWGGEAGVAYDANY
HQVGDTIDNLNTEAYLLNSKATAFAVATYANDLSTIPKP^MTTAVKRANWGHMHRRTMPKKRQTAHRH
AAKGCFHSRVEQ (Seq ID **no** 8)

The sequence absent the underlined leader sequence is denoted SEQ ID NO: 25.

[00292] SEQ ID NO: 9 = Nucleotide sequence of the pTrex8gM_TRI03 1 expression construct.

CTAGAGTTGTGAAGTCGGTAATCCCGCTGTATAGTAATACGAGTCGCATCTAAATACTCCGAAGCTGCTGCGAACCCGGAGAATC
GAGATGTGCTGGAAAGCTTCTAGCGAGCGGCTAAATTAGCATGAAAGGCTATGAGAAATTCTGGAGACGGCTTGTTGAATCATGG
CGTTCCATTCTTCGACAAGCAAAGCGTTCCGTCGCAGTAGCAGGCACTCATTCCCGAAAAAACTCGGAGATTCCTAAGTAGCGAT
GGAACCGGAATAATATAATAGGCAATACATTGAGTTGCCTCGACGGTTGCAATGCAGGGGTACTGAGCTTGGACATAACTGTTCC
GTACCCCACCTCTTCTCAACCTTTGGCGTTTCCCTGATTCAGCGTACCCGTACAAGTCGTAATCACTATTAACCCAGACTGACCGG
ACGTGTTTTGCCCTTCATTTGGAGAAATAATGTCATTGCGATGTGTAATTTGCCTGCTTGACCGACTGGGGCTGTTCGAAGCCCGA

ATGTAGGATTGTTATCCGAACTCTGCTCGTAGAGGCATGTTGTGAATCTGTGTCGGGCAGGACACGCCTCGAAGGTTCACGGCAA
GGGAAACCACCGATAGCAGTGTCTAGTAGCAACCTGTAAAGCCGCAATGCAGCATCACTGGAAAATACAAACCAATGGCTAAAA
GTACATAAGTTAATGCCTAAAGAAGTCATATACCAGCGGCTAATAATTGTACAATCAAGTGGCTAAACGTACCGTAATTTGCCAA
CGGCTTGTGGGGTTGCAGAAGCAACGGCAAAGCCCCACTTCCCCACGTTTGTTTCTTCACTCAGTCCAATCTCAGCTGGTGATCCC
CCAATTGGGTCGCTTGTTTGTTCCGGTGAAGTGAAAGAAGACAGAGGTAAGAATGTCTGACTCGGAGCGTTTTGCATACAACCAA
GGGCAGTGATGGAAGACAGTGAAATGTTGACATTCAAGGAGTATTTAGCCAGGGATGCTTGAGTGTATCGTGTAAGGAGGTTTGT
CTGCCGATACGACGAATACTGTATAGTCACTTCTGATGAAGTGGTCCATATTGAAATGTAAGTCGGCACTGAACAGGCAAAAGAT
TGAGTTGAAACTGCCTAAGATCTCGGGCCCTCGGGCCTTCGGCCTTTGGGTGTACATGTTTGTGCTCCGGGCAAATGCAAAGTGTG
GTAGGATCGAACACACTGCTGCCTTTACCAAGCAGCTGAGGGTATGTGATAGGCAAATGTTCAGGGGCCACTGCATGGTTTCGAA
TAGAAAGAGAAGCTTAGCCAAGAACAATAGCCGATAAAGATAGCCTCATTAAACGGAATGAGCTAGTAGGCAAAGTCAGCGAAT
GTGTATATATAAAGGTTCGAGGTCCGTGCCTCCCTCATGCTCTCCCCATCTACTCATCAACTCAGATCCTCCAGGAGACTTGTACA
CCATCTTTTGAGGCACAGAAACCCAATAGTCAACCATCACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCAGACCTTCGGTGC
TTTTCTCGTTTCCTTCCTCGCCGCCAGGTAAGTTGGCCTTGATGAACCATATCATATATCGCCGAGAAGTGGACCGCGTGCTGAGA
CTGAGACAGCGGCCTGGCCGCGGCCAACGGACCTGGATGGGATTGGAAGCCCCCCGTCCACCCCAAGGTCCTCCCCCAGATGATC
CACCTCTGGGACCTCATGCACGGCGCCCAGAAGCTCGAAGATTTCGCCTACGCCTACCCCGAGCGCAACCGCGTCTTTGGCGGCC
CTGCCCACGAGGACACCGTCAACTACCTCTACCGCGAGCTGAAGAAGACCGGCTACTACGACGTCTACAAGCAGCCCCAGGTCCA
CCAGTGGACCCGAGCCGATCAGGCCCTCACCGTCGACGGCAAGAGCTACGTCGCCACCACCATGACCTACAGCCCCAGCGTCAAC
GTCACCGCCCCTCTCGCCGTCGTCAACAACCTCGGCTGCGTCGAGAGCGACTACCCCGCCGACCTCAAGGGCAAGATCGCCCTCG
TTTCTCGCGGCGAGTGCCCCTTCGCCACCAAGTCTGTCCTCAGCGCCAAGGCTGGCGCCGCTGCCGCTCTCGTCTACAACAACATC
GAGGGCAGCATGGCCGGCACCCTCGGCGGACCTACTTCTGAGCTGGGCCCCTACGCCCCCATTGCCGGCATTTCTCTCGCCGACG
GCCAGGCCCTCATCCAGATGATTCAGGCCGGCACCGTCACCGCCAACCTCTGGATCGACAGCAAGGTCGAGAACCGCACCACCTA
CAACGTCATTGCCCAGACCAAGGGCGGCGACCCCAACAACGTCGTCGCTCTCGGCGGCCACACCGACTCTGTTGAGGCTGGCCCT
GGCATCAACGACGACGGCAGCGGCATCATCAGCAACCTCGTCGTCGCCAAGGCCCTCACCCGCTTCAGCGTCAAGAACGCCGTCC
GCTTCTGCTTCTGGACCGCCGAAGAGTTCGGCCTCCTCGGCAGCAGCTACTACGTCAACAGCCTCAACGCCACCGAGAAGGCCAA
GATCCGCCTCTACCTCAACTTCGACATGATCGCCAGCCCCAACTACGCCCTCATGATCTACGACGGCGACGGCAGCGCCTTCAACC
TCACTGGCCCTGCTGGCAGCGCCCAGATCGAGCGCCTCTTCGAGGACTACTACAAGAGCATCCGCAAGCCCTTCGTCCCCACCGA
GTTCAACGGCCGCAGCGACTACGAGGCCTTCATCCTCAACGGCATCCCCGCTGGCGGCATCTTCACTGGCGCCGAGGCCATCAAG
ACCGAGGAACAGGCCAAGCTGTTCGGCGGCCAGGCTGGCGTCGCCCTCGATGCCAACTACCACGCCAAGGGCGACAACATGACC
AACCTCAACCGCGAGGCCTTCCTCATCAACAGCAAGGCCACCGCCTTCGCCGTCGCCACCTACGCCAACTCCCTCGACAGCATCC
CCAGCCGCAACATGAGCACCGTCGTCAAGCGCAGCCAGCTTGAGCAGGCCAAGAAGTCCACCCCCCACACCCACACTGGCGGCA
CCGGCTGCTACAAGGACCGCGTCGAACAGTAAGACCCAGCTTTCTTGTACAAAGTGGTGATCGCGCCAGCTCCGTGCGAAAGCCT
GACGCACCGGTAGATTCTTGGTGAGCCCGTATCATGACGGCGGCGGGAGCTACATGGCCCCGGGTGATTTATTTTTTTTGTATCTA
CTTCTGACCCTTTTCAAATATACGGTCAACTCATCTTTCACTGGAGATGCGGCCTGCTTGGTATTGCGATGTTGTCAGCTTGGCAAA
TTGTGGCTTTCGAAAACACAAAACGATTCCTTAGTAGCCATGCATTTTAAGATAACGGAATAGAAGAAAGAGGAAATTAAAAAA
AAAAAAAAAACAAACATCCCGTTCATAACCCGTAGAATCGCCGCTCTTCGGCTAGCTAGTTACGCTTGTTTATTTACGACAAGATC
TAGAAGATTCGAGATAGAATAATAATAATAACAACAATTTGCCTCTTCTTTCCACCTTTTCAGTCTTACTCTCCCTTCTGACATTGA
ACGCCTCAATCAGTCAGTCGCCTTGTACTTGGCACGGTAATCCTCCGTGTTCTTGATATCCTCAGGGGTAGCAAAGCCCTTCATGC
CATCGATAATGTCATCCAGAGTGAGGATGGCAAAGATGGGGATGCCGTACTCCTTCCTCAGCTCGCCAATGGCACTCGGTCCAGG
CTTGGAGTCGTCGCCATCCGCAGCGGGGAGCTTCTCCATGCGGTCCAGGGCCACGACGATGCCGGCGACGATGCCGCCCTCCTTG
GTGATCTTCTCAATGGCGTCCCTCTTGGCGGTGCCGGCGGTGATGACGTCGTCGACAATCAGGACCCTCTTGCCCTTGAGCGAAGC
GCCGACGATGTTGCCGCCCTCGCCGTGGTCCTTGGCCTCCTTGCGGTCAAACGAGTAGGAGACGCGGTCCAGGTTCTGGGGCGCC
AGCTCGCCGAGCTTGATGGTGATGGCGGAGCACAGCGGGATGCCCTTGTAGGCCGGGCCGAAGACGATGTCGAACTCTAGGCCG
GCCTTCTCCTGGGCCTCGATGATGGTCTTTGCAAAGGCGGAGGCGATGGCGCCGGCGAGGCGCGCCGTGTGGAATTCGCCCGCGT
TGAAGAAGTAGGGGGATATCCGCTTGGACTTGAGCTCGAAGCTGCCAAACTTGAGGACGCCGCCGTCGATGGCGGATTTGAGGA
AGTCCTGCTTGTAGGCAGGCAGCTGGGAGGTGGTAGCCATTCTGTTGGATTTGGATAGTGTCCTTATTCTCTGATTTGAACAGTAG
ATCAGGACGAGTGAGAGGGATGCAGAGGTTGGATTGGAGTGGTTGAGCTATAAAATTTAGAGGCGCGCCGTATCGAGTTTTCACA
TGGAAGTCAAAGCGTACAGTGCGAGCTTGTACGTTGGTCTTAGTATCCCACAAGCTTCTGTCTAGGTATGATGATGGCTATAAGTC

ACCCAAGGCAGAACTCATCTTGAAGATTGTCTAGAGTGATTTTACCGCTGATGAAATGACTGGACTCCCTCCTCCTGCTCTTATAC
GAAAAATTGCCTGACTCTGCAAAGGTTGTTTGTCTTGGAAGATGATGTGCCCCCCCATCGCTCTTATCTCATACCCCGCCATCTTTC
TAGATTCTCATCTTCAACAAGAGGGGCAATCCATGATCTGCGATCCAGATGTGCTTCTGGCCTCATACTCTGCCTTCAGGTTGATG
TTCACTTAATTGGTGACGAATTCAGCTGATTTGCTGCAGTATGCTTTGTGTTGGTTCTTTCCAGGCTTGTGCCAGCCATGAGCGCTT
TGAGAGCATGTTGTCACCTATAAACTCGAGTAACGGCCACATATTGTTCACTACTTGAATCACATACCTAATTTTGATAGAATTGA
CATGTTTAAAGAGCTGAGGTAGCTTTAATGCCTCTGAAGTATTGTGACACAGCTTCTCACAGAGTGAGAATGAAAAGTTGGACTC
CCCCTAATGAAGTAAAAGTTTCGTCTCTGAACGGTGAAGAGCATAGATCCGGCATCAACTACCTGGCTAGACTACGACGTCAATT
CTGCGGCCTTTTGACCTTTATATATGTCCATTAATGCAATAGATTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCCC
AATTTCGCAGATCAAAGTGGACGTTATAGCATCATAACTAAGCTCAGTTGCTGAGGGAAGCCGTCTACTACCTTAGCCCATCCATC
CAGCTCCATACCTTGATACTTTAGACGTGAAGCAATTCACACTGTACGTCTCGCAGCTCTCCTTCCCGCTCTTGCTTCCCCACTGGG
GTCCATGGTGCGTGTATCGTCCCCTCCTTAATTAAGGCCATTTAGGCCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGA
GCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTC
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CTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCT
GCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGAT
TAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGT
ATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTG
GTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCT
CAGTGGAACGAAAACTCACGTTAAGGCCTGCAGGGCCGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTT
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CCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACC
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TGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAA
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TTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAA
AACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTAT
TGAAGCATTTATCAGGGTTATTGTCTCATGGCCATTTAGGCCT (Seq ID no 9)

[00293] SEQ ID NO: 10 = Nucleotide sequence of the pTrex8gM_TRI032 expression construct.

