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(54) Title: CHEESEMAKING WITH RECOMBINANT ASPARTIC PROTEASE

(57) Abstract

A process of producing cheese in improved yields, wherein a recombinant aspartic protease, derived from *Rhizomucor miehei* or *Rhizomucor pusillus*, is added to milk in sufficient amounts to effect clotting of the milk, after which the resulting curd is processed in a manner known *per se* for making cheese.

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CHEESEMAKING WITH RECOMBINANT ASPARTIC PROTEASE

FIELD OF INVENTION

This invention relates to a process for producing cheese in improved yields.

BACKGROUND OF THE INVENTION

In the production of cheese it is necessary to coagulate the milk in order to be able to separate the casein from the whey. Products containing rennin, which is a milk coagulating enzyme isolated from calf stomachs, have for many years been used for this purpose. Shortage of calf stomachs has in the last decades resulted in intense searches for other milk coagulating enzymes. Today, bovine pepsin, porcine pepsin, as well as microbial enzymes are being used commercially. The most useful among the microbial rennets are Rhizomucor miehei rennet and Rhizomucor pusillus rennet.

SUMMARY OF THE INVENTION

In this invention it is surprisingly found that the glycosylation of the aspartic protease can give an increase in cheese yield of 0.2% compared with the native enzyme. An increase in cheese yield of 0.2% would mean an extra 2 kg of cheese per one ton of cheese having a value of about 8 - 10 US$ (prices of the dairy).

Accordingly, the present invention relates to a process of producing cheese in improved yields, wherein a recombinant aspartic protease is added to milk in sufficient amounts to effect clotting of the milk, after which the resulting curd is processed in a manner known per se for making cheese.

The term "recombinant aspartic protease" is applied to (pro)chymosin or microbial aspartic protease produced in a host organism transformed with DNA coding for the protease. The

host organism may conveniently be a fungus, e.g. a yeast or a filamentous fungus.

The term "filamentous fungus" is intended to include fungi belonging to the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes or fungi imperfecti, including Hyphomycetes such as the genera Aspergillus, Trichoderma, Penicillium, Fusarium or Humicola.

DETAILED DISCLOSURE OF THE INVENTION

Protease

According to this invention it is preferred to use recombinant aspartic protease from Mucorales (e.g. Rhizomucor, in particular Rhizomucor miehei or Rhizomucor pusillus) expressed in Aspergillus or Trichoderma in a process of producing cheese in improved yields.

A DNA sequence encoding bovine prochymosin or preprochymosin may for instance be obtained as described in EP 215 594. A DNA sequence encoding Rhizomucor miehei aspartic protease may be isolated as described in EP 238 023.

DNA sequences encoding functions facilitating gene expression typically comprise a promoter, transcription initiation sites, and transcription termination and poly-adenylation functions.

The promoter which may be preceded by upstream activating sequences and enhancer sequences as known in the art may be any DNA sequence exhibiting a strong transcriptional activity in filamentous fungi and may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase or a cellulase.

Examples of suitable promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral α-amylase, A. niger acid stable α-amylase, A. niger glucoamylase, Rhizomucor miehei lipase, A. oryzae alkaline protease or A. oryzae triose phosphate isomerase.

The filamentous fungus host organism may conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of Aspergillus sp., such as A. niger, A. nidulans or A. oryzae. The use of A. oryzae in the production of recombinant proteins is extensively described in e.g. EP 238 023.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The techniques used to transform a fungal host cell may suitably be adapted from the methods of transforming A. nidulans described in, for instance, Yelton et al., Proc. Natl. Acad. Sci. USA 81, 1984, pp. 1470-1474, or EP 215 594, or from the methods of transforming A. niger described in, for instance Buxton et al., Gene 37, 1985, pp. 207-215 or US 4,885,249, or from the method of transforming A. oryzae described in EP 238 023. In the process of the present invention, the host cell is transformed with a vector system comprising a DNA sequence coding for a selection marker which is capable of being incorporated in the genome of the host organism on transformation, but which is either not expressed by the host before transformation or expressed in amounts which are not sufficient to permit growth under selective conditions. Transformants can then be selected and isolated from non-transformants on the basis of the incorporated selection marker.

Suitable selection markers are derived from the A. nidulans or A niger argB gene, the A. nidulans trpC gene, the A. nidulans amdS gene, the Neurospora crassa pyr4 or DHFR genes, or the A. niger or A. oryzae niaD gene.

