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A METHOD FOR HYDROLYSING PROTEINS

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(71) Applicant(s)
NOVO NORDISK A/S

(72) Inventor(s)
PER MUNK NIELSEN; PETER HVASS; KIM HANSEN; GITTE BUDOLFSEN

(74) Attorney or Agent
SPRUSON & FERGUSON , GPO Box 3898, SYDNEY NSW 2001

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It has now been found that a proteolytic enzyme preparation derived from *Aspergillus oryzae* and supplied from Novo Nordisk A/S under the tradename Flavourzyme™ possesses excellent protein hydrolysing properties, for example is it possible to obtain a high degree of hydrolysis and a non-bitter hydrolysate.

Accordingly, the present invention provides a method for hydrolysis of proteins by incubation with a proteolytic preparation derived from *Aspergillus oryzae* and supplied by Novo Nordisk A/S, Denmark under the tradename Flavourzyme™.

In its second aspect, the invention provides protein hydrolysates obtained by the method of the invention.

In further aspects, the invention provides food and non-food products comprising a protein hydrolysate of the invention.

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CLAIM

1. A method for hydrolysing a protein by incubating with a proteolytic enzyme preparation, which proteolytic preparation is derived from *Aspergillus oryzae* and comprises at least five proteolytic components each having an approximate molecular weight, respectively, selected from 23 kD, 27 kD, 31 kD, 32 kD, 35 kD, 38 kD, 42 kD, 47 kD, 53 kD, and 100 kD, and by which method there is obtained a degree of protein hydrolysis (DH) of higher than 60%.

15. The method according to any one of the preceding claims, in which the protein is incubated with the proteolytic preparation in combination with one or more other protease preparations.

33. The food product according to claim 32, being an ingredient of mother milk substitute; a cheese flavour product; an enzymatically produced HVP; a protein enriched dietetic product; a soup-, bouillon- or meat-flavour product; a meat extract product; or a hydrolysate for improving the production of a starter culture.

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(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NIELSEN, Per, Munk [DK/DK]; Rytterstien 29A, DK-3400 Hillerød (DK). HVASS, Peter [DK/DK]; Toftebæksvej 54 st. tv., DK-2800 Lyngby (DK). HANSEN, Kim [DK/DK]; Kastanjevej 30, DK-4632 Bjæverskov (DK). BUDOLFSEN, Gitte [DK/DK]; Lyøvej 3, 3. tv., DK-2000 Frederiksberg (DK).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

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(54) Title: A METHOD FOR HYDROLYSING PROTEINS

(57) Abstract

A method for hydrolysing a vegetable or animal protein by incubating with a proteolytic enzyme preparation derived from *Aspergillus oryzae* and comprising at least five proteolytic components each having an approximate molecular weight, respectively, selected from 23 kD, 27kD, 31 kD, 32 kD, 35 kD, 38 kD, 42 kD, 47 kD, 53 kD, and 100 kD, for example Flavourzyme™, provides a protein hydrolysate useful in or as a food product such as mother milk substitute, cheese, HVP, meat extract, flavouring agent, and process aid for fermentation of food products, or a non-food product such as pet food, cosmetics. By the method is obtained a high degree of hydrolysis (DH), flavour development, and a high protein solubility (PSI).

A METHOD FOR HYDROLYSING PROTEINS

TECHNICAL FIELD

The present invention relates to a method of hydrolysing proteins, a protein hydrolysate obtained by the method, and food and non-food products containing the protein hydrolysate.

BACKGROUND ART

Methods of hydrolysing proteins have been described in e.g. GB Patent 1,507,380, US Patent 3,723,250, and International Patent Application Nos. WO 89/00272, WO 92/13964, and WO 90/05462.

There is a need for methods of hydrolysing proteins leading to a high degree of protein hydrolysis and to hydrolysates with excellent organoleptic properties.

SUMMARY OF THE INVENTION

It has now been found that a proteolytic enzyme preparation derived from *Aspergillus oryzae* and supplied from Novo Nordisk A/S under the tradename Flavourzyme™ possesses excellent protein hydrolysing properties, for example is it possible to obtain a high degree of hydrolysis and a non-bitter hydrolysate.

Accordingly, the present invention provides a method for hydrolysis of proteins by incubation with a proteolytic preparation derived from *Aspergillus oryzae* and supplied by Novo Nordisk A/S, Denmark under the tradename Flavourzyme™.

In its second aspect, the invention provides protein hydrolysates obtained by the method of the invention.

In further aspects, the invention provides food and non-food products comprising a protein hydrolysate of the invention.

Brief Description of Drawings

The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows the degree of hydrolysis (%DH) vs. time of hydrolysis (hours) achieved by the method of the invention applied to sodium caseinate in the pH range of from pH 5 to pH 9 (■: pH 5, ▲ pH 6, ○ pH 7, □ pH 8, • pH 9;

Fig. 2 shows the degree of hydrolysis (%DH) of soy protein isolate after 22 hours of hydrolysis according to the method of invention (■: Flavourzyme™, ▲: Corolase™ 7092, ◇: Corolase™ 7093, ◆: Alcalase™ , □: Neutrase™); and

Fig. 3 shows the degree of hydrolysis (%DH) of Nacaseinate after 22 hours of hydrolysis according to the method of invention (■: Flavourzyme™, ▲: Corolase™ 7092, ◇: Corolase™ 7093, ◆: Alcalase™ , □: Neutrase™).

