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(54) Title: METHOD FOR OBTAINING A DAIRY ALTERNATIVE FOOD PRODUCT WITH IMPROVED FOAMING

(57) Abstract: Provided herein is the use of an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase for obtaining a dairy alternative food product with improved foaming properties.

METHOD FOR OBTAINING A DAIRY ALTERNATIVE FOOD PRODUCT WITH IMPROVED FOAMING

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 use of a specific endopeptidase and a protein deamidase for obtaining a dairy alternative food product with improved organoleptic properties, in particular improved foaming properties.

BACKGROUND OF THE INVENTION

The number of people pursuing a vegan, vegetarian, or non-dairy diet for health reasons has increased in recent years. Further, food products made from animals' milk, such as cows' milk, are increasingly recognized for their high environmental costs. These factors are leading to a greater demand for dairy alternative food products for foods traditionally derived from milk, including milk, cheese, and yogurt.

Some examples of a dairy alternative food product which have received great attention in recent years are food products based on almond, oat, pea and soy. The rising popularity of these plant substrates can be partly attributed to their numerous health benefits, low allergenicity properties, and the growing interest in sustainable and ethical food choices. For example oats and almonds are perceived as healthy for a number of reasons: They are a great source of important vitamins, minerals, fibres (β-glucans), antioxidants, as well as essential amino acids. Health benefits which have been associated with intake of oats and almonds include weight loss, lower blood cholesterol levels and reduced risk of heart disease. Also peas and soy are attractive options for health-conscious consumers. Peas, for example, present a lower risk of causing allergic reactions compared to traditional dairy products, which is a significant advantage for individuals with lactose intolerance or specific food allergies.

Dairy alternative food products may be derived from plant material with high starch content. In general, to convert the high-starch plant material into a dairy alternative food product, the starch must be hydrolyzed. The conversion of the starch typically includes a gelatinization step in which starch granules are dissolved to form a viscous suspension, a liquefaction step in which the starch is partially hydrolyzed with a concomitant loss in viscosity, and optionally a saccharification step which involves the production of glucose and maltose by further hydrolysis.

The use of enzymes to improve conversion of plant material, such as high-starch plant material, into plant-based dairy alternative food products remains a topic of interest in the industry

and includes finding improved methods for obtaining plant-based dairy alternative food products with organoleptic properties matching those of conventional dairy products, such as cow's milk.

WO 2014/123466 A1 discloses enzymatic modification of oat material using amylases and protein deamidation.

WO 2008/131008 A2 and WO 2009/155557 A2 disclose modification of soy protein using proteases.

US 2022/0046939A1 discloses processes for producing plant based milk substitutes, such as oat milks or oat creamers, comprising use of endoproteases and divalent cationic salts.

It is an object of the present invention to identify improved methods for producing plant-based dairy alternative food products with improved organoleptic properties, such as, e.g., improved foaming properties.

SUMMARY OF THE INVENTION

The present inventors have found that by treating a slurry of a plant material in water with a combination of enzymes, including an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase, more protein is solubilized, i.e. total protein as well as soluble protein is increased in the final product, and an improved plant-based dairy alternative food product with superior foaming properties is obtained. The improvement in foaming properties is seen, e.g., in the resulting foam having better stability, a higher density of micro-foam, higher volume, higher coherency, and/or a smoother texture compared to a foam produced from a plant-based dairy alternative food product that has not been treated with an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase.

The invention therefore provides a method for obtaining a dairy alternative food product, the method comprising the steps of:

(a) obtaining a slurry of a plant material in water; and

(b) treating the slurry of step (a) with an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase to obtain the dairy alternative food product.

The invention also provides the use of an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase in the production of a dairy alternative food product to improve foaming properties.

By using a combination of an endopeptidase having a specificity for cleaving after Arg and/or Lys, such as, e.g., a trypsin-like endopeptidase from Fusarium spp., and a protein deamidase, such as, e.g., a protein deamidase obtained from Chryseobacterium spp., a plant-based dairy alternative food product, such as, e.g., an oat-based beverage, is obtained which has improved foaming properties and stability when used in for example an acidic food matrix, such as, e.g., coffee or tea. Thus, the combination of enzymes as claimed herein will enable producers of dairy alternative food products, such as producers of oat-based beverages, to obtain a dairy

alternative food product having superior foaming properties. Such a product may be an oat-based drink for barista applications..

The invention therefore also provides a method for obtaining an oat-based dairy alternative food product, the method comprising the steps of:

(a) obtaining a slurry of an oat material in water; and

(b) treating the slurry of step (a) with an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase to obtain the dairy alternative food product.

The use of a specific endopeptidase combined with a protein deamidase gives even more soluble protein and better foaming than when the enzymes are used alone. Furthermore, sensory properties are not affected negatively by the combined use of the specific endopeptidase and protein deamidase. This is surprising in view of previous disclosures, such as that of WO 2014/123466 A1, which discloses that the use of a protease in combination with protein deamidase should be avoided due to adverse effects on organoleptic properties of the resulting drink.

Without wishing to be bound by any particular theory, the inventors believe that the unique properties of the dairy alternative food product obtained in the methods according to the present invention can be explained by the specificity of the endopeptidase of the invention. In particular, the endopeptidase of the present invention does not suffer from the same limitations as other less specific or non-specific proteases, such as the broad-spectrum endopeptidase Neutrase® sold by Novozymes A/S, which do not improve foaming and can result in undesirable organoleptic properties, such as bitterness. While the specific endopeptidase alone can improve foaming, it is further postulated that the combined use of a specific endopeptidase and a protein deamidase gives even more protein solubilization and further improves foaming properties of the drinks obtained in the methods disclosed herein. This has also been shown, for examples, in Examples 5 and 10 herein, wherein use of the specific endopeptidase and protein deamidase as disclosed herein, resulted in oat- and pea-based beverages having more soluble protein and improved foaming capability and stability, without giving rise to bitter off-notes in the obtained food products.

The invention further provides a dairy alternative food product comprising a mixture of polypeptide fragments each having primarily either an arginine residue or lysine residue at the carboxyl terminus.

SEQUENCES

SEQ ID NO: 1: Trypsin-like endopeptidase from Fusarium oxysporum.

SEQ ID NO: 2: Endopeptidase from Fusarium spp.

SEQ ID NO: 3: Endopeptidase from Kutzneria albida.

SEQ ID NO: 4: Endopeptidase from Trichoderma reesei.

SEQ ID NO: 5: Endopeptidase from Actinosynnema mirum.

SEQ ID NO: 6: Endopeptidase from Kribbella flavida.

SEQ ID NO: 7: Endopeptidase from Fusarium solani.

SEQ ID NO: 8: Endopeptidase from Kribbella flavida.

SEQ ID NO: 9: Endopeptidase from Aspergillus niger.

SEQ ID NO: 10: Endopeptidase from Aspergillus oryzae.

SEQ ID NO: 11: Endopeptidase from Shewanella woodyi.

SEQ ID NO: 12: Endopeptidase from Porcinus.

SEQ ID NO: 13: Lysine-specific endopeptidase from Achromobacter lyticus.

SEQ ID NO: 14: Protein deamidase derived from Chryseobacterium viscerum (the strain has formerly been referred to as Chryseobacterium sp-62563) having the mature polypeptide sequence shown as SEQ ID NO: 15.

SEQ ID NO: 15: Mature polypeptide sequence of protein deamidase derived from Chryseobacterium viscerum.

SEQ ID NO: 16: Protein deamidase derived from Chryseobacterium proteolyticum having the mature polypeptide sequence shown as SEQ ID NO: 17.

SEQ ID NO: 17: Mature polypeptide sequence of protein deamidase derived from Chryseobacterium proteolyticum.

SEQ ID NO: 18: Protein deamidase derived from Chryseobacterium gambrini having the mature polypeptide sequence shown as SEQ ID NO: 19.

SEQ ID NO: 19: Mature polypeptide sequence of protein deamidase derived from Chryseobacterium gambrini.

SEQ ID NO: 20: Protein deamidase derived from Chryseobacterium culicis having the mature polypeptide sequence shown as SEQ ID NO: 21.

SEQ ID NO: 21: Mature polypeptide sequence of protein deamidase derived from Chryseobacterium culicis.

SEQ ID NO: 22: Protein deamidase derived from Chryseobacterium defluvii having the mature polypeptide sequence shown as SEQ ID NO: 23.

SEQ ID NO: 23: Mature polypeptide sequence of protein deamidase derived from Chryseobacterium defluvii.

SEQ ID NO: 24: Oat peptide fragment #1 from Example 9.

SEQ ID NO: 25: Oat peptide fragment #2 from Example 9.

SEQ ID NO: 26: Oat peptide fragment #3 from Example 9.

SEQ ID NO: 27: Oat peptide fragment #4 from Example 9.

SEQ ID NO: 28: Oat peptide fragment #5 from Example 9.

SEQ ID NO: 29: Oat peptide fragment #6 from Example 9.

SEQ ID NO: 30: Oat peptide fragment #7 from Example 9.

SEQ ID NO: 31: Oat peptide fragment #8 from Example 9.

SEQ ID NO: 32: Oat peptide fragment #9 from Example 9.

SEQ ID NO: 33: Oat peptide fragment #10 from Example 9.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with this detailed description, the following definitions apply. Note that the singular forms “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise.

As used herein, the terms “drink”, “milk” and “beverage” are used interchangeably and have the same meaning.

Unless defined otherwise or clearly indicated by context, all percentages are percentage by weight (percent w/w or “% (w/w)”).

Unless defined otherwise or clearly indicated by context, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The term “dairy alternative food product” refers to a food product which can be used as a substitute for a dairy food product. The dairy alternative food product is a plant-based composition which may or may not be combined with additional food ingredients to produce the dairy alternative food product. The dairy alternative food product can be ingested by humans or animals, including domesticated animals, such as, e.g., companion animals. The dairy alternative food product may be an aqueous solution or suspension of a plant-based dairy alternative powder, a concentrate or an isolate. Or the plant-based dairy alternative food product may be any other suitable preparation obtained from a plant, such as, e.g., an aqueous suspension of a flour or the like obtained from a plant, such as from a part of a plant. The plant-based dairy alternative food product may be a combination of any of the above. In some embodiments, the additional food ingredients added to the dairy alternative food product may be any food ingredient deemed useful by a practitioner of skill in the art. The additional food ingredient may be a solid or liquid ingredient. The additional food ingredient may or may not be plant-based. In some embodiments, the additional food ingredient is water.

The dairy alternative food product may be fortified with a plant-based dairy alternative powder, such as, e.g., soymilk powder, or with concentrated or isolated protein, such as, e.g., soy/pea protein isolate or soy/pea protein concentrate. In an embodiment, the dairy alternative food product is fortified, such as, e.g., an oat-based drink fortified with pea protein.

Preferably, the dairy alternative food product has a protein content of at least 0.5% (w/w).

Preferably, the dairy alternative food product has a protein content of at most 4% (w/w).

In a preferred embodiment, the dairy alternative food product has a protein content of about 1% (w/w), such as about 1.5% (w/w), such as about 2% (w/w).

Preferably, the dairy alternative food product has a lipid content of at least 1% (w/w).

Preferably, the dairy alternative food product has a lipid content of at most 5% (w/w).

The additional food ingredients which may be added to the dairy alternative food product, include, but are not limited to, e.g., lipids, such as oils, in particular plant oils, sugars, such as

sucrose, proteins, various forms of synthetic amino acids, dietary fibres, salts, minerals, flavouring agents, vitamins, and any combination thereof.

In an embodiment, lipid is added to the dairy alternative food product. The lipid may be a plant oil or a mixture of plant oils. The lipid may be selected from rapeseed oil, flaxseed oil, safflower oil, flaxseed oil, soybean oil, olive oil, sunflower oil, palm oil and combinations thereof. In one embodiment the lipid is rapeseed oil, sunflower oil or a combination thereof. The selection of suitable lipid depends on the type of plant-based dairy alternative food product desired.

The lipid may be added in an amount of between about 1% to about 5% (w/w), such as about 3% (w/w), relative to the weight of the final product.

In an embodiment, salt is added to the dairy alternative food product. The salt may be sodium chloride, dicalcium carbonate, dicalcium phosphate, tricalcium phosphate, calcium carbonate, and any combination thereof. The salt may also be dipotassium phosphate.

In an embodiment, vitamins and/or minerals are added to the dairy alternative food product. The vitamins may be vitamin A, vitamin C, vitamin D, vitamin E, vitamin B12, thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), vitamin B6, vitamin K, folic acid (vitamin B9, and mixtures thereof. The mineral may be calcium, phosphorous, magnesium, sodium, potassium, chloride, iron, zinc, iodine, selenium, copper and mixtures thereof.

The dairy alternative food product may be standardized and/or homogenized. The dairy alternative food product may be pasteurized or otherwise heat-treated.

Dairy alternative food products include plant-based beverages, creamers, cheeses, ice creams and yogurts. In some embodiments, the dairy alternative food product is a plant-based beverage. Examples of plant-based beverages include almond drinks, soy drinks, cashew drinks, macadamia drinks, coconut drinks, pea drinks, rice drinks, quinoa drinks, flax drinks, hemp drinks, oat drinks and drinks comprising any combination thereof. In an embodiment, the dairy alternative food product is an almond drink, a soy drink, a pea drink, a rice drink, an oat drink, or any combinations thereof. In a preferred embodiment, the dairy alternative food product is an oat-based beverage. In another preferred embodiment, the dairy alternative food product is a pea- or soy-based beverage. In another preferred embodiment, the dairy alternative food product is an almond-based beverage.

The dairy alternative food product of the invention is derived from a plant material, which is or is derived from the edible portions of a plant. In some embodiments, the plant material is derived from edible portions of a plant which are high in starch. In some embodiments, the edible portion of the plant may be tubers, roots, stems, cobs, legumes, fruits, or seeds, such as, e.g., nuts. In some embodiments, the plant is a cereal and the plant material is or is derived from the cereal grain, also referred to as the whole grain. In further embodiments, the cereal grain may be from corn, rice, millet, milo, quinoa, or oat. In yet further embodiments, the cereal grain may be from barley, wheat, buckwheat, or rye. In some embodiments, the plant material is or is derived from a tuber or root (including rhizomes), such as a potato, sweet potato, cassava, tiger

nut (chufa nut), canna, or tapioca. In some embodiments, the plant material is or is derived from a fruit, such as banana, jack fruit, or bread fruit. In some embodiments, the plant material is or is derived from a hemp, fava, sago, pea, or bean plant.

In some embodiments, the plant material is heat treated. In some embodiments, the plant material is dehydrated. In some embodiments, the plant material is de-hulled, ground, wet milled, and/or dry milled. In some embodiments, the plant material is almond flour, corn flour, rice flour, barley flour, wheat flour, buckwheat flour, millet flour, quinoa flour, oat flour, rye flour, potato flour, sweet potato flour, cassava flour, tiger nut flour, tapioca flour, hemp flour, pea flour, soy flour, bean flour, de-hulled oats, de-hulled barley, de-hulled wheat, de-hulled peas, de-hulled beans, or a combination or any thereof. In some embodiments, the plant material is smashed or ground to produce a paste.

