Proteolysis and consistency of Meshanger cheese

Promotor: dr. H. Mulder, oud-hoogleraar in de zuivelbereiding en de melkkunde.

L. de Jong

Proteolysis and consistency of Meshanger cheese

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
hoogleraar in de organische scheikunde,
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Abstract

Jong, L. de (1978) Proteolysis and consistency of Meshanger cheese. Doctoral thesis, Wageningen. (viii) + 15 p., 2 figs, 15 refs, Eng. and Dutch summaries. Agric. Res. Rep. 875 p. 3-4; Neth. Milk Dairy J. 29 (1975): 162-168; 30 (1976): 242-253; 31 (1977): 314-327; 32 (1978): 15-25; 31 (1977): 296-313; 32 (1978): 1-14.
Also: Agric. Res. Rep. (Versl. landbouwk. Onderz.) 875 p. 27-43.

Proteolysis in Meshanger cheese, estimated by quantitative polyacrylamide gel electrophoresis is discussed. The conversion of α_{s1}-casein was proportional to rennet concentration in the cheese. Changes in consistency, after a maximum, were correlated to breakdown of α_{s1}-casein. The changes in structure of the cheese during ripening, studied by electron microscopy, looked similar to those reported for Camembert cheese. Softening of cheese with high moisture content was due to rennet breakdown of α_{s1}-casein. Tests with substrates of sodium paracaseinate, calcium paracaseinate–calcium phosphate complex and a synthetic complex of casein, calcium hydroxide and phosphoric acid revealed that Na^+ and Ca^{2+} both influenced rennet proteolysis in those systems. Their interaction was considerable and depended on the type of substrate. In cheeses of lower moisture contents than Meshanger cheese, firmness was primarily regulated by volume fraction of protein in the fat-free cheese. Differences in protein breakdown cannot simply be attributed to differences in moisture content.

Free descriptors: cheese varieties, Noordhollandse Meshanger cheese, soft cheese, cheese ripening, cheese model substrates, enzymes, rennet, chymosin, gel electrophoresis, rheology, light microscopy, electron microscopy, cheese structure.

This thesis will also be published as Agricultural Research Reports 875.

Stellingen

1. De werkwijze van Deißmann ter berekening van eiwitconcentraties uit lichtabsorptie van gekleurde eiwitbanden op polyacrylamide gels leidt in principe tot onjuiste resultaten.

Ricarda Deißmann, Veränderungen in den Proteinfraktionen von Milch während technologischer Prozesse, Dissertation, Justus Liebig-Universität, Gießen, BRD, 1977.

2. In het „Keuringsreglement-Z.K.B. voor Melkpoeder" dient een apart criterium opgenomen te worden voor het onoplosbaarheidscijfer van instantmelkpoeders.

Keuringsreglement-Z.K.B. voor Melkpoeder.

3. Bij het verklaren van het verschil tussen de mate waarin vers gewonnen melk en koud bewaarde melk door centrifugeren kunnen worden ontroomd wordt teveel de nadruk gelegd op de bewaartemperatuur en te weinig op de bewaarduur.

O. Aule, Svenska Mejeritidningen, 1966, 157-160.

4. De consistentie van kaas, en zeker die van kaas met een hoog vochtgehalte, is afhankelijk van de afbraak van α_{s1}-caseïne.

Dit proefschrift.

5. Gezien de sterke mate waarin de diffusie van chymosine in kaas – ook in kaas met een hoog vochtgehalte – wordt geremd, dient men bij de bereiding van kaas waarbij het concentreren van de vetvrije melkdrogestof plaats heeft met behulp van ultrafiltratietechnieken extra aandacht te besteden aan de gelijkmatige verdeling van het toe te voegen stremsel.

Dit proefschrift.

6. Bakri heeft onvoldoende aangetoond dat de afbraak van caseïnes in oplossingen gecorreleerd is met de lichtverstrooiing veroorzaakt door de gevormde, niet-oplosbare afbraakprodukten.

M. B. A. Bakri, The caseins and rennin action, Thesis, Washington State University, Washington, USA, 1968.

7. Uit bloed afkomstige lipoproteïnen hebben invloed op de mate van vetsplitsing die door melklipase wordt veroorzaakt tijdens gekoeld bewaren van rauwe melk.

H. B. Castberg & P. Solberg, Meieriposten 63(1974) 961-975.

8. De beschouwing van Ernstrom over de achtergronden van zijn methode ter bepaling van stremselconcentraties in kaas is onzorgvuldig.

C. A. Ernstrom, Dairy & Ice Cream Field 159(1976) 43-46.

9. Wanneer men spreekt over *het* iso-elektrische punt van een eiwit, gaat men volledig voorbij aan de betekenis van het begrip iso-elektrisch punt.

o.a.: P. Teppema & F. Brouwer, Neth. Milk Dairy J. 30(1976) 79-94.

10. Modellen van caseïnemicellen waarin aan het kolloïdale calciumfosfaat geen of slechts een ondergeschikte rol is toebedacht inzake de structuur en stabiliteit van de micel, zijn onjuist.

D. G. Schmidt & T. A. J. Payens, Surface and colloid science, Volume 9: 165-229, John Wiley & Sons, 1976.

11. De produkten verkregen uit melk of melkderivaten door toepassing van membraanprocessen zouden vooral gebruikt moeten worden voor het samenstellen van andere dan gerenommeerde zuivelprodukten.

Proefschrift van L. de Jong
Proteolysis and consistency of Meshanger cheese
Wageningen, 31 mei 1978

Woord vooraf

Van de personen die bijgedragen hebben aan de totstandkoming van dit proefschrift wil ik met name mijn promotor prof. dr. H. Mulder noemen. Ik heb zijn voortdurende aanmoedigingen en adviezen bijzonder op prijs gesteld.

Aliza de Groot-Mostert heeft op diverse niveaus een belangrijke bijdrage geleverd, waarvoor ik erg dankbaar ben.

De stichting J. Mesdagfonds, Kaascontrolestation „Friesland", te Leeuwarden, heeft met aanzienlijke financiële steun dit onderzoek mogelijk gemaakt.

Met de medewerkers van de sectie Zuiveltechnologie en melkkunde van de vakgroep Levensmiddelentechnologie heb ik steeds bijzonder plezierig samengewerkt, evenals met de medewerkers van de algemene dienst van genoemde vakgroep.

The influence of the moisture content on the consistency and the protein breakdown of cheese
L. de Jong, Neth. Milk Dairy J. 32 (1978): 1-14.

Introduction

1 Introduction and motivation

In our laboratory Noordhollandse Meshanger cheese, a Dutch soft cheese serves as a model for studying cheese ripening (1, 2, 3). The cheese is characterized among other things by its fast ripening (about 14 days), not complicated by the action of a surface flora, and a soft, smooth, consistency, salient for the mature cheese. Prolonged ripening leads to the ultimate liquefaction of the cheese.

The interest for Meshanger cheese in our laboratory has been stimulated by the necessity to have a system available to study the processes involved in the protein ripening of cheese traditionally experienced as important. During the investigations it became clear that it could very well serve as model to study the relation between protein breakdown and cheese consistency.

The salient softening of Meshanger cheese differs in an important and principal way from that of well known soft cheeses as Camembert and Brie cheese. The latter have a change in consistency from the outer parts to the inner part of the cheese, whereas Meshanger starts to soften in its centre, proceeding to the rind.

The consistency of cheese can be expected to be regulated by its moisture content and the properties of the only solid continuous phase in cheese, the protein matrix. The discontinuous globular fat, which has some influence on the consistency of Meshanger cheese just after preparation, is not likely to contribute to its softening.

The preparation of cheese, in essention a process in which the larger part of the milk dry matter is concentrated, involves the conversion of κ-casein by chymosin. Due to this conversion and in the presence of sufficient Ca^{2+} the protein matrix mentioned above is constructed from the paracasein micelles. A three dimensional protein network forms in which the milk fat globules are embedded. The mass contracts and extrudes whey. The conditions during these steps of cheesemaking determine the properties that develop during ripening. The properties of the constructional components, the paracasein micelles, composed of submicelles consisting mainly of α_{s1}-, β-, and para-κ-casein molecules (4), largely determine the properties of the matrix. Those properties depend on physico-chemical conditions which start to change

Agric. Res. Rep. 875 p. 3-4

Cheese ripening studied in model systems. I. Introductory part

H. Mulder, A. Noomen and L. de Jong

1 Methods to study cheese ripening

The ripening of cheese is a complex of many complicated processes, in which especially protein, lactose and fat are transformed. The result is a product with the desired appearance, consistency and flavour. Together with the physical, chemical and microbial properties of the milk, the method of making and the ripening conditions determine the sort of cheese that is formed. The intricacy of all this is demonstrated by the great number of cheese varieties, so that research on cheese ripening is no simple matter. Most workers in this field study the ripening of one particular cheese; hence they are tied to the whole complex of conditions that leads to a cheese of the quality desired for the sort. Therefore every kind of cheese must be investigated separately.

Nevertheless, cheeses that are quite different still have much in common in ripening and we believe that a general, but simple model would be useful to study the main ripening processes, from which the ripening of special cheeses can be deduced. Among possible approaches to such a model are simplifying the preparation of a known cheese, resulting in a cheese with a less complicated ripening (stripped cheese), and synthesizing cheese-like systems from the necessary components (simulated cheese).

2 Stripped cheese as model

As basis we chose the soft Noordhollandse Meshanger cheese. This cheese ripens in about 2 weeks; the preparation is simple; its flavour is very mild. The stripped Meshanger cheese ripens without a surface flora. If desired it can be acidified with a lactone instead of with lactic acid bacteria; the milk is pasteurized. At first sight such a simple stripped cheese may seem ideal. However it has restrictions. Its composition depends on the properties of the milk; a possible influence for instance of whey components on ripening is not yet clear. Furthermore such stripped cheeses contain rennet enzymes, enzymes like milk protease etc.. The action of lactic acid bacteria may cause complications. A stripped cheese that contains no active rennet enzymes and no milk protease can be prepared, but its structure can differ significantly from that of normal cheese because of the severe treatment necessary to inactivate

enzymes. This treatment involves high pasteurization temperatures and manipulation with the calcium ion concentration.

Another way to prepare such a model cheese is not to work the cheese curd into cheese in the normal way, but to add ingredients like lactic acid and salt to curd (artificially composed stripped cheese). However there still are uncertainties, such as the influence of the whey enclosed in the curd.

3 Models composed from pure ingredients

The more radical way for making a model is to compose it from the individual components. The most important are calcium paracaseinate–calcium phosphate complex, lactic acid, NaCl and water. Enzymes or other substances, whose influence on ripening is to be studied, can be added. The attraction of these simulated cheeses is that their composition is exactly known. However they also have restrictions, for instance whey is left out. Using whey instead of water may reintroduce unknowns. Of course the structure of these systems differs from that of normal cheese.

One could continue, as many investigators did, and investigate the conversion of pure components of cheese, for instance by studying the proteolytic activity of enzymes in solutions of casein salts. We found however that one cannot extrapolate to cheese as, for instance, calcium has a considerable influence on casein breakdown. So the composition and properties of model systems should resemble those of cheese as closely as possible. All models, however, have some restrictions. The choice depends on the problem to be studied.

4 Motivation and approach

The motivation of our study was to gain knowledge on cheese ripening, especially on proteolysis and its consequences for cheese consistency. The factors thought to be most important were studied in the types of models mentioned. The influence of rennet (chymosin) was studied in all models whereas to study the action of milk protease the preferred system was simulated cheese. The relation between consistency and proteolysis was studied in the stripped Meshanger cheese, as was the relation between structure and consistency.

To study protein breakdown two methods were adapted. For estimation of the soluble nitrogen components, this was an extraction method and the unattacked proteins were estimated by quantitative electrophoresis.

Further details about motivation, results and conclusions of the work are reported in full in:

II – Proteolysis in soft cheese, studied on Meshanger cheese and cheese mo-

dels, reported by A. Noomen

and

III – Proteolysis and consistency of Meshanger cheese, reported by L. de Jong.

2 Experimental

Polyacrylamide gel electrophoresis (PAE) was carried out in an E-C vertical gel cell. The method used was derived from the manual (1) and from Thompson et al. (2) and Melachouris (3).

2.1 Preparation of the polyacrylamide gel

The gel buffer contained 46 g Tris per litre and some concentrated HCl to adjust the pH to 8.9. The gel solution was prepared by dissolving 54 g urea, 14 g Cyanogum 41 (E-C Apparatus Corporation) and 0.2 ml N,N,N',N'-tetramethylethylene diamine in the gel buffer and making up the volume to 200 ml.

As gel support a plug was used. This plug was formed in the E-C cell, supported at an angle of 45°, by polymerizing 30 ml of the gel solution, with 30 mg ammonium persulphate as a catalyst. After 1 h of polymerizing, the water (5 ml) used to protect the gel solution from the air was decanted. The plug surface was rinsed with gel solution. 110 ml gel solution with 110 mg ammonium persulphate was used to form the running gel with slots as described in the manual (1). The gel solution was left to polymerize at room temperature for at least one night.

2.2 The electrophoretic run

The electrode buffer containing 0.7 g Tris and 2.9 g glycine per 1 litre (pH 8.5) was precooled to 4 °C.

Tap water cooling was switched on. After a 20-min initial run at 200 V, the excess of gel and the slot-former were removed.

Samples of 50 μl, containing 0.5 % protein dissolved in a Tris-HCl buffer pH 8.5 with 8 M urea, were applied to the slots. The run took exactly 3 h at a constant voltage of 280 V.

2.3 Gel slab staining and de-staining

The gel slabs were stained by immersion for 1 h in a mixture of methanol: water: acetic acid = 25:65:10 containing 0.1 % amido black 10B. The stain was thoroughly mixed every 5 min and renewed every 20 min. For good results the mixing and renewing are very important since otherwise staining irregularities will occur, introducing considerable misinterpretations in a quantitative follow-up; this is due to diffusion of urea and buffer ions from the gel slab into the stain. For rapid and reproducible removal of excess of stain from the gel slabs the E-C de-staining equipment was used. The destaining agent was 7 % acetic acid. The destaining grid, used without filter

paper, was moved up and down every 5 min. After 20 min the acetic acid was regenerated over the activated charcoal. Total de-staining took 40 min. The gel slabs were stored in 7 % acetic acid. If the de-staining was performed exactly as described the gel slab obtained showed no de-staining artefacts and it was perfectly transparent, ready for immediate densitometry measurements.

2.4 Densitometry

With the aid of a Vitatron TLD 100/Hg densitometer the optical density of the stained protein bands was determined with a 600 nm filter and a 0.16 mm diaphragm in the read-out unit, while the sample holder oscillated with an amplitude of 1.5 mm.

The relative protein concentration was calculated from the integration signs on the densitograms.

2.5 Gel filtration

Exclusion chromatography in a gel column was carried out according to Foster & Green (4) on different types of Sephadex G.

3 Results and discussion

The results obtained with gel filtration were unsatisfactory. Differences in break-down, easily detected with polyacrylamide gel electrophoresis (PAE), were scarcely demonstrable by gel filtration. Therefore this method was abandoned for the present purpose. A more detailed account will be given in a subsequent report.

PAE gave excellent results. To interpret the results, some aspects need to be further considered. Unattacked α_s and β caseins as present in cheese after pressing gave a pattern as shown in Fig. 1. During the ripening of cheese, part of the protein is broken down. With the method described the concentration of a protein component can be derived from the densitograms. As long as the optical density remains constant no change in the concentration is observed and the protein is considered to be unattacked. Protein which contains the same Rf value is considered to be unattacked, and breakdown is calculated from the decrease in the concentration as compared to the original value. This would be a perfect criterion as long as the Rf value of the proteolytic products differs from that of the original proteins. Rf values are mainly defined by the charge of the protein. Protein breakdown not involving a change of charge is unlikely and the calculation described is permitted if the following conditions are fulfilled.

