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(54) Title: PROCESS FOR THE PRODUCTION OF AN INFANT FORMULA

(57) Abstract: The present invention provides a process to produce a casein hydrolysate which comprises a hydrolysis process comprising at least three enzyme fermentations of casein - a hydrolysis by the addition of a subtilisin; - a hydrolysis by the addition of an aminopeptidase mixture such as an Aspergillus niger or oryzae enzyme complex; and - a hydrolysis by the addition of a proline specific endoprotease and whereby between the enzyme treatment no inactivation of the enzymes takes place.



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PROCESS FOR THE PRODUCTION OF AN INFANT FORMULA**Field of the invention**

The present invention relates to a process to produce an infant formula or ingredients thereof as well as the infant formula or ingredients thereof such as hydrolyzed milk.

Background of the invention

Milk proteins consist of a casein and a whey protein fraction. The casein fraction is the main source of amino acids, calcium and phosphate all of which are required for growth of the newborn. The whey protein fraction also is a source of amino acids and in addition it contains several bioactive and putative health promoting proteins such as immunoglobulins, folate binding protein, lactoferrin, lactoperoxidase and lysozyme. Whereas adults rarely exhibit bovine milk allergies, such milk allergies are quite common among infants. Cow milk allergy is often encountered during the first months of life and within a week after the introduction of cow milk. Eventually 2-7.5 % of new born infants develop a cow milk allergy. The caseins and the beta-lactoglobulin from whey form the major cow milk allergens.

Because cow milk allergy among infants is relatively common, it is not surprising that special infant formula products have been developed containing highly hydrolyzed cow milk proteins. As a result of the enzymatic hydrolysis, antigenic cow milk epitopes are destroyed hereby reducing potential allergenic reactions. To accommodate the needs of the various groups of allergic individuals, formula are being marketed incorporating cow milk proteins hydrolyzed to different extents. Highly hydrolyzed hypoallergenic products have been available for a number of decades. Relatively new are products containing protein hydrolysates with low degrees of hydrolysis (DH). The latter products are intended to slowly accustom the immune system of infants to cow milk antigens. In fact these are prophylactic products aimed at minimising the risks of

developing a cow milk allergy. Ideally all hydrolysates, ranging from highly hydrolyzed to such hardly hydrolyzed prophylactic products, should provide a good taste and exhibit good shelf stabilities.

5 The gastro-intestinal digestion and subsequent assimilation of dietary proteins is a very efficient process. After ingestion and swallowing of proteinaceous foodstuff, it reaches the stomach where it is mixed with acid and the endoproteases pepsin and chymosin. Whereas adults secrete mainly the broadly active pepsins, the highly selective chymosin interacts with the casein fraction only. As chymosin represents a major gastric protease in newborn, it is not surprising that the clotting of cow milk casein differs from
10 the clotting of human milk casein in the stomach of infants. Moreover, the composition of human milk differs from the composition of cow milk so that also the gastric emptying and the metabolization pocesses differ.

Occasional opening of the pylorus allows the acidified and partly hydrolyzed food to flow from the stomach into the small intestine. In the first part of the small intestine i.e.
15 in the duodenum, the pH of the stomach contents is raised by bicarbonate and bile as well as pancreatic juice are added. The pancreatic juice contains an additional set of proteases, i.e. the endoproteases trypsin, chymotrypsin and elastase as well as carboxypeptidases to further degrade the peptides and polypeptides present. The peptides and free amino acids formed are absorbed mainly in the duodenum and the
20 jejunum.

The whey and casein proteins have strongly different amino acid compositions. The casein fraction is relatively rich in proline residues and due to this, casein hydrolysates tend to be notoriously bitter and easily precipitate from the aqueous solution. These disadvantages of casein hydrolysates can only be overcome with
25 superior technical skills and have resulted in a situation in which whey hydrolysates have become popular whereas enzymatically hydrolyzed caseins find limited application only. However, nature provides amino acids to the newborn in the form of milk and eighty percent of the amino acids present in milk are present in the form of casein.

Nowadays the protein part of infant formula is often based on cow's milk proteins.
30 In infant formula with hydrolysed protein, most often in the form of whey protein hydrolysates. However, whereas cow milk incorporates whey proteins beta-lactoglobuline and alpha-lactalbumine, in human milk the beta-lactoglobuline fraction is absent.

Whereas children often have no problems, for example allergenicity problems, with the consumption of cow's milk, babies, on the other hand, are much more receptive for allergenicity issues created by the consumption of cow's milk. One reason for this could be that because of their limited hydrolytic capacity, specific protein fragments, can survive the digestion process. For example because their digestive capacities were developed for consuming mother's milk and not for consuming cow's milk.

Summary of the invention

The present invention provides a process to produce a casein hydrolysate which is especially suitable as ingredient in hypoallergenic infant formula. Also other uses like protein drinks are part of the present invention. It is believed that hydrolyzed casein is an essential and versatile ingredient that can find application in infant formula as well as various other protein drinks. The present process is directed to produce a casein hydrolysate containing for 60 to 90 wt%, preferably 65 to 85 wt%, free amino acids plus peptides having a MW of less than 500 Da. This casein hydrolysate preferably contains 5 to 30 wt%, preferably 8 to 25 wt% of free amino acids. In general this hydrolysate will therefore contain for 55 to 80 wt%, preferably 60 to 75 wt% of peptides having a MW of less than 500 Da. The free amino acids content of the hydrolysate will in general be between 8 and 30 wt%, preferably between 10 and 25 wt%. Wt% is based on total protein content.

The present invention discloses a casein hydrolysis process comprising at least three enzyme treatments to guarantee a good taste and a hypoallergenic character whereby between the enzyme additions no intentional inactivation of the enzymes present takes place. The first enzyme treatment is a subtilisin addition, followed by the addition of an aminopeptidase comprising mixture, such as an *Aspergillus niger* or *oryzae* enzyme complex, and finally a proline specific endoprotease is added.

Detailed description of the invention

A "peptide" or "oligopeptide" is defined herein as a chain of at least two amino acids that are linked through peptide bonds. The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires. A "polypeptide" is defined herein as a chain containing more than 30 amino acid residues. All (oligo)peptide and polypeptide formulas or sequences herein are written from left to right in the direction from amino-terminus to carboxy-

terminus, in accordance with common practice. A protein is defined as used herein as the non-hydrolyzed protein. Moreover, especially when protein is discussed in general, protein can also mean the total of polypeptides, peptides, and free amino acids. A “water-soluble” peptide is a peptide which is soluble in water at a pH of 5.0.

5 The one-letter and three-letter code of amino acids used herein is commonly known in the art and can be found in Sambrook, et al. ((1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

10 By protein hydrolysate, hydrolysate, or hydrolysed protein is meant the product that is formed by enzymatic or microbial hydrolysis of the protein. An enriched hydrolysate being a fraction of the protein hydrolysate for example enriched in selected peptides or wherein peptides or polypeptides have been removed from the hydrolysate. So an enriched hydrolysate is preferably a mixture of peptides (or a peptide mixture).

15 The protein hydrolysate of the present invention has in general a DH of between 7 and 50, preferably a DH of between 9 and 40 and most preferably between 10 and 30. The Degree of Hydrolysis (DH) as obtained during incubation with the various protolytic mixtures was monitored using a rapid OPA test (Nielsen et al. (2001) J. Food Sci. 66:642-646). The degree of hydrolysis is the extent to which peptide bonds are broken by the enzymatic hydrolysis reaction.

20 The internationally recognized schemes for the classification and nomenclature of all enzymes from IUBMB include proteases. The updated IUBMB text for protease EC numbers can be found at the internet site: <http://www.chem.qmw.ac.uk/iubmb/enzyme/EC3/4/11/>. In this system enzymes are defined by the fact that they catalyze a single reaction. This has the important implication that
25 several different proteins are all described as the same enzyme, and a protein that catalyses more than one reaction is treated as more than one enzyme. The system categorises the proteases into endo- and exoproteases. The terms “protease”, “proteinase” and “peptidase” are used interchangeably herein. Endoproteases are those enzymes that hydrolyze internal peptide bonds, exoproteases hydrolyze peptide bonds adjacent to a
30 terminal α -amino group (“aminopeptidases”), or a peptide bond between the terminal carboxyl group and the penultimate amino acid (“carboxypeptidases”). The endoproteases are divided into sub-subclasses on the basis of catalytic mechanism. There are sub-subclasses of serine endoproteases (EC 3.4.21), cysteine endoproteases (EC 3.4.22),

aspartic endoproteases (EC 3.4.23), metalloendoproteases (EC 3.4.24) and threonine endoproteases (EC 3.4.25).

The aminopeptidases are in EC class 3.4.11. Sub-classification is on the basis of the relative efficiency with which the 20 different amino acids are removed. Aminopeptidases with a narrow and a broad specificity can be distinguished. Aminopeptidases can sequentially remove single amino-terminal amino acids from protein and peptide substrates. Aminopeptidases with a narrow specificity exhibit a strong preference for the type of amino acid residue at the P1 position that is liberated from the substrate peptide. Aminopeptidases of broad specificity are capable of releasing a range of different amino acids at the N-terminal or P1 positions (according to Schechter's nomenclature: Schechter, I. And Berger, A. 1967. Biochem Biophys Res Commun 27:157-162). Carboxypeptidases can sequentially remove single carboxy-terminal amino acids from protein and peptide substrates. Comparable with the situation for the endoproteases, carboxypeptidases are divided into sub-subclasses on the basis of catalytic mechanism. The serine-type carboxypeptidases are in class EC 3.4.16, the metallo-carboxypeptidases in class EC 3.4.17 and the cysteine-type carboxypeptidases in class EC 3.4.18. The value of the EC list for proteases resides in providing standard terminology for the various types of protease activity and especially in the assignment of a unique identification number and a recommended name to each protease.

Subtilisin (EC 3.4.21.62)

Subtilisin is a serine endopeptidase and is an example of peptidase family S8. Subtilisin includes subtilisin BPN' (also subtilisin B, subtilopeptidase B, subtilopeptidase C, Nagarse, Nagarse proteinase, subtilisin Novo, bacterial proteinase Novo) and subtilisin Carlsberg (subtilisin A, subtilopeptidase A, alcalase Novo). Similar enzymes are produced by various *Bacillus subtilis* strains and other *Bacillus* species which are also included. Subtilisin is commercially available under names Alcalase® or Protex®.

An enzyme preparation rich in aminopeptidases

Enzyme preparations incorporating an aminopeptidase activity are commercially available such as Flavourzyme® 1000L (Novozymes, Denmark) and Sumizyme® FP (Shin Nihon, Japan). Both enzyme preparations are obtained from *Aspergillus* species. Both Flavourzyme® and Sumizyme® FP are known to be complex enzyme preparations that contain several aminopeptidolytic enzyme activities besides non-specified endoproteolytic and carboxypeptidolytic activities. However, other enzyme preparations relatively rich in aminopeptidolytic activities may be used as well in the process of the

present invention, for example enzyme preparations such as Peptidase™ 436P-P436P and Peptidase 433P-P433P, both commercially available from Biocatalysts (Wales, UK). Aminopeptidases are also known to be produced by other microorganisms than *Aspergilli*, for example *Bacilli* and *Lactobacilli* are known to produce various aminopeptidases. For the process according to the present invention, aminopeptidases obtained from *Aspergilli* are preferred.

Proline-specific endoprotease (EC 3.4.21.26)

WO 02/45524 describes a proline-specific endoprotease obtainable from *Aspergillus niger*, which can be advantageously used in the present invention. The *A. niger* derived enzyme cleaves preferentially at the carboxyterminus of proline, but can also cleave at the carboxyterminus of hydroxyproline and, be it with a lower efficiency, at the carboxyterminus of alanine. WO 2002/45524 also teaches that there exists no clear homology between this *A. niger* derived enzyme and the known prolyl oligopeptidases from other microbial or mammalian sources. In contrast with known prolyl oligopeptidases, the *A. niger* enzyme has an acid pH optimum. The secreted *A. niger* enzyme appears to be a member of family S28 of serine peptidases rather than the S9 family into which most cytosolic prolyl oligopeptidases have been grouped (Rawlings, N.D. and Barrett, A.J.; Biochim. Biophys. Acta 1298 (1996) 1-3). The *A. niger* proline-specific protease is a "true" endoprotease in the sense that it is capable of hydrolysing intact proteins as well as peptides. Preferably, the *A. niger* derived enzyme preparation is used as a pure enzyme.

Especially suited for the present invention is an enzyme:

- having proline-specific endoprotease activity and
- having an amino-acid sequence identical to sequence NO:2 of WO 2002/45523 or having an amino acid sequence which has at least 80%, preferably at least 90% amino acid sequence identity with amino acids 1 to 526 of sequence NO:2 of WO 2002/45523. The level of identity between amino acid sequences is determined by the method mentioned in WO 2002/45523 page 15.

Process conditions

The subtilisin treatment is a hydrolysis process at between 30 and 65 °C, preferably between 40 and 60°C and more preferably between 50 and 60°C. The incubation period of this subtilisin step takes between 30 min and 10 h, preferably between 1 h and 8 h and more preferably between 1.5 h and 5 h. The pH is between 4 and 8, preferably between 5 and 7.

The aminopeptidase treatment is a hydrolysis process at between 30 and 65 °C, preferably between 40 and 60°C and more preferably between 50 and 60°C. The incubation period with this aminopeptidase takes between 30 min and 15 h, preferably between 1 h and 10 h and more preferably between 2 h and 8 h. The pH is between 4 and 8, preferably between 5 and 7.

The proline specific endoprotease treatment is a hydrolysis process at between 30 and 65 °C, preferably between 40 and 60°C and more preferably between 50 and 60°C. The incubation period with this proline specific endoprotease step takes between 1 h and 24 h, preferably between 3 h and 20 h and more preferably between 5 h and 16 h. The pH is between 3 and 7, preferably between 4 and 6.

As disclosed above, first the subtilisin step takes place. After the addition of the aminopeptidase preparation, so in the aminopeptidase step, the subtilisin is still active. After the addition of the proline specific endoprotease, so in the proline specific endoprotease step, the aminopeptidase and even the subtilisin might still be active.

The present process has several advantages over other processes. An important advantage of the present invention is the use of microbial enzymes only. In general animal-derived, pancreatic preparations incorporating proteolytic activity are used to hydrolyze the various milk proteins. A typical example of such a pancreas derived proteolytic activity is trypsin. Trypsin (EC 3.4.21.4) is a serine protease found in the digestive system of many vertebrates, where it serves to hydrolyze dietary proteins into smaller peptides which eventually are taken up in the blood stream. Apart from trypsin, pancreatic preparations also incorporate a number of other proteases. Obviously the use of such animal derived proteolytic enzyme mixtures will be less desirable for kosher or halal products. Moreover animal derived products may be linked to diseases like BSE. The present invention preferably replaces all these commonly used, animal-based, pancreatic proteases with microbial derived or produced alternatives. Therefore the hydrolysate of the invention is advantageously produced by using enzymes which are produced in or by a microorganism such as a microbial host cell, and preferably originate from a microorganism.

At the end of the present hydrolysis process, all enzymatic activities are inactivated and the resulting hydrolysate can be pasteurised or sterilised, for example using an UHT (ultra high temperature) treatment. The enzyme inactivation step can be a heat treatment comprising heating to a temperature of at least 85°C for at least 10 minutes. Such heat treatment is preferably carried out at an acidic pH value, preferably

between 3 and 7. If higher temperatures or more extreme pH values are used, shorter periods may be feasible.. The temperature treatment can take place directly after the hydrolysis process but can also take place after optionally drying and/or purifying the hydrolysate.

5 Optionally, non-solubilised material from the final product is removed, for example, by decantation or low speed centrifugation at for example 2000-4000 g. Alternatively, MF (Micro Filtration), UF (Ultra Filtration) might be used to remove the larger parts present in the hydrolysate or the hydrolysate can be filtered using for example diatomaceous earth or fiberglass filters. Preferably this removal of non-
10 solubilised material takes place by UF. Optionally the filtered final hydrolysate(s) may be purified on a resin or can be treated with activated charcoal or with nanofiltration, ion exchange or electro dialysis to remove a surplus of salts and/or off taste components.

 Finally the hydrolysate can be concentrated or dried using standard industrial concentrators and driers such as evaporators, fluidized bed driers or spray driers.
15 Preferably the obtained product is in a concentrated and/or granular form. The hydrolysate may be used in food formulations before or after concentration or drying.

 The commercially available hypoallergenic infant formula based on casein hydrolyzates can be divided into different catagories. A first category incorporates a severely hydrolysed casein fraction which results in a product with a very high free
20 amino acids content, that is, typically more than 50 wt%. This is also reflected in high Degree of Hydrolysis (DH) values. An example of such a product is Nutramigen (Mead Johnson, Evansville, IN, USA). A disadvantage of such a high free amino acid content is a strong tasting product with a high osmotic value. Additionally the digestive system of newborns get less habituated to the digestion of peptides. Another category of infant
25 formula incorporates casein hydrolyzates with less free amino acids and having a relatively low DH value. Although such infant formula can be assumed to be better to adapt the digestive system of newborns to larger peptides and proteins, the lower DH values may lead to some residual peptides with an allergenic potential, that is, such product may not always be hypoallergenic. The process according to the present
30 invention yields casein hydrolysates combining the advantages of both product categories without the disadvantages thereof. The hydrolysed casein obtained according to the present process incorporates relatively low levels of free amino acids, has a relatively low DH, and at the same time has a high amount of small peptides. Most importantly the present hydrolysate is hypoallergenic and has an excellent taste.

The protein composition of the casein hydrolysate of the invention typically exists of:

- a fraction having MW > 5000 Da: 0.001 to 1 wt%, preferably 0.1 to 1 wt%;
- a fraction having 5000 < MW < 1500 Da: 0.1 to 10 wt%, preferably 1 to 8 wt%;
- 5 - a fraction having 1500 < MW < 500 Da: 9 to 35 wt%, preferably 12 to 30 wt%; and
- a fraction having MW < 500 Da: 60 to 90 wt%, preferably 65 to 85 wt%;

(the fraction MW < 500 Da includes the free amino acids, the free amino acids in the protein composition are between 8 to 30 wt%, preferably between 10 and 25 wt%). Wt% is based on total (dry) protein content. The amounts of free amino acids, dipeptides and tripeptides can be determined as described in the Materials and Methods section. Advantageously in the hydrolysate of the invention the weight ratio between the sum of the di- and tripeptides and free amino acids is between 1.7 and 4, preferably between 1.8 and 3. Commercially available infant formulas showed ratios of less than 1.5 and sometimes even less than 1. These formulas contain hydrolysates that are extensively hydrolysed to make sure that hardly no larger peptides or oligopeptides remain present in the hydrolysate, but also resulting in an high amount of free amino acids in the hydrolysate. The present invention is directed to have an high amount of di- and tripeptides in the hydrolysate which is expressed in the above discussed ratio.

The hydrolysate is advantageously hypoallergenic. Hypoallergenicity is determined according to the method described in the Materials and Methods section.

According to another aspect of the invention the hydrolysate is non-bitter. Bitterness is determined according to the method described in the Materials and Methods section.

According to yet another aspect of the present invention, a product comprising the hydrolysate of the invention is a beverage such as a sports drink, a soft drink or a health drink, or a food, preferably a dietetic food, such as a product for elderly or for slimming people, clinical nutrition or an infant formula such as a term or follow-on product . Preferably this product is an infant formula.

Obviously the hydrolysate can be subjected to treatments with enzymes other than proteases such as lactases to remove traces of lactose present. The hydrolysate can also be fermented with different types of starter or probiotic cultures or can be combined with all kinds of ingredients such as oils, fats, emulsifiers, carbohydrates, fruit concentrates, flavours, colorants, alcohol, carbon dioxide, thickeners, acidulants, antioxidants, herbs or herb extracts, health promoting compounds like vitamins or

provitamins or bioactive or peptides containing tryptophane or specific amino acids to formulate a product which is in line with the marketing needs.

Materials and Methods

5 **Materials**

Casein, edible acid casein was obtained Fonterra, New Zealand

Proline-specific endoprotease from *A. niger*

10 Overproduction and chromatographic purification of the proline specific endoprotease from *Aspergillus niger* was accomplished as described in WO 02/45524. The *A. niger* proline specific endoprotease activity was tested using Z-Gly-Pro-pNA (Fluka 96286) as a substrate at 37 °C in a citrate/disodium phosphate buffer pH 4.6. The reaction products were monitored spectrophotometrically at 405 nM. The increase in absorbance at 405 nm in time is a measure for enzyme activity. A Proline Protease Unit
15 (PPU) is defined as the quantity of enzyme that releases 1 µmol of p-nitroanilide per minute under the conditions specified and at a substrate concentration of 0.37mM Z-Gly-Pro-pNA.

Flavourzyme®100L, Novozymes

20 Alcalase® Novozymes, Protex® 6L. Genencor/ Danisco

Degree of Hydrolysis

 The Degree of Hydrolysis (DH) as obtained during incubation with the various protolytic mixtures was monitored using a rapid OPA test (Nielsen, P.M.; Petersen, D.;
25 Dambmann, C. Improved method for determining food protein degree of hydrolysis. *Journal of Food Science* **2001**, 66, 642-646).

Quantifying free amino acids

 To quantify the level of free amino acids in the supernatants of the casein
30 hydrolysates prepared according the procedure outlined in Example 4, the following method was used. By applying the conventional UV detection for monitoring free amino acids after pre-column AccQ-Tag derivatization, a serious interference with small peptides occurs. We used the AccQ-Tag®_{ultra} method of Waters (Milford MA, US). In this method, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) is used as a reagent

for the quantitative conversion of primary and secondary amino acids into the corresponding carbamide derivatives. In our quantitative UPLC/ tandem MS based method, pre-column AccQ-Tag[®]_{ultra} derivatization circumvents ion suppression effects from co-eluting endogeneous dipeptides. LC-MS/MS was applied for monitoring diagnostic ions of the free amino acid derivatives, i.e. the protonated carbamide derivative and one daughter ion (m/z 171), that result from the loss of the amino acid from the corresponding carbamide derivative. By monitoring the above-mentioned MS/MS transition, free amino acids are selectively monitored, also in the presence of peptides from the hydrolysate.

To measure the free amino acids in the supernatants of the casein hydrolysates prepared according to Example 4, the following protocol for sample preparation and derivatization was used:

-Dissolve 40-300 mg of protein hydrolysate in 50 ml 0.1 N HCl

-Transfer 100 μ l of this solution into a vial

-Add 100 μ l of internal standard (IS) solution (containing 1 mg of each amino acid, which is totally ¹³C isotope-labelled, per 50 ml)

-Mix or stir vigorously

-Transfer 10 μ l of this sample/IS solution into a test tube

-Add 70 μ l borate buffer (from the Waters AccQ-Tag ultra reagent package) and mix

-Add 20 μ l reagent solution (from the Waters AccQ-Tag ultra reagent package) and mix immediately

-Transfer the derivative solution to an injection vial

-Heat the vial at 55°C for 10 minutes and mix

-Inject 1 μ l into the UPLC system.

Subsequent UPLC-MS/MS analyses were performed on an Ultra high-Pressure Liquid Chromatograph (UPLC) combined with a Xevo TQ mass spectrometer from Waters under the following conditions.:

UPLC

Column: Acquity UPLC BEH C₁₈, 150 x 2.1 mm I.D. (1.7 μ m)

Flow: 0.4 ml/min

Mobile phase : Solvent A: Water /AccQ.Tag Eluent A (95:5 % v/v)

Solvent B: AccQ.Tag Eluent B

Injection volume : 1 μ l partial loop with needle overfill

Injection loop: 2 μ l
 Column temperature: 43°C
 Tray temperature: 20°C
 Runtime: 60 min
 Gradient :

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Time (min)	Solvent A (%)	Solvent B (%)	Curve
0	99.9	0.1	
1.14	99.9	0.1	linear
2.00	98.5	1.5	linear
5.50	98.1	1.9	linear
6.50	98.0	2.0	convex
10.00	97.6	2.4	linear
12.00	96.0	4.0	linear
20.00	88.0	12.0	linear
35.00	85.0	15.0	convex
36.00	2.0	98.0	linear
38.00	2.0	98.0	linear
39.00	99.9	0.1	linear
60.00	99.9	0.1	

MS:

Ion mode : ESI positive
 Capillary voltage : 1.5 V
 Cone voltage: 30 V
 Extractor voltage: 3.0 V
 LM resolution: 3
 HM resolution: 15
 Ion energy: 0.5 eV
 Desolvation temperature: 600°C
 Source temperature: 150°C

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	Cone gas flow:	50 L/hr
	Desolvation gas flow:	500 L/hr
	Dwell time:	10 msec
	Interscan delay:	5 msec
5	Interchannel delay:	20 msec

Quantifying di- and tripeptides

To roughly determine the molecular weight (MW) distribution of oligopeptides in a protein hydrolysate, usually size exclusion chromatography (GPC) is used. However, for the MW range < 500 Da this technique provides insufficient information about the distribution between free amino acids, di- and tripeptides. Quantitative analysis of di- and tripeptides in general by LC-MS is hampered by the fact that the proton affinity differs a factor > 100 among peptides.

We determined total di- and tripeptides by quantitative LC/MS method in which we took advantage of a pre-column derivatization and exact mass analysis using a LTQ orbitrap mass spectrometer. Using the AccQ-Tag[®]_{ultra} derivatization technique (see above) it was possible to significantly reduce the difference in proton affinity between the various peptides. The derivatization was carried out according to the following procedure:

- Prepare a 1mg/ml protein hydrolysate solution in MilliQ
- 20 - Transfer 10 µl of this solution in a test tube
- Add 70 µl borate buffer (from the Waters AccQ-Tag ultra reagent package) and mix
- Add 20 µl reagent solution (from the Waters AccQ-Tag ultra reagent package) and mix
- 25 - Transfer the solution to an injection vial
- Heat the vial at 55 °C for 10 minutes and mix
- Inject a volume into the LC/MS system.

Subsequent analyses were performed using an Accela HPLC (in high pressure Mode, coupled with a LTQ/orbitrap mass spectrometer from Thermo Electron). An Agilent SB-C18 column (1.8 µm, 2.1*50mm) was used at 55 °C for separation of the peptides, with gradient elution, starting at 96% A (0.1 % Formic acid (FA)) and increasing to 30 %B (0.1 % FA in Acetonitrile (ACN)) in 7 minutes, directly equilibrating at 96 % A for three minutes. The flow-rate was 0.4 ml/min, and the injection volume was 5 µl. Samples were stored at 4°C upon analysis. The LTQ/orbitrap was operated in

ESI/pos mode and scanning from m/z 100-1500 at a resolution of 60000. The mass accuracy was kept < 2ppm by daily calibrating the orbitrap.

Using a wide variety of dipeptide standards, first the deviation of the proton affinity (expressed in peak area) was determined before/after derivatization. A total of 20 different dipeptides, selected on the basis of their hydrophobicities, were used and after subsequent derivatization and LC-MS analysis the peak area per pMole dipeptide was calculated. The analysis was performed in 5-fold. Typically for the 20 dipeptides a RSD of <30 % was obtained whereas for the non-derivatized dipeptides a RSD of > 80 % was obtained. On the basis of a similar measurement of 20 different derivatized tripeptides a RSD of 40% was obtained. The di- and tri-peptides present in the casein hydrolysate were determined in MS mode using accurate mass detection.

To calculate the absolute amount of total di- tri-peptides in the casein hydrolysate prepared according to the procedure described in Example 4, known amounts of some di- and tri-peptides (which do not occur in the amino acid sequence of the caseine) were added to the sample as standard. For quantification automatically all theoretical exact masses of the di and tri peptides were plotted as ion chromatograms. After integration all peak areas of the individual di- and tripeptides were summed, whereas also after integration all peak areas of the standard di- and tripeptides were summed. The ratio of these areas results in the quantification of total di- and tripeptides.

β -Casein Enzyme Linked Immuno Sorbent Assay (ELISA)

Samples are dissolved and diluted, in order to fit within the calibration curve, in Phosphate Buffered Saline (PBS, BupHtm, Thermo, 28327) pH 7.4 and using Protein LoBind Eppendorf tubes to avoid sticking of the casein to the plastics. The diluted samples are adhered to the surface of a 96-wells microtiterplate (MTP, Nunc Maxisorb VWR 735-0034). After adherence of the samples for 16 hours at 4°C, the plates are washed using PBS and the plates are incubated with a SEA Block Blocking Buffer (Thermo, 37527) to block all free binding places. The β -casein antigen of the coated sample material is determined in 4 steps.

First step, incubation with the primary affinity purified Rabbit anti-Bovine casein (Gentaur, RCAS-10A RCAS-10A), diluted to the appropriate dilution made in the SEA Block Blocking Buffer for 30 minutes at room temperature. After the incubation period, the MTP plate is washed 4 times with PBST (PBS supplemented with 2.5 % Surfact-Amps[®] 20, Thermo 28320).

For the second step, the plate is incubated with a biotinylated affinity purified goat-anti-rabbit IgG polyclonal antibody (Thermo 32054) for 30 minutes at room temperature. After the incubation period the MTP plate is washed 4 times with PBST.

For the third step the MTP plate is incubated for 30 minutes at room temperature with a streptavidin-HRP (Horse Radish Peroxidase) conjugate (HyCult biotechnology, HE101). After the incubation period, the MTP plate is 3 times washed with PBST and 1 time with PBS.

The fourth step is the colour development, this is established by the incubation with TMB (3,3',5,5'-tetramethylbenzidine, Thermo,34028) as substrate. The latter is converted in a blue colour by means of HRP and after stopping the incubation after 30 minutes with 1M H₂SO₄ (VWR S9852.5000). Its intensity is measured at 450 nm, using a standard MTP plate reader, and is a measure for the amount of β -casein present.

Calculation

Prepare a calibration curve by plotting the absorbance at 450 nm versus the known amount of β -casein of the standards. This calibration curve must be fitted according linear regression $y = ax + b$, in which y = the absorbance, x = amount of β -casein, a = slope of the calibration curve, and b = intercept of the calibration curve. This curve is used to determine the β -casein concentration in unknown samples.

Calculate the β -casein concentration of the unknown samples as follows:

$$\frac{((A_{450} - b) / a)}{C(\text{sample})} = \text{Casein}(\text{concentration})$$

Whereby:

A_{450} = Absorbance at 450 nm

b = Intercept of calibration curve

a = Slope of the calibration curve

$C(\text{sample})$ = Concentration sample in mg/ml

Example 1

Non-bitter casein hydrolysates.

A suspension of 10% (w/w) of casein is subjected to a three-step enzymatic hydrolysis. In the first step, the casein is incubated with the serine endoprotease Protex 6L (Genencor, Leiden, The Netherlands). Incubation takes place at a pH of 6.5 at 55 °C

using 5 ml of enzyme per kg of casein. After 2 hours of incubation, the Flavourzyme is added using 1 ml of enzyme per kg of casein. This incubation was done at 55 °C. After 4 hours of incubation the pH is lowered to 4.5 and 1 PPU of proline-specific endoprotease/gram of protein is added. The incubation is continued for another 9 hours at 55 °C. A typical DH value at this stage is 28 and the liquid has a negligible bitterness as judged following the procedure described in the Materials & Methods section. After a brief heat shock to inactivate all enzymatic activities, the liquid is evaporated to obtain a concentrated liquid containing approximately 30% dry matter. The process was run two times resulting in batches EWR0904 and EWR0908.

10 The protein composition of the produced casein hydrolysate EWR0904 was:

- 0.1 wt% having MW > 5000 Da;
 - 4 % having 5000<MW<1500 Da;
 - 21 % having 1500<MW<500 Da %; and
 - 75% having MW < 500 Da
- 15 - A ratio total di- and tripeptides\ total free amino acids is > 1.9

Product	Total free amino acids % (w/w) on protein	Total di- and tripeptides % (w/w) on protein	Weight ratio between the sum of di- and tripeptides and free amino acids	Elisa expressed as casein in mg/kg protein
Nutramigen®	55.0	14.3	0.26	659
Friso® Allergy Care	14.1	19.3	1.37	125
Batch EWR0908	11.5*	32.2*	2.80	188
Batch EWR0904	19.9	38.9	1.95	182

* On powder

Sensoric evaluation

20 The casein hydrolysate has been assessed by a taste panel for bitterness, odour and taste, next to a number of commercial infant formulae products.