In preferred embodiments, the plant material is oat material. In further embodiments, the plant material is oat flour, de-hulled oats, or a combination thereof. In still further embodiments, the oat material may be oat flour, such as heat-treated oat flour, or it may be milled oat kernels, such as de-hulled and heat-treated oat kernels, which have been wet-milled, or it may be any other oat material known in the art. The oat material may be heat treated. In a preferred embodiment, the oat material is oat flour, preferably heat-treated oat flour.

In another preferred embodiment, the plant material is a leguminous material. In a further preferred embodiment, the plant material is a pea or soy material. The pea or soy material may be a pea or soy flour, a pea or soy protein concentrate, or a pea or soy protein isolate.

In another preferred embodiment, the plant material is an almond material. The almond material may be an almond flour, an almond paste, an almond protein concentrate, or an almond protein isolate.

In the methods of the invention, the plant material is suspended in an aqueous solution to produce a slurry, where the ratio of plant material to aqueous solution is 1:3 to 1:8 (w/w). In some embodiments, the ratio of plant material to aqueous solution is 1:4 to 1:6 (w/w). In some embodiments, the ratio of plant material to aqueous solution is 1:1 to 1:4 (w/w).

Preferably, the plant material has a protein content of at least 5% (w/w).

Preferably, the plant material has a protein content of at most 25% (w/w), such as at most 20% (w/w).

In a preferred embodiment, the plant material has a protein content of about 15% (w/w).

Alternatively, when the plant material is a protein concentrate or a protein isolate, it may have a protein content of up to 90% (w/w).

In some embodiments, the plant material is a pea protein isolate or a soy protein isolate having a protein content in the range of 75-85% (w/w).

In some embodiments, the protein isolate has a low or medium initial protein solubility. In some embodiments, the pea protein isolate or soy protein isolate has a low or medium initial protein solubility. In the context of the invention, protein isolates (PIs) are grouped into three

groups according to their initial protein solubility, namely low solubility PIs, medium solubility PIs, and high solubility PIs.

In the context of the invention, a low solubility protein isolate has an initial soluble protein content up to 20% (w/w), e.g. an initial soluble protein content of 5-15% (w/w). Protein isolates having a low initial solubility is be understood as a protein isolate wherein only a small portion of the protein is dissolvable in solution without any enzymatic treatment, in particular without any protein deamidase treatment.

A medium solubility protein isolate has an initial soluble protein content greater than 20% (w/w) and up to 50% (w/w). In an embodiment, the medium solubility protein isolate has an initial protein solubility of between 25-40% (w/w). Protein isolates having a medium initial solubility is be understood as protein isolates exhibiting a moderate ability to dissolve in solution when no enzymatic treatment is performed, such as when no protein deamidase is used to treat the protein isolate.

A high solubility protein isolate has an initial soluble protein content greater than 50% (w/w). In an embodiment, the high solubility protein isolate has an initial protein solubility of between 55-75% (w/w). Protein isolates having high initial solubility is be understood as protein isolates wherein a substantial portion of the protein can be dissolved in solution without any enzymatic or similar treatment. The improvement in soluble protein content for high solubility protein isolates is expected to have a lower benefit from the enzymatic treatment according to the invention, in particular from the protein deamidase treatment.

The initial protein solubility may be determined using a method as exemplified in Example 10 herein or similar methods for protein solubility measurements. In some embodiments, the protein isolate has a low or medium initial protein solubility as determined using a method as exemplified in Example 10. In some embodiments, the pea protein isolate or soy protein isolate has a low or medium initial protein solubility as determined using a method as exemplified in Example 10.

In some embodiments, the slurry of plant material in water has a protein content in the range of 1-15% (w/w), such as in the range of 2-10% (w/w).

In the methods of the invention, the slurry of a plant material in water may be treated with one or more hydrolyzing enzymes selected from the group of pectinases, hemicellulases, xylanases, beta-glucanases, mannanases, glucanases, glucoamylases, iso-amylases, alpha-amylases, beta-amylases, and mixtures thereof. In a preferred embodiment, the hydrolyzing enzyme is alpha-amylase, beta-glucanase, glucoamylase or any combination thereof. The hydrolyzing enzyme may be added simultaneously and/or sequentially with the specific endopeptidase and protein deamidase. In the context of the present invention, the one or more hydrolyzing enzymes may be selected within the same group of enzymes, such as, e.g., within the group of alpha-amylases, and/or may be selected from different groups of hydrolyzing enzymes, such as, e.g., from the group of alpha-amylases and from the group of beta-glucanases.

The alpha-amylase may be any alpha-amylase suitable for the methods according to the invention. In some embodiments, the alpha-amylase is a bacterial endo-alpha-amylase, preferably obtained from, or a variant of, an endo-alpha-amylase obtained from, Bacillus, preferably from Bacillus amyloliquefaciens. An example of a bacterial endo-alpha-amylase is BAN® available from Novozymes A/S.

In some embodiments, the alpha-amylase is a fungal endo-alpha-amylase, preferably obtained from, or a variant of, an endo-alpha-amylase obtained from, Aspergillus, preferably from Aspergillus oryzae. An example of a fungal endo-alpha-amylase is Fungamyl® available from Novozymes A/S.

In the context of the present invention, the term “variant” means a polypeptide having enzymatic 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

enzymatic 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 labelling, 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.

In some embodiments, the alpha-amylase is a raw starch hydrolyzing alpha-amylase. A raw starch hydrolyzing alpha-amylase, also known as a raw starch degrading alpha-amylase, as used herein refers to an enzyme that can directly degrade raw starch granules below the gelatinization temperature of starch. Sources of raw starch degrading enzymes include enzymes obtained from Aspergillus spp. such as Aspergillus oryzae, Aspergillus niger and Aspergillus kawachii. Examples of such raw starch degrading enzymes include the raw starch degrading enzymes described in WO 2005/003311, WO 2006/0692, WO 2006/060289 and WO 2004/080923.

In some embodiments, the raw starch degrading alpha-amylase is an acid alpha-amylase. An “acid alpha-amylase” is an alpha-amylase (4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) which when added in an effective amount has activity at a pH in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0. A source of a raw starch degrading acid alpha-amylase is the acid alpha amylase from Aspergillus niger disclosed as "AMYA_ASPNG" in the Swiss-prot/TREMBL database under the primary accession no. P56271 and described in more detail in WO 1989/01969 (Example 3). The Aspergillus niger acid alpha-amylase is also shown as SEQ ID NO: 1 in WO 2004/080923 (Novozymes A/S) which is hereby incorporated by

reference. A suitable commercially available acid fungal alpha-amylase derived from Aspergillus niger is the product SP288 (SEQ ID NO:1 of U.S: Patent No. 7,244,597; avail-able from Novozymes A/S). Other sources of acid alpha-amylases include those derived from a strain of the genera Rhizomucor and Meripilus, such as a strain of Rhizomucor pusillus (WO 2004/055178) or Meripilus giganteus. In yet another embodiment, the acid alpha-amylase is derived from Aspergillus kawachii and is disclosed by Kaneko et al., J. Ferment. Bioeng. 81:292-298(1996) "Molecular-cloning and determination of the nucleotide-sequence of a gene encoding an acid-stable alpha-amylase from Aspergillus kawachii"; and further as EMBL:#AB008370. In some embodiments, the raw starch degrading alpha amylase possesses a carbohydrate binding module (CBM) which binds to starch. In some embodiments, the CBM binds preferentially to starch, particularly to thermally untreated, granular starch. Such a CBM may also be referred to as a starch binding domain (SBD). SBDs are known to be in 15 CBM families, namely CBM20, 21, 25, 26, 34, 41, 45, 48, 53, 68, 69, 74, 82, and 83.

In some embodiments, the raw starch degrading alpha amylase may be a hybrid alpha-amylase comprising a starch-binding domain (SBD) and an alpha-amylase catalytic domain (CD). A hybrid alpha-amylase may also comprise an alpha-amylase catalytic domain (CD), a starch binding domain (SBD), and a linker connecting the CD and SBD, as is known in the art.

In an embodiment the catalytic domain is derived from a strain of Aspergillus kawachii. Examples of hybrid alpha-amylases include the ones disclosed in WO 2005/003311, U.S. Patent Publication no. 2005/0054071 (Novozymes), and US Patent No. 7,326,548 (Novozymes) which is hereby incorporated by reference. Examples also include those enzymes disclosed in Table 1 to 5 of the examples in US Patent No. 7,326,548, and in U.S. Patent Publication no. 2005/0054071 (Table 3 on page 15), such as, an Aspergillus niger alpha-amylase catalytic domain (CD) with Aspergillus kawachii linker and starch binding domain (SBD).

Other acid alpha-amylase include the enzymes disclosed in WO 2004/020499 and WO 2006/069290 and the enzymes disclosed in WO 2006/066579 as SEQ ID NO:2 (hybrid A. niger alpha-amylase+CBM), SEQ ID NO:3, or SEQ ID NO:4 (JA129). Hybrid alpha-amylase consisting of Rhizomucor pusillus alpha-amylase with Aspergillus niger glucoamylase linker and SBD disclosed as V039 in Table 5 in WO 2006/069290.

In some embodiments, the alpha-amylase is a heat tolerant bacterial alpha-amylase preferably obtained from, or a variant of, a heat tolerant endo-alpha-amylase obtained from, Bacillus, preferably from Bacillus licheniformis or Bacillus stearothermophilus.

Examples of heat tolerant bacterial alpha-amylases are Termamyl® Classic or Termamyl® SC available from Novozymes A/S. "Heat tolerant" in the context of the present invention means that the enzyme can resist irreversible thermal inactivation.

In some embodiments, the hydrolyzing enzyme is a glucoamylase (also known as amylo-glucosidase or AMG). One Glucoamylase Unit (AGU) is defined as the amount of enzyme, which

hydrolyses 1 micromole maltose per minute under the standard conditions 37°C, pH 4.3, substrate: maltose 23.2 mM, buffer: acetate 0.1 M, reaction time 5 minutes.

In some embodiments, the hydrolyzing enzyme is a maltogenic amylase. The maltogenic alpha-amylase (EC 3.2.1.133) may be from a bacteria, such as, e.g., from Bacillus. An example of a bacterial maltogenic amylase is Maltogenase® available from Novozymes A/S.

In some embodiments, the hydrolyzing enzyme is a xylanase. The xylanase may be of microbial origin, e.g., derived from a bacterium or fungus, such as a strain of Aspergillus, in particular of A. aculeatus, A. niger, A. awamori, or A. tubigensis, from a strain of Trichoderma, e.g., T. reesei, or from a strain of Humicola, e.g., H. insolens. Suitable commercially available xylanase preparations for use in the present invention include PANZEA BG, PENTOPAN MONO BG and PENTOPAN 500 BG (available from Novozymes A/S), GRINDAMYL POWERBAKE (available from Danisco), and BAKEZYME BXP 5000 and BAKEZYME BXP 5001 (available from DSM).

In some embodiments, the hydrolyzing enzyme is a beta-glucanase. A beta-glucanase may only have beta-glucanase activity or may have other enzymatic activities as well. In some embodiments, the enzyme having beta-glucanase activity may be a preparation of an endo-alpha-amylase obtained from Bacillus, such as, e.g., from Bacillus amyloliquefaciens, which has beta-glucanase side activity. In some embodiments, the enzyme having beta-glucanase activity may be in a cellulolytic enzyme preparation. In further embodiments, the cellulolytic enzyme preparation may be obtained from Trichoderma reesei. Examples of enzyme preparations having beta-glucanase activity include BAN®, Celluclast®, or Ultraflo® Prime, each available from Novozymes A/S. These enzyme preparations are considered to comprise a beta-glucanase.

The enzymes used in the methods of the invention may be added to the slurry comprising the plant material in any suitable form, such as in the form of a liquid, in particular a stabilized liquid, or it may be added as a substantially dry powder or granulate. Granulates may be produced, e.g., as disclosed in US Patent No. 4,106,991 and US Patent No. 4,661,452. Liquid enzyme preparations may, for instance, be stabilized by adding a sugar or sugar alcohol or lactic acid according to established procedures. Other enzyme stabilizers are well-known in the art.

The enzymes may be added to the slurry comprising the plant material in any suitable manner, such as individual components (separate or sequential addition of the enzymes) or addition of the enzymes together in one step or in one composition.

The enzymes are added to the slurry and the mixture is held at a temperature in the range of 20-60°C, so that the plant material is hydrolyzed by the enzymes to produce a hydrolyzed plant material.

In some embodiments, the slurry is held at different temperatures depending on the enzymes added to the slurry. In some embodiments, the enzymes treatment is carried out in two steps with different temperatures, referred to hereinafter as a two-step process. In the context of the present invention, a two-step process comprises holding the slurry at different temperatures

for certain time periods, wherein the temperature in a given step is set to match the enzyme(s) added to the slurry according to optimal temperature for enzymatic activity. For example, the slurry may, in one step, be heated to a temperature in the range of 70-90°C, one or more first enzyme(s), for example one or more alpha-amylases, may be added and the slurry kept at this temperature range for a time period, such as, e.g., for 30 minutes. The slurry may then be cooled to a temperature in the range of 20-60°C, such as, e.g., 50-60°C, followed by the addition of one or more second enzyme(s) different from the first enzyme(s), such as for example the protein deamidase and/or the endopeptidase having a specificity for cleaving after Arg and/or Lys, and the slurry be kept at the temperature range for, e.g., 30 minutes. Alternatively, in the context of the present invention, a process referred to hereinafter as a one-step process may be carried out, comprising the steps of providing a slurry held a temperature in the range of 5-20°C, adding to the slurry an enzyme having activity in the range of 5-65°C, heating the slurry to a temperature in the range of 65-90°C, adding a further enzyme having enzymatic activity in the temperature range 65-90°C, wherein the entire process lasts 90-180 minutes.

The process used, including temperature ranges, pH and the length of enzymatic treatment, will vary depending on the plant material and the enzymes added to the slurry. The skilled person will know how to determine the best process parameters based, e.g., on the plant material and enzymes used.

For treatment of the slurry with the specific endopeptidase and protein deamidase, the slurry is held at a temperature in the range of 20-60°C to allow for hydrolysis of the plant material. In some embodiments, the slurry is held at a temperature between 25-40°C, 30-45°C, 35-50°C, 40-55°C, 50-60°C, or 50-65°C. In further embodiments, the slurry is held at a temperature of about 20°C, 25°C, about 30°C, about 35°C, about 40°C, about 45°C, about 50°C, about 55°C, or about 60°C.

In some embodiments, the slurry having added to it the specific endopeptidase and the protein deamidase is held at 20-60°C for at least 10 minutes to allow for enzymatic hydrolysis of the plant material. In some embodiments, the slurry is held for about 10, about 15, about 20, about 25, about 30, about 60, about 120, about 180, about 240 or at least about 240 minutes to allow for enzymatic hydrolysis of the plant material. In some embodiments, the slurry is held for at least about 10, 30, or 60 minutes. In some embodiments, the slurry is held for 30 minutes. In some embodiments, the slurry is held for 60 minutes. In some embodiments, the slurry is held for 30-60 minutes.

