Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2967538 A1 2016/06/09

(21) **2 967 538**

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) **A1**

(86) **Date de dépôt PCT/PCT Filing Date:** 2015/12/01
(87) **Date publication PCT/PCT Publication Date:** 2016/06/09
(85) **Entrée phase nationale/National Entry:** 2017/05/11
(86) **N° demande PCT/PCT Application No.:** EP 2015/078177
(87) **N° publication PCT/PCT Publication No.:** 2016/087427
(30) **Priorité/Priority:** 2014/12/01 (EP14195682.1)

(51) **Cl.Int./Int.Cl.** ***C12P 21/06*** (2006.01), ***A23J 3/00*** (2006.01)

(71) **Demandeur/Applicant:**
NOVOZYMES A/S, DK

(72) **Inventeur/Inventor:**
LYNGLEV, GITTE BUDOLFSEN, DK

(74) **Agent:** WILSON LUE LLP

(54) **Titre : PROCEDE DE PRODUCTION D'UN HYDROLYSAT DE PROTEINES**
(54) **Title: METHOD FOR PRODUCING A PROTEIN HYDROLYSATE**

(57) **Abrégé/Abstract:**
The present invention relates to a method of producing a protein hydrolysate comprising a step of enzymatic protein hydrolysis performed at high temperature.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 June 2016 (09.06.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/087427 A1

(51) International Patent Classification:
C12P 21/06 (2006.01) *A23J 3/00* (2006.01)

(21) International Application Number:
PCT/EP2015/078177

(22) International Filing Date:
1 December 2015 (01.12.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
14195682.1 1 December 2014 (01.12.2014) EP

(71) Applicant: **NOVOZYMES A/S** [DK/DK]; Krogshoejvej 36, 2880 Bagsvaerd (DK).

(72) Inventor: **LYNGLEV, Gitte Budolfsen**; Egernvej 39, 2000 Frederiksberg (DK).

(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))*

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

WO 2016/087427 A1

(54) **Title**: METHOD FOR PRODUCING A PROTEIN HYDROLYSATE

(57) **Abstract**: The present invention relates to a method of producing a protein hydrolysate comprising a step of enzymatic protein hydrolysis performed at high temperature.

METHOD FOR PRODUCING A PROTEIN HYDROLYSATE

Reference to sequence listing

This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to enzymatic production of protein hydrolysates.

BACKGROUND OF THE INVENTION

Protein hydrolysates are mixtures of polypeptides, oligopeptides and/or amino acids that are manufactured from protein sources using partial or complete hydrolysis. There is a growing interest in protein hydrolysate preparations which have many uses, e.g., in human nutrition, e.g., as ingredients in energy drinks, weight-control and sports nutrition products, as a source of nutrition for elderly and underweight patients, and in the flavour industry. The protein could be derived from plants, e.g., soy, wheat or corn, or from animals, e.g., milk, eggs, meat or fish.

Production of protein hydrolysates in the food industry involves enzymatic, acid or alkali protein hydrolysis. Chemical hydrolysis is difficult to control and reduces the nutritional quality of the products. Enzymatic hydrolysis works without destructing amino acids and by avoiding the extreme temperatures and pH levels required for chemical hydrolysis, the nutritional properties of the protein hydrolysates remain largely unaffected.

However, for enzymatically hydrolysed protein, the protein yield and the degree of hydrolysis (DH) obtained are often limited.

A key factor limiting the yield and degree of hydrolysis is the conformation of the substrate protein to be hydrolysed. Not unfolded proteins, e.g. globular proteins, will often be more difficult to degrade than unfolded proteins, as it is more difficult for the proteases to degrade folded proteins. A variety of reagents and conditions can cause denaturation and result in the disruption of the secondary and tertiary structure of the protein. Heat can be used to disrupt hydrogen bonds and non-polar hydrophobic interactions a.o. being responsible for the secondary- and tertiary structure; the heating causes the molecules to vibrate so rapidly and violently that these bonds/interactions are disrupted.

It is common practice to unfold or denature the substrate protein by performing a heat treatment before addition of the proteolytic enzymes; however this is not an optimal method as the unfolding or denaturation often requires a high temperature at which the proteolytic enzymes applied are not stable and/or not at their optimum for activity. Especially the exopeptidases have a low thermostability. Lowering the temperature after unfolding will enable the proteins to re-aggregate in a way which will reduce the efficiency of the proteolytic degradation.

It is an object of the present invention to provide protein hydrolysates having improved properties, such as a high solubility, a high degree of hydrolysis, a high protein yield and/or a pleasant flavour.

SUMMARY OF THE INVENTION

The present invention provides a two-step procedure, where the substrate protein is first degraded by an endopeptidase at a temperature which is sufficiently high to both ensure that the substrate protein is unfolded/denatured and to also prevent the substrate protein from re-aggregating. This hydrolysis is carried out by use of a thermostable endopeptidase. Preferably, the thermostable endopeptidase is nonspecific. Being nonspecific enables the endopeptidase to degrade the substrate protein to relatively small peptides with limited ability to re-aggregate.

After this first hydrolysis step performed at high temperature, the temperature is lowered and a second hydrolysis step is performed using a protease preparation having a high aminopeptidase activity. Preferably, the protease preparation also has a high carboxypeptidase activity. Such second hydrolysis step provides a deep hydrolysis of the peptides.

The invention provides a method for producing a protein hydrolysate, comprising:

a) adding to a composition comprising substrate protein a thermostable endopeptidase;

b) performing a first hydrolysis step by incubating the composition of step a) for at least 10 minutes at a temperature of at least 75°C;

c) adding to the compostion of step b) a protease preparation having an aminopeptidase activity of at least 200 LAPU/g; and

d) performing a second hydrolysis step by incubating the composition of step c) for at least 10 minutes at a temperature which is at least 10°C lower than the temperature used in step b).

The invention also provides a method for producing a protein hydrolysate, comprising:

a) adding to a composition comprising substrate protein a thermostable endopeptidase which (i) has at least 60% sequence identity to the polypeptide of SEQ ID NO: 3, (ii) is encoded by a polynucleotide having at least 60% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) is a variant of the polypeptide of SEQ ID NO: 3 comprising a substitution, deletion, and/or insertion at one or more positions; and

b) incubating the composition of step a) for at least 10 minutes at a temperature of at least 75°C.

DETAILED DESCRIPTION OF THE INVENTION

Substrate protein

The substrate protein to be used in the methods of the present invention may be a soy protein. A variety of soy protein materials may be used. In general, the soy protein material may be derived from whole soybeans in accordance with methods known in the art. The whole soybeans may be standard soybeans (i.e., non genetically modified soybeans), genetically modified soybeans, or combinations thereof. Suitable examples of soy protein material include soy extract, soy milk, soy milk powder, soy curd, soy flour, isolated soy protein, soy protein concentrate, and mixtures thereof.

The soy protein may be a soy protein isolate (also called isolated soy protein or ISP). In general, soy protein isolates have a protein content of at least about 90% soy protein on a moisturefree basis. The soy protein isolate may comprise intact soy proteins or it may comprise partially hydrolyzed soy proteins.