The first condition is that the Rf value of the breakdown products of α_s

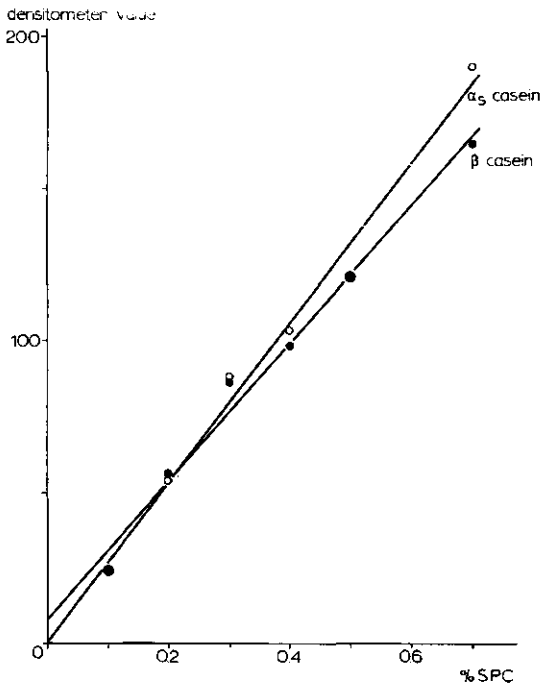

Fig. 4. Densitometer values from the gel shown in Fig. 3 against the concentration of sodium paracaseinate (% SPC).

broken down. The products from α_s casein hydrolysis moved in front of the original band without interfering with the detection.

The second condition is that the optical density has to be proportional to the protein concentration in the sample. To check this 50 μl of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.7 % sodium paracaseinate solutions were studied. Fig. 4 shows densitometer values for α_s and β casein against sample concentration and we see that the correlation is satisfactory. The standard deviation free from regression (hence the standard error of estimate) corresponds to 0.028 % protein for α_s casein and to 0.027 % for β casein. Hence the calculation of concentrations from the integration pattern obtained would be permitted.

Finally we would like to remark that the quantity of amido black bound per gram of substance may differ between break-down products and the original protein. Calculations on those bands, particularly when comparisons are made with those of the original proteins, may be erroneous.

In conclusion, it can be stated that PAE as described here is a useful method for the quantitative study of proteolysis. This may be demonstrated by Fig. 1 and 5, which show the electropherogram and the derived relative protein

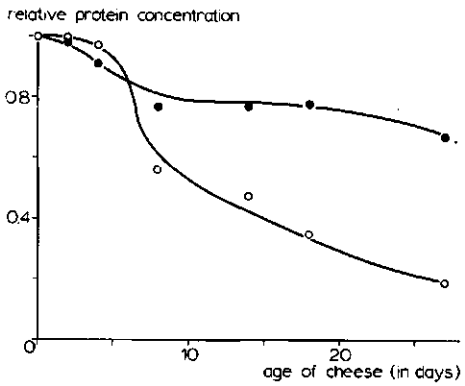

Fig. 5. Relative concentration of the unattacked a_s (○) and β casein (●) at different stages of ripening of the cheese of Fig. 1.

concentration. The samples were taken at different stages of ripening from a type of soft cheese.

Acknowledgment

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For technical assistance in the experiments I owe many thanks to Aliza de Groot-Mostert.

Samenvatting

L. de Jong, *De kwantitatieve gel-elektroforetische bepaling van kaasrijping*

Gel-elektroforese wordt dikwijls gebruikt voor de kwalitatieve bepaling van eiwitafbraak in kaas. In deze publikatie wordt een kwantitatieve uitwerking van polyacrylamide-gel-elektroforese (PAE) beschreven. Aangetoond wordt dat PAE toegepast op de beschreven manier in kombinatie met een geschikte densitometrische methode veel informatie geeft over het in kaas voorkomen van onopgeloste stikstofverbindingen.

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3. N. Melachouris, *J. Dairy Sci.* 52 (1969) 456.
4. P. M. D. Foster & Margaret L. Green, *J. Dairy Res.* 41 (1974) 259.

Neth. Milk Dairy J. 30 (1976): 242-253

Protein breakdown in soft cheese and its relation to consistency. 1. Proteolysis and consistency of 'Noordhollandse Meshanger' cheese

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Summary

This paper on the relation between proteolysis and consistency of soft cheese deals with the softening of 'Noordhollandse Meshanger' cheese. The ripening of this cheese is uncomplicated, which makes it suitable to serve as a model for this study. The proteolysis during the ripening was studied with the aid of polyacrylamide gel electrophoresis. The course of the consistency during ripening is discussed as well as the relation between consistency and the breakdown of α_{s1} casein.

1 Introduction

Lately the research on the technology and ripening of Meshanger cheese in our department resulted in a description of the ripening of this old Dutch cheese by Noomen & Mulder (1) and Noomen (2).

The reconstruction of the production technique made it possible to prepare this soft type cheese on a pilot plant scale. The cheese is easy to prepare and it shows a relatively simple type of 'ripening'. The major characteristic of this ripening is a softening starting in the centre of the cheese, slowly continuing outwards. The ripening proceeds rapidly: within 2 weeks the cheese is ready for consumption while after 3-4 weeks it has completely liquefied. It does not develop a pronounced taste. The ripening is not complicated by the action of a surface flora nor of a micelium growing throughout the cheese. This uncomplicated ripening, compared with, for example, the ripening of Camembert cheese, Limburger cheese, Kernhem cheese, Butterkäse, Bel Paeso and Port Salut, provides an opportunity to study the phenomenon of the softening of

[1] Present address: Netherlands Institute for Dairy Research (NIZO), Ede, the Netherlands.

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Neth. Milk Dairy J. 31 (1977): 314-327

Protein breakdown in soft cheese and its relation to consistency. 2. The influence of the rennet concentration

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Summary

The relation between the breakdown of α_{s1} casein, the proportion of enclosed rennet and the consistency of Meshanger cheese was studied. It was demonstrated that a linear relation exists between the rennet concentration and the decomposition rate of α_{s1} casein during the ripening of Meshanger cheese.

The relation between α_{s1} casein breakdown and changes in consistency is also described. In cheese without α_{s1} casein breakdown no softening occurs. It is emphasized that views with respect to the softening of cheese being caused by the action of proteolytic enzymes of its surface flora are very questionable. The softening of cheeses with high moisture contents must primarily be attributed to the decomposition of α_{s1} casein by rennet.

1 Introduction

In the first paper of this series on the proteolysis and consistency of soft cheese (1) a description of processes occurring during the ripening of a model type soft cheese, Meshanger cheese, was given. It was emphasized that there could be a close relation between consistency and paracasein breakdown. Furthermore a suggestion was made that commercial rennet could act as a general proteolytic agent. This paper deals with the elaboration of this idea by studying the influence of the rennet concentration in cheese on the processes occurring during its ripening, chiefly the proteolysis and the changes in consistency.

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Table 1. Preparation schedule and composition of the cheeses. (See text for further explanation.)

Block	Code	Rennet quantities			Rennet contents (ml/kg cheese protein)	Protein content (%)	Fat content (%)
		used for clotting (ml/100 litres milk)	added before electrical heating (ml/100 litres milk)	added during the stirring of the whey/curd mass (litres/ 80 litres)			
I	N	40			1.346	16.2	20.6
	2N	80			3.795	16.1	21.2
	RF	40			n.d.[1]	17.5	15.8
	N+½	40		0.50	4.393	16.3	20.1
	N+1	40		1.00	5.987	15.2	19.3
II	½N	20			0.740	16.9	20.6
	N	40			1.310	15.8	19.5
	N+½	40		0.53	5.069	15.9	20.0
	RF	40			n.d.[1]	18.1	21.5
	RFR	40	40		2.845	16.0	18.6
III	N	40			—	15.4	18.4
	RFR	20	20		—	16.5	18.8

[1] n.d.: not detectable.

2 Methods and materials

Meshanger cheese was prepared and ripened, as described by Noomen (2), in our pilot plant. The milk was supplied by the University dairy farm. The cheese was produced in batches of 4 to 6. Different levels of enclosed rennet were obtained by varying the amount of rennet used for the clotting of the cheese milk and by adding rennet during the stirring of the curd/whey mixture after the cutting of the curd. Table 1A gives a survey of the way in which the basic prescription was adapted to obtain cheeses with different levels of enclosed rennet. In the same table the symbols used to indicate certain variations in cheesemaking are given; N stands for Meshanger cheese prepared according to the normal method. ½ N indicates cheese prepared with half the normal quantity of rennet to set the milk, the number behind the plus sign indicates the amount of rennet in litres added during the stirring of the curd/whey mixture. The experiments were carried out in three blocks each indicated by one of the three first roman numbers I, II and III. Thus ½ N II indicates a cheese prepared in the second block with half the normal quantity of rennet to clot the milk.

To obtain cheese in which rennet is not acting as a general proteolytic enzyme the technique described by Visser (3) was applied. This method is based on the heat inactivation of the rennet used to split the $\varkappa$ casein in the milk. In order to achieve this heat inactivation clotting of the milk was prevented by decreasing the Ca concentration by means of ion exchange. We shall call this Ca-depleted milk. After rennet inactivation by pasteurization the Ca content was re-established by the addition of a solution of $CaCl_2$ to the milk at a temperature below 10 °C. Heating this milk (by passing an electric current through it) to 30 °C gave a gel which was very suitable for working into cheese. The starter was added just after the $CaCl_2$. To establish that the processes necessary to obtain rennet-free cheese (RF) did not interfere with normal cheese ripening it was necessary to prepare cheese according to Visser's method in which rennet acted as general proteolytic agent. This was done by adding rennet again to the treated milk just before the warming (to induce normal milk coagulation) was started. These cheeses are indicated by RFR. Rennet concentrations are given in Table 1A.

Most of the other methods used were described previously (1), as was gel electrophoresis (4).

3 Results

All the cheeses were analysed for moisture and NaCl content. Those results

which agree with those reported previously (1) are not reported again in any detail.

Table 1B gives the protein content, the fat content and the content of rennet enclosed in the cheese. The rennet content is expressed per kg of cheese protein. When the quantity of rennet used for preparation is compared with the quantity of enclosed rennet (cf. Table 1) it can be seen that larger amounts of rennet used to set the milk resulted in higher rennet concentrations in the cheese. In the cheeses prepared from Ca-depleted milk to which rennet was added afterwards (RFR II), the quantity of enclosed rennet is much higher than in normal Meshanger cheese. The pH at which the treated milk clotted (6.30) was much lower than the normal clotting pH, because lactic acid was added together with the $CaCl_2$ solution, after the heat inactivation of the rennet in the milk. The addition of lactic acid was necessary to ensure formation of a gel, suitable for the cheese preparation, instead of a precipitate (3). This lower pH, however, must have caused, according to Stadhouders

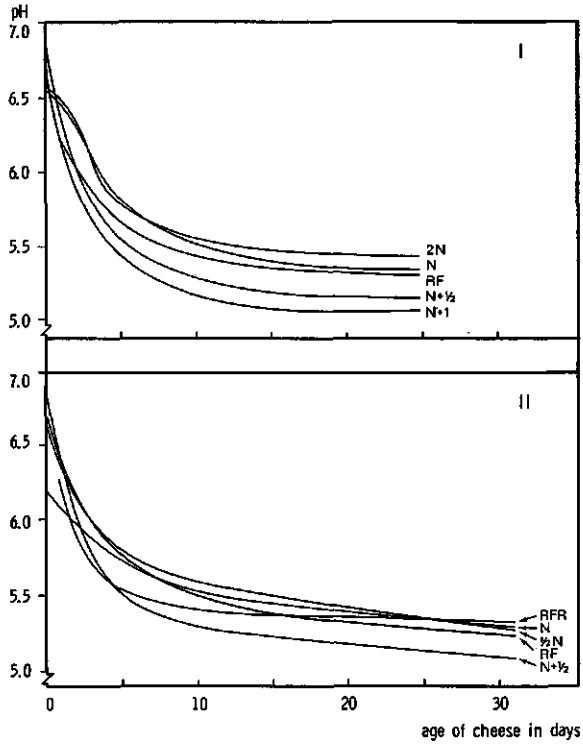

Fig. 1. The course of the pH of a number of Meshanger cheeses with different amounts of enclosed rennet. Abbreviations are explained in the text.

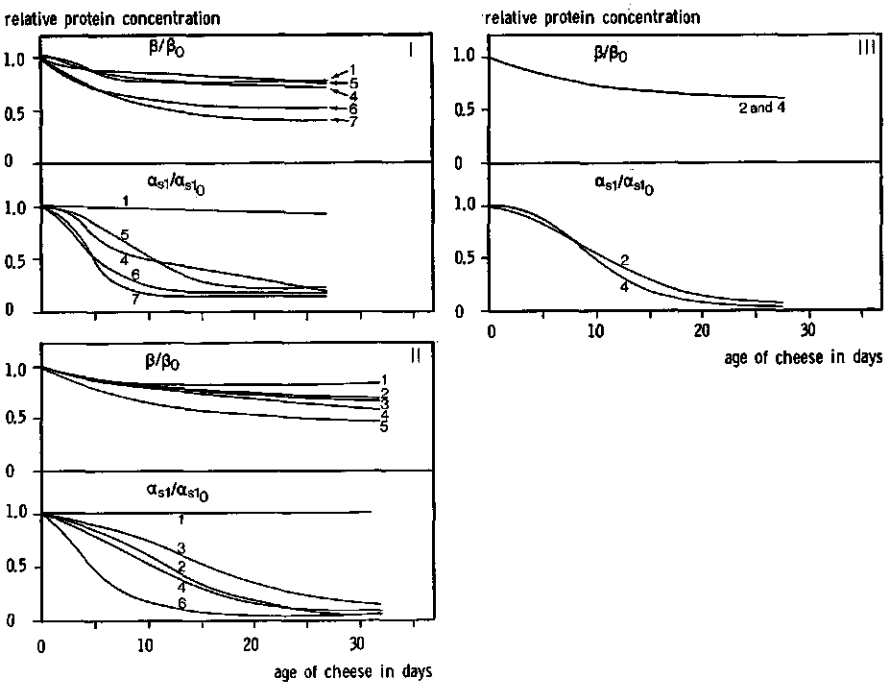

Fig. 2. The breakdown of α_{s1} and β casein during the ripening of Meshanger cheese in relation to the rennet concentration. 1=RF, 2=RFR, 3=½N, 4=N, 5=2N, 6=N+½, 7=N+1. The abbreviations are explained in the text.

(5), the enclosure of a larger quantity of rennet during the setting of the milk. To reduce this high level of enclosed rennet, as compared with that in normal cheese, less rennet was added in the preparation of RFR III. In the 'rennet-free cheeses' (RF) no rennet was detectable. The addition of rennet during stirring of the whey/curd mass, in the experiments N+½ and N+1 as indicated in Table 1, resulted in an unexpectedly low increase of the rennet content of the cheeses, compared with that obtained by using twice the normal quantity of rennet to set the milk as in the experiments 2N. Addition of rennet during the stirring of the curd/whey mixture appeared to be ineffective. This will be due to the low diffusion rate of rennet in curd, the short stirring time (about 10 minutes) and the continuing syneresis. The effective diffusion coefficient of rennet is assumed to be at least 10 times lower than that of lactose in the curd.

The course of the pH in the centre of the cheeses was determined at different times. Typical curves are given in Fig. 1. It can be seen that notable differences exist between the cheeses prepared with different amounts of ren-

net. It seems that the quantity of rennet enclosed affected the rate of decrease of the pH. This could be due to stimulation of the starter bacteria by the rennet or to a stimulation by breakdown products from the paracasein formed more rapidly at higher rennet concentrations. Pearce (6) suggested that higher rennet contents do not influence the course of the pH during cheese ripening but this may be true only for cheeses with a fast fall in pH; for cheeses with a slow pH decrease, such as Meshanger, the effect appears to be considerable.