The DNA sequences coding for the aspartic protease, promoter and terminator may be inserted in a vector containing the selection marker, or it may be inserted in a separate vector for introduction into the host cell. In the present context, the term "vector system" is intended to include a single vector or two or more vectors which together contain the total DNA information to be introduced into the host cell. The vector or vectors may be linear or closed circular molecules. In a preferred embodiment of the process of the invention, the

vector system comprises two vectors, one carrying the DNA sequence coding for the selection marker, and the other carrying the DNA sequences encoding the heme protein, the preregion and the functions facilitating gene expression.

The extent of glycosylation of the recombinant aspartic protease has surprisingly been found to be higher than the glycosylation of the aspartic proteases obtained from naturally occurring Rhizomucor strains. More specifically ,the novel recombinant aspartic protease is characterized in that the carbohydrate content is of at least 50% more than the native aspartic protease, preferably the carbohydrate content is of a level of 100% more than the native aspartic protease. The term "native aspartic protease" is intended to indicate the protease produced by an organism in nature (e.g. in calf stomach or by a microorganism such as Rhizomucor).

Differentiation and identification of the native aspartic protease and of the recombinant aspartic protease is possible through measurement of their glycosylation.

It has formerly been described that Aspergillus glycosylates Humicola lipase in a different way from the native Humicola lipase (see EP 305 216), but it is new that Aspergillus also glycosylates Rhizomucor aspartic protease differently from the native Rhizomucor aspartic protease, and specifically that by this process the carbohydrate content of the aspartic protease increases by 50 -100%.

Analysis of the Amount of N-bound Glycosylation

The amount of N-bound glycosylation is determined as the difference in molecular weights between the aspartic protease and a deglycosylated aspartic protease. The deglycosylation is performed by Endoglycosidase H (from Genzyme) (removes N-bound glycosylation) using 1 mg/ml protease and a reaction time of 18 hour at 37°C, the Endo H is dosed so that no additional deglycosylation is obtained at increased Endo H dosages. The MW weight is measured by SDS-PAGE.

The amount of N-bound glycosylation is expressed in kD glycosylation per mole protein.

Cheesemaking

Any type of milk, in particular milk from ruminants such as cows, sheep, goats or camels, may be used as the starting material in the process of the invention, e.g. as reconstituted milk, whole milk, concentrated whole milk or skimmilk.

The milk may be concentrated in various ways such as by evaporation or spray-drying, but is preferably concentrated by membrane filtration, i.e. ultrafiltration in which molecules with a molecular weight of up to 20,000 are allowed to pass the membrane, optionally with diafiltration before or after ultrafiltration, or possibly hyperfiltration in which molecules of a molecular weight of up to 500 are allowed to pass the membrane. For a more detailed description of the ultrafiltration process, see for instance Quist et al., Beretning fra Statens Mejeriforsøg, 1986.

A starter culture may be added to the milk before or simultaneously with the addition of the recombinant aspartic acid in the present invention. The starter culture is a culture of lactic acid bacteria used, in conventional cheesemaking, to ferment the lactose present in the milk and to cause further decomposition of the clotted casein into smaller peptides and free amino acids as a result of their production of proteases and peptidases. The starter culture may be added in amounts which are conventional for the present purpose, i.e. typically amounts of about 1×10^4 - 1×10^5 bacteria/g of cheese milk, and may be added in the form of freeze-dried, frozen or liquid cultures. When the milk employed in the process of the invention is concentrated milk, it is preferred to add the starter culture after concentrating the milk, although this is not an absolute requirement, as the starter bacteria will be retained during filtration.

After adding the milk clotting enzyme the subsequent steps in the cheesemaking process, i.e. further salting, pressing, and ripening the curd, may be conducted in the traditional way of producing cheese, e.g. as described by R.

Scott, Cheesemaking in Practise, 2nd Ed., Elsevier, London, 1986.

It is at present contemplated that most types of cheese may advantageously be prepared by the process of the invention.

The present invention relates to an aspartic protease preparation in liquid, stabilized, spray-dried, vacuum-dried, freeze-dried or granulated form, or immobilized on a suitable carrier.

The various ways in which the enzyme preparation may be formulated are well known in the enzyme art, cf. for instance K. Aunstrup et al., "Production of Microbial Enzymes", in Microbial Technology (H.J. Peppler and D. Perlman, Eds.), 2nd Ed., Vol I, Academic Press 1979, pp 295-297.

In the process of the present invention the amount of recombinant aspartic protease will vary according to the degree of concentration of the milk, but the enzyme will usually be added in an amount of 1 - 10 KRU per 1 l of whole milk.