Detailed Disclosure of the Invention

Characterization of Flavourzyme™ showed that the proteolytic *Aspergillus oryzae* preparation comprises several proteolytic components. It appeared that the preparation comprises five or more proteolytic enzyme components, each of which may have any of the following approximate molecular weights: 23 kD, 27 kD, 31 kD, 32 kD, 35 kD, 38 kD, 42 kD, 47 kD, 53 kD, and 100 kD.

Accordingly, the present invention provides a method for hydrolysing a protein by incubating with a proteolytic enzyme preparation, which proteolytic preparation is derived from *Aspergillus oryzae* and comprises at least five proteolytic components each having an approximate molecular weight, respectively, selected from 23 kD, 27 kD, 31 kD, 32 kD, 35 kD, 38 kD, 42 kD, 47 kD, 53 kD, and 100 kD, and by which method there is obtained a degree of protein hydrolysis (DH) of higher than 60%.

In a preferred embodiment, the protein is incubated with a proteolytic preparation derived from *Aspergillus oryzae* and comprising at least five proteolytic components having the approximate molecular weights 23 kD, 31 kD, 35 kD, 38 kD, and 53 kD, respectively.

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The molecular weight of the protease components in the proteolytic preparation was determined by using SDS polyacrylamide gel electrophoresis (SDS-PAGE) in a manner known to persons skilled in the art. In this way, the molecular weight of each protease component was determined.

The method of the invention is able to perform extensive protein hydrolysis of proteins, and the method leads to non-bitter hydrolysates and hydrolysates having pronounced soup-flavour/meat-flavour.

The extent of the protein hydrolysis may be determined by the degree of hydrolysis achieved. In the context of this invention, the degree of hydrolysis (DH) is defined by the following formula:

$$DH = \frac{\text{Number of peptide bonds cleaved}}{\text{Total number of peptide bonds}} \times 100\ \%$$

The DH may be calculated according to *Adler-Nissen*; Enzymic Hydrolysis of Food Proteins; Elsvier Applied Science Publishers Ltd. (1986), p. 122.

By using the method of the invention it may be possible to obtain a DH which is higher than 35%, preferably higher than 60%, more preferably higher than 70%, most preferably higher than 80%.

By the method of the invention it may also be possible to obtain a high degree of protein solubility. The degree of protein solubility may be described by way of a Protein Solubility Index (PSI) as described by *Adler-Nissen, op. cit.*

In a preferred embodiment, a protein solubility higher than 50% PSI, preferably higher than 70% PSI, more preferably higher than 90% PSI, is obtained.

The protein or proteinaceous material which may advantageously be hydrolysed by the present method may be any vegetable protein such as soy protein, grain proteins, e.g. wheat gluten or zein, rape seed protein, alfalfa protein, pea protein, fabaceous bean protein, cotton seed protein or sesame seed protein, or any animal protein or proteinaceous material

such as milk protein, whey protein, casein, meat protein, fish protein, blood protein, egg white or gelatin.

To obtain a satisfactory degree of hydrolysis, the proteolytic enzyme may suitably be added to the protein or proteinaceous material in an amount of 0.05-15 AU/100 g of protein, in particular 0.1-8 AU/100 g of protein.

The incubation may be performed at a pH from between about 4 and about 10, preferably between about 5 and about 9. As shown in Example 2, the method of the invention performs excellent even at extreme pH conditions, i.e. at pH values in all of the range of 5 to 9.

The incubation may be performed at any convenient temperature at which the enzyme preparation does not become inactivated, i.e. in the range of from about 20°C to about 70°C.

In accordance with established practice, the proteolytic enzyme preparation may suitably be inactivated by increasing the temperature of the incubation mixture to above about 70°C, or by decreasing the pH of the incubation mixture to below about 4.0.

In yet another preferred embodiment, incubation of a protein or proteinaceous substrate may be performed with a combination of Flavourzyme™ and one or more other protease preparations.

Preferred protease preparations comprises neutral or alkaline proteases. Examples of suitable neutral proteases are neutral proteases derived from *Bacillus*, preferably from *Bacillus subtilis*, such as the enzyme preparation supplied by Novo Nordisk, Denmark, under the tradename Neutrase™. Examples of suitable alkaline proteases are alkaline proteases derived from *Bacillus*, preferably from *Bacillus licheniformis*, such as the enzyme preparation supplied by Novo Nordisk, Denmark, under the tradename Alcalase™ and containing Subtilisin A (Subtilisin Carlsberg) as the active component.

The incubation carried out in the method of the invention may also be performed with a combination of Flavourzyme™ and one or more other lipase preparations.