3.1 The decomposition of paracasein during the ripening

Fig. 2 gives the relative protein concentration as a function of the age of cheeses. The roman figures used to indicate the different parts of the figure refer to the blocks of experiments mentioned above. The course of the curves obtained is as expected in view of the results already published (1). In general, it can be stated that the proteolysis was more extensive when the rennet concentration was higher, both for the decomposition of α_{s1} casein and for β casein. The proteolysis of α_{s1} casein took place in all experiments in which the cheeses contained active rennet, giving an almost complete breakdown of this casein. The rate of protein breakdown is remarkable in those cases when rennet was added during the stirring of the whey/curd mass.

The experiments with the rennet-free (RF) cheeses clearly showed that no appreciable breakdown of α_{s1} casein occurred if no active rennet was present. Attention must be drawn to the slight breakdown of β casein in the rennet-

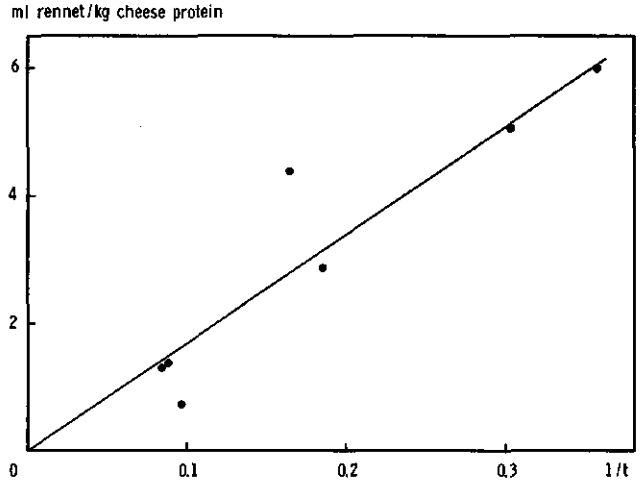

Fig. 3. The relation between rennet concentration and breakdown rate of α_{s1} casein in Meshanger cheese. Explanation in the text.

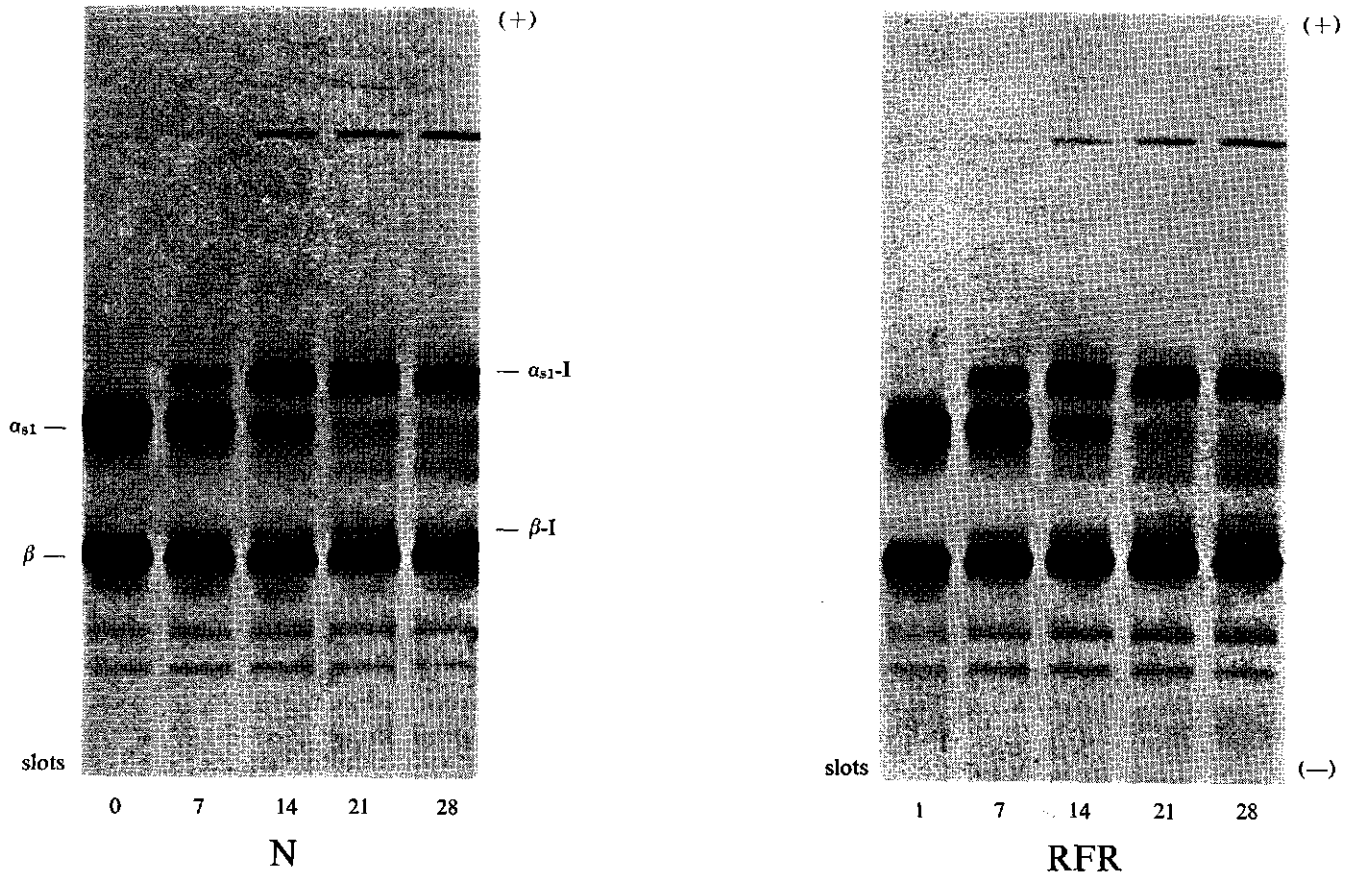

Fig. 4. Polyacrylamide gel electropherograms of Meshanger cheeses prepared as explained in the text. The numbers refer to the age of cheese in days.

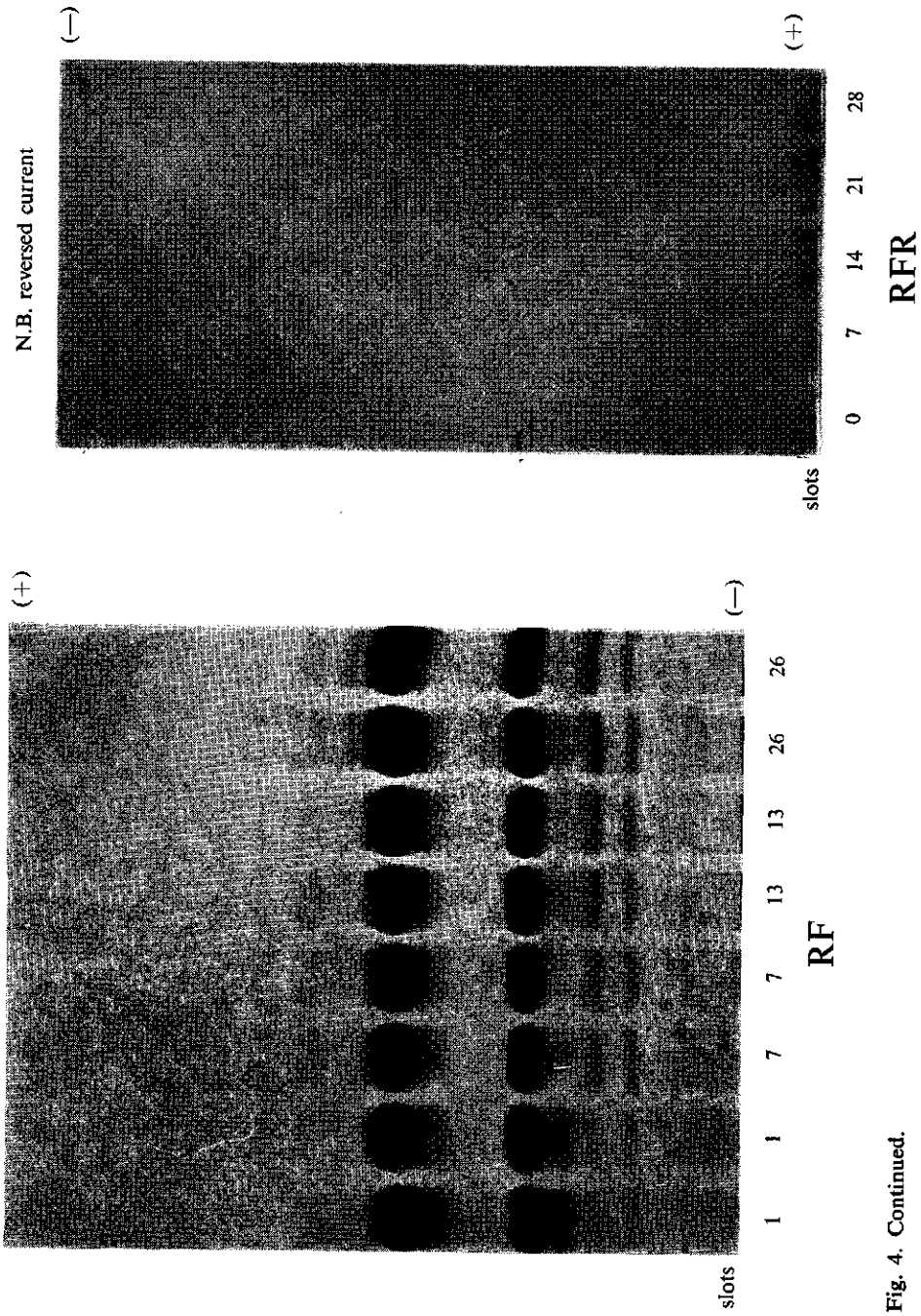

Fig. 4. Continued.

free cheese. This proteolysis may have been due to other proteolytic enzymes, such as milk proteases and exo-protease of the starter bacteria. The cheeses RFR prepared according to Visser's method, with rennet addition before the dielectrical heating, showed a proteolysis of α_{s1} and β casein similar to that in normal prepared Meshanger cheese. This indicates that the process used for the preparation of rennet-free cheese does not alter the liability of α_{s1} and β casein to be broken down. A quick look at Fig. 2 could give the impression that the proteolysis of α_{s1} casein differs at different rennet concentrations. If we remember, however, the considerable physico-chemical changes occurring during the early days of the ripening of the cheese, it will become clear that a mere comparison of the shape of the curves is not particularly useful. To compare the different breakdown rates of α_{s1} casein in relation to the rennet concentration, we may calculate the time necessary to break down half the quantity of substrate and compare it with the enzyme concentration. But unequivocal results are only obtained if the conditions with regard to, for example, pH and temperature are fixed. In our case, many factors varied, the most important for this problem being pH and salt content whose influence on the rate of proteolysis is well-known (7). Therefore this method cannot be applied, at least not when starting from the time of clotting of the cheese milk. To make the problem manageable it was assumed that under the ripening conditions of Meshanger cheese no great changes occurred in the reaction rate as soon as the pH had dropped below 5.4. From the pH/time graphs (Fig. 1) for each cheese the time at which this occurs can be obtained. From the curves representing the proteolysis (Fig. 2) the quantity of α_{s1} casein still unattacked at this moment can be derived. Taking this quantity as the starting concentration the time needed to reduce it by half can be calculated. This lapse of time (t) was taken as a measure of the rate of proteolysis. In Fig. 3 the enzyme concentration is plotted against $1/t$, and both quantities appeared to be proportional. The point at the origin of the graph represents the breakdown in rennet-free cheeses which, as can be seen in Fig. 2, was negligible while no rennet was detectable in these cheeses (Table 1B).

Fig. 4 shows polyacrylamide gels used for the quantitative detection of the protein breakdown, and they demonstrate the qualitative proteolysis in the N, RF and RFR cheeses. The patterns shown make it clear that the proteolysis in N and RFR occurred in exactly the same way in both experiments. This leads to the conclusion that not only the degree but also the type of protein breakdown is the same during the ripening of N and RFR cheese. On the gel representing the patterns of RF cheese of different ages no breakdown products could be detected. The last gel shows the pattern obtained

by electrophoresis with reversed current. Comparison with the one shown previously (1) domonstrates that there are no differences between the protein substances with a positive charge at pH 8.9 of N and RFR cheese. This indicates once more that there are no differences in proteolysis induced by applying the technique necessary to obtain 'rennet-free cheese'.

3.2 The change in firmness during ripening

Curves representing the firmness during the ripening of the cheeses are in Fig. 5. The roman numbers refer to the blocks already mentioned. The softening of the normal cheeses N was a expected and described previously (1).

The cheeses with unusual rennet contents (see Table 1B), showed remarkable differences. The experiments in blocks I and II show that the rennet

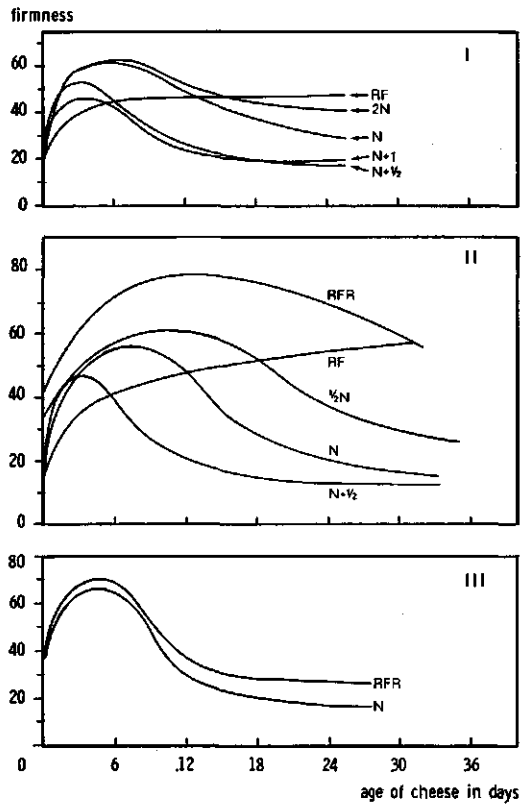

Fig. 5. The course of the firmness of Meshanger cheeses in relation to the amount of enclosed rennet. The abbreviations are explained in the text.

content influenced the rate of softening. Rennet contents lower than that in N cheese gave slower softening while an increase of rennet content gave a faster softening. When no active rennet was present no softening occurred, as can be seen from RF I and RF II.

In two cases a deviation was found. In experiment 2N, cheeses having a higher rennet content than normal softened more slowly and remained more firm than the normal cheese. The RFR II cheese did this in a even more pronounced manner. By comparison of the dry matter contents and the pH levels of these cheeses and N cheese it follows that the deviation might be due to high pH and low moisture content. The influence of moisture content and pH on cheese properties is of course well known; more information on this subject will be given in a subsequent paper.

However, as the RFR cheese, being the control of our experiments, differed from normal cheese, this experiment had to be repeated. The result of these experiments are in block III. The only difference between RFR II and RFR III, ignoring moisture content and pH, is that, for the preparation of the latter, less rennet was added before dielectrical heating of the treated milk. This should result in a lower rennet content in the cheese. The softening and the other processes during the ripening of RFR III and N III cheese were identical as can be seen from Figs 2, 4 and 5. These results give further confirmation of the similarity of N and RFR cheese, in spite of the complicated treatment necessary to perform experiments with RF and RFR cheese.

Fig. 5 shows rather large differences between the maximum firmness (F_{max}) of the cheeses produced by the different treatments. Because the course of the moisture content during the ripening of the different comparable batches of cheese did not differ there had to be another reason for this phenomenon. We concluded previously (1) that the height of the maximum is the result of two processes: (a) an increasing firmness caused by the change in chemicophysical conditions in the cheese, and (b) a decrease in firmness caused by the breakdown of α_{s1} casein. The latter effect, being stronger than the former one, causes the softening of the cheese and its ultimate liquefaction. The different batches of cheese contain different quantities of rennet (cf. Table 1B) and, as is indicated above, the protein decomposition rate is related to the rennet content. Therefore the decrease of the firmness in cheese with a high rennet content will start before the cheese has reached a level of high firmness. Roughly speaking, this effect causes F_{max} to be lower at higher contents of enclosed rennet.