The soy protein may be a soy protein concentrate. In general, soy protein concentrates have a protein content of about 65% to less than about 90% on a moisture-free basis. Alternatively, the soy protein may be a soy protein concentrate blended with a soy protein isolate to substitute for a portion of the soy protein isolate as a source of soy protein material. Typically, if a soy protein concentrate is substituted for a portion of the soy protein isolate, the soy protein concentrate is substituted for up to about 40% of the soy protein isolate by weight, at most, and more preferably is substituted for up to about 30% of the soy protein isolate by weight.

The soy protein may be soy flour. In general, soy flour has a protein content of about 49% to about 65% on a moisture-free basis. The soy flour may be defatted soy flour, partially defatted soy flour, or full fat soy flour. The soy flour may be blended with soy protein isolate or soy protein concentrate.

The substrate protein to be used in a method of the present invention material may be derived from a plant other than soy. By way of non-limiting example, suitable plants include amaranth, arrowroot, barley, buckwheat, canola, cassava, channa (garbanzo), legumes, lentils, lupin, maize, millet, oat, pea, potato, rice, rye, sorghum, sunflower, tapioca, triticale, wheat, and mixtures thereof. Especially preferred plant proteins include barley, canola, lupin, maize, oat, pea, potato, rice, wheat, and combinations thereof. In one embodiment, the plant protein material may be canola meal, canola protein isolate, canola protein concentrate, and combinations thereof. In another embodiment, the plant protein material may be maize or corn protein powder, maize or corn protein concentrate, maize or corn protein isolate, maize or corn germ, maize or corn gluten, maize or corn gluten meal, maize or corn flour, zein protein, and combinations thereof. In still another embodiment, the plant protein material may be barley powder, barley protein concentrate, barley protein isolate, barley meal, barley flour, and combinations thereof. In an alternate embodiment, the plant protein material may be lupin flour, lupin protein isolate, lupin protein concentrate, and combinations thereof. In another alternate embodiment, the plant protein material may be oatmeal, oat flour, oat protein flour, oat protein isolate, oat protein concentrate, and combinations thereof. In yet another embodiment, the plant protein material may be pea flour, pea protein isolate, pea protein concentrate, and combinations thereof. In still another embodiment, the plant protein material may be potato protein powder, potato protein isolate, potato protein concentrate, potato flour, and combinations thereof. In a further embodiment, the plant protein material may be rice flour, rice meal, rice protein powder, rice protein isolate, rice protein concentrate, and combinations thereof. In another alternate embodiment, the plant protein material may be wheat protein powder, wheat gluten, wheat germ, wheat flour, wheat protein isolate, wheat protein concentrate, solubilized wheat proteins, and combinations thereof.

The substrate protein to be used in a method of the present invention material may be derived from an animal source. In one embodiment, the animal protein material may be derived from eggs. Non-limiting examples of suitable egg proteins include powdered egg, dried egg solids, dried egg white protein, liquid egg white protein, egg white protein powder, isolated ovalbumin protein, and combinations thereof. Egg proteins may be derived from the eggs of chicken, duck, goose, quail, or other birds. In an alternate embodiment, the protein material may be derived from a dairy source. Suitable dairy proteins include non-fat dry milk powder, milk protein isolate, milk protein concentrate, acid casein, caseinate (e.g., sodium caseinate, calcium caseinate, and the like), whey protein isolate, whey protein concentrate, and combinations thereof. The milk protein material may be derived from cows, goats, sheep, donkeys, camels, camelids, yaks, water buffalos, etc. In a further embodiment, the protein may be derived from the muscles, organs, connective tissues, or skeletons of land-based or aquatic animals. As an example, the animal protein may be gelatin, which is produced by partial hydrolysis of collagen extracted from the bones, connective tissues, organs, etc, from cattle or other animals.

The substrate protein to be used in a method of the present invention material may be a combination of two or more of the protein materials listed above.

In a preferred embodiment, the substrate protein is selected from soy protein, wheat gluten protein or whey protein. In a more preferred embodiment, the substrate protein is soy protein.

The substrate protein to be used in the methods of the present invention is typically mixed or dispersed in water to form an aqueous composition comprising substrate protein.

The composition comprising substrate protein may have a dry matter content of at least 1% (w/w), preferably at least 5% (w/w), more preferably at least 8% (w/w). The composition comprising substrate protein may have a dry matter content of 1-75% (w/w), preferably 5-40% (w/w), more preferably 8-30% (w/w).

The pH of the composition comprising substrate protein may be adjusted and monitored according to methods generally known in the art. The pH of the composition may be adjusted and maintained at from about pH 5 to about pH 10. In one embodiment, the pH of the composition may be adjusted and maintained at from about pH 6.5 to about pH 9. In a preferred embodiment, the pH of the compostion may be adjusted and maintained at about pH 7-8. In another embodiment, the pH of the composition comprising substrate protein is not adjusted.

Thermostable endopeptidase

In the methods of the present invention, a thermostable endopeptidase is added to the composition comprising substrate protein.

Endopeptidase activity may be determined by using one of the assays of the Examples.

A thermostable endopeptidase in the context of the present invention may be defined as an endopeptidase, which after incubation for 15 minutes at 80°C and a pH where the endopeptidase exhibits at least 40% of its maximum activity has a residual activity of at least 80% relative to its activity after incubation at 37°C.

A thermostable endopeptidase in the context of the present invention may be defined as an endopeptidase, which after incubation for 15 minutes at 80°C and pH 9 has a residual activity of at least 80% relative to its activity after incubation at 37°C.

The residual activity may be determined using the Residual Activity Measurement assay as described in the Examples.

Preferably, the thermostable endopeptidase is an endopeptidase, which after incubation for 15 minutes at 90°C and a pH where the endopeptidase exhibits at least 40% of its maximum activity has a residual activity of at least 80% relative to its activity after incubation at 37°C. More preferably, the thermostable endopeptidase is an endopeptidase, which after incubation for 15 minutes at 95°C and a pH where the endopeptidase exhibits at least 40% of its maximum activity has a residual activity of at least 80% relative to its activity after incubation at 37°C.

The thermostable endopeptidase may be an endopeptidase, which after incubation for 15 minutes at 80°C, preferably 90°C, more preferably 95°C, and pH 9 has a residual activity of at least 80% relative to its activity after incubation at 37°C.

The thermostable endopeptidase may have at least 20%, preferably at least 30%, at least 40%, at least 50%, at least 60%, at least 70% or at least 80%, of the endopeptidase activity of the polypeptide of SEQ ID NO: 3. The skilled person will know how to determine the endopeptidase activity, e.g., by using one of the assays of the Examples.

In a preferred embodiment, the thermostable endopeptidase has at least 20%, preferably at least 30%, at least 40%, at least 50%, at least 60%, at least 70% or at least 80%, of the endopeptidase activity of the polypeptide of SEQ ID NO: 3 after incubation at 80°C and pH 9 for 15 minutes, where the endopeptidase activity is measured according to the Relative Activity Assay.