CTAGAGTTGTGAAGTCGGTAATCCCGCTGTATAGTAATACGAGTCGCATCTAAATACTCCGAAGCTGCTGCGAACCCGGAGAATC
GAGATGTGCTGGAAAGCTTCTAGCGAGCGGCTAAATTAGCATGAAAGGCTATGAGAAATTCTGGAGACGGCTTGTTGAATCATGG
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GGAACCGGAATAATATAATAGGCAATACATTGAGTTGCCTCGACGGTTGCAATGCAGGGGTACTGAGCTTGGACATAACTGTTCC
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ATGTAGGATTGTTATCCGAACTCTGCTCGTAGAGGCATGTTGTGAATCTGTGTCGGGCAGGACACGCCTCGAAGGTTCACGGCAA
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GTACATAAGTTAATGCCTAAAGAAGTCATATACCAGCGGCTAATAATTGTACAATCAAGTGGCTAAACGTACCGTAATTTGCCAA
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CCAATTGGGTCGCTTGTTTGTTCCGGTGAAGTGAAAGAAGACAGAGGTAAGAATGTCTGACTCGGAGCGTTTTGCATACAACCAA
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CTGCCGATACGACGAATACTGTATAGTCACTTCTGATGAAGTGGTCCATATTGAAATGTAAGTCGGCACTGAACAGGCAAAAGAT
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CACCGAGCCTTTGGAAGCTCTGGCCACAACGCCACCGTCGACTTTCTCTACTACACCCTCAAGGCCCTCGACTACTACAACGTCAC
CAAGCAGCCCTTCAAGGAAATCTTCAGCAGCGGCACCGGCAGCCTCACCGTGGACGGCGAGGACATCGAGGCCGAGACTCTCAC
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CCTCAGCTTTGACGGCCTCCCTCGCGTCGACGGCAAGAAGCGACGTTGGGACGCTCACCGAGCCCGCATGCTCAAGCGATCTGCT
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GCGCGCCGTGTGGAATTCGCCCGCGTTGAAGAAGTAGGGGGATATCCGCTTGGACTTGAGCTCGAAGCTGCCAAACTTGAGGACG
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TGGACTCCCTCCTCCTGCTCTTATACGAAAAATTGCCTGACTCTGCAAAGGTTGTTTGTCTTGGAAGATGATGTGCCCCCCCATCGC
TCTTATCTCATACCCCGCCATCTTTCTAGATTCTCATCTTCAACAAGAGGGGCAATCCATGATCTGCGATCCAGATGTGCTTCTGGC
CTCATACTCTGCCTTCAGGTTGATGTTCACTTAATTGGTGACGAATTCAGCTGATTTGCTGCAGTATGCTTTGTGTTGGTTCTTTCC

AGGCTTGTGCCAGCCATGAGCGCTTTGAGAGCATGTTGTCACCTATAAACTCGAGTAACGGCCACATATTGTTCACTACTTGAATC
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CTTCCCGCTCTTGCTTCCCCACTGGGGTCCATGGTGCGTGTATCGTCCCCTCCTTAATTAAGGCCATTTAGGCCGTTGCTGGCGTTT
TTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGA
TACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCT
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ATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTC
TTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGA
AAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTC
ACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTC
ATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGGCCATTTAGGCCT (Seq ID no 10)

[00294] SEQ ID NO: 11 = Nucleotide sequence of the pTrex8gM_TRI033 expression construct.

CTAGAGTTGTGAAGTCGGTAATCCCGCTGTATAGTAATACGAGTCGCATCTAAATACTCCGAAGCTGCTGCGAACCCGGAGAATC
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TATAGCATCATAACTAAGCTCAGTTGCTGAGGGAAGCCGTCTACTACCTTAGCCCATCCATCCAGCTCCATACCTTGATACTTTAG
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CTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAA
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TTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACC
CACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCA
AAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTG
TCTCATGGCCATTTAGGCCT (Seq ID no 11)

[00295] SEQ ID NO: 12 = Nucleotide sequence of the pTrex8gM_TRI034 expression construct.

CTAGAGTTGTGAAGTCGGTAATCCCGCTGTATAGTAATACGAGTCGCATCTAAATACTCCGAAGCTGCTGCGAACCCGGAGAATC
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CTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGGCCATTTAGGCCT (Seq ID no 12)

[00296] SEQ ID NO: 13 = Nucleotide sequence of the pTrex8gM_TRI035 expression construct.

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[00297] SEQ ID NO: 14 = Nucleotide sequence of the pTrex8gM_TRI036 expression construct.

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TTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTT
GAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGGCCATTTAGGCCT (Seq ID no 14)

[00298] SEQ ID NO: 15 = Nucleotide sequence of the pTrex8gM_TRI037 expression construct.

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[00299] SEQ ID NO: 16 = Nucleotide sequence of the pTrex8gM_TRI038 expression construct.

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TTCCCCACTGGGGTCCATGGTGCGTGTATCGTCCCCTCCTTAATTAAGGCCATTTAGGCCGTTGCTGGCGTTTTTCCATAGGCTCCG
CCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC
CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGC
GCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTC
AGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCAC
TGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGG
ACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCG
CTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACG
GGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGCCTGCAGGGCCGATTTTGGTCATGAGATTATCAAAAAGGATCTTCAC
CTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAA
TCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGG
GAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGC
CAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGT
AAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTT
CATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCG
ATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGT
AAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGT
CAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAG
GATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTC
TGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCT
TTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGGCCATTTAGGCCT (Seq ID no 16)

Example 2 - Operational pH- and temperature optimum of the PepN 2 from *Neosartorya fischeri* (TRI031) in gluten hydrolyses

[00300] The liberation of glutamic acid is an important quality parameter in vegetable protein hydrolyses such as soy or gluten hydrolyses. The liberated glutamic acid can be perceived as the so-called "umami-flavor". In order to determine the effect of the initially applied pH in gluten hydrolyses in terms of glutamic acid and glutamine liberation, the initial pH of a gluten hydrolyses was adjusted just after the liquefaction of the gluten after 15 - 30 min with 1 M HC1. Gluten was treated with Glutaminase (Amano, Japan). In addition, gluten was treated with FoodPro Alkaline Protease, FoodPro PNL as well as PepN 2 (Neosartorya fischeri). After 20 hr, the hydrolysis was terminated employing ultrafiltration (10 kDa cut-off, Sartorius Stedium Biotech, Gottingen, Germany). The permeate was applied for the enzymatic glutamic acid analyses (enzymatic L-glutamic acid analysis kit, Roche, Mannheim, Germany). As shown in Figure 2, the PepN 2 performed more efficient within the pH range of 7.0 - 9.0 compared to pH 6.0.

[00301] In order to determine the effect of the applied temperature during the gluten hydrolyses in terms of glutamic acid and glutamine liberation, the initial pH was adjusted with 1 M HC1 to pH 7.0 just after the liquefaction of the gluten suspension after 15 - 30 min. A temperature range from 40 - 60°C was applied as shown in Figure 3. Gluten was combined with Glutaminase (Amano, Japan). In addition, gluten was combined FOODPRO® Alkaline Protease, FOODPRO® PNL as well as PepN 2 *(Neosartoryafischeri).* After 20 h of hydrolysis, the hydrolysis was terminated employing ultrafiltration (10 kDa cut-off, Sartorius Stedium, Gottingen, Germany). The permeate was applied for the enzymatic glutamic acid analyses (enzymatic L-glutamic acid analysis kit, Roche, Mannheim, Germany). The PepN 2 from *Neosartoryafischeri* performed most efficient at 60°C.

Example 3 - Comparison of the PepN 2 from *Neosartoryafischeri* (TRI031) and *Aspergillus clavatus* (TRI035) in terms of degree of hydrolyses

[00302] The degree of hydrolyses (DH) describes the relative amount of cleaved peptides bounds compared to an acid hydrolyses of the same amount of protein conducted e.g. in 6 M HCL at ~120°C for -24 h. The higher the DH can therefore be applied to assess the efficiency of amino acid liberation of a general PepN such as a PepN 1 or a PepN 2 type. The cell free culture

broth was concentrated (10 kDa cut-off, Sartorius, Gottingen, Germany) and desalted applying a disposal desalting column (PD10, GE, Miinchen, Germany) as described by the manufacturer. As substrates were pre-hydrolysed 10% (w/w) Na-caseinate (DMK, Germany), whey protein isolate (WPI; Aria, Viby, Denmark), soy protein isolate (SPI; SUPRO® 760, DuPont, Brabrand, Denmark) and gluten suspensions were applied. The pre-hydrolysis was conducted with 1% (w/wprotein) of FOODPRO® Alkaline Protease, 1% ($w/w_{prot\ ei_n}$) FOODPRO® PNL for the Na-Caseinate, the WPI as well as the SPI suspension. All pre-hydrolyses were conducted at 55°C for 18 h followed by an inactivation step at 95°C for 20 min. Each applied PepN 2 has been inactivated at 95 C for 15 min as a control of the elimination of any carryover of amino acids from the culture broth to the final hydrolyses as well as for the sufficient inactivation of the applied endopeptidases. The hydrolyses were conducted in a 96-well micro titer plate format and consisted of 150 µL of the pre-hydrolyzed protein suspension combined with 50 µL of a PepN 2 stock solution as indicated in Table 1 below. The hydrolyses were conducted at 50°C for 20 hr, subsequently, the hydrolysis were stopped by the addition of 20 µL 2 M TCA (Trichloroacetic acid, Sigma-Aldrich, Schnelldorf, Germany). The efficiency of the amino acid liberation of the PepN 2 types from *Neosartoryafischeri* and *Aspergillus clavatus* can be seen in Table 1 (B) and (A). In particular, for the substrates, Na-caseinate, WPI, and SPI, significantly higher DHs were achieved upon the application of the PepN 2 types from *N. fischeri* and *A. clavatus.* In addition, the achieved DH at a PepN 2 stock solution concentration could be significantly increased. For the gluten hydrolyses, an increased DH was noticed in the protein range from 0.02 - 0.3 mg/mL PepN 2.

Table 1: Comparison of the degree of hydrolyses of the PepN 2 types from *N. fischeri* and *A. clavatus.*

A

		PepN 2 from *Neosartorya fischeri* (mg/mL)										
Substrate	water	inactivated enzyme at the	0.02	0.03	0.04	0.05	0.1	0.15	0.2	0.25	0.3	0.35

		highest cone.										
			Degree of hydrolysis [%]									
Casein	7	8	14	15	16	20	19	21	23	22	24	24
WPI	7	8	17	19	19	21	23	28	24	26	27	29
SPI	5	6	11	13	14	15	17	18	19	19	19	22
Gluten	3	4	10	13	15	14	17	18	19	19	21	20

B

		PepN 2 from *Aspergillus clavatus* (mg/mL)										
Substrate	water	inactivated enzyme at the highest cone.	0.02	0.03	0.04	0.05	0.1	0.15	0.2	0.25	0.3	0.35
			Degree of hydrolysis [%]									
Casein	8	8	16	19	19	20	21	23	24	24	24	27
WPI	7	8	19	20	21	22	23	27	25	28	29	29
SPI	6	6	11	12	13	14	16	15	17	21	21	21
Gluten	3	4	12	14	15	15	17	18	17	19	19	22

Example 4 - Gluten hydrolyses applying two endopeptidases in combination with the PepN 2 candidates TRI032, TRI033, TRI034, TRI035, TRI037 and TRI038

[00303] A pre-hydrolysed gluten suspension was prepared by addition of FOODPRO® Alkaline Protease (FPAP) and FOODPRO® PNL (FPPNL). The hydrolysis was conducted at 55 °C. After ~18 hr, the hydrolysis was terminated by heating to 90 °C for 10 min. After cooling to 50°C, glutaminase was applied to the gluten. Subsequently, 150 µL of the pre-hydrolyzed gluten was transferred to each well of a 96-well microtiter plate. The glutamic acid liberation was conducted applying 50 µL (protein concentration: 0.5 mg/mL) of TRI032, TRI033, TRI034, TRI035, TRI037, and TRI038. The hydrolysis was performed for 18 hr and terminated by addition of 20 µL 2 M TCA. The terminated hydrolyses were filtered (0.22 mm) and further

analyzed with the enzymatic glutamic acid analyses kit (Roche, Mannheim, Germany). The liberated glutamic acid concentrations are shown in Figure 4.

Example 5 - Hydrolysis of proline containing peptide

[00304] The peptide WHWLQLKPGQPMY was hydrolysed with TRI03 1 and TRI035. The peptide (1 mg/mL) was incubated with 1 ug/mL of the aminopeptidase in 20 mM CPB-Buffer (20 mM Citric acid, 20 mM Phosphate, 20 mM Boric acid) at 55°C. Aliquots (50 µL) were stopped with 50 ul 5% TFA at the indicated time points and subjected to LC-MS analysis.

[00305] The present results show that TRI03 1 and TRI035 can hydrolyze the peptide WHWLQLKPGQPMY (Figure 5) to KPGQPMY (Figure 6) and further down to QPMY (Figure 7). That is in contrast to what has been described previously for *Aspergillus oryzae* PepN 2 (A.M. Blinkovsky *et al., Biochimica et Biophysica Acta,* 1480 (2000) 171-181, where it was claimed that the Aspergillus oryzae PepN 2 does not hydrolyze any of the X-Pro bonds examined including those of the peptide WHWLQLKPGQPMY.) However, we also find that their enzyme cleaves the peptide down to QPMY (Figure 7).

[00306] *Data Acquisition:* Capillary LC-MS/MS analyses were performed using an Agilent 1100 LC system (Agilent Technologies) interfaced to a LTQ Orbitrap Classic hybrid mass spectrometer (Thermo Scientific, Bremen, Germany). Samples were loaded onto a 15 cm Phenomenix Jupiter 4µ Proteo 90A, C4 analytical column. Separation was performed at a flow rate of 16 µL/ηιη using a 10 min gradient of 0-40% Solvent B $H_20/CH_3CN/$ HCOOH (50/950/0.65 v/v/v) into the IONMAX® ion source (Thermo Scientific, San Jose). The LTQ Orbitrap Classic instrument was operated in a data-dependent MS/MS mode. The peptide masses were measured by the Orbitrap (MS scans were obtained with a resolution of 60 000 at m/z 400), and up to 2 of the most intense peptide m/z were selected and subjected to fragmentation using CID in the linear ion trap (LTQ). Dynamic exclusion was enabled with a list size of 500 masses, duration of 40 s, and an exclusion mass width of ±10 ppm relative to masses on the list.

