Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese

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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,
in het openbaar te verdedigen
op vrijdag 25 november 1977
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

Centre for Agricultural Publishing and Documentation

Wageningen - 1977

Abstract

Visser, F. M. W. (1977) Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. Doctoral thesis, Wageningen. (viii) + 32 p., 2 figs, 1 table, 79 refs, Eng. and Dutch summaries, Neth. Milk Dairy J. 30 (1976): 41–54; 31 (1977): 120–133, 188–209, 210–239, 247–264, 265–276.
Also: Agric. Res. Rep. (Versl. landbouwk. Onderz.) 870, p. II-1 – II-32

A method for the aseptic manufacture of cheeses, free either from rennet or from rennet and starter, is described, allowing the action of starter bacteria and milk protease to be studied without the interference of rennet. These cheeses, together with aseptic starter-free cheeses, were used to elucidate the contribution of rennet, starter bacteria and milk protease to breakdown of protein and to development of bitterness and cheese flavour during ripening of Gouda cheese. Their combined action was studied in normal aseptic cheeses, allowing the estimation of possible interactions. Different amounts of enclosed rennet and different starter cultures, either 'bitter' or 'non-bitter', were used in the cheeses. Proteolysis was characterized by different analytical methods.

Free descriptors: aseptic cheesemaking, rennet-free cheese, starter-free cheese, rennet-and starter-free cheese, Gouda cheese, cheese ripening, rennet, starter bacteria, milk protease, proteolysis, bitter flavour, cheese flavour, (bitter) peptides, amino acids, gel electrophoresis of cheese.

This thesis will also be published in Agricultural Research Reports 870.

Stellingen

1. Bij de ontwikkeling van geïmmobiliseerde enzympreparaten voor de stremming van kaasmelk gaat men voorbij aan het belang van een zekere hoeveelheid ingesloten stremsel voor de rijping van kaas.

M. J. Taylor, T. Richardson & N. F. Olson, J. Milk Fd. Technol. 39 (1976) 864.

2. De opmerkelijke stabiliteit van proteolytische enzymsystemen tijdens de rijping van kaas kan, in het bijzonder bij stremsel, mede te danken zijn aan een door bepaalde kaascomponenten uitgeoefende beschermende werking.

3. Bezwaren tegen de bepaling van de stremselactiviteit in kaas met behulp van een extractiemethode zijn ongegrond, indien ze gebaseerd zijn op een mogelijk storende invloed van zuurselproteïnasen.

F. M. W. Visser, Neth. Milk Dairy J. 30 (1976) 41.
M. L. Green, J. Dairy Res. 44 (1977) 159.
J. Stadhouders, G. Hup & C. B. van der Waals, Neth. Milk Dairy J. 31 (1977) 3.

4. Bij de produktie van een Goudse kaas die behalve door de afwezigheid van het gebrek 'bitter' tevens wordt gekenmerkt door de aanwezigheid van voldoende kaassmaak, is de keuze van een geschikt zuursel van primair belang.

Dit proefschrift.

5. Het verdient aanbeveling bij de bepaling van het kiemgetal van gekoeld bewaarde, rauwe melk het verdunde monster een intensievere mengbehandeling te geven dan op dit moment gebruikelijk is.

I. E. Te Whaiti & T. F. Frijer, N. Z. J. Dairy Sci. Technol. 12 (1977) 51.

6. De frequent geciteerde uitspraak van Reiter et al., dat melkprotease in kaas aminozuren vormt, is weliswaar juist, maar vindt volstrekt onvoldoende steun in de door hen beschreven experimenten.

B. Reiter, Y. Sorokin, A. Pickering & A. J. Hall, J. Dairy Res. 36 (1969) 65.

7. De antagonistische werking van melkzuurbacteriën op meerdere soorten pathogene en bederfverwekkende micro-organismen in gefermenteerde levensmiddelen verdient meer aandacht en toepassing buiten de zuivelindustrie.

C. Daly, W. E. Sandine & P. R. Elliker, J. Milk Fd. Technol. 35 (1972) 349.

8. Bij de bepaling van de voluminositeit van caseïnemicellen met behulp van viscosimetrie dient bij lagere temperaturen rekening te worden gehouden met een viscositeitsverhogende werking van dissociërend β-caseïne.

S. M. Sood, K. S. Sidhu & R. K. Dewan, Milchwissenschaft 31 (1976) 470.
M. Noelken & M. Reibstein, Arch. Biochem. Biophys. 123 (1968) 397.

9. Het valt te betwijfelen of de uitgangspunten van de logaritmische afstervingstheorie in overeenstemming zijn met het optreden van subletale beschadigingen bij het vernietigen van micro-organismen.

10. Het in de Warenwet gehanteerde Federgetal als norm voor de samenstelling van vleeswaren dient te worden vervangen door kwaliteitseisen waarin het rechtstreeks bepaalde eiwitgehalte en het bindweefselgehalte zijn opgenomen.

11. Het dient als onjuist te worden aangemerkt aan bepaalde voedingsmiddelen een gunstige dan wel ongunstige invloed op de gezondheid toe te schrijven; een voedingsmiddelenpakket mag slechts in zijn geheel en in relatie tot de gebruikers worden beoordeeld.

Stellingen behorend bij het proefschrift van Ir. F. M. W. Visser, getiteld 'Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese'.

Woord vooraf

De voltooiing van dit proefschrift biedt mij de gelegenheid dank te betuigen aan allen die hebben bijgedragen aan de totstandkoming ervan.

Op de eerste plaats dank ik mijn moeder die mij de mogelijkheid heeft geboden te studeren. Tevens past bij deze gelegenheid een woord van dank aan al degenen, van onderwijzers tot professoren, die aan mijn vorming hebben bijgedragen. Hooggeleerde Pilnik, uw deskundigheid en enthousiasme op het gebied van de levensmiddelenchemie zijn daarbij vooral van belang geweest.

Geachte promotor, hooggeleerde Vos, u bood mij de mogelijkheid dit onderzoek uit te voeren op het Laboratorium voor Zuiveltechnologie en melkkunde, waarvoor ik u grote dank verschuldigd ben. Uw stimulerende begeleiding en de ruime mate van vrijheid bij het onderzoek heb ik ten zeerste gewaardeerd.

Het Kaascontrolestation 'Friesland' te Leeuwarden ben ik erkentelijk voor de financiële steun die het via de Stichting 'J. Mesdagfonds' gedurende vier jaar verstrekte.

Dat ik met veel plezier zal terugdenken aan deze periode is te danken aan alle medewerkers van de sectie Zuiveltechnologie en melkkunde en van de Centrale Dienst Biotechnion. Goede herinneringen bewaar ik in het bijzonder aan de samenwerking met mevrouw A. E. A. de Groot-Mostert en de heren J. L. Uitentuis en H. J. van der Stege. Beste Aliza, Jan en Henk, zonder jullie enthousiasme en daadwerkelijke steun zou het onderzoek veel minder voorspoedig verlopen zijn.

Het aseptisch bereiden van kaas zou niet mogelijk geweest zijn zonder de medewerking bij de aseptische melkwinning van het Melkhygiënisch Onderzoek Centrum te Wageningen en de afdeling Veeteelt van de Landbouwhogeschool. Met name ir. Tj. de Vries, J. Nuninga, J. Hagens, R. Uiterwijk en H. Knol ben ik zeer erkentelijk. Beste Hooyer, jouw voor- en nazorg bij de melkwinning en kaasbereiding stelde ik zeer op prijs. Het NIZO te Ede dank ik in de persoon van dr. J. Stadhouders onder andere voor het zeer vroegtijdig beschikbaar stellen van de stremselbepalingsmethode in kaas.

Ir. W. O. van Middendorp en mevrouw ir. T. van Pijkeren leverden een bijdrage aan het onderzoek in het kader van hun doctoraalstudie, terwijl de samenwerking met ir. A. J. Vos eveneens zeer nuttig was.

De medewerking van de dames J. van Asperen, Geke Nass en Laura Vlug, die het typewerk verzorgden, werd zeer op prijs gesteld. Dank betuig ik eveneens aan de heren J. van de Beek en C. Rijpma, die het foto- en tekenwerk van dit proefschrift verzorgden. De medewerkers van het Pudoc, in het bijzonder de heer D. van der Heij, ben ik erkentelijk voor hun inspanningen om de publikaties en dit proefschrift op tijd en verzorgd het licht te laten zien.

Ten slotte deel ik dit proefschrift met jou Ine, omdat ik zonder jouw aanmoediging en begrip dit werk nooit voltooid zou hebben.

Curriculum vitae

Na het behalen van het diploma Gymnasium-β aan het Thomas a Kempis College te Arnhem, begon ik in 1966 mijn studie aan de Landbouwhogeschool te Wageningen. Als studierichting koos ik Levensmiddelentechnologie. In januari 1973 werd met lof het ingenieursdiploma behaald met als hoofdvak de Levensmiddelenchemie en de bijvakken Levensmiddelenmicrobiologie (verzwaard) en Technische microbiologie. Daarna verrichtte ik, met financiële steun van het Mesdagfonds te Leeuwarden, op de afdeling Zuiveltechnologie en melkkunde van de Landbouwhogeschool te Wageningen het onderzoek waarvan de resultaten in dit proefschrift zijn samengevat. Sedert juli 1977 ben ik werkzaam bij de Zuid-Nederlandse Melkindustrie b.v., D.M.V., te Veghel.

Contents

Introduction

1. Ripening of cheese

The development of a high quality cheese is primarily dependent on a controlled fermentation of the concentrated milk constituents. Although this process starts during manufacture, for most cheese varieties a longer or shorter period of maturation is required before consumption. During this ripening the lactose, the protein and the fat are involved in a complex series of biochemical reactions, resulting in the development of the desired flavour and the optimum physical characteristics.

Gouda cheese is a semi-hard, full-cream cheese variety that can be consumed from about 1 month of ripening onwards, but that is often kept for a longer ripening time up to 1 year and more. Its production is of great economic importance for the Dutch dairy industry. In 1976 the production amounted about 260 000 tons. Although large amounts (about 50%) are exported from the Netherlands, Gouda cheese is produced in considerable proportions in other countries too.

Normal Gouda cheese is manufactured from low-pasteurized cow's milk, using calf rennet to clot the milk. In other countries rennet substitutes are being used increasingly. As a rule mixed-strain starter cultures containing *Streptococcus cremoris* strains together with aroma producing streptococci or leuconostocs are used. Edam cheeses, that have a lower fat content and a different shape, are manufactured in an corresponding way and therefore can be expected to show a ripening process almost identical with that in Gouda cheese.

2. Breakdown of protein

Decomposition of protein during ripening governs the conversion of the rubbery and coarse original curd into a more or less smooth-bodied, homogeneous substance. Especially in soft types of cheese like Camembert, Limburger and Meshanger, these changes in consistency are outstanding (1).

Proteolysis, however, also influences the flavour of the cheese since many breakdown products – amino acids and peptides in particular – are considered to be significant contributors to cheese flavour directly, or to be precursors for other characteristic taste and aroma components (2,3). Moreover they are implicated in certain flavour defects of the cheese, especially bitterness (4,5,6).

Proteolysis, therefore, is a determinative process for the quality of nearly all ripened cheese types, whether a short-ripened soft type of cheese, like Camembert, or a long-ripened hard variety like Cheddar cheese.

Since most of the soluble serum proteins and the glycomacropeptide of κ-casein are lost with the whey during manufacture of Gouda cheese, paracasein is the principal substrate for the proteolytic enzymes during the ripening. It constitutes more than 96% of the protein in cheese.

Paracasein, however, is not a homogeneous substrate but is a complex of at least three major components, α_{s1}-casein, β-casein and para-κ-casein. They represent about 50, 35 and 10% of the total paracasein in cheese respectively. All three are relatively hydrophobic phosphoproteins with molecular weights of about 23 600 (α_{s1}), 24 000 (β) and 12 300 (para-κ). The primary structure is elucidated at this moment and was reviewed by Mercier et al (7).

In Gouda cheese four main groups of agents could be responsible for the degradation of paracasein during ripening.

- The coagulant (rennet). Rennet contains the proteolytic enzyme rennin (chymosin). A certain proportion of the rennet used to clot the milk is normally incorporated in the curd and survives the making of Gouda cheese into the ripening period (8).
- The starter bacteria or their enzymes. Starter bacteria were shown to possess a rather complex system of endo- and exopeptidase activities (9). Several authors reviewed literature on the proteolytic activity of lactic acid bacteria used in cheese manufacture (10,11,12).
- The indigenous milk protease. Cow's milk was shown to contain a proteolytic enzyme system of non-bacterial origin (13,14), that survives pasteurization of the cheese milk (15). Being associated with the casein fraction of milk, it will finish up in the cheese.
- Non-starter micro-organisms or their enzymes. These organisms can originate from the raw milk or enter the cheese by multifarious and haphazard contamination during manufacture. Apart from bacteria like lactobacilli, that always grow to high numbers in Gouda cheese, cells or enzymes from a variety of other micro-organisms may be found in Gouda cheese.

Most bacteria involved in cheese ripening are equipped with a proteolytic enzyme system consisting of various proteinases and peptidases of intracellular and extracellular location. The effective activity of these enzymes in cheese is dependent, inter alia, on maximum cell count reached and on time and degree of cell-lysis. The rennet used in cheesemaking is a crude enzyme extract from calf stomachs that may contain impurities of others proteolytic enzymes than rennin (e.g. pepsin).

Thus proteolysis during ripening of a normal Gouda cheese is an extremely complex process that is operated by multifarious enzymes. The activity of each can be influenced by products formed by the others.

Because different proteolytic enzymes act together, one would expect that during cheese ripening a mixture of breakdown products would develop with different molecular weights, ranging from hardly attacked casein fractions to the small amino acids.

For the assay of protein breakdown, different analytical methods are available. Because of the heterogeneity of the products, no one method is however available to characterize the progress of proteolysis completely, expressing it in one distinguis-

hing number or value. The characterization of proteolysis will often depend on the scope of study and the method used. In study of cheese consistency, for instance, analysis of the larger insoluble protein fractions will be relevant. In cheese flavour research, analysis of smaller soluble breakdown products and amino acids will give information. For this Schormüller (16) introduced the two terms 'extent' and 'depth' of proteolysis.

The hydrolysis of protein in cheese has long been assessed by measuring the nitrogen soluble in a certain extraction liquid, separating the compounds by properties like solubility in water, in trichloroacetic acid solutions of different concentrations, solubility at pH 4.6, and precipitability by calcium (17,18,19). Among the disadvantages of these methods are that it is not clear what exactly is being measured and that what is being measured may vary between cheeses.

In the last decade, gel electrophoresis has been used increasingly. Usually polyacrylamide or starch gels have been used (20, 21, 22). Disappearance of the different casein components and formation of some larger breakdown products can so be followed.

Gel filtration has been proposed too to analyse proteolysis in cheese but such methods are less sensitive than electrophoresis (23,24,25). Gel filtration is also suitable for analysis of low molecular weight, soluble fractions (25,26) but attention should be paid that conditions during chromatography encourage dissociation.

The total amount and pattern of free amino acids in cheese have long been used to follow proteolysis (19,27,28).

Occasionally different chromatographic or other methods have been described in the literature.

The ultimate description of proteolysis would be to identify all the peptides and amino acids formed and thereby the peptide bonds cleaved. A restricted number of (bitter) peptides have indeed been isolated and identified from cheese (6,29).

Over the years, much effort has been spent in describing protein breakdown and flavour development in various types of cheese, as is testified by numerous publications. Usually cheeses made in commercial or pilot plant were employed in these investigations. Although adventitious growing bacteria could always exert various influences on the ripening of these cheeses, rennet and starter bacteria together were thougt to play the central role in protein breakdown in Gouda cheese (30). Native milk protease was thought to be of limited influence only (30).

Nowadays research on cheese proteolysis knows different angles of incidence.

Because of the complexity of both the substrate and the enzyme systems in cheese, many investigations are performed with milk, solutions of casein components or synthetic peptides as model substrates to elucidate the separate proteolytic actions of rennin (31, 32, 33), starter bacteria or their cell extracts (10, 34, 35, 36), and isolated milk protease (13,14). Although fundamental knowledge, especially about the specificity of rennin, has been gained in this way, these results need not reflect the activity of the enzyme systems in cheese itself. Studies with more 'cheese-like' model systems, like cheese slurries and paracaseinate-phosphate complex, have been reported too (37, 38).

Another approach is to unravel the proteolytic system of starter bacteria involved in cheesemaking by isolating, characterizing and locating the different enzyme

activities of the cultured cells (9,11,39,40).

Research on proteolysis and flavour development in cheese itself has made considerable progress since aseptic cheesemaking was introduced, obviating the uncertain and changing effects of adventitious bacteria (28,41,42,43). The major role of starter bacteria in development of flavour in Gouda (44,45) and Cheddar cheeses (46) was demonstrated. Lawrence et al. (12) recently reviewed literature in this field. By aseptic techniques, the central role of rennet and starter bacteria in proteolysis of Cheddar and Gouda cheese was convincingly proved (28,44). Chemical acidification – instead of a starter – was first introduced by Mabbit et al (47). Ever since, that technique or modifications of it have been used to characterize qualitatively the action of rennet alone in cheese (19,25,28,44,48).

3. The 'bitter' defect in cheese

Although a sligt bitter taste can be desired in some foods and beverages (grape fruits, beer etc.), it is generally judged as a defect. Today bitterness is one of the most frequently occurring defective flavours in cheese. It has long been recognized as a major defect in Gouda and Cheddar cheese but also occurs in diverse other sorts of cheese. Although the frequency of other cheese defects has been decreased greatly in the past decades by better hygienic and technological control of the cheesemaking process, the defect 'bitter' is still an actual problem. The occurrence of bitter off-flavours has been a main hindrance to the introduction of a number of other procedures in cheesemaking such as the use of high-pasteurized cheesemilk, the introduction of alternative rennets and the use of fast-acidifying starter strains, for instance.

The bitter defect in cheese is attributable to the accumulation of bitter-tasting peptides from casein components during ripening (4,5,6). Since a high average hydrophobicity appears to be a common property of bitter peptides (49,50,51), the strongly hydrophobic caseins (7) are extremely susceptible to liberation of bitter peptides on hydrolysis. Use of casein hydrolysates in other foods is hindered by these bitter flavours.

Thus the occurrence of bitter defects in cheese is closely interwoven with the protein breakdown during ripening.

During the last decade, many studies on the origin of this problem have been reported and reviewed (52,53). By far, the majority of the research was on Gouda and Cheddar cheese. Rennet and certain starter bacteria were shown to determine accumulation of these bitter peptides, as well as general proteolysis. However the exact roles of these enzyme systems in the formation and degradation of bitter peptides in cheese and their interaction, if any, are still hypothetical. Stadhouders and coworkers (54) emphasized the important role of rennet in the production of bitter compounds in Gouda cheese. However Lowrie et al. (12,55) considered the starter to be responsible for bitter formation in Cheddar cheese.

Until now most of the researchers have used normal 'open-vat' cheeses and have examined manufacturing conditions affecting bitterness in cheese (54, 56, 57). Other investigators succeeded in isolation and chemical identification of bitter peptides from the cheese itself (6,58), while many others employ model systems of casein to

study the bitter peptides formed by different proteolytic enzymes (33,59,60). Such experiments may be helpful for a better understanding of the complex problem of bitter flavour in cheese. Another field of research is the study of starter bacteria outside the cheese – often in milk cultures – to characterize properties associated with the production or degradation of bitter peptides (40,61,62,63).

Aseptic cheesemaking techniques to study bitterness have been employed only by Lowrie et al (46) for Cheddar cheese.

4. Purpose of the research and approach

Both rennet and starter bacteria are recognized to play the major roles in protein breakdown during ripening of Gouda cheese and in development of cheese flavour and bitter defects. Native milk protease was earlier thought to be of minor importance but recently received considerable attention as a possible agent in cheese proteolysis (15,64). Although there are several theories, partly controversial, about the role of these enzyme systems in proteolysis and bitter development, their exact contributions and interactions are not well understood.

The purpose of our investigations was to study in cheese the separate and combined action of rennet and starter bacteria especially in proteolysis and bitter defect. Only after assessing their separate contributions can a reliable estimate be made of possible interactive forces between the enzyme systems. Since the problems of cheese proteolysis and bitterness run parallel and since the defect 'bitter' is essentially a proteolytic problem, it is feasible to investigate both themes together.

With the technique for the aseptic manufacture of Gouda cheese combined with the aseptic milking of selected cows, both developed at our laboratory by Kleter (43,65), one can restrict the proteolytic activity in cheese to rennet, starter bacteria and milk protease.

To study the separate action of starter bacteria in cheese, we had to develop a method of aseptic cheesemaking by which rennet was excluded from the ripening process. Nevertheless the cheese should be normal in composition and should offer starter bacteria and their enzymes a normal substrate during ripening.

The use of an artificially acidifying agent, like δ'-gluconic acid lactone, during manufacture allowed us to eliminate the activity of the starter bacteria in cheese. The use of an aseptic cheesemaking technique is prerequisite for the manufacture of an edible starter-free cheese. We had to face a number of problems concerning asepsis, composition and rennet retention before we could make a reliable quantitative estimate of rennet action in this way. Several authors employed a similar technique.

Finally, the use of the chemical acidification during 'rennet-free' cheesemaking would give a cheese in which only milk protease is active during ripening and would allow study of this enzyme system in cheese alone.

Table 1 lists different types of aseptically made cheese types used in our studies on the separate and combined action of rennet, starter bacteria and milk protease during ripening of Gouda cheese.

Both 'bitter' and 'non-bitter' starter bacteria were used for cheesemaking to compare the degradation of protein during the ripening of bitter and non-bitter cheeses.

Hardly any studies on cheese ripening have included an estimate of the amount of rennet in the investigated cheeses. A more quantitative approach to the role of rennet in cheese ripening is possible only if the amount of rennet acting in the cheeses is known exactly, especially since retention of rennet is extremely sensitive to variation in cheesemaking conditions (54). We always determined the amount of rennet present in our cheeses (66).

Table 1. Survey of the different aseptically made cheeses used during the investigations.

Type of cheese	Proteol. enzyme systems active on ripening
1. 'normal' aseptic	rennet starter bacteria milk protease
2. aseptic starter-free (ASF)	rennet milk protease
3. aseptic rennet-free (ARF)	starter bacteria milk protease
4. aseptic rennet- and starter-free (ARSF)	milk protease

As a control, the cheeses were analysed for components like moisture, fat, salt and pH and were checked for the absence of unwanted micro-organisms. The growth and survival of starter bacteria was monitored during cheesemaking and ripening too.

Proteolysis was monitored during the ripening in different ways, characterizing both the primary casein components and the smaller breakdown products. The amount of breakdown products was estimated in a soluble-nitrogen fraction and the molecular weight (MW) distribution of the peptides in this fraction was analysed by gel filtration. The amount and composition of free amino acids in cheese - the ultimate products of proteolysis – were monitored as well. The data on breakdown products and on primary attack of α_{s1} and β-casein (obtained by polyacrylamide gel electrophoresis) reflected well the 'extent' and 'depth' of proteolysis.

To monitor the development of bitterness and cheese flavour, the different cheeses were submitted to a taste panel.

Bitter peptides were extracted from the cheeses and analysed by means of gel filtration.

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Method for the manufacture of rennet-free cheese

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Received: 17 November 1975

Summary

A method is described for the aseptic manufacture of cheese, which excludes any effect of rennet on the ripening process. By reducing the calcium plus magnesium content of the milk below 0.100 % by means of ion exchange, normal renneting could proceed without clotting. After completing the enzymatic phase of rennin action, the enzyme was inactivated completely by pasteurization of the milk at 72 °C for 20 seconds. By cooling the milk to 4 - 5 °C calcium could be returned to the milk without clotting. Clotting was obtained by dielectrical warming to 30 °C.

The milk had to be adjusted to a calcium plus magnesium content of about 0.160 % and a pH of 6.30 - 6.34 to avoid on the one hand flocculation of the milk at 4 - 5 °C, and on the other hand to get a homogeneous, firm gel on warming. The gel obtained was used for the manufacture of rennet-free cheese. In this cheese the role of starter bacteria and milk protease on the ripening can be studied.

The final cheeses prepared by this method showed normal starter development and calcium content.

1 Introduction

During the manufacture and ripening of cheese several enzyme systems can play a role in the breakdown of proteins and the development of flavour. These enzymes originate from the milk itself, the rennet or the micro-organisms. The latter are added intentionally (starter) or get into the cheese in another way (lactobacilli, micrococci, etc.).

To investigate the role of rennet and starter bacteria in the 'bitter' defect of Gouda cheese we need methods of cheese manufacture that make it possible to study the action of the above-mentioned enzymes separately. In researches into other aspects of cheese ripening such methods can also be useful. Investigations on the activity of proteolytic enzymes in milk and casein substrates are important, but it is questionable whether the results of such model experiments are representative of the activity of these enzymes in the cheese itself.

By the use of an aseptic cheesemaking technique, developed by Kleter (1, 2),

we are able to restrict the proteolytic activity to milk protease, rennet, starter bacteria and – if required – added micro-organisms.

The use of artificial acidification makes it possible to eliminate the activity of starter bacteria. The study of this type of cheese gives us information about the role of rennet in cheese ripening and particularly in the development of the 'bitter' defect.

In this paper we describe the development of an aseptic cheesemaking technique in which the influence of rennet on the ripening process is excluded. In these cheeses we can examine the role of starter bacteria on cheese ripening and at the same time elucidate differences between 'bitter' and 'non-bitter' starter strains.

Application of artificial acidification in this new cheesemaking technique makes it possible to study the activity of milk protease on its own.

The starting point for the development of this technique was that the resulting cheese should be as similar as possible to a normal cheese in relation to its composition, because proteolytic activity can be dependent on the physical structure of the casein, pH, salt content, etc. (3, 4). Because of this it is not possible to use acid coagulation for this technique of cheesemaking.

2 Principles of the method

Clotting of milk is a result of the activity of the enzyme rennin (chymosin) contained in the rennet extract. It is a process in which three reactions are involved. The primary, enzymatic reaction is the hydrolysis of a specific bond in $\varkappa$-casein. As a result the $\varkappa$-casein loses its ability to stabilize the remaining paracaseinate complex. In the secondary phase aggregation of the destabilized paracasein occurs in the presence of calcium ions and a clot will form. For this reaction the presence of the enzyme is not necessary. The tertiary reaction, following clotting, is a slow, gradual hydrolysis of the casein components. This proteolysis can contribute greatly to the ripening of cheese and it is for this reason that we want to eliminate this phase in some of our experiments. Therefore we shall have to inactivate the rennin after the first, but before the third phase.

The enzymatic reaction can be separated from the coagulation in two ways:

1. The coagulation reaction has a higher temperature coefficient than does the primary reaction (5). Therefore the enzyme can act upon milk at a low temperature (0 - 10 °C) without coagulation. When the temperature is subsequently raised to 30 - 40 °C the milk clots rapidly.
2. The coagulation cannot proceed in the absence of calcium ions. When the enzyme has acted in 'low-calcium' milk the addition of calcium salt results in

immediate clotting. Some workers (6, 7) have tried to develop a continuous cheesemaking process on this principle.

Using insolubilized enzymes to clot milk could be a technique for the manufacture of rennet-free cheese. The enzymatic reaction must be carried out below 10 °C and the enzyme can be separated from the milk afterwards. To permit second-stage clotting the milk must then be warmed. Green & Crutchfield (8) prepared insoluble derivates of rennin and chymotrypsin with Agarose and amino-ethyl cellulose. The decrease of the specific activity and the solubilization of the rennin during the process, however, have until now been the main objections to application of these materials.

The principle of the method described in this paper is that the enzyme acts upon milk with a reduced calcium content. After inactivation of the rennin we add calcium to the milk at a temperature below 10 °C without coagulation. Warming the milk to 30 °C without agitation then results in clotting of the milk. The gel obtained can be made into rennet-free cheese.

Fig. 1 gives a summary of the different steps in the process. Fig. 2 shows the flow sheet of the process that can be carried out aseptically. The successive steps will be described in the next section.

3 Description of the method

3.1 Analyses and materials used

3.1.1 Calcium. In milk, whey, ultrafiltrate and cheese extract (see Section 3.1.2) we determined the quantity of calcium plus magnesium by a direct

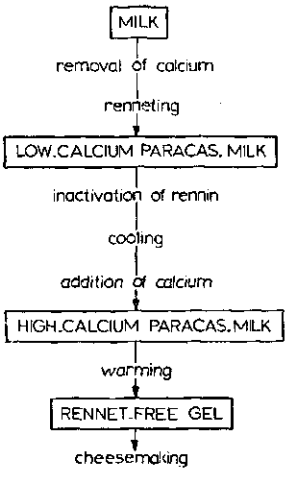

Fig. 1. Schematic reproduction of the subsequent steps of milk treatment to obtain a rennet-free gel.

Fig. 1. Schematisch overzicht van de opeenvolgende melkbehandelingen voor het verkrijgen van een stremselvrij gel.

Fig. 2. Flow sheet of the process for the aseptic manufacture of rennet-free cheese.

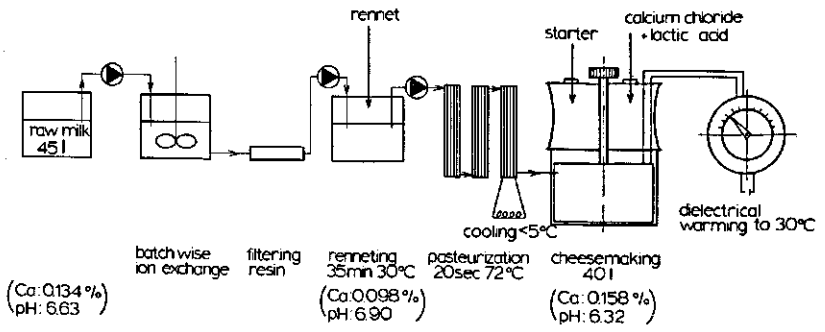

Fig. 2. Processchema bij de aseptische bereiding van stremselvrije kaas.

complexometric titration. The titration was carried out at pH 10 with Complexon III (0.01 *M*) in the presence of magnesium-Titriplex. Eriochrome black T was used as indicator. Any reference in our experiments to 'calcium' content implies the content of calcium plus magnesium determined as above. The magnesium content of normal milk is 0.013 % (9), i.e. about 10 % of the calcium content.

3.1.2 Phosphorus. To estimate the inorganic phosphate content of cheese approximately 2 g finely ground cheese were dissolved in 20 ml ammonia (5 %). A 15 % trichloroacetic acid (TCA) solution was added to make up the total volume to 100 ml. After one hour the mixture was filtered through a fine filter paper. In the clear filtrate the phosphorus content was estimated according to International Standard FIL-IDF 33 A, from clause 7.2.6. The filtrate was also used for the calcium determination of the cheese.

3.1.3 Non-protein nitrogen (NPN). To 25 ml of milk 25 ml of 4 % and 24 % TCA solution were added, respectively. After one hour the mixtures were filtered through a fine filter paper (Schleicher and Schüll, No 1575). Nitrogen was determined in the clear filtrate by a micro-Kjeldahl procedure.

3.1.4 Ultrafiltration. To obtain ultrafiltrates of various milk samples we used an Amicon concentrator, type CH 3. The membrane used was type HIDP 10 (Amicon).

3.1.5 pH. The pH was determined by means of a Radiometer PHM 601. We used a combined electrode, type GK 2301 C.

3.1.6 Rennet. Dutch commercial rennet was used with a guaranteed clotting power of 10 000 units.

3.1.7 Resin. For the ion exchange process we used Dowex 50 W-X 8 in the Na^+ form (Fluka A.G.) with a particle size of 0.3 - 0.8 mm. The resin is separated from the milk on Scrynel NY 265 HD precision netting (Merrem and La Porte).

3.2 Removal of calcium

To reduce the calcium content of the milk we used an ion exchange technique employing the strongly acid cation resin Dowex 50 W-X 8 in the Na^+ form. Wet sterilization (30 min, 121 °C) proved to have no harmful effect on the exchange properties of this resin.

In our process a batchwise method of exchange was most suitable. It has the additional advantage of a better guarantee of sterility than did a column technique. We always used 2.5 % (w/v) resin and a temperature of 30 - 33 °C. Dependent on the stirring capacity the process time needed was 10 - 30 min. To stop the reaction the resin was filtered from the milk on sterilizable netting.

We determined the amount of 'calcium' that should be removed to give a stable, unflocculated milk after normal renneting (35 min, 30 °C; see Section 3.3) and subsequent pasteurization (20 s, 72 °C, see Section 3.4).

Normal milk contains 0.134 % calcium plus magnesium (9). Reducing the 'calcium' content to 0.100 % or lower always proved to be sufficient, although some milks showed stability at 0.110 % 'calcium'. If a more severe heat

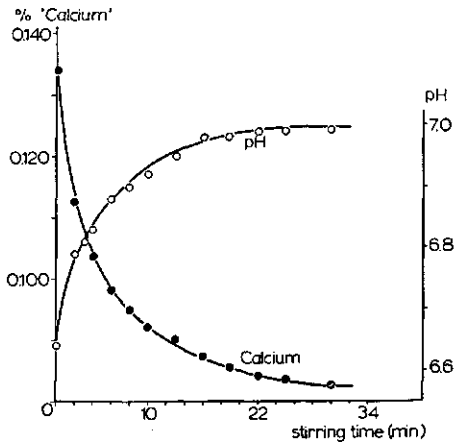

Fig. 3. The course of the 'calcium' content and pH of milk during batchwise ion exchange at 30 °C with 2.5 % Dowex 50 W-X8 in the Na^+ form.

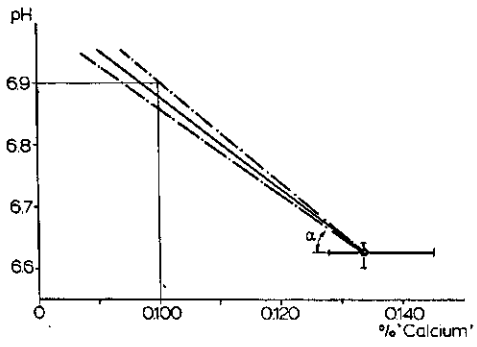

Fig. 4. Relation between pH and 'calcium' content of milk on treatment with Dowex ion exchange resin in the Na^+ form. The lines are representative for raw milk with an average pH and 'calcium' content. The range observed in pH and 'calcium' content for raw milk is indicated.
Drawn line: $tg\alpha = 0.756$ (average); broken lines (extremes): $tg\alpha = 0.69$ (lower line) and $tg\alpha = 0.81$ (upper line).

treatment or a more extended renneting procedure were applied, the 'calcium' would have to be decreased further.

Fig. 3 shows the course of the 'calcium' concentration and the pH during the exchange process for an arbitrary, but representative milk sample. Exchanging the 'calcium' for sodium causes an increase in the pH of the milk. This increase varies linearly with the amount of 'calcium' removed from the milk. During a period of one year we found the mean increase to be 0.76 pH units per 0.1 % exchanged 'calcium', with a variation of 0.69 - 0.81. In Fig. 4 these relations are outlined for raw milk with a 'calcium' content of 0.134 % and a pH of 6.63, the average values we found in our milk. It can be seen that exchanging up to pH 6.90 is necessary to adjust to a 0.100 % 'calcium' level mentioned above. The variation possible after exchanging is 0.0943 - 0.100 % 'calcium'.

For milk with a different pH or calcium content it is possible to calculate the pH to be reached from Fig. 4. We indicated in this graph the variation we found in our milk during the period of one year.

Some of the magnesium will also have been removed from the milk. This however will be less than 10 % of the amount of calcium exchanged.

3.3 Action of rennin

After the resin treatment of the milk the conversion of casein into paracasein by rennin proceeds without coagulation of the milk.

In normal cheesemaking rennin forms a clot in about 30 min at 30 °C, indicating that the primary phase has been largely achieved within this time. However, in our experiments the enzymatic reaction proceeds in calcium-poor milk with a somewhat increased pH. To control the primary reaction in this milk we followed the increase in NPN soluble in 2 % and 12 % TCA, respecti-

vely. Comparison of the reaction rates in normal and in resin-treated milk at 4 °C (to avoid sampling problems) indicated that there was hardly any difference. This agrees with the results of Verma & Gehrke (10). Moreover McGann showed (11) that the calcium ion concentration had no influence on the primary phase. Other workers (12, 13) demonstrated the same for colloidal calcium phosphate.

After the action of 0.025 % rennet for 35 min at 30 °C we observed an increase of 125 mg NPN and 50 mg NPN soluble in 2 % and 12 % TCA, respectively. These values indicate that in 35 min the primary reaction has been largely achieved (14).

In our cheesemaking experiments we let the rennin (0.025 %) act upon the milk for 35 min at 30 °C before starting the inactivation (see Section 3.4). This inactivation takes some time, the rennin being allowed to act for 35 - 50 min. The NPN increases in this milk proved to be 134 mg and 63 mg NPN soluble in 2 % and 12 % TCA per litre of milk, respectively, indicating that the primary phase had proceeded far enough to make normal clotting possible subsequently.

It seems unlikely that the rennin develops any other proteolytic activities in this time than splitting of the macropeptide from κ-casein (15, 16, 17, 18).

3.4 Inactivation of rennin

After the enzymatic phase, the rennin has to be inactivated completely. For this purpose we pasteurized the milk for 20 s at 72 °C, a process that in any case is necessary for the manufacture of cheese. According to Verhey (19) this pasteurization should be severe enough to destroy the rennin.

In our ion-exchanged milk, containing 2.5 % rennet which had been desalted by dialysis, we estimated the rennin inactivation by determining the clotting power of the milk.

A treatment of 20 s at 60 °C was found to inactivate 99 % of the rennin. After 20 s at 72 °C we could never demonstrate any rennin activity. 0.25 % of the original clotting power would have been demonstrable. In milk treated as above and made free of milk protease by a heat treatment, we could not demonstrate any proteolytic activity after pasteurizing for 20 s at 72 °C. We incubated the milk for 2 weeks at 30 °C. 1 % residual activity could have been measured.

Moreover we could not demonstrate any activity in cheese made according to the method described in this paper. We used the NIZO technique (20) for the determination of rennin activity in cheese. We should have been able to measure 1 % of the quantity normally present in Gouda cheese.

3.5 *Clotting*

3.5.1 Addition of calcium. In order to coagulate the treated milk after pasteurization we have to add calcium to the milk. For this purpose the milk (40 - 45 litres) was cooled to 4 - 5 °C in the cooling section of the pasteurizer and then pumped into the aseptic cheesemaking vat (2). A sterile, cooled solution of calcium chloride was then slowly added with agitation of the milk. This solution also contained some lactic acid for the pH adjustment of the milk. Clotting occurred on warming the milk without any agitation.

We observed that the addition of the originally exchanged amount of calcium did not result in the formation of a firm gel on warming. However the addition of too great an amount of calcium resulted in immediate flocculation of the milk at 4 - 5 °C giving on warming a spongy gel that is unusable for cheesemaking.

We performed small-scale stability experiments with cooled milk treated according to Sections 3.2, 3.3 and 3.4 in order to learn the conditions which yield a milk that does not coagulate at 4 - 5 °C but yet gives a good gel on warming. To 500 ml of this milk we slowly added 25 ml cold solution containing a precalculated quantity of calcium chloride and lactic acid. The flocculation time was examined at 4 - 5 °C and stable milks were warmed dielectrically to 30 °C in a 500-ml container to assess the gel properties.

On the basis of these experiments we came to the following conclusions about the stability of the cold milk against flocculation:

a. At a fixed excess of calcium the stability increases with decreasing pH of the milk after addition.

b. Increasing the added excess of calcium decreases the stability at a fixed pH after addition. Table 1 gives an example of the stability of a milk sample at different pH values and calcium contents.

Table 1. Example of the stability[1] of a treated milk sample at 4 - 5 °C in dependence of the pH and the 'calcium' content.

pH	% 'Calcium' after addition		
	0.155	0.165	0.175
6.52	–	–	–
6.43	+	–	–
6.39	+	–	–
6.30	+	+	–
6.21	+	+	+

[1] + = stability for at least 1½ h after addition; – = unstable within 5 min after addition.

Table 2. The cold stability of a treated milk sample at different pH levels, in dependence of the proceeding of the enzymatic reaction of rennin action.

Rennin action		Stability[1] of treated milk at 4 - 5 °C			
time (min)	increase NPN[3] (mg/litre milk)	pH[2] 6.51	pH 6.36	pH 6.29	pH 6.20
15	29	+	+	+	+
35	48	–	+	+	+
90	57	–	–	+	+
180	67	–	–	±	+

[1] + = stability for at least 1½ h after addition; – = unstable within 5 min after addition.
[2] pH after addition of calcium; 'calcium' is adjusted to 0.160 %.
[3] NPN soluble in 12 % TCA.

c. The addition of phosphoric acid instead of lactid acid decreases the stability.
d. The stability depends on the type of milk. Particularly in the period May – August the milk proved to be more stable against flocculation than during the autumn. It is possible that this effect is based on the same properties of milk as was the phenomenon observed by Arentzen (21). He found that in the autumn the coagulation phase on warming the milk, renneted at 0 - 5 °C, was much shorter than in the spring.
e. Rennin action being more extended, the stability of the milk decreased. Table 2 gives a summary of the stability against flocculation of July milk at a constant calcium content of 0.160 % and different pH values in dependence of the renneting time. Before the primary rennin action was completed these milks could coagulate into a gel on warming up.

Taking the view that the conversion of casein into paracasein has to be nearly completed, a 'calcium' content of 0.155 - 0.160 % and a pH of 6.30 - 6.34 proved to be the most suitable combination in our experiments to obtain a stable milk and a firm gel after warming.

At an increased pH there is more risk of flocculation at 4 - 5 °C, particularly in the autumn. Decreasing the excess of calcium results in too weak a gel.

3.5.2 Warming up. In a dielectrical heating system the milk was warmed to 30 °C without any agitation for the formation of a homogeneous gel. For this purpose we assembled two circular, stainless steel electrodes (Ø 410 mm, distance 250 mm) in the aseptic cheesemaking vat. These electrodes could be folded and dismantled to enable any manipulations during cheesemaking. The electrodes were insulated from the rest of the apparatus with teflon. The milk

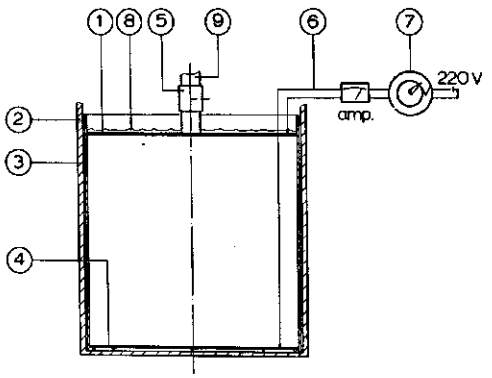

Fig. 5. The dielectrical heating assembly in the aseptic cheesemaking apparatus.
1. upper stainless steel electrode; 2. wall of the cheese vat; 3. silicone rubber insulation wall; 4. lower stainless steel electrode; 5. teflon insulation; 6. electricity wire; 7. adjustable electrical resistance; 8. level of the milk; 9. central shaft.

was insulated from the wall of the vat by a sterilizable silicon-rubber wall. The upper electrode was placed just under the surface of the milk, the lower one on the bottom of the vat, but insulated from it. Figure 5 shows the heating assembly of the apparatus.

At a voltage of 60 - 40 V a current of about 20 A went through the milk. In this way 40 litres of milk were warmed from 5 °C to 30 °C in about one hour.

3.6 Cheesemaking

When the gel was formed it was cut coarsely at first so that the electrodes could be folded up. Thereafter normal aseptic cheesemaking could proceed in the apparatus.

In making cheese from this rennet-free gel two points have to be taken into account:

a. the syneresis of the curd is less than with normal cheesemaking;

b. the cheese tends to become too acid, possibly owing to a decreased buffering capacity; the application of a more intensive curd washing overcomes this problem.

The starter was added to the cold milk just before warming. The clotting and curd treatment took somewhat more time than usual and we added therefore half the quantity of starter normally used. The growth and decay rate of the starters in these cheeses scarcely differed from normal aseptic cheeses. In particular, the maximum populations reached in the cheese by different starters were characteristically the same as in normal aseptic cheeses.

In some experiments we added rennet to the cold milk just before warming up. We could demonstrate that in these 'control cheeses' proteolysis and

Table 3. 'Calcium' and inorganic phosphorus content of rennet-free cheeses in comparison with cheeses made in a normal way. Average results of 10 cheeses from different cheesemaking experiments.

	'Calcium' % on dry matter	Range	Inorg. phosphorus % on dry matter	Range
Normal cheese	1.36	1.23-1.50	0.49	0.43-0.54
Rennet-free cheese	1.38	1.18-1.46	0.46	0.41-0.51

development of the 'bitter' defect were not altered in relation to normal aseptic cheeses.

3.7 Calcium balance

The rennet-free cheeses made according to the described method proved to have a 'calcium' and inorganic phosphate content that showed hardly any deviation from cheeses made in the normal way. Table 3 gives the average results. The values resemble those found for Gouda cheeses (22).

Although our cheese-milk contained excess of calcium (0.160 %), this was not recovered in the cheeses. The first whey in rennet-free cheesemaking contained 0.081 % 'calcium' in contrast to normal first whey containing 0.051 %. The rest of the excess of calcium disappeared during the washing procedure. Calcium determinations in the ultrafiltrates of milk in different stages of treatment confirmed that a great part of the excess of calcium was present in our cheesemilk in soluble form. Table 4 gives the average results of several experiments.

The soluble calcium decreased less than did the total calcium after ion exchange. Therefore some calcium must have been withdrawn from the micelles. Of the added quantity of calcium 65 % was recovered in the ultrafiltrate, explaining the high calcium content of the whey. In particular, the lower pH

Table 4. Distribution of 'calcium' in milk at different stages of treatment. Average results of several experiments.

	pH	% total 'calcium'	% soluble 'calcium'[1]
Raw milk	6.63	0.133	0.047
Resin-treated milk	6.90	0.095	0.038
Cheese milk	6.31	0.160	0.080

[1] Soluble 'calcium' is measured in the ultrafiltrates.

(6.30 - 6.35) would have favoured the increase of the proportion of soluble calcium. The somewhat increased sodium chloride content of our cheese-milk can also have dissolved some calcium from the micelles. However, according to Puri & Parkash (23) this would have been only about 0.001 % calcium.

Several workers (24, 25) have reported that 1/3 to 1/4 of the calcium added to milk will be recovered in the ionic state. If more than 5 mmol of calcium per litre were added, all the added calcium would appear in the ionic state. From this we may conclude that our cheese-milk contained a very high concentration of calcium ions.

Pearce (26) pointed out that the clotting time is independent of the calcium ion concentration when this exceeds 2.5 mmol. He also concluded that the calcium content in the micelles is more important for the stability than is the ionic content.

Pyne (27) observed that the colloidal calcium phosphate increases the sensivity to calcium ions of the calcium paracaseinate with which it is associated. As a result the temperature at which coagulation can occur is also decreased. In our milk at normal pH (>6.4) coagulation already occurred at 4 - 5 °C after addition of calcium. Evidently, both the re-introduction of calcium phosphate into the micelles and the high calcium ion concentration increased the sensitivity of this milk to coagulation. In addition, the calcium content of the micelles appeared to be more important for the cold stability than did the ionic concentration during our experiments.

At an increased calcium ion concentration (28) coagulation can already proceed before the primary conversion of casein is completed. This could explain the results mentioned in Section 3.5.1e.

4 Discussion

A technique is described for the manufacture of a rennet-free coagulum that can be used for the manufacture of all types of rennet-free cheese, both hard and soft varieties. In this way it is possible to study the role of starter bacteria on their own in cheese ripening processes.

Applying artificial acidification with lactic acid and δ'-gluconic acid lactone, it also proved to be possible to make cheeses in which the influence of both rennin and starter bacteria was eliminated. In this way the role of the enzyme milk protease on the ripening could be studied.

The method of manufacture of cheese described in this paper offers a good guarantee that the cheese will have a composition which is similar to that of a normal cheese. The absolute absence of the rennin enzyme in these cheeses and the aseptic conditions of the process are also guaranteed.

Acknowledgments

The author is indebted to Mr H. J. van der Stege and Mr J. L. Uitentuis for their valuable technical assistance.

This study was made possibly by the financial support of the Stichting J. Mesdagfonds, instituted by Kaascontrolestation Friesland, Leeuwarden.

Samenvatting

F. M. W. Visser, *Een methode voor de bereiding van stremsel-vrije kaas*

Bij het onderzoek van de kaasrijping en met name van het gebrek 'bitter' in kaas bestaat er behoefte aan kaasbereidingsmethoden waarmee de werking van de verschillende proteolytische enzymen in kaas gescheiden kan worden.

In dit artikel wordt een methode beschreven voor de aseptische bereiding van kaas waarin het stremsel geen invloed heeft op het rijpingsproces. Op deze wijze kan de rol die zuursels en melkprotease bij de kaasrijping spelen goed bestudeerd worden. Fig. 1 en 2 geven een overzicht van de methode.

Door met behulp van ionenwisseling het 'calcium'-gehalte (calcium + magnesium) van melk tot beneden 0,100 % te verlagen, kon het stremsel op melk inwerken zonder dat stremming optrad. Na de omzetting van caseïne in paracaseïne werd het stremsel volledig geïnactiveerd door de melk te pasteuriseren (20 s bij 72 °C). Na koeling van de melk tot 4 - 5 °C kon weer calcium worden toegevoegd, zodat bij opwarmen van de melk stremming optrad. Dit opwarmen tot 30 °C gebeurde met behulp van dielectrische verwarming, zodat de melk in volstrekte rust bleef.

De op te warmen melk bleek een 'calcium'-gehalte van 0,160 % en een pH van 6.30 - 6.34 te moeten hebben om enerzijds vlokking bij 4 - 5 °C te vermijden, anderzijds een stevig gel bij opwarmen te verkrijgen.

Het ontstane, stremselvrije gel kan tot verschillende kaassoorten verwerkt worden.

De uiteindelijke kazen vertoonden bij gebruik van een zuursel een normale ontwikkeling van deze flora. De gehaltes aan calcium en anorganisch fosfaat van de kazen waren normaal.

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Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 1. Description of cheese and aseptic cheesemaking techniques

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Received: 24 January 1977

Summary

To study aspects of cheese ripening, aseptically made cheeses were used in which the protein is decomposed during ripening through the separated and combined actions of rennet, starter bacteria and milk protease. This paper describes the aseptic cheesemaking techniques used and the average composition of normal, rennet-free, starter-free and rennet- and starter-free aseptic cheeses. The pH, moisture, salt and fat contents resembled those of Gouda-type cheese and, when starter was used, the calcium and inorganic phosphorus also showed normal levels.

The losses of calcium phosphate and the rennet retention in the curd proved to be very much dependent on the chemical acidification procedure used in the starter-free cheesemaking process. Extremely high rennet levels and low calcium and phosphorus contents were observed in starter-free cheeses acidified early in the process, according to the frequently used Mabbitt method (9). A method of chemical acidification is described that overcomes these disadvantages to a large extent.

The rennet contents of all cheeses are reported.

The outgrowth and survival of seven different starter strains in aseptic Gouda-type cheeses were followed. Characteristic differences were observed.

The next paper will present results on proteolysis, bitterness and cheese flavour.

1 Introduction

During the manufacture and ripening of cheese a gradual decomposition of casein takes place due to the combined action of various proteolytic enzymes. This proteolysis is directly involved in the development of the consistency and flavour of the ripening cheese, two very important quality aspects. The products of proteolytic breakdown are important constituents of mature

cheese flavours, both desirable and undesirable, and are precursors of flavour and aroma components.

It is very difficult to draw definite conclusions from normal, open vat, cheesemaking experiments when studying proteolysis and flavour development during the ripening. Aseptic cheesemaking techniques have enabled cheese to be made under controlled bacteriological conditions, obviating the uncertain and changing effects of adventitious bacteria. Mabbitt et al. (1) were the first to develop an aseptic vat technique in 1959. Until now, different approaches of aseptic cheesemaking have been reported for Cheddar (2, 3, 4) and soft cheese types (5). Kleter (6) described an apparatus for making Gouda-type cheese under strict aseptic conditions, and he also used aseptic milking of selected cows in order to obtain milk with an extremely low bacterial count (7). In normal aseptic cheeses only rennet, milk protease and proteolytic enzymes from starter bacteria are active during ripening.

In the different aspects of ripening (consistency, flavour, bitterness, etc.) it is desirable to unravel the influences of rennet and starter bacteria, which, until now, have been believed to be the determinative proteolytic enzyme systems in cheese (8).

The contribution of rennet (or rennet substitutes) may be assessed by the use of aseptic cheeses in which acidulation is simulated by δ'-gluconic acid lactone. This technique was introduced for Cheddar cheese by Mabbitt et al. (9) and ever since has been used by several workers in studies on cheese proteolysis (2, 3, 10, 11, 12). O'Keeffe et al. (13) proposed an adapted acidulation technique and questioned the validity of results obtained with Mabbitt's method. Lebars and co-workers (5, 14) produced an aseptic model curd on these principles, and recently Kleter (15) reported on an aseptic starter-free Gouda-type cheese.

Any application of these methods without using aseptic cheesemaking techniques (16, 17) is largely useless in our opinion.

Conclusions on the contribution of rennet in proteolysis are, however, hard to draw from starter-free cheeses without knowing the exact amounts of the rennet acting in the cheese.

In a previous publication (18) we described a method for the aseptic manufacture of rennet-free cheese. Using this method and the above mentioned cheesemaking techniques we were able to study the separate actions of rennet, starter bacteria and milk protease in cheese and to estimate their contributions to Gouda cheese ripening. In subsequent papers the results of these investigations on proteolysis generally, and on the 'bitter' defect and cheese flavour in particular, will be dealt with.

In this report details will be given of the applied cheesemaking techniques,

the composition of the experimental cheeses and some factors known to be important in these studies, such as rennet retention, calcium content and starter growth.

2 Materials and methods

2.1 Starter cultures

Six strains of *Streptococcus cremoris* were used. The strains E8, HP and Wg2 were obtained from the Netherlands Institute for Dairy Research (NIZO) at Ede. The strains AM1, AM2 and Z8 came from the New Zealand Dairy Research Institute.

One mixed strain starter with the index Bos was also used. This starter, known as BD type, was from the NIZO collection. Stock cultures of each strain were stored in skim milk at – 40°C. Deep-frozen cultures were sub-cultured twice before inoculating (1 %) 240 ml autoclaved (10 min, 121 °C) skim milk (10 %, OXOID). This milk was incubated at 20 °C for 20 hours before transferring to the cheese milk.

2.2. Rennet

One batch of Dutch commercial calf veal rennet was used with a clotting power of 10 000 Soxhlet units. Portions of rennet were sterilized by Seitz filtration.

2.3 Milk

Milk was obtained from the dairy herd of the University. For aseptic cheese-making experiments portions of 100 litres of milk were drawn aseptically (7) from 8 - 10 selected cows. For normal cheesemaking mixed milk from the complete herd was used. Before cheesemaking the milk was pasteurized for 20 s at 72 °C. Aseptically drawn milk was not standardized for fat content because of possible difficulties in maintaining asepsis.

2.4 Cheese manufacture

The apparatus and techniques for Gouda-type cheesemaking under aseptic conditions were essentially the same as described by Kleter (6, 15). If possible, cheeses were made in synchronous trials by coupling the stirring/cutting mechanism of the two cheese vats. If parallel experiments were not possible – e.g. when different amounts of rennet were used – cheeses that were to be compared were manufactured one after the other on the same day and from one portion of milk. In nearly all aseptic cheesemaking experiments the setting temperature was 28 °C and cooking was done at 28 - 30 °C. The aseptic

procedure limited the application of much higher cooking temperatures, the final cheeses otherwise becoming too dry.

Normal non-aseptic cheesemakings were performed in open 40-litre vats of the same geometry as the aseptic vats. Cheesemaking was as usual for Gouda cheese including cooking temperatures of 33 - 34 °C unless otherwise indicated.

Brining and ripening conditions were the same as described before (15). All cheeses were stored for the period of six months.

2.5 Compositional analyses of cheese

Compositional analyses of cheeses were carried out at 9 days after manufacture in a sector sample which had been very finely grated.

The pH was estimated in a compressed sample of cheese immediately after grating by means of a Radiometer with combined electrode.

The moisture, fat and salt contents were determined according to standard methods as described before (15).

Inorganic phosphate and calcium contents of the cheeses were determined in a 15 % trichloroacetic acid filtrate of the cheese as described before (18).

2.6 Bacteriological analyses

Bacteriological examination of raw and pasteurized milk was carried out as described by Kleter & de Vries (7). For bacteriological enumerations in cheese a borer sample was taken (6 - 11 g), ensuring complete asepsis. Samples were diluted 20 times with sterile 2 % trisodium citrate solution in an Atomix blender (MSE, London). Blending was carried out at full speed for 2 minutes to homogenize the sample and to reduce streptococcal chains to uniform size prior to plating (19). Blended samples were decimally diluted with quarter strength Ringer (OXOID) and pour plates were made on different media. The total viable count was made on Plate Count Agar (DIFCO) plus 0.1 % skim milk powder and Tomato Dextrose Agar (20). In our aseptic cheeses these counts represented the total starter counts. Cheeses were checked for coliforms and lactobacilli on Violet Red Bile Agar (DIFCO) and Rogosa SL Agar (DIFCO), respectively. Non-sugar requiring bacteria were counted on Nutrient Agar (DIFCO).

2.7 Estimation of the rennet content of cheese

Cheeses of between two and six months of age were analysed for rennet content. We used the NIZO method (21), adapted in some details to our circumstances. The Netherlands standard rennet was used as a reference (obtained from Rijkszuivelstation at Leiden).

2.8 Redox potential

In some cheeses the redox potential was measured at 1 - 2 months of age by means of a Radiometer and a platinum electrode embedded for 2 days in the cheese.

3 Results and discussion

3.1 Bacteriological examinations

Over a period of 3½ years about 6500 litres of milk for cheesemaking experiments were drawn aseptically in 74 milkings. The bacteriological quality of the raw milk portions is presented in Table 1. Coliform bacteria and lactobacilli were always absent from 2 ml of raw milk.

In pasteurized (20 min, 72 °C) milk samples, taken from the cheesemaking vats, no bacteria could be detected in 1 ml on PCA. In aseptically made cheeses to which starter bacteria had been added no contaminant bacteria could be detected in 0.1 g of cheese. We could conform to the same degree of sterility for two series of starter-free cheeses. In Section 3.2.3 we will report on problems with thermoduric bacteria in these cheeses.

3.2 Manufacture and composition of cheeses

3.2.1 Normal aseptic cheeses. Cheeses containing both rennet, milk protease and starter bacterial enzymes as the only proteolytic systems we shall call 'normal' aseptic cheeses. The manufacture was the same as described before (15) and conformed essentially to Gouda cheesemaking. 10 ml of rennet and 240 ml of starter culture were added to 40 litres of sterile milk. Renneting time was about 30 min and total processing up to drainage of the second whey took 100 - 115 min.

Cheesemaking experiments with starter cultures added were done with

Table 1. Distribution of bacteriological counts in aseptically drawn milk portions used for cheesemaking over the period 1973-1976.

Total count/ml	Frequency
1- 10	15
11- 50	33
51-100	12
100-200	10
200-800	4

differing amounts of rennet, varying from 30 ml down to 3.5 ml per 40 litres of cheesemilk. Process temperatures were not altered when high or low rennet levels were used, nor were calcium chloride or other chemicals added to the milk. In these experiments the extent of dry stirring was adapted to obtain cheeses with normal composition.

3.2.2 Aseptic rennet-free (ARF) cheeses. The method for the aseptic manufacture of rennet-free cheese has been described before (18). In this method the rennet is inactivated after completing its primary enzymatic action in low-calcium milk but before the coagulation stage. After addition of calcium chloride, starter culture and warming a rennet-free coagulum is obtained which is used for cheesemaking.

The stirring was adapted to the slower syneresis of the rennet-free curd, the total processing time being about 35 % longer than for normal cheesemaking. The amount of added water was raised at cooking to obtain a normal pH in the final cheeses.

21 rennet-free cheeses were made with 7 different starter strains. In a number of experiments a fresh portion of rennet was added to the milk just before warming up. In these 'control cheeses' the influence of ARF milk treatment on the ripening could be checked.

3.2.3 Aseptic starter-free (ASF) cheeses. During the manufacture of starter-free cheese acidification was simulated by the addition of δ'-gluconic acid lactone (GAL) and lactic acid.

We normally used an ASF cheesemaking technique in which the GAL was added to the curd as late as possible in the Gouda process, i.e. after complete drainage of the last whey. About 600 g of sterile GAL powder were added per vat and mixed very thoroughly with the curd. Up to the addition of the

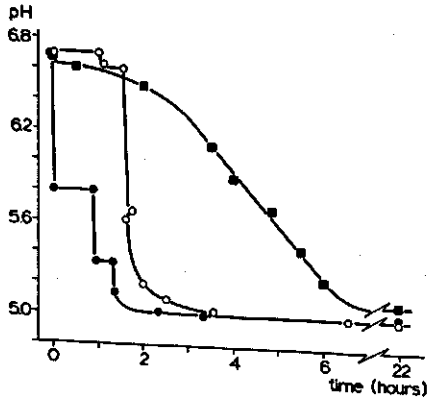

Fig. 1. Decrease in pH during ASF cheesemaking experiments as compared with normal aseptic cheesemaking. ■ normal aseptic cheesemaking (starter acidification); ○ ASF-gal cheesemaking; ● ASF-lac cheesemaking.

GAL normal cheesemaking procedures were used. Sometimes 5 - 10 ml of lactic acid (85 %) were added during cooking. Cheeses made in this way will be indicated as ASF-gal cheeses.

In a number of experiments another method of chemical acidification was applied. Here the pH of the cheese milk was adjusted to 5.8 with lactic acid. At stirring, about 60 ml lactic acid were added and finally 150 g of GAL were added to the curd. These cheeses will be indicated as ASF-lac cheeses; this process is very close to the technique for ASF-Cheddar cheesemaking employed by several other workers (2, 9, 12).

Fig. 1 shows the development of pH during the manufacture of both types of ASF cheese as compared to normal cheesemaking. In ASF cheesemaking experiments the amounts of added rennet were varied from 2 ml to 20 ml per 40 litres of milk.

In a number of ASF cheeses we detected spore-forming bacteria grown up to counts of 10^5 - 10^6 per gram of cheese after two weeks of ripening. Identification of isolated strains showed that most were of the species *B. subtilis* and *B. licheniformis*. Further studies made clear that these thermoduric bacteria were often present in the aseptically drawn, raw milk in counts of 1 per 5 - 20 ml. We could prove that these bacteria originated from the teat skin of the cow in spite of intensive disinfection with iodophor solution and alcohol. They arrived in the milk because of rinsing effects in the teat cups during machine milking. More intensive cleaning procedures or the application of plastic sprays on the teats were not able to overcome the problem completely. In portions of milk drawn with cannulas we could not detect these spore-forming bacteria, but scaling-up the cannula milking for 50 - 100 litres of milk proved to be difficult.

In the period 1974-1975 we succeeded in making 9 ASF cheeses which were completely free from bacteria during the ripening period of 6 months.

In the 1976 season we heat-shocked the raw milk for 20 s at 63 °C and added 0.1 % sterile L-alanine after cooling to 37 °C, according to Martin & Blackwood (22), in order to force germination of the *Bacillus* spores. After 15 min the milk was pasteurized as usual and made into ASF cheese. We succeeded in making a series of 10 sterile ASF cheeses in this way.

Needless to say, only sterile ASF cheeses were accepted for the reported cheese ripening experiments.

Obviously the few thermoduric bacteria present in the pasteurized milk were unable to grow in the presence of starter bacteria, possibly due to the inhibitory properties of the lactic acid bacteria (23, 24).

Until now all investigators have reported the addition of considerable amounts of antibiotics to repress the outgrowth of thermoduric bacteria in

Table 2. Average composition of different aseptic cheese types at 9 days after manufacture.[1]

Cheese type		Number of cheeses	pH	% moisture	% fat in dry matter	% salt in moisture
Normal aseptic	normal rennet	30	5.07 (0.05)	43.7 (1.85)	48.0 (2.20)	4.4 (0.60)
	high rennet	10	5.01 (0.04)	45.5 (1.50)	48.6 (1.42)	4.4 (0.61)
	low rennet	11	5.07 (0.05)	43.4 (1.50)	48.8 (2.09)	4.1 (0.88)
ARF		21	5.08 (0.06)	46.6 (2.37)	48.0 (3.34)	4.5 (0.85)
ASF	gal	14	5.12 (0.10)	41.6 (1.42)	46.0 (0.68)	4.5 (0.55)
	lac	5	5.23 (0.06)	43.5 (1.91)	—	5.2 (0.60)
ARSF		3	5.11 (0.08)	45.5 (0.56)	—	4.9 (0.90)

[1] Values between brackets: standard deviation for cheeses.

aseptic starter-free cheeses (5, 4, 12, 10). Apart from the fact that the addition is not desirable for cheese tasting, the antibiotics used were not able to repress outgrowth absolutely in our ASF cheeses, neither did the addition of sodium nitrite to the milk.

We estimated the redox potential of our normal aseptic cheeses as –150 mV, while ASF cheeses were about 30 mV. The reduction of the redox potential by starter bacteria did not appear to be the growth-limiting factor for thermoduric bacteria.

3.2.4 Aseptic rennet- and starter-free (ARSF) cheeses. To obtain cheeses in which only milk protease would be active during the ripening we applied a combination of the ASF-gal and ARF cheesemaking procedures. Normal ARF cheesemaking was followed – without the addition of starter – until GAL was added to the curd.

3.2.5 Composition of the cheeses. In Table 2 the average composition for the different aseptic cheese types is presented.

It can be seen that ARF cheeses have at nine days a somewhat higher moisture content than the other cheeses. This was due to the retarded syneresis of the curd under ARF cheesemaking conditions. On the other hand the addition of GAL to the curd decreased the moisture content of ASF-gal cheeses. This could be corrected during the ripening by earlier waxing of the cheeses. During the ripening all cheeses gradually lost moisture down to an average content of 33 % at six months of age. ARF cheeses then averaged 34.3 % moisture. In six months the pH of the cheeses increased by about 0.13 units.

Table 3. Content of calcium plus magnesium and of inorganic phosphorus for different aseptic cheese types.

Cheese	Ca + Mg in dry matter		Phosphate in dry matter	
	average	range	average	range
Normal aseptic	1.42	1.23 - 1.58	0.54	0.43 - 0.65
ARF	1.49	1.31 - 1.60	0.53	0.43 - 0.67
ASF-gal	1.24	1.08 - 1.38	0.50	0.41 - 0.57
ASF-lac	0.75	0.55 - 0.85	0.32	0.29 - 0.37
ARSF	1.20	1.13 - 1.27	0.42	0.41 - 0.44

Table 3 gives the 'calcium' and inorganic phosphate contents of the cheese types investigated.

As can be seen, a considerable amount of calcium and phosphorus is lost during the ASF-lac cheesemaking process. The decrease of the pH of the cheese milk to 5.8, early in the process, will have been the particular culprit. Recently O'Keeffe et al. (13) assumed that application of the Mabbitt method (9) for starter-free Cheddar cheesemaking would cause a large loss of calcium from the cheese due to the excessive early pH decrease in the milk, and they proposed an adapted acidification technique.

In our ASF-gal cheeses and ARSF cheeses only a slightly decreased calcium content was observed. Inorganic phosphate contents were even within the range of normal aseptic cheeses. This must be attributed to the addition of GAL at a time when by far the most of the whey had already been drained. The calcium and inorganic phosphate contents of normal aseptic and ARF cheeses resembled Gouda cheese levels.

3.3 Rennet content of the cheeses

Our normal aseptic cheeses made with 10 ml rennet per 40 litres of milk contained as an average 280 μl rennet per kg of cheese at 9 days. This value corresponds with the rennet content normally found in Gouda cheese when cooking temperatures during the manufacture are not too high (21).

We determined the rennet content in 25 normal aseptic cheeses, the standard deviation between cheesemakings being 30 μl.

Fig. 2 shows that the rennet retention is linearly related to the amount of rennet added to the milk. This relation was also observed for the ASF cheese types. However, a somewhat increased retention of rennet was observed in ASF-gal cheeses as compared to normal cheeses. Extremely high rennet retention was observed in ASF-lac cheeses.

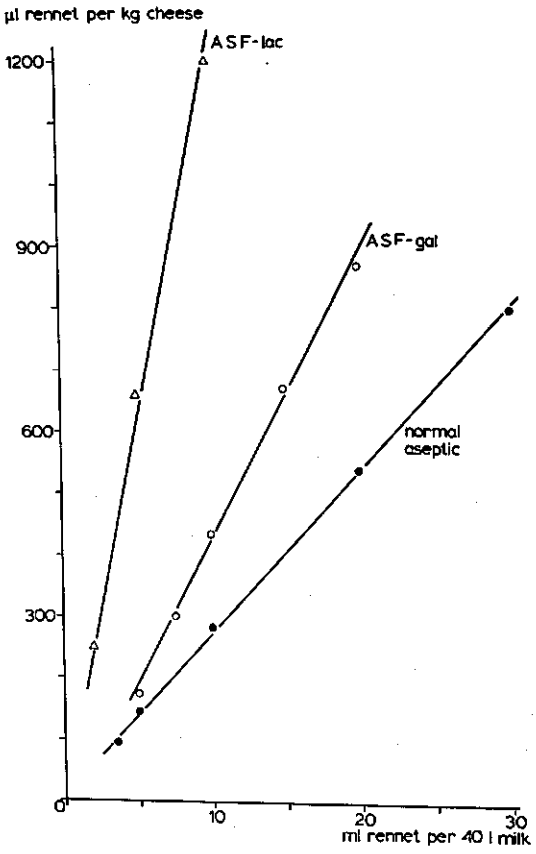

Fig. 2. Relation between the amount of rennet used for cheese-making and the retention of the enzyme in normal aseptic cheeses, ASF-gal cheeses and ASF-lac cheeses.

Stadhouders (21) has already pointed out that the rennet retention in the curd is greatly influenced by the pH of the milk and the acidification rate of the starter bacteria. Retention greatly increased with decreasing milk pH and with greater starter activity.

The increased rennet retentions in the ASF cheeses must certainly be attributed to the same causes.

In our opinion these increased rennet levels in ASF cheeses have certain consequences for the results obtained by other workers with ASF cheeses, especially for those made according to the Mabbitt method (12). The relatively much increased proteolysis in these cheeses – observed by O'Keeffe et al. (13) – can be attributed to a great extent to the high levels of rennet enclosed in these cheeses. Extensive results on the proteolysis in these cheeses will be presented in subsequent papers. The interpretation of results on proteolysis

in ASF cheeses seems very difficult to us without knowing the exact amount of rennet in the cheese.

As was indicated before (18) no rennet could be detected in cheeses manufactured according to the ARF and ARSF methods.

3.4 Starter growth and survival

Fig. 3 gives the growth and survival characteristics for the 7 starter strains as determined in normal aseptic cheeses. The strains AM1 and AM2 survived relatively poorly in our cheeses. Martley & Lawrence (26) observed the same for Cheddar cheese.

The outgrowth of several starter strains in our cheeses is shown in Table 4. For AM1 and AM2 cheeses colony counts were also made after 6 hours, but the counts proved not to be much higher than presented in Table 4. As can be seen, strains Wg2 and Z8 reached relatively high numbers in the curd. The outgrowth of strain AM1 was low as compared to other starters.

Outgrowth and survival characteristics of starter bacteria during cheese ripening have been implicated in the development of Cheddar cheese flavour

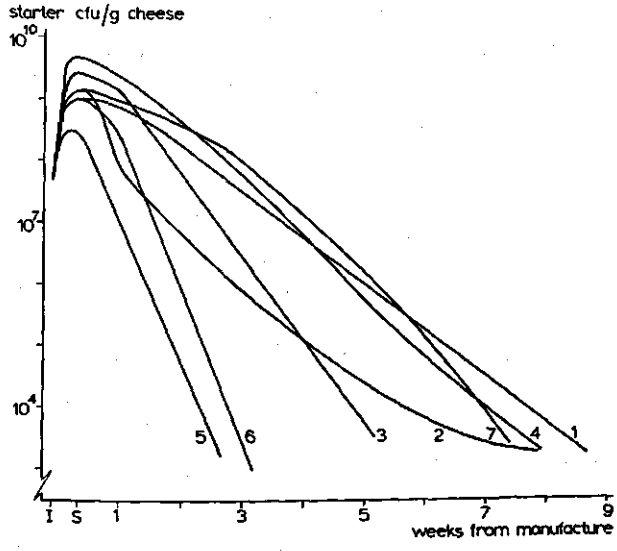

Fig. 3. Starter populations during the manufacture and ripening of aseptic Gouda-type cheeses. Plate counts of samples I (milk after inoculation with starter) and S (cheese immediately before brining) are expressed as per gram of finished cheese.
1 = E8; 2 = HP; 3 = Z8; 4 = Wg2; 5 = AM1; 6 = AM2; 7 = Bos. cfu = colony-forming units.

Table 4. Starter colony counts in normal aseptic and ARF cheeses 24 hours after manufacture.

Starter strain		cfu*/gram cheese × 10^7					
		normal aseptic cheeses			ARF cheeses		
		average		range	average		range
1	E8	(7)**	97	51 - 145	(3)**	91	50 - 127
2	HP	(7)	146	60 - 310	(6)	234	125 - 420
3	Z8	(4)	264	230 - 295	(1)	303	—
4	Wg2	(6)	471	320 - 670	(3)	386	300 - 480
5	AM1	(3)	30	16 - 36	(2)	39	23 - 55
6	AM2	(2)	100	82 - 116	(2)	81	37 - 125
7	Bos	(2)	143	134 - 153	(2)	126	94 - 158

* cfu = colony-forming units.
** Number of cheeses investigated.

as well as of bitter flavour (25, 26, 2, 10). In the next paper the results on cheese flavour and bitterness for our cheeses will be discussed in relation to the starter characteristics presented.

Table 4 also shows that no basic differences were observed in the outgrowth of our starter strains in normal aseptic and ARF cheeses. The rates at which numbers declined during ripening were also comparable, showing that ARF cheeses are reliable for the investigation of the role of starter bacteria in cheese ripening.

Acknowledgments

The aseptic milking was carried out in co-operation with the Milk Hygiene Research Centre (MOC), Wageningen and the Animal Husbandry Departmentof the Agricultural University.

Thanks are due to Mr J. L. Uitentuis for assistance in the manufacture of the cheeses and to Mr W. O. van Middendorp for work on the thermoduric bacteria.

Stichting J. Mesdagfonds, instituted by Kaascontrolestation Friesland. Leeuwarden supported this study financially.

Samenvatting

F. M. W. Visser, *De bijdrage van de enzymen van stremsel en zuursel en van melkprotease aan de eiwitafbraak en smaakontwikkeling in Goudse kaas. 1. Beschrijving van de kazen en de aseptische kaasbereidingstechnieken*

Voor het rijpingsonderzoek werd gebruik gemaakt van aseptisch bereide kazen waarin de eiwitafbraak wordt veroorzaakt door de gescheiden en gecombineerde werking van resp. stremsel, zuursel en melkprotease. In dit artikel worden de daarvoor gebruikte aseptische kaasbereidingstechnieken beschreven evenals de samenstelling van de verschillende kazen. De pH, vocht-, zout- en vetgehalten bleken overeen te komen met die van een normale Goudse kaas. Ook het calcium- en anorganisch fosfaatgehalte van de kazen bleek normaal, met uitzondering van de zuurselvrije kazen, waarbij deze gehalten sterk afhankelijk bleken te zijn van de gebruikte chemische verzuringsmethode. Door middel van stremselbepalingen in kaas werd aangetoond dat deze afhankelijkheid ook bestond voor de ingesloten hoeveelheid stremsel. Bij de veelgebruikte methode volgens Mabbitt (9) werd veel meer stremsel ingesloten dan bij een normale kaasbereiding. Bovendien werd in op deze wijze bereide kaas ca. 40 % minder calciumfosfaat aangetroffen.

De door ons beschreven verzuringsmethode voor zuurselvrije kaas komt aan bovengenoemde bezwaren grotendeels tegemoet.

De groei en afsterving van zeven verschillende zuurselstammen in aseptische kaas werd gevolgd. Er bleken karakteristieke verschillen tussen de zuursels te bestaan.

In volgende artikelen wordt ingegaan op de eiwitafbraak, kaassmaak en bitterheid van de hierbovengenoemde kazen.

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Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 2. Development of bitterness and cheese flavour

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Received: 14 April 1977

Summary

The development of bitterness and cheese flavour was studied in aseptically made, Gouda-type cheeses in which the actions of rennet, starter bacteria and milk protease were separated (23). At the same time different aspects of bitterness development were studied in normal aseptic cheeses, such as the influence of varying amounts of rennet both with 'bitter' and 'non-bitter' starter cultures.

It was observed that

– rennet, when present in the concentration found in normal Gouda cheese, is capable of producing bitter starter-free cheeses after some months of ripening. The application of larger amounts of rennet makes the bitterness appear earlier and be more intensive;
– 'bitter' starters, as contrasted to 'non-bitter' starters, are able to produce a distinctly bitter rennet-free cheese;
– milk protease on its own does not contribute significantly to bitterness in our Gouda-type cheeses;
– mutual stimulation in the formation of bitter peptides by rennet and starter bacteria is not necessarily an important factor;
– non-bitter' starters have the power to degrade considerable quantities of bitter peptides to non-bitter products in our cheese. 'Bitter' starters seem to possess this property to a far less extent;
– the growth of a starter to high cell densities in the cheese does not appear to be the determinative factor for the occurrence of bitter flavour in Gouda cheese;
– neither rennet nor milk protease contribute noticeably to the development of cheese flavour;
– 'non-bitter' starters develop important levels of cheese flavour in rennet-containing cheeses; distinct cheese flavour was formed without the help of rennet. 'Bitter' strains have far less potency to do so.

A mechanism for the development of the bitter defect in Gouda cheese is discussed.

1 Introduction

Nowadays bitterness is one of the most frequently occurring defective flavours in cheese. It has long been recognized as a major defect in Gouda and Ched-

dar cheese, but it also occurs in other cheeses. A recent survey of French cheese manufacturers showed that soft-cheese types such as Camembert and blue cheeses such as Roquefort are also sensitive to the development of bitterness (1). Cheeses made from goat or ewe milk seem to be less susceptible to bitter defects (2). Reports exist on bitter defects in Cottage cheese (3), Emmentaler (4), Japanese yeast-ripened cheese (5), Cacciotta (6), White pickled cheese (7), Gruzinskii (8), etc., indicating the universality of this problem. There are many studies reporting bitterness in cheese made with various rennet substitutes (9, 10, 11).

By far most of the research on the bitter defect has been carried out on Cheddar and Gouda cheese. It has been found that the defect is attributable to the formation and accumulation of bitter tasting peptides liberated from casein components (12, 13, 14) probably resulting from the proteolytic action of rennet, starter bacteria or other micro-organisms. Although, through this knowledge, progress has been made in minimalizing the risk of bitterness in Cheddar and Gouda cheese, the exact roles of the different proteolytic enzyme systems in the development of this defect are still hypothetical and the subject of some controversial theories.

Czulak (15) suggested that rennet is responsible for the production of bitter peptides in Cheddar cheese. These peptides would accumulate in the cheese if starter bacteria lacked the peptidases to degrade them. At that time a similar theory was developed for Gouda cheese bitterness (16).

An alternative theory was introduced for Cheddar cheese by Lowrie & Lawrence (17). In their opinion rennet merely produces non-bitter peptides of high molecular weight that are converted to bitter peptides by starter proteinases. All starter strains would have the potential to produce bitter cheese, the occurrence of bitterness in the final cheese depending upon the ability of starter bacteria to multiply to high cell densities under the manufacturing conditions used. Experiments with Camembert (18) seem to support this theory. Factors affecting the bitter defect in Cheddar cheese were also shown to be important in Cheddar flavour development (19).

Stadhouders & Hup (20) published a tentative scheme for the formation and breakdown of bitter peptides in Gouda cheese, paying special attention to the amount of rennet retained in the cheese. They suggest that the synergistic effect of bitter peptides produced by rennet and those produced by the specific proteinase system of bitter starters would be important for the development of bitter flavour in Gouda cheese. In their opinion a non-bitter starter has the ability to degrade bitter peptides.

Apparently some essential differences exist in the development of bitter flavour in Gouda and Cheddar cheese with regard to the roles played by

rennet and starter bacteria. As was remarked recently by Lawrence (21), controlled Gouda cheesemaking trials with aseptic vats would need to be carried out to resolve this problem of bitter flavour development satisfactorily.

Until now, only Lowrie et al. (19) have applied aseptic cheesemaking techniques to study bitterness in Cheddar cheese.

Because it is very difficult to unravel the various contributions of starter bacteria and rennet to cheese ripening processes by means of normal cheesemaking experiments, we employed aseptically made cheeses in which the action of these enzyme systems could be separated. The special cheesemaking techniques used have been described in previous papers (22, 23).

The present study reports on the development of bitterness and cheese flavour in aseptic Gouda-type cheeses, ripened through the separated and combined action of rennet, starter bacteria and milk protease.

2 Materials and methods

2.1 Cheeses

Normal aseptic, aseptic starter-free (ASF), aseptic rennet-free (ARF), aseptic rennet- and starter-free (ARSF) and open-vat cheeses were manufactured as described previously (22, 23). The average composition and rennet contents of the cheeses were presented earlier (23). All the cheeses approached Gouda-type composition. Ripening took place at 13-14 °C for the period of 6 months.

2.2 Starter cultures

Streptococcus cremoris strains with index E8, Wg2, HP, AM1, AM2 and Z8 were used (23). Mixed-strain starter Bos, used in Dutch cheese factories, was also employed. The ability to give bitter flavour or not was the criterion for selection of these strains. In a number of aseptic cheesemakings we paired a 'bitter' and a 'non-bitter' strain just before addition to the cheese vat. The total volume of the starter mixture was always 240 ml per vat (as normal).

An E8 starter concentrate was prepared aseptically. After the addition of sodium citrate the cells were centrifuged from the bulk starter culture. After 3 hours (20 °C) culturing of the suspended sediment in milk, the concentrate was introduced into the cheese vat, yielding 10 times more starter bacteria than normal.

Lactobacilli were isolated from non-bitter, open-vat Wg2 cheese. A mixture of 3 strains – not further identified – was added to the cheese milk in an experiment. After this addition the cheese milk contained 300 lactobacilli per ml. Growth and survival curves of all the starter strains in aseptic cheeses were given earlier (23).

2.3 Rennet

Dutch commercial calf-veal rennet with a guaranteed clotting power of 10 000 units was used for the manufacture of our cheeses. In a limited number of ASF cheesemakings rennet, especially made from stomachs of new-born calves only, was used. This preparation was supplied by the 'Coöperatieve Stremsel- en Kleurselfabriek', Leeuwarden.

2.4 Organoleptic evaluations

A laboratory panel of 7-10 selected graders evaluated the cheeses for bitterness and cheese flavour. Bitterness in all cheeses was judged at 1, 3 and 6 months of age. Cheese flavour was graded only in limited numbers of cheeses, mostly at 3 and 6 months. The intensity of bitterness was scored on a 6-point scale according to Emmons et al. (24):

1 = no detectable bitterness;
2 = barely detectable bitterness;
3 = distinct bitterness, but at low level;
4 = moderate bitterness;
5 = very strong bitterness;
6 = unpalatable bitterness.

For the grading of cheese flavour we used a similar scale with the following descriptions:

1 = no detectable cheese flavour;
2 = barely detectable cheese flavour;
3 = distinct cheese flavour, but at a low level;
4 = substantial cheese flavour;
5 = strong cheese flavour;
6 = very strong cheese flavour.

Panel reliability was checked periodically by random duplication of samples.

Panel members were tested for their sensitivity to detect bitterness in aqueous solutions of quinine sulphate.

To estimate interactions between cheese flavour and bitterness detection, bitter-cheese extracts or quinine sulphate were mixed with non-bitter cheeses of different age which had different levels of cheese flavour. Mixing was done by passing the cheeses through a grinder six times. Bitter-cheese extracts were prepared according to Harwalkar (25), elimination of astringent fractions included.

2.5 Statistics

Differences in bitter scores within pairs of parallel cheeses were tested for significance by using the Wilcoxon matched-pairs signed-rank test (26).

3 Results

3.1 Development of bitterness

3.1.1 Normal aseptic cheeses. 'Normal' aseptic cheeses will refer to aseptically made cheeses manufactured with the addition of both rennet and starter bacteria.

Table 1 shows the average results for bitterness observed in 28 aseptic cheeses made wit a normal amount of rennet and seven different starter cultures. Some of the cheeses were made in parallel experiments, and the rennet concentration in these cheeses was similar to that in normal Gouda cheese. Although some variation in the composition of the aseptic cheeses had to be allowed, the aseptic cheesemaking procedure proved to give very consistent results with regard to the development of bitterness. The starters can be divided into four cultures (E8, AM1, AM2, Bos) always producing non-bitter Gouda-type cheese, two strains (HP, Wg2) resulting in very bitter cheese and strain Z8 gradually developing bitterness during the ripening.

Since maximum starter populations reached in the cheese have been implicated directly in the development of bitterness in Cheddar cheese (17, 19, 27, 28) we have summarized these maxima as observed in our aseptic cheeses (23) in Table 1. 'Bitter' starter strains Wg2 and Z8 grew to the highest num-

Table 1. Bitterness of normal aseptic cheeses made with different starter strains and normal rennet amounts.

Starter strain	Number of experiments	Average bitter score at			cfu $\times 10^{9}$**
		1 month	3 months	6 months	
E8	5	1.3 (0.16)*	1.3 (0.18)	1.5 (0.17)	0.97
AM1	3	1.7 (0.23)	1.5 (0.28)	1.4 (0.42)	0.30
AM2	3	1.9 (0.06)	1.6 (0.22)	1.5 (0.22)	1.00
Bos	2	1.6 (0.21)	1.5 (0.14)	1.6 (0.14)	1.43
HP	6	4.4 (0.42)	4.6 (0.42)	4.6 (0.69)	1.46
Wg2	6	3.6 (0.41)	4.3 (0.39)	4.4 (0.66)	4.71
Z8	3	1.7 (0.23)	2.8 (0.33)	3.7 (0.20)	2.64

* Values between brackets: standard deviation of cheese productions.
** Colony counts in cheese at 24 hours after manufacture, expressed as colony forming units per gram of cheese (23).

Table 2. Bitterness in normal aseptic E8 cheese as influenced by the level of outgrowth of the starter in the cheese.

Cheese	Composition 9 days			cfu after 24 h $\times 10^9$	Bitter score at		
	pH	% moist.	% S/M*		1 month	3 months	6 months
E8	5.01	44.7	4.1	0.72	1.2	1.2	1.4
E8 conc.	5.01	43.9	4.4	2.5	2.2	2.0	1.9

* % salt in moisture.

bers in the cheese. E8, AM2, Bos and 'bitter' strain HP reached about equal numbers in the cheese. AM1 showed distinctly lower maximum populations than the other starters.

The maximum population reached by strain E8 was raised by adding an E8-concentrated culture to the cheese milk. From the same portion of milk a normal E8 cheese was made synchronously in the twin cheese-vat. Development of acidity was equalized in both vats with sodium hydroxide solution. As can be seen from Table 2 a threefold increase in maximum population was not sufficient to result in distinctly bitter cheese although some increase could be noticed in the bitter scores.

In a number of experiments we paired 'non-bitter' starter E8 with 'bitter' strain Wg2 or HP. Each cheesemaking experiment consisted of two synchronous cheesemakings from the same portion of milk. The results are presented in Table 3. The use of equal volumes of E8 and Wg2 resulted in non-bitter cheeses. However, on raising the amount of Wg2 in the mixture to 3/4, bitter cheese was obtained, the defect being less intensive as compared with

Table 3. Bitterness in normal aseptic cheeses made with combinations of starter strains.

Exp. No	Starter	Culture per vat (ml)	cfu/ml* t=0 $\times 10^6$	cfu/g** 24 h $\times 10^9$	Average bitter score at		
					1 month	3 months	6 months
1	Wg2	120	4.0	3.3	3.2	3.6	3.2
	E8/Wg2	120/120 (1/1)	12.0	2.3	1.7	1.1	1.2
2	E8/Wg2	120/120 (1/1)	8.5	1.3	1.6	1.2	1.4
	E8/Wg2	60/180 (1/3)	9.9	5.0	2.6	2.6	1.7
3	HP	240	6.0	1.4	3.6	4.5	4.1
	E8/HP	80/160 (1/2)	5.0	1.0	2.5	3.4	3.2

* Number of starter bacteria present in the cheese milk just after starter addition.
** Number of starter bacteria present in the cheese after 24 hours.

Table 4. Bitterness in normal aseptic Wg2 cheese as influenced by the addition of lactobacilli to the milk.

Cheese	Composition after 9 days			Lactobacilli per g of cheese at 2 months ($\times 10^6$)	Bitter score at		
	pH	% moist.	% S/M		1 month	3 months	6 months
Wg2	5.12	45.5	4.2	0	3.1	4.4	3.6
Wg2 + lactobacilli	5.14	45.1	4.2	13	3.1	4.4	3.7

normal Wg2 cheeses. Pairing E8 and HP also decreased the bitterness significantly in comparison with normal HP cheese but the 'debittering' did not seem to be as effective as in E8/Wg2 pairing.

In a parallel experiment aseptic Wg2 cheese was made. In one cheese-vat a mixture of lactobacilli – adventitious bacteria always growing in Dutch cheese types – was added to the cheese milk, and normal Wg2 cheese was manufactured synchronously in the twin vat. As can be concluded from Table 4, the development of bitterness was not influenced by the abundant growth of these lactobacilli.

To study the influence of rennet on the development of bitterness in normal aseptic cheeses varying amounts of rennet were used in parallel cheesemaking experiments both with 'bitter' and 'non-bitter' starter strains. Table 5 summarizes the results. Doubling the amount of rennet used to make E8 cheese proved to result in non-bitter cheeses. However, on triplicating the rennet level, problems with bitterness can occur. Especially in the younger cheeses it was difficult to obtain consistent results with this amount of rennet. Nevertheless in all cheeses a decrease in the initial bitterness seemed to occur during ripening. The starter AM1 clearly developed bitterness in combination with threefold rennet levels.

Decreasing the amount of rennet used for 'bitter'-starter cheeses in parallel experiments resulted in significantly less intensive bitterness ($P < 0.05$), but even with amounts down to 1/3 of the normal quantity bitterness was still clearly detectable in the cheeses. It is difficult to estimate the intensification of bitter flavour in HP cheeses with increasing rennet levels because of the relatively high bitter scores for normal-rennet HP cheeses. During the first months of ripening of Z8 cheeses distinctly higher bitter scores were observed with the double amount of rennet.

Table 5. Bitterness in normal aseptic cheeses as influenced by the amount of rennet.

Starter strain	Rennet content of cheese*	Number of experiments	Average bitter score at		
			1 month	3 months	6 months
E8	½ n	2	1.0 (0.00)**	1.0 (0.00)	1.0 (0.00)
	n	5	1.3 (0.16)	1.3 (0.18)	1.5 (0.17)
	2 n	3	1.6 (0.45)	2.2 (0.72)	1.5 (0.32)
	3 n	3	3.6 (1.10)	2.5 (0.98)	2.1 (0.35)
AM1	n	3	1.7 (0.23)	1.5 (0.28)	1.4 (0.42)
	3 n	1	4.1 —	4.4 —	3.4 —
HP	1/3 n	2	2.7 (0.17)	3.1 (0.75)	2.6 (0.00)
	½ n	2	3.6 (0.28)	4.0 (0.19)	3.2 (0.87)
	n	6	4.4 (0.42)	4.6 (0.42)	4.6 (0.69)
	2 n	1	4.0 —	4.9 —	4.9 —
Wg2	1/3 n	2	2.9 (0.46)	2.4 (0.46)	2.4 (0.28)
	n	6	3.6 (0.41)	4.3 (0.39)	4.4 (0.66)
Z8	½ n	1	1.4 —	2.5 —	2.5 —
	n	3	1.7 (0.23)	2.8 (0.33)	3.7 (0.20)
	2 n	2	2.7 (0.46)	4.2 (0.07)	3.6 (0.07)

* n = normal amount of rennet retained in the curd: 280 μl per kg cheese (for rennet retentions in cheeses see Ref. 23).
** Values between brackets: standard deviations for cheese productions.

3.1.2 Aseptic starter-free (ASF) cheeses. The only proteolytic enzyme systems active during the ripening of ASF cheese are rennet and native milk protease. In particular, the role of rennet in the formation of bitter peptides can be studied in these cheeses, on condition that the retention of rennet in these cheeses is known (23).

Table 6. Bitterness in aseptic starter-free cheeses made with different amounts of rennet.

Rennet in cheese*	Number of experiments	Average bitter score at		
		1 month	3 months	6 months
92 ∼ (1/3 n)	1	1.5 —	1.3 —	1.3 —
175 ∼ (3/5 n)	3	1.4 (0.15)**	1.5 (0.15)	1.9 (0.47)
300 ∼ (n)	5	1.7 (0.19)	2.6 (0.19)	3.2 (0.50)
430 ∼ (1½ n)	2	1.9 (0.44)	3.0 (0.40)	3.1 (0.25)
670 ∼ (2½ n)	2	3.7 (0.14)	4.3 (0.35)	3.7 (0.14)
895 ∼ (3 n)	3	3.3 (0.15)	4.1 (0.45)	4.1 (0.40)

* μl rennet per kg cheese at 9 days (280 μl/kg is normal (23)).
** Values between brackets: standard deviation of cheese productions.

Table 7. Bitterness in aseptic rennet-free cheeses made with different starter strains.

Starter strain	Number of experiments	Average bitter score at		
		1 month	3 months	6 months
E8	4	1.3 (0.10)**	1.7 (0.46)	1.5 (0.17)
AM1	3	1.4 (0.25)	1.3 (0.10)	1.4 (0.47)
AM2	2	1.3 (0.21)	1.3 (0.07)	1.5 (0.21)
Bos	2	1.2 (0.14)	1.2 (0.07)	1.5 (0.21)
HP	4	3.0 (0.48)	3.9 (0.45)	3.1 (0.81)
Wg2	5	2.3 (0.27)	3.2 (0.58)	2.9 (0.86)
Z8	2	1.6 (0.14)	2.1 (1.27)	2.1 (0.00)
—*	1	1.2 —	1.3 —	1.3 —

* No starter added: ARSF experiment.
** Values between brackets: standard deviation of cheese productions.

Table 6 shows the average bitter scores of ASF cheeses depending on the amounts of rennet retained in these cheeses. Rennet concentrations of 3/5 and 1/3 of the amount normally present in our Gouda cheeses proved to be insufficient to raise the bitterness over the threshold level during ripening for 6 months. A considerable number of ASF cheeses was made which contained rennet levels corresponding to those in Gouda cheese. At this concentration rennet appeared to produce bitter peptides gradually, the cheese at one month not yet showing bitterness but at 6 months being distinctly bitter.

Increased amounts of rennet, starting from about twice normal, resulted in bitter cheese from just 1 month old. Particularly with higher amounts of rennet some cheese tasters reported a somewhat different bitter sensation in these ASF cheeses as compared with normal aseptic, bitter cheeses. The bitter perception seemed to take place more quickly, the aftertaste being not so persistent.

We could not detect significant differences in bitterness between ASF cheeses made with normal commercial calf rennet and those made with the special preparation.

3.1.3 Aseptic rennet-free (ARF) cheeses. The only proteolytic enzymes active during the ripening of ARF cheeses are those from starter bacteria and milk protease. The contribution of starter bacteria to flavour development can be studied in these cheeses.

22 ARF cheeses were made by using the 7 starter cultures mentioned before. Seven pairs of cheese were made in parallel experiments to compare starter strains directly. Table 7 reviews the average bitterness levels developing on ripening. The four starters judