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(54) Title: PROCESS FOR MAKING A MILK-BASED PROTEIN HYDROLYSATE

(57) Abstract: The present invention relates to an enzymatic process for making a milk-based protein hydrolysate and use of such hydrolysate, e.g., in an infant formula composition.

PROCESS FOR MAKING A MILK-BASED PROTEIN HYDROLYSATE

Reference to sequence listing

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

5 **FIELD OF THE INVENTION**

The present invention relates to an enzymatic process for making a milk-based protein hydrolysate and use of such hydrolysate, e.g., in an infant formula composition.

BACKGROUND OF THE INVENTION

Infant formulae have been developed, which allow for substituting breast feeding of infants.

10

Such infant formulae should wholly satisfy the nutritional requirements of infants until the introduction of appropriate complementary feeding. Further, taste is important, as at least the parents prefer infant formulae having a non-bitter taste. Infant formulae which instead of ordinary cow's milk comprise hydrolyzed milk protein, e.g. partially hydrolyzed whey protein or 15 extensively hydrolyzed casein, are often used as such formulae are less allergenic and still have an acceptable taste.

Processes for preparation of partial hydrolysates described in the literature often comprise use of pancreatic enzymes such as trypsin preparations produced by extraction of porcine 20 pancreatic tissue (see, e.g., WO9304593 A1, US5039532 A). Some of the processes described comprise use of a mixture of trypsin and chymotrypsin. E.g., EP0353122 A discloses a process for preparing a hypoallergenic whey protein hydrolysate using a mixture of trypsin and chymotrypsin, wherein the ratio of the chymotrypsin/trypsin activities is between 1.5 and 3.0. In EP0631731 A1, a mixture of trypsin and chymotrypsin having a trypsin to chymotryp- 25 sin ratio of 1.3 to 18 in USP units, more preferably 4 to 6, is said to typically result in a hydrolysate of desirable properties.

For several reasons, use of proteolytic enzymes produced from a microorganism may confer benefits. For example, enzyme production from a microorganism is efficient and easy to con- 30 trol. Therefore, such enzymes can be produced in large quantities and at high purity. Also, use of microbial enzymes will help overcoming increasing QA related difficulties as regards extraction of enzymes from an animal source.

One object for the present inventors has been to develop a process for making a milk-based

protein hydrolysate with microbially produced enzymes which has a low allergenicity. Another object has been to develop a process for making a milk-based protein hydrolysate with microbially produced enzymes which has a protein fragment profile having similarity to the protein fragment profile of hydrolysates prepared with extracted preparations comprising trypsin and chymotrypsin. Another object has been to develop a process for making a milk-based protein hydrolysate with microbially produced enzymes which has an acceptable taste. In particular, it would be highly desirable to have a partial whey protein hydrolysate which has low allergenicity, which has a protein fragment profile having similarity to the protein fragment profile of hydrolysates prepared with extracted preparations comprising trypsin and chymotrypsin, and/or which has an acceptable taste, in particular as regards bitterness.

SUMMARY OF THE INVENTION

The present inventors have surprisingly found that microbially produced enzymes can be used for production of a milk-based protein hydrolysate, e.g., for inclusion into an infant formula.

The invention therefore relates to a process for the preparation of a milk-based protein hydrolysate comprising treatment of a solution of a milk-based proteinaceous material with

a) a trypsin-like endopeptidase produced from a microorganism, and

b) at least one other endopeptidase produced from a microorganism.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows UV-chromatograms of *Fusarium* trypsin (bottom trace) compared with porcine trypsin (upper trace) and the peptide sequence identity of some major peaks are displayed.

Fig. 2 shows UV-chromatograms of *Brachysporiella* protease (bottom trace) compared with bovine chymotrypsin (upper trace) and the peptide sequence identity of some major peaks are displayed.

DETAILED DESCRIPTION OF THE INVENTION

In the process according to the invention, a milk-based proteinaceous material is used as starting material. Such milk-based proteinaceous material may, e.g., consist of whey-based proteinaceous material, casein or mixtures of whey-based proteinaceous material and casein. The whey-based proteinaceous material may be sourced from a whey obtained from cheese making, particularly a sweet whey such as that resulting from the coagulation of casein by rennet. The whey-based proteinaceous material may also be used in the form of concentrates in the range of 35-80 % protein as obtained by ultrafiltration (UF whey), or from

whey protein isolates. This whey-based proteinaceous material, optionally, may also be demineralized by ion exchange and/or electrodialysis (ED whey). In a preferred embodiment, the milk-based proteinaceous material is whey protein concentrate (WPC).

5 The casein source may be acid casein or non-fat milk solids.

The whey-based proteinaceous material and/or the casein may be used, e.g., in the form of liquid concentrates or powders.

10 In a preferred embodiment, the milk-based proteinaceous material is a whey-based proteinaceous material. In a more preferred embodiment, the source of such whey-based proteinaceous material is sweet whey from which the caseino-glyco-macropeptide (CGMP) has been removed or whey protein isolate. In an even more preferred embodiment, the source of such whey-based proteinaceous material is sweet whey from which the caseino-glyco-
15 macropeptide has been removed.

Removal of the CGMP from sweet whey results in a protein material with a threonine content closer to that of human milk. This modified sweet whey can then be supplemented with those amino acids in respect of which it has a low content (principally histidine and arginine). A
20 process for removing CGMP from sweet whey is described in EP 880902.

For the process of the invention, the proteinaceous material is diluted or reconstituted to solutions or suspensions, preferably comprising around 2-35% by weight of proteinaceous material, more preferably around 5-30% by weight.

25

In the process of the invention, the milk-based proteinaceous material is treated with at least two endopeptidases, which have both been produced from a microorganism.

Such endopeptidases to be used in the process of the invention may be produced from a mi-
30 croorganism of any genus. For purposes of the present invention, the term “produced from” as used herein in connection with a given organism shall mean that the polypeptide used according to the invention is produced by fermentation of a cell of the given organism. The polypeptide may be native to the organism from which it is produced or it may be produced heterologously from a host organism in which a nucleotide sequence encoding the polypep-
35 tide has been inserted.

One of the endopeptidases to be used in a process according to the invention is a trypsin-like

endopeptidase. The term "trypsin-like endopeptidase" is defined herein as an endopeptidase which preferentially cleaves peptides or proteins at the C-terminal side of the L-isomer of arginine and/or lysine.

5 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.

10 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.

15 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.

20 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. 25

A trypsin-like endopeptidase according to the invention in a preferred embodiment has a Trypsin ratio of more than 100, wherein the Trypsin ratio is determined as the activity of the enzyme when cleaving after Arg or Lys (whichever is the larger) divided by the activity of the enzyme when cleaving after any one of Ala, Asp, Glu, Ile, Leu, Met, Phe or Val (whichever is 30 the larger). I.e., in a preferred embodiment, a trypsin-like endopeptidase according to the invention has a specificity for cleaving after Arg 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 or Val (whichever is the larger). Such activity measurements to determine the Trypsin ratio should be performed at a pH-value where the activity of the endopeptidase is at 35 least half of the activity of the endopeptidase at its pH optimum. The Trypsin ratio may be determined as described in Example 1 of the present application.

Typically, such trypsin-like endopeptidase has optimal proteolytic activity at a pH from about 6.0 to about 11.0, preferably at a pH from about 8 to about 10, and at a temperature from about 40°C to about 70°C, preferably at a temperature from about 45°C to about 65°C or from about 45°C to about 60°C.

5

In a preferred embodiment, the trypsin-like endopeptidase is a fungal endopeptidase. In a more preferred embodiment, the trypsin-like endopeptidase is derived from a strain of *Fusarium*, preferably *Fusarium oxysporum*. It may, e.g., have the amino acid sequence of the mature polypeptide of SEQ ID NO: 2 of the present application (SWISSPROT No. P35049). A
10 trypsin-like endopeptidase from *Fusarium oxysporum* having the amino acid sequence shown as amino acids 25-248 of SEQ ID NO: 2 has previously been described (US5,288,627; US5,693,520).

In one embodiment, the trypsin-like endopeptidase is derived from *Fusarium solani*, e.g.
15 AP977S having the amino acid sequence shown as SEQ ID NO: 4 of the present application (GENESEQP: ADZ80577). In another embodiment, the trypsin-like endopeptidase is derived from *Fusarium* cf. *solani*, e.g. AP971 having the amino acid sequence shown as SEQ ID NO: 6 of the present application.

20 For purposes of the present invention, the term “derived from” as used herein in connection with deriving a polynucleotide or a polypeptide from a given source (i.e., a biological organism) may mean that the polynucleotide (or the polynucleotide encoding the polypeptide) is identical to or a variant of a polynucleotide sequence naturally present in that source organism, irrespective if the polynucleotide sequence has been inserted into or the polypeptide is
25 produced by another organism.

In a preferred embodiment, the trypsin-like endopeptidase is produced from a fungus. In a more preferred embodiment, the trypsin-like endopeptidase is produced from a strain of *Fusarium*.

30

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

i) a polypeptide comprising an amino acid sequence having at least 60% identity to the mature polypeptide of any of SEQ ID NOs: 2, 4 or 6;

35 ii) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with (i) the mature polypeptide coding sequence of any of SEQ ID NOs: 1, 3 or 5, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of any

of SEQ ID NOs: 1, 3 or 5, or (iii) a full-length complementary strand of (i) or (ii);
iii) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% identity to the mature polypeptide coding sequence of any of SEQ ID NOs: 1, 3 or 5; and
5 iv) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of any of SEQ ID NOs: 2, 4 or 6.

The term “mature polypeptide” is defined herein as a polypeptide having endopeptidase activity that is in its final form following translation and any post-translational modifications,
10 such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc.

The term “mature polypeptide coding sequence” is defined herein as a nucleotide sequence that encodes a mature polypeptide having endopeptidase activity.

15 The mature polypeptide of SEQ ID NO: 2 may be amino acids 25-248. The mature polypeptide of SEQ ID NO: 4 may be amino acids 26-251. The mature polypeptide of SEQ ID NO: 6 may be amino acids 18-250.

For purposes of the present invention, the degree of identity between two amino acid se-
20 quences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends in Genetics 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLO-
25 SUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:
(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)
For purposes of the present invention, the degree of identity between two deoxyribonucleo-
30 tide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, supra) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, supra), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) sub-
35 stitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:
(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in

Alignment)

In one preferred embodiment of the invention, the trypsin-like endopeptidase comprises an amino acid sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98% identity to the mature polypeptide of SEQ ID NO: 2. In a more preferred embodiment of the invention, the trypsin-like endopeptidase comprises the amino acid sequence of the mature polypeptide of SEQ ID NO: 2. In another more preferred embodiment of the invention, the trypsin-like endopeptidase comprises amino acids 25 to 248 of SEQ ID NO: 2. In an even more preferred embodiment of the invention, the trypsin-like endopeptidase consists of the amino acid sequence of the mature polypeptide of SEQ ID NO: 2. In another even more preferred embodiment of the invention, the trypsin-like endopeptidase consists of amino acids 25 to 248 of SEQ ID NO: 2.

In another preferred embodiment of the invention, the trypsin-like endopeptidase comprises an amino acid sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98% identity to the mature polypeptide of SEQ ID NO: 4. In a more preferred embodiment of the invention, the trypsin-like endopeptidase comprises the amino acid sequence of the mature polypeptide of SEQ ID NO: 4. In another more preferred embodiment of the invention, the trypsin-like endopeptidase comprises amino acids 26 to 251 of SEQ ID NO: 4. In an even more preferred embodiment of the invention, the trypsin-like endopeptidase consists of the amino acid sequence of the mature polypeptide of SEQ ID NO: 4. In another even more preferred embodiment of the invention, the trypsin-like endopeptidase consists of amino acids 26 to 251 of SEQ ID NO: 4.

In another preferred embodiment of the invention, the trypsin-like endopeptidase comprises an amino acid sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98% identity to the mature polypeptide of SEQ ID NO: 6. In a more preferred embodiment of the invention, the trypsin-like endopeptidase comprises the amino acid sequence of the mature polypeptide of SEQ ID NO: 6. In another more preferred embodiment of the invention, the trypsin-like endopeptidase comprises amino acids 18 to 250 of SEQ ID NO: 6. In an even more preferred embodiment of the invention, the trypsin-like endopeptidase consists of the amino acid sequence of the mature polypeptide of SEQ ID NO: 6. In another even more preferred embodiment of the invention, the trypsin-like endopeptidase consists of amino acids 18 to 250 of SEQ ID NO: 6.

In another preferred embodiment of the invention, the trypsin-like endopeptidase is encoded by a polynucleotide that hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions, with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) a full-length complementary strand of (i) or (ii). (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, New York).

In another preferred embodiment of the invention, the trypsin-like endopeptidase is encoded by a polynucleotide that hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions, with (i) the mature polypeptide coding sequence of SEQ ID NO: 3, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 3, or (iii) a full-length complementary strand of (i) or (ii).

In another preferred embodiment of the invention, the trypsin-like endopeptidase is encoded by a polynucleotide that hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions, with (i) the mature polypeptide coding sequence of SEQ ID NO: 5, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 5, or (iii) a full-length complementary strand of (i) or (ii).

In the context of the present invention, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS preferably at 45°C (very low stringency), more preferably at 50°C (low stringency), more preferably at 55°C (medium stringency), more preferably at 60°C (medium-high stringency), even more preferably at 65°C (high stringency), and most preferably at 70°C (very high stringency).

In another preferred embodiment of the invention, the trypsin-like endopeptidase is encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98%, identity to the mature polypeptide coding sequence of SEQ ID NO: 1.

In another preferred embodiment of the invention, the trypsin-like endopeptidase is encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98%, identity to the mature polypeptide coding sequence of SEQ ID NO: 3.

In another preferred embodiment of the invention, the trypsin-like endopeptidase is encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98%, identity to the mature polypeptide coding sequence of SEQ ID NO: 5.

In another preferred embodiment of the invention, the trypsin-like endopeptidase is a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of any of SEQ ID NOs: 2, 4 or 6.

In the context of the present invention, such variant may be an allelic (natural) variant or it may be an artificial variant. It may comprise a substitution, deletion, and/or insertion of one or more (or several) amino acids of a mature polypeptide. Preferably, amino acid changes are 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 one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 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.

The concentration of trypsin-like endopeptidase is preferably 1,000-1,000,000 USP Trypsin Units per g milk-based protein, more preferably 5,000-500,000, and most preferably 25,000-250,000.

One USP Trypsin Unit is the activity causing a change in absorbance at 253 nm of 0.003 at

pH 7.6 and 25°C using N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrate.

The specific activity may vary quite significantly among different trypsin-like endopeptidases, but the skilled person will easily be able to determine in which amount the trypsin-like endopeptidase is to be used, e.g. based on the degree of hydrolysis.

The ratio of trypsin-like endopeptidase to milk-based protein is preferably 0.01-10% weight/weight, more preferably 0.01-5%, more preferably 0.05-2.5%, even more preferably 0.5-1%, and most preferably around 0.75%.

In a process according to the present invention, a milk-based proteinaceous material is treated with a trypsin-like endopeptidase and at least one other endopeptidase.

In a preferred embodiment, the at least one other endopeptidase is a serine endopeptidase.

In another preferred embodiment, the at least one other endopeptidase has an activity which is less specific than the trypsin-like endopeptidase.

In another preferred embodiment, the at least one other endopeptidase has an activity which resembles the activity of mammalian chymotrypsin, e.g., chymotrypsin extracted from porcine pancreatic tissue.

In another preferred embodiment, the at least one other endopeptidase has a higher specificity for cleaving at the carboxy-terminal side of either of tyrosine, phenylalanine, tryptophan, leucine, methionine or histidine than for cleaving on the carboxy-terminal side of any other natural amino acid.

In another preferred embodiment, the at least one other endopeptidase has a specificity for cleaving at the carboxy-terminal side of at least one of tyrosine, phenylalanine, tryptophan, leucine, methionine or histidine, which is at least 3-fold higher, preferably at least 5-fold higher, than its specificity for cleaving at the carboxy-terminal side of either one of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, isoleucine, lysine, proline, serine, threonine and valine.