If there were no softening due to α_{s1} casein breakdown, the batches of cheeses produced according to the normal procedure (except for the use and the addition of different quantities of rennet), would theoretically reach an

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Aliza de Groot-Mostert carried out many analyses on the cheeses, which were prepared by Jan Uitentuis.

Samenvatting

L. de Jong, *Het verband tussen de eiwitafbraak en de consistentie van zachte kaas. 2. De invloed van de stremselconcentratie*

Nadat in een voorgaand artikel (1) een beschrijving is gegeven van het reologisch gedrag van Meshanger kaas wordt in dit artikel het verband tussen de afbraak van α_{s1}-caseine, de hoeveelheid ingesloten stremsel en de consistentie van deze kaas behandeld. Met behulp van een techniek die het mogelijk maakt het gedrag van kaas te bestuderen zonder dat daarin actief stremsel aanwezig is, wordt aangetoond dat er een lineair verband bestaat tussen de stremselconcentratie en de afbraak van α_{s1}-caseïne. Bovendien wordt het verband tussen de afbraak van α_{s1}-caseïne en consistentieveranderingen gedetailleerd beschreven. In kazen waarin geen afbraak van α_{s1}-caseïne plaatsvindt, vindt ook geen vervloeiing plaats. Er wordt uiteengezet dat theorieën, die het vervloeien van zachte kazen, zoals Camembert, toeschrijven aan de activiteit van proteolytische enzymen van de schimmels die op het oppervlak van de kaas groeien, achterhaald zijn. Het vervloeien van kazen met een hoog vochtgehalte moet toegeschreven worden aan de afbraak van α_{s1}-caseïne onder invloed van stremsel.

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Protein breakdown in soft cheese and its relation to consistency. 3. The micellar structure of Meshanger cheese

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Key words: cheese structure, microscopy, electron microscopy, protein breakdown, consistency

Summary

The structure of Meshanger cheese was studied by fluorescence and interference contrast microscopy as well as electron microscopy. Structural changes in the protein matrix during the ripening are described and compared with those in other soft and in hard cheeses. It is indicated that there are no visible differences in structural changes during the ripening of soft and hard cheese. The consequences concerning cheese ripening and casein micelle structure theory are discussed.

1 Introduction

Of the three major components of cheese, fat, water and protein, the last one primarily constitutes the structural matrix of the cheese. This protein matrix is formed after the proteolytic conversion by rennet of $\varkappa$ casein into para-$\varkappa$ casein (1, 2). The paracasein micelles form a gel in the presence of Ca^{2+} and the curd obtained can be transformed into cheese in many ways. The conditions during this transformation determine the properties and ripening of the cheese.

Meshanger cheese (4, 5, 6) is prepared and ripened under such conditions that its consistency changes very fast; it liquefies within 3 weeks. The softening is governed by the rate of conversion of α_{s1} casein, which constitutes about 38 % of the whole casein (3), into its primary decomposition product α_{s1}-I.

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In previous papers (8, 9) we showed the close relation between the chemical change of α_{s1} casein and the softening of Meshanger cheese; the relation with changes in the micro-structure of the cheese had not yet been dealt with.

The micro-structure of cheese, soft as well as hard, has been studied by light and electron microscopy (10-19). The papers of Knoop et al. (20-23) on Camembert cheese structure and that of Mulder et al. (24) dealing with the structure of hard cheese present a good survey of our present knowledge.

This paper presents a description of the structural changes during the ripening of Meshanger cheese, by various microscopical techniques. Implications concerning casein micelle structure are discussed.

2 Methods and material

2.1 Cheese preparation

The Meshanger cheeses were taken from the normal production in the pilot plant of our laboratory. The cheese was prepared according to Noomen (6).

2.2 Microscopy

2.2.1 Fluorescence microscopy

Cubical samples with edges of a about 5 mm were taken from the centre of the cheeses. With solid carbon dioxide the unfixed cubes were rapidly frozen in a droplet of water on the sample holder of a Reichert microtome. Sections of approximately 2 μm were cut with a pre-cooled knife and stretched on a droplet of water on a microscope slide. Water was used, since no differences were observed between preparations made with water, cheese whey and salt solutions. The sections were air-dried and stained by immersion in a solution of acridine orange (0.1 %). After rinsing with water the air-dried sections were embedded in Canada balsam and studied with a Zeiss WL microscope, equipped for fluorescence with excitation from below. The excitation filter had a cut-off wavelength of about 400 nm; the barrier filter transmitted light above 530 nm. The pictures were photographically recorded.

2.2.2 Interference contrast microscopy

The slices prepared for fluorescence microscopy (see Section 2.2.1) were also studied with the interference contrast technique. We used the Zeiss microscope mentioned before, fitted with Nomarski interference contrast condensers and objectives.

2.2.3 Electron microscopy

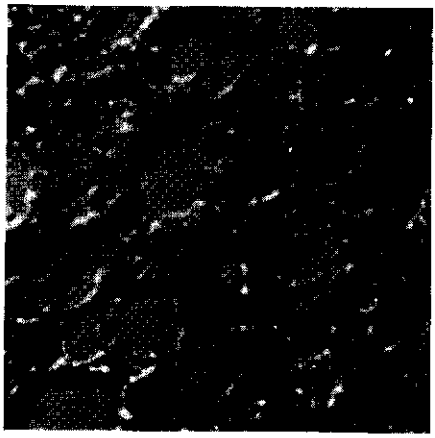
2.2.3.1 Embedding technique. From the centre of pre-cooled cheeses cubes were cut with edges of about 1 mm. After fixation in a solution of 4 % formalin with a pH equal to that of the cheese, the cubes were post-fixed in a 1 % solution of OsO_4 in a veronal buffer with a pH equal to that of the cheese. After one hour the cubes were rinsed with water and dehydrated in a series of ethanol-water mixtures with increasing ethanol concentration. After treatment with propylene oxide the cubes were embedded in Epon 812 and kept for about 12 hours at temperatures of 34, 45 and 60 °C.

Sections $\sim$ 50 nm thick were cut with glass knives on a LKB ultra-microtome Ultrotome III and post-stained with lead citrate according to Reynolds (25). Electron micrographs were made with a Philips EM 300 at the Technical and Physical Engineering Research Service (TFDL), Wageningen, the Netherlands.

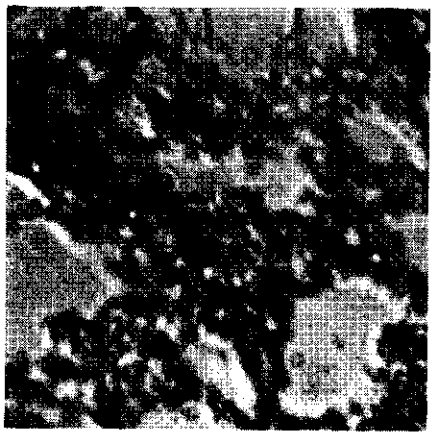
2.2.3.2 Freeze fracturing. Pieces (about 1 × 1 × 1 mm), were cut from the centre of cheeses, mounted on specimen holders, frozen in melting freon and stored in liquid nitrogen. Freeze fracturing was done in a Baltzer freeze etching unit (BAF 301). The fractured surface was coated with a thin layer of Pt and C, approximately 2 nm thick, followed by a supporting layer of C. The replicas were rinsed by immersion in HNO_3 and NH_3 solutions and mounted on uncoated 400 mesh grids. Electron micrographs were taken as described in Section 2.2.3.1.

3 Results

Micrographs of Meshanger cheese at 0, 7, 14, 21 and 29 days after preparation are shown in Fig. 1. The pictures were made by the fluorescence technique described in Section 2.2.1. Although the resolving power with the fluorescence technique is better than in normal light microscopy it is still only of the same order as the average diameter of the paracasein micelles: about 100 nm (7). The picture at 0 days shows that the protein matrix of the cheese contains individual particles sticking together and forming a network. The best impression of this network is seen where small strands of protein 'cross a black hole'. The holes have contained the milk fat globules which have disappeared during the preparation of the slices. The size of the holes, 0.3 - 5 μm, is well within the range mentioned by Mulder & Walstra (27) for milk fat globules: diameter 0.1 - 10 μm. This indicates that the preparation technique does not grossly alter the dimensions of the objects studied. Some of the holes mentioned above may have been filled with whey. The micrographs from cheeses 7 and 14 days old show that the protein part had become more dense. The holes in the matrix

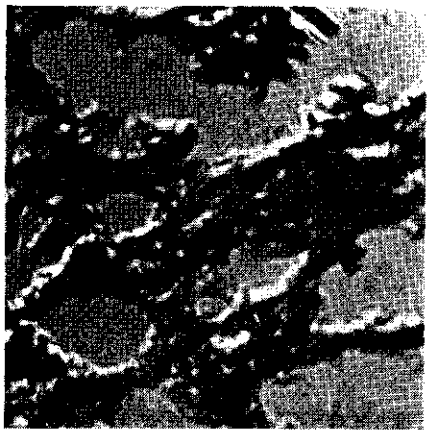


0 days



7 days

14 days



21 days

29 days

Fig. 2. Interference contrast micrographs of Meshanger cheese. Microscope objective × 100, N.A. 1.00; magnification × 3400.

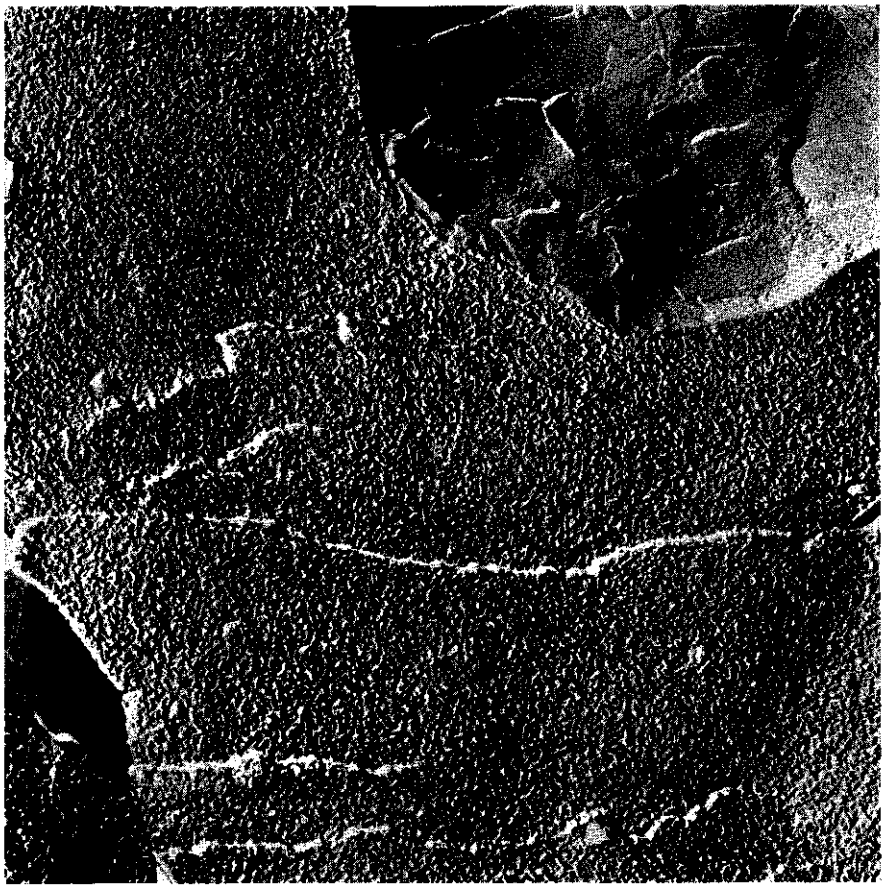


Fig. 3. Meshanger cheese at 28 days of age. Electron micrograph (freeze fracturing) by TFDL. Magnification × 44 000.

Peters (22). Their explanation that the proteolytic enzymes of the surface flora would be responsible for Camembert softening is questionable, as was reported previously (9). Both the electron micrographs and the photomicrographs demonstrate that fat globules exist in the mature cheese, which indicates that contrary to for example Cheddar cheese, the fat is and remains a discontinuous phase during the ripening (14).

Fig. 3 shows an electron micrograph of a sample of Meshanger cheese at the age of 33 days prepared according to the freeze fracturing procedure (Section 2.2.3.2). The picture shows the protein mass and part of a milk fat globule. The protein seems to consist of small spherical units, whose diameter is approximately equal to that of submicelles.

4 Discussion

The photomicrographs obtained with the fluorescence and the interference contrast technique both show the same picture of structural changes during the ripening of Meshanger cheese, a soft cheese with a high moisture content. It is striking that these structural changes completely agree with those reported by Mulder et al. (24), concerning Gouda and Edam cheese. In both cases a structure composed of small units changed into a smooth structure without recognizable internal organization.

Fig. 5 shows an electron micrograph of an embedded specimen of Gouda cheese. It is published by courtesy of Dr Schmidt, NIZO, Ede, the Netherlands. Comparison between this picture and Fig. 4 does not reveal any difference between the structure of mature Meshanger and Gouda cheese. Other photos by D. G. Schmidt (to be published) show no difference between immature soft and hard cheese. Meshanger cheese, however, softens while Gouda and Edam cheese do have a change in consistency (29) but without softening.

Since the structural changes during the ripening of hard and soft cheeses seem similar and are governed by the breakdown of their protein, under comparable physico-chemical conditions in the cheese (pH, salt content and Ca ion concentration) and at a similar degree of α_{s1} casein decomposition, the

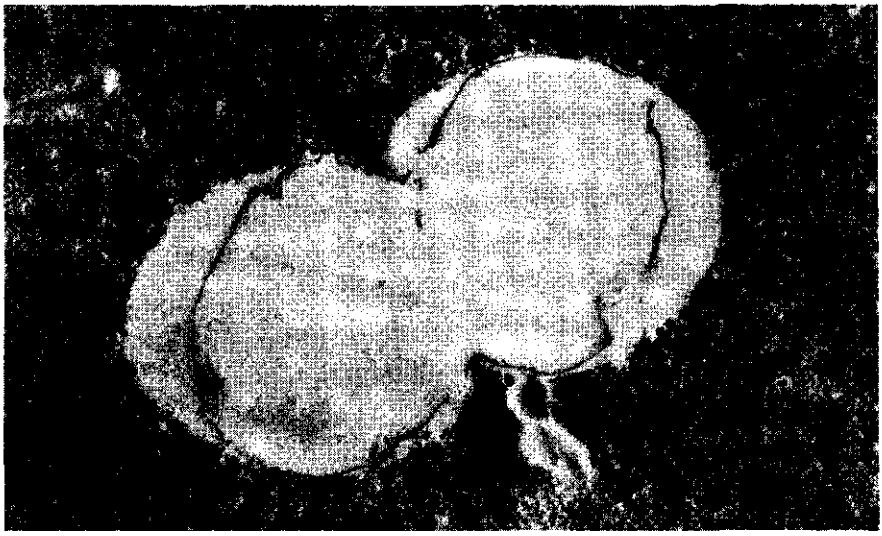


Fig. 5. Gouda cheese (12 weeks of age). Electron micrograph (embedding technique) by TFDL. Magnification × 31 000. By courtesy of Dr D. G. Schmidt (NIZO, Ede, the Netherlands).

moisture content regulates the rheological behaviour of the mass of partly broken down protein.