[00307] *Label free quantification:* The RAW files were accessed with the program Skyline 2.6.0.7176(MacLean, B., *etal,* "Skyline: an open source document editor for creating and analyzing targeted proteomics experiments", *Bioinformatics, 2010,* 26(7): p. 966-8) which uses the MSI intensities to build chromatograms *(Schilling, B., etal,* "Platform-independent and label-free quantitation of proteomic data using MSI extracted ion chromatograms in skyline: application to protein acetylation and phosphorylation", *Mol Cell Proteomics,* 2012. 11(5): p.

202-14). The precursor isotopic import filter was set to a count of three, (M, M+1, and M+2) at a resolution of 60,000 and the most intense charge state was used. Peptide sequences of the substrate as well as cleavage products were typed into Skyline and intensities were calculated in each sample. Figures were directly copied from the program (See Figure 8).

Example 6: Comparison of the degree of hydrolyses of the PepN 2 from *A. clavatus* to the PepN from *A. oryzae* and *L. helveticus* ATCC 12046

[00308] Enzyme activities of PepN 1 *Lactobacillus helveticus* ATCC® 12046, PepN 1 *Aspergillus oryzae* and from PepN 2 from *Aspergillus clavatus* (TRI035) were determined according to Stressler, Eisele *et al.* (2013, *infra).* PepN 1 from *L. helveticus* was expressed and purified as described by Stressler, Eisele *etal.* (2013, *infra).*

[00309] PepN 1 from *A. oryzae* was purified from FLAVOURZYME® 500L (Sigma-Aldrich, Schnelldorf, Germany) after desalting on PDio columns equilibrated in 20 mM Bis-Tris, pH 6.5. The sample was purified by anion exchange chromatography, on a Source Q15, XK26/15 (SQ15) column (GE -Lifesciences, USA) and equilibrated with 20 mM Bis/Tris, pH 6.5 (buffer A). The sample (30 mL) was loaded to the column at a flow rate of 7 mL/min. the column was washed with buffer A and bound proteins were eluted with a linier gradient of 0-0.5 M NaCl in 20 mM Bis/Tris, pH 6.5 (50 min). During the entire run, fractions of approx. 13 mL were collected and kept on ice. Fractions with the highest aminopeptidase activity were combined, desalted on PD10 in 20 mM Bis-Tris, pH 6.0 and subjected to a second run on a Poros Q20 HR26/10, XK26/10 column (GE -Lifesciences, USA) equilibrated with 20 mM Bis/Tris, pH 6.0 (buffer A). The PepNl sample was loaded to the column at a flow rate of 4 ml/min. the column was washed with buffer A and bound proteins were eluted with a linier gradient of 0-0.25 M NaCl in 20 mM Bis/Tris, pH 6.0 (30 min). During the entire run, fractions of approx. 8 mL were collected and kept on ice. The purified PepN 1 from *A. oryzae* was found to have a molecular weight of about 40 kDa according to SDS-PAGE electrophoresis.

[00310] The PepN 1 and 2 aminopeptidases were standardized to the activity range of 2.5 - 200 nkat*mL^{-1}. For protein hydrolyses, 1% (W/W_{WPI}) FOODPRO® Alkaline Protease (DuPont, Brabrand, Denmark) and 1% (w/wwpi) FOODPRO® PNL (DuPont, Brabrand, Denmark) were added to the whey protein isolate suspension (WPI; LACPRODAN® 9224, Aria Ingredients, Viby, Denmark) with an initial pH of 7.0. Following the endopeptidases addition, the WPI was

mixed and 200 µL were transferred to each well of a 96-well microtiter plate. Subsequently, 5 µL of PepN solution were added as indicated in the Table 2. The hydrolysis was conducted at 50 °C without pH control and terminated after 18 hr by addition of 20 µL of 2 M TCA. Prior to the determination of the DH (Nielsen, Petersen *et al.*, 2001, *infra)*, all samples were filtered 0.22 µm.

[00311] It was found that PepN 2 from *A. clavatus* achieved high DH than PepN 1 from *A. oryzae* at all dosages and higher DH than PepN 1 from *L. helveticus* at the four highest activity dosages tested. And at the two highest dosages PepN 2 from *A. clavatus* produced at least 25% higher DH than the PepN 1 aminopeptidases.

Table 2: Comparison of the degree of hydrolyses of the PepN 2 from *A. clavatus* to the PepN 1 from *L. helveticus* and *A. oryzae*

PepN dosage [nkat/mL] in stock solution									
0	**2,5**	**5**	**10**	**25**	**50**	**75**	**100**	**200**	
Degree of hydrolysis [%]									
14	**14**	15	**16**	**16**	**18**	**16**	**21**	**19**	**PepN 1: *A. oryzae***
13	**18**	18	**20**	**23**	**23**	**24**	**28**	***27***	**PepN 1: *L helveticus***
14	**15**	17	**17**	**20**	**24**	**26**	**35**	**36**	**PepN 2: A. *clavatus***

Example 7: Influence of sodium chloride concentration on glutamic acid liberation in soy and gluten hydrolyses

[00312] Sodium chloride is frequently applied in the food industry to reduce the microbial contamination risk in protein hydrolyses. Pre-hydrolysed 10% (w/w) soy (SUPRO® 760, DuPont, Brabrand, Denmark) and gluten (Sigma-Aldrich, Schnelldorf, Germany) was prepared by addition of 1% ($w/w_{protein}$) FOODPRO® Alkaline Protease (DuPont, Brabrand, Denmark) and 1% (w/wprotein) FOODPRO® PNL (DuPont, Brabrand, Denmark). The hydrolyses were performed at 50°C at pH 7.0 (without pH control) for 18 hr (heat inactivation after 18 hr; 90°C; 10 min). Subsequently, the hydrolyses were divided in sodium chloride containing (185 mM) and non-salt containing hydrolyses. 150 µL of each pre-hydrolysed protein suspension was

combined with differently standardized *Aspergillus clavatus* PepN 2 as indicated in Table 3. The hydrolyses were conducted at 50°C without pH control and terminated after 18 hr by addition of 20 µL of 2 M TCA. Prior to the determination of the DH (Nielsen, Petersen *et al.* 2001, *infra),* all samples were filtered 0.22 µm. As shown in Table 3, the addition of 185 mM NaCl had no influence on the determined degree of hydrolysis in soy and gluten hydrolyses.

Table 3: Influence of 185 mM NaCl on the degree of hydrolyses in soy and gluten hydrolyses

Protein											
SOY					Gluten						
Protein concentration [mg/mL]											
water	0.2	0.3	0.4	0.5	0.1	0.2	0.25	0.3	0.4	0.5	
Degree of hydrolysis											
1	1	11	12	12	12	14	16	17	16	17	18	reference
1	1	10	11	11	12	14	15	15	16	17	18	185 mM NaCl

[00313] Nielsen, P. M., *et al.* (2001), *Journal of Food Science* ***66****(5):* 642-646.

[00314] Stressler, T., *et al.* (2013), *PLoS ONE* 8(7).

Example 8: Product inhibition of PepN2 TRI031 and TRI035 vs. TRI063 (*A. oryzae)*

[00315] In this assay enzyme activity is determined by hydrolysis of the substrate H-Ala-nitroanilide (pNA). The absorbance of released pNA is determined over time at a wavelength of 405 nm using a microtiter plate reader.

[00316] As materials buffer 20 mM CPB buffer (20 mM of Na-citrate, Na-phosphate and Na-borate) pH 9.0 was used. Enzyme samples of PepN 2 TRI031 and TRI035 were used. 20 mg H-Ala-pNA substrate (BACHEM, L-1070) was solubilized in 1 mL DMSO (Dimethyl Sulphoxide; Sigma cat# D2650). 96 well plates Costar assay plate 9017 (Corning Inc) were

applied and read in a VERSAMAX® reader from Molecular Devices. For the inhibition studies 10 mL of a 20 mg/mL solution were made of each of the amino acids: lysine, histidine, leucine, tryptophan, proline, glycine, serine, asparagine, threonine, aspartic acid, glutamic acid. Tryptophan and proline were dissolved in DMSO. All other amino acids were dissolved in buffer. The final inhibitor mix was made by combining 500 µl of each amino acid solution.

[00317] A 96 well plate was placed on ice and 180 µl buffer, 15 µl diluted enzyme and 20 µl inhibitor mix were added to wells in the plate and mixed. Each plate contained all four enzymes at inhibitor mix concentrations of 0.017, 0.085, 0.128, 0.170, 0.426, 0.851, 1.702, 2.553 mg/mL. The reaction was started by adding 20 µl substrate. Only one substrate concentration was used per plate. After initiating the reaction, the plate was, as quickly as possible, placed in the microplate reader, set to 30°C, and absorbance at 405 nm was measured with 30 sec intervals for 30 min. Six plates were run with one substrate concentrations on each plate (either 0.017, 0.170, 0.426, 0.851, 1.702, 2.553 mg/mL). For determination of the apparent inhibition constant (Ki), data was exported to GraphPad Prism, which fitted Michaelis-Menten curves for each inhibitor concentration and based on this calculated Ki in mg/ml. The unit was converted to mM by using an average inhibitor molecular weight of 133.9 g/mol. Apparent Ki values for product inhibition and respective standard deviations (SD) for the different enzymes are listed in Table 4.

Table 4

	Ki (mM)	SD
TRI031	5.67	0.85
TRI035	3.06	0.59
TRI063 (A. oryzae)	1.20	0.36

[00318] From Table 4 it is seen that PepN 2 TRI03 1 and TRI035 have the highest apparent Ki values for product inhibition, meaning that they are less inhibited by product compared to TRI063 from A. oryzae.

Example 9: PepN2 and PepNl activity on Glu-pNA and Gln-pNA substrates

[00319] H-Glu-Nitroanilid (pNA) and H-Gln-Nitroanilid (pNA) were used as substrates and the release of p-Nitroanilid (pNA) was measured by absorbance at 405 nm. As buffer 20 mM CPB-Buffer (20 mM Citric acid, 20 mM Na-phosphate, 20 mM Boric acid) pH 9.0 was used. 10 mg H-Glu-pNA and H-Gln-pNA (from BACHEM or Schafer N; Copenhagen, Denmark) were dissolved in 1 ml of DMSO (Dimethyl Sulphoxide from SIGMA; cat # D2650).

[00320] For running the assay 80 μL buffer, 10 μL H-Glu-pNA or H-Gln-pNA substrate and 10 uL appropriately diluted PepN 2 was incubated in Costar assay plate 9017 (Corning Inc.) at 45°C and measured at 405 nm every 30 sec for 30 min in a VERSAMAX® microplate reader (Molecular Devices) running with SoftMaxPro 5.4. 1 software. Activity was determined as the maximal slope of the linear part of the curve determined over 10 time points.

[00321] The assay was run for purified samples of PepN 2 TRI03 1 and TRI035 as well as purified PepN 1 L. helveticus (Stressler, Eisele *et al., supra)* and non-purified PepN 1 A. sojae (COROLASE® LAP, AB Enzymes) which all had comparable activity on Ala-pNA or Leu-pNA (run as described for Ala-pNA in Example H, but without inhibitor). As shown in Table 5 the two PepN 2 aminopeptidases showed activity on Glu- and Gln-pNA in contrast to the two PepN 1 enzymes that showed no significant activity. This indicates that PepN 2 aminopeptidases are much more effective in Glu and Gin release than PepN 1 aminopeptidases.

Table 5. Activity of PepN 2 and PepN 1 on Glu-pNA and Gln-pNA substrates

	Vmax (mU/min)	
	Glu-pNA	Gln-pNA
PepN 2 TRI031	50.8	75.9
PepN 2 TRI035	20.1	98.7
Pep N 1 L. helveticus	0.02	0
PepN 1 Corolase LAP	0	0

Example 10 - Hydrolysis of peptide with proline in position 2

[00322] The peptide library XPAAAR (X being all amino acids except cysteine) was hydrolysed with enzyme samples of TRI032, TRI035, TRI063 (A. oryzae) as well as COROLASE® LAP. The peptide library XPAAAR (1 mg/mL) was incubated with 1 ug/mL of the aminopeptidase in 3 x20 mM CPB-Buffer (20 mM Citric acid, 20 mM Phosphate, 20 mM Boric acid) at 55°C. 50 µï¿½ aliquots were stopped with 50 ul 5% TFA at the indicated time points and subjected to LC-MS analysis.

[00323] *Data Acquisition:* Capillary LC-MS/MS analyses were performed using an Agilent 1100 LC system (Agilent Technologies) interfaced to a LTQ Orbitrap Classic hybrid mass spectrometer (Thermo Scientific, Bremen, Germany). Samples were loaded onto a 15 cm Phenomenex Jupiter 4µ Proteo 90A, C4 analytical column. Separation was performed at a flow rate of 16 µL/ηιη using a 10 min gradient of 0-40% Solvent B $H_20/CH_3CN/$ HCOOH (50/950/0.65 v/v/v) into the IONMAX® ion source (Thermo Scientific, San Jose). The LTQ Orbitrap Classic instrument was operated in a data-dependent MS/MS mode. The peptide masses were measured by the Orbitrap (MS scans were obtained with a resolution of 60 000 at m/z 400), and up to 2 of the most intense peptide m/z were selected and subjected to fragmentation using CID in the linear ion trap (LTQ). Dynamic exclusion was enabled with a list size of 500 masses, duration of 40 s, and an exclusion mass width of ±10 ppm relative to masses on the list.