In some embodiments, a lipid is added to the slurry of plant material to obtain a slurry with a lipid content of about 1% to about 5% (w/w), such as about 3% (w/w). The lipid may be added before, during or after the slurry of plant material is treated with enzymes. In one embodiment, the lipid is added before the enzymatic treatment of the slurry of plant material. In one embodiment, the lipid is added after the enzymatic treatment of the slurry of plant material. The lipid may be selected from rapeseed oil, flaxseed oil, safflower oil, flaxseed oil, soybean oil, olive

oil, sunflower oil, palm oil and combinations thereof. In one embodiment the lipid is rapeseed oil, sunflower oil, or a mixture thereof.

After treatment with the enzymes, the enzymes may be inactivated. The enzymes may be inactivated at any step after hydrolysis. In some embodiments, the enzymes are inactivated before or after the dairy alternative food product has been separated into solid and liquid streams. In other embodiments, the enzymes are inactivated after additional food ingredients have been added to the harvested liquid stream.

In some embodiments, the enzymes are inactivated by a heat treatment. In some embodiments, the heat treatment is 85-95°C for 5-30 minutes. In further embodiments, the heat treatment is 85-95°C for 10 minutes. In some embodiments, the heat treatment is 95°C for 5, 10, 15, 20, 25, or 30 minutes.

In some embodiments, the enzymes are inactivated by an Ultra High Temperature (UHT) treatment. The UHT treatment may be direct or indirect. In some embodiments, the UHT treatment is 135-154°C for 1-10 seconds. In further embodiments, the UHT treatment is 140-150°C for 3, 4, 5, 6, 7, 8, 9, or 10 seconds. In further embodiments, the UHT treatment is 140-145°C for 3, 4, 5, 6, 7, 8, 9, or 10 seconds. In some embodiments, the UHT treatment is 143°C for 4, 5, 6, 7, or 8 seconds.

After enzyme inactivation, the dairy alternative food product, such as the harvested liquid stream, may be cooled.

The dairy alternative food product may be separated into a solid and a liquid stream, for example by centrifugation. Centrifugation may occur in a decanter centrifuge. Following centrifugation, the liquid stream may be harvested or collected and used as a liquid dairy alternative food product. The liquid stream may still comprise some solid matter. In some embodiments, the liquid stream comprises 1-80% solids. In further embodiments, the liquid stream comprises 1-10%, 5-20%, 10-25%, 20-35%, 25-40%, 30-45%, 35-50%, 40-55%, 45-60%, 50-65%, 55-70%, 60-75%, or 65-80% solids. In some embodiments, the liquid stream comprises 10-15% solids.

In some embodiments, the liquid stream is further processed to remove water, also referred to as concentrated. Concentration also increases the relative amounts of solids in the concentrated liquid stream. In some embodiments, water removal will concentrate the products of hydrolysis, namely the released sugars. Concentration may occur by evaporation of the water in the liquid stream. In some embodiments, the concentrated liquid stream comprises 10-100% solids. In further embodiments, the concentrated liquid stream comprises 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80%-90%, or 90-100% solids. In some embodiments, water removal will increase the viscosity of the dairy alternative food product.

In some embodiments, the liquid stream is used directly as a plant-based dairy alternative food product. Additional food ingredients may be added to the liquid stream to produce the dairy alternative food product. In some embodiments, the liquid stream is derived from oat material.

For example, an oat-derived liquid stream may be formulated using for instance sodium chloride (NaCl), oil and flavouring agents. It may be homogenized. It may be UHT or ESL treated and aseptically packed. The final product may be sold as a plant-based dairy alternative beverage, such as an oat beverage.

The plant-based dairy alternative food product obtained in the methods of the present invention has improved foaming properties. In the context of the present invention, the term "foaming" or "foamability" refer to the ability of the plant-based dairy alternative food product to produce and maintain a volume of foam or volume of froth after foaming. Furthermore, the term "foaming" refers to the introduction of air into a material, such as a dairy alternative food product. For the present invention, foaming is typically achieved through whipping or beating the dairy alternative food product, sometimes with the aid of steam, such as, e.g., by using the steam wand of an espresso machine. Foaming may also be produced by whisking, shaking, or whipping. Electric milk frothers can also be used to produce the foam. The foam may be further characterized as "microfoam". Microfoam has microscopic, uniform bubbles, can be shiny, and is slightly thickened. Microfoam has a consistency which resembles wet paint. Microfoam is preferred for latte art, as the microfoam provides definition and stability to the patterns and/or etchings. An important quality of the foam is its "coherency". Foam coherence is determined by the interaction of the foam-liquid interphase, where the foam adheres to the surface of the liquid milk. No or poor foam coherency results in the foam lagging behind the liquid milk when the foamed milk is poured, so that blobs of foam will linger in the foamer and then plop out. Foam with very good coherency pours smoothly from the foamer, so that the foam and the liquid pour simultaneously or nearly simultaneously and there is no or little residual foam lagging toward the end of the pour.

The volume of foam may be determined by any method known in the art for evaluation of such. For example, one method of determining the volume of foam is to place the dairy alternative food product in a milk frother or foamer (e.g., a Nespresso Milk Frother, Nespresso USA Inc., New York, NY), and foaming the dairy alternative food product. The volume, e.g., as measured by the height of the foam or froth, and the quality of foam or froth (amount and type of bubbles, smoothness of foam/froth, etc.) may be observed visually and recorded over time. A relevant measurement for recording may be volume of milk/foam mix after foaming. The foam produced with the dairy alternative food product of the present invention collapse slowly, i.e., it is stable over time, and is largely unaffected by its addition into for example an acidic food matrix, such as coffee. Thus, the dairy alternative food product of the present invention is particularly useful in coffee applications, such as in barista applications. Dairy alternative food products suitable for barista application may be also be referred to as barista beverages. "Barista beverage" is a beverage which may comprise additional ingredients to create a beverage which produces a better foam, with high coherency for consistent pouring and the creation of latte art.

Plant-based barista beverages may have more fat and/or more protein compared to standard plant-based dairy alternative beverages.

In the context of the present invention an “acid/acidic food matrix” is a coffee or tea drink with a pH in the range of 4-6, such as a pH in the range of about 4.5-5.5, e.g., such as a pH in a range of about 4.8-5.1. For example, the food matrix may be a highly acidic coffee having pH below 5.0. Examples of highly acidic coffee includes, but are not limited to, espresso and ristretto. The food matrix may also be a tea beverage, such as tea beverage based on Chai tea blends, spiced tea blends, black teas, such as, e.g., Assam and Darjeeling black teas, green teas, earl grey, oolong tea, and rooibos tea. Such tea beverages may be used to prepare chai lattes. The dairy alternative food product of the present invention may be used for any barista application. In preferred embodiments, the acidic food matrix is a hot or heated beverage, such as a heated coffee drink or a heated tea drink.

For some applications, such as barista application in foamed coffee beverages, foaming is desirable. The invention therefore relates to the use of an endopeptidase having specificity for cleaving after Arg and/or Lys alone or in combination with a protein deamidase in the production of a plant-based dairy alternative food product for barista applications. In preferred embodiments, the endopeptidase with specificity for cleaving after Arg and/or Lys is used alone or in combination with a protein deamidase in the production of an oat drink for coffee to improve foaming properties of the oat drink. In other preferred embodiments, the endopeptidase with specificity for cleaving after Arg and/or Lys is used alone or in combination with a protein deamidase in the production of a pea, soy or almond drink for coffee to improve foaming properties of the pea, soy or almond drink. In a preferred embodiment, the endopeptidase having a specificity for cleaving after Arg and/or Lys and the protein deamidase is used in combination in the production of a dairy alternative food product to improve foaming properties.

The treatment with the endopeptidase and the protein deamidase may be performed simultaneously or sequentially. E.g., the protein deamidase may be added first to the slurry of plant material and after some time, such as, e.g., 30-60 minutes, the endopeptidase is added. Or the treatment with the endopeptidase may be performed first and then the protein deamidase is added sequentially.

Alternatively, the endopeptidase and protein deamidase may be added at the same time or essentially at the same time in the process. In a preferred embodiment, the slurry of plant material is treated with the endopeptidase and protein deamidase simultaneously.

The dairy alternative food product according to the invention comprises a mixture of polypeptide fragments each having primarily either an arginine residue or a lysine residue at the carboxyl terminus.

In some embodiments, the dairy alternative food product comprises an enzymatically deamidated plant material and a mixture of polypeptide fragments each having primarily either an arginine residue or a lysine residue at the carboxyl terminus. In the context of the present

invention, the term “enzymatically deamidated plant material” means a plant material treated with a protein deamidase for deamidation. A person skilled in the art will know of suitable analytical methods to determine enzymatic deamidation of a plant material. An example of one such method is exemplified in Example 1 disclosed herein by the measurement of free ammonium content (step 2 of the Assay procedure).

Without wishing to be bound by any particular theory, the inventors believe that the peptide fragment profile of the dairy alternative food product obtained using the methods disclosed herein contributes to the superior benefits reported herein, including, but not limited to, the improved foaming properties and organoleptic properties, such as reduced bitterness, of the final food product.

In one embodiment, the dairy alternative food product is an oat-based dairy alternative food product comprising a mixture of polypeptide fragments each having primarily either an arginine residue or a lysine residue at the carboxyl terminus. In a further embodiment, the oat-based dairy alternative food product is characterized by comprising at least one or more of the peptide fragments according to SEQ ID NOs 25, 27, 28, 29, 32 and 33 as the most abundant peptide fragment(s) determined using the method of Example 9.

In another embodiment, the dairy alternative food product is an almond-based dairy alternative food product comprising a mixture of polypeptide fragments each having primarily either an arginine residue or a lysine residue at the carboxyl terminus.

In another embodiment, the dairy alternative food product is a pea- or soy-based dairy alternative food product comprising a mixture of polypeptide fragments each having primarily either an arginine residue or a lysine residue at the carboxyl terminus.

Using the method of Example 9, the inventors have surprisingly found that by treating a dairy alternative food product, such as, e.g. an oat-based beverage as tested in Example 9, with a combination of the endopeptidase and protein deamidase according to the invention, the most abundant peptide fragments are the peptide fragments according to SEQ ID Nos: 25, 27, 28, 29, 32 and 33. Since the peptide fragments of SEQ ID Nos: 25, 27, 28, 29, 32 and 33 are only isolated from samples of the dairy alternative food product treated with the combination of the specific protease and protein deamidase, it can be concluded that a dairy alternative food product having a peptide fragment profile different from alternative dairy alternative food products prepared using methods and enzymes different from those disclosed herein, is obtained. Thus, a dairy alternative food product obtained using the methods disclosed herein and having the peptide fragment profile as described herein, can be differentiated from alternative dairy alternative food products. Such comparisons can be carried out, e.g., using a method such as that disclosed in Example 9.

Endopeptidase

In the methods of the invention, a slurry of a plant material in water is treated with an endopeptidase having specificity for cleaving after Arg and/or Lys.

An endopeptidase having specificity for cleaving after Lys and/or Arg may be defined as an endopeptidase having a preference, preferably a strong preference, for cleaving after one or both of Arg (Arginine) and Lys (Lysine) in a peptide or protein chain. The skilled person will know whether or how to determine whether a certain endopeptidase is specific or not. One example of how to determine specificity of an endopeptidase is disclosed in Example 9 herein.

In a preferred embodiment, the endopeptidase is a trypsin-like endopeptidase. Trypsin-like endopeptidase are a subfamily of serine proteases, characterized by their ability to cleave peptide bonds where the carboxyl side (C-terminal side) is a lysine or arginine residue. A trypsin-like endopeptidase is an endopeptidase having specificity for cleaving after Lys and/or Arg.

In a preferred embodiment, the trypsin-like endopeptidase preferentially cleaves peptides or proteins at the C-terminal side of arginine and lysine. This means that the endopeptidase has a higher specificity for cleaving after both of arginine and lysine than it has for cleaving after any other amino acid.

In another preferred embodiment, the trypsin-like endopeptidase preferentially cleaves peptides or proteins at the C-terminal side of arginine or lysine. This means that the endopeptidase has a higher specificity for cleaving after any of arginine or lysine than it has for cleaving after any other amino acid.

In another preferred embodiment, the trypsin-like endopeptidase preferentially cleaves peptides or proteins at the C-terminal side of arginine. This means that the endopeptidase has a higher specificity for cleaving after arginine than it has for cleaving after any other amino acid.

In another preferred embodiment, the trypsin-like endopeptidase preferentially cleaves peptides or proteins at the C-terminal side of lysine. This means that the endopeptidase has a higher specificity for cleaving after lysine than it has for cleaving after any other amino acid.

In another preferred embodiment, the trypsin-like endopeptidase specifically cleaves peptides or proteins at the C-terminal side of arginine and lysine.

In preferred embodiments, the endopeptidase is a trypsin-like endopeptidase selected from the group consisting of:

i) a polypeptide having a polypeptide sequence which is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% identical to any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12; and

ii) a variant of the polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 comprising a substitution, deletion, and/or insertion at one or more positions.

In a more preferred embodiment, the endopeptidase is a trypsin-like endopeptidase selected from the group consisting of:

i) a polypeptide having a polypeptide sequence which is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% identical to SEQ ID NO: 1; and

ii) a variant of the polypeptide of SEQ ID NO: 1 comprising a substitution, deletion, and/or insertion at one or more positions.

The trypsin-like endopeptidase is preferably derived from a strain of Fusarium, more preferably from Fusarium oxysporum.

In an embodiment, the endopeptidase is a lysine-specific endopeptidase, preferably a lysine-specific endopeptidase selected from the group consisting of:

i) a polypeptide having a polypeptide sequence which is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% identical to SEQ ID NO: 13; and

ii) a variant of the polypeptide of SEQ ID NO: 13 comprising a substitution, deletion, and/or insertion at one or more positions.

The lysine-specific endopeptidase is preferably derived from a strain of Achromobacter, more preferably from Achromobacter lyticus.

For purposes of the present invention, the sequence identity between two polypeptide sequences is determined as the output of "longest identity" 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 6.6.0 or later. The parameters used are a gap open penalty of 10, a gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. In order for the Needle program to report the longest identity, the -nobrief option must be specified in the command line. The output of Needle labelled "longest identity" is calculated as follows:

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

In the context of the present invention, a trypsin-like endopeptidase is an endopeptidase which specifically cleaves on the carboxy terminal side of arginine or lysine. I.e., it specifically cleaves on the carboxy terminal side of arginine or lysine or both. In a preferred embodiment, the trypsin-like endopeptidase specifically cleaves on the carboxy terminal side of arginine and lysine.

In the context of the present invention, a lysine-specific endopeptidase is an endopeptidase which specifically cleaves on the carboxy terminal side of lysine. A lysine-specific endopeptidase may also be termed a lysyl-specific endopeptidase.

Preferably, the trypsin-like or lysine-specific endopeptidase has a specificity for cleaving after Arg and/or Lys (whichever is the larger) which is at least 100-fold higher than its specificity for cleaving after any one of Ala, Asp, Glu, Ile, Leu, Met, Phe, Tyr or Val (whichever is the larger).