Rennet Strength

1 RU is the Novo unit of rennet strength. 1 KRU = 1000 RU. The rennet strength is determined by using a modified Berridge method (N.J. Berridge, Analyst, 77, 1952, p. 57) where the rennet strength is determined by using a Rennilase® (Rhizomucor miehei aspartic protease available from Novo Nordisk A/S) powder standard as a reference.

The principle of the method is that the enzyme acts upon a solution of skimmed milk powder containing calcium chloride under standard conditions. The time needed from the addition of the enzyme until the reaction mixture begins to show flocculation is measured. This time is approximately inversely proportional with the enzyme concentration and is compared with the flocculation time for a sample with known enzyme strength. These times should not differ substantially from each other, and they should amount to 5.0 min ± 0.5 min.

The determination of strength is carried out visually and is performed at 30°C. "Berridge Substrate" consisting of 12

g of spray-dried skimmed milk powder dissolved in 100 ml of 0.01 M calcium chloride solution is used. The substrate must stand for at least 1 hour before it can be used.

The analysis is performed in a glass water bath in which the test tubes by means of a motor are slowly rotated about their longitudinal axes with an inclination of about 30° relative to the water surface. A film of milk will thereby continuously be formed on the tube above the milk surface, and it is easy to see the flocculation in this film as soon as it starts.

Sometimes local clottings can take place in the milk. These clottings are not to be confused with the actual flocculation which is characterized by the adhesion of small flakes of casein to the test tube near the milk surface.

About half an hour before the analysis is carried out place stoppered test tubes containing 10 ml of milk substrate in the constant temperature water bath at 30°C ± 0.1°C. The flocculation time changes slightly with the time in which the substrate is kept warm. Now add with a syringe one ml of the diluted enzyme solution to each of the 3 test tubes and simultaneously activate a stop watch for each tube. The stop watch is started immediately after the enzyme solution has been injected into the test tube. The enzyme solution is injected against the wall of the tube to avoid foaming of the milk. Immediately after the addition of the enzyme, the test tube is closed with a clean, dry stopper and inverted 3 times so as to wash all the enzyme off the tube wall and into the milk. The first test tube may immediately thereafter be placed on the rotating spindle. At the point in time when small flakes of coagulum start being deposited on the tube the stop watch for the first tube is stopped, the tube is replaced with tube No. 2 etc. Try to achieve a flocculation time between 4.5 and 5.5 min. The flocculation time for the 3 test tubes must not vary more than 0.1 min (6 sec.), and the average is used to calculate the strength of the rennet.

Calculation of the Rennet Strength

$$\text{Strength of sample} = \frac{\text{Strength of standard} \times \text{Flocculation time of dilut. of stand.}}{\text{Flocculation time of sample}} \times \frac{\text{Dilution of sample}}{\text{Dilution of standard}}$$

The modified Berridge method is only suited for the determination of rennet strengths of rennets without added calcium salts.

The invention is further illustrated in the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Cheese Yield Using Aspergillus oryzae produced Aspartic Protease and Rennilase® XL

Enzymes

Aspergillus oryzae produced protease: Transformant p777 (described in EP 489 718) was fermented for approx. 5 days in a conventional agitated and aerated 2.5 m^3 fermentor on a medium containing soya grits supplied with maltodextrin during growth, the pH was kept below 6.0 by addition of phosphoric acid. The aspartic protease was recovered from the fermentation broth by conventional filtration, ultrafiltration, and evaporation techniques. The aspartic protease was destabilized as described in US Patent 4,357,357. The aspartic protease concentrate was diluted to 54.63 KRU/g.

Rhizomucor miehei produced aspartic protease: Rennilase® XL 51.42 KRU/g was used (available from Novo Nordisk A/S).

Both enzymes were used so that the first sign of flocculation appeared after about 15 min. Dosages were kept at constant level throughout the experiment. Variations in the

coagulability of the milk was compensated by varying cutting times.

Cheesemaking in Beakers

To a 5 l beaker 4000 g of whole pasteurized milk (available from MD Foods, Karolinevej 1, DK-4200 Slagelse, Denmark) was added.

A starter culture CHL 113 batch 010691 (available from Chr. Hansen, Bøge Alle 10, DK-2970 Hørsholm, Denmark) was incubated for 16 hours in sufficient amount of milk at room temperature and frozen in 40 g portions, enough portions for the entire experiment.