In a preferred embodiment, the thermostable endopeptidase is a nonspecific endopeptidase. The skilled person will know if an endopeptidase is a specific endopeptidase, which, e.g., cleaves after Arg or Lys, or if it is a nonspecific endopeptidase. A nonspecific endopeptidase may also be characterized as an endopeptidase having a broad specificity.

A nonspecific endopeptidase may be characterized in that incubation of 0.5% (w/w) BSA with the endopeptidase for 4 hours at a temperature and pH where the endopeptidase exhibits at least 40% of its maximum activity results in a degree of hydrolysis of at least 10%, preferably at least 15%.

In some embodiments, the thermostable endopeptidase has been isolated.

In some embodiments, the thermostable endopeptidase has a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. The thermostable endopeptidase may differ by up to 10 amino acids, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, from the mature polypeptide of SEQ ID NO: 2.

In the context of the present invention, the term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N terminal processing, C terminal truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide is amino acids 111 to 523 of SEQ ID NO: 2. It is known in the art that a host cell may produce a mixture of two of more different mature polypeptides (i.e., with a different C terminal and/or N terminal amino acid) expressed by the same polynucleotide. It is also known in the art that different host cells process polypeptides differently, and thus, one host cell expressing a polynucleotide may produce a different mature polypeptide (e.g., having a different C terminal and/or N terminal amino acid) as compared to another host cell expressing the same polynucleotide.

The mature polypeptide of SEQ ID NO: 2 has been experimentally determined as amino acids 111-523 using SDS-PAGE in-gel digest and liquid chromatography and high resolution mass spectrometry. A peptide map has been made covering 68% of the mature sequence including both the N-terminal and the C-terminal.

In the context of the present invention, the term “sequence identity” is a measure of the relatedness between two amino acid sequences or between two nucleotide sequences.

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

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

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, supra) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, supra), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

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

In some embodiments, the thermostable endopeptidase has a sequence identity to the polypeptide of SEQ ID NO: 3 of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. The thermostable endopeptidase may differ by up to 10 amino acids, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, from the polypeptide of SEQ ID NO: 3.

In some embodiments, the thermostable endopeptidase comprises or consists of the amino acid sequence of SEQ ID NO: 3 or an allelic variant thereof.

In some embodiments, the thermostable endopeptidase is encoded by a polynucleotide having a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of at least

60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

In the context of the present invention, the term “mature polypeptide coding sequence” means a polynucleotide that encodes a mature polypeptide having endopeptidase activity. In one aspect, the mature polypeptide coding sequence is nucleotides 331 to 1569 of SEQ ID NO: 1.

In some embodiments, the thermostable endopeptidase is a variant of the mature polypeptide of SEQ ID NO: 2 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions. In some embodiments, the number of amino acid substitutions, deletions and/or insertions introduced into the mature polypeptide of SEQ ID NO: 2 is up to 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In some embodiments, the thermostable endopeptidase is a variant of the polypeptide of SEQ ID NO: 3 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions. In some embodiments, the number of amino acid substitutions, deletions and/or insertions introduced into the polypeptide of SEQ ID NO: 3 is up to 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In the context of the present invention, the term “variant” means a polypeptide having endopeptidase activity comprising an alteration, i.e., a substitution, insertion, and/or deletion, at one or more (e.g., several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion means removal of the amino acid occupying a position; and an insertion means adding one or more (e.g., several) amino acids, e.g., 1-5 amino acids, adjacent to and immediately following the amino acid occupying a position.

The amino acid changes may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the groups of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R. L. Hill, 1979, In, The Proteins, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may affect the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in a polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, Science 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for endopeptidase activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., 1996, J. Biol. Chem. 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., 1992, Science 255: 306-312; Smith et al., 1992, J. Mol. Biol. 224: 899-904; Wlodaver et al., 1992, FEBS Lett. 309: 59-64. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, Science 241: 53-57; Bowie and Sauer, 1989, Proc. Natl. Acad. Sci. USA 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman et al., 1991, Biochemistry 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire et al., 1986, Gene 46: 145; Ner et al., 1988, DNA 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness et al., 1999, Nature Biotechnology 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

A thermostable endopeptidase to be used in a method of the present invention may be obtained from microorganisms of any genus. For purposes of the present invention, the term “obtained from” as used herein in connection with a given source shall mean that the polypeptide encoded by a polynucleotide is produced by the source or by a strain in which the polynucleotide from the source has been inserted. In one aspect, the polypeptide obtained from a given source is secreted extracellularly.

The thermostable endopeptidase may be obtained from an organism characterized as a hyperthermophile. The endopeptidase may be obtained from a hyperthermophilic bacterium, e.g., from *Thermotoga, Thermosipho, Fervidobacterium, Aquifex, Calderobacterium, Thermocrinis*, or it may be obtained from archaea, e.g., from *Sulfolobus, Metallosphaera, Acidianus, Stygiolobus, Sulfurococcus, Sulfurisphaera, Thermoproteus, Pyrobaculum, Thermofilum, Thermocladium, Caldivirga, Desulfurococcus, Staphylothermus, Sulfophobococcus, Stetteria, Aeropyrum, Ignicoccus, Thermosphaera, Thermodiscus, Pyrodictium, Hyperthermus, Pyrolobus, Thermococcus, Pyrococcus, Archaeoglobus, Ferroglobus, Methanothermus, Methanococcus, Methanopyrus.*

In some preferred embodiments, the thermostable endopeptidase may be obtained from *Pyrococcus*. In some preferred embodiments, the thermostable endopeptidase may be obtained from *Pyrococcus furiosus*.

Strains of *Pyrococcus* are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

The thermostable endopeptidase may be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) or DNA samples obtained directly from natural materials (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms and DNA directly from natural habitats are well known in the art. A polynucleotide encoding the thermostable endopeptidase may then be obtained by similarly screening a genomic DNA or cDNA library of another microorganism or mixed DNA sample. Once a polynucleotide encoding a thermostable endopeptidase has been detected with the probe(s), the polynucleotide can be isolated or cloned by utilizing techniques that are known to those of ordinary skill in the art (see, e.g., Sambrook et al., 1989, supra).

In some preferred embodiments, the thermostable endopeptidase may be a variant of an endopeptidase obtained from *Pyrococcus*. In some preferred embodiments, the thermostable endopeptidase may be a variant of an endopeptidase obtained from *Pyrococcus furiosus*.

The thermostable endopeptidase may be a variant of an endopeptidase obtained from any organism, such as from an organism characterized as a hyperthermophile, e.g., from one of the hyperthermophilic organisms listed above. Alternatively, the thermostable endopeptidase may be a variant of an endopeptidase obtained from an organism which is not a hyperthermophile, such as a variant having a higher thermostability.

In some embodiment, the endopeptidase is an S8 endopeptidase, e.g., an S8 protease from *Pyrococcus*, preferably from *Pyrococcus furiosus*.

In some embodiments, the thermostable endopeptidase has a sequence identity to the polypeptide of SEQ ID NO: 4 of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. The thermostable endopeptidase may differ by up to 10 amino acids, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, from the polypeptide of SEQ ID NO: 4.