In an embodiment, the trypsin-like or lysine-specific endopeptidase has a specificity for cleaving after Arg and/or Lys (whichever is the larger) which is at least 10-fold, such as at least 20-fold or at least 50-fold, higher than its specificity for cleaving after any one of Ala, Asp, Glu, Ile, Leu, Met, Phe, Tyr or Val (whichever is the larger). In another embodiment, the trypsin-like or lysine-specific endopeptidase has a specificity for cleaving after Arg and/or Lys (whichever is the

larger) which is at least 200-fold, such as at least 500-fold or at least 1000-fold, higher than its specificity for cleaving after any one of Ala, Asp, Glu, Ile, Leu, Met, Phe, Tyr or Val (whichever is the larger).

Preferably, such determination of specificities should be performed at a pH-value where the activity of the endopeptidase is at least half of the activity of the endopeptidase at its pH optimum. Preferably, any such relative specificities are to be determined using Suc-AAP-X-pNA substrates as described in Example 3 of WO 2008/125685 which is incorporated by reference.

Preferably, a trypsin-like endopeptidase to be used in the method of the invention is classified in EC 3.4.21.4.

Preferably, a lysine-specific endopeptidase to be used in the method of the invention is classified in EC 3.4.21.50.

Any endopeptidase, in particular any specific endopeptidase, such as any trypsin-like or lysine-specific endopeptidase, can be used in the method of the invention. The origin of such endopeptidase to be used in the method of the invention is not important for a successful outcome.

The endopeptidase to be used in the method of the invention may be derived from any source. It may be derived from an animal, e.g., it may be a porcine or a bovine trypsin. Such porcine or bovine trypsin may have been extracted, e.g., from porcine or bovine pancreas, or it may have been expressed in a microorganism, such as in a filamentous fungus or yeast, or in a bacterium.

The endopeptidase to be used in the method of the invention may be derived from a microorganism, such as from a filamentous fungus or yeast, or from a bacterium.

In a preferred embodiment, the endopeptidase is derived from a fungus. In another preferred embodiment, the endopeptidase is derived from a bacterium.

The endopeptidase may be extracellular. It may have a signal sequence at its N-terminus, which is cleaved off during secretion.

The endopeptidase may be derived from any of the sources mentioned herein.

The term “derived” means in this context that the enzyme may have been isolated from an organism where it is present natively, i.e. the polypeptide sequence of the endopeptidase is identical to a native polypeptide. The term “derived” also means that the enzyme may have been produced recombinantly in a host organism, the recombinantly produced enzyme having either a polypeptide sequence which is identical to a native enzyme or having a modified polypeptide sequence, e.g. having one or more amino acids which are deleted, inserted and/or substituted, i.e. a recombinantly produced enzyme which is a mutant of a native polypeptide sequence. Within the meaning of a native enzyme are included natural variants. Furthermore, the term “derived” includes enzymes produced synthetically by, e.g., peptide synthesis. The term “derived” also encompasses enzymes which have been modified e.g. by glycosylation, phosphorylation etc., whether in vivo or in vitro. With respect to recombinantly produced enzymes the term “derived

from" refers to the identity of the enzyme and not the identity of the host organism in which it is produced recombinantly.

The endopeptidase may be obtained from a microorganism by use of any suitable technique. For instance, an enzyme preparation may be obtained by fermentation of a suitable microorganism and subsequent isolation of an endopeptidase preparation from the resulting fermented broth or microorganism by methods known in the art. The endopeptidase may also be obtained by use of recombinant DNA techniques. Such method normally comprises cultivation of a host cell transformed with a recombinant DNA vector comprising a DNA sequence encoding the endopeptidase and the DNA sequence being operationally linked with an appropriate expression signal such that it is capable of expressing the enzyme in a culture medium under conditions per-mitting the expression of the enzyme and recovering the enzyme from the culture. The DNA sequence may also be incorporated into the genome of the host cell. The DNA sequence may be of genomic, cDNA or synthetic origin or any combinations of these, and may be isolated or synthesized in accordance with methods known in the art.

The endopeptidase may be purified. The term "purified" as used herein covers endopeptidase enzyme protein essentially free from insoluble components from the production organism. The term "purified" also covers endopeptidase enzyme protein essentially free from insoluble com-ponents from the native organism from which it is obtained. Preferably, it is also separated from some of the soluble components of the organism and culture medium from which it is derived. More preferably, it is separated by one or more of the unit operations: filtration, precipitation, or chromatography.

Preferably, the endopeptidase is purified from its production organism. More preferably, the endopeptidase is purified from its production organism meaning that the endopeptidase preparation does not comprise living production organism cells.

Accordingly, the endopeptidase may be purified, viz. only minor amounts of other proteins being present. The expression "other proteins" relate in particular to other enzymes. The term "purified" as used herein also refers to removal of other components, particularly other proteins and most particularly other enzymes present in the cell of origin of the endopeptidase. The endopeptidase may be "substantially pure", i.e., free from other components from the organism in which it is produced, i.e., e.g., a host organism for recombinantly produced endopeptidase. Preferably, the endopeptidase is an at least 40% (w/w) pure enzyme protein preparation, more preferably at least 50%, 60%, 70%, 80% or even at least 90% pure.

The term endopeptidase includes whatever auxiliary compounds may be necessary for the enzyme's catalytic activity, such as, e.g., an appropriate acceptor or cofactor, which may or may not be naturally present in the reaction system.

The endopeptidase may be in any form suited for the use in question, such as, e.g., in the form of a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a protected enzyme.

The enzymes dosage will depend on parameters such as the temperature, the incubation time and the dairy alternative recipe. The skilled person will know how to determine the optimal enzyme dosage.

A trypsin-like or lysine-specific endopeptidase to be used in the method of the invention may be added at a concentration of 10-2000 KMTU/kg substrate protein, preferably 25-1500 KMTU/kg substrate protein, more preferably 35-1000 KMTU/kg substrate protein, even more preferably 40-750 KMTU/kg substrate protein.

Trypsin-like and lysine-specific endopeptidases hydrolyse the chromophoric substrates Ac-Arg-p-nitro-anilide (Ac-Arg-pNA) and/or Ac-Lys-p-nitro-anilide (Ac-Arg-pNA). The liberated pNA produces an absorption increase at 405 nm, which is proportional to enzyme activity. One KMTU is equivalent to the amount of enzyme that produces 1 micromole p-nitroaniline per minute, when Ac-Arg-pNA or Ac-Lys-pNA is incubated with the enzyme at pH 8.0 at 37°C. The activity may be determined relative to a standard of declared strength.

Protein deamidase

In the methods of the invention, a slurry of a plant material in water is treated with a protein deamidase.

In the present invention, a protein deamidase refers to an enzyme having an effect of directly acting on an amide group of a side chain of an amino acid that constitutes a protein to cause deamidation and release ammonia without cleaving a peptide bond of the protein and crosslinking proteins.

The term “deamidase” means a protein-glutamine glutaminase (also known as glutaminylpeptide glutaminase) activity, as described in EC 3.5.1.44, which catalyses the hydrolysis of the gamma-amide of glutamine substituted at the carboxyl position or both the alpha-amino and carboxyl positions, e.g., L-glutaminylglycine and L-phenylalanyl-L-glutaminylglycine. Thus, deamidases can deamidate glutamine residues in proteins to glutamate residues and are also referred to as protein glutamine deamidase. Deamidases comprise a Cys-His-Asp catalytic triad (e.g., Cys-156, His-197, and Asp-217, as shown in Hashizume et al. “Crystal structures of protein glutaminase and its pro forms converted into enzyme-substrate complex”, Journal of Biological Chemistry, vol. 286, no. 44, pp. 38691–38702) and belong to the InterPro entry IPR041325.

Deamidase may also include a protein asparaginase that directly acts on an amide group of a side chain of an asparagine residue contained in a protein to release ammonia and thus converts the asparagine residue into an aspartate residue. In the present invention, as a protein deamidase, any one of the protein glutaminase and the protein asparaginase can be used, or both can be used in combination. One example of the protein deamidase used in the present invention is a protein glutaminase.

A protein deamidase 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 embodiment, the polypeptide obtained from a given source is secreted extracellularly.

The types or origins of the protein deamidase used in the present invention are not particularly limited. Examples of the protein deamidase includes protein deamidases derived from Chryseobacterium genus, Flavobacterium genus, Empedobacter genus, Sphingobacterium genus, Aureobacterium genus, or Myroides genus.

In some embodiments, the protein deamidase may be derived from Chryseobacterium genus, such as Chryseobacterium viscerum, C. gambrini, C. culicis, C. defluvii, or C. proteolyticum. In some embodiments, the deamidase in the methods of the invention is derived from or obtained from Chryseobacterium viscerum.

EP1839491 discloses cloning of a protein glutaminase from Chryseobacterium proteolyticum expressed in Corynebacterium glutamicum. Deamidases are also commercially available, e.g., protein glutaminases derived from Chryseobacterium genus, for example, "Amano PG500” (manufactured by Amano Enzyme Inc.).

The term “derived” means in this context that the enzyme may have been isolated from an organism where it is present natively, i.e. the polypeptide sequence of the protein deamidase is identical to a native polypeptide. The term “derived” also means that the enzyme may have been produced recombinantly in a host organism, the recombinantly produced enzyme having either a polypeptide sequence which is identical to a native enzyme or having a modified polypeptide sequence, e.g. having one or more amino acids which are deleted, inserted and/or substituted, i.e. a recombinantly produced enzyme which is a mutant of a native polypeptide sequence. Within the meaning of a native enzyme are included natural variants. Furthermore, the term “derived” includes enzymes produced synthetically by, e.g., peptide synthesis. The term “derived” also encompasses enzymes which have been modified e.g. by glycosylation, phosphorylation etc., whether in vivo or in vitro. With respect to recombinantly produced enzymes the term “derived from” refers to the identity of the enzyme and not the identity of the host organism in which it is produced recombinantly.

The protein deamidase may be obtained from a microorganism by use of any suitable technique. For instance, an enzyme preparation may be obtained by fermentation of a suitable microorganism and subsequent isolation of a protein deamidase preparation from the resulting fermented broth or microorganism by methods known in the art. The protein deamidase may also be obtained by use of recombinant DNA techniques. Such method normally comprises cultivation of a host cell transformed with a recombinant DNA vector comprising a DNA sequence encoding the protein deamidase and the DNA sequence being operationally linked with an appropriate

expression signal such that it is capable of expressing the enzyme in a culture medium under conditions per-mitting the expression of the enzyme and recovering the enzyme from the culture. The DNA sequence may also be incorporated into the genome of the host cell. The DNA sequence may be of genomic, cDNA or synthetic origin or any combinations of these, and may be isolated or synthesized in accordance with methods known in the art.

The protein deamidase may be purified. The term "purified" as used herein covers protein deamidase enzyme protein essentially free from insoluble components from the production organism. The term "purified" also covers protein deamidase enzyme protein essentially free from insoluble components from the native organism from which it is obtained. Preferably, it is also separated from some of the soluble components of the organism and culture medium from which it is derived. More preferably, it is separated by one or more of the unit operations: filtration, precipitation, or chromatography.

Preferably, the protein deamidase is purified from its production organism. More preferably, the protein deamidase is purified from its production organism meaning that the protein deamidase preparation does not comprise living production organism cells.

Accordingly, the protein deamidase may be purified, viz. only minor amounts of other proteins being present. The expression “other proteins” relate in particular to other enzymes. The term "purified" as used herein also refers to removal of other components, particularly other proteins and most particularly other enzymes present in the cell of origin of the protein deamidase. The protein deamidase may be "substantially pure", i.e., free from other components from the organism in which it is produced, i.e., e.g., a host organism for recombinantly produced protein deamidase. Preferably, the protein deamidase is an at least 40% (w/w) pure enzyme protein preparation, more preferably at least 50%, 60%, 70%, 80% or even at least 90% pure.

The term protein deamidase includes whatever auxiliary compounds may be necessary for the enzyme's catalytic activity, such as, e.g., an appropriate acceptor or cofactor, which may or may not be naturally present in the reaction system.

The protein deamidase may be in any form suited for the use in question, such as, e.g., in the form of a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a protected enzyme.

For example, protein deamidases can be obtained from a culture broth of the above-described microorganisms.

Protein deamidases are produced by microbial cells in an inactive proform, which comprises a propeptide domain tightly bound to a deamidase domain. The proform is expressed as a fusion protein, which has reduced deamidase activity to protect the viability of the host cell. In nature, the fusion protein is post-processed to remove the propeptide and release the active deamidase outside of the host cell. However, in recombinant expression systems, the fusion protein is secreted outside of the host cell as an inactive proform comprising the propeptide. The propeptide may then be enzymatically cleaved off to separate it from the mature deamidase. The

protein deamidases of the methods and compositions of the present invention are mature deamidases where the propeptide has been removed. In some embodiments, the propeptide was cleaved enzymatically by an endopeptidase. In some embodiments, the propeptide may still be present in the composition comprising the mature deamidase.

The recombinant, mature protein deamidases used in the methods of the invention comprises the polypeptide of SEQ ID NOs: 15, 17, 19, 21, and 23. Each mature protein deamidase is derived from a deamidase proform polypeptide, which comprises the polypeptide of SEQ ID NO: 14, 16, 18, 20, and 22, respectively. The proform polypeptide comprises a propeptide at the N-terminal end, fused to a deamidase which is the same as that of the polypeptide of SEQ ID NOs: 15, 17, 19, 21, or 23. The propeptide may be enzymatically cleaved from the proform polypeptide to release the mature deamidase. Naturally occurring propeptide sequences are provided in the proform polypeptide.

The methods and compositions of the invention include a mature deamidase and optionally a second polypeptide which is derived from the propeptide of a deamidase. The second polypeptides described herein are mutated variants of the naturally occurring propeptides. These variant propeptide sequences have been found to bind less strongly to their corresponding deamidase, so that they are more easily enzymatically cleaved off after recombinant expression and secretion from of the host cell. The polypeptides of SEQ ID NOs: 14 to 23 are derived from Chryseobacterium spp. and are described in PCT application PCT/EP2023/055936; filed March 8, 2023, herein incorporated by reference.

After expression of the proform polypeptide in a recombinant expression system, a site-specific endopeptidase is used to cleave off the propeptide, leaving an active, mature deamidase. In some embodiments, the cleaved propeptide is not purified away from the mature deamidase. Therefore, the propeptide may be present in the composition with the mature deamidase.

According to a preferred embodiment the protein deamidase applied in the process of the invention is derived from or obtained from a Chryseobacterium species, e.g., Chryseobacterium proteolyticus or Chryseobacterium viscerum.

In the context of the present invention, the term “mature polypeptide” means a polypeptide in its mature form following N terminal processing (e.g., removal of signal peptide). A "signal peptide" is a sequence of amino acids attached to the N-terminal portion of a protein, which facilitates the secretion of the protein outside the cell. The mature form of an extracellular protein lacks the signal peptide, which is cleaved off during the secretion process.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NO: 15.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NO: 17.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NO: 19.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NO: 21.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NO: 23.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to a mature polypeptide of SEQ ID NO: 14.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to a mature polypeptide of SEQ ID NO: 16.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to a mature polypeptide of SEQ ID NO: 18.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to a mature polypeptide of SEQ ID NO: 20.