In another preferred embodiment, the at least one other endopeptidase has a higher specificity for cleaving at the carboxy-terminal side of each of at least three amino acids from the group consisting of tyrosine, phenylalanine, tryptophan, leucine, methionine and histidine

than for cleaving on the carboxy-terminal side of arginine.

In another preferred embodiment, the at least one other endopeptidase has a higher specificity for cleaving at the carboxy-terminal side of each of at least three amino acids from the group consisting of tyrosine, phenylalanine, tryptophan, leucine, methionine and histidine than for cleaving on the carboxy-terminal side of lysine.

In another preferred embodiment, the at least one other endopeptidase has a higher specificity for cleaving at the carboxy-terminal side of each of tyrosine, phenylalanine, tryptophan, leucine, methionine and histidine than for cleaving on the carboxy-terminal side of both of arginine and lysine.

In another preferred embodiment, the at least one other endopeptidase has a Chymotrypsin ratio of at least 3, preferably at least 5. A Chymotrypsin ratio of at least 5 means that the activity of the enzyme when cleaving after one of Phe, Leu or Met (whichever is the larger) is at least five times higher than the activity when cleaving after any one of Ala, Arg, Asp, Glu, Ile, Lys or Val (whichever is the larger). I.e., the at least one other endopeptidase has a specificity for cleaving after one of Phe, Leu or Met (whichever is the larger) which is at least 3-fold higher, preferably at least 5-fold higher, than its specificity for cleaving after any one of Ala, Arg, Asp, Glu, Ile, Lys or Val (whichever is the larger). Such activity measurements to determine the Chymotrypsin ratio 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. The Chymotrypsin ratio may be determined as described in Example 1 of the present application.

In another preferred embodiment, the at least one other endopeptidase is a bacterial endopeptidase. In a more preferred embodiment, the at least one other endopeptidase is derived from a strain of *Nocardiopsis*, preferably from *Nocardiopsis sp*. NRRL 18262 (previously described in, e.g., WO 88/03947). It may, e.g., have the amino acid sequence of the mature polypeptide of SEQ ID NO: 8 of the present application. The DNA and amino acid sequences of the protease derived from *Nocardiopsis sp*. NRRL 18262 have previously been published in, e.g., DK patent application no. 1996 00013.

In another more preferred embodiment, the at least one other endopeptidase is derived from *Metarhizium*, preferably *Metarhizium anisopliae*, e.g. having the amino acid sequence of the mature polypeptide of SEQ ID NO: 10 of the present application (TREMBL:Q9Y843). In another more preferred embodiment, the at least one other endopeptidase is derived from *Brachysporiella*, preferably *Brachysporiella gayana*, e.g. having the amino acid sequence of

the mature polypeptide of SEQ ID NO: 12 of the present application (CGMCC 0865). The DNA and amino acid sequences of the proteases derived from *Metarhizium anisopliae* and *Brachysporiella gayana* have previously been published in, e.g., WO04072279.

5 In another preferred embodiment, the at least one other endopeptidase is produced from a bacterium.

In another preferred embodiment of the invention, the at least one other endopeptidase is selected from the group consisting of:
10 i) a polypeptide comprising an amino acid sequence having at least 60% identity to the mature polypeptide of any of SEQ ID NOs: 8, 10 or 12;
ii) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with (i) the mature polypeptide coding sequence of any of SEQ ID NOs: 7, 9 or 11, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of any
15 of SEQ ID NOs: 7, 9 or 11, or (iii) a full-length complementary strand of (i) or (ii);
iii) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% identity to the mature polypeptide coding sequence of any of SEQ ID NOs: 7, 9 or 11; and
iv) a variant comprising a substitution, deletion, and/or insertion of one or more (several)
20 amino acids of the mature polypeptide of any of SEQ ID NOs: 8, 10 or 12.

The mature polypeptide of SEQ ID NO: 10 may be amino acids 187-374. The mature polypeptide of SEQ ID NO: 12 may be amino acids 190-375.

25 In another preferred embodiment of the invention, the at least one other endopeptidase comprises an amino acid sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98% identity to the mature polypeptide of SEQ ID NO: 8. In a more preferred embodiment of the invention, the at least one other endopeptidase comprises the
30 amino acid sequence of the mature polypeptide of SEQ ID NO: 8. In another more preferred embodiment of the invention, the at least one other endopeptidase comprises amino acids 1 to 188 of SEQ ID NO: 8. In an even more preferred embodiment of the invention, the at least one other endopeptidase consists of the amino acid sequence of the mature polypeptide of SEQ ID NO: 8. In another even more preferred embodiment of the invention, the at least one
35 other endopeptidase consists of amino acids 1 to 188 of SEQ ID NO: 8.

In another preferred embodiment of the invention, the at least one other endopeptidase com-

prises an amino acid sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98% identity to the mature polypeptide of SEQ ID NO: 10. In a more preferred embodiment of the invention, the at least one other endopeptidase comprises the amino acid sequence of the mature polypeptide of SEQ ID NO: 10. In another more preferred embodiment of the invention, the at least one other endopeptidase comprises amino acids 187 to 374 of SEQ ID NO: 10. In an even more preferred embodiment of the invention, the at least one other endopeptidase consists of the amino acid sequence of the mature polypeptide of SEQ ID NO: 10. In another even more preferred embodiment of the invention, the at least one other endopeptidase consists of amino acids 187 to 374 of SEQ ID NO: 10.

In another preferred embodiment of the invention, the at least one other endopeptidase comprises an amino acid sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98% identity to the mature polypeptide of SEQ ID NO: 12. In a more preferred embodiment of the invention, the at least one other endopeptidase comprises the amino acid sequence of the mature polypeptide of SEQ ID NO: 12. In another more preferred embodiment of the invention, the at least one other endopeptidase comprises amino acids 190 to 375 of SEQ ID NO: 12. In an even more preferred embodiment of the invention, the at least one other endopeptidase consists of the amino acid sequence of the mature polypeptide of SEQ ID NO: 12. In another even more preferred embodiment of the invention, the at least one other endopeptidase consists of amino acids 190 to 375 of SEQ ID NO: 12.

In another preferred embodiment of the invention, the at least one other endopeptidase is encoded by a polynucleotide that hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions, with (i) the mature polypeptide coding sequence of SEQ ID NO: 7, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 7, or (iii) a full-length complementary strand of (i) or (ii).

In another preferred embodiment of the invention, the at least one other endopeptidase is encoded by a polynucleotide that hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions, with (i) the mature polypeptide coding sequence of SEQ ID NO: 9, (ii) the genomic DNA sequence comprising the mature polypeptide

coding sequence of SEQ ID NO: 9, or (iii) a full-length complementary strand of (i) or (ii).

In another preferred embodiment of the invention, the at least one other endopeptidase is encoded by a polynucleotide that hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions, with (i) the mature polypeptide coding sequence of SEQ ID NO: 11, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 11, or (iii) a full-length complementary strand of (i) or (ii).

In another preferred embodiment of the invention, the at least one other endopeptidase is encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98%, identity to the mature polypeptide coding sequence of SEQ ID NO: 7.

In another preferred embodiment of the invention, the at least one other endopeptidase is encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98%, identity to the mature polypeptide coding sequence of SEQ ID NO: 9.

In another preferred embodiment of the invention, the at least one other endopeptidase is encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 85% or at least 90%, even more preferably at least 95%, and most preferably at least 98%, identity to the mature polypeptide coding sequence of SEQ ID NO: 11.

In another preferred embodiment of the invention, the trypsin-like endopeptidase is a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of any of SEQ ID NOs: 8, 10 or 12.

The concentration of the at least one other endopeptidase is preferably 100-100,000 USP Chymotrypsin Units per g milk-based protein, more preferably 500-50,000, and most preferably 1,000-20,000.

One USP Chymotrypsin Unit is the activity causing a change in absorbance at 237 nm of 0.0075 at pH 7.0 and 25°C using N-acetyl-L-tyrosine ethyl ester (ATEE) as substrate.

The specific activity may vary quite significantly among different endopeptidases, but the skilled person will easily be able to determine in which amount the at least one other endopeptidase is to be used, e.g. based on the degree of hydrolysis.