In some embodiments, the thermostable endopeptidase comprises or consists of the amino acid sequence of SEQ ID NO: 4 or an allelic variant thereof.

In some embodiments, the thermostable endopeptidase is a variant of the mature polypeptide of SEQ ID NO: 4 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions. In some embodiments, the number of amino acid substitutions, deletions and/or insertions introduced into the mature polypeptide of SEQ ID NO: 4 is up to 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In some preferred embodiments, the thermostable endopeptidase may be obtained from *Nocardiopsis prasina*.

In some embodiments, the thermostable endopeptidase may be a variant of an endopeptidase obtained from *Nocardiopsis*. In some embodiments, the thermostable endopeptidase may be a variant of an endopeptidase obtained from *Nocardiopsis prasina*.

In some embodiments, the thermostable endopeptidase may be a serine protease, e.g., a serine protease from *Nocardiopsis*, preferably from *Nocardiopsis prasina*.

In some embodiments, the thermostable endopeptidase has a sequence identity to the mature polypeptide of SEQ ID NO: 6, e.g., to amino acids 183-368 of SEQ ID NO: 6, of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. The thermostable endopeptidase may differ by up to 10 amino acids, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, from the mature polypeptide of SEQ ID NO: 6.

In some embodiments, the thermostable endopeptidase comprises or consists of the amino acid sequence of the mature polypeptide of SEQ ID NO: 6 or an allelic variant thereof.

In some embodiments, the thermostable endopeptidase is a variant of the mature polypeptide of SEQ ID NO: 6 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions. In some embodiments, the number of amino acid substitutions, deletions and/or insertions introduced into the mature polypeptide of SEQ ID NO: 6 is up to 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In some embodiments, the thermostable endopeptidase has a sequence identity to the polypeptide of SEQ ID NO: 8 of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least

80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. The thermostable endopeptidase may differ by up to 10 amino acids, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, from the polypeptide of SEQ ID NO: 8.

In some embodiments, the thermostable endopeptidase comprises or consists of the amino acid sequence of the polypeptide of SEQ ID NO: 8 or an allelic variant thereof.

In some embodiments, the thermostable endopeptidase is a variant of the polypeptide of SEQ ID NO: 8 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions. In some embodiments, the number of amino acid substitutions, deletions and/or insertions introduced into the polypeptide of SEQ ID NO: 8 is up to 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In some preferred embodiments, the thermostable endopeptidase may be obtained from *Thermobifida*. In some preferred embodiments, the thermostable endopeptidase may be obtained from *Thermobifida fusca*.

In some embodiments, the thermostable endopeptidase may be a variant of an endopeptidase obtained from *Thermobifida*. In some embodiments, the thermostable endopeptidase may be a variant of an endopeptidase obtained from *Thermobifida fusca*.

In some embodiments, the thermostable endopeptidase may be an S1A protease, e.g., an S1A protease from *Thermobifida*, preferably from *Thermobifida fusca.*

The thermostable endopeptidase may be one of the variants of a protease from *Thermoascus aurantiacus* having a higher thermostability which are disclosed in WO 2011/072191.

The amount of thermostable endopeptidase added to the composition comprising substrate protein can and will vary depending upon the source of the substrate protein, the desired degree of hydrolysis, and the duration of the hydrolysis reaction. The amount of thermostable endopeptidase may range from about 1 mg of enzyme protein to about 5000 mg of enzyme protein per kilogram of dry matter. In another embodiment, the amount may range from 10 mg of enzyme protein to about 2000 mg of enzyme protein per kilogram of dry matter. In yet another embodiment, the amount may range from about 50 mg of enzyme protein to about 1000 mg of enzyme protein per kilogram of dry matter.

<u>Hydrolysis of substrate protein with thermostable endopeptidase</u>

After addition of the thermostable endopeptidase, the composition is incubated for at least 10 minutes at a temperature of at least 75°C. In some preferred embodiments, the composition is incubated for at least 30 minutes, e.g., for at least 1 hour or at least 2 hours. In some preferred embodiments, the composition is incubated for 10 minutes to 20 hours, e.g., for 30 minutes to 10 hours or for 1-8 hours.

In some preferred embodiments, the composition is incubated at a temperature of at least 80°C, e.g., at least 85°C, at least 90°C or at least 95°C. In some preferred embodiments, the composition is incubated at a temperature of 75-120°C, e.g., 80-115°C, 85-110°C, 90-105°C or 95-100°C.

Second hydrolysis with a protease preparation having aminopeptidase activity

In some aspects of the present invention, after the first hydrolysis step with the thermostable endopeptidase, a protease preparation having an aminopeptidase activity of at least 200 LAPU/g is added to the composition and a second hydrolysis step is performed by incubating for at least 10 minutes at a temperature which is at least 10°C lower than the temperature used in the first hydrolysis step.

After the first hydrolysis step, the pH of the composition may be adjusted according to methods generally known in the art. The pH of the composition may be adjusted and maintained during the second hydrolysis step at from about pH 5 to about pH 10. In one embodiment, the pH of the composition may be adjusted and maintained at from about pH 7 to about pH 9, e.g., at about pH 8. In a preferred embodiment, the pH of the composition is not adjusted before the second hydrolysis step.

The protease preparation may have an aminopeptidase activity of at least 300 LAPU/g, preferably at least 500 LAPU/g, more preferably at least 1000 LAPU/g. One LAPU (leucine amino peptidase) is defined as the amount that hydrolyzes 1mmol L-leucine-p-nitroanilide per minute at 37°C, pH 8.0. The absorption increase of the product p-nitroaniline is measured at 405 nm and is proportional to the enzyme activity.

The protease preparation may have an aminopeptidase activity of 200-5000 LAPU/g, preferably 500-2000 LAPU/g.

Preferably, the protease preparation is added in a total amount of at least 1 LAPU/g dry matter, preferably at least 5 LAPU/g dry matter, more preferably at least 8 LAPU/g dry matter.

Preferably, the protease preparation has a carboxypeptidase activity of at least 5 CPDU/g, preferably at least 10 CPDU/g, more preferably at least 20 CPDU/g. One CPDU (carboxypeptidase unit) is defined as the amount of enzyme that hydrolyzes 1 µmole N-(3-[2-furyl]acryloyl)-Ala-Lys per minute at 37°C, pH 5.8. The absorption decrease is measured at 340nm and is proportional to the enzyme activity.

The protease preparation may have a carboxypeptidase activity of 5-2000 CPDU/g, preferably 10-1000 CPDU/g.

Preferably, the protease preparation is added in a total amount of at least 0.02 CPDU/g dry matter, preferably at least 0.1 CPDU/kg dry matter, more preferably at least 0.15 CPDU/kg dry matter.

In a preferred embodiment, the protease preparation comprises at least five proteolytic components each having an approximate molecular weight, respectively, selected from 23 kD, 27 kD, 31 kD, 32 kD, 35 kD, 38 kD, 42 kD, 47 kD, 53 kD, and 100 kD. In another preferred embodiment, the protease preparation comprises at least five proteolytic components having the approximate molecular weights 23 kD, 31 kD, 35 kD, 38 kD and 53 kD, respectively.