In one embodiment the deamidase is selected from a polypeptide having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to a mature polypeptide of SEQ ID NO: 22.

A protein deamidase to be used in the methods of the invention may be added at a concentration of 0.01-20 IPA(U)/g substrate protein, such as 0.1-12 IPA(U)/g substrate protein, 0.5-7 IPA(U)/g substrate protein.

Deamidase (protein glutaminase) activity was measured using the assay described in Example 1. The activity assay consists of two separate de-coupled parts: (1) an enzymatic step wherein ammonia is formed by the catalytic action of the protein deamidase; and (2) a non-enzymatic detection step, wherein the ammonia formed in step (1) is derivatized to a blue indophenol compound with an absorption maximum at 630 nm. The amount of enzyme producing 1 µmol ammonia per minute at 37°C is defined as 1 unit (given in Indophenol Assay Unit: IPA(U)). The activity may be determined relative to a standard of declared strength.

The invention is further defined by the following numbered embodiments:

Embodiment 1. Method for obtaining a dairy alternative food product, the method comprising the steps of:

(a) obtaining a slurry of a plant material in water; and

(b) treating the slurry of step (a) with an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase to obtain the dairy alternative food product.

Embodiment 2. Method of embodiment 1, wherein the endopeptidase and protein deamidase is added simultaneously or sequentially.

Embodiment 3. Method of any one of embodiments 1 or 2, further comprising treating the slurry of step (a) with one or more hydrolyzing enzymes selected from the group of pectinases, hemicellulases, xylanases, beta-glucanases, mannanases, glucanases, glucoamylases, iso-amylases, alpha-amylases and beta-amylases, and mixtures thereof.

Embodiment 4. Method of embodiment 3, wherein the hydrolyzing enzyme is an alpha-amylase, a beta-glucanase, a glucoamylase or any combination thereof.

Embodiment 5. Method of any of the preceding embodiments, wherein the treatment of step (b) is carried out at a temperature in the range of 20-60°C.

Embodiment 6. Method of any of the preceding embodiments, wherein the treatment of step (b) is carried out at a temperature in the range of 50-60°C.

Embodiment 7. Method of any of the preceding embodiments, wherein the treatment of step (b) last for at least 10 minutes, at least 30 minutes or at least 60 minutes.

Embodiment 8. Method of any of the preceding embodiments, further comprising the addition of a lipid during step (a) or step (b), or after step (b), to obtain a slurry or a dairy alternative food product having a lipid content of 1-5% (w/w).

Embodiment 9. Method of embodiment 8, wherein the lipid content is 3% (w/w).

Embodiment 10. Method of any of embodiments 8 or 9, wherein the lipid is an oil.

Embodiment 11. Method of embodiment 10, wherein the oil is a plant oil selected from the group of rapeseed oil, sunflower oil, or a mixture thereof.

Embodiment 12. Method of any of the preceding embodiments, further comprising the steps of:

(c) separating the dairy alternative food product into solid and liquid streams;

(d) harvesting the liquid stream as a liquid dairy alternative food product; and

(e) optionally inactivating the enzymes.

Embodiment 13. Method of embodiment 12, wherein the enzymes are inactivated by a heat treatment, such as an Ultra-High Temperature (UHT) treatment.

Embodiment 14. Method of any of the preceding embodiments, wherein the plant material has a protein content of 5-25% (w/w).

Embodiment 15. Method of any of the preceding embodiments, wherein the plant material has a protein content of 5-20% (w/w), more preferred of 10-20% (w/w), most preferred of 10-15% (w/w).

Embodiment 16. Method of any embodiments 1-13, wherein the plant material has a protein content in the range of 75-85% (w/w).

Embodiment 17. Method of any of the preceding embodiments, wherein the plant material is derived from almond, soy, cashew, macadamia, coconut, pea, bean, rice, quinoa, flax, hemp or oat.

Embodiment 18. Method of any of the preceding embodiments, wherein the plant material is derived from soy, cashew, macadamia, coconut, pea, bean, rice, quinoa, flax, hemp or oat.

Embodiment 19. Method of any of the preceding embodiments, wherein the plant material is derived from almond, oat, pea or soy, preferably from almond, oat and pea.

Embodiment 20. Method of any of the preceding embodiments, wherein the plant material is an oat material.

Embodiment 21. Method of any of the preceding embodiments, wherein the dairy alternative food product is an almond drink, a soy drink, a cashew drink, a macadamia drink, a coconut drink, a pea drink, a rice drink, a quinoa drink, a flax drink, a hemp drink or an oat drink.

Embodiment 22. Method of any of the preceding embodiments, wherein the dairy alternative food product is a soy drink, a cashew drink, a macadamia drink, a coconut drink, a pea drink, a rice drink, a quinoa drink, a flax drink, a hemp drink or an oat drink.

Embodiment 23. Method of any of the preceding embodiments, wherein the dairy alternative food product is an almond drink, a soy drink, a pea drink, or an oat drink, preferably an almond drink, a pea drink or an oat drink.

Embodiment 24. Method of any of the preceding embodiments, wherein the dairy alternative food product is an oat beverage, such as an oat beverage for use in coffee.

Embodiment 25. Method of any of the preceding embodiments, wherein one or more additional food ingredients are added to the dairy alternative food product.

Embodiment 26. Method of embodiment 25, wherein the additional food ingredient is selected from the group of lipids, sugars, proteins, various forms of synthetic amino acids, dietary fibres, salts, minerals, flavouring agents, vitamins, and any combination thereof.

Embodiment 27. Method of any of the preceding embodiments, wherein the dairy alternative food product is for use in an acidic food matrix.

Embodiment 28. Method of embodiment 27, wherein the acidic food matrix is a coffee drink or a tea drink.

Embodiment 29. Method of any of embodiments 27 or 28, wherein the acidic food matrix is coffee.

Embodiment 30. Method of any of embodiments 27-29, wherein the acidic food matrix is a heated coffee drink.

Embodiment 31. Method of any of the preceding embodiments, wherein the endopeptidase is a trypsin-like endopeptidase or a lysine-specific endopeptidase.

Embodiment 32. Method of any of the preceding embodiments, wherein the endopeptidase is a trypsin-like endopeptidase, preferably a microbially derived trypsin-like endopeptidase.

Embodiment 33. Method of any of embodiments 31-32, wherein the trypsin-like endopeptidase is derived from a strain of Fusarium, preferably from Fusarium oxysporum.

Embodiment 34. Method of any of embodiments 31-33, wherein the trypsin-like endopeptidase is selected from the group consisting of:

i) a polypeptide comprising a polypeptide sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12; and

ii) a variant of the polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 comprising a substitution, deletion, and/or insertion at one or more positions.

Embodiment 35. Method of any of embodiment 31-34, wherein the trypsin-like endopeptidase is selected from the group consisting of:

i) a polypeptide comprising a polypeptide sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NO: 1; and

ii) a variant of the polypeptide of SEQ ID NO: 1 comprising a substitution, deletion, and/or insertion at one or more positions.

Embodiment 36. Method of any of embodiments 1-31, wherein the endopeptidase is a lysine-specific endopeptidase, preferably a microbially derived lysine-specific endopeptidase.

Embodiment 37. Method of embodiment 36, wherein the lysine-specific endopeptidase is derived from a strain of Achromobacter, preferably from Achromobacter lyticus.

Embodiment 38. Method of any of embodiments 36-37, wherein the lysine-specific endopeptidase is selected from the group consisting of:

i) a polypeptide comprising a polypeptide sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NO: 13; and

ii) a variant of the polypeptide of SEQ ID NO: 13 comprising a substitution, deletion, and/or insertion at one or more positions.

Embodiment 39. Method of any of the preceding embodiments, wherein the protein deamidase is derived from or obtained from a Chryseobacterium species, more preferably from Chryseobacterium proteolyticus or Chryseobacterium viscerum.

Embodiment 40. Method of any of the preceding embodiments, wherein the protein deamidase comprises a polypeptide sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NOs: 15, 17, 19, 21, or 23.

Embodiment 41. Method of any of the preceding embodiments, wherein the protein deamidase comprises the polypeptide sequence of SEQ ID NOs: 15, 17, 19, 21, or 23.

Embodiment 42. Method of any of the preceding embodiments, wherein the dairy alternative food product has one or more improved properties compared to a dairy alternative

food product obtained using the same method but without addition of an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase, wherein the one or more improved properties are selected from:

i) an improved foaming volume and/or foam height;

ii) an improved foam stability;

iii) an improved foam coherency and/or a smoother texture of the foam;

iv) reduced off-notes, such as reduced bitterness; and

v) improved stability and/or reduced feathering when mixed with an acidic food matrix, such as a coffee drink or a tea drink.

Embodiment 43. Method for obtaining an oat-based dairy alternative food product, the method comprising the steps of:

(a) obtaining a slurry of an oat material in water; and

(b) treating the slurry of step (a) with an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase to obtain the oat-based dairy alternative food product.

Embodiment 44. Method of embodiment 43, further comprising the steps of:

(c) separating the oat-based dairy alternative food product into solid and liquid streams;

(d) harvesting the liquid stream; and

(e) optionally inactivating the enzymes.

Embodiment 45. Method of any of embodiments 43 or 44, further comprising treating the slurry of step (a) with an alpha-amylase and/or a beta-glucanase.

Embodiment 46. Method for obtaining a legume-based dairy alternative food product, the method comprising the steps of:

(a) obtaining a slurry of a leguminous material in water; and

(b) treating the slurry of step (a) with an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase to obtain the legume-based dairy alternative food product.

Embodiment 47. Method of embodiment 46, wherein the leguminous material is obtained or derived from pea, soy, or a combination thereof.

Embodiment 48. Method of any of embodiments 46-47, wherein the leguminous material is a protein isolate or a protein concentrate.

Embodiment 49. Method of any of embodiments 46-48, wherein the leguminous material is characterized by having a low or medium initial protein solubility as determined using a method as exemplified in Example 10.

Embodiment 50. Method of any of embodiments 46-49, wherein the legume-based dairy alternative food product has one or more improved properties compared to a legume-based dairy alternative food product obtained using the same method but without addition of an

endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase, wherein the one or more improved properties are selected from:

i) an improved foaming volume and/or foam height;

ii) an improved foam stability;

iii) an improved foam coherency and/or a smoother texture of the foam;

iv) reduced off-notes, such as reduced bitterness; and

v) improved stability and/or reduced feathering when mixed with an acidic food matrix, such as a coffee drink or a tea drink.

Embodiment 51. Method of any of embodiments 46-50, wherein the dairy alternative food product is a pea-based dairy alternative food product, preferably wherein the leguminous material is a pea protein concentrate or a pea protein isolate.

Embodiment 52. Method for obtaining an almond-based dairy alternative food product, the method comprising the steps of:

(a) obtaining a slurry of an almond material in water; and

(b) treating the slurry of step (a) with an endopeptidase having a specificity for cleaving after Arg and/or Lys to obtain the almond-based dairy alternative food product.

Embodiment 53. Method for obtaining an almond-based dairy alternative food product, the method comprising the steps of:

(a) obtaining a slurry of an almond material in water; and

(b) treating the slurry of step (a) with an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase to obtain the almond-based dairy alternative food product.

Embodiment 54. Use of an endopeptidase having a specificity for cleaving after Arg and/or Lys in the production of a dairy alternative food product to improve one or more properties of the dairy alternative food product selected from:

i) an improved foaming volume and/or foam height;

ii) an improved foam stability;

iii) an improved foam coherency and/or a smoother texture of the foam;

iv) reduced off-notes, such as reduced bitterness; and

v) improved stability and/or reduced feathering when mixed with an acidic food matrix, such as a coffee drink or a tea drink.

Embodiment 55. Use of an endopeptidase having a specificity for cleaving after Arg and/or Lys in the production of a dairy alternative food product to improve foaming properties.

Embodiment 56. Use according to any of embodiments 54-55, wherein the dairy alternative food product is an almond-, oat-, or legume-based dairy alternative food product, preferably an almond-, oat-, soy- or pea-based dairy alternative food product.

Embodiment 57. Use according to any of embodiments 54-56, wherein the dairy alternative food product is an almond-, oat- or pea-based dairy alternative food product.

Embodiment 58. Use according to any of embodiments 54-57, wherein the dairy alternative food product is an oat-based dairy alternative food product.

Embodiment 59. Use according to any of embodiments 54-58, wherein the endopeptidase having specificity for cleaving after Arg and/or Lys is a trypsin-like or a lysine-specific endopeptidase, preferably a trypsin-like endopeptidase.

Embodiment 60. Use of an endopeptidase having a specificity for cleaving after Arg and/or Lys in the production of a pea-based dairy alternative food product to improve foaming volume, to improve foaming stability, and/or to reduce off-notes, such as reduce bitterness, of the pea-based dairy alternative food product.

Embodiment 61. Use according to embodiment 60, wherein the endopeptidase having a specificity for cleaving after Arg and/or Lys is a trypsin-like endopeptidase.

Embodiment 62. Use of an endopeptidase having a specificity for cleaving after Arg and/or Lys in the production of an almond-based dairy alternative food product to improve foaming volume, to improve foaming stability, and/or to improve stability and/or reduce feathering when mixing the almond-based dairy alternative food product with an acidic food matrix, such as a coffee drink or a tea drink.

Embodiment 63. Use of an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase in the production of a dairy alternative food product to improve one or more properties of the dairy alternative food product selected from:

i) an improved foaming volume and/or foam height;

ii) an improved foam stability;

iii) an improved foam coherency and/or a smoother texture of the foam;

iv) reduced off-notes, such as reduced bitterness; and

v) improved stability and/or reduced feathering when mixed with an acidic food matrix, such as a coffee drink or a tea drink.

Embodiment 64. Use of an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase in the production of a dairy alternative food product to improve foaming properties.

Embodiment 65. Use according to any of embodiments 63-64, wherein the dairy alternative food product is an almond-, oat-, or legume-based dairy alternative food product, preferably an almond-, oat-, pea- or soy-based dairy alternative food product.

Embodiment 66. Use according to any of embodiments 63-65, wherein the dairy alternative food product is an oat beverage, such as an oat beverage for use in coffee.

Embodiment 67. Use of an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase in the production of a pea-based dairy alternative food product to improve foaming volume, to improve foaming stability, and/or to reduce off-notes, such as reduce bitterness, of the pea-based dairy alternative food product.

Embodiment 68. Dairy alternative food product comprising a mixture of polypeptide fragments each having primarily either an arginine residue or a lysine residue at the carboxyl terminus.

Embodiment 69. Dairy alternative food product of embodiment 68, further comprising enzymatically deamidated plant material.

Embodiment 70. Dairy alternative food product of any of embodiments 68-69, wherein the dairy alternative food product is a plant-based beverage, such as a plant-based beverage for barista application.

Embodiment 71. Dairy alternative food product of any of embodiments 68-70, wherein the dairy alternative food product has one or more improved properties compared to a dairy alternative food product obtained using the same method as embodied in any of embodiments 1-42 but without addition of the endopeptidase having a specificity for cleaving after Arg and/or Lys and the protein deamidase, wherein the one or more improved properties are selected from:

i) an improved foaming volume and/or foam height;

ii) an improved foam stability;

iii) an improved foam coherency and/or a smoother texture of the foam;

iv) reduced off-notes, such as reduced bitterness; and

v) improved stability and/or reduced feathering when mixed with an acidic food matrix, such as a coffee drink or a tea drink.