$CaCl_2$, 0.8 g per portion and a thawed portion of starter culture are added. After 30 min ripening time the milk coagulating enzyme in an amount of 10.9 KRU per 4000 g of milk was introduced. Both enzymes were used in each trial, and their order was not randomized. Flocculation time was around 15 min, and the cutting time was approx. 2 times flocculation time plus 1 min. After a healing time of 4 min and a stirring time of 15 min the curd and whey were transferred to a mould with cheese-cloth and left to drain overnight.

Analysis

Well mixed total whey was filtered through one layer of gauze to remove curd particles. Total N was determined using a modified Kjeldal method on a Tecator Digestor and Kjeltec 1003 Distilling System. Protein was expressed as N x 6.38. All analysis were done in triplicate.

A paired t test was used on n = 61 differences in total protein content of whey, using $t = x_{mean}/(s^2/n)^{1/2}$ as an estimate, where X is the difference in total whey protein between the corresponding experiments with the two enzymes, and S^2 is the estimated variation of X, $t_{0.95}(60\%) = 1.67$, $t_{0.995}(60\%) = 3.23$.

Results

In Table 1 some parameters of manufacture of Feta Cheese can be found. Coagulation times averaged 15.41 min for aspartic protease and 15.35 min for Rennilase XL. Δprotein was calculated as the difference between the amount of Rennilase XL Whey times protein % deducting the amount of Aspartic Protease Whey times protein %. Average protein loss was found to be 30.30 g with aspartic protease whey and 30.50 g with Rennilase XL whey. These figures could mean an increase in cheese yield of 0.2%, when changing from Rennilase XL to aspartic protease.

X_{mean} on Δprotein was found to be -0.1953 and s to be 0.4642 and n = 61, and thus an estimate for t can be calculated as $t_o = -0.1953/(0.4642^2/61)^{½} = -3.2860$, which means that the hypothesis that no difference between yields can be rejected with very high probability ($p < .001$).

Table 1 **Some parameters of manufacture of Feta Cheese**

parameter	**mean**	**n**	**s**
Milk, amount	4000 g		constant
Starter	1%		constant
Milk coagulating enzyme KRU/4000 g			
Aspartic protease	10.9	61	constant
Rennilase®XL	10.9	61	constant
$CaCl_2$	0.02%		constant
Times min			
ripening	30	122	constant
flocculation			
Aspartic protease	15.41	61	±0.63
Rennilase XL	15.35	60	±0.64
cutting time			
Aspartic protease	31.79	61	±1.25
Rennilase XL	31.82	60	±1.21
healing time	4		constant
stirring	15		constant
scooping	10		constant
scooping to press	120		constant
pH			
milk	6.69	122	-
setting	6.57	122	±0.05
whey			
Aspartic protease	6.53	56	±0.14
Rennilase XL	6.53	51	±0.14
Temperatures °C			
setting	33		
Weights g			
milk	4000	122	constant
whey	3299.3	122	±27.5
curd	685.7	117	±20.6

Recovery %	
outputs/inputs	99.9
whey/outputs	82.8
curd/outputs	17.2

EXAMPLE 2

Deglycosylation by EndoH of Aspartic Protease Expressed from Rhizomucor miehei and Aspergillus oryzae

Aspartic protease is a glycosylated protein both when expressed from R. miehei and from A. oryzae. Endoglycosidase H can liberate the glyco part from N-glycosylated proteins by specific hydrolysis between two N-acetylglucosamine molecules which are bound to Asn in N-glycosylated proteins.

Samples

The Aspergillus oryzae produced aspartic protease (produced as described in Example 1) was purified from the culture broth by the method described in Journal of Chromatography 476 (1989) 227-233.

The Rhizomucor miehei produced aspartic protease was purified from Rennilase 150® L (available from Novo Nordisk A/S) by the method described in Journal of Chromatography 476: (1989) 227-233.

Experimental:

Aspergillus oryzae produced aspartic protease and Rhizomucor miehei produced aspartic protease were incubated at a concentration of 1 mg/ml with EndoH (genzyme) for 18 hours at 37°C in 100 mM phosphate buffer at pH 6.0. The degree of hydrolysis was determined from the change in MW by SDS-PAGE in 5 - 20% gradient gels.

Results:

The experiment showed that 0.02 U/ml EndoH was sufficient for almost total deglycosylation, and that the MW of

the aspartic protease molecule did not change when incubated without EndoH.

The MW of the deglycosylated aspartic protease was 38.3 kD both from R. miehei and A. oryzae, whereas the glycosylated enzyme from R. miehei showed a MW of 41.5 kD, and the glycosylated enzyme from A. oryzae showed a MW of 44.7 kD.