Sensoric evaluations were carried out by a panel trained in detecting and ranking various levels of bitterness and other attributes typical of milk protein hydrolysates. During the sessions the taste trials were performed 'blind' and the attributes were scored on a scale from low (0) to very high (+++). The temperature of the samples when served was 37°C.

25

The following products were analysed:

The casein hydrolysate in infant nutrition base and the commercial products Nutramigen® (Mead Johnson) and Friso® Allergy Care (frieslandfoods). The products were dissolved in water according to the instruction on the packages.

Results of the sensoric evaluations are depicted in the table underneath.

	The present casein hydrolysate in infant nutrition base	Nutramigen®	Friso® Allergy Care
General odour	+	++	+++
General taste	++	+++	++
Bitterness	+	++	++
Animal feed	+	++	++
Bouillon	+	++	+++
Aftertaste	+	+++	++

The taste of the three solutions were evaluated by a panel of experienced tasters. The hydrolysate of the invention was found to be the best of the three formula, indicated by lower scores on the attributes 'general odour', 'general taste', 'bitterness', 'animal feed', 'bouillon', and 'aftertaste'. The Nutramigen product was scored highest on the attributes 'general taste', 'bitterness', 'animal feed', and 'aftertaste'. The Friso scored better than Nutramigen on 'general taste' and 'aftertaste', but was scored higher on the attributes 'general odour' and 'bouillon'.

The tasters were unanimous in their conclusion that the infant nutrition base with the hydrolysate of the invention was significantly less bitter than the two commercial products alone, and in addition taste and odour attributes were largely improved.

CLAIMS

- 5 1. A casein hydrolysate comprising for 60 to 90 wt%, preferably 65 to 85 wt%, of the amino acids and peptides having a MW of less than 500 Da and whereby the free amino acids content of the hydrolysate is between 8 and 20 wt%, preferably between 10 and 18wt%.
- 10 2. A casein hydrolysate according to claim 1 which comprises for 9 to 35 wt%, preferably 12 to 30 wt%, of the amino acids and peptides having a MW of between 1500 and 500 Da.
3. A casein hydrolysate according to claim 1 or 2 which comprises for 0.1 to 10 wt%, preferably 1 to 8 wt%, of the amino acids and peptides having a MW of between 1500 and 5000 Da.
- 15 4. A casein hydrolysate according to any one of claims 1 to 3 which comprises for 0.001 to 1 wt%, preferably 0.1 to 0.5 wt%, of the amino acids and peptides having a MW of more than 5000 Da.
5. A casein hydrolysate according to any one of claims 1 to 4 whereby the weight ratio between the sum of di- and tripeptides and free amino acids is between 1.7 and 4, preferably between 1.8 and 3.
- 20 6. A casein hydrolysate according to any one of claims 1 to 5 which has a DH (Degree of Hydrolysis) of between 7 and 50, preferably between 9 and 40, more preferably between 10 and 30.
7. A casein hydrolysate according to any one of claims 1 to 4 which is prepared using enzymes produced in a microorganism, and preferably originate from a microorganism.
- 25 8. A casein hydrolysate according to any one of claims 1 to 4 which is hypoallergenic or which is non-bitter.
9. A process to produce a casein hydrolysate according to claim 1 which comprises a hydrolysis process comprising at least three enzyme fermentations of casein
- 30 - a hydrolysis by the addition of a subtilisin;
- a hydrolysis by the addition of an aminopeptidase mixture such as an *Aspergillus niger* or *oryzae* enzyme complex ; and
- a hydrolysis by the addition of a proline specific endoprotease

and whereby between the enzyme treatment no inactivation of the enzymes takes place.

10. A process of claim 4 wherein the hydrolysis by subtilisin takes place at 30 to 65 °C, preferably between 40 and 60 °C, more preferably between 50 and 60 °C.

5 11. A process of claim 4 or 5 wherein the hydrolysis by aminopeptidases takes place at 30 to 65 °C, preferably between 40 and 60 °C, more preferably between 50 and 60 °C.

12. A process of any one of claims 4 to 6 wherein the hydrolysis by subtilisin takes place at 30 to 65 °C, preferably between 40 and 60 °C, more preferably between 50 and 60 °C.

10 13. A product comprising the hydrolysate of any one of claims 1 to 8 or 11, which is a beverage such as a sports drink, a soft drink or a health drink, or a food, preferably a dietetic food, such as a product for elderly or for slimming people or an infant formula such as a term or follow-on product .

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/071676

A. CLASSIFICATION OF SUBJECT MATTER

INV. A23L1/305 A23J3/34 A23L1/29 C12N9/48
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 A23J C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

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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

"E" earlier application or patent but published on or after the international filing date

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"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

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INTERNATIONAL SEARCH REPORT

International application No
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