Embodiment 72. Dairy alternative food product of any of embodiments 68-71, wherein the dairy alternative food product is an almond-, oat-, or legume-based dairy alternative food product, preferably an almond-, oat, pea-, or soy-based dairy alternative food product.

Embodiment 73. Dairy alternative food product of any of embodiments 68-72, wherein the dairy alternative food product is an oat-based dairy alternative food product.

Embodiment 74. Dairy alternative food product of any of the embodiments 68-73, further comprising a protein deamidase comprising a polypeptide sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NOs: 15, 17, 19, 21, or 23.

Embodiment 75. Oat-based dairy alternative food product characterized by comprising one or more of the peptide fragments of SEQ ID NOs 25, 27, 28, 29, 32 and 33 as the most abundant peptide fragment determined using the method of Example 9.

Embodiment 76. Oat-based dairy alternative food product of embodiment 75, further comprising enzymatically deamidated oat material.

Embodiment 77. Oat-based dairy alternative food product of any of embodiments 75-76, further comprising a protein deamidase comprising a polypeptide sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to SEQ ID NOs: 15, 17, 19, 21, or 23.

Embodiment 78. Legume-based dairy alternative food product comprising a mixture of polypeptide fragments each having primarily either an arginine residue or a lysine residue at the carboxyl terminus.

Embodiment 79. Legume-based dairy alternative food product of embodiment 78, further comprising enzymatically deamidated leguminous material.

Embodiment 80. Legume-based dairy alternative food product of embodiment 79, characterized by having higher soluble protein, reduced bitterness, improved foaming volume and/or improved foam stability compared to a legume-based dairy alternative food product obtained using a method as embodied in any of embodiments 1-42 but without addition of the endopeptidase having a specificity for cleaving after Arg and/or Lys and the protein deamidase.

Embodiment 81. Legume-based dairy alternative food product of any of embodiments 79-80, wherein the legume-based dairy alternative food product is a pea- or soy-based dairy alternative food product.

Embodiment 82. Almond-based dairy alternative food product comprising a mixture of polypeptide fragments each having primarily either an arginine residue or a lysine residue at the carboxyl terminus.

Embodiment 83. Almond-based dairy alternative food product of embodiment 82, characterized by having an improved foaming volume, improved foaming stability, and/or improved stability and/or reduced feathering when mixed with an acidic food matrix, such as a coffee drink or a tea drink, compared to an almond-based dairy alternative food product obtained using a method as embodied in embodiment 52 but without addition of the endopeptidase having a specificity for cleaving after Arg and/or Lys.

Embodiment 84. Almond-based dairy alternative food product of any of embodiments 82-83, further comprising enzymatically deamidated almond material.

Embodiment 85. Almond-based dairy alternative food product of embodiment 84, wherein the enzymatically deamidated almond material is obtained using the method as embodiment in embodiment 53.

Embodiment 86. Oat-based dairy alternative food product obtainable by the method of any of embodiments 1-45.

Embodiment 87. Legume-based dairy alternative food product obtainable by the method of any of embodiments 1-42 and 46-51.

Embodiment 88. Pea-based dairy alternative food product obtainable by the method of any of embodiments 1-42 and 46-51.

Embodiment 89. Soy-based dairy alternative food product obtainable by the method of any of embodiments 1-42 and 46-50.

Embodiment 90. Almond-based dairy alternative food product obtainable by the method of any of embodiments 1-42 and 52-53.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention as well as combinations of one or more of the embodiments.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties. The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

EXAMPLES

Materials

Enzymes

The following enzymes are used throughout the examples:

Protease TL: Trypsin-like endopeptidase from Fusarium oxysporum having the sequence of SEQ ID NO: 1.

AC3: Trypsin-like endopeptidase from Achromobacter lyticus having the sequence of SEQ ID NO: 13.

Porcine trypsin: Trypsin-like Endopeptidase from Porcinus having the sequence of SEQ ID NO: 12.

Protease CTL: Chymotrypsin-like protease from Nocardiopsis sp. NRRL 18262 previously disclosed as SEQ ID NO: 8 in WO 2010/112546 A1.

Protein deamidase: Protein glutaminase derived from Chryseobacterium viscerum (the strain has formerly been referred to as Chryseobacterium sp-62563) having the mature polypeptide sequence shown as SEQ ID NO: 15. Cleavage of the propeptide was achieved by treating the deamidase of SEQ ID NO: 14 with a site-specific endopeptidase. The site-specific endopeptidase used was a glutamyl endopeptidase from Bacillus licheniformis. The resulting active deamidase after maturation was the polypeptide shown in SEQ ID NO: 15. The Chryseobacterium viscerum strain was isolated from a soil sample collected in Sibhult, Sweden in September 2013.

Example 1: Protein deamidase activity assay

The protein deamidase activity assay consists of two separate de-coupled parts:

1) An enzymatic step wherein ammonia is formed by the catalytic action of the protein deamidase; and

2) A non-enzymatic detection step wherein the ammonia formed in step (1) is derivatized to a blue indophenol compound with an absorption maximum at 630 nm.

In step (1), the ammonia is developed by the deamidating action of the protein deamidase. In step (2), the generated ammonia reacts with phenol to form dioxyphenylamine under alkaline conditions. The reaction is catalysed by sodium pentacyanonitrosylferrate(III) (sodium nitroprusside). "Color Reagent solution A" contains phenol and sodium nitroprusside. "Color Reagent Solution B" provides alkaline reaction conditions. The intermediate is then oxidized by addition of sodium hypochlorite ("Color Reagent Solution C") to form indophenol blue. This compound absorbs visible light at 630 nm. The enzyme activity is then calculated using a standard curve.

Assay Procedure:

Step (1) Enzymatic step with ammonia formation

Reagents:

Assay dilution solution: 0.2 M Na-phosphate buffer, 0.01% Triton X-100, pH 6.5.

Assay buffer: Same as above. Used to prepare stock solution and diluted sample of protein deamidase (referred to in the following as "enzyme").

Substrate solution: 30 mM Z-Gln-Gly (Merck C6154-1G) in assay dilution solution (check pH after dissolution).

Stop solution: 0.4M TCA.

Standard: NH_4Cl (Ammonium Standard for IC, Merck 59755-100ML, 1000 mg/L NH_4^+ in water) diluted in assay dilution solution (see also "Standard curve" section).

Dissolve/dilute enzyme product in assay buffer and prepare suitable dilution resulting in a linear assay response.

Incubation:

1. Add 10 µL of diluted enzyme samples in triplicates to the wells of a 96-well microtiter plate (MTP).
2. Add 100 µL of substrate solution to each well.
3. For blank samples add 100 µL 0.4M TCA solution.
4. Seal the plate using transparent plate sealer.
5. Incubate the plate for 10 minutes at 37°C, 500 rpm, on a thermomixer equipped with a lid heating function.
6. To stop the reaction, carefully add 100 µL 0.4M TCA solution (except for the blank samples, which already contain TCA).

Total reaction volume: 210 µL

Step (2) Ammonia detection step

Reagents:

Color reagent A: 4% (w/v) Phenol, 0.015% (w/v) sodium pentacyanonitrosylferrate(III) dihydrate (sodium nitroprusside) ($Na_2[Fe(CN)_5NO]\cdot 2H_2O$).

Color reagent B: 5% (w/v) Potassium hydroxide.

Color reagent C: 28% (w/v) Potassium carbonate, 6% (v/v) sodium hypo-chlorite (Sigma-Aldrich 239305-25ml, < 5% available Cl_2).

Incubation:

1. Transfer 15 µL from each well from step (1) into a new 96-well MTP.
2. Transfer 45 µL Milli-Q water to each well.
3. To each well, add 30µL of color reagent B (on lab table, shake gently by hand to mix).
4. To each well, add 60µL of color reagent A (on lab table, shake gently by hand to mix).
5. To each well, add 60µL of color reagent C (on lab table, shake gently by hand to mix).
6. Color development: Carefully seal the plate and leave it on lab table for 30 minutes.
7. Carefully transfer the MTP to a plate reader and measure absorbance at 630 nm.

Total reaction volume: 210 µL

Standard curve:

Standard stock solution: 1000 mg NH_4^+/L.

The standard curve is prepared by adding dilutions of the ammonium standard in the assay dilution buffer in the ammonia detection step. That is, mixing 15 µL diluted ammonia standard with 45 µL water and then add the color reagents in the order given above; B, A, and C.

The amount of enzyme producing 1 µmol ammonia per minute at 37°C is defined as 1 unit (Indophenol Assay Unit; IPA(U)):

$$\frac{IPA(U)}{mL} = C_{NH4^+}(\frac{mg}{L}) * \frac{1\ mol}{18.04\ g} * \frac{V_{reaction}\ (\mu L)}{V_{enzyme}\ (\mu L)} * \frac{1}{10\ min.} * \frac{V_{NH3\ detection}\ (\mu L)}{V_{NH3\ generated}\ (\mu L)} * DF$$

where

$$C_{NH4^+}(\frac{mg}{L}) = C_{NH4+}^{predil.}\left(\frac{mg}{L}\right) * \frac{V_{NH4+,predil.}\ (\mu L)}{V_{NH3\ detection}(\mu L)}$$

which can be shortened to

$$\frac{IPA(U)}{mL} = C_{NH4+}^{predil.}(\frac{mg}{L}) * \frac{21}{180.04} * DF\ \frac{\mu mol}{mL * min.}$$

where

- C_{NH4+} is the ammonia concentration in the reaction solution derived from the ammonium standard curve (i.e., taking into account the dilution of the prediluted ammonium standard solution in the ammonia derivatization step).

- 18.04 is the molecular mass of ammonium used for the standard solution.
- $V_{reaction}$ is the reaction volume in the well when ammonia is generated (210 µL).
- V_{enzyme} is the volume of enzyme solution added to the well when ammonia is generated (10 µL).
- $V_{NH3}\ detection$ is the reaction volume in the well when ammonia is detected (210 µL).

Example 2: Testing of protein deamidase and protease TL in production of oat-based beverages

15% w/w oat flour (13% protein) was mixed with deionized water and placed in a Vorwerk Thermomixer with stirring. Total amount of the mix was 2000 g. BAN® 480L (0.2% w/w on oat flour) was added and the suspension incubated at 80°C for 30 min. The liquefied solution was then distributed into 190 g subsamples (with continuous stirring to ensure homogenous samples), cooled and incubated at 53°C for 30 minutes with stirring after addition of 0.2% w/w on oat flour Fungamyl® 800L for further saccharification. 1.21 IPA(U) per g oat protein of protein deamidase and increasing amount of protease TL (0, 42, 70, 98, 140 KMTU/kg oat protein) was added as shown in Table 1 below.

After incubation the slurry was heat treated at 90°C for 10 minutes for enzyme inactivation. The solution was then centrifuged at 1200g/10 minutes at RT and the supernatant formulated with 3% rapeseed oil and 0.08% NaCl and homogenized in the Vorwerk Thermomixer (speed 10/1 min).

Total protein, soluble protein and foaming capacity were measured in the final beverage. Total protein was measured directly in the final beverage, while soluble protein was measured after centrifugation of a small sample at 21,000g for 10 minutes. Protein determination was done using a LECO analyzer (Dumas determination of nitrogen after combustion, reduction and detection of N2 using a conductivity detector). Foaming capacity was measured by frothing 120 g final beverage in a Kitchen frother (Severin) at 55°C using the full cup coffee program. After frothing the sample was poured into a 250 ml glass cylinder and the total volume and the liquid volume noted. Foam volume after 0 minutes and after 10 minutes (ΔFoam) was calculated as Total volume – Liquid Volume.

Results are given in Table 1 below.

Table 1: Total protein, soluble protein and foam measured in the final oat beverage.

All samples contain 0.2% w/w on oat flour BAN® 480L and 0.2% w/w on oat flour Fungamyl® 800L. Samples were centrifuged at 1200g for 10 min.

Sample	Protein deamidase IPA(U)/g protein	Protease TL KMTU/kg protein	Total protein, in beverage, % w/w	Soluble protein in beverage, %w/w	ΔFoam, 0 min	ΔFoam, 10 min
1	0	0	0.61	0.11	35	38
2	1.21	0	0.98	0.53	130	84

3	1.21	42	1.00	0.57	167	94
4	1.21	70	1.04	0.63	170	101
5	1.21	98	1.03	0.62	165	93
6	1.21	140	1.06	0.63	165	93

As seen in Table 1, total protein, soluble protein and foaming capacity increased significantly when adding protein deamidase (sample 2) compared to the blank sample (sample 1, no enzymes added). Adding increasing amounts of protease TL on top of protein deamidase (samples 3-6) resulted in a further increase in protein content and foaming capacity for the lowest tested dosage of protease TL compared to protein deamidase alone, while higher dosages of protease TL had limited effect.

Example 3: Testing of protein deamidase and protease TL or Neutrase® 0.8L in production of oat-based beverages

The experiment was run as described above for Example 2, combining protein deamidase with either protease TL (specific endopeptidase) or Neutrase® 0.8L (broad spectrum endopeptidase) with the following minor changes: total amount of oat suspension was 1000 g for both liquefaction and saccharification, saccharification temperature was 55°C, and separation of the oat beverage was done running pulsed centrifugation, 3x pulses at 1200g.

Results are given in Table 2 below.

Table 2: Total protein, soluble protein and foam measured in the final oat-based beverage. All samples contain 0.2% w/w on oat flour BAN® 480L and 0.2% w/w on oat flour Fungamyl® 800L.

Sample	Protein deamidase IPA(U)/g protein	Protease TL KMTU/kg oat protein	Neutrase® 0.8L % w/w on protein	Total protein, in beverage, % w/w	Soluble protein in beverage, %w/w	ΔFoam, 10 min
1	0	0	0	1.15	0.15	24
2	1.21	0	0	1.27	0.64	115
3	1.21	0	0.025	1.37	0.59	99
4	1.21	70	0	1.40	0.78	143

As seen from the results in Table 2, total protein increased when combining protein deamidase with protease TL or Neutrase® 0.8L compared to when protein deamidase were added alone. Soluble protein only increased with protease TL, while Neutrase® 0.8L showed a drop in soluble protein. Foam capacity improved with protease TL in combination with protein deamidase compared to protein deamidase alone, while Neutrase® 0.8L in combination with protein deamidase reduced foaming capacity.

Example 4: Testing of protein deamidase and protease TL in higher dosages in production of oat-based beverages

The experiment was run as described above for Example 2, combining protein deamidase and higher dosages of protease TL with the following modifications: total amount of oat suspension was 1800 g for liquefaction and 225 g for saccharification. Saccharification temperature was 55°C. 1 IPA(U)/g oat protein of protein deamidase and increasing amount of protease TL (56, 280, 700, 1400 KMTU/kg oat protein) was added according to Table 3 below. Separation of the oat beverage was done running pulsed centrifugation, 3x pulses at 1200g.

Results are given in Table 3 below.