Preferred lipase preparations comprises fungal lipases. Examples of suitable fungal lipases are lipases derived from *Mucor*, preferably from *Rhizomucor miehei*, such as the enzyme preparation supplied by Novo Nordisk, Denmark, under the tradename Palatase™M; and lipases derived from *Aspergillus*, preferably from *Aspergillus niger* such as the enzyme preparation supplied by Novo Nordisk, Denmark, under the tradename Palatase™A.

In another aspect, the present invention provides a protein hydrolysate obtained by the method of the invention.

Determination of AU

The proteolytic activity may be determined with haemoglobin as substrate.

In the Anson-Haemoglobin method for the determination of proteolytic activity denatured haemoglobin is digested, and the undigested haemoglobin is precipitated with trichloroacetic acid (TCA). The amount of TCA soluble product is determined with phenol reagent, which gives a blue colour with tyrosine and tryptophan.

One Anson Unit (AU) is defined as the amount of enzyme which under standard conditions (i.e. 25°C, pH 7.5 and 10 min. reaction time) digests haemoglobin at an initial rate, so that there is liberated per minute an amount of TCA soluble product which gives the same colour with phenol reagent as one milliequivalent of tyrosine.

A folder AF 4/5 describing the analytical method in more detail is available from Novo Nordisk A/S, DK-2880 Bagsvaerd, Denmark, upon request which folder is hereby included by reference.

Determination of LU

Assay for lipase activity :

A substrate for lipase was prepared by emulsifying glycerine tributyrat (MERCK) using gum-arabic as emulsifier.

Lipase activity was assayed at pH 7 using pH stat method. One unit of lipase activity (LU/mg) is defined as the amount needed to liberate one micromole fatty acid per minute.

Step 1:- Centrifuge the fermentation supernatant, discard the precipitate. Adjust the pH of the supernatant to 7 and add gradually an equal volume of cold 96 % ethanol. Allow the mixture to stand for 30 minutes in an ice bath. Centrifuge and discard the precipitate.

Step 2:- Ion exchange chromatography. Filter the supernatant and apply on DEAE-fast flow (Pharmacia TM) column equilibrated with 50 mM tris-acetate buffer pH 7. Wash the column with the same buffer till absorption at 280 nm is lower than 0.05 OD. Elute the bound enzymatic activity with linear salt gradient in the same buffer (0 to 0.5 M NaCl) using five column volumes. Pool the fractions containing enzymatic activity .

Step 3:- Hydrophobic chromatography. Adjust the molarity of the pool containing enzymatic activity to 0.8 M by adding solid Ammonium acetate. Apply the enzyme on TSK gel Butyl- Toyopearl 650 C column (available from Tosoh Corporation Japan) which was pre-equilibrated with 0.8 M ammonium acetate. Wash the unbound material with 0.8 M ammonium acetate and elute the bound material with distilled water.

Step 4:- Pool containing lipase activity is diluted with water to adjust conductance to 2 mS and pH to 7. Apply the pool on High performance Q Sepharose (Pharmacia) column pre-equilibrated with 50 mM tris -acetate buffer pH 7. Elute the bound enzyme with linear salt gradient.

Food Products

In a further aspect, the present invention relates to food products comprising a protein hydrolysate of the invention.

The amount of protein hydrolysate incorporated in the food product will typically be in the range of 1-30% by weight.

An important food product of the present invention is an ingredient of a mother milk substitute for infants. Due to the high degree of hydrolysis obtained by the method of the invention, the protein hydrolysates of the invention may advantageously be incorporated in mother milk substitutes, the hydrolysate having a significantly lower allergenicity than unhydrolysed milk proteins. The milk substitute may be formulated in substantially the same way as that indicated in the prior literature for products of this type (cf. for instance EP Patent Application 322,589) with the exception that the protein hydrolysate included in the known products is replaced by the protein hydrolysate of the present invention.

The food product of the invention may also include the protein hydrolysate of the invention as a protein supplement or to provide other properties of the food product. Thus, the protein hydrolysate incorporated in the food product may for instance be based on meat or scrap meat (e.g. the so-called mechanically recovered meat, i.e. meat remaining on bones after the regular pieces of meat have been carved from animal carcases in the slaughterhouse. For a more detailed description of the general procedure, see applicant's co-pending International Patent Application WO 90/05462) rendered from bone by subjecting crushed bone to the method of the invention. Other proteinaceous by-products from the meat industry may also be used.

The resulting protein hydrolysate may then suitably be added to emulsified meat products, e.g. sausages or patés, or as flavour ingredients in soups or other food products.

A food product obtained from cow milk by the method of invention is preferably a cheese flavour product. The extensive hydrolysis of the milk protein surprisingly leads to flavour compounds with very distinct cheese flavour. Such cheese flavour products of the invention preferably find application in snack products, imitation cheese products, or as enhancers of cheese flavour in general. The cheese flavour may be adjusted by use of lipases according to well established practice.

A traditional way of producing Hydrolysed Vegetable Protein (HVP) for use as flavour products is based on cooking in hydrochloric acid at a high temperature and for a long time. This is known to cause unwanted formation of chloric containing compounds. By the process of the invention it is now possible to obtain enzymatically produced HVP products, which are considered more healthy products. The high DH obtainable by the process of the invention leads to desirable flavour characteristics and flavour enhancing properties.

The process of the invention may also be used for protein enrichment of dietetic products due to the non-bitter flavour obtained by the process. Also advantageous is the very high protein solubility in a very broad pH range that is associated with the process of the invention.

It has surprisingly been found that an excellent fermentation medium may be produced by subjecting milk to the method of the present invention. Such milk hydrolysates may be used for acceleration of the fermentation in yoghurt production or in the production of lactic acid starter bacteria cultures, by addition in relatively small amounts. The shortening of process time increases production capacity and reduces the risk of infection, e.g. bacteriophage infection.

Accordingly, the method of the invention may be used in combination with a fermentation process, preferably a fermentation process for the production of a food product. Thus, the proteolytic enzyme preparation, e.g. Flavourzyme™, used in the present method or the protein hydrolysate of the invention or both may be added in a fermentation process, preferably a food fermentation process, for enhancing the productivity of the fermentation process. Examples of such fermentation processes are processes involving or fermenting fish (fish sauce), cocoa beans or soy (such as soy sauce, tempeh, miso). The addition of the proteolytic enzyme preparation or the addition of protein hydrolysate may be useful for decreasing the total process time, i.e. increasing the rate of fermentation.

Non-food products

The process of the invention may also find application in the non-food area. The flavour characteristics obtained by the method of invention may advantageously be used for production of pet food.

Production of very high DH-hydrolysates from gelatine improves gelatine products for incorporation into cosmetics, e.g. creams and shampoos.

The beneficial effect as fermentation medium mentioned above is expected to make the hydrolysates of the invention useful for other fermentations as well.

The following examples further illustrate the present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Beef Hydrolysis

In this example two experiments were carried out using the following enzyme preparations (indicating % w/w based on protein content):

Preparation A) 2% Flavourzyme™ (2.30 AU/g)

Preparation B) 1% Flavourzyme™, 2% Neutrase™ 0.5L, and 0.15% Alcalase™ 2,4L.

All enzymes are available from NOVO NORDISK A/S, Denmark. Flavourzyme™ is a proteolytic preparation derived from *Aspergillus oryzae*, Neutrase™ 0.5L is a proteolytic preparation derived from *Bacillus subtilis*, and Alcalase™ 2.4L is a proteolytic preparation derived from *Bacillus licheniformis*.

408 g of beef were minced twice and mixed with 392 g of water to a protein content of 10 % w/w. The mixture was blended for 30 sec. and heated to 55°C. Initial osmolality (mOsm) and soluble dry matter (°Brix) were measured, and the subsequent hydrolysis was monitored by these values.

After four hours of incubation the enzymes were inactivated by heating at 90°C for 30 min. The hydrolysates

were subjected to centrifugation at 3000 xG for 15 min. and weighed. The centrifugate was stored in a refrigerator until the next day, and the lipid phase separated off and weighed. pH in centrifugate was 5.85.

The results are shown in Tables 1 and 2 below.

Table 1
Initial Osmolality (mOsm) and Soluble Dry Substance (°Brix)

Time	Preparation A			Preparation B		
min.	mOsm/kg	ΔmOsm/kg	°Brix	mOsm/kg	ΔmOsm/kg	°Brix
0	203	0	3.9	202	0	4.5
15				360	158	9.9
45	490	287	11.1	484	282	11.1
60	534	331	11.6			
90	576	373	12.3	534	332	12.3
120	623	420	12.7	579	377	12.8
180	685	482	13.5	624	422	13.3
240	726	523	13.8	663	461	13.7

Table 2
Preparation A

Mixture	Weight g	Protein %	Protein g	Protein yield %	DS* %
Meat/Water	800	10	80		15
Defatted centrifugate	689	9.46	65.2	81.5	10.6

* Dry Substance

As calculated from the Tables, the dosages used in these experiments Flavourzyme™ increase yields from usually 45%

to 81.5%, and the solubility increases from usually 57% to 89.9% PSI. A degree of hydrolysis 70.2% DH was achieved.

The hydrolysates were served before a tasting panel in a 4% solution. The meat flavour was most pronounced in hydrolysates obtained with Preparation A.

EXAMPLE 2

Hydrolysis at Various pH

In this experiment the method of the invention was performed at pH values in the range of 5-9 with sodium caseinate as substrate.

An 8% (w/w) solution of sodium caseinate (Miprodan™ 30, obtained from MD FOODS Amba, Denmark) was prepared by heating to 80°C. To 800 g of this solution 1 g methyl-4-hydroxybenzoate and 0.15 g propyl-4-hydroxybenzoate were added, and the temperature of the solution was lowered to 50°C. pH was adjusted to the desired value with 4 N NaOH or 4 N HCl.

1% w/w based on the protein content Flavourzyme™ (2.30 AU/g, NOVO NORDISK A/S, Denmark) was added the solution, and the incubation was allowed to proceed to maximal degree of hydrolysis (about 22 hours).

At pH 9.00, 8.00, and 7.00 the hydrolysis was carried out in a pH stat, and the incubation monitored by consumption of NaOH. At pH 6.00 and 5.00 (initial pH) the hydrolysis was monitored by decreased pH and increased osmolality.

Samples were obtained after 0.25, 1, 2, 4.5, and 22 hours of incubation. The enzyme was inactivated by heating to 85°C for 3 minutes, followed by cooling in ice-water.

The results are presented in Fig. 1. As evident from this Figure, the process of the invention is capable of exerting hydrolysis in the pH range of from pH 5 to pH 9.

EXAMPLE 3

Comparison Example

In this experiment the degree of hydrolysis (DH) obtained by the process of invention (incubation with Flavourzyme™) was compared to the DH obtained by incubation with other known proteolytic preparations.

The experiment was carried out at 50°C as a non-pH stat hydrolysis (no adjustments of pH during hydrolysis), starting at pH 7.00 adjusted with 4N NaOH. To ensure equal enzyme dosage each hydrolysis was carried out using the same proteolytic activity as expressed in AU/g (cf. above for determination of AU).

Two protein raw materials were tested:

1) Na-caseinate (supplied by MD Foods Amba, Denmark)
2) Soy protein isolate (supplied by Protein Technologies International, U.S.A.).

2500 g of an 8% (w/w based on protein content) solution of protein raw material was prepared. The protein solution was heat treated at 85°C for 3 minutes and cooled to hydrolysing temperature 50°C.

Samples of 400g were prepared for hydrolysis.

The following enzymes and enzyme dosages were used:

Enzyme	Activity, AU/g	Dosage per 400g
Flavourzyme, Novo Nordisk A/S	3.36	0.321 g
Neutrase 0.5L, Novo Nordisk A/S	0.484	2.231 g
Alcalase 2.4L, Novo Nordisk A/S	2.58	0.419 g
Corolase 7093, Röhm GmbH	0.094	11.489 g
Corolase 7092, Röhm GmbH	1.54	1.426 g

The hydrolysis was carried on for 22 hours.

The degree of hydrolysis was measured after 5 and 22 hours of hydrolysis, respectively. The results are shown in fig. 2 (soy protein isolate) and fig. 3 (Na-caseinate). From the results it is evident that hydrolysis by using Flavourzyme clearly results in the highest degree of hydrolysis.

EXAMPLE 4

Cheese Flavour Production

Whole milk was heat treated for 2 min at 100°C and subsequently cooled to 50°C. Four portions of 200 ml each were prepared (indicating % w/w based on protein content):

1) 1% Flavourzyme™ (2.30 AU/g)

2) 1% Flavourzyme™ (2.30 AU/g) + 0.15% Palatase™ M 200L (200 LU/g from NOVO NORDISK A/S, Denmark)

3) 0.15% Palatase™ M 200L (200 LU/g from NOVO NORDISK A/S, Denmark)

4) Control

The hydrolysis was carried out for 20 hours at 50°C. Intensive cheese flavour developed in samples 1, 2, and 3, while the control was almost neutral.

The flavour-samples 1, 2, and 3, were now diluted in cream cheese, and a fourth sample was prepared as a control.

1.1) 0.5 g of sample No. 1 + 20 g of cheese

2.1) 6.0 g of sample No. 2 + 20 g of cheese

3.1) 6.0 g of sample No. 3 + 20 g of cheese

4.1) 6.0 g of fresh milk + 20 g of cheese.

Sample No. 1.1 (Flavourzyme™) appeared to have a very strong and intense flavour and a taste of well ripened cheese. Sample No. 2.1 (Flavourzyme™ + Palatase™) appeared to have a well balanced cheese flavour. Compared to sample No. 1.1 and No. 2.1, sample No. 3.1 turned out to give the weakest taste impression. The reference, sample No. 4.1 was evaluated being sour/sourish.

In conclusion it is possible to add the enzymes Flavourzyme™ and the combination of Flavourzyme™ and Palatase™ directly into the milk to form excellent cheese flavour.

The traditional way of producing cheese flavour is carried out by adding lipases and proteases to cheese. That is, the use of Flavourzyme™ and Flavourzyme™ in combination with Palatase™ creates a new and much more simple way of producing cheese flavour.

EXAMPLE 5

Accelerated Yoghurt Fermentation

Whole milk was heat treated for 2 min at 100°C and subsequently cooled to 50°C. Two portions of 200 ml were prepared (indicating % w/w based on protein content):

1) 1% Flavourzyme™ (2.30 AU/g)

2) Control

The hydrolysis was carried out for 20 hours at 50°C. Then the enzymes were denatured by heat treatment at 80°C for 5 min.

Skim milk was heat treated at 90°C for 2 min and three samples of 200 ml were prepared.

1.1) 200 ml of milk + 10 ml of sample No. 1

2.1) 200 ml of milk + 10 ml of sample No. 2

3.1) 200 ml of milk

The temperature was adjusted to 41°C, and yoghurt culture YC DVS from Chr. Hansens Lab. was added to all three samples in a level of 10^6 bacteria per g.

The fermentation course was followed by measuring pH, cf. Table 4, below.

Table 4

Acidification Course

(pH)	1) Flavour-zyme™	2) Blind	3) Blind/Blind
Initial pH value	6.6	6.5	6.5
pH after 200 min	5.9	6.4	6.4
pH after 360 min	4.4	5.9	6.3

It appears from the pH measurements that the addition of Flavourzyme™ hydrolysed milk accelerates the fermentation, meaning that the production time of fermented milk products can be reduced considerably.

The claims defining the invention are as follows:

1. A method for hydrolysing a protein by incubating with a proteolytic enzyme preparation, which proteolytic preparation is derived from *Aspergillus oryzae* and comprises at least five proteolytic components each having an approximate molecular weight, respectively, selected from 23 kD, 27 kD, 31 kD, 32 kD, 35 kD, 38 kD, 42 kD, 47 kD, 53 kD, and 100 kD, and by which method there is obtained a degree of protein hydrolysis (DH) of higher than 60%.

2. The method according to claim 1 in which the proteolytic preparation comprises at least five proteolytic components having the approximate molecular weights 23 kD, 31 kD, 35 kD, 38 kD and 53 kD, respectively.

3. The method according to claim 1 or claim 2, by which there is obtained a degree of protein hydrolysis (DH) higher than 70%.

4. The method according to claim 3 in which the protein hydrolysis (DH) is higher than 80%.

5. The method according to claim 1 or claim 2, by which there is obtained a degree of protein solubility (Protein Solubility Index (PSI)) higher than 50%PSI.

6. The method according to claim 5, wherein the protein solubility obtained is higher than 70%PSI.

7. The method according to claim 5, wherein the protein solubility obtained is higher than 90%PSI.

8. The method according to any one of the preceding claims, in which the protein is a vegetable protein.

9. The method according to claim 8, in which the vegetable protein is selected from soy protein; grain protein; rape seed protein; alfalfa protein; pea protein; fabaceous bean protein; cotton seed protein; and sesame seed protein.

10. The method according to claim 9, wherein the grain protein is selected from wheat gluten and zein.

11. The method according to any one of claims 1 to 7, in which the protein is an animal protein.

12. The method according to claim 11, in which the animal protein is selected from milk protein; whey protein; casein; meat protein; fish protein; blood protein; egg white, and gelatin.

13. The method according to any one of the preceding claims, in which the incubation is performed at a pH of between about 4 and about 10.

14. The method according to claim 13, in which the incubation is performed at a pH of between 5 and about 9.

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15. The method according to any one of the preceding claims, in which the protein is incubated with the proteolytic preparation in combination with one or more other protease preparations.

16. The method according to claim 15, in which the protease preparation is a neutral protease derived from *Bacillus*.

17. The method according to claim 16, in which the neutral protease is derived from *Bacillus subtilis*.

18. The method according to claim 15, in which the protease preparation is an alkaline protease derived from *Bacillus*.

19. The method according to claim 18, in which the alkaline protease is derived from *Bacillus licheniformis*.

20. The method according to any one of the preceding claims, in which the protein is incubated with the proteolytic preparation in combination with one or more lipase preparations.

21. The method according to claim 20, in which the lipase preparation comprises a fungal lipase derived from *Mucor*.

22. The method according to claim 21, in which the fungal lipase is derived from *Rhizomucor miehei*.

23. The method according to any one of claims 20 to 22, in which the lipase preparation comprises a fungal lipase derived from *Aspergillus*.

24. The method according to claim 23 in which the fungal lipase is derived from *Aspergillus niger*.

25. The method according to any one of the preceding claims, wherein the protein is hydrolysed and simultaneously fermented to a food product.

26. A method for hydrolysing a protein by incubating with a proteolytic enzyme preparation, substantially as hereinbefore described with reference to any one of the Examples but excluding the Comparative Examples.

27. A protein hydrolysate obtained by the method according to any one of the preceding claims.

28. The protein hydrolysate according to claim 27, wherein the hydrolysed protein is an animal protein selected from the group consisting of milk protein, whey protein, casein, meat proteins, fish protein, blood protein, egg white and gelatin.

29. The protein hydrolysate according to claim 27, wherein the hydrolysed protein is a vegetable protein selected from the group consisting of soy protein; grain protein; rape seed protein; alfalfa protein; pea protein; fabaceous bean protein; cotton seed protein; and sesame seed protein.

30. The protein hydrolysate according to claim 29, wherein the grain proteins are selected from wheat gluten and zein.

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31. A protein hydrolysate, substantially as hereinbefore described with reference to any one of the Examples but excluding the Comparative Examples.

32. A food product comprising a protein hydrolysate according to any one of claims 27 to 31.

33. The food product according to claim 32, being an ingredient of mother milk substitute; a cheese flavour product; an enzymatically produced HVP; a protein enriched dietetic product; a soup-, bouillon- or meat-flavour product; a meat extract product; or a hydrolysate for improving the production of a starter culture.

34. A non-food product comprising a protein hydrolysate according to any one of claims 27 to 31.

35. The non-food product according to claim 24, being a pet food, a cosmetic, or a fermentation broth.

Dated 26 June, 1997
Novo Nordisk A/S

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

AUST

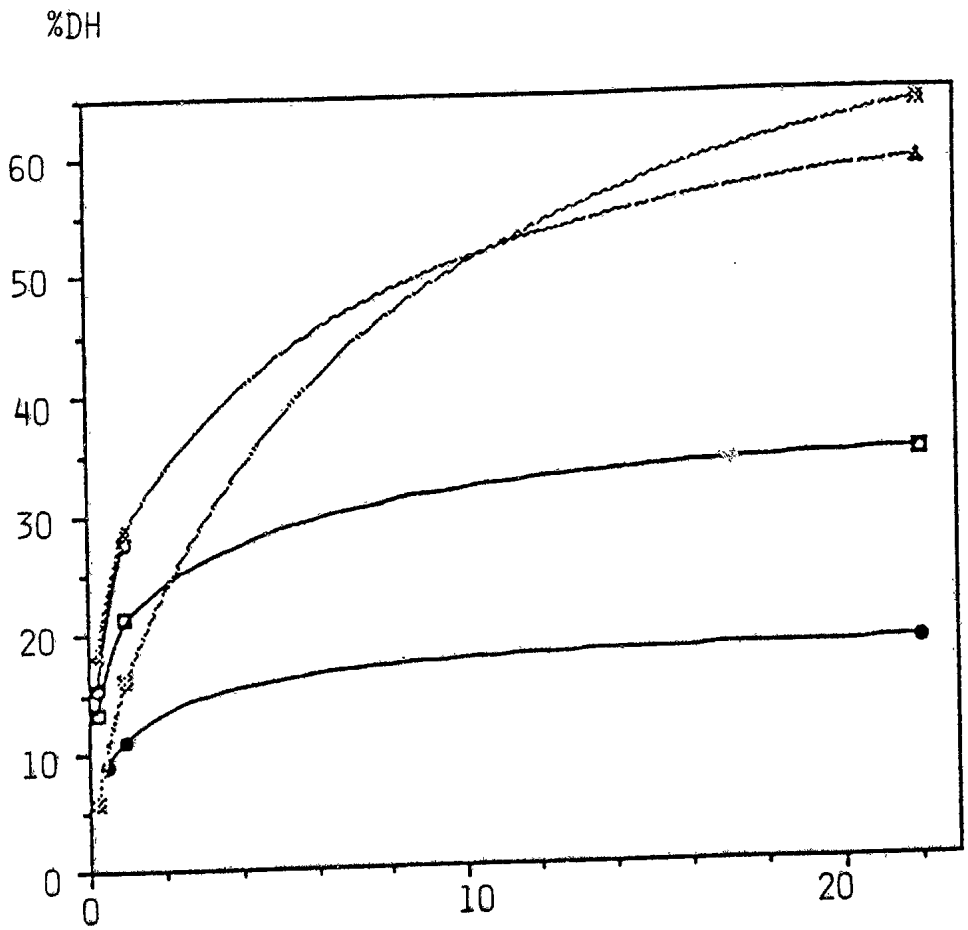

Fig. 1

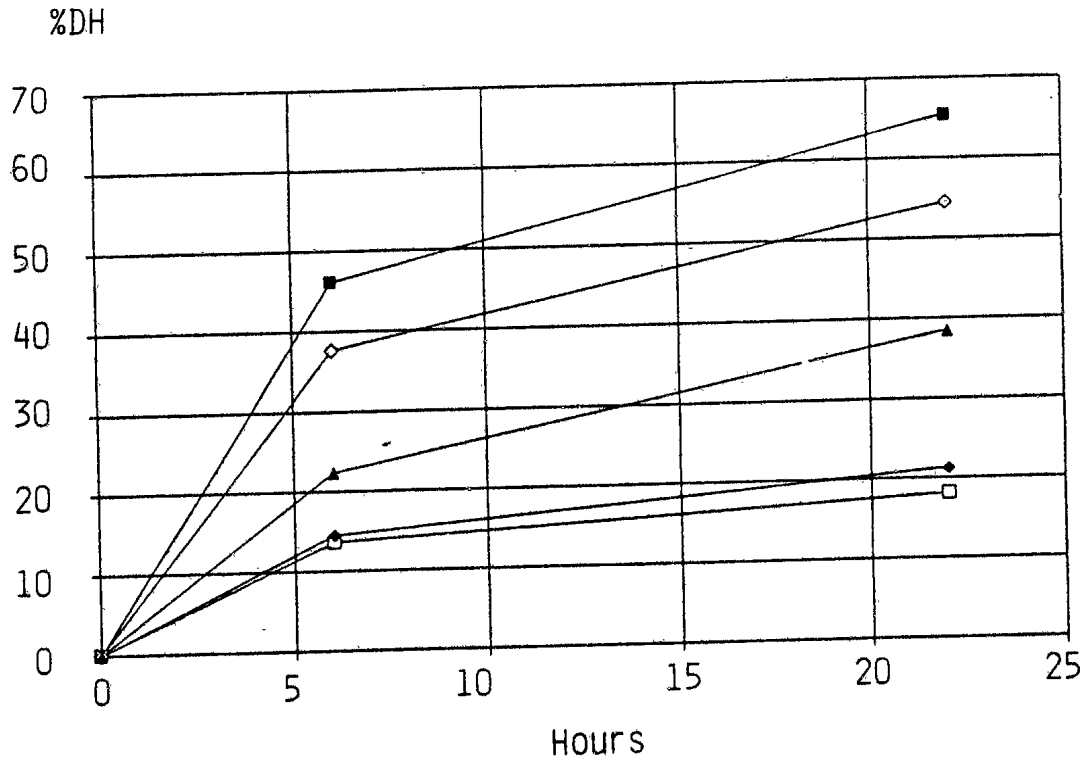

Fig. 2

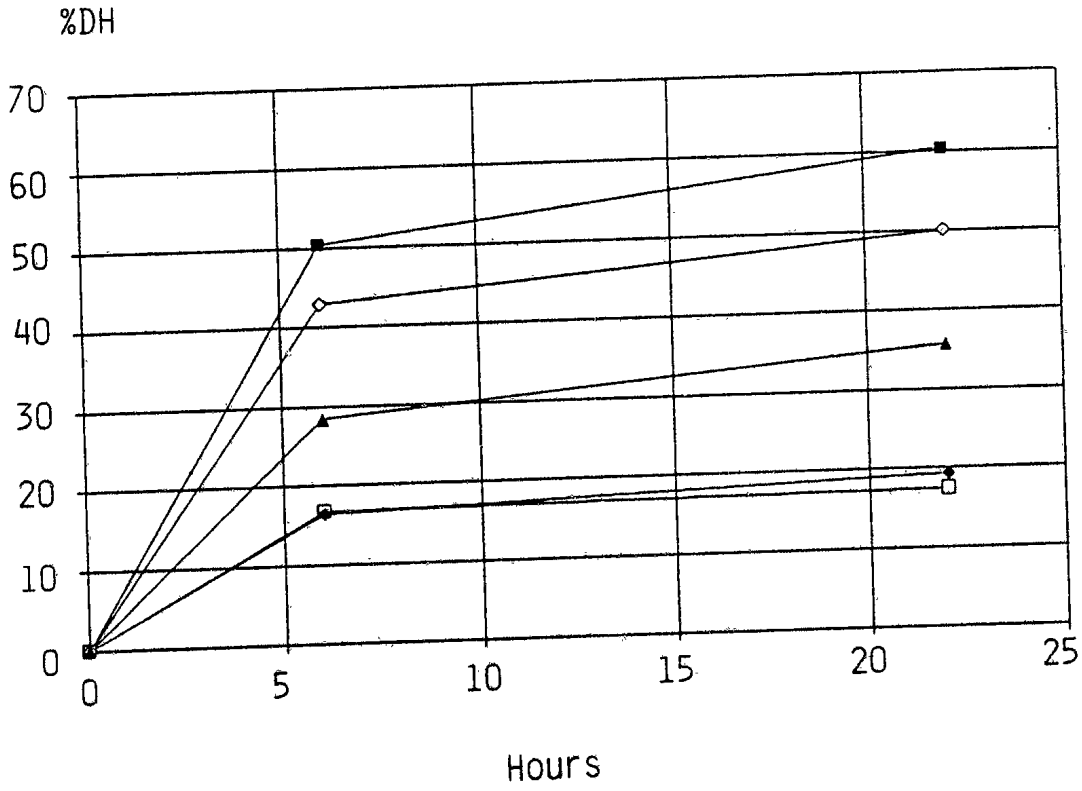

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00165

A. CLASSIFICATION OF SUBJECT MATTER

IPC : C12N 9/62, A23J 3/34
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC : A23J, A23L, C12N, C12R

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

SE,DK,FI,NO classes as above

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

EPOQUE, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE, A1, 3306009 (RÖHM GMBH), 23 August 1984 (23.08.84), whole document ---	1-21
X	EP, A1, 0429760 (SOCIETE DES PRODUITS NESTLE S.A.), 5 June 1991 (05.06.91), whole document ---	1-21

☐ 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" erlier 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
22 July 1994	29 -07- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Jack Hedlund Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/07/94

International application No.
PCT/DK 94/00165

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CH-A,B- 662475	15/10/87
		FR-A,B- 2541308	24/08/84
		NL-A- 8304449	17/09/84
EP-A1- 0429760	05/06/91	SE-T3- 0429760	
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		CH-A- 679542	13/03/92
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		DE-U- 6900066	04/02/93
		JP-A- 3168066	19/07/91
		US-A- 5141757	25/08/92