Preferably the protease preparation is dervied from a fungus, more preferably a filamentous fungus.

In a preferred embodiment, the protease preparation is dervied from *Aspergillus*, preferably from *Aspergillus oryzae*.

The protease preparation may be the protease preparation derived from *Aspergillus oryzae* which is described in WO94/25580.

The protease preparation may be the protease preparation derived from *Aspergillus oryzae* supplied from Novozymes A/S under the tradename Flavourzyme®. The protease preparation may be the protease preparation Protease A ”Amano” 2 SD from a strain of *Aspergillus oryzae* (Amano).

After the first hydrolysis step, the temperature of the compostion is adjusted to a temperature which is at least 10°C lower than the temperature used in the first hydrolysis step. The temperature may be adjusted before, during or after the addition of the protease preparation. Preferably, the temperature is adjusted before addition of the protease preparation.

After addition of the protease preparation, the composition is incubated for at least 10 minutes at such temperature. In some preferred embodiments, the composition is incubated for at least 30 minutes, preferably for at least 1 hour, e.g., for at least 2 hours or for at least 4 hours after addition of the protease preparation. In some preferred embodiments, the composition is incubated for 10 minutes to 72 hours, e.g., for 1-48 hours or for 2-24 hours after addition of the protease preparation.

In some preferred embodiments, the composition is incubated at a temperature of 30-65°C, e.g., 35-60°C, 40-60°C or 45-55°C.

An enzyme capable of converting Gln to Glu may be added at the same time as or after the addition of the protease preparation. It may be, e.g., a glutaminase or a gamma-glutamyl-transpeptidase.

Protein hydrolysate obtained

The degree of hydrolysis of a protein hydrolysate obtained by a method of the present invention can and will vary depending upon the source of the substrate protein, the protease(s) used, and the degree of completion of the hydrolysis reaction.

The degree of hydrolysis (DH) refers to the percentage of peptide bonds cleaved versus the starting number of peptide bonds. For example, if a starting protein containing five hundred peptide bonds is hydrolyzed until fifty of the peptide bonds are cleaved, then the DH of the resulting hydrolysate is 10%. The degree of hydrolysis may be determined using the trinitrobenzene sulfonic (TNBS) colorimetric method or the orthophthaldialdehyde (OPA) method, as detailed in the examples. The higher the degree of hydrolysis, the greater the extent of protein hydrolysis.

If the substrate protein is soy protein, the degree of hydrolysis of a protein hydrolysate obtained by a method of the present invention may be at least 10%, more preferably at least 15%, at least 20% or at least 30%. In some embodiments, the degree of hydrolysis of the protein hydrolysate is 10-100%, preferably 15-80% or 20-60%. Preferably, the degree of hydrolysis is determined using the OPA method.

The solubility of a protein hydrolysate obtained by a method of the present invention can and will vary depending upon the source of the source of the substrate protein, the protease(s) used, and the pH of the composition. The solubility is a measure of the solubility of the solids (i.e., polypeptide fragments) in the protein hydrolysate. The amount of soluble solids may be estimated by measuring the amount of solids in solution before and after centrifugation (e.g., about 500-1000 x g for about 5-10 min). Alternatively, the amount of soluble solids may be determined by estimating the amount of protein in the composition before and after centrifugation using a technique well known in the art, such as, e.g., a bicinchoninic acid (BCA) protein assay or by measuring the protein content of the supernatant (obtained by centrifugation at 1200 for 5 min) relative to the protein content of the whole sample as described in the Examples.

Preferably, the solubility of a protein hydrolysate obtained by a method of the present invention is at least 60%, more preferably at least 65% or at least 70%. In some embodiments, the solubility of the protein hydrolysate is 60-100%, preferably 65-100% or 70-100%.

EXAMPLES

The experimental Thermostable protease (PFus) used in the Examples is the endopeptidase of SEQ ID NO: 3.

Example 1

Hydrolysis of soy bean meal (SBM) with experimental Thermostable protease (PFus) in comparison with the state of the art enzyme, Alcalase 2.4L

A hydrolysis assay has been performed in 12% soy solution. The solution was prepared by suspending 42 g soy bean meal in 308 g demineralized water (Milli Q water). pH was adjusted to pH 8 by 4N NaOH. 40 g SBM suspensions were heated to 70°C and 95°C, respectively; 0.25% Alcase 2.4 L was added to the 70°C SBM suspension and 50 or 100mg ep/kg SBM of PFus was added to the 95°C SBM suspension. A control with no enzyme added has been included for both temperatures. The samples were incubated for 30, 60 and 120 min with stirring. Small amounts, i.e. 1.5 ml of each of the samples were withdrawn after 30, 60 and 120 min and the enzymatic hydrolysis was stopped immediately for PFus by placing the samples in an ice bath and for the Alcalase samples by heating the samples at 95°C for 15 min. Samples were in general frozen until analysis and handled on ice when in use. %DH was measured in duplicate on the suspension by OPA and solubility was measured in duplicate by the BCA method.

The degree of hydrolysis (%DH) of each of the hydrolysates was determined by using the o-phtaldialdehyde (OPA) assay. For this, each hydrolysate (and non-hydrolyzed starting material) was diluted to 2.5% dry matter and afterwards diluted 1:20. 20 µl aliquot of each sample/standard was transferred to microtiter plates (MTP) and 200 µl OPA reagent was added (OPA reagent: The following reagents are weighed in 100 ml measuring flask and dissolved in milli Q water, milli Q water added up to 100 ml: 0.504 g Sodium bicarbonate, 0.4293 g Sodium carbonate decahydrate, 100 mg Sodium dodecyl sulphate (SDS), 88 g di-thiotritol (DTT), 80 mg o-phthaldehyde (OPA) dissolved in 2 ml 96% ethanol). The absorbance was measured at 340 nm. A standard curve with L-serine (0-0.5 mg/ml) was also included. The degree of of hydrolysis was calculated as related to the serine standard.

Solubility analysis: The ratio of soluble solids of each hydrolysates was determined by measuring the soluble protein using bicinchoninic acid (BCA) based protein assay (e.g Micro BCA Protein Assay Kit; Sigma BCA1). BCA was measured on the very sample and on supernatants. Full samples are diluted to 2.5 % dry matter and 1.5 ml of the 2.5% dry matter samples are centrifuged 10 min at 500G. All samples are diluted 1:20. BSA (bovine serum albumin) standard dilution (0 – 1.0 mg/ml) was included in each MTP. 20µL of each sample and standard are transferred to MTP (Micro Titer Plates). 160 µL BCA reagent (8 ml Bicinchoninic Acid + Copper sulphate 4% w/v solution) is added. MTP plates are incubated 30 min at 37°C and the absorbance at 582 nm is measured. The absorbance at 582 nm is directly proportional to the protein concentration and the solubility is then calculated as the protein concentration in the supernatant relative to the very sample.

The results appear from the table below. It is clearly seen that on all aspects %DH and solubility of the samples treated with the thermostable protease PFus are superior to Alcalase.

Table 1: *Comparison of PFus and Alcalase 2.4L on the two parameters %DH and Solubility*

	Solubility %	DH%
Process ref 70 °C, 120 min	67	6.5
0.25 % Alcalase, 30 min	80	9.7
0.25 % Alcalase, 60 min	80	10.6
0.25 % Alcalase, 120 min	73	12.4
Process ref 95 °C, 120 min	71	6
PFus 50 mg/kg, 30 min	86	10
PFus 50 mg/kg, 60 min	82	12.2
PFus 50 mg/kg, 120 min	89	13.4
PFus 100 mg/kg, 30 min	88	10.7
PFus 100 mg/kg, 60 min	90	12.1
PFus 100 mg/kg, 120 min	87	14.2

Example 2

Hydrolysis of soy bean meal (SBM) with a combination of experimental Thermostable protease (PFus) and Flavourzyme 1000L in comparison with the state of the art enzyme, Alcalase 2.4L and Flavourzyme 1000L.

A hydrolysis assay has been performed in a two-step hydrolysis procedure. 12% soy solution was prepared by suspending 42 g soy bean meal in 308 g demineralized water (Milli Q water). pH was adjusted to pH 8 by 4N NaOH. 30 g SBM suspensions were heated to 70°C and 95°C, respectively, 0.25% Alcase 2.4 L was added to the 70°C SBM suspension and 100mg ep/kg SBM of PFus was added to the 95°C SBM suspension. A control with no enzyme added has been included. The samples were incubated for 120 min with stirring. All samples were then adjusted to 50°C and 0.5, 1.5 and 3.0% Flavourzyme 1000L (having at least 1000 LAPU/g and at least 20 CPDU/g), respectively, was added. The samples were left for 20h at 50°C. Small amounts, i.e. 1.5 ml of each of the samples were withdrawn after 2, 4 and 20 hours after start and the enzymatic hydrolysis was stopped immediately either by placing the samples in an ice bath or by heating the samples at 100°C for 10 min. Samples were in general frozen until analysis and handled on ice when in use. %DH was measured in duplicate on the suspension by OPA and solubility was measured in duplicate by the BCA method. The results appear from the table below.

It is clearly seen that on the analyzed parameters, %DH and solubility for the thermostable enzyme either alone or in combination with Flavourzyme is superior to Alcalase.

Table 2: *Comparison of PFus and Alcalase2.4L in combination with Flavourzyme 1000L on the two parameters %DH and Solubility*

	Solubility%	DH%
PFus 100 mg/kg, 2 h	88	10.1
PFus+0.5% Flavourzyme, 4 h	80	16.9
PFus+1.5% Flavourzyme, 4 h	79	23.8
PFus+3% Flavourzyme, 4 h	86	29.2
0.25% Alc, 2 h	71	9.7
Alc+0.5% Flavourzyme, 4 h	57	16.3
Alc+1.5% Flavourzyme, 4 h	64	19.7
Alc+3% Flavourzyme, 4 h	65	22.8
PFus+0.5% Flavourzyme, 20 h	96	24.6
PFus+1.5% Flavourzyme, 20 h	85	35.2
PFus+3% Flavourzyme, 20 h	67	40
Alc+0.5% Flavourzyme, 20 h	56	23.9
Alc+1.5% Flavourzyme, 20 h	54	31.5
Alc+3% Flavourzyme, 20 h	61	39.6

Example 3

Performance of PFus in comparison with Alcalase 2.4L for wheat gluten hydrolysis

15% wheat gluten, Tereos Syrah was prepared by suspending 75 g gluten in 425 g Milli Q water, pH is adjusted to pH 8.0 with 4N NaOH. For each test sample 100 g suspension was heated to 70°C and 95°C, respectively; 0.25% Alcase 2.4 L was added to the 70°C gluten suspension and 100 mg ep/kg gluten of PFus was added to the 95°C gluten suspension. A control with no enzyme added has been included. The samples were incubated for 60, 120 and 240 min with stirring. After 240 min the samples with PFus and Alcalase, respectively, were split in two 50 g samples. All samples were then adjusted to 50°C and 1.0 or 3.0% w/w Flavourzyme 1000L was added to each sample. The samples with + 3% Flavourzyme were left for 4h at 50°C and the samples with + 1% Flavourzyme were left for 16h at 50°C. Small amounts, i.e. 1.5 ml of each of the samples were withdrawn after each step 60, 120, 240 min, 4 h, 16 h and the enzymatic hydrolysis was stopped immediately either by placing the samples in an ice bath or by heating the samples at 100°C for 10 min. Samples were in general frozen until analysis and handled on ice when in use. %DH was measured in duplicate on the suspension by OPA and solubility was measured in duplicate by the BCA method. The results appear from the table below. Solubility for the thermostable enzyme either alone or in combination with Flavourzyme is superior to Alcalase.

Table 3: Wheat gluten hydrolysis: *Comparison of PFus and Alcalase2.4L alone and in combination with Flavourzyme 1000L on the two parameters %DH and Solubility*

samp. No		Solubility %	DH%
19	Untreated Gluten	66	0.1
1	Process ref 70°C - 60 min	75	0.3
2	Alcalase - 60 min	70	4.1
3	Process ref 95°C - 60 min	59	0.4
4	PFus - 60 min	91	2.1
5	Process ref 70°C - 120 min	79	0.3
6	Alcalase - 120 min	77	4.4
7	Process ref 95°C - 120 min	74	0.4
8	PFus - 120 min	97	2.9
9	Process ref 70°C - 240 min	82	0.3
10	Alcalase - 240 min	77	5
11	Process ref 95°C - 240 min	70	0.7
12	PFus - 240 min	110	2.6
13	Process ref 70°C – 240 min + 17 h	47	0.2
15	Process ref 95°C – 240 min + 17 h	45	1
14	Alcalase - 240 + 1 % FZ	57	25.3
16	PFus - 240 + 1 % FZ	82	21.7
17	Alcalase- 4h + 3 % FZ 4h	66	17.5
18	PFus - 4h + 3 % FZ 4h	77	15.3

Example 4

Characterization of the experimental Thermostable protease (PFus)

pH Activity Profile

Assay principle:

A kinetic Suc-AAPF-pNA assay was used for recording the pH-activity profile. The increase in OD_{405} was monitored over time as a measure of the protease activity.

Assay buffers:

100 mM succinic acid, 100 mM HEPES, 100 mM CHES, 100 mM CABS, 1 mM $CaCl_2$, 150mM KCl, 0.01% Triton X-100 adjusted to pH-values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 with HCl or NaOH.

Assay substrate solution:
50 mg Suc-AAPF-pNA (Bachem L-1400) dissolved in 1.0 mL DMSO and further diluted 45x with 0.01% Triton X-100.