Table 3: Total protein, soluble protein and foam measured in the final oat-based beverage. All samples contain 0.2% w/w on oat flour BAN® 480L and 0.2% w/w on oat flour Fungamyl® 800L.

Sample	Protein deamidase IPA(U)/g protein	Protease TL KMTU/kg oat protein	Total protein, in beverage, % w/w	Soluble protein in beverage, %w/w	ΔFoam, 0 min	ΔFoam, 10 min
1	0	0	1.10	0.24	68	53
2	1	0	1.31	0.77	142	90
3	0	1400	1.19	0.39	135	75
4	1	56	1.35	0.83	154	101
5	1	280	1.40	0.84	203	139
6	1	700	1.43	0.94	222	145
7	1	1400	1.47	0.98	214	132

As seen from the results shown in Table 3, higher dosages of protease TL combined with protein deamidase further increased total and soluble protein as well as the foaming capacity.

Example 5: Testing of protein deamidase and protease TL in production of oat-based beverages in pilot scale

400 kg oat groats was scalded at 90°C for 45 minutes (45% w/w oat groats). Water and BAN® 480L (0.2% w/w on oat dry matter) was added bringing the dry matter to 20-25%. The slurry was then milled and incubated at 70°C. After 30 minutes the mix was cooled to 55°C by adding cold water, bringing the final dry matter to ~19 %. Fungamyl® 800L (0.15% w/w on oat dry matter) was added and protease TL and protein deamidase was added as shown in Table 4. The slurry was incubated at 55°C for 30 minutes and then heat-treated at 90°C for 5 sec for enzyme inactivation. The slurry was separated using a decanter and formulated with 3% sunflower seed oil and 0.08% NaCl. The beverage was UHT treated and homogenized.

Total protein, soluble protein and foaming capacity were measured in the final beverage as described in Example 2.

Results are given in Table 4 below.

Table 4: Total protein, soluble protein and foam measured in the final oat-based beverage. All samples contain 0.2% w/w on oat flour BAN® 480L and 0.15% w/w on oat flour Fungamyl® 800L.

Sample	Protein deamidase IPA(U)/g protein	Protease TL KMTU/kg oat protein	Total protein, in beverage, % w/w	Soluble protein in beverage, %w/w	ΔFoam, 0 min	ΔFoam, 10 min
1	0	0	0.76	0.28	45	35
2	0	1400	0.95	0.43	85	65
3	1.21	70	1.12	0.67	220	127

As seen from the data shown in Table 4, total and soluble protein increased by 25% and 54%, respectively, when adding protease TL compared to the control with only BAN® 480L and Fungamyl® 800L. When adding protein deamidase and protease TL increases were even higher, reaching 47% and 140%. Foaming capacity also improved in samples with protease TL alone (~x2) and in the combined treatment with protease TL and protein deamidase (~x4-5).

Sensory tests:

In order to evaluate if there was a difference in sensory properties when adding protease TL +/- protein deamidase, a difference-from-control test was carried out. The panellists were served a labelled reference (Control, sample #1) and asked to compare this to the other samples (#2 and #3). In this study, the panellists compared the labelled reference to 3 samples which also included the reference (this time unlabelled). This served as an experimental control for noise. The samples were served in a balanced, randomized design. The key output from a Difference-from-control test is a mean score for each test sample and the unlabelled reference. Total number of panellists for the test was 16.

Based on the response from the panellists no significant difference was found between the control and samples #2 and #3 ($p>0.05$).

It was therefore concluded, that using a specific endopeptidase, such as a trypsin-specific endopeptidase (protease TL) in the production of an oat-based beverage, does not affect sensory aspects of the beverage, despite the fact that proteases are often known to introduce bitterness.

Example 6: Testing of protease TL in production of oat-based beverages

In another series of experiments, the effect of adding protease alone in the production of plant-based beverages was tested using an oat-based beverage as an example.

15% w/w oat flour (13% protein) was mixed with deionized water and placed in a Vorwerk Thermomixer with stirring. Total amount was 1500 g. BAN® 480L (0.2% w/w on oat flour) was added and the suspension incubated at 80°C for 30 min. The liquefied solution was then distributed into 225g subsamples (with continuous stirring to ensure homogenous samples), cooled and incubated at 50°C for 30 minutes with stirring after addition of 0.2% w/w on oat flour

Fungamyl® 800L for further saccharification. Increasing amounts of protease TL (56, 280, 700, 1400, 3500, 7000, 35000 KMTU/kg oat protein) was added according to the table below.

After incubation the slurry was heat-treated at 90°C for 10 minutes for enzyme inactivation. The solution was centrifuged using pulsed centrifugation, 3x pulses at 1200g, and the supernatant formulated with 3% rapeseed oil and 0.08% NaCl and homogenized in the Vorwerk Thermomixer (speed 10/1 min).

Results are given in Table 5 below.

Table 5: Total protein, soluble protein and foam measured in the final oat-based beverage. All samples contain 0.2% w/w on oat flour BAN® 480L and 0.2% w/w on oat flour Fungamyl® 800L.

Sample	Protease TL KMTU/kg oat protein	Total protein, in beverage, % w/w	Soluble protein in beverage, %w/w	ΔFoam, 0 min
1	0	1.02	0.25	42
2	56	1.03	0.29	50
3	280	1.07	0.36	88
4	700	1.11	0.37	110
5	1400	1,13	0,40	120
6	3500	1.08	0.39	107
7	7000	1.04	0.44	119
8	35000	1.04	0.54	123

Addition of a trypsin-like endopeptidase (protease TL) resulted in significantly more soluble protein in the final oat-based beverage. Total protein also increased but to a lesser extent. Foaming capacity improved, showing 3x more foam at highest enzyme dosage.

Example 7: Testing of Alcalase® 2.4L or Neutrase® 0.8L in production of oat-based beverages

Experiment was run as described above for Example 6, except the two broad-spectrum endopeptidases, Alcalase® 2.4L FG and Neutrase® 0.8L, were tested instead of the specific endopeptidase (protease TL).

Results are given in Tables 6A and 6B below.

Table 6A: Total protein, soluble protein and foam measured in the final oat-based beverage. All samples contain 0.2% w/w on oat flour BAN® 480L and 0.2% w/w on oat flour Fungamyl® 800L.

Sample	Alcalase® 2.4L, % product w/w on protein	Total protein, in beverage, % w/w	Soluble protein in beverage, %w/w	ΔFoam, 0 min
1	0	0.97	0.28	36
2	0.002	0.95	0.27	40
3	0.008	0.98	0.27	32

4	0.02	0.76	0.28	33
5	0.04	0.68	0.33	24
6	0.2	0.77	0.37	25
7	0.5	0.99	0.47	30
8	1	0.88	0.43	26

Table 6B: Total protein, soluble protein and foam measured in the final oat-based beverage. All samples contain 0.2% w/w on oat flour BAN® 480L and 0.2% w/w on oat flour Fungamyl® 800L. Separation was done using 3x pulsed centrifugation at 1200g.

Sample	Neutrase® 0.8L, % product w/w on protein	Total protein, in beverage, % w/w	Soluble protein in beverage, %w/w	ΔFoam, 0 min
1	0	1.03	0.26	44
2	0.005	1.00	0.24	39
3	0.024	1.01	0.29	41
4*	0.06	1.13*	0.26*	59*
5	0.12	0.83	0.30	39
6	0.6	0.73	0.40	36
7	1.5	0.73	0.47	34
8	3	0.79	0.49	31

*It is unclear why Sample 4 treated with Neutrase® 0.8L (Table 6B) is out of trend but is likely to be an outlier and does not detract from the overall conclusions of the experiment.

Thus, and as seen in Tables 6A and 6B, none of the broad-spectrum proteases (Alcalase® 2.4L and Neutrase® 0.8L) tested showed any improvement in foaming and instead foaming capacity tended to show a drop. Total protein also dropped with dosage of both of the broad-spectrum proteases while soluble protein increased.

Example 8: Testing of alternative specific proteases in production of oat-based beverages

The experiment was run as described above for Example 6, except that temperature during saccharification was 55°C, and protease TL was replaced by two alternative specific proteases: AC3 protease and porcine trypsin.

Results are given in Tables 7A and 7B below.

Table 7A: Total protein, soluble protein and foam measured in the final oat-based beverage. All samples contain 0.2% w/w on oat flour BAN® 480L and 0.2% w/w on oat flour Fungamyl® 800L.

Sample	AC3, mg ep/g protein	Total protein, in beverage, % w/w	Soluble protein in beverage, % w/w	ΔFoam, 0 min
1	0	1.07	0.20	37
2	0,016	1.13	0.40	67
3	0,08	1.09	0.41	95
4	0,2	1.27	0.51	116
5	0,4	1.20	0.48	129

Table 7B: Total protein, soluble protein and foam measured in the final oat-based beverage. All samples contain 0.2% w/w on oat flour BAN® 480L and 0.2% w/w on oat flour Fungamyl® 800L.

Sample	Porcine trypsin, mg ep/g protein	Total protein in beverage, % w/w	Soluble protein in beverage, %w/w	ΔFoam, 0 min
1	0	1.14	0.29	37
2	0,016	1.17	0.38	71
3	0,08	1.27	0.44	100
4	0,2	1.26	0.46	114
5	0,4	1.27	0.51	119

Use of the specific trypsin-like proteases, AC3 and porcine trypsin, clearly improved foaming, reaching triple amount of foam at highest dosage. Total protein increased a little (~10%) with protease dosage, while soluble protein showed higher increases (75-150%).

The inventors have found that the specificity of the endopeptidase has significant effect on the foaming ability of the plant-based dairy alternative food product. In particular, in comparative studies testing the endopeptidase according to the present invention and two alternative endopeptidases with different specificities and properties, i.e. a glutamyl endopeptidase from Bacillus licheniformis and a metalloendopeptidase from Thermoascus aurantiacus, it was found that only the herein claimed endopeptidase resulted in the improved foaming of a plant-based beverage. It was thus concluded that in order to obtain the beneficial effects on foaming, the endopeptidase used in the methods claimed herein must have a specificity for cleaving after Arg and/or Lys.

Example 9: Peptide analysis of oat-based beverages

Samples were prepared as in Example 2, except oil content of the supernatant was 0.8%. Mass spectrometry analysis of the oat-based beverages after homogenization was performed to identify peptides generated by degradation of oat proteins by the protease.

Diagnostic peptide analysis by LC-MS/MS:

Tandem liquid chromatography-mass/mass spectrometry (LC-MS/MS, also known as MS2) was used for analysis. Analysis was carried out on a TIMS-TOF Pro 2 MS spectroscope (Bruker) equipped with an Evosep One LC system (Evosep). During analysis, molecules were ionized in the first spectrometer (MS1) and separated based on the mass/charge ratio. Selected ionized molecules were then fragmented further and introduced into the second spectrometer (MS2) which again separated the analytes by mass/charge ratio. The second fragmentation step made it possible to identify and separate ions that had very similar mass/charge ratios after the first ionization step thus increasing detection accuracy. The data from the LC-MS/MS analysis

were searched against a protein database derived from the oat genome. Searching was carried out using the MASCOT search engine (MASCOT server version: 2.5, Matrix Science), which is the standard software tool for protein identification by LC-MS/MS (Current Protocols in Protein Science 25.2.1-25.2.19).

MASCOT search settings:

- Tryptic enzymatic digest;
- Max missed cleavages:1;
- Peptide charge 2+, 3+ and 4+;
- Peptide tolerance +/- 20 ppm;
- MS/MS tolerance +/- 0.05 Da;
- Fixed modifications: carbamidomethyl (C);
- Variable modifications: oxidation (M), deamidation (N,Q)

In Table 8, the identity and response of the ten most abundant peptide fragments in the different oat beverage samples are shown.

All oat beverage samples were treated with BAN® 480L and Fungamyl® 800L as previously described in Example 2, and was further characterized by:

Sample 1: no protein deamidase or protease TL added.

Sample 2: PD (1.21 IPA(U)/g oat protein).

Sample 3: PD (1.21 IPA(U)/g oat protein) + protease TL (42 KMTU/kg oat protein).

Sample 4: PD (1.21 IPA(U)/g oat protein) + protease TL (70 KMTU/kg oat protein).

Sample 5: PD (1.21 IPA(U)/g oat protein) + protease TL (98 KMTU/kg oat protein).

Sample 6: PD (1.21 IPA(U)/g oat protein) + protease TL (140 KMTU/kg oat protein).

Table 8: Ten most abundant peptide fragments in oat-based beverage samples (1-6) treated with different combinations of enzymes.

	Sample no.					
Peptide fragment	1	2	3	4	5	6
ALPVDVIANAYR (SEQ ID NO: 24)	1855994	306843	6000253	6246795	15388720	6909406
VAIMEANPR (SEQ ID NO: 25)			959319	906115	2627371	856063
VIGAQTESAFLR (SEQ ID NO: 26)	157148	17647	277635	368094	1170144	249116
HWPLPPFGGDSR (SEQ ID NO: 27)			720281	652255	2264249	18742
STYNLLEQRPTIANR			283786	192157	676715	219889

(SEQ ID NO: 28)						
TNPNSMVSHIAGK (SEQ ID NO: 29)			756999	727112	2012126	695526
ALPIDVLANAYR (SEQ ID NO: 30)	135652		478012	525654	1511361	525118
TIQGELGGFLGSQEGQK (SEQ ID NO: 31)	114502		452300	423424	1133184	377704
LVLPGELAK (SEQ ID NO: 32)					1255613	412498
AQEAGAGGGAAT TAGGGTTR (SEQ ID NO: 33)			312454	266440	857583	305335

As seen from Table 8, samples treated with protease TL contain a high number of peptides typically generated by proteases with a trypsin-like specificity, whereas sample 1 and 2 without protease TL had very few of these peptides. Thus, by using the method of Example 9, it was concluded that treating an oat-based dairy alternative food product, such as, e.g. an oat-based beverage, with a combination of the endopeptidase and protein deamidase according to the invention, the most abundant peptide fragments isolated from the sample are the peptide fragments according to SEQ ID Nos: 25, 27, 28, 29, 32 and 33. In addition to being the most abundant, these peptide fragments of SEQ ID Nos: 25, 27, 28, 29, 32 and 33 were only isolated from the oat-based dairy alternative food product treated with the combination of the protease and protein deamidase according to the invention and were not identified when the same food product was treated with protein deamidase alone (Sample 2) or with neither of the two enzymes (Sample 1). These data thus support the conclusion that a plant-based dairy alternative food product with a unique peptide fragment profile is obtained when using the methods disclosed herein.

Example 10: Testing of protein deamidase and proteases in production of pea-based beverages

Pea milk preparation

Three different types of pea protein isolates (PPIs) were used in this example:

PPI1: AGT FYPP-85-C, protein content 82%

PPI2: Pisane C9, protein content 79%

PPI3: Roquette Nutralys S85F 2.0, protein content 82%

To prepare the pea milk samples, each of three PPIs (PPI1, PPI2, PPI3) was suspended in deionized water to a final protein content of 10% and mixed with magnetic stirrer, separately. Different enzymes were added to these isolate solutions and incubation of the isolate solutions were conducted at 60°C for 1 hour, 150rpm shaker.