In a previous paper (9) we reported that Meshanger cheese without active rennet enclosed does not have any perceptible α_{s1} casein breakdown and as a consequence no softening. Unfortunately the technique for preparing 'rennet-free cheese' was not yet available when the research reported in this paper was performed. Therefore no micrographs of those cheeses can be presented at this time. It is likely that those cheeses do not show any structural change after a few days of ripening, and that the structure will remain unaltered. The observations of Knoop & Peters (20) who describe a continuing change of the coagulum in time may be explained by the protein breakdown by rennet, which they did not consider. This proteolysis is considerable (30) and even stronger at higher incubation temperature (31). More experiments with systems in which no active rennet is present should be performed to obtain results which permit definite conclusions to be drawn.

Since the building elements of the protein matrix of the freshly formed cheese are paracasein micelles, understanding the cheese structure requires knowledge of casein micelle structure. The micelle is thought to be constructed of submicelles (7). As has already been mentioned (8, 9) the degree of breakdown of α_{s1} casein, about 38 % of the protein in the micelles, determines the consistency of Meshanger cheese. A few remarks may however be made. Knoop & Peters (28) demonstrated that, in contrast to former opinions, renneted milk did coagulate at low temperatures (4 °C), although very slowly as was to be expected. The coagulum thus formed could not be electron microscopically distinguished from gels produced at higher temperatures. As β casein is said to diffuse to a great extent out of the casein micelle at low temperatures (7, 32-38), this implies on the one hand that β casein does not play an important structural role in the casein micelle and on the other that it favours the idea of α_{s1} casein as a structure former of the casein submicelle.

Schipper (39) reported on the function of phosphate in relation to calcium in the building of the calcium caseinate-calcium phosphate complex in milk. Schmidt & Buchheim (40) and Schmidt et al. (41) have reported that the casein micelle disintegrates into submicelles when the calcium is removed by dialysis. At low pH values calcium is also removed from the micelles which results in their disintegration. Monib (42) has reported that, at pH 5.2, 50-60 % of the calcium present in Edam cheese is dissolved in the cheese serum. This dissolution will cause a change in calcium bonds in the paracasein micelles. However, Meshanger cheese without active rennet enclosed (9) reveals no softening at pH 5.2 whereas normal Meshanger cheese softens rapidly when α_{s1} casein is broken down. Whatever exact mechanism may be respons-

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The proteolytic action of rennet on different casein substrates under various conditions

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Summary

The decomposition of α_{s1} and β casein by rennet in a substrate containing sodium paracaseinate was determined using a quantitative gel electrophoretic method. The conditions during incubation were varied with respect to pH, and Na and Ca content. To obtain more information on the influence of added Ca, experiments were performed with calcium paracaseinate-calcium phosphate complex and a similar 'synthetic complex' prepared from acid precipitated casein. The effects of pH, [Na+] and [Ca++] were considerable and mutally dependent. The results obtained did not permit conclusions to be drawn on the mechanism of the simulation and inhibition of the proteolytic reaction by Ca, although the results are important for the elucidation of the decomposition of the cheese protein.

1 Introduction

In the course of a study of the proteolysis during the ripening of Noordhollandse Meshanger cheese, a soft cheese used in our laboratory as a model for studying cheese ripening, we were confronted with a striking difference between the rate of breakdown of α_{s1} and β casein, and with the high rate of the overall proteolysis in this cheese compared with that in hard cheese (1, 2, 3). The difference in the decomposition rate demanded our special attention as we now know that proteolytic decomposition in soft cheese, as well as in hard cheese, can be attributed to a great extent to the action of rennet (3). However, from the experiments with cheese it could not be decided which factors were responsible for the existing differences. As both α_{s1} and β casein can be split by rennet (for example Fox (4)), it appears likely that conditions during the ripening of the cheese determine the differences in proteolysis.

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The most striking differences in physico-chemical conditions between cheeses are those of pH, salt and moisture content. Furthermore the Ca ion concentration of the cheese serum, which varies with the type of cheese, also changes due to the salt uptake but primarily due to the decrease of the pH (5). The influence of the moisture content on the rate of proteolysis will be dealt with in a subsequent paper.

Reports on the proteolysis of caseins by chymosin are numerous, but the analytical methods used often give information only on the proteolytic products soluble in a certain liquid. Other papers treat variables other than those in which we are interested. Moreover many of the investigations were performed to obtain information on the decomposition products of low molecular weight, while we primarily want to know which part of the casein has not yet been attacked by rennin.

If electrophoretic methods were used to study this subject a quantitative interpretation of the results was not given. Hence we had to carry out even the more obvious experiments, such as those on the effects of pH and salt.

The aim of the research reported in this paper can best be described as the collection of information, in model experiments, which can help us to elucidate the differences in proteolysis mentioned above. We shall focus on the influence of pH, and Na and Ca concentration, using quantitative polyacrylamide gel electrophoresis as the analytical method to detect proteolysis.

2 Methods and materials

2.1 Sodium paracaseinate

Sodium paracaseinate was prepared from milk delivered by the university dairy farm. The pH of the skim milk was decreased to about 4.6 with 1 *N* HCl. The precipitate obtained was washed with demineralized water and dissolved with the aid of 1 *N* NaOH. This procedure was repeated once. To the solution of sodium caseinate so obtained (pH 6.6) commercial rennet was added. After being kept on 30 °C for 30 min, the mixture was pasteurized at 72 °C for 20 s to inactivate the rennet. The paracaseinate was precipitated twice more as described above, and then spray-dried. The sodium paracaseinate obtained was canned.

2.2 Calcium phosphate-calcium paracaseinate complex

The calcium phosphate-calcium paracaseinate complex used in the experiments, further referred to as 'complex' was prepared according to Noomen (13).

2.3 Casein

The casein used was prepared as described by Schipper (8).

2.4 Incubation

2.4.1 Incubation of sodium paracaseinate. A solution of 25 g sodium paracaseinate in 1 litre demineralized water, containing 200 mg thimerosal – ((carboxyphenyl)thio) ethylmercury sodium salt – per litre to prevent microbiological activity, was heated for 30 min at 80 °C to prevent milk protease activity during incubation. After cooling to room temperature, quantities of 80 ml of this solution were transferred to conical flasks with screw caps. The desired quantity of NaCl was added and the pH was adjusted to 5.2 with 0.1 *N* HCl unless otherwise indicated. After cooling in ice water, 0.04 ml of commercial rennet, strength 10 000, was added. The flasks were rotated in a water-bath at a temperature of 13 °C. After 16 h the enzymatic reaction was stopped by adding urea to a concentration of 8 *M*, and the pH was increased with 0.1 *N* NaOH to 8.5. After dilution to a protein content of approximately 0.5 % with a buffer of pH 8.5 (Tris-HCl) containing 8 *M* urea, samples were taken for an electrophoretic separation of the proteins (see Section 2.5).

If this experiment had to be performed with different Ca concentrations, the concentration of the stock protein solution was doubled; 40 ml of this solution were mixed with 40 ml of a solution containing twice the required quantity of Ca and 200 mg thimerosal per litre.

This procedure was tested in preliminary experiments. It appeared that pH adjustment needed much attention. In spite of the precautions taken, the pH after incubation differed from the desired value. Fig. 1 shows the result of an experiment, without rennet, with sodium paracaseinate substrates with different pH values. The pH before incubation, after readjustment during 2 h, is plotted against the pH after incubation. Obviously the protein substrates, in the low pH region, did not quite reach an equilibrium in 2 h. If the experiments were performed with NaCl dissolved in the substrates the deviation was less, while greater difficulties were encountered in the experiments with Ca additions. The pH values referred to in this paper are those measured after incubation.

The substrates used to check the pH before and after incubation were also used to control whether a mere incubation at a certain pH influenced the electrophoretic pattern. From the densitogram it was learned that incubation at different pH values does not result in significant differences in the protein quantities before and after incubation.

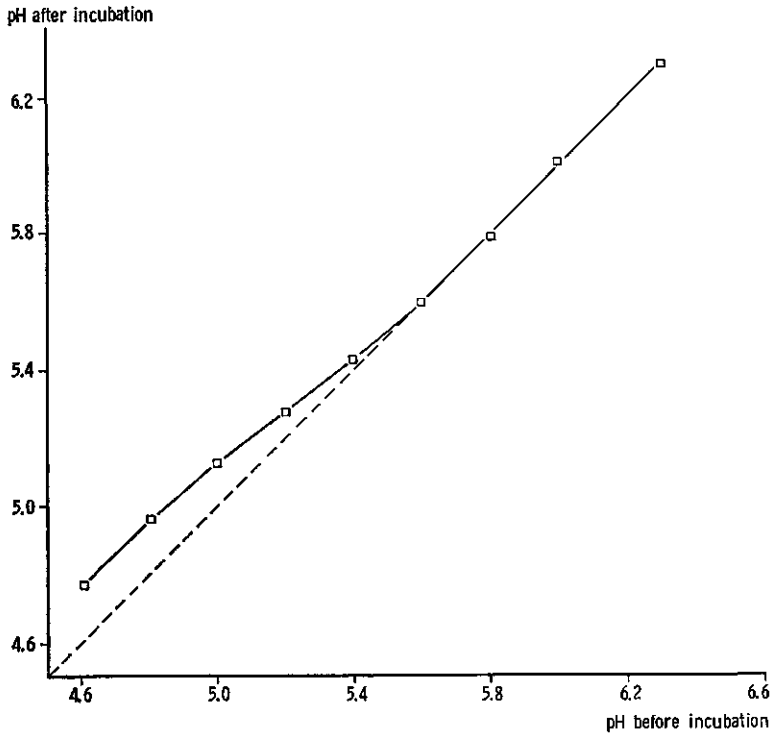

Fig. 1. The change of the pH during incubation of sodium paracaseinate substrates.

We also checked whether Ca interfered with the electrophoretic pattern obtained. The pattern of sodium paracaseinate incubated with rennet and Ca (II) was the same as that obtained in an experiment in which Ca was removed, after incubation, by dialysation (IV). The addition of Ca just before an electrophoretic separation of a sample incubated with rennet (III) did not influence the pattern obtained, as can be seen when it is compared with the one obtained without Ca addition (I). The roman numerals between brackets refer to the patterns in Fig. 2.

2.4.2 The incubation of 'complex' with rennet. The experiments with 'complex' as the protein constituent were performed as described for sodium paracaseinate in the previous section.

2.4.3 The incubation of a synthetic calcium phosphate-calcium caseinate complex with rennet. With the aid of a solution of $Ca(OH)_2$ in a solution containing 33 % sucrose according to Hoffman and Gortner (6), casein was

carefully dissolved. The pH was adjusted to 6.7. At this pH solutions of $Ca(OH)_2$ and H_3PO_4 were added simultaneously, using such a procedure as necessary to maintain a pH of 6.7. The procedure used is identical to that described by Mulder & Schipper (7) and Schipper (8). With this procedure a synthetic Ca caseinate-Ca phosphate complex is produced. This complex can serve as a model for the similar complex in milk. After the desired adjustments and additions with respect to Ca and Na content, and pH were made, the suspension obtained was incubated and analysed as described in Section 2.4.1.

2.5 Polyacrylamide gel electrophoresis and densitometry

Polyacrylamide gel electrophoresis and densitometry were performed as described previously (9). The values obtained by densitometry were corrected for the difference in protein concentration due to the different quantities of acid and base necessary to adjust the pH of the substrates. From the corrected densitometrical values the quantities of α_{s1} and β casein were cal-

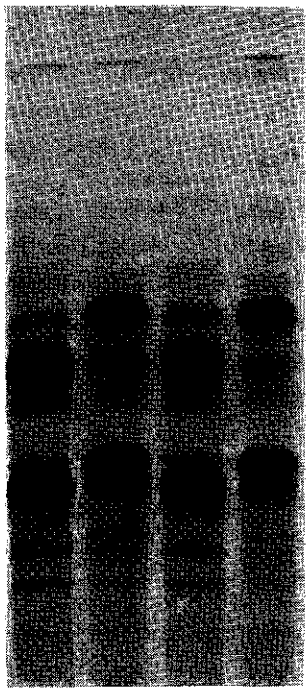

Fig. 2. The pattern obtained by an experiment performed to demonstrate the absence of Ca effect on the electrophoretic separation. Further explanation in the text.

CASEIN PROTEOLYSIS BY RENNET

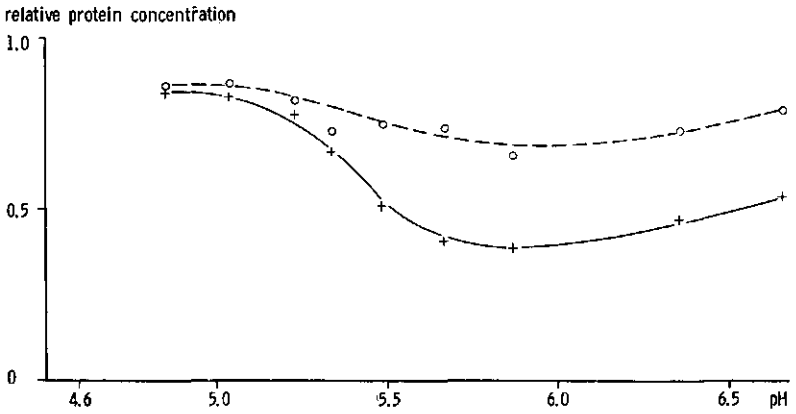

Fig. 5. The decomposition of α_{s1} (o) and β casein (+) by rennet as a function of the pH during incubation.

tions on sodium paracaseinate substrates, incubated as described in Section 2.4.1, with different contents of Na, Ca or Na and Ca. Therefore all further experiments were made with the one lot of Dutch commercial rennet with a strength of 10 000.

3.2 Experiments with sodium paracaseinate substrates

3.2.1 The influence of the pH. Substrates composed as described in Section 2.4.1 were adjusted to different pH levels and incubated with rennet.

Fig. 5 shows the result of a representative experiment. Throughout the whole pH range tested the proteolysis of β casein was more extensive than that of α_{s1} casein. The α_{s1} casein breakdown under these conditions is, compared to the decomposition of β casein, less pH-dependent.

The β casein breakdown has an optimum near pH 5.85 and is more extensive in the higher than in the lower pH region. The qualitative interpretation of the gel showed that the breakdown products formed at different pH levels were the same as those seen in Fig. 3. Of course the amounts of proteolytic products differed according to the pH of the substrate.

3.2.2 The influence of NaCl. Substrates with sodium paracaseinate as protein component were composed as described in Section 2.4.1. Before the adjustment of the pH to 5.2, NaCl was added. Fig. 6 shows the result of the electrophoretic separation. The NaCl concentrations given in this figure were calculated from the quantities added to the substrates. The sodium of the sodium

paracaseinate and the chloride from the diluted hydrochloric acid used to adjust the pH were neglected. Low concentrations of NaCl favoured the breakdown of β casein, but 11 % NaCl almost completely inhibited the proteolysis of this casein. Maximum breakdown of α_{s1} casein occurred at 3-5 % NaCl; higher concentrations had an inhibitory effect but did not stop the reaction as in the case of β casein. The formation of proteolytic products from α_{s1} casein was even more extensive at 11 % NaCl than at concentrations below 3 %. The addition of NaCl did not affect the pattern of the decomposition of α_{s1} and β casein.

3.2.3 The effect of simultaneous addition of NaCl and $CaCl_2$. The experiments reported in the preceding paragraphs were planned to obtain information on the rennet proteolysis of casein under physico-chemical conditions comparable to those in ripening Meshanger cheese. But the cheese has a more complicated composition than the model substances, and at least one

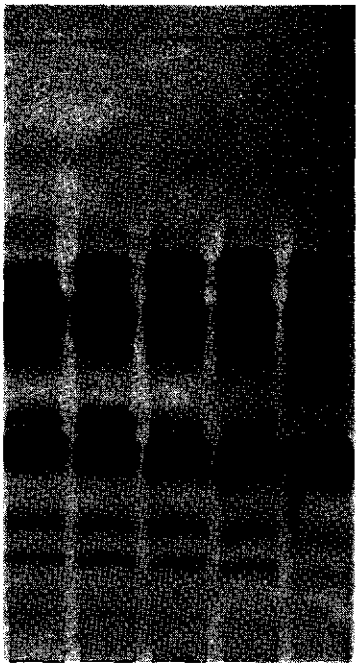

Fig. 6. An example of the influence of NaCl concentration on the proteolysis of sodium paracaseinate by rennet.