The N-bound glycosylation content of the A. oryzae expressed aspartic protease is then 6.4 kD (44.7 kD minus 38.3 kd) per mole protein, being 100% more than the 3.2 kD (41.5 kD - 38.3 kD) N-bound glycosylation per mole Rennilase (R. miehei expressed aspartic protease).

EXAMPLE 3

Glycolysation of Aspartic Protease

Samples

The Aspergillus oryzae produced aspartic protease (produced as described in Example 1) was purified from the culture broth by the method described in Journal of Chromatography 476 (1989) 227-233.

The Rhizomucor miehei produced aspartic protease was purified from Rennilase 150® L (available from Novo Nordisk A/S) by the method described in Journal of Chromatography 476: (1989) 227-233.

Both the purified enzymes were freeze dried.

Determination of the Glycosylation

25 µg samples were hydrolysed in 2M TFA (trifluoroacetic acid) in a micro-oven.

The monosaccharides were separated and detected by a Dionex high performance anionic exchange chromatography (HPAEC), the separations were performed by an isocratic elution in 16 mM NaOH. The monosaccharides were detected by pulsed amperometric detection (ref: Rocklin and Pohl;, J. Liq. Chromatography 6: 1577-1590).

The analysed samples are equivalent to 20 µg dry-matter (sample).

The samples were analysed in triplicate.

Results

	Rhizomucor miehei produced aspartic protease	Aspergillus oryzae produced aspartic protease
Galactosamin	0	0
Glucoseamin	265	630
Galactose	170	500
Glucose	200	100
Mannose	1300	2860

The results are given in relative amounts.

It is observed that there are significant differences in the sugar content between the A. oryzae expressed aspartic protease and the R. miehei expressed aspartic protease, and that the recombinant aspartic protease has an N-bound glucoseamin, galactose and mannose content of 100% more than the native protease.

CLAIMS

1. A process of producing cheese in improved yields, wherein a recombinant aspartic protease is added to milk in sufficient amounts to effect clotting of the milk, after which the resulting curd is processed in a manner known per se for making cheese.

2. A process according to claim 1, wherein the gene coding for the aspartic protease is derivable from a filamentous fungus.

3. A process according to claim 2, wherein the filamentous fungus is of the order Mucorales.

4. A process according to claim 2, wherein the filamentous fungus is of the genus Rhizomucor.

5. A process according to claim 2, wherein the filamentous fungus is Rhizomucor miehei or Rhizomucor pusillus.

6. A process according to any of claims 1 - 5, wherein the aspartic protease is produced by recombinant DNA techniques in a filamentous fungus.

7. A process according to claim 6, wherein the filamentous fungus belongs to the genus Aspergillus or Trichoderma.

8. A process according to claim 7, wherein the filamentous fungus is a strain of A. oryzae, A. niger, A. nidulans, or A. awamori, or wherein the filamentous fungus is a strain of Trichoderma reesei.

9. A process according to any of claims 6 - 8, wherein the recombinant aspartic protease has a different glycosylation from the native protease.

10. A process according to claim 9, wherein the recombinant protease has an N-bound carbohydrate content of at least 50% more than the native protease, preferably an N-bound carbohydrate content of 100% more than the native protease.

11. A process according to claim 1 - 10, wherein said aspartic protease is added in an amount of 1 - 10 KRU per 1 l of milk.

12. A process according to any of claims 1 - 11, wherein the milk is whole milk or concentrated milk.

13. A process according to any of claims 1 - 12, wherein a starter culture is added to the milk before or simultaneously with the addition of the recombinant aspartic protease.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00163

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A23C 19/032, C12N 15/80
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)

IPC5: A23C, C12N

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)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TIBTECH, Volume 7, October 1989, Gunter Saunders et al, "Heterologous gene expression in filamentous fungi", see page 2, table 4	1-13
Y	EP, A1, 0489718 (NOVO NORDISK A/S), 10 June 1992 (10.06.92), see abstract	1-13
Y	US, A, 3212905 (KEI ARIMA ET AL), 19 October 1965 (19.10.65), whole document	1-13

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

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 1994	01 -08- 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.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0489718	10/06/92	DE-D,T- 3788524	11/05/94
		EP-A,B- 0238023	23/09/87
		SE-T3- 0238023	
US-A- 3212905	19/10/65	DE-A- 1442118	13/03/69
		DE-A- 1692334	29/06/72
		GB-A- 970331	00/00/00
		US-A- 3151039	00/00/00