A portion of each pea protein isolate solution was saved for protein solubility measurement (see below).

The pea milk was formulated according to the general recipe provided in Table 9 below. The ingredients were mixed well in a VORWERK Thermomix set at a rotation speed set of 2.5 and 60°C heating. Then the samples were homogenized at rotation speed 8 for 3 minutes at 60°C.

Table 9: General pea milk recipe and ingredients

Lab pea milk ingredient	Lab pea milk recipe, based on weight (g)
PPI solution	3.4%
Gellan gum	0.04%
Calcium (from $CaCO_3$)	0.186%
Sucrose	3.4%
Oil (soybean salad oil)	1.9%

Following formulation, each pea milk sample was pasteurized at 90°C for 10 minutes under agitation. Then, each pea milk sample was transferred to a fridge for storage at 4°C until analysing.

Protein solubility measurement

Protein solubility was measured in the supernatant of a pea protein isolate solutions (before formulation to pea milk) after centrifugation at 15,000g for 10 minutes. Protein content determination was done using a LECO analyzer (Dumas determination of nitrogen after combustion, reduction and detection of N2 using a conductivity detector). Protein factor was 6.25. The results from the protein solubility measurements are summarized in Table 10.

Table 10: Protein solubility of pea protein solutions prepared using three different commercially available pea protein isolates (PPI1, PPI2 and PPI3)

Commercial pea protein isolate (PPI)	Enzyme combination	Deamidase dosage, IPA(U)/g protein	Protease dosage (KMTU/g for TL and PROT/g for CTL)	Soluble protein/g protein (%)

PPI1	Control (no enzyme)	0	0	11
	Protein deamidase	2	0	16.9
	Protein deamidase	4	0	18.8
	Protein deamidase + Protease TL	2	0.28	34.4
	Protease TL	0	0.28	32
PPI2	Control (no enzyme)	0	0	22.0
	Protein deamidase	2	0	29.6
	Protein deamidase	4	0	31.0
	Protease CTL	0	150	53.8
	Protein deamidase + Protease CTL	2	150	65.5
	Protease TL	0	0.28	50.8
	Protein deamidase + Protease TL	2	0.28	70.3
PPI3	Control (no enzyme)	0	0	57.8
	Protein deamidase	2	0	66.7
	Protein deamidase	4	0	68.5
	Protein deamidase + protease TL	2	0.28	64.0
	Protease TL	0	0.28	49.8

The initial solubility ranking (without any enzyme treatment) was PPI1 (11%, low soluble protein) < PPI2 (22%, medium soluble protein) < PPI3 (57.8%, high soluble protein). The pea protein isolates (PPIs) were thus grouped according to their initial protein solubility as follows:

Low Solubility: The PPIs that fall under this category have initial soluble protein content up to 20%. This suggest that only a small portion of the protein is dissolvable in solution without any protein deamidase treatment.

Medium Solubility: The PPIs that fall under this category have initial soluble protein content greater than 20% and up to 50%. These proteins exhibit a moderate ability to dissolve in solution when no protein deamidase treatment is performed.

High Solubility: The PPIs that fall under this category have initial soluble protein content of more than 50%, indicating a substantial portion of the protein can be dissolved in solution without any protein deamidase treatment. The improvement in soluble protein content for protein isolates belonging to this group is expected to have a lower benefit from the protein deamidase treatment.

Treatment with protein deamidase alone and in combination with protease TL could improve the protein solubility in the tested pea protein solutions. Using protease TL alone could improve the solubility for the low and medium soluble PPIs (such has PPI1 and PPI2). For the low (e.g. PPI1) and medium (e.g. PPI2) soluble PPIs, the combination of protein deamidase and

protease showed significant synergistic effect on protein solubility improvement (34.4% for PPI1 and 70.3% for PPI2).

The samples treated with protease CTL, both with/without protein deamidase, showed strong bitterness in sensory tests, while for the samples treated with protease TL, there was no significant sensory difference in bitterness.

Foaming determination

Each pea milk sample was subjected to foaming using a Severin milk foaming machine set at a soymilk pattern.

The initial net foam height (not including liquid) was measured at time point 0 min as foaming capability. Foam height at time point 10 minutes was measured as foaming stability. The results from the foaming determination are summarized in Tables 11-13.

Foaming results

Foaming results from testing PPI1, PPI2 and PPI3 are summarized below with reference to Tables 11, 12 and 13, respectively.

As can be seen from Table 11, for the samples of pea milk prepared from a low initial soluble protein isolate (PPI1), the use of protein deamidase alone, protease TL alone and the combination of both could significantly improve the foaming capability (0min) and foaming stability (10min) of the pea milk samples. In particular, the use of protease TL alone gave rise to the best foaming capability (660%) and foaming stability (670%) improvement.

Table 11: Pea milk prepared from PPI1 solution

Commercial pea protein isolate (PPI)	Enzyme combination	Deamidase dosage, IPA(U)/g protein	Protease dosage, KMTU/g protein	0min foam height, mL	10min, foam height, mL
PPI1	Control	0	0	12	7
	Protein deamidase	2	0	21	12
	Protein deamidase	4	0	38	16
	Protease TL	0	0.28	92	54
	Protein deamidase + protease TL	2	0.28	58	34

As can be seen from Table 12, for the samples of pea milk prepared from a medium initial soluble protein isolate (PPI2), the use of protein deamidase alone, protease TL alone, protease

CTL alone, and the combinations could significantly improve the foaming capability (0min) and foaming stability (10min) of the pea milks. In particular, treatment with protease TL alone performed best on pea milk prepared from PPI2 and shows 75% improvement of foaming capability and 116% improvement of foaming stability. For all of the pea milk samples prepared by treatment with protease CTL, a bitterness off flavour was reported.

Table 12: Pea milk prepared from PPI2 solution

Commercial pea protein isolate (PPI)	Enzyme combination	Deamidase dosage, IPA(U)/g protein	Protease dosage (KMTU/g for TL and PROT/g for CTL)	0min, foam height, mL	10min, foam height, mL
PPI2	Control	0	0	79	53.7
	Protein deamidase	2	0	103	75.0
	Protein deamidase	4	0	110	81.5
	Protease CTL	0	150	118	115.5
	Protein deamidase + Protease CTL	2	150	129	116.5
	Protease TL	0	0.28	139	116.0
	Protein deamidase + Protease TL	2	0.28	114	121.0

As can be seen from Table 13, for the samples of pea milk prepared from a high initial soluble protein isolate (PPI3), the foaming property of the pea milk prepared without any enzymes was already quite good and the treatment with the protein deamidase alone, the protease TL alone and the combination of both showed only slight improvements in foaming capability (0min), but more significant improvements in foaming stability (10min). In particular, the combination of protein deamidase and protease TL improved foaming capability with 7% and foaming stability with 37% compared to the control pea milk. The use of protease TL alone only showed foaming stability improvement compared to the control.

Table 13: Pea milk prepared from PPI3 solution

Commercial pea protein isolate (PPI)	Enzyme combination	Deamidase dosage, IPA(U)/g protein	Protease dosage, KMTU/g protein	0min, foam height, mL	10min, foam height, mL
PPI3	Control	0	0	157	97

	Protein deamidase	2	0	160	106
	Protein deamidase	4	0	161	105
	Protease TL	0	0.28	159	124
	Protein deamidase + Protease TL	2	0.28	168	133

Example 11: Testing of protease TL in production of almond-based beverage with improved foaming

Almond paste (Almond paste from KoRo, ingredients: 100% almond, protein content: 21-25%) was added to deionized water to a final protein concentration of 8% and the slurry was mixed. Protease was added as indicated in Table 14, and the samples held at 55°C for 1 hour.

After incubation, the samples were diluted to 1% protein and 0.1% sodium chloride was added. The beverage samples were then heat-treated for enzyme inactivation at 90°C for 15 minutes, cooled down and homogenized using an Ultraturrax.

Foaming capacity was measured by foaming 120 g of beverage sample in a Severin milk frother using the full cup program at 55°C. After frothing, the beverage samples were poured into 250 mL glass cylinders and the total volume and the liquid volume noted after initial phase separation (approx. 0-1 min). Foam volume (ΔFoam) was calculated as “Total volume – Liquid Volume” after 1 minute, and foam stability after 10 minutes.

Coffee stability was evaluated by pouring 100 g of foamed beverage sample into 20 g of Espresso coffee. Precipitation or curdling was observed during 10 minutes and scored using a scale of 1-7, with 1 indicating a stable drink with no curdling/precipitation and 7 indicating an unstable drink with a lot of curdling/precipitation.

Table 14: Foaming capacity and coffee stability results

Sample #	Protease dosage, % w/w on protein	ΔFoam volume (mL), t = 0 min	ΔFoam volume (mL), t = 10 min	Stability in coffee, t = 0 min	Stability in coffee, t = 10 min
1	0	164	117	6	7
2	0	168	123	6	7
3	0.75	205	166	1	6
4	0.75	207	170	1	6

Use of the protease improved foaming capacity and stability of the almond beverage samples. Stability in espresso coffee was improved during the first couple of minutes or delayed compared to the untreated sample, but final stability after 10 minutes was not satisfactory. This could be partly explained from the highly acidic espresso coffee used for this experiment.

CLAIMS

1. Method for obtaining a dairy alternative food product, the method comprising the steps of:

(a) obtaining a slurry of a plant material in water; and

(b) treating the slurry of step (a) with an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase to obtain the dairy alternative food product, wherein the endopeptidase having a specificity for cleaving after Arg and/or Lys is a trypsin-like or lysine-specific endopeptidase.

2. Method of claim 1, further comprising treating the slurry of step (a) with one or more hydrolyzing enzymes selected from the group of pectinases, hemicellulases, xylanases, beta-glucanases, mannanases, glucanases, glucoamylases, iso-amylases, alpha-amylases, beta-amylases, and mixtures thereof.

3. Method of claim 2, wherein the hydrolyzing enzyme is alpha-amylase, beta-glucanase, glucoamylase or any combination thereof.

4. Method of any of the preceding claims, wherein the treatment of step (b) is carried out at a temperature in the range of 20-60°C, such as in the range of 50-60°C.

5. Method of any of the preceding claims, further comprising the addition of a lipid during step (a) or (b) or after step (b) to obtain a slurry or a dairy alternative food product having a lipid content of 1-5% (w/w) based on the weight of the slurry or dairy alternative food product, optionally wherein the lipid is an oil, preferably a plant oil.

6. Method of any of the preceding claims, further comprising the steps of:

(c) separating the dairy alternative food product into solid and liquid streams;

(d) harvesting the liquid stream as a liquid dairy alternative food product; and

(e) optionally inactivating the enzymes.

7. Method of any of the preceding claims, wherein the plant material has a protein content of 5-20% (w/w), such as 10-15% (w/w).

8. Method of any of the preceding claims, wherein the plant material is derived from almond, soy, cashew, macadamia, coconut, pea, bean, rice, quinoa, flax, hemp or oat.

9. Method of any of the preceding claims, wherein the dairy alternative food product is an almond drink, a soy drink, a cashew drink, a macadamia drink, a coconut drink, a pea drink, a bean drink, a rice drink, a quinoa drink, a flax drink, a hemp drink or an oat drink.

10. Method of any of the preceding claims, wherein the dairy alternative food product is for use in coffee.

11. Method of any of preceding claims, wherein the endopeptidase is a trypsin-like endopeptidase derived from a strain of Fusarium, more preferably from Fusarium oxysporum, or a lysine-specific endopeptidase derived from a strain of Achromobacter, more preferably from Achromobacter lyticus.

12. Method for obtaining an oat-based dairy alternative food product, the method comprising the steps of:

(a) obtaining a slurry of an oat material in water; and

(b) treating the slurry of step (a) with an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase to obtain the oat-based dairy alternative food product, wherein the endopeptidase having a specificity for cleaving after Arg and/or Lys is a trypsin-like or lysine-specific endopeptidase.

13. Use of an endopeptidase having a specificity for cleaving after Arg and/or Lys and a protein deamidase in the production of a dairy alternative food product to improve foaming properties, wherein the endopeptidase having a specificity for cleaving after Arg and/or Lys is a trypsin-like or lysine-specific endopeptidase.

14. Use according to claim 13, wherein the dairy alternative food product is an oat beverage, such as an oat beverage for coffee.

15. Dairy alternative food product comprising a mixture of polypeptide fragments each having primarily either an arginine residue or a lysine residue at the carboxyl terminus, and further comprising enzymatically deamidated plant material.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/103410

A. CLASSIFICATION OF SUBJECT MATTER

A23L2/38(2021.01)i; A23J3/34(2006.01)i; A23C11/10(2021.01)i

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)

IPC: A23L, A23J, A23C

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)

CNABS, DWPI, CNTXT, ENTXT, VEN, CNKI, ISI web of science, Pubmed, Baidu, NCBI,NOVOZYMES A/S, HENDRIKSENHanne Vang, endopeptidase, Arg, Lys, deamidase, trypsin-like, lysine-specific, plant, hydrolyzing enzyme, Fusarium oxysporum, Achromobacter lyticus, oat, foaming

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2008131008 A2 (SOLAE, LLC et al.) 30 October 2008 (2008-10-30) claims 16-20, 25-30, description paragraphs 26, 37-39, examples 1, 19	1-15
Y	WO 2021260067 A2 (SOCIÉTÉ DES PRODUITS NESTLÉ S.A.) 30 December 2021 (2021-12-30) claims 1-3, 9, 10, 12, description paragraphs 6-10, 32	1-15
Y	US 2022338503 A1 (Amano Enzyme Inc., et al.) 27 October 2022 (2022-10-27) the abstract, claims 1-15	1-12, 15
A	US 2022338503 A1 (Amano Enzyme Inc., et al.) 27 October 2022 (2022-10-27) the abstract, claims 1-15	13-14
Y	WO 2022045152 A1 (Amano Enzyme Inc., et al.) 03 March 2022 (2022-03-03) the abstract, claims 1-19	1-12, 15
A	WO 2022045152 A1 (Amano Enzyme Inc., et al.) 03 March 2022 (2022-03-03) the abstract, claims 1-19	13, 14

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2024	**20 September 2024**

Name and mailing address of the ISA/CN	Authorized officer
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2022148567 A1 (NOVOZYMES A/S) 14 July 2022 (2022-07-14) the abstract, claims 1-15	1-12, 15
A	WO 2022148567 A1 (NOVOZYMES A/S) 14 July 2022 (2022-07-14) the abstract, claims 1-15	13, 14
A	WO 2021004817 A1 (NOVOZYMES A/S) 14 January 2021 (2021-01-14) the abstract, claims 1-15	1-15
A	WO 9743910 A1 (NOVO NORDISK A/S, et al.) 27 November 1997 (1997-11-27) the abstract, claims 1-33	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/103410

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. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2024/103410

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
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				EP	2146586	A2	27 January 2010
				WO	2008131008	A3	18 December 2008
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				WO	2021260067	A3	24 February 2022
				BR	112022025422	A2	24 January 2023
				MX	2022016269	A	15 February 2023
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				AR	122737	A1	05 October 2022
				CL	2022003641	A1	04 August 2023
				US	2023225378	A1	20 July 2023
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