4 Discussion

The experiments reported in Section 3.2.1 dealing with sodium paracaseinate showed a marked relation between the proteolysis of sodium paracaseinate by rennet and the pH during incubation. This effect has been reported previously by several authors (4, 15, 16, 17). Ledford et al. (17) unfortunately did not report experiments at a pH below 5.5. Fox (4) studied a greater pH range at different temperatures. If the differences in experimental conditions are taken into account, his results show the same tendency as ours: at 13 °C the proteolysis of β casein goes faster than that of α_{s1} casein. In our experiments, however, the decomposition of β casein is more dependent on the pH.

Therefore we must assume that the rate of proteolysis during cheese ripening is markedly influenced by the course of the pH during cheese ripening. This observation is of minor importance for cheeses such as Gouda and Cheddar with a fast fall in pH, but for an explanation of the proteolytic processes occuring in cheeses with a pH development such as in Meshanger (2) it seems to be important, mainly during the first week after preparation when the pH of the centre of the cheese decreases slowly to about 5.2. The most important aspect of the experiments is however the difference in breakdown observed between α_{s1} and β casein at different pH levels. This may lead to an explanation of the differences in breakdown rates between α_{s1} and β casein during the ripening of different kinds of cheese.

The remaining experiments were performed at a pH of about 5.2, unless otherwise indicated. At this pH experiments were performed to collect information on the influence of other factors on the proteolysis. The reported influence of Na additions agrees with Fox & Walley's (19) trials though attention must be drawn to differences in incubation temperature. Again there is the possibility of explaining differences in breakdown between α_{s1} and β casein during cheese ripening from the results of these experiments. The results of the experiments with different Ca concentrations do not allow a decision to be made on the mechanism of the stimulation and inhibition of the decomposition of α_{s1} and β casein, although is seems evident that the Ca bound to the protein as well as the Ca dissolved in the substrate liquid does influence the reaction.

It is questionable whether the influence of Ca on the proteolysis of α_{s1} and β casein can be further elucidated in this type of model experiment. It is preferable to perform experiments with cheeses with different Ca contents to study this phenomenon.

Furthermore we wish to emphasize that the use of data collected in model experiments with dilute suspensions of casein, to explain cheese ripening

phenomena, should be done very carfully. As indicated in this paper, small changes in substrate concentrations do have a considerable effect on the results obtained. Interpretation of proteolysis during cheese ripening based on results of the experiments mentioned may lead to false theories. It seems that studies on this subject can best be made, no matter how much labour is involved, with cheeses produced under strictly controlled circumstances, as described for instance by Kleter (19) and Visser (20), and with starters with a very low proteolytic activity or with artificial acidification.

Since the rennet concentration depends on the pH of the cheese milk (21) much attention had to be given to its estimation (12) to prevent wrong conclusions being drawn due to misinterpretations of the amounts of rennet enclosed during the preparation of the cheese.

Acknowledgment

This investigation was made possible because of a considerable grant by the Stichting J. Mesdagfonds, Kaascontrolestation 'Friesland', Leeuwarden, the Netherlands.

Samenvatting

L. de Jong en Aliza E. A. de Groot-Mostert, *De afbraak, onder verschillende omstandigheden, van diverse caseïne substraten door stremsel*

De afbraak van α_{s1}- en β-caseïne onder invloed van stremsel werd gevolgd met een kwantitatieve gelelektroforetische methode. De pH en de Na- en Ca-gehalten tijdens de incubatie werden gevarieerd. Om meer informatie te krijgen over met name de invloed van de toevoeging van Ca werden, nadat in eerste instantie natriumparacaseïnaat was gebruikt, ook experimenten uitgevoerd met een uit ondermelk gewonnen calciumparacaseïnaat-calciumfosfaat-complex en met een dergelijk complex samengesteld uitgaande van zure caseïne. Het bleek dat de pH, [Na+] en [Ca++] grote invloed hadden op het verloop van de proteolytische reactie.

De resultaten van de experimenten lieten niet toe conclusies te trekken over het mechanisme van de beïnvloeding door Ca van de eiwitafbraakreactie. De verkregen resultaten zijn van belang voor de verklaring van de proteolytische reacties in kaas en wel in die zin dat voorzichtigheid geboden is bij pogingen om deze processen te verklaren op grond van resultaten van modelexperimenten. Aangegeven wordt dat betrouwbare experimenten uitgevoerd kunnen worden met kaas mits aan een aantal voorwaarden wordt voldaan.

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The influence of the moisture content on the consistency and protein breakdown of cheese

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Key words: consistency, protein breakdown, moisture content, enzyme diffusion

Summary

Cheeses with moisture contents between 40 and 60 % were prepared with methods derived from the production method for Meshanger cheese. The protein breakdown during the ripening of these cheeses is discussed, as well as the change in the firmness. The relation between the volume fraction of the protein in the fat-free cheese (φ_p) and the firmness is given. Attention is paid to the possibility of the chymosin molecule diffusing in the moisture of the cheese, which is thought to be composed of globular fat and protein particles. It was concluded that the transport of chymosin in the cheese moisture is very small compared with that of sodium and chlorine ions in the same medium.

1 Introduction

In previous papers (1, 2) we reported on the change in consistency of Meshanger cheese; it appeared to be regulated largely by the breakdown of α_{s1} casein by rennet. Circumstantial evidence was obtained indicating that the moisture content of the Meshanger cheese had to be above a certain limit and that its pH should not deviate too much from 5.2 to make the desirable softening possible.

To study the effect of the moisture content on cheese properties especially its consistency during ripening we used the Meshanger cheese developed in this laboratory (3, 4) as a model. Its preparation was adapted as necessary to obtain cheese with a lower moisture content.

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Table 1. Preparation schedules of the cheeses. After pasteurization (72 °C, 20–25 s) of the milk, 20 g KNO_3, 10 ml starter *(S. lactis)* and 40 ml rennet (strength 10 800) were added per 100 litres cheese milk.

	H basic schedule*	*1L*	*2L*	*3L*	*5L*	*6L*
Fat content of the cheese milk (%)	3.0±0.1	3.10	3.10	3.07	3.12	3.08
Coagulation temp. (°C)	29.5—31.0	29.3	30.2	29.2	30.3	30.0
Coagulation time (min)	20—25	30	30	31	30	28
Cutting time (min)	5	20	25	22	20	17
Sedimentation time (min)	—	5	11	12	15	18
Whey removed (%)	—	17	36	36	47	~50
Stirring temp. (°C)	30	31.3	32.0	34.0	37.0–34.5	38.0–35.5
Stirring time (min)	6—7	75	49	77	65	45
Sedimentation time (min)	10	11	14	18	16	15
Moulds (E: Edam, G: Gouda)	E	G	G	G	G	G
Brining time (h)	**	12	17	17	30	36

* All cheeses denoted *H* were prepared according to the basic schedule.
** Cf. Ref. 5.

2 Methods and materials

2.1 Cheese preparation

The cheeses were produced from morning milk, supplied by the University dairy farm, as indicated in Table 1. The preparation of Meshanger cheese, described in detail by Noomen (5), is summarized in the column 'basic schedule *H*' of Table 1. Cheeses with lower moisture contents were obtained by adapting the 'basic schedule', as indicated in the other columns. Cheeses will be identified by the alphanumeric code at the head of the table. Cheeses with the same numerical code are produced from the same milk on the same day. *H* refers to 'high moisture content'. *L* to 'low moisture content'. Each batch consisted of 5-7 cheeses. They ripened at 13 °C.

2.2 Analytical methods

The methods used for the estimation of dry matter content, fat content, pH, protein content, NaCl content, rennet content and consistency were described previously (1), as was gel electrophoresis (6, 1).

3 Results

Table 2 shows the fat content, the protein content and the rennet concentration of the cheeses. The ratio between the fat content and the protein con-

tent of *L* and *H* cheeses with the same number (i.e. produced from the same milk) is equal except for *6H* and *6L*. For the *6L* cheeses the ratio is lower which may be due to some fat loss as a result of the high stirring temperature and long stirring time during production (cf. Table 1). The low quantities of enclosed rennet in the cheeses with the lowest moisture contents are due to the higher temperatures used during their production as was inferred by Stadhouders (7). Fig. 1 shows the moisture contents of the cheeses at different ages. There were some differences between the moisture contents of the normal Meshanger cheeses, the *H* cheeses, but they were all considerably higher than those of the *L* cheeses. After 28 days the moisture content of the different cheeses was between 60 and 40 %. The mass fraction of the protein matrix in the fat-free cheese (μ_p) (Table 2) covered the range between 0.18 and 0.32; $\mu_p = 0.32$ is about the maximum obtainable in a full-cream cheese.

Fig. 2 shows the course of the firmness (F) of the cheeses. All the observations concerning the *H* cheeses are plotted but as these curves all show similar shapes only one is drawn. The causes of the maximum in F in these cheeses after a few days of ripening have been discussed previously (1). As is to be expected, the firmness curves of the *L* cheeses are at a higher level than those of the *H* cheeses. The maximum in the graphs of the cheeses with the lowest moisture content, *5L* and *6L*, is remarkable, as well as the lack of a maximum in the graphs of the cheese *1L*, *2L* and *3L*.

Table 2. Some data of the cheeses.

Batch and type[1]	Fat (% in DM)	Protein (% in DM)	Rennet concentration, (strength 10800) (ml/kg cheese protein)	μ_p[2]	φ_p[3]	Y[4] (nm)
1H	43.9	41.4		0.22	0.33	2.8
2H	44.4	41.2	1.134	0.22	0.33	2.8
3H	45.7	39.8	1.595	0.21	0.32	3.0
5H	46.2	34.2	1.837	0.19	0.29	3.5
6H	47.5	33.3	1.478	0.18	0.27	3.9
1L	45.6	43.3		0.27	0.41	1.8
2L	46.2	42.9	0.853	0.28	0.42	1.7
3L	47.6	42.2	1.529?	0.30	0.45	1.6
5L	49.2	36.3	0.429	0.29	0.44	1.5
6L	49.0	38.0	0.363	0.32	0.48	1.2

[1] The code is explained in Section 2.1.
[2] Mass fraction of the cheese protein in the fat-free cheese at 3–4 weeks.
[3] Volume fraction of the cheese protein in the fat-free cheese, $\varphi_p = 1.5 \times \mu_p$. Specific volume of casein: 1.5 ml/g (Ref. 10).
[4] Mean free distance between protein particles calculated as indicated in Section 4.

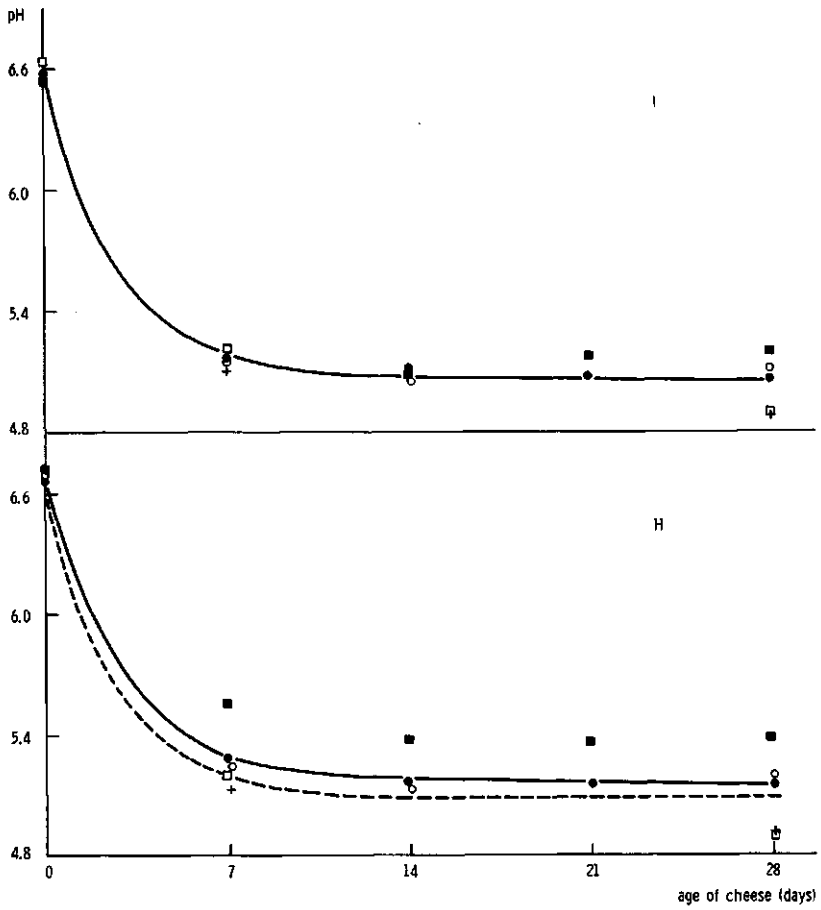

Fig. 3. The pH of the cheeses during the ripening. The curves give the averages within each group. For comparison the *L* curve is also drawn (broken line) next to the *H* curve. Further explanation in the caption of Fig. 1.

rate of proteolysis of α_{s1} casein in particular (2), we should not jump to such a conclusion. This will be further commented on below. The decomposition of β casein is not clearly different between the *H* and *L* groups. Both groups show a considerable variation, which may be due to different contents of milk proteases (8), since there is a clear correlation with batch numbers. The age of the cheeses at which 0.5, 0.6, 0.7 part of the α_{s1} casein was broken down was derived from Fig. 4. The firmness (F) at these times, obtained from Fig. 2, was plotted against the corresponding dry matter content, as derived from Fig. 1 (Fig. 6). Batch 6*H* was taken, as typical of the *H* group. From Fig. 6 it

can be concluded that the firmness of the cheese is closely related to its dry matter content, even if the protein breakdown and its influence on the firmness of the cheese is taken into account. Changes in dry matter content cause greater changes in firmness at a high than at a low dry matter content.

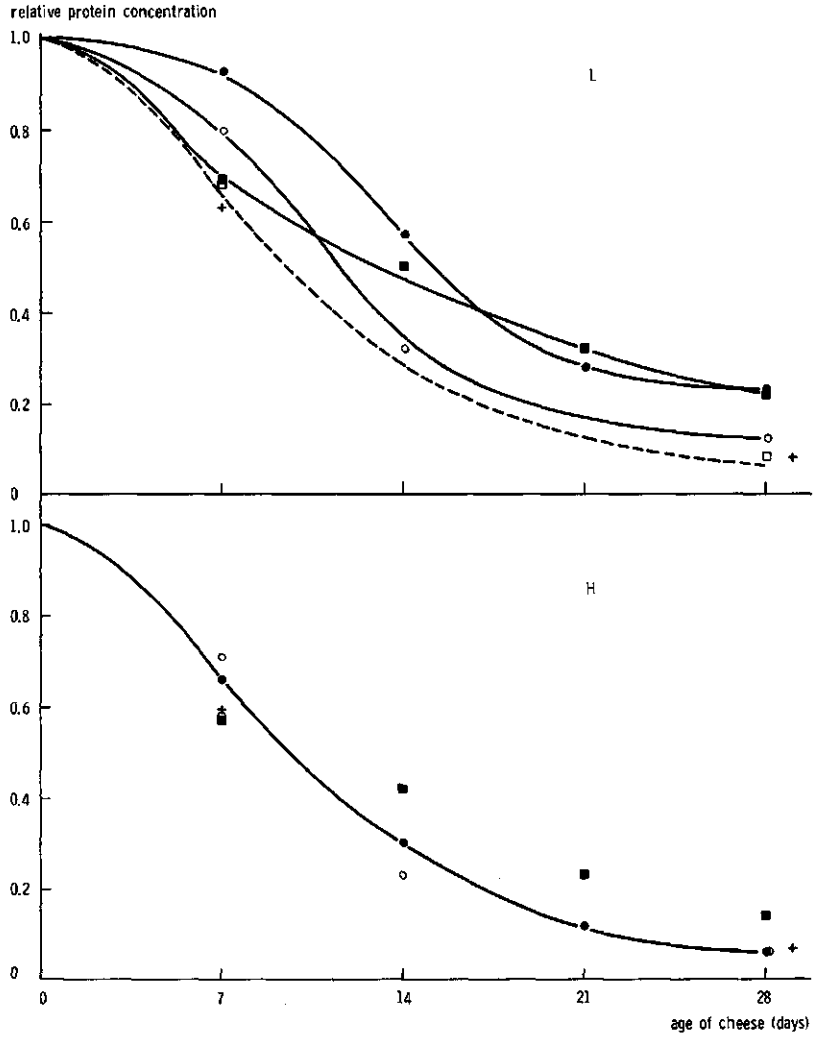

Fig. 4. The quantities of unattacked α_{s1} casein during the ripening of the cheeses. For the *H* cheeses a curve is drawn and repeated (dotted line) among the curves for the *L* cheeses. The curves for *1L* and *2L* are almost identical with the dotted line, and are not drawn. Further explanation in the caption of Fig. 1.