Assay conditions:
The purified protease stock solution was diluted using 0.01% Triton X-100 in order to ensure an adequate response at the selected assay conditions. 20 µL protease solution was mixed with 100 µL assay buffer in a microtiterplate. The assay was started by adding 100 µL pNA substrate solution and the increase in OD_{405} was monitored over time.

pH	Relative Activity (%)
2	0.2
3	0.3
4	4.7
5	31.6
6	69.7
7	82.4
8	82.7
9	84.5
10	90.3
11	100.0

pH Stability as Evaluated by Residual Activity Measurements

Assay principle:
A kinetic Suc-AAPF-pNA assay was used for obtaining the pH stability profile at 37°C. The increase in OD_{405} was monitored over time as a measure of the protease activity.

Incubation buffers:

100 mM succinic acid, 100 mM HEPES, 100 mM CHES, 100 mM CABS, 1 mM $CaCl_2$, 150mM KCl, 0.01% Triton X-100 adjusted to pH-values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 with HCl or NaOH.

Assay buffer:

0.5 M Tris/HCl, 2mM $CaCl_2$, pH 9.0.

Assay substrate solution:

50 mg Suc-AAPF-pNA (Bachem L-1400) dissolved in 1.0 mL DMSO and further diluted 45x with 0.01% Triton X-100.

Assay conditions:

The purified protease stock solution was diluted using incubation buffer at selected pH's in order to ensure the correct incubation pH and a protease concentration of approx. 0.1 mg/mL. This was followed by incubation at 37°C for 2 hrs. Then the protease samples were transferred to the same pH (pH 9), before assay for residual activity, by dilution in assay buffer to ensure an adequate response in the assay for residual activity. The residual activity in the samples was measured by mixing 20 µL protease solution with 100 µL assay buffer in a microtiterplate. The assay was started by adding 100 µL pNA substrate. The increase in OD_{405} was monitored over time. The reference sample was kept at 5°C throughout the incubation step. The data listed in the table below are the residual activities relative to the activity recorded for the sample incubated at 5°C.

pH	Residual Activity (%)
2	1.5
3	1.0
4	96.3
5	95.7
6	101.5
7	102.1
8	100.8
9	99.1
10	98.7

11	104.1
5°C	100.0

Temperature Stability as Evaluated by Residual Activity Measurements

Assay principle:

A kinetic Suc-AAPF-pNA assay was used for obtaining the temperature-stability profile at pH 9. The increase in OD_{405} was monitored over time as a measure of the protease activity.

Assay buffer:

100 mM succinic acid, 100 mM HEPES, 100 mM CHES, 100 mM CABS, 1 mM $CaCl_2$, 150 mM KCl, 0.01% Triton X-100, pH 9.0.

Assay substrate solution:

50 mg Suc-AAPF-pNA (Bachem L-1400) dissolved in 1.0 mL DMSO and further diluted 45x with 0.01% Triton X-100.

Assay conditions:

The purified protease stock solution was diluted using incubation buffer in order to ensure the correct assay pH and a protease concentration of approx. 0.1 mg/mL. This was followed by incubation at selected temperatures for 15 min. After incubation the residual activity in the samples was measured by mixing 20 µL protease solution with 100 µL assay buffer in a microtiterplate. The assay was started by adding 100 µL pNA substrate. The increase in OD_{405} was monitored over time. The data listed in the table below are the residual activities relative to the activities recorded for the incubations at 37°C.

Temperature (°C)	Residual Activity (%)
37	99.0
50	99.8
60	100.0
70	99.9
80	99.5

90	97.9
99	84.6

Temperature-Activity Profile

Assay principle:

An endpoint Suc-AAPF-pNA assay was used for obtaining the temperature-activity profile at pH 9. OD_{405} was recorded as a measure of protease activity.

Assay buffer:

100 mM succinic acid, 100 mM HEPES, 100 mM CHES, 100 mM CABS, 1 mM $CaCl_2$, 150 mM KCl, 0.01% Triton X-100, pH 9.0.

Assay substrate solution:

50 mg Suc-AAPF-pNA (Bachem L-1400) dissolved in 1.0 mL DMSO and further diluted 50x with the assay buffer.

Assay conditions:

The purified protease stock solution was diluted using 0.01% Triton X-100 in order to ensure an adequate response at the selected assay conditions. 200 µL of assay substrate solution were pipetted in an Eppendorf tube and placed on ice. 20 µL of the diluted protease solution was added and the assay started by transferring the Eppendorf tube to an Eppendorf thermomixer, which was set to the assay temperature. The tube was incubated for 15 minutes at the highest shaking rate (1400 rpm). The incubation was stopped by transferring the tube to the ice bath and adding 600 µL 500 mM succinic acid, pH 3.5. 200 µL supernatant was transferred to a microtiter plate and OD_{405} read as a measure of peptidase activity. A buffer blind was included in the assay (instead of enzyme).

Temperature (°C)	Relative Activity (%)
15	5.9
25	7.4
37	13.6
50	26.4

60	40.4
70	53.1
80	81.3
90	93.5
99	100.0

Example 5

Endo-Protease Activity Assay

Assay principle:

An endpoint-assay using the Protazyme AK substrate (AZCL-casein) or Protazyme OL substrate (AZCL-collagen), both from Megazyme. OD_{590} is recorded after terminating the reaction. The increase in absorbance reflects solubilized, dye-coupled casein/collagen fragments and is a measure of the protease endo-activity. Protazyme OL is particularly suited for proteases with acidic pH optima.

Assay buffer:

Selected to fit the requirements of the endo-protease to be tested (e.g. inclusion of known required cofactors). A broad, cocktail buffer serving many needs could be: 100 mM succinic acid, 100 mM HEPES, 100 mM CHES, 100 mM CABS, 1 mM $CaCl_2$, 150 mM KCl, 0.01% Triton X-100 adjusted to pH-values 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 with HCl or NaOH.

Assay substrate suspension:

A Protazyme AK or Protazyme OL tablet (from Megazyme) was suspended in 2.0 mL 0.01% Triton X-100 by gentle stirring.

Assay conditions:

500 μL of the assay substrate suspension and 500 μL assay buffer were mixed in an Eppendorf tube and placed on ice. 20 μL protease sample (diluted in 0.01% Triton X-100) was added. The assay was initiated by transferring the Eppendorf tube to an Eppendorf thermomixer, which was set to the assay temperature. The tube was incubated for 15 minutes on the Eppendorf thermomixer at its highest shaking rate (1400 rpm.). The incubation was stopped by transferring the tube back to the ice bath. Then the tube was centrifuged in an ice-cold centrifuge for a few

minutes and 200 µL supernatant was transferred to a microtiter plate. OD_{590} was read as a measure of protease activity. A buffer blind was included in the assay (instead of enzyme).

Example 6

Hydrolysis of soy bean meal (SBM) with either of experimental thermostable protease from *Thermobifida fusca* (*T. fusca* protease), thermostable serine protease from *Nocardiopsis prasina* (*Nocardiopsis* protease) or state of the art enzyme Alcalase 2.4L each combined with Flavourzyme 1000L

The *Nocardiopsis* protease has the amino acid sequence of SEQ ID NO: 4.

The wild-type DNA and amino acid sequence of the *T. fusca* protease are shown as SEQ ID NOs: 5 and 6. The protease has been expressed in *Bacillus subtilis* with a suitable signal sequence replacing the native one and with an HQHQHQH-tag in the C-terminal. The amino acid sequence of the expression construct is shown as SEQ ID NO: 7. The N-terminal has been determined as AAIIGGN (amino acids 183-189 of SEQ ID NO: 6 and 179-185 of SEQ ID NO: 7) using Edman degradation. SEQ ID NO: 8 shows the mature amino acid sequence of the *T. fusca* protease based hereon.

Both the *Nocardiopsis* protease and the *T. fusca* protease have a temperature optimum at or above 80°C.

A hydrolysis assay has been performed in a two-step hydrolysis procedure. 12% soy solution was prepared by suspending 12 g soy bean meal in 88 g demineralized water (Milli Q water). The solution was stirred for 30 min. before pH was adjusted to 8.0 with 4 N NaOH.

For each treatment, 5 g substrate solution was weighed out in 5 ml Eppendorf tubes and heated in an Eppendorf thermomixer, mixing speed = 1000 rpm. Proteases were added at room temp. immediately before heating-up to the optimum temperature of the 3 enzymes: Alcalase at 70°C, *Nocardiopsis* protease at 80°C and *T. fusca* protease at 80°C. The times to reach the optimum temperature range from 10-30 min. Alcalase 2.4 L was added at a dosage of 5.7 AU/kg protein, *T. fusca* protease at 200 mg/kg and *Nocardiopsis* protease at 100 mg/kg After 4 hours 1.5 ml samples are taken out and placed on ice. The temperature of the remaining sample materials were decreased to 50°C and Flavourzyme 1000L was added at 1.5%. After 16 hours the samples were placed on ice. All samples (4 hours and 20 hours) were stored in a freezer until analysis was carried out. All samples were analysed for %DH as described in example 1 and % solubility as described below. % solubility was analysed by measuring the protein content of the supernatant (obtained by centrifugation at 1200 for 5 min) relative to the protein content of the whole sample. The protein content of both the whole sample and the supernatant were analysed by a LECO FP628. LECO analysis is based on detection of the nitrogen content by combustion analysis. The nitrogen convertion factor applied is 6.25.

Table 4: *Comparison of Thermostable T. fusca protease, Nocardiopsis protease and Alcalase 2.4L in combination with Flavourzyme 1000L on the two parameters %DH and Solubility*

	Solubility %		**%DH**	
	4h	**4+16h**	**4h**	**4+16h**
Alcalase 2.4 L 70°C	**84+/-0.1**		**9.8+/-0.3**	
***Nocardiopsis* prot 80°C**	**89+/-0.05**		**9.8+/-0.14**	
***T. fusca* prot 80°C**	**90+/-0.03**		**11.5+/-0.8**	
Alcalase 2.4L 70°C + Flavourzyme 1000L		**55+/-0.2**		**29.5+/- 0.8**
***Nocardiopsis* prot 80°C + Flavourzyme 1000L**		**67+/-0.1**		**32.6+/-0.0**
***T. fusca* prot 80°C + Flavourzyme 1000L**		**78+/-0.5**		**31.3+/-0.5**

The solubility of the soy samples for the thermostable enzymes either alone or in combination with Flavourzyme were observed as superior to Alcalase. The %DH is also either the same or higher for the thermostable enzymes compared to Alcalase.

CLAIMS

1. A method for producing a protein hydrolysate, comprising:

a) adding to a composition comprising substrate protein a thermostable endopeptidase;

b) performing a first hydrolysis step by incubating the composition of step a) for at least 10 minutes at a temperature of at least 75°C;

c) adding to the compostion of step b) a protease preparation having an aminopeptidase activity of at least 200 LAPU/g; and

d) performing a second hydrolysis step by incubating the composition of step c) for at least 10 minutes at a temperature which is at least 10°C lower than the temperature used in step b).

2. The method of claim 1, wherein the thermostable endopeptidase is a nonspecific endopeptidase.

3. The method of claim 2, wherein the nonspecific endopeptidase is characterized in that incubation of 0.5% (w/w) BSA with the endopeptidase for 4 hours at a temperature and pH where the endopeptidase exhibits at least 40% of its maximum activity results in a degree of hydrolysis of at least 10%.

4. The method of any of the preceding claims, wherein the thermostable endopeptidase is an endopeptidase, which after incubation for 15 minutes at 80°C and pH 9 has a residual activity of at least 80% relative to its activity after incubation at 37°C.

5. The method of any of the preceding claims, wherein the thermostable endopeptidase (i) has at least 60% sequence identity to the polypeptide of SEQ ID NO: 3, (ii) is encoded by a polynucleotide having at least 60% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) is a variant of the polypeptide of SEQ ID NO: 3 comprising a substitution, deletion, and/or insertion at one or more positions.

6. The method of any of the preceding claims, wherein the thermostable endopeptidase (i) has at least 60% sequence identity to the polypeptide of SEQ ID NO: 8, (ii) is encoded by a polynucleotide having at least 60% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 5, or (iii) is a variant of the polypeptide of SEQ ID NO: 8 comprising a substitution, deletion, and/or insertion at one or more positions.

7. The method of any of the preceding claims, wherein the thermostable endopeptidase (i) has at least 60% sequence identity to the polypeptide of SEQ ID NO: 4, or (ii) is a variant of the polypeptide of SEQ ID NO: 4 comprising a substitution, deletion, and/or insertion at one or more positions.

8. The method of any of the preceding claims, wherein the protease preparation added in step c) is a protease preparation from *Aspergillus*, preferably *Aspergillus oryzae*.

9. The method of any of the preceding claims, wherein the protease preparation added in step c) comprises at least five proteolytic components each having an approximate molecular weight, respectively, selected from 23 kD, 27 kD, 31 kD, 32 kD, 35 kD, 38 kD, 42 kD, 47 kD, 53 kD, and 100 kD.

10. The method of any of the preceding claims, where in step d) the compostition is incubated at a temperature of 30-65°C.

11. The method of any of the preceding claims, wherein an enzyme capable of converting Gln to Glu is added at the same time or after step c).

12. The method of any of the preceding claims, wherein the protein hydrolysate obtained in step d) has a degree of hydrolysis of at least 10%.

13. A method for producing a protein hydrolysate, comprising:

a) adding to a composition comprising substrate protein a thermostable endopeptidase which (i) has at least 60% sequence identity to the polypeptide of SEQ ID NO: 3, (ii) is encoded by a polynucleotide having at least 60% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) is a variant of the polypeptide of SEQ ID NO: 3 comprising a substitution, deletion, and/or insertion at one or more positions; and

b) incubating the composition of step a) for at least 10 minutes at a temperature of at least 75°C.

14. The method of any of the preceding claims, wherein the substrate protein is selected from soy protein, wheat gluten protein or whey protein.

15. The method of any of the preceding claims, wherein the composition comprising substrate protein has a dry matter content of at least 1% (w/w).