[00324] Label free quantification: The RAW files were accessed with the program Skyline 2.6.0.7176 (MacLean, B., *et al., supra)* which uses the MSI intensities to build chromatograms (Schilling, B., *et al, supra).* The precursor isotopic import filter was set to a count of three, (M, M+1, and M+2) at a resolution of 60,000 and the most intense charge state was used. Peptide sequences of the substrate as well as cleavage products were typed into Skyline and intensities were calculated in each sample. Figure 10 was generated on the basis the sum of the peak areas of the three isotopes (M, M+1, and M+2). A substrate standard curve (with serial 2 fold increases in concentration from Std 3.125 to Std 50) was included and shows a linear relationship between substrate concentration and peak area.

[00325] LC-MS results (Figure 10) show that TRI032 and TRI035 can hydrolyze the peptide TPAAAR over time to less than half the concentration within 2 hr incubation, whereas TRI063 *{A. oryzae)* and COROLASE® LAP show no hydrolysis of TPAAAR within 12 hr.

[00326] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way

of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS:

1. An isolated polypeptide having aminopeptidase activity, selected from the group consisting of:

(a) a polypeptide having an amino acid sequence which has at least 80% identity with the amino acid sequence of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, or SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8; and

(b) a fragment of (a), wherein the fragment has aminopeptidase activity.

2. The polypeptide of claim 1, comprising an amino acid sequence which has at least 90% identity with the amino acid sequence of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, or SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8.

3. The polypeptide of claim 1, comprising the amino acid sequence of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, or SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8 or a fragment thereof.

4. The polypeptide of claim 3, comprising the amino acid sequence of SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 5, or SEQ ID NO: 6, or SEQ ID NO: 7, or SEQ ID NO: 8.

5. The polypeptide of any of claims 1 to 4, wherein said fragment comprises any one of SEQ ID NOs: 18 to 25.

6. An isolated polypeptide having aminopeptidase activity which has a predicted mature sequence with more than 13 residues on the N-terminal side of the conserved residue I/V in position 67 as shown in the sequence alignment of Figure 9, or a fragment thereof, wherein the fragment has aminopeptidase activity.

7. An isolated polypeptide that has aminopeptidase activity, and which has decreased product inhibition compared to the aminopeptidase of SEQ ID NO: 17.

8. The isolated polypeptide of claim 7 wherein said polypeptide is as defined in any one of claims 1 to 6.

9. An isolated polypeptide that has aminopeptidase activity, and which is capable of hydrolyzing a polypeptide with a proline residue in position 2, as numbered from the N-terminus.

10. The isolated polypeptide of claim 9, which is capable of hydrolyzing a polypeptide with a proline residue in position 2, as numbered from the N-terminus, to less than half the starting concentration within 2 hours of incubation.

11. The isolated polypeptide of claim 9 or 10, wherein said isolated polypeptide is as defined in any one of claims 1 to 8.

12. An isolated nucleic acid sequence comprising a nucleic acid sequence which encodes the polypeptide of any of claims 1 to 8.

13. A nucleic acid construct comprising the nucleic acid sequence of claim 12 operably linked to one or more control sequences which direct the production of the polypeptide in a suitable expression host.

14. A recombinant expression vector comprising the nucleic acid construct of claim 13, a promoter, and transcriptional and translational stop signals.

15. A recombinant host cell comprising the nucleic acid construct of claim 13.

16. The recombinant host cell of claim 15, wherein the host cell is a *Trichoderma* cell, preferably a *Trichoderma reesei* cell.

17. A method for producing the polypeptide of any of claims 1 to 11 comprising (a) cultivating a strain to produce a supernatant comprising the polypeptide; and (b) recovering the polypeptide.

18. A method for producing the polypeptide of any of claims 1 to 11 comprising (a) cultivating a host cell of claims 15 or 16 under conditions suitable for production of the polypeptide; and (b) recovering the polypeptide.

19. A method for producing the polypeptide of any of claims 1 to 11 comprising (a) cultivating a homologous recombinant cell, having incorporated therein a new transcription unit comprising a regulatory sequence, an exon, and/or a splice donor site operably linked to a second exon of an endogenous nucleic acid sequence encoding the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

20. The method of claim 19, wherein the host cell is a *Trichoderma* cell, preferably a *Trichoderma reesei* cell.

21. A method for the production of a protein hydrolysate comprising subjecting a proteinaceous substrate to a polypeptide of any of claims 1 to 11.

22. The method of claim 21, further comprising subjecting said proteinaceous substrate to an endopeptidase.

23. The method of claim 21 or 22, wherein the hydrolysate is enriched in Leu, Gly, Glu, Ser, Asp, Asn, Pro, Cys, Ala, and/or Gin.

24. The method of claim 23, wherein the hydrolysate is enriched in Glu and/or Gin.

25. A method for obtaining from a proteinaceous substrate a protein hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues, comprising subjecting the substrate to a deamidation process and a polypeptide of any of claims 1 to 11.

26. The method of claim 25, further comprising subjecting the substrate to one or more unspecific acting endo- and/or exo-peptidase enzymes.

27. A composition comprising a polypeptide of any of claims 1 to 11 and a suitable carrier.

28. A method of producing malted cereal comprising:

a) providing a cereal grain,

b) adding to the cereal an effective amount of the polypeptide of any of claims 1 to 11 during a malting process; and

c) obtaining a malted cereal.

29. A method for the production of free amino nitrogen (FAN) during brewing comprising adding an effective amount of the polypeptide of any of claims 1 to 11 during a brewing process.

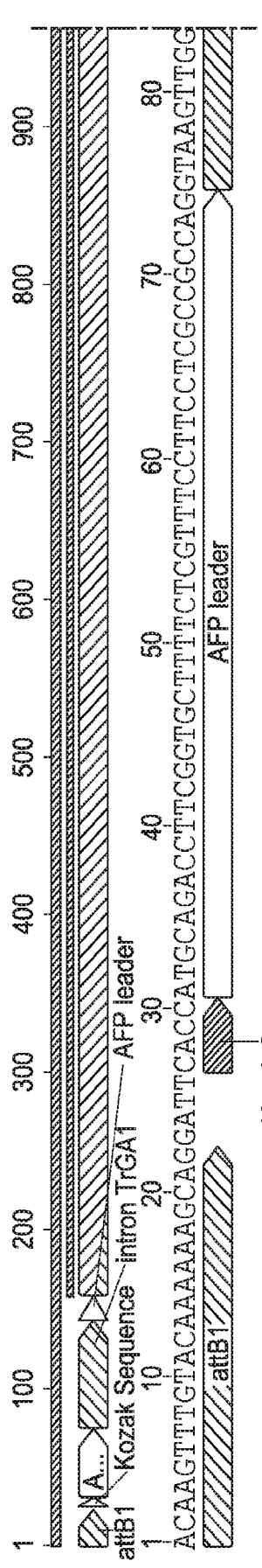


FIG. 1

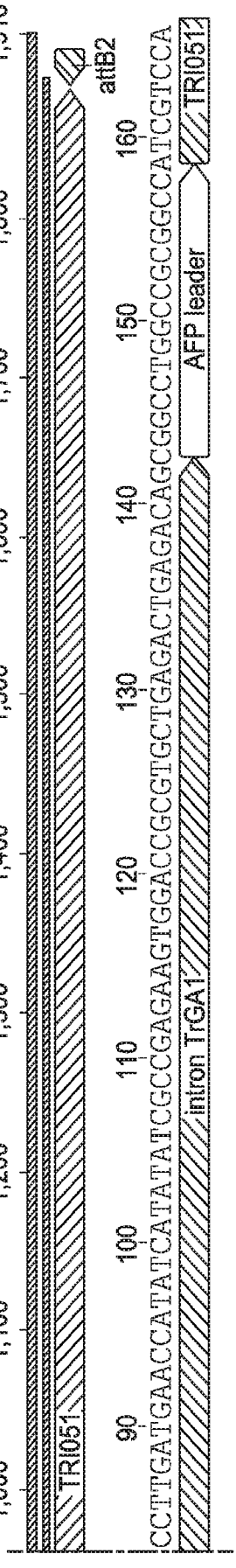


FIG. 1 Continued

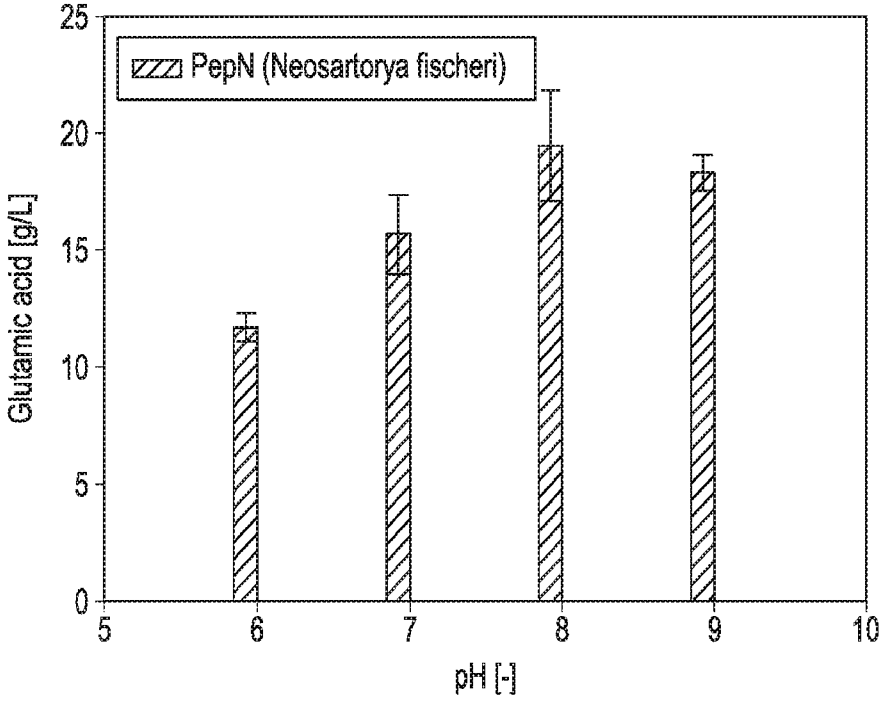

PepN (Neosartorya fischeri)
Glutamic acid [g/L]
pH [-]
0
5
10
15
20
25
5
6
7
8
9
10

FIG. 2

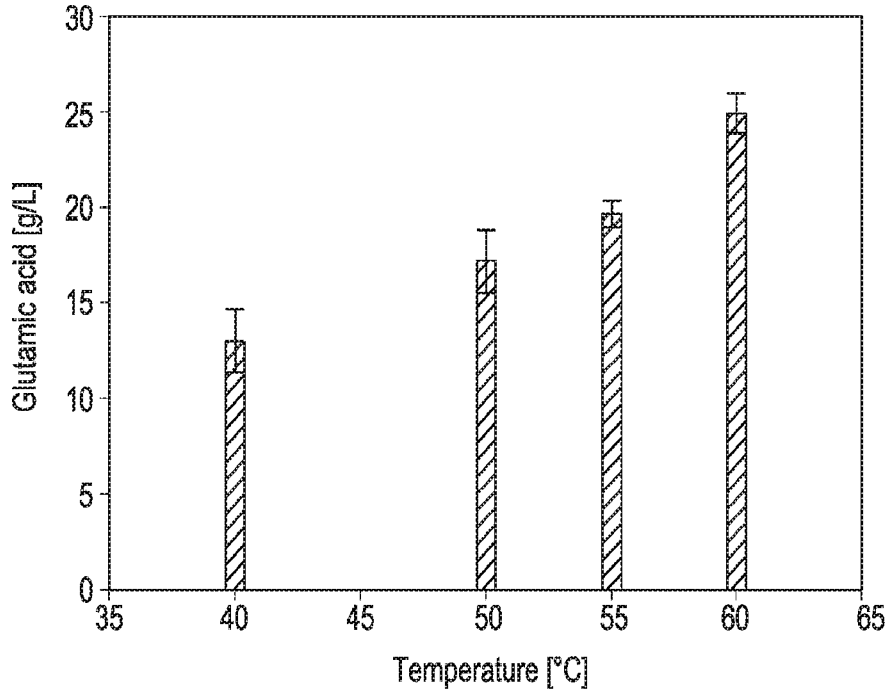

Glutamic acid [g/L]
Temperature [°C]
0
5
10
15
20
25
30
35
40
45
50
55
60
65

FIG. 3

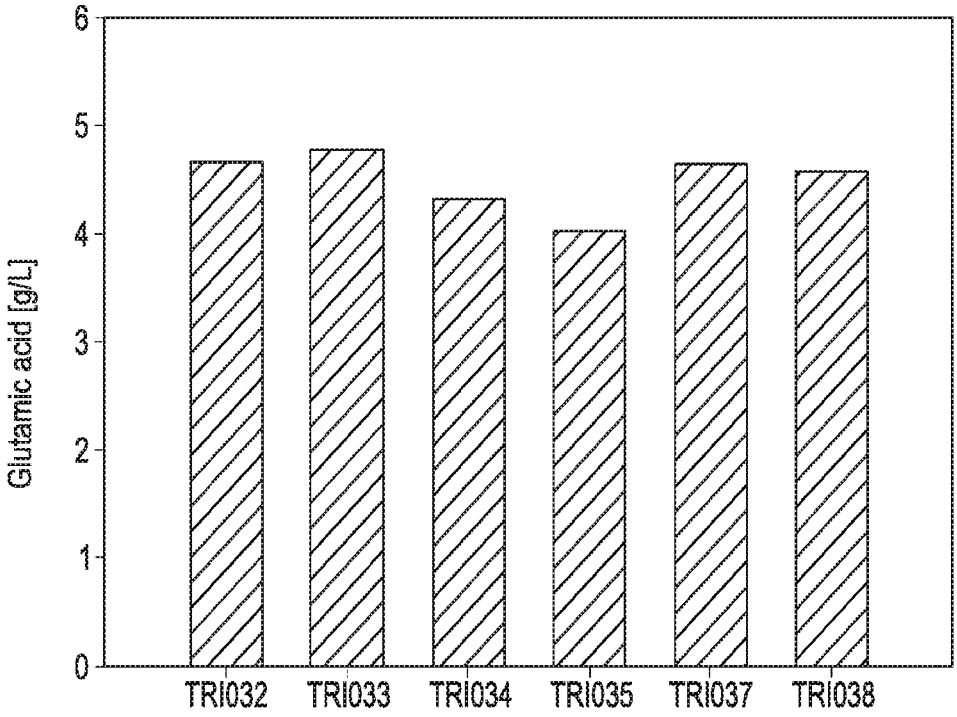

Glutamic acid [g/L]
0
1
2
3
4
5
6
TRI032
TRI033
TRI034
TRI035
TRI037
TRI038

FIG. 4

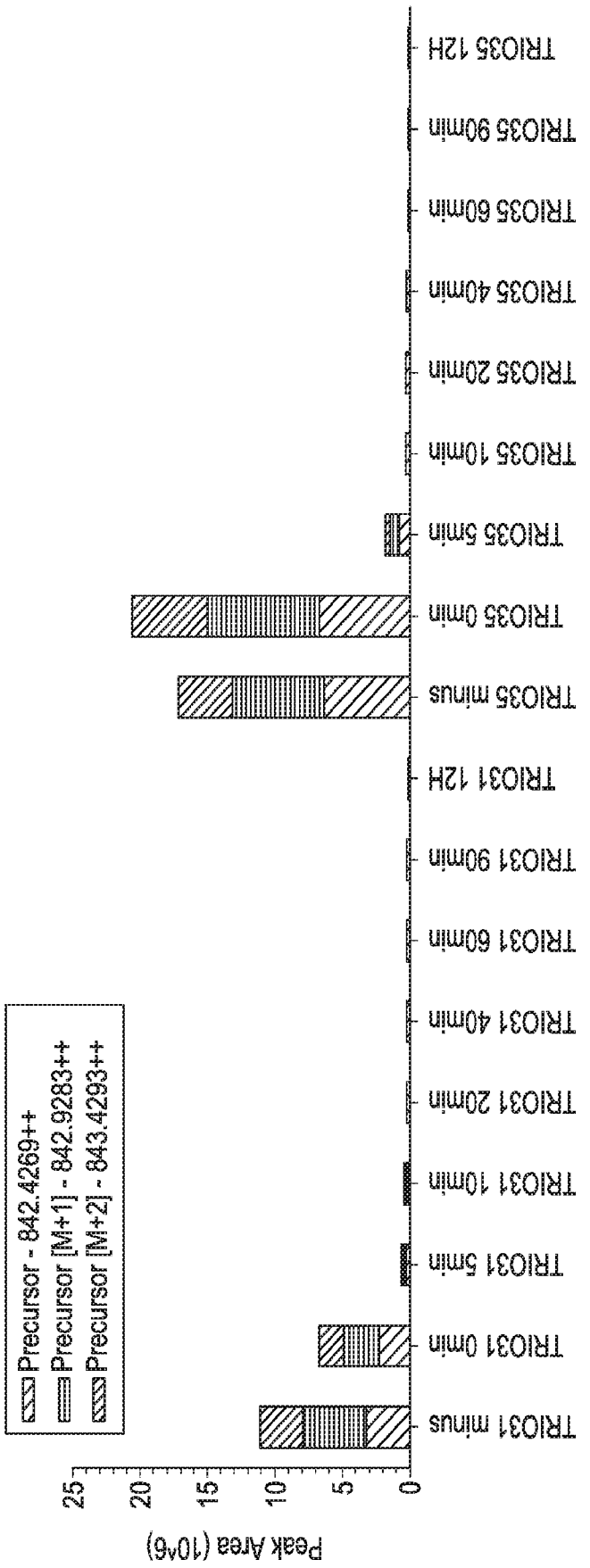

Peak Area (10^6)
0
5
10
15
20
25
Precursor - 842.4269++
Precursor [M+1] - 842.9283++
Precursor [M+2] - 843.4293++
TRIO31 minus
TRIO31 0min
TRIO31 5min
TRIO31 10min
TRIO31 20min
TRIO31 40min
TRIO31 60min
TRIO31 90min
TRIO31 12H
TRIO35 minus
TRIO35 0min
TRIO35 5min
TRIO35 10min
TRIO35 20min
TRIO35 40min
TRIO35 60min
TRIO35 90min
TRIO35 12H
Replicate

FIG. 5

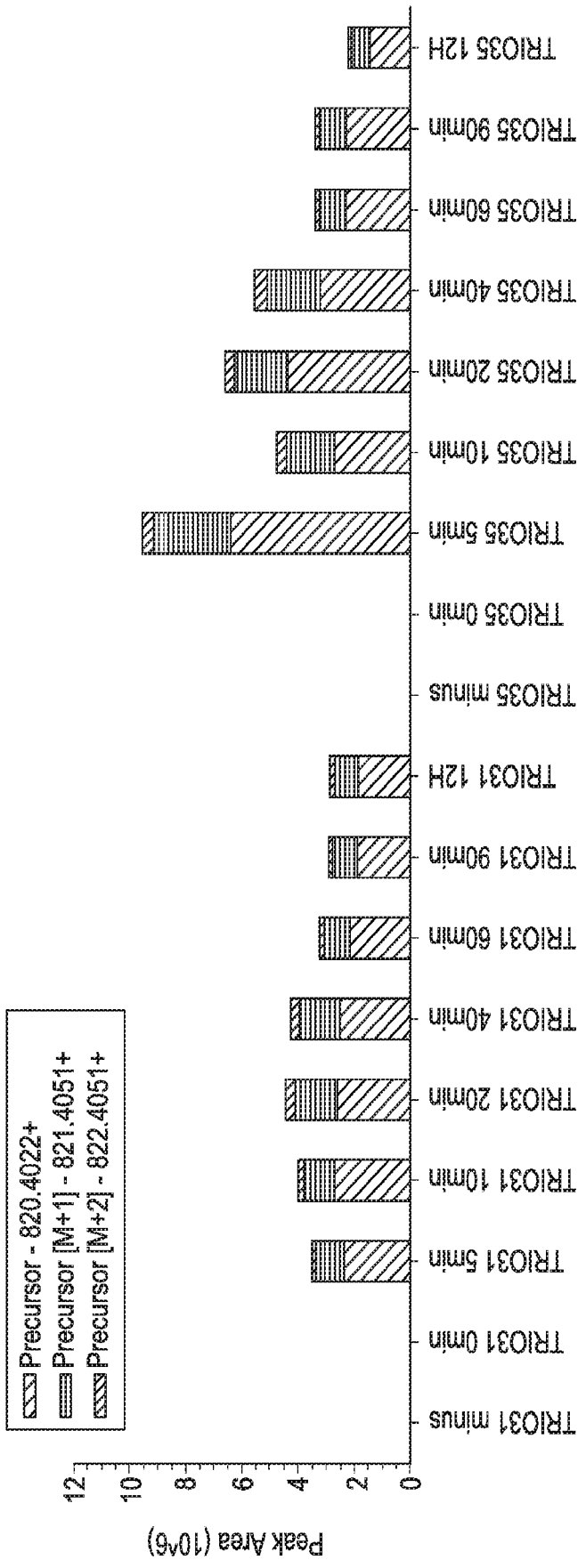

Precursor - 820.4022+
Precursor [M+1] - 821.4051+
Precursor [M+2] - 822.4051+
Peak Area (10^6)
0
2
4
6
8
10
12
TRIO31 minus
TRIO31 0min
TRIO31 5min
TRIO31 10min
TRIO31 20min
TRIO31 40min
TRIO31 60min
TRIO31 90min
TRIO31 12H
TRIO35 minus
TRIO35 0min
TRIO35 5min
TRIO35 10min
TRIO35 20min
TRIO35 40min
TRIO35 60min
TRIO35 90min
TRIO35 12H
Replicate

FIG. 6

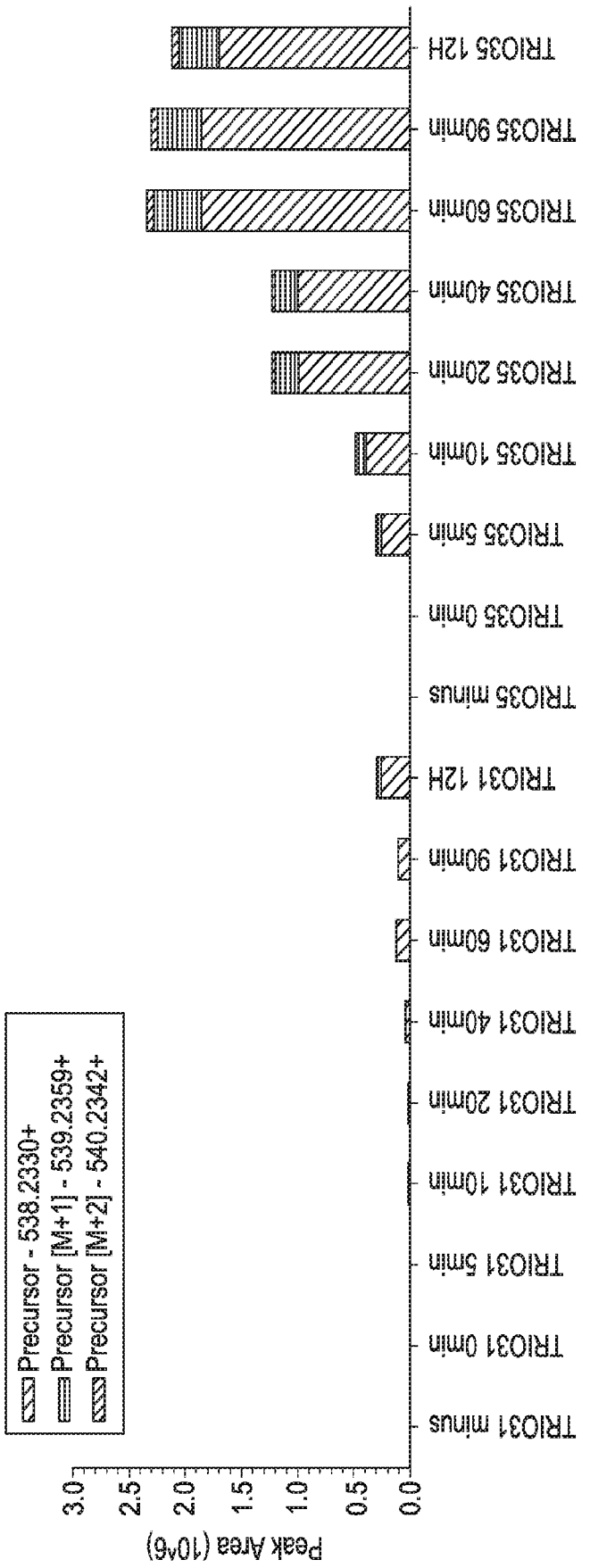

Precursor - 538.2330+
Precursor [M+1] - 539.2359+
Precursor [M+2] - 540.2342+
Peak Area (10^6)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TRIO31 minus
TRIO31 0min
TRIO31 5min
TRIO31 10min
TRIO31 20min
TRIO31 40min
TRIO31 60min
TRIO31 90min
TRIO31 12H
TRIO35 minus
TRIO35 0min
TRIO35 5min
TRIO35 10min
TRIO35 20min
TRIO35 40min
TRIO35 60min
TRIO35 90min
TRIO35 12H
Replicate

FIG. 7

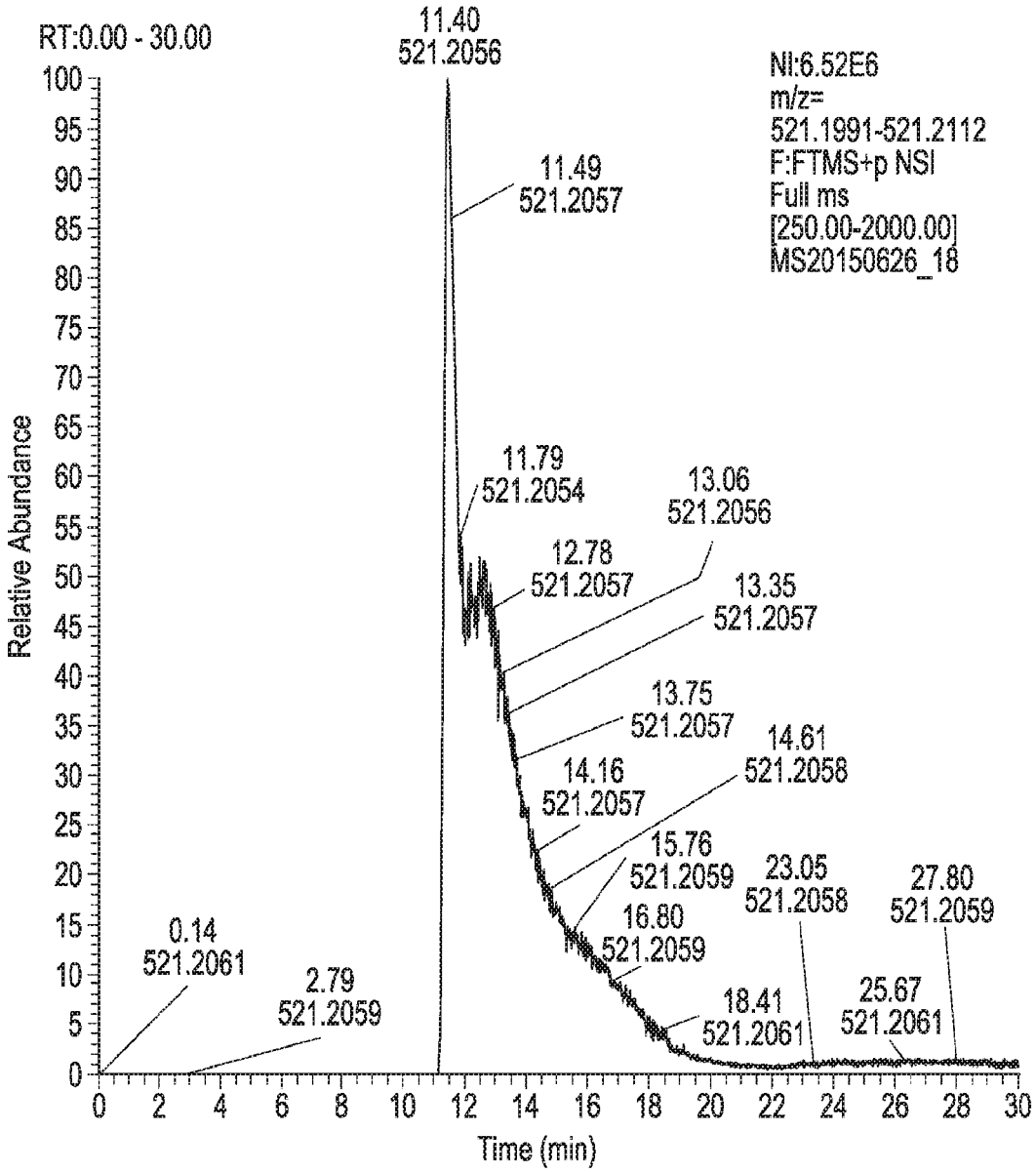

RT:0.00 - 30.00
11.40
521.2056
NI:6.52E6
m/z=
521.1991-521.2112
F:FTMS+p NSI
Full ms
[250.00-2000.00]
MS20150626_18
11.49
521.2057
11.79
521.2054
13.06
521.2056
12.78
521.2057
13.35
521.2057
13.75
521.2057
14.61
521.2058
14.16
521.2057
15.76
521.2059
23.05
521.2058
27.80
521.2059
16.80
521.2059
0.14
521.2061
2.79
521.2059
18.41
521.2061
25.67
521.2061
Relative Abundance
Time (min)

FIG. 8A

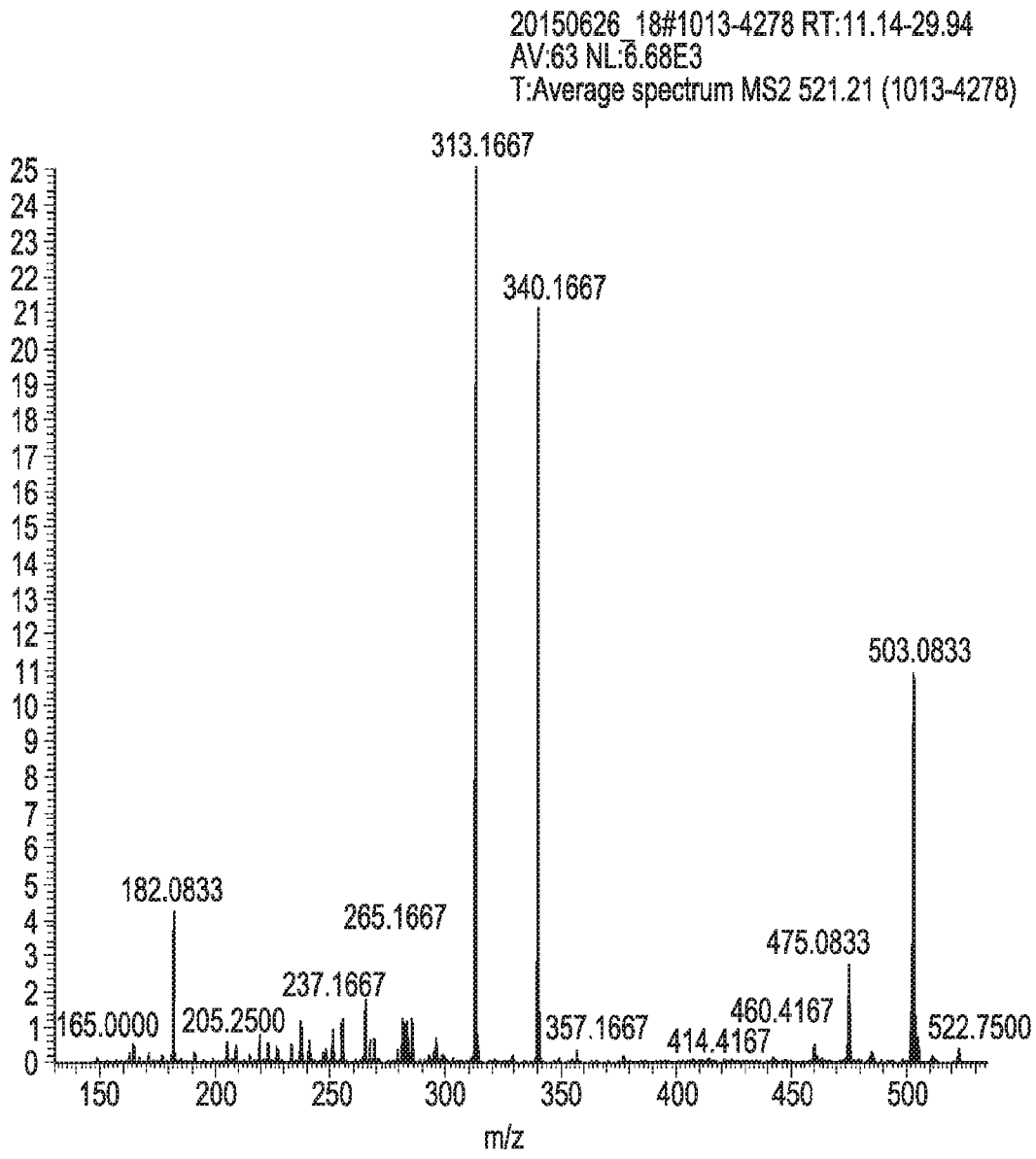

20150626_18#1013-4278 RT:11.14-29.94
AV:63 NL:6.68E3
T:Average spectrum MS2 521.21 (1013-4278)
313.1667
340.1667
503.0833
182.0833
265.1667
475.0833
237.1667
460.4167
165.0000
205.2500
357.1667
414.4167
522.7500
m/z

FIG. 8B

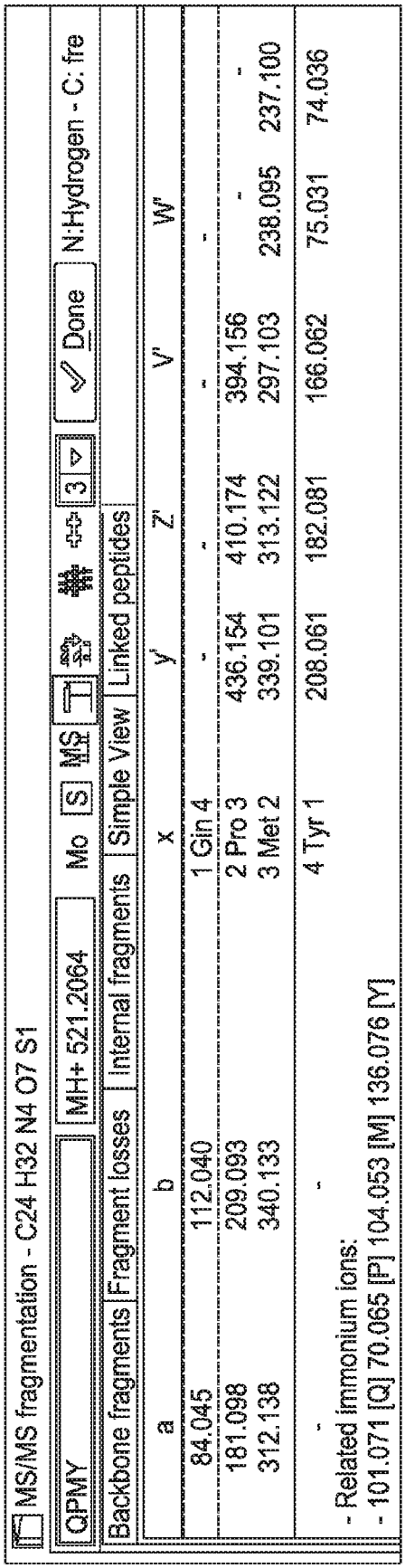

MS/MS fragmentation - C24 H32 N4 O7 S1
QPMY
MH+ 521.2064
Mo
S
MS
3
Done
N:Hydrogen - C: fre
Backbone fragments
Fragment losses
Internal fragments
Simple View
Linked peptides
a
b
x
y'
Z'
V'
W'
84.045 112.040 1 Gln 4 - - - -
181.098 209.093 2 Pro 3 436.154 410.174 394.156 -
312.138 340.133 3 Met 2 339.101 313.122 297.103 238.095 237.100
- - 4 Tyr 1 208.061 182.081 166.062 75.031 74.036
- Related immonium ions:
- 101.071 [Q] 70.065 [P] 104.053 [M] 136.076 [Y]

FIG. 8C

FIG. 9A	FIG. 9B
FIG. 9C	FIG. 9D
FIG. 9E	FIG. 9F

FIG. 9

1 10

1. TRI030(A new protein sequence entered manually)
2. EEH10739 (Ajellomyces capsulatus G186AR-EEH10739)
3. EGC45709 (Ajellomyces capsulatus H88-EGC145703)
4. EQL38596 (Ajellomyces dermatitidis ATCC 26199 - EQL38596)
5. EEQ88518 (Ajellomyces dermatitids ER-3-eeq88518)
6. XP_002627256 (Ajellomyces dermatitidIs SLH14081-XP_0026....
7. EGE77314 (Ajellomyces dermatitidis ATCC 18188 -EGE77314)
8. EEH19247 (Paracoccidioides brasiliensis Pb03 - EEh19247)
9. EEH37734 (Paracoccidioides sp. 'lutzii Pb01 - EEH37734
10. TRI031 (A new protein sequence entered manually)
11. XP_001258675 (Neosartorya fischeri NRRL 181 -XP_0012586 ...
12. XP_748386 (Aspergillus fumigatus Af293-XP_748386)
13 AAR96059 (Aspergillus fumigatus AAR96059)
14. TRI035 (A new protein sequence entered manually)
15. XP_001273779 (Aspergillus clavatus NRRL 1-XP_001273779)
16. TRI063 (A new protein sequence entered manually)
17. XP_001819545 (Aspergillus oryzae RIB10-XP_001819545)
18. AAN94152_novo_patent
19. KGO56599 (Penicillium expansum - KG056599)
20.KGO44619 (Penicillium expansum - KG044619)
21. KGO68951 (Penicillium italicum - KGO68951)
22. XP _002567159 (Penicillium rubens Wisconsin 54-1255-XP_0...
23 CDM29328 (Penicillium roqueforti FM164 - CDM29328)
24. EPS27203 (Penicillium oxalicum 114-2 EPS27203)
25. TRI037 (A new protein sequence entered manually)
26. XP_001217759 (Aspergillus terreus NIH2624 - XP_001217759)
27. TRI038 (A new protein sequence entered manually)
28. XP_681714 (Aspergillus nidulans FGSC A4-XP_681714)
29. XP 002844235 (Arthroderma otae CBS 113480 - XP-0028442...
30. EZF31821 (Trichophyton interdigitale H6-EZF3181821)
31. A7UI09 (Trichophyton equinum -A7UI09)
32. A7UI10 (Trichophyton tonsurans- A7UI10)
33. XP-003024240 (Trichophyton verucosum HKI 0517- XP_0030...
34. XP_003013309 (Arthnroderma benhamiae CBS 112371-XP_00 ...
35. XP_003231107 (Trichophyton rubrum CBS 118892 -XP_00323 ...
36. XP_002582878 (Uncinocarpus reesii 1704-XP-002582878)
37. XP_001240466 (Coccidioides immitis RS - XP_001240466)
38. XP_003068016 (Coccidioides posadasii C735 delta SOWgp-x...

FIG. 9A

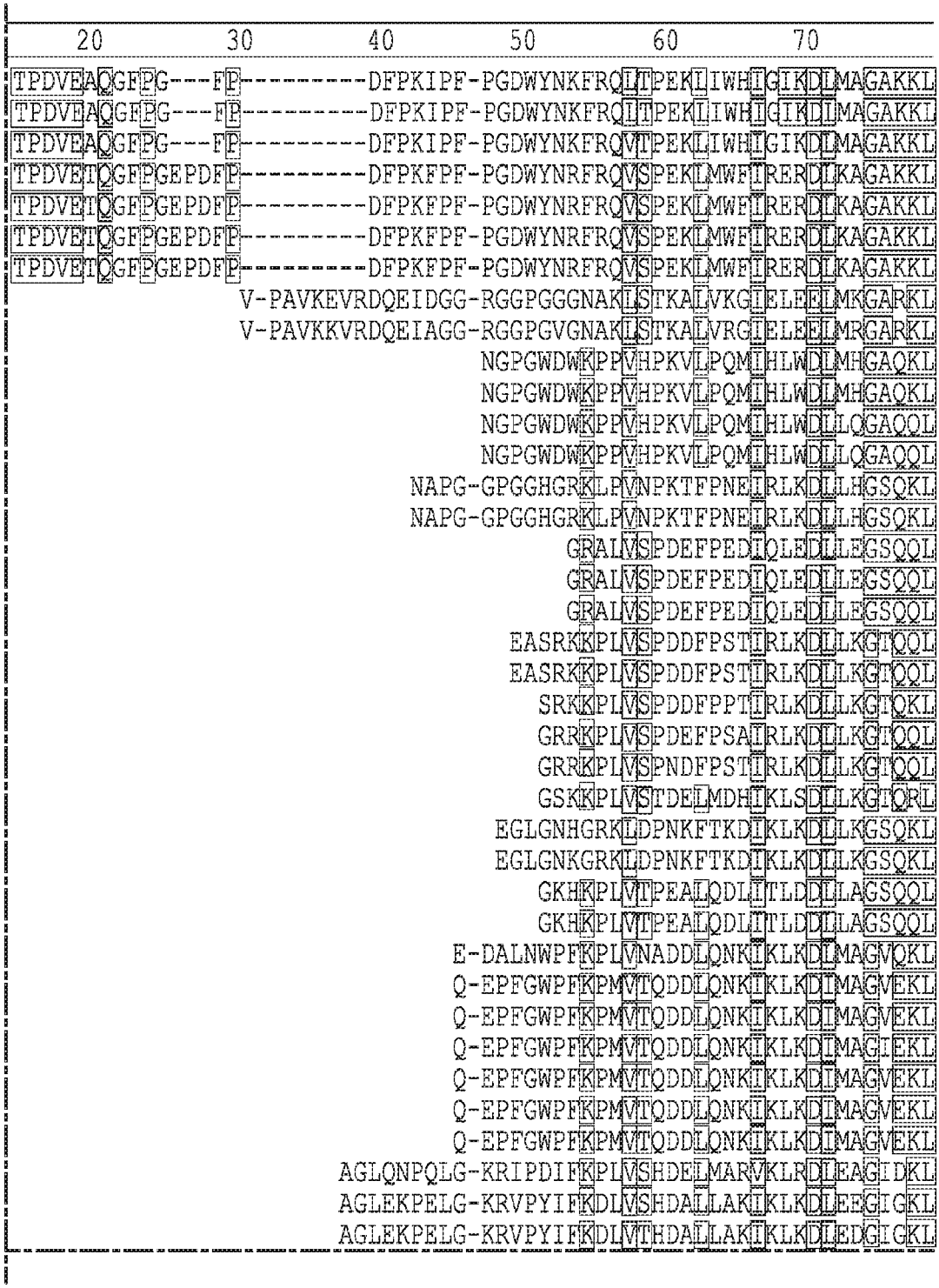


FIG. 9B

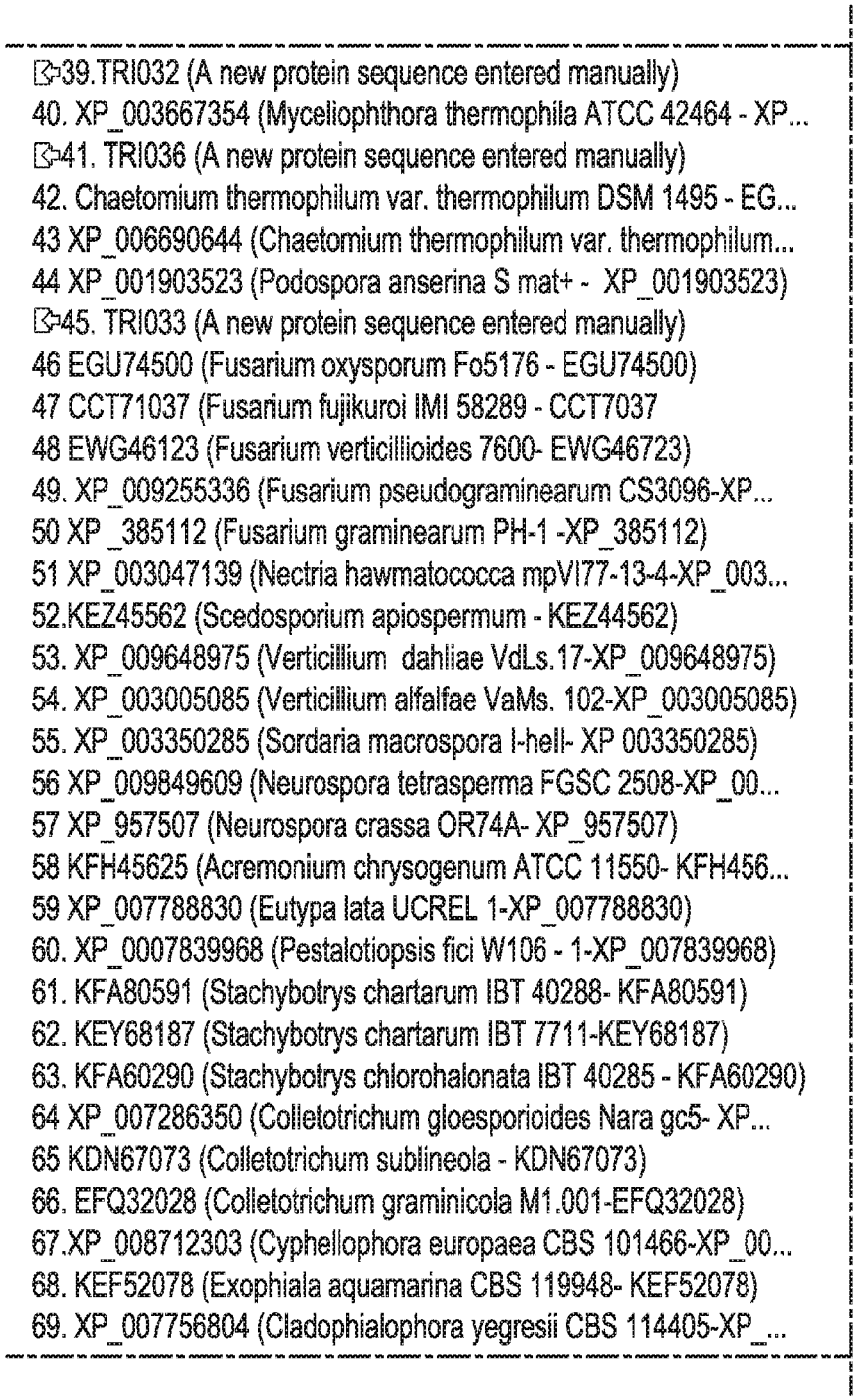
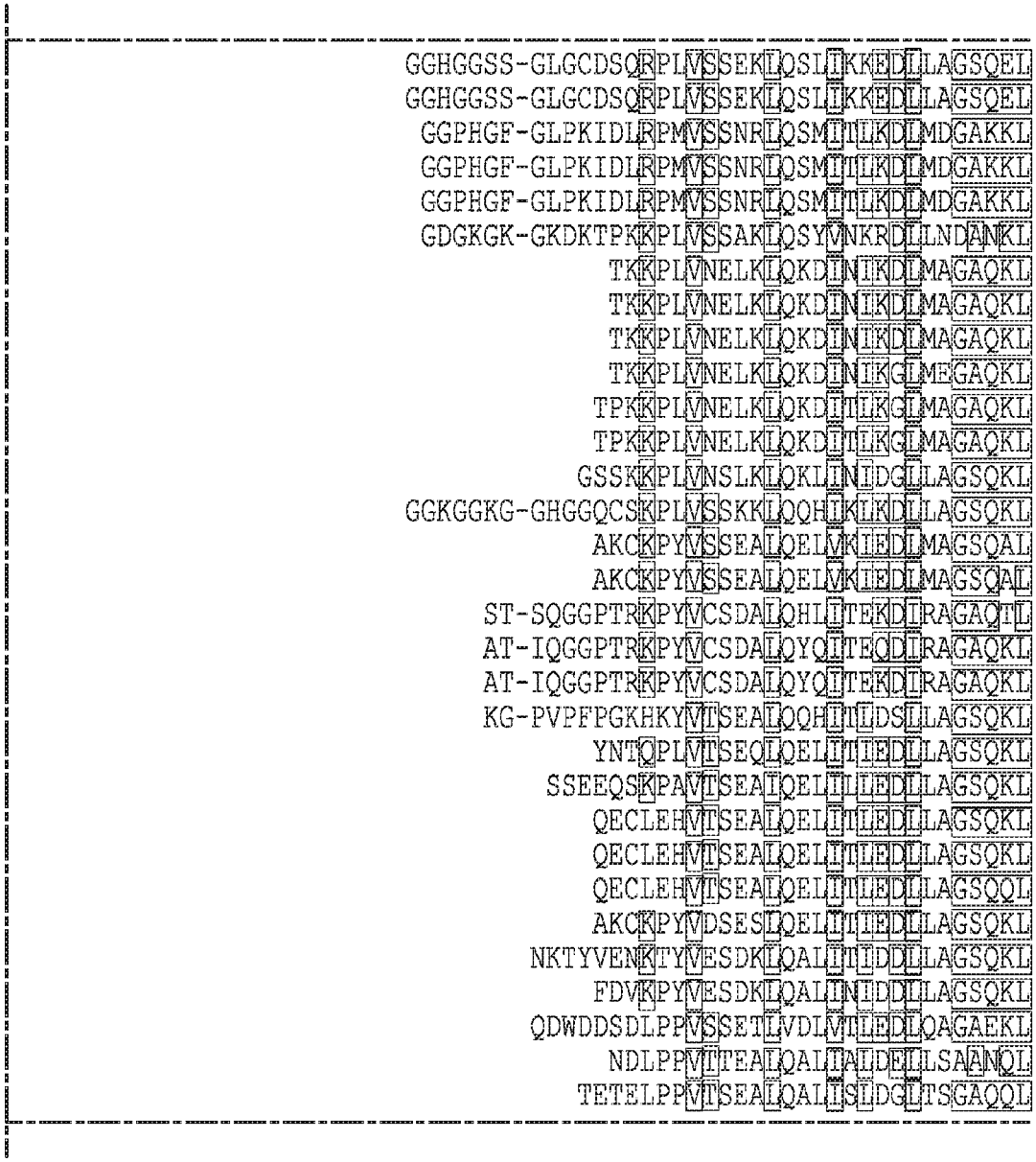

39.TRI032 (A new protein sequence entered manually)
40. XP_003667354 (Myceliophthora thermophila ATCC 42464 - XP...
41. TRI036 (A new protein sequence entered manually)
42. Chaetomium thermophilum var. thermophilum DSM 1495 - EG...
43 XP_006690644 (Chaetomium thermophilum var. thermophilum...
44 XP_001903523 (Podospora anserina S mat+ - XP_001903523)
45. TRI033 (A new protein sequence entered manually)
46 EGU74500 (Fusarium oxysporum Fo5176 - EGU74500)
47 CCT71037 (Fusarium fujikuroi IMI 58289 - CCT7037
48 EWG46123 (Fusarium verticillioides 7600- EWG46723)
49. XP_009255336 (Fusarium pseudograminearum CS3096-XP...
50 XP _385112 (Fusarium graminearum PH-1 -XP_385112)
51 XP_003047139 (Nectria hawmatococca mpVI77-13-4-XP_003...
52.KEZ45562 (Scedosporium apiospermum - KEZ44562)
53. XP_009648975 (Verticillium dahliae VdLs.17-XP_009648975)
54. XP_003005085 (Verticillium alfalfae VaMs. 102-XP_003005085)
55. XP_003350285 (Sordaria macrospora I-hell- XP 003350285)
56 XP_009849609 (Neurospora tetrasperma FGSC 2508-XP_00...
57 XP_957507 (Neurospora crassa OR74A- XP_957507)
58 KFH45625 (Acremonium chrysogenum ATCC 11550- KFH456...
59 XP_007788830 (Eutypa lata UCREL 1-XP_007788830)
60. XP_0007839968 (Pestalotiopsis fici W106 - 1-XP_007839968)
61. KFA80591 (Stachybotrys chartarum IBT 40288- KFA80591)
62. KEY68187 (Stachybotrys chartarum IBT 7711-KEY68187)
63. KFA60290 (Stachybotrys chlorohalonata IBT 40285 - KFA60290)
64 XP_007286350 (Colletotrichum gloesporioides Nara gc5- XP...
65 KDN67073 (Colletotrichum sublineola - KDN67073)
66. EFQ32028 (Colletotrichum graminicola M1.001-EFQ32028)
67.XP_008712303 (Cyphellophora europaea CBS 101466-XP_00...
68. KEF52078 (Exophiala aquamarina CBS 119948- KEF52078)
69. XP_007756804 (Cladophialophora yegresii CBS 114405-XP_...

FIG. 9C


GGHGGSS-GLGCDSQRPLVSSEKLQSLIKKEDLLAGSQEL
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GGPHGF-GLPKIDLRPMVSSNRLQSMITLKDLMDGAKKL
GGPHGF-GLPKIDLRPMVSSNRLQSMITLKDLMDGAKKL
GGPHGF-GLPKIDLRPMVSSNRLQSMITLKDLMDGAKKL
GDGKGK-GKDKTPKKPLVSSAKLQSYVNKRDLLNDANKL
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TKKPLVNELKLQKDINIKDLMAGAQKL
TKKPLVNELKLQKDINIKDLMAGAQKL
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GSSKKPLVNSLKLQKLINIDGLLAGSQKL
GGKGGKG-GHGGQCSKPLVSSKKLQQHIKLKDLLAGSQKL
AKCKPYVSSEALQELVKIEDLMAGSQAL
AKCKPYVSSEALQELVKIEDLMAGSQAL
ST-SQGGPTRKPYVCSDALQHLITEKDIRAGAQTL
AT-IQGGPTRKPYVCSDALQYQITEQDIRAGAQKL
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KG-PVPFPGKHKYVTSEALQQHITIDSLLAGSQKL
YNTQPLVTSEQLQELITIEDLLAGSQKL
SSEEQSKPAVTSEAIQELILLEDLLAGSQKL
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QECLEHVTSEALQELITLEDLLAGSQKL
QECLEHVTSEALQELITLEDLLAGSQQL
AKCKPYVDSESLQELITIEDLLAGSQKL
NKTYVENKTYVESDKLQALITIDDLLAGSQKL
FDVKPYVESDKLQALINIDDLLAGSQKL
QDWDDSDLPPVSSETLVDLVTLEDLQAGAEKL
NDLPPVTTEALQALIALDELLSAANQL
TETELPPVTSEALQALISLDGLTSGAQQL

FIG. 9D

70. XP_008726648 (Cladophialophora carrionii CBS 160.54-XP_0...	
71. XP_003302914 (Pyrenophora teres f.teres 0-1- XP_003302914)	
72. XP_001939352 (Pyrenophora tritici-repentis Pt-1C-BFP- XP_00...	
73. XP_007711902 (Bipolaris zeicola 26-R-13-XP_007711902)	
74. EUN32952(Bipolaris victoriae FI3 - EUN32952)	
75. XP_007693445 (Bipolaris oryzae ATCC 44560-XP_007693445)	
76. EMD95569 (Bipolaris maydis C5 - EMD95569)	
77. XP_007694276 (Bipolaris sorokiniana ND90Pr-XP_007694276)	
78. XP_008025353 (Setospherie turcica ET28A - XP_008025353)	
79. XP_007799086 (Phaeosphaeria nodorum SN15- XP_017990...	
80. XP_003837343 (leptopaeria maculans JN3 - XP+00383743)	
81.XP_007584460 (Neofusicoccum parvum UCRNP2 - XP_00758...	STPAGHVHAVRPA
82. EKG22544 (Macrophomina phaseolina MS6- EKG22544)	VRPA
83. XP_007779904 (Coniosporium apollinis CBS 100218- XP_007...	SSPS
84.XP_007877829(Pseusozyma flocculosa PF-1-XP_007877829)	
85. GAC92761 (Pseudozyma hubeiensis SY62 - GAC92761)	
86. EST06929 (Pseudozyma brasiliensis GHG001 - EST06929)	
87. CBQ71642 (Sporisoriym reilianum SRZ2 - CBQ71640)	
88 XP_762603 (Ustilago maydis 521 - XP_762603)	
89. GAC71207 (Pseudozyma antarctica T-34 - GAC71207)	
90. XP_003856003 (Zymoseptoria tritici IPO323 - XP_003850003)	TNVDGPIEILEVR
91 EMF10676(Sphaerulina musiva SO2202 - EMF10676)	
92. XP 007580302 (Neofusicoccum parvum UCRNP2 - XP_00785...	
93. EKG11208 (Macrophomina phaseolina MS6-EKG11208)	
94 XP_007777605 (Coniosporium apollinis CBS 100218 - XP_007...	
95.XP_00800916(Endocarpon pusillum Z07020 - XP_007800916)	
96. XP_001791850 (Phaeospaeria nodorum SN15-XP 0017918...	
97 XP 008086247 (Glarea lozoyensis ATCC 20868 - XP_0080862...	
98.XP_008081131 (Glarea lozoyensis ATCC 20868 - XP_0080811...	
99. XP_001833866 (Caprinopsis cinlerea okayama7#130 - XP_001...	
100 XP _002840011 (Tuber melanosporum Mel28 - XP_28400011)	
101 XP_007799116 (Eutypa lata UCREL 1 - XP_007799116)	
☞102. TRI034 (A new protein sequence entered manually)	
103. Fusarium oxysporum f. sp. Cubense race 1 - ENH69875 (Mod...	

FIG. 9E

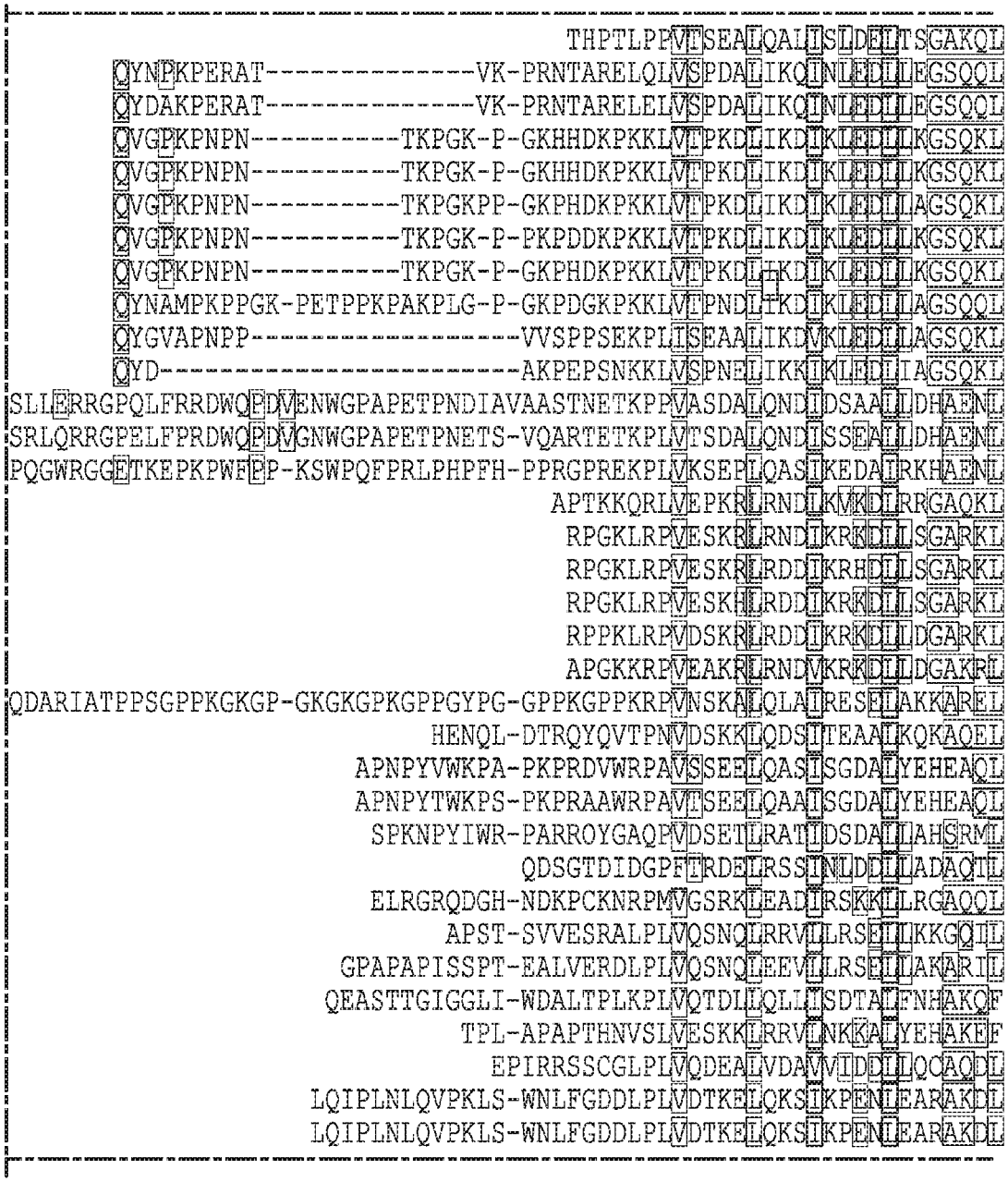

FIG. 9F

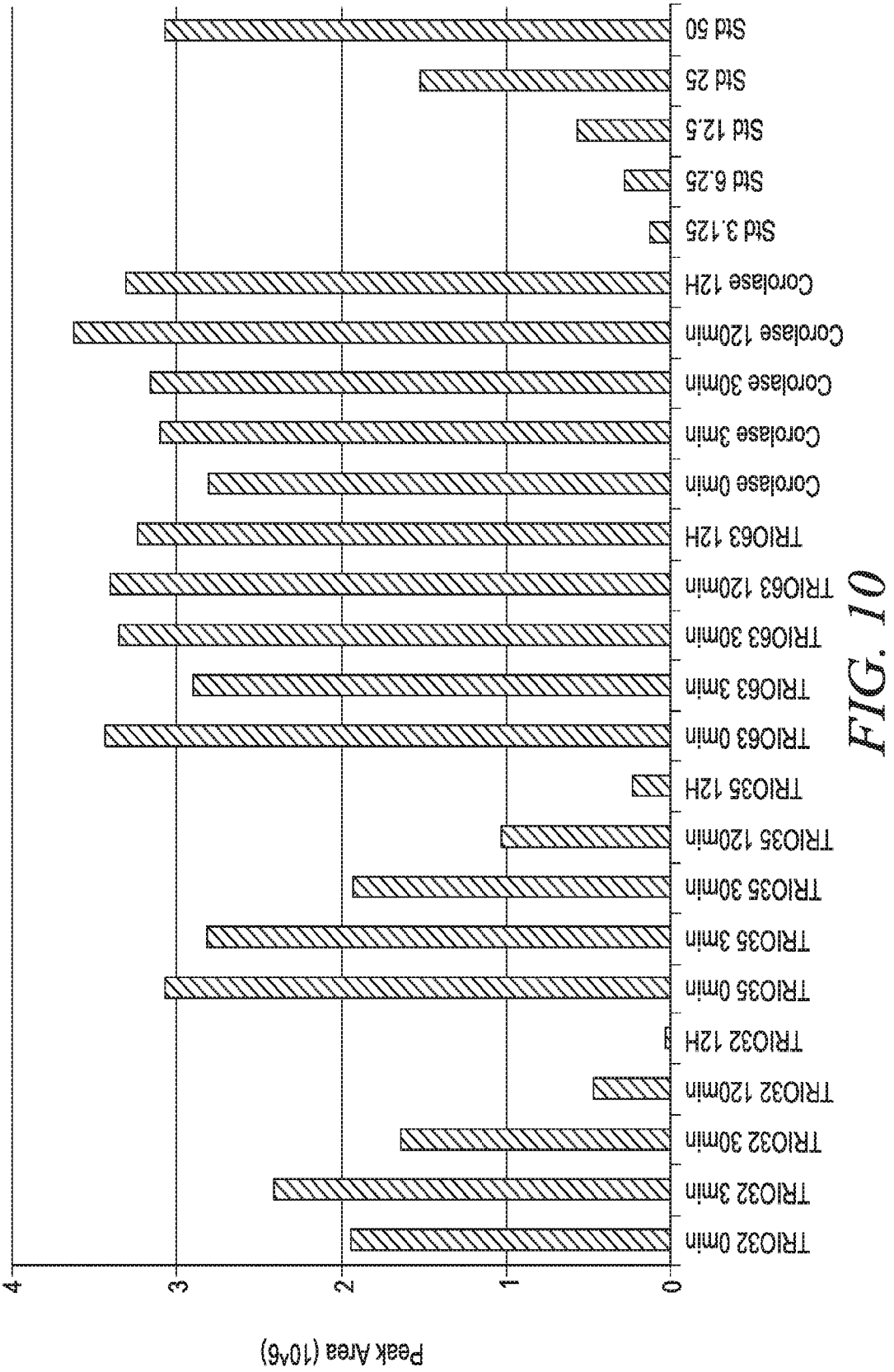
Peak Area (10^6)
0
1
2
3
4
TRIO32 0min
TRIO32 3min
TRIO32 30min
TRIO32 120min
TRIO32 12H
TRIO35 0min
TRIO35 3min
TRIO35 30min
TRIO35 120min
TRIO35 12H
TRIO63 0min
TRIO63 3min
TRIO63 30min
TRIO63 120min
TRIO63 12H
Corolase 0min
Corolase 3min
Corolase 30min
Corolase 120min
Corolase 12H
Std 3.125
Std 6.25
Std 12.5
Std 25
Std 50

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/039494

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P21/06 C12N9/48 C12N15/80
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P C07K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/150376 Al (DANISCO US INC GENENCOR DIV [US] ; CERVIN MARGUERITE A [US] ; KIM STEVEN) 11 December 2008 (2008-12-11) page 2; figure lb; sequence 4 page 8 -----	4, 16-20
X	W0 97/29179 Al (GIST BROCADES BV [NL] ; LAR0YE MARIE PAULE [FR] ; S0UPPE JEROME [FR]) 14 August 1997 (1997-08-14) page 6, line 13 - line 20 page 7, line 20 - line 24 example 7 page 9 -----	21-29
X	W0 03/102195 Al (DSM IP ASSETS BV [NL] ; EDENS LUPP0 [NL] ; DEKKER PETRUS JACOBUS THE0D0R) 11 December 2003 (2003-12-11) the whole document -----	21-29

-/- ·

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

X I See patent family annex.

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

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

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

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

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 September 2016	14/10/2016

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

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/039494

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13fer1 (a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13fer1 (a)).

      [ ] on paper or in the form of an image file (Rule 13fer1 (b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/039494

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