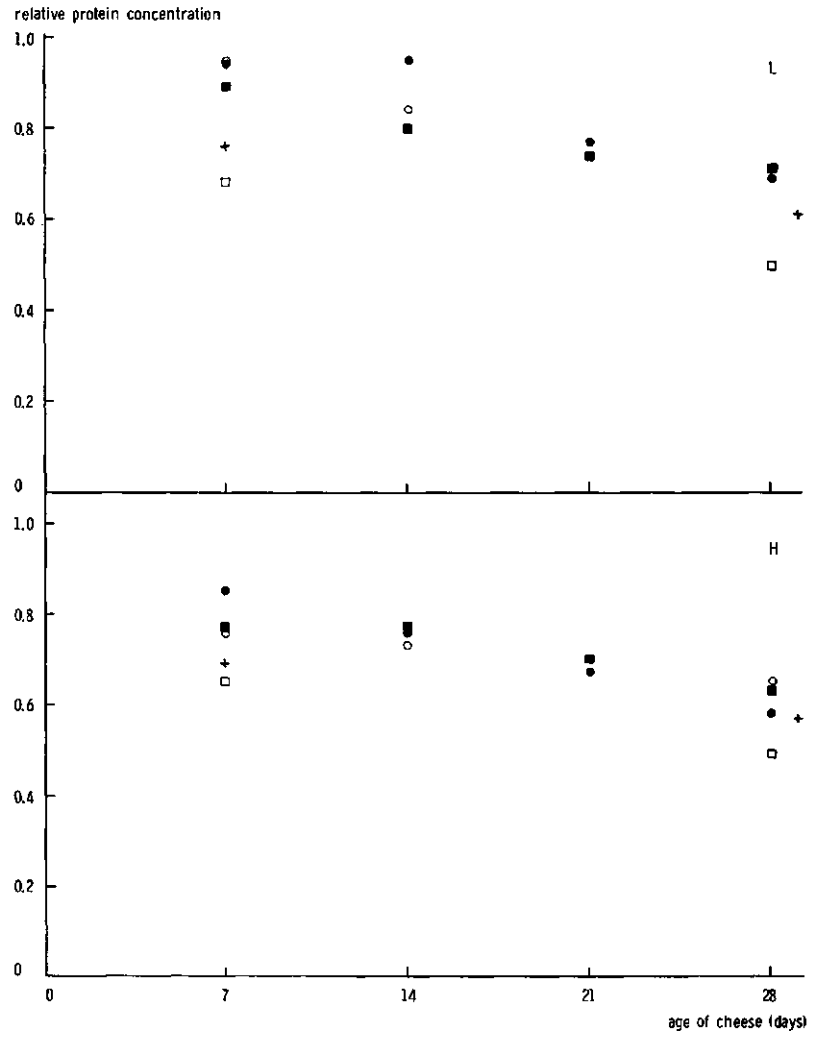

Fig. 5. The quantities of unattacked β casein during the ripening of the cheeses. The relative concentration at 0 days equals 1. Explanation of the symbols in the caption of Fig. 1.

In Fig. 7 in which F is plotted against μ_p (Table 2), the influence of the protein fraction of the cheese on the firmness of the cheese is also very clearly demonstrated. Since the discontinuous fat in these cheeses may not be expected to contribute much to the firmness, Fig. 7 gives an even better impression of the relation between protein content and firmness than does Fig. 6. If the protein matrix of the cheese is assumed to be constructed from para-

casein micelles (spherical parts), an idea well supported by numerous electron microscopic pictures (survey, Ref. 9), which remain globular at least at the beginning of the ripening, it is logical to look for the relation between the volume fraction of the paracasein micelles in the fat-free cheese, φ_p (Table 2), and F. To calculate φ_p a certain value for the specific volume ν of casein micelles, for example 1.6 ml per gram dry casein as reported by Schmidt et al. (10), should be used. It should be realized that differences exist between casein

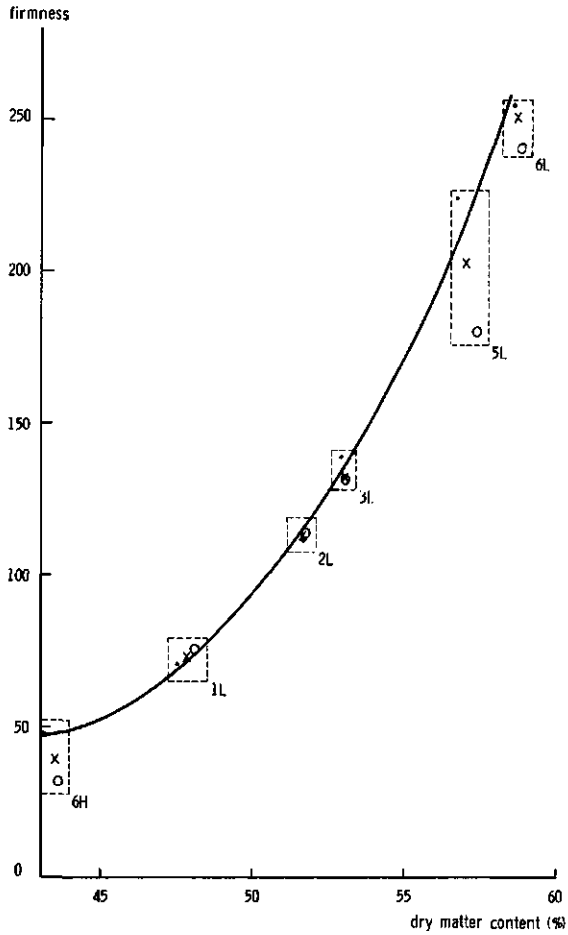

Fig. 6. The relation between the firmness and the dry matter content of the cheeses. The line is drawn with regard to the points (·) indicating the firmness of the cheese at the time that 50 % of the α_{s1} casein had been broken down. × indicates the firmness when 60 % of the α_{s1} casein is broken down, ○ the situation at 70 % decomposition. Further explanation in the text.

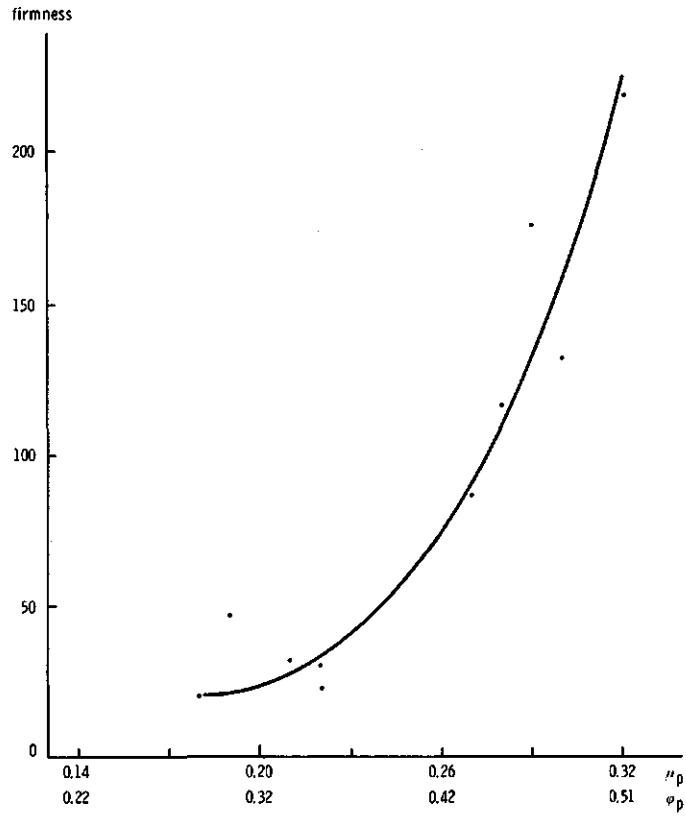

Fig. 7. The relation between the firmness and the mass fraction (μ_p) and the volume fraction (φ_p) of the protein in the fat-free cheese.

micelles in cow's milk and the paracasein micelles in cheese. In Fig. 7 we have also indicated $\varphi_p = \mu_p \times 1.6$. Whether the influence on F by the protein is studied by μ_p or φ_p does not make any significant difference to the result. Using φ_p however is fundamentally more correct.

4 Discussion

The experiments with cheeses with different moisture contents were performed in such a way that the only intended compositional difference between the batches just after preparation was that in moisture content. Due to the procedure required to obtain low moisture contents, however, as already indicated, the rennet content of these cheeses was decreased in comparison to those in cheeses with high moisture contents. The decomposition of paracasein, how-

ever, is not only dependent on the rennet concentration; the amounts of dissolved calcium and sodium, for example, will also affect the reaction rate as demonstrated previously (11). Even if it is accepted that the effect of various factors on the breakdown reaction of α_{s1} casein in our cheeses cannot be known exactly, the remaining quantity of unattacked α_{s1} casein is at most 25 % (cf. Fig. 4) after one month of ripening. A comparable value was reported recently by Visser & de Groot-Mostert (12) for Gouda cheese containing rennet as the only proteolytic enzyme. This indicates that an important change in the protein fraction of cheese, viz the conversion of α_{s1} casein into its primary decomposition product α_{s1}-I, has taken place after 1 month of ripening at 13 °C, no matter what its moisture content is. Assuming the rheological properties of cheese to be largely dependent on this conversion and on its moisture content, the desired consistency can be obtained within 1 month. This may perhaps open up possibilities of speeding up the ripening of some types of cheese (including flavour formation).

To understand the kinetics of the protein breakdown, the following consideration may be important. About 80 % of the clotting strength of Dutch commercial rennet (10 000) can be attributed to the activity of chymosin. According to de Koning (13), purified chymosin has a clotting strength of 9×10^6 to 12×10^6 units/mg (average 10.5×10^6). Hence our rennet would have contained about 0.76 mg chymosin per ml. Assuming its molecular weight to be 34 000 (13) and its partial specific volume 0.73 ml/g (14), the radius of the globular chymosin molecule is calculated to be about 2.15 nm. The ratio between chymosin molecules and the various other particles present in cheese with high and with low moisture content (55 and 40 % respectively) was calculated (Table 3).

As was indicated previously (1, 2) in Meshanger cheese prepared with the only slightly proteolytic *S. lactis*, the breakdown of α_{s1} casein into its primary decomposition product α_{s1}-I can be attributed to the proteolytic activity of the rennet and is proportional to the rennet concentration in cheese. This proportionality is common in enzymatic reactions in liquid substrates as long as the enzyme concentration is low compared with the substrate concentration. From a biochemical point of view, however, cheese is not a liquid. It is a 'solid' system with as can be seen in Table 3 low numbers of chymosin molecules compared with α_{s1} casein molecules ($\sim 1 : 5 \times 10^5$). The chymosin is dissolved in the cheese moisture, but its substrate consists of a solid protein mass composed of paracasein micelles containing submicelles. Due to the great difference between the number of submicelles and chymosin molecules displacement of the latter will be necessary to enable

Table 3. Calculation of the ratio of the numbers of protein particles or molecules to chymosin molecules, in two imaginary cheeses with high and low moisture content.

	High moisture content	Ratio to chymosine molecules	Low moisture content	Ratio to chymosine molecules
Moisture content (%)	55		40	
Rennet content (μl/kg cheese)	250		250	
Chymosin content[1] (mg/kg cheese)	0.19		0.19	
Number of chymosin molecules/kg cheese[2]	3.4×10^{15}	1	3.4×10^{15}	1
Protein content (%)	14		23	
Number of paracasein micelles/kg cheese[3]	1.8×10^{17}	54	3×10^{17}	89
Number of submicelles/kg cheese	3.6×10^{20}	1.1×10^{5}	6×10^{20}	1.8×10^{5}
Number of α_{s1} casein molecules/kg cheese[4]	1.4×10^{21}	4.2×10^{5}	2.3×10^{21}	6.9×10^{5}
x chymosin[5] (nm)	640		640	

[1] Rennet contains about 0.76 mg chymosin/ml as is explained in the text.

[2] $\frac{0.19}{1000} \times \frac{1}{34 \cdot 10^3} \times 6 \cdot 10^{23} = 3.4 \times 10^{15}$

[3] A casein micelle contains at average about 2000 submicelles, each containing 10 casein monomers. 1 gram paracasein (average mol.wt 23 000) contains 2.6×10^{19} monomers (para)-casein.

[4] α_{s1} casein is about 39% of whole casein (cf. Ref. 8).

[5] The mean distance x between chymosin molecules of which there are N per unit volume of cheese: $x = N^{-1/3}$.

continued breakdown of the α_{s1} casein in the submicelles.

A simplified model for transport processes in cheese is proposed by Geurts et al. (15), the cheese is thought to be constructed of separate small protein spheres and globular fat. This model served very well to explain the influence of the moisture content of the cheese on the transport phenomena observed. To get an impression of the possibilities for chymosin molecules to diffuse in such cheese model systems, we calculated the mean free distance Y between protein spheres, with diameters d of the same order as those of submicelles (10 nm). We used the equation $Y/d \approx 0.225\ (\varphi_m/\varphi\text{-}1)$ as inferred by Walstra (16). Y is given in Table 2. The mean free distance is conceived to be a measure of the average pore width between the spheres of the protein matrix. Even the pores with the largest diameter Y (cf. Table 2) are smaller than the calculated diameter of the chymosin molecule (4.3 nm). Pores with a larger diameter than the average will exist. Furthermore it should be realized that the pore width is subject to changes due to the Brownian movement of the proteins and to the proteolysis in the system. The transport of chymosin molecules, however, will be extremely difficult.

Preliminary experiments were performed to detect the transport of alkaline phosphatase in Meshanger cheese (17). This enzyme was chosen since sensitive methods were available for its detection, and it is not present in cheese prepared from pasteurized (72 °C, 20 s) milk. The transport of the enzyme appeared to follow roughly Fick's laws of diffusion. A pseudo diffusion coefficient (D*) of approximately 0.003 cm^2/day was found. Geurts, Walstra & Mulder (15) reported D* to be about 0.2 cm^2/day for NaCl in cheese moisture. Because the radius of the chymosin molecule will be about equal to that of alkaline phosphatase both molecules will have a comparable D*. Therefore the transport of chymosin in cheese moisture will be very slow as compared with that of NaCl in the same medium. The other factors taken into account by Geurts et al. (15) are likely to cause a further delay of the diffusion of the chymosin. Since the mean distance between chymosin molecules is about 640 nm (Table 3), it will be very difficult for the chymosin molecule to reach all the casein molecules and to serve as a catalyst for their decomposition reaction. The slow transport of the chymosin molecule in the cheese matrix may be responsible for the delay in the breakdown of its protein if compared with that in liquid substrates under comparable conditions.

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Samenvatting

L. de Jong, *De invloed van het vochtgehalte op de consistentie en de eiwitafbraak van kaas*

Uitgaande van de bereidingswijze van Meshanger kaas werden kazen bereid met vochtgehaltes op 28 dagen tussen 40 en 60 %. De eiwitafbraak in deze kazen wordt besproken evenals het verloop van de stevigheid tijdens de rijping. Het verband tussen de volumefractie van het eiwit in de vetvrije kaas (φ_p) en de stevigheid wordt aangegeven. Bij de bespreking wordt aandacht gegeven aan de mogelijkheden die het chymosine-molecuul heeft om zich in de kaas die opgebouwd wordt gedacht uit vet- en eiwitbolletjes te verplaatsen. Berekend werd dat deze verplaatsing in het kaasvocht zeer gering zal zijn vergeleken bij die van keukenzout.

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Discussion and conclusions

1 Proteolysis in Meshanger cheese

The electrophoretic separation of the different caseins in cheese on polyacryl amide gels, followed by a densitometrical estimation, proved satisfactoryly for the qualitative and quantitative reproduction of the decomposition of α_{s1}- and β-casein during cheese ripening.

After a slow start, which may be due to the high pH at the beginning of ripening of Meshanger cheese, the decomposition of α_{s1}-casein accelerated until about the 8th day. After about 2 weeks half had disappeared. Conversion into α_{s1}-I (1), the primary decomposition product of α_{s1}-casein, was completed after about 30 days. The α_{s1}-I was further decomposed at a slower rate and at 30 days still much was left in the cheese. Decomposition of β-casein was fast during the first days of ripening, becoming negligible after 5 days. The breakdown product with an electrophoretic mobility only slightly higher than that of the original β-casein is likely to be β-I (2). Significant changes in the protein fractions with an electrophoretic mobility less than β-casein were not observed.

The fastest moving breakdown products increase in amount during the ripening. These are presumably products formed by the decomposition of α_{s1}-I.

Normally electrophoresis is under alkaline conditions in the presence of urea. The caseins and their decomposition products mentioned above then have a negative charge and move towards the positive pole. Electrophoresis with reversed current yielded a slowly moving diffuse band that increased with ripening. This positive charged product was not further identified.

2 Consistency

The course of the change of the firmness of the Meshanger cheese during the ripening (Fig. 1) estimated with the Allo Kramer shear press may be remarkable at first sight; however, it is very well explainable. The increase in firmness just after preparation will be due to

- decrease in moisture content,
- salt uptake,

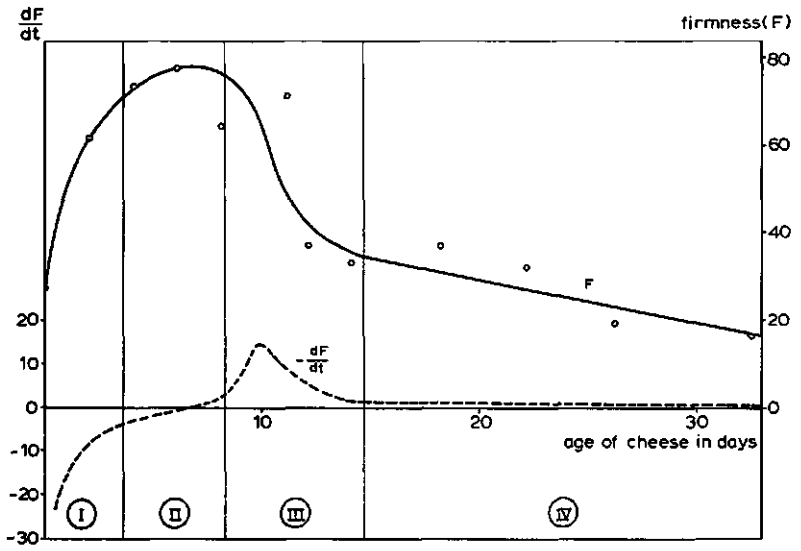

Fig. 1. The course of the firmness during the ripening of Meshanger cheese.

– decrease in pH,
– solidification of the fat as the cheese cools from production temperature (30 °C) to the temperature of the ripening room (13 °C).
After a maximum it decreased rather rapidly. This softening of the cheese is attributable to the decomposition of the protein matrix of the cheese, and especially to the breakdown of the α_{s1}-casein component, by the chymosin present in the Dutch commercial rennet used.

3 Trials with different rennet concentrations

To collect more information on the relation between the proteolysis of α_{s1}-casein and the development of cheese consistency, Meshanger cheese was made with different amounts of enclosed rennet. Cheeses without detectable active rennet were prepared by a technique developed in our laboratory (3). In cheeses without rennet, no α_{s1}-casein was broken down and in the cheeses with increasing content of rennet breakdown increased proportionally. Except at the very beginning of the ripening when large changes in pH and NaCl concentration occur, decomposition of α_{s1}-casein was proportional to firmness.

4 Microstructure

By light microscopy at magnification 2200-3400 changes in the protein struc-

ture of soft cheese could not be distinguished from those of hard cheese. They were characterized as a transition from a network of separate particles into a smooth uniform mass without recognizable structure.

Electron-micrographs at magnification 10 500, 34 000 and 70 000 revealed a more detailed structure. The paracasein micelles remained visible from just after the preparation of the cheese until 7 days of ripening but lost their globular shape. The position of milk fat globules was clearly visible (Fig. 2). In the mature cheese, the micelles completely disappeared, only particles with a diameter an order of magnitude less than casein micelles remained visible. These structural changes are identical with those reported during the ripening of Camembert cheese (4). It seems reasonable to attribute softening of Camembert cheese to the same process as in Meshanger cheese: breakdown of α_{s_1}-casein by rennet enzymes. Older opinions that the softening of Camembert cheese be due to the proteolytic enzymes from the surface flora

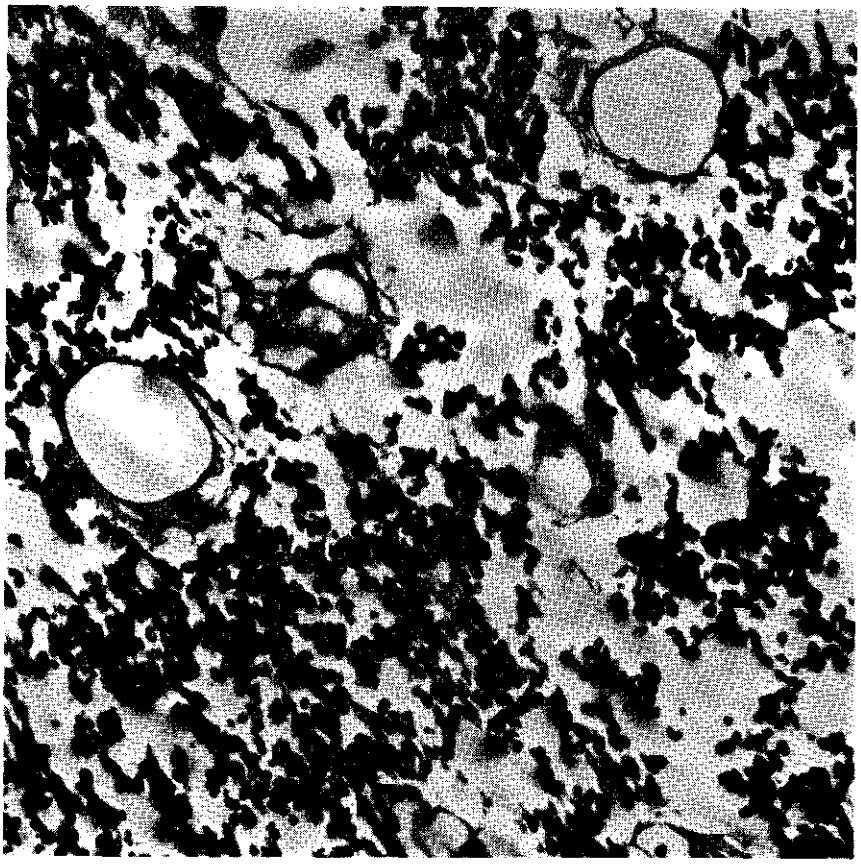

Fig. 2. Meshanger cheese after 7 days ripening. Magnification × 10 500. Electronmicrograph by TFDL.

(e.g. 4) seem questionable moreover because the diffusion of those enzymes into the cheese is very difficult as is indicated below.

5 Proteolysis in synthetic models

In substrates with sodium paracaseinate as protein constituent was no difference between proteolysis caused by Dutch commercial rennet and that caused by the enzyme preparation extracted from the abomasum of newly born calves. By adjusting the pH and the NaCl concentration of those substrates optima for the proteolytic reaction of rennet on sodium paracaseinate were found. Also the influence of Ca was tested. It affected proteolytic breakdown of both α_{s1}-casein and β-casein. To elucidate the processes involved, tests were set up with calcium phosphate-calcium paracaseinate instead of sodium paracaseinate and also with a synthetic complex of calcium caseinate and calcium phosphate (5, 6). Even then the exact mechanism of the mutual influences between Na^+, Ca^{2+} concentrations and proteolysis could not be fully explained. Proteolysis of caseins under different conditions is difficult to study in such suspensions, since the state of protein is not known exactly, so that data are difficult to interpret in connection with cheese ripening.

6 Moisture content and consistency

To see whether the results with Meshanger cheese were applicable to the ripening of other cheeses, cheeses with lower moisture contents were prepared. Proteolysis was the same in cheeses with different moisture contents and consistency of those cheeses was determined primary by the volume fraction of protein matrix in the fat-free cheese.

7 Diffusion of enzymes in cheese

To get an impression of the kinetics of the proteolysis in cheese, the diffusion of chymosin was calculated with a simplified theoretical model (7). In that model the radius of the chymosin molecule was so large relative to the pores in cheese that transport would be impeded.

In test to detect the transport of alkaline phosphatase in soft cheese (8) was revealed that compared to NaCl (7), the pseudo diffusion coefficient of NaCl (about 20 mm^2/day) was much greater than of phosphatase (0.4 mm^2/day).

As the molar mass of chymosin and alkaline phosphatase are similar, their transport equations may be assumed to be similar. This further indicates that diffusion of chymosin in cheese will be difficult and may explain the differences in breakdown rates estimated in cheese and in liquid substrates. Presumably enzymes from a surface flora could only diffuse slowly into a cheese.

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Summary

Samenvatting

De bereiding en de rijping van Noordhollandse Meshanger kaas, een zachte kaas met een hoog vochtgehalte die tot omstreeks 1940 in Noord-Holland werd gemaakt, is uitgebreid bestudeerd in ons laboratorium. Nadat de bereidingstechniek was gereconstrueerd bleek deze kaas zeer geschikt om te gebruiken als modelsysteem om de vervloeiing van kaas te bestuderen. De bereidingswijze is eenvoudig en de kaas rijpt zonder oppervlakteflora in ongeveer 2 weken, na 4 weken is de kaas geheel vervloeid.

Uitgegaan werd van de gedachte dat de consistentie-eigenschappen van kaas voornamelijk bepaald zullen worden door de eigenschappen van de eiwitmatrix, de enige continu fase in de kaas die vast is. Deze eiwitmatrix wordt gevormd indien, bij een voldoende hoge concentratie van calciumionen, de $\varkappa$-caseïne in melk door bijvoorbeeld stremsel wordt omgezet in para-$\varkappa$-caseïne. De matrix bestaat uit paracaseïnemicellen die op hun beurt weer opgebouwd zijn uit submicellen (voornamelijk bestaande uit α_{s1}-, β- en para-$\varkappa$-caseïne en calciumfosfaat).

De veranderingen die deze eiwitmatrix ondergaat tijdens de kaasrijping werden op drie manieren bestudeerd.

– Met behulp van een instrument waarmee de kracht gemeten kon worden die nodig is om een tamelijk groot stuk kaas door een rooster te persen werden de consistentie-eigenschappen bepaald.

– Met behulp van een kwantitatieve polyacrylamidegel-elektroforese-methode werd nagegaan welk deel van de oorspronkelijke eiwitmoleculen nog aanwezig was.

– Ten slotte werd de structuur van de eiwitmatrix bestudeerd met zowel licht- als elektronenmicroscopie.

Het bleek dat de door ons verder ontwikkelde elektroforesetechniek heel geschikt was voor het kwantitatief weergeven van de eiwitafbraak in kaas.

De afbraak van α_{s1}-caseïne tijdens de rijping van Meshanger verloopt de eerste dagen langzaam. Daarna volgt een periode van ongeveer 10 dagen waarin de afbraak sneller gaat. Na 30 dagen is alle α_{s1}-caseïne omgezet, voornamelijk in het afbraakprodukt dat karakteristiek is voor de proteolyse door stremsel: α_{s1}-I. De afbraak van β-caseïne verloopt in het begin sneller dan die van α_{s1}-caseïne, na ca. 5 dagen echter komt de afbraak praktisch tot stilstand.

Het gebruikte zuursel heeft nauwelijks proteolytische eigenschappen.

In kazen bereid volgens een op ons laboratorium ontwikkelde techniek waarin geen actief stremsel aanwezig was, werd geen afbraak van α_{s1}-caseïne waargenomen. Het bleek dat deze afbraak rechtevenredig was met de stremselconcentratie in de kaas en dat de consistentieveranderingen, nadat eerst een bepaalde maximale stevigheid was bereikt, weer evenredig waren met de hoeveelheid niet omgezet α_{s1}-caseïne.

De veranderingen in de structuur van de eiwitmatrix tijdens de rijping bleken overeen te komen met die in harde kaas gerapporteerd worden. Uit een meer gedetailleerd beeld dat verkregen werd met elektronenmicroscopie kon dezelfde conclusie getrokken worden. Bovendien kwamen de structuurveranderingen tijdens de rijping overeen met die zoals beschreven voor Camembertkaas.

Het lijkt er op dat het vervloeien van zachte kazen toegeschreven moet worden aan de omzetting van α_{s1}-caseïne door stremsel. Opvattingen waarin de proteolytische enzymen van een oppervlakteflora een belangrijke rol spelen zijn dubieus.

Berekeningen aan de hand van een theoretisch model toonden aan dat de diffusie van chymosine in kaas zeer traag verloopt omdat de straal van het chymosinebolletje ongeveer gelijk is aan de straal van de poriën die verondersteld werden in de kaas te bestaan.

Experimenten die uitgevoerd werden met alkalische fosfatase toonden aan dat de pseudo-diffusiecoëfficient voor dit enzym in kaasvocht (0,4 mm^2/dag) veel kleiner is dan die voor NaCl in een dergelijk systeem (20 mm^2/dag). Aangezien alkalische fosfatase en chymosine een overeenkomstig molecuulgewicht hebben is dit een nadere aanwijzing voor de moeilijke verplaatsing van chymosine in kaas.

Om snel na te gaan wat voor invloed o.a. natrium- en calciumionen hebben op de proteolyse door stremsel, werden experimenten uitgevoerd met substraten met natriumparacaseïnaat, calciumparacaseïnaat-calciumfosfaat-complex en een dergelijk complex samengesteld uit caseïne, calciumhydroxide en fosforzuur. Afgeleid kon worden dat zowel natrium als calcium een grote invloed hebben op het verloop van de proteolyse en bovendien dat het verloop van de reactie afhankelijk was van het gebruikte substraat. Het feitelijke mechanisme van de reactie kon niet worden opgehelderd, wel werd geconcludeerd dat voor dergelijke experimenten modelsystemen die de omstandigheden in kaas beter benaderen geschikter zijn.

De experimenten met kazen met een vochtgehalte lager dan dat van Meshanger leerde ons dat de stevigheid van deze kazen in de eerste plaats bepaald wordt door de volumefractie van het eiwit in de vetvrije kaas en tevens dat de verschillen in eiwitafbraaksnelheid tussen kazen niet eenvoudig toe te schrijven zijn aan verschillen in vochtgehalte.