The ratio of the at least one other endopeptidase to milk-based protein is preferably 0.001-1% weight/weight, more preferably 0.001-0.5%, more preferably 0.005-0.25%, even more preferably 0.02-0.1%, and most preferably around 0.05%.

Preferably, the at least one other endopeptidase is added at a concentration which is between 2% and 50% of the concentration of trypsin-like endopeptidase added based on the weight of the endopeptidases, more preferably between 5% and 20%, even more preferably between 5% and 15%, and most preferably about 10%.

Preferably, the activity of the trypsin-like endopeptidase measured in USP Trypsin Units is between 5-fold and 500-fold higher, more preferably between 10-fold and 200-fold higher, than the activity of the at least one other endopeptidase measured in USP Chymotrypsin Units.

An optional preliminary step prior to hydrolysis is pre-heating of the solution or suspension of milk-based proteinaceous material, e.g., to ensure denaturation of whey protein fractions, e.g., serum albumin (BSA), α-lactalbumin, β-lactoglobulin and immunoglobulins (particularly IgG). This step usually results in a diminished residual antigenicity when assessed immunochemically (as described hereinafter). In a preferred embodiment, a pre-treatment step is performed which comprises heating of the proteinaceous material at about 75-95°C for about 5-30 minutes. In another preferred embodiment, a pre-treatment step is performed which comprises heating of the proteinaceous material at above 135°C for about 1-5 seconds. In another preferred embodiment, a pre-treatment step is performed which comprises heating of the proteinaceous material at about 130°C for about 30-60 seconds.

The skilled person will know which conditions to preferably apply for the hydrolysis reaction. It may be carried out, e.g., as disclosed in US5039532 or EP0631731A1. It may, e.g., be conducted at a temperature of about 40°C to 60°C, during 1 to 6 hours, at pH values within the range 6.5 to 8.5, preferably 6.5 to 8.

In a preferred embodiment, following a first treatment with the peptidases, the proteinaceous material is further subjected to a second proteolytic hydrolysis followed by endopeptidase inactivation. In a more preferred embodiment, the proteinaceous material is subjected to a heat treatment in between the first and the second proteolytic hydrolysis as disclosed in 5 US5039532.

Irrespective of the conditions of the hydrolysis, the hydrolysate preferably is subjected to an additional step of inactivation of the endopeptidases. This peptidase inactivation in a preferred embodiment comprises a heat treatment of about 0.1 to 30 min at a temperature of 10 about 70 to 110°C, preferably 75 to 95°C. Alternatively, the endopeptidases may be inactivated by sterilization at ultra-high temperature (e.g., at about 130°C for about 30-60 seconds).

The protein hydrolysate obtained may be further clarified. It may be stored in a liquid state. 15 The hydrolysate may also be ultrafiltrated, it may be concentrated, e.g., by evaporation, and it may be dried, e.g., by spray drying or lyophilization.

In a preferred embodiment, the protein hydrolysate obtained has a moderate degree of hydrolysis. In another preferred embodiment, the protein hydrolysate obtained is a partial hy-20 drolysate. In another preferred embodiment, the protein hydrolysate obtained has a degree of hydrolysis of between 5 and 30%, preferably between 10 and 25% and more preferably between 12 and 20%. A particularly preferred degree of hydrolysis is around 14%. Another particularly preferred degree of hydrolysis is around 15%.

25 The degree of hydrolysis (DH) expresses the extent of the protein hydrolysis obtained by the method. In the context of the invention, the degree of hydrolysis (DH) is defined as follows:

DH = (Number of peptide bonds cleaved / Total number of peptide bonds) x 100 %

30 Degree of hydrolysis (DH) of the protein hydrolysate obtained may be measured spectrophotometrically according to the method of Church, F. C. et al. (1983) Spectrophotometric Assay Using *o*-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolated Milk Proteins, *J. Dairy Sci.* **66**: 1219-1227.

35 The molecular weight distribution of the peptides in the protein hydrolysate obtained may be determined, e.g., by size exclusion chromatography (SEC). In a preferred embodiment, the hydrolysate of the invention is comprised of peptides where less than 1% on a weight-basis

has a molecular weight of above 20,000 kDa.

The hydrolysate obtained by the method of the invention is preferably devoid of detectable intact milk protein. The absence of intact milk protein in the hydrolysate may be demonstrated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Direct comparison between a hydrolysate and the non-hydrolyzed protein starting material can be made in the same gel. In a preferred embodiment, the hydrolysate of the invention is comprised of peptides where less than 1% on a weight-basis is intact milk-based protein.

The residual antigenicity of the hydrolysate obtained by the method of the invention may be determined using an enzyme-linked immunosorbent assay (ELISA). Non-hydrolyzed milk protein is immobilized on a solid phase at concentrations that fall within the linear dose response range established in the assay. Hydrolysate preparations are similarly immobilized. Subsequent, sequential incubations with rabbit anti-cow milk protein and an enzyme conjugate reactive with rabbit IgG reveals the presence of antigenically recognizable proteins and peptides. Results obtained with the hydrolysate are compared on a mass basis to those obtained with the non-hydrolyzed protein starting material. The percent antigenicity reduction of the hydrolysate is then calculated.

In a preferred embodiment, the protein hydrolysate obtained by a process according to the invention has a reduction in antigenicity of at least about 80%, preferably at least about 85%, more preferably at least about 90% or at least about 95%, most preferably at least about 98%, and even most preferably the reduction in antigenicity is at least about 99%, relative to the corresponding non-hydrolyzed milk-based proteinaceous material as measured by ELISA.

In a preferred embodiment, the protein hydrolysate obtained by a process according to the invention is for an infant formula composition.

In a preferred embodiment, the invention relates to a process for the preparation of an infant formula composition, which comprises the steps of obtaining a milk-based protein hydrolysate as disclosed above and which further comprises a step of including the milk-based protein hydrolysate into an infant formula composition.

Such infant formula composition may be in the form of a powder, a concentrated liquid, or a ready-to-use liquid.

Such infant formula composition preferably contains ingredients which are designed to meet the nutritional needs of a human infant. Thus, in addition to a protein hydrolysate obtained according to a process of the invention, the infant formula should preferably contain a lipid source, a carbohydrate source and other nutrients such as vitamins and minerals. Typically, animal oils, vegetable oils, starch, sucrose, lactose and/or corn syrup solids may be added to the formula to supply part or all of the above nutrients.

It is preferred that an infant formula composition comprising a protein hydrolysate of the invention is nutritionally complete. By the term "nutritionally complete" is meant that the composition contains adequate nutrients to sustain healthy human life for extended periods.

In a preferred embodiment, an infant formula composition comprising a protein hydrolysate of the invention additionally includes free arginine in an amount of from 0.1 to 2 % by weight of protein and/or free histidine in an amount of from 0.1 to 3 % by weight of protein. Addition of free arginine and/or free histidine is particularly preferred if modified sweet whey or whey protein isolate is used as the milk-based proteinaceous material in the process of the invention.

In another preferred embodiment, an infant formula composition comprising a protein hydrolysate of the invention is in the form of a nutritionally complete composition comprising an amount of milk-based protein hydrolysate of at least 5% dry solid, preferably about 10 to 30% dry solid.

EXAMPLES

Example 1

Microbial alternative to extracted porcine pancreatic trypsin preparation

Pancreatic Trypsin Novo 6.0S (available from Novozymes A/S – in the following PTN) is a product produced by extraction of porcine pancreatic tissue. The main components in PTN are trypsin and chymotrypsin with a ratio trypsin:chymotrypsin of at least 12.5:1 (activity basis, i.e. USP Trypsin Units:USP Chymotrypsin Units).

To identify a suitable microbial alternative to an extracted enzyme preparation like PTN, the activity at pH 9 of different microbial proteases on 10 different Suc-AAPX-pNA substrates available from Bachem (X = A, R, D, E, I, L, K, M, F and V) was measured. Based on these measurements, the Trypsin ratio (TR) and the Chymotrypsin ratio (CR) for each microbial

protease were calculated. The Trypsin ratio and the Chymotrypsin ratio are defined as follows:

TR = max activity on Suc-AAP(R/K)-pNA / max activity on Suc-AAPnon(R/K)-pNA
5 CR = max activity on Suc-AAP(F/L/M)-pNA / max activity on Suc-AAPnon(F/L/M)-pNA

Trypsin ratio:

Trypsins are specific serine endopeptidases that cleave on the carboxy terminal side of either an arginine residue or a lysine residue, i.e. they have a strict preference for R or K in the
10 P1 position. Therefore, a reasonable definition of a trypsin-like protease is that the Trypsin ratio is > 100, meaning that the activity on any of the 8 other Suc-AAPnon(R/K)-pNA substrates are less than 1% of the activity on the best Suc-AAP(R/K)-pNA substrate.

Chymotrypsin ratio:

15 Chymotrypsins are known to have a less strict preference for cleaving on the carboxy terminal side of either aromatic amino acid residues (Trp, Tyr or Phe) or the hydrophobic amino acid residues Leu, Met and His. As compared to trypsin, chymotrypsin is a less specific endopeptidase, a reasonable definition of a chymotrypsin-like protease is that the Chymotrypsin ratio is > 5, meaning that the activity on any of the 7 other Suc-AAPnon(F/L/M)-
20 pNA substrates are less than 20% of the activity on the best Suc-AAP(F/L/M)-pNA substrate.

Suc-AAPX-pNA assay:

Proteases: PTN
25 Porcine trypsin (UNIPROT:P00761)
Fusarium trypsin (trypsin-like protease from *Fusarium oxysporum*, SEQ ID NO: 2)
Bovine TLCK-treated chymotrypsin (Sigma, C-3142)
Alcalase (available from Novozymes A/S)
30 Bacillopeptidase F (from *B. licheniformis*, UNIPROT:Q65JX1)
Brachysporiella protease (from *Brachysporiella gayana*, SEQ ID NO: 12)
Esperase (available from Novozymes A/S)
Metarhizium protease (from *Metarhizium anisopliae*, SEQ ID NO: 10)
Nocardiopsis Protease (from *Nocardiopsis sp.* NRRL 18262, SEQ ID NO: 8)
35 Savinase (available from Novozymes A/S)

Substrates: Suc-AAPA-pNA (Bachem L-1775)

Suc-AAPR-pNA (Bachem L-1720)
Suc-AAPD-pNA (Bachem L-1835)
Suc-AAPE-pNA (Bachem L-1710)
Suc-AAPI-pNA (Bachem L-1790)
5 Suc-AAPL-pNA (Bachem L-1390)
Suc-AAPK-pNA (Bachem L-1725)
Suc-AAPM-pNA (Bachem L-1395)
Suc-AAPF-pNA (Bachem L-1400)
Suc-AAPV-pNA (Bachem L-1770)

10 Temperature: Room temperature (25°C)

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

15 Assay: 20 µl peptidase dilution (diluted in 0.01% Triton X-100) was placed in a well in a Microtiter plate. The assay was started by adding 200 µl pNA substrate (50 mg dissolved in 1.0 ml DMSO and further diluted 90x with the Assay buffer). The initial increase in OD405 was monitored as a measure of the peptidase activity. If a linear (or near linear) plot was not achieved in the 4 minutes
20 measuring time, the peptidase was diluted further and the assay was repeated.

Data:

Suc-AAPX-pNA	Porcine Trypsin	Fusarium Trypsin	Bovine Chymo-trypsin	Alcalase	Bacillopeptidase F	Brachysporiella Protease	Esperase	Metarhizium Protease	Nocardiopsis Protease	Savinase
Suc-AAPA-pNA	0.0000	0.0000	0.0009	0.0250	1.0000	0.0185	0.1608	0.0237	0.1297	0.1031
Suc-AAPR-pNA	1.0000	1.0000	0.0062	0.0118	0.5819	0.0456	0.0086	0.0374	0.0906	0.0013
Suc-AAPD-pNA	0.0000	0.0000	0.0001	0.0005	0.7323	0.0084	0.0139	0.0075	0.0007	0.0004
Suc-AAPI-pNA	0.0000	0.0000	0.0007	0.0003	0.0023	0.0009	0.0019	0.0007	0.0003	0.0001

Suc-AAPM-pNA	0.0003	0.0000	0.3476	0.3758	0.6546	0.4439	1.0000	0.4210	0.7808	0.9177
Suc-AAPV-pNA	0.0000	0.0000	0.0004	0.0003	0.3544	0.0027	0.0009	0.0020	0.0137	0.0006
Suc-AAPL-pNA	0.0000	0.0000	0.2244	0.8650	0.0557	0.2838	0.6591	0.1940	0.1800	0.1789
Suc-AAPE-pNA	0.0000	0.0000	0.0003	0.0029	0.0144	0.0007	0.0060	0.0004	0.0000	0.0082
Suc-AAPK-pNA	0.5140	0.5307	0.0003	0.0190	0.1562	0.0219	0.0052	0.0264	0.0754	0.0097
Suc-AAPF-pNA	0.0006	0.0000	1.0000	1.0000	0.0089	1.0000	0.3889	1.0000	1.0000	1.0000
Max of Suc-AAP(R/K)-pNA	1.0000	1.0000	0.0062	0.0190	0.5819	0.0456	0.0086	0.0374	0.0906	0.0097
Max of Suc-AAPnon(R/K)-pNA	0.0006	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Trypsin ratio	**1750**	**>10000**	**0.006**	**0.019**	**0.58**	**0.046**	**0.009**	**0.037**	**0.091**	**0.010**
Max of Suc-AAP(F/L/M)-pNA	0.0006	0.0000	1.0000	1.0000	0.6546	1.0000	1.0000	1.0000	1.0000	1.0000
Max of Suc-AAPnon(F/L/M)-pNA	1.0000	1.0000	0.0062	0.0250	1.0000	0.0456	0.1608	0.0374	0.1297	0.1031
Chymotrypsin ratio	**0.0006**	**0.0000**	**160**	**40**	**0.65**	**22**	**6.2**	**27**	**7.7**	**9.7**

The reported activity data for each endopeptidase in the Table is relative to the activity for the best Suc-AAPX-pNA substrate.

5 Conclusion:

Using the above definition of a trypsin-like protease as a protease having a Trypsin ratio of > 100, Porcine trypsin (TR = 1750) and *Fusarium* trypsin (TR > 10,000) are trypsin-like proteases. The rest of the tested proteases are not trypsin-like proteases.

10 Using the above definition of a chymotrypsin-like protease as a protease having a Chymotrypsin ratio of > 5, Bovine chymotrypsin (CR = 160), Alcalase (CR = 40), *Brachysporiella* protease (CR = 22), Esperase (CR = 6.2), *Metarhizium* protease (CR = 27), *Nocardiopsis* protease (CR = 7.7) and Savinase (CR = 9.7) are chymotrypsin-like proteases. The rest of

the tested proteases are not chymotrypsin-like proteases.

An alternative definition of a chymotrypsin-like protease would be to calculate the sum of the squares of the relative activity differences to chymotrypsin. Using this definition, the *Metarhizium* protease comes out as the most chymotrypsin-like serine protease, followed by *Brachysporiella* protease, *Nocardiopsis* protease, Savinase and Alcalase (data not shown).

Example 2

Hydrolysis of WPC with combinations of microbial endopeptidases

The next step was to see if it is possible to get the same degree of hydrolysis (DH) of a Whey Protein Concentrate (WPC) using a mixture of microbial endopeptidases as what can be obtained for PTN when the two enzyme mixtures are dosed equally.

Hydrolysis experiments:

The hydrolysis assay we have performed was a two step hydrolysis procedure. 8.4% WPC (containing 80% whey protein) was suspended in pH 8.0 buffer. The first hydrolysis step was performed by adding half of the enzyme dose and incubating the solution with agitation for 2 hours at 55°C. Then the partly hydrolysed whey protein substrate was denatured by a short heating treatment (5 minutes at 90°C). The second hydrolysis step was performed by adding the other half of the enzyme dose and incubating the solution with agitation for 30 minutes at 55°C. Finally, the enzyme reaction was stopped and the enzymes inactivated by another short heating treatment (10 minutes at 90°C). The final concentration of whey protein/peptides was 6.4% WP. By using this assay we should be able to compare PTN with a microbial alternative.

As H^+ is produced during hydrolysis in the alkaline range, we checked pH for some of the highest enzyme dosages. We couldn't detect any drop in pH, indicating that our pH 8.0 buffer is strong enough to control pH during hydrolysis.

First we tested 4 different doses of PTN (0.625, 1.25, 2.5 and 5.0 mg trypsin/g whey protein). For the lowest dose the whey protein hydrolysate was very viscous and almost impossible to get out of the Eppendorf tube and therefore DH wasn't determined for this hydrolysate. An SDS-PAGE analysis of the PTN hydrolysates showed that at least for the 5.0 mg trypsin/g whey protein dose, most of the intact proteins were degraded. On the following SDS-PAGE

gel, equal amounts of protein/peptides are loaded on each lane. DH = 9.4% for the 5.0 mg trypsin/g whey protein PTN dosage.

We then ran some hydrolysis experiments with purified trypsins (Porcine trypsin and *Fusa-*
5 *rium* trypsin). We couldn't get a DH above approx. 6.5%, even at relatively high dosages. See the Data section below.

Finally, we ran a third series of hydrolysis experiments where we kept the dosage of the *Fusarium* trypsin constant at 5.0 mg trypsin/g whey protein and varied the chymotrypsin-like
10 protease dosages (0.25, 0.5 and 1.0 mg protease/g whey protein). Here we obtained DH-values in the same range as PTN dosed equally (DH = 9.4%).

It is seen that the effect on DH of increasing dosages of the second chymotrypsin-like protease have different effects depending on the second protease. The combination of *Fusarium*
15 trypsin and Glutamyl peptidase BL from *Bacillus licheniformis* (which is not a chymotrypsin-like protease) seems to level off as was seen for *Fusarium* trypsin alone.

There is a correlation between the DH-values (see Data section below) and the appearance of the whey protein hydrolysates on SDS-PAGE gels. A combination of *Fusarium* trypsin and
20 *Nocardiopsis* protease seem (from the SDS-PAGE gels – not shown) to be the best serine protease combination to remove intact proteins from the WPC substrate.

Hydrolysis assay:

25 *Endopeptidases used:*

Enzyme	Conc. (mg/ml)	
	Trypsin	Chymotrypsin
PTN 6.0S	187 mg/g (1310 USP/mg)	18.7 mg/g (84 USP/mg)
Porcine trypsin	3.1	
Fusarium trypsin	8.0	
Metarhizium protease		2.6
Brachysporiella protease		10.6
Alcalase		19.6

Nocardiopsis protease		8.8
Glutamyl peptidase BL	3.7	

For calculation of the trypsin and chymotrypsin content in PTN 6.0S, we have used a specific activity for Porcine trypsin = 7000 USP/mg and a specific activity for Porcine chymotrypsin = 4500 USP/mg. The enzyme concentration in the other preparations was estimated from A280/E280. PTN was dissolved in 20 mM HEPES/NaOH, 5 mM $CaCl_2$, pH 8. Other enzymes were used “as is” after thawing.

Hydrolysis:

84 mg/ml WPC (LactProdan80 from Arla comprising about 80% protein of total dry matter) was suspended in 1.0 M HEPES/NaOH, 50 mM $CaCl_2$, pH 8.0 and the pH was adjusted to pH 8.0 with 27% NaOH. 1000 µl of this suspension was placed in an Eppendorf tube on ice. 25 µl Peptidase solution was added and the Eppendorf tube was transferred to an Eppendorf thermomixer prewarmed to 55°C. The tube was incubated for 120 minutes on the thermomixer (55°C, 1400 rpm). The tube was then transferred to another Eppendorf thermomixer which was prewarmed to 90°C and incubated for 5 minutes on this thermomixer (90°C, 1400 rpm). The tube was transferred back to the first 55°C Eppendorf thermomixer and after 5 minutes (to bring the hydrolysis mixture back to 55°C), 25 µl Peptidase solution was added and the tube was incubated for 30 minutes on the thermomixer (55°C, 1400 rpm). Finally, the tube was again transferred to the 90°C thermomixer and incubated for 10 minutes on this thermomixer (90°C, 1400 rpm).

The hydrolysate (with 64 mg/ml whey protein) was analysed by SDS-PAGE (not shown) and DH was measured using the OPA method.

SDS-PAGE:

The hydrolysate was diluted 5x in 0.1% SDS. 20 µl of this dilution was mixed with 20 µl 2xSDS-PAGE sample buffer with reducing agent. This mixture was boiled and 10 µl was applied to a 4-20% Tris-glycine gel.

OPA method for measuring DH:

The hydrolysate was diluted 80x in 0.01% Triton X-100. 30 µl of this dilution was transferred to a MicroTiterPlate (MTP) and 225 µl freshly prepared OPA reagent was added and after 2 minutes the absorbance at 340 nm was read in a MTP reader. The response of unknown samples were compared with a serine standard dilution series and expressed as mg/ml ser-

ine. "OPA response" was calculated as "mg/ml serine in the hydrolysate" relative to "mg/ml substrate". The "OPA response" of the Enzyme blank was subtracted for calculation of DH.

OPA reagent:

5 3.81 g Di-sodium tetraborate (Merck 6308) and 1.00 g SDS (BIO-RAD 161-0301) was dissolved in 80 ml deionised water. Just before use, 80 mg o-phtaldialdehyde (Merck 821027) dissolved in 2.0 ml ethanol was added and 1.0 ml 10% (w/v) DTE (Merck 24511). Finally, the volume was adjusted to 100 ml with deionised water.

10 Data:

Dosage			Total "OPA response" (%)	DH (%)
Enzyme	Trypsin	Chymotrypsin		
	(mg/g WP)	(mg/g WP)		
Blank	0	0	9.4	0.0
PTN	1.25	0.125	16.6	7.2
PTN	2.5	0.25	17.3	7.9
PTN	**5**	**0.5**	**18.8**	**9.4**
Porcine trypsin	2.5		15.8	6.4
Porcine trypsin	5		16.0	6.6
Fusarium trypsin	5		15.5	6.1
Fusarium trypsin	10		15.9	6.5
Blank	0	0	9.5	0.0
Fusarium trypsin + *Metarhizium*	5	0.25	18,1	8.6
Fusarium* trypsin + *Metarhizium	**5**	**0.5**	**19.3**	**9.8**
Fusarium trypsin + *Metarhizium*	5	1.0	21.6	12.1
Fusarium trypsin + *Brachysporiella*	5	0.25	17.3	7.8
Fusarium trypsin + *Brachysporiella*	5	0.5	17.9	8.4
Fusarium* + *Brachysporiella	**5**	**1.0**	**19.1**	**9.6**
***Fusarium* trypsin + Alcalase**	**5**	**0.25**	**19.6**	**10.1**
Fusarium trypsin + Alcalase	5	0.5	22.4	12.9

Fusarium trypsin + Alcalase	5	1.0	24.1	14.6
Fusarium* trypsin + *Nocardiopsis	**5**	**0.25**	**19.1**	**9.6**
Fusarium trypsin + *Nocardiopsis*	5	0.5	20.0	10.5
Fusarium trypsin + *Nocardiopsis*	5	1.0	21.0	11.5
***Fusarium*+Glutamyl peptidase BL**	**5**	**0.25**	**18.9**	**9.4**
Fusarium + Glutamyl peptidase BL	5	0.5	19.4	9.9
Fusarium + Glutamyl peptidase BL	5	1.0	19.8	10.3

Conclusion:

It seems to be possible to get the same degree of hydrolysis (DH) of WPC using a microbial PTN-replacer (dosed as PTN). It is also obvious from the results that a trypsin-like protease
5 alone will not be able to give the same DH as PTN.

Example 3

Cleavage specificity analysis of Fusarium trypsin and Brachysporiella endopeptidase

10 Introduction:

The proteolytic cleavage specificity of the microbial trypsin-like endopeptidase, *Fusarium* trypsin, was compared with porcine trypsin. And the proteolytic cleavage specificity of the microbial chymotrypsin-like endopeptidase from *Brachysporiella* was compared with the proteolytic specificity of TLCK treated porcine chymotrypsin. (TLCK inactivates trypsin activity
15 without effecting chymotrypsin).

The cleavage specificity analyses were performed by incubation of the described endopeptidases with the denatured model substrate bovine beta-lactoglobulin A. The sequences of the resulting proteolytic peptides were determined by online RP-HPLC-ESI-Orbitrap MS/MS
20 combined with a tandem mass spectrometry data analysis program used for protein identification (SEQUEST) and a database containing only bovine beta-lactoglobulin A.

Samples:

25 Proteases: Porcine trypsin (UniProt accession: P00761)

Fusarium trypsin (trypsin-like protease from *Fusarium oxysporum*, SEQ ID NO: 2)

Bovine TLCK treated chymotrypsin (Sigma, C-3142)
Brachysporiella protease (from *Brachysporiella gayana*, SEQ ID NO. 12)

Substrate: Beta-lactoglobulin A, from bovine milk (Sigma L7880 097K7010)

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Proteolysis:
Denaturation of beta-lactoglobulin A:
An amount of ca. 11 mg Beta-lactoglobulin A from bovine milk (Sigma L7880 097K7010) was dissolved in 1 ml buffer (100 mM ammoniumacetat, 1 mM $CaCl_2$ pH8. The disulfide bridges
10 of beta-lactoglobulin were reduced by addition of dithiothreitol (DTT, CAS number 3483-12-3) to a final concentration of 20 mM. The mixture was incubated at room temperature (25°C) for 30 min. Subsequently 2-Iodoacetamide (CAS number 144-48-9) was added to a final concentration of 55 mM. The latter mixture was incubated in darkness and at room temperature for 30 min.

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The denatured beta-lactoglobulin A was buffer changed to 100 mM ammoniumacetat, 1 mM $CaCl_2$ pH8 prior to incubation with the proteases. Buffer change was performed on a PD-10 Desalting column from GE-healthcare (Sephadex G-25 column material). First the column was equilibrated with 25 ml 100 mM ammoniumacetat, 1mM $CaCl_2$ pH8. The beta-
20 lactoglobulin sample volume was adjusted to a final volume of 2.5 ml with equilibration buffer and loaded on the column. Beta-lactoglobulin A was eluted with 3.5 ml 100 mM ammoniumacetat, 1mM $CaCl_2$ pH8. The final protein concentration of the bufferchanged beta-lactoglobulin was determined to 2 mg/ml by spectrophotometric analysis at 280 nm and an estimated extinction (A280) coefficient of 1.

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Proteolytic incubation:
An amount of 400 µg beta-lactoglobulin corresponding to 190 µl solution was incubated at 40°C with the following amount of proteases:

Porcine trypsin	8 µg
Fusarium trypsin	16 µg
Bovine TLCK treated chymotrypsin	4 µg
Brachysporiella protease	4 µg

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The proteolysis process was stopped after 18 hours by addition of aqueous solution of 10%
35 trifluoroacetic acid (TFA, CAS number 76-05-1) to a final concentration of 1% TFA in the sample. All samples were stored at -20°C prior analysis by LC-MS/MS

LC-MS/MS method:

All proteolytic samples were analyzed on a RP-HPLC-ESI-Orbitrap MS/MS system consisting of Waters C18 column (ACQUITY UPLC® BEH C18, 1.7 µm, 2.1 x 100 mm), an Accela liquid chromatography system from Thermo Scientific and a LTQ Orbitrap XL ETD hybrid mass
5 spectrometer from Thermo Scientific. A volume of 20 µl was injected onto the column. The peptides were separated by the following gradient:

Solvent A: 0.1% formic acid (CAS number 64-18-6) in UHQ water and solvent B: 0.1% formic acid in acetonitrile (CAS number 75-05-8)
10

Time (min)	%B solvent
0	5
2	5
49	50
51	90
53	90
55	5
60	5

The eluting peptides were monitored online by a UV-detector at 214 nm and subsequently online by a mass spectrometer in MS/MS mode. The resulting UV-chromatograms are shown in Figures 1 and 2. The precursor ion in the mass spectrometer was detected at a resolution
15 of 30,000 (FWHM) and the fragment ions at a resolution of 15,000 (FWHM). The resulting MS and MS/MS spectra were analyzed with SEQUEST software (algorithm subject to U.S. patents 6,017,693 and 5,538,897) via Proteome Discoverer (version 1.0, Thermo Fisher Scientific) against beta-lactoglobulin. The cleaving enzyme was defiened as “No-enzyme” with “unspecific”cleavage specificity. Proteolytic fragments of some of the high intensive UV-
20 peaks were identified as indicated in the Figures 1 and 2.

Results:

For comparison the UV-chromatogram of the *Fusarium* trypsin assay (bottom trace) is displayed in Figure 1 together with porcine trypsin assay (upper trace) and the sequence iden-
25 tity of some major peptides are displayed. Likewise, in Figure 2, the UV-chromatogram of *Brachysporiella* protease assay (bottom trace) is displayed together with bovine chymotryp-

sin (upper trace). In all cases, the identified peptides have been detected in both the upper and the bottom trace.

Conclusion:

5 In Figure 1, common proteolytic peptides of bovine beta-lactoglobulin A are detected for *Fusarium* trypsin and porcine trypsin, thus the two endoproteases both have trypsin like specificity. In Figure 2, some common proteolytic peptides of bovine beta-lactoglobulin A are detected for *Brachysporiella* protease and bovine chymotrypsin.

CLAIMS

1. A process for the preparation of a milk-based protein hydrolysate comprising treatment of a solution of a milk-based proteinaceous material with
   a) a trypsin-like endopeptidase produced from a microorganism, and
   5 b) at least one other endopeptidase produced from a microorganism.

2. A process according to claim 1, wherein the trypsin-like endopeptidase is derived from a strain of *Fusarium*.

10 3. A process according to any of the preceding claims, wherein the trypsin-like endopeptidase is selected from the group consisting of:
   i) a polypeptide comprising an amino acid sequence having at least 60% identity to the mature polypeptide of any of SEQ ID NOs: 2, 4 or 6;
   ii) a polypeptide encoded by a polynucleotide that hybridizes under at least low strin-
15 gency conditions with (i) the mature polypeptide coding sequence of any of SEQ ID NOs: 1, 3 or 5, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of any of SEQ ID NOs: 1, 3 or 5, or (iii) a full-length complementary strand of (i) or (ii);
   iii) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having
20 at least 60% identity to the mature polypeptide coding sequence of any of SEQ ID NOs: 1, 3 or 5; and
   iv) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of any of SEQ ID NOs: 2, 4 or 6.

25 4. A process according to any of the preceding claims, wherein the ratio of trypsin-like endopeptidase to milk-based protein is 0.01-10% weight/weight, preferably 0.01-5%, more preferably 0.05-2.5%, even more preferably 0.5-1%.

5. A process according to any of the preceding claims, wherein the at least one other
30 endopeptidase has a higher specificity for cleaving at the carboxy-terminal side of tyrosine, phenylalanine, tryptophan, leucine, methionine and histidine than for cleaving on the carboxy-terminal side of arginine and lysine.

6. A process according to any of the preceding claims, wherein the at least one other
35 endopeptidase has a Chymotrypsin Ratio of at least 3.

7. A process according to any of the preceding claims, wherein the at least one other endopeptidase is derived from a strain of *Nocardiopsis, Metarhizium* or *Brachysporiella*.

8. A process according to any of the preceding claims, wherein the at least one other
5 endopeptidase is selected from the group consisting of:
i) a polypeptide comprising an amino acid sequence having at least 60% identity to the mature polypeptide of any of SEQ ID NOs: 8, 10 or 12;
ii) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with (i) the mature polypeptide coding sequence of any of SEQ ID
10 NOs: 7, 9 or 11, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of any of SEQ ID NOs: 7, 9 or 11, or (iii) a full-length complementary strand of (i) or (ii);
iii) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% identity to the mature polypeptide coding sequence of any of SEQ ID NOs:
15 7, 9 or 11; and
iv) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of any of SEQ ID NOs: 8, 10 or 12.

9. A process according to any of the preceding claims, wherein the ratio of the at least one
20 other endopeptidase to milk-based protein is 0.001-1% weight/weight, preferably 0.001-0.5%, more preferably 0.005-0.25%, even more preferably 0.02-0.1%.

10. A process according to any of the preceding claims, wherein the at least one other endopeptidase is added at a concentration which is between 2% and 50% of the concen-
25 tration of trypsin-like endopeptidase added based on the weight of the endopeptidases.

11. A process according to any of the preceding claims, wherein hydrolysis is conducted at a temperature of about 40°C to 60°C, during 1 to 6 hours, at pH values within the range 6.5 to 8.5.

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12. A process according to any of the preceding claims, wherein the protein hydrolysate obtained has a degree of hydrolysis of between 5 and 30%, preferably between 10 and 25% and more preferably between 12 and 20%.

35 13. A process according to any of the preceding claims, wherein the protein hydrolysate obtained is comprised of peptides where less than 1% on a weight-basis has a molecular weight of above 20,000 kDa.

14. A process according to any of the preceding claims, wherein the protein hydrolysate obtained has a reduction in antigenicity of at least about 80%, preferably at least about 85%, more preferably at least about 90% or at least about 95%, most preferably at least about 98%, and even most preferably at least about 99%, relative to the corresponding non-hydrolyzed milk-based proteinaceous material as measured by ELISA.

15. A process for the preparation of an infant formula composition according to any of the preceding claims, which further comprises a step of including the milk-based protein hydrolysate obtained into an infant formula composition.

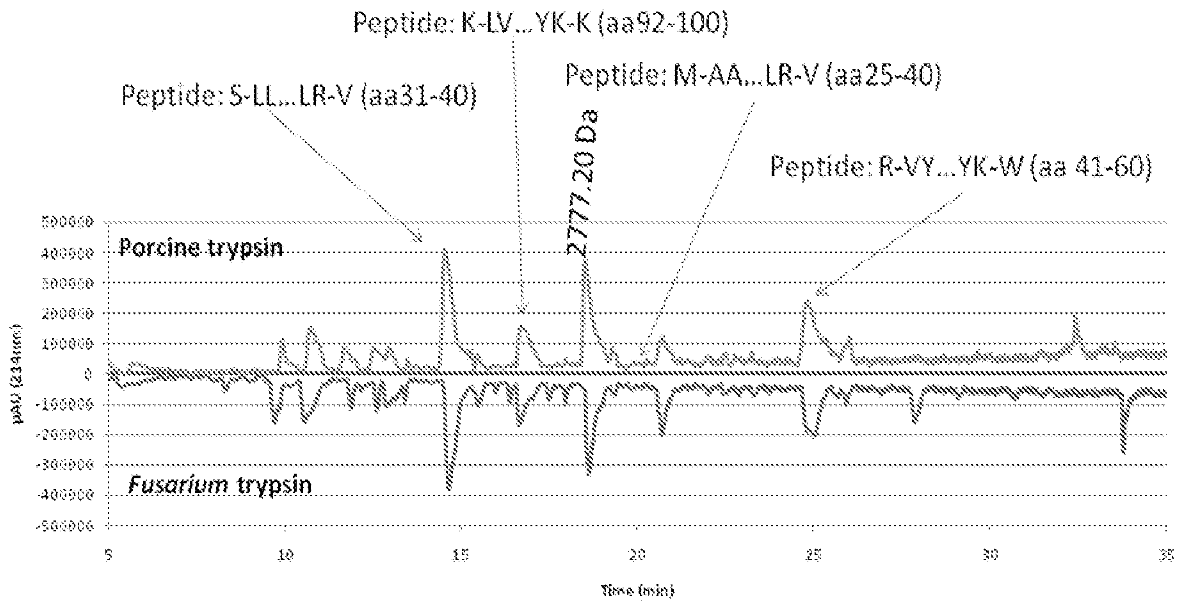

Figure 1

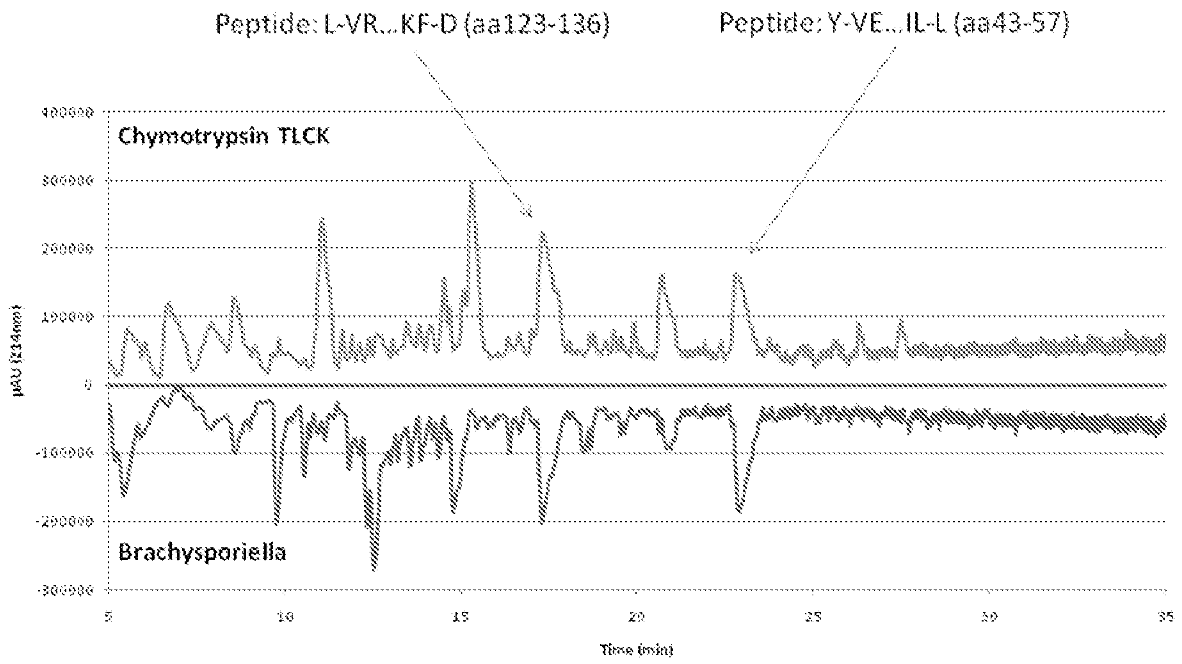

Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/054290

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23L1/305 A61K38/01 C12N9/52 C12N9/58 C12N9/64
C12N9/76
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23L A23K C07K C12N A61K

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 practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2008/254505 A1 (BUDOLFSEN GITTE [DK] ET AL) 16 October 2008 (2008-10-16) the whole document -----	1-15
A	WO 03/059083 A1 (FRAUNHOFER GES FORSCHUNG [DE]; SCHAEFER CHRISTIAN [DE]; WAESCHE ANDREA) 24 July 2003 (2003-07-24) the whole document -----	1-15
A	WO 93/24020 A1 (NOVO NORDISK AS [DK]; NIELSEN PER MUNK [DK]; HVASS PETER [DK]) 9 December 1993 (1993-12-09) the whole document -----	1-15
A	US 6 036 983 A (NIELSEN PER MUNK [DK]) 14 March 2000 (2000-03-14) the whole document -----	1-15
	-/--	

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

[X] 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

"E" earlier document 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
21 July 2010	29/07/2010

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/054290

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92/21248 A1 (DANMARK PROTEIN AS [DK]) 10 December 1992 (1992-12-10) the whole document	1-15
X	JP 6 343422 A (MEIJI MILK PROD CO LTD) 20 December 1994 (1994-12-20) the whole document	1-15
X	EP 0 353 122 A1 (COOP AGRICOLES LAIT UNION [FR]) 31 January 1990 (1990-01-31) cited in the application the whole document	1,4-6, 9-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/054290

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

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

   a. (means)
      - [ ] on paper
      - [X] in electronic form

   b. (time)
      - [X] in the international application as filed
      - [ ] together with the international application in electronic form
      - [ ] subsequently to this Authority for the purpose of search

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/054290

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
US 2008254505	A1	16-10-2008	AU	2008237912	A1	23-10-2008
			EP	2146589	A2	27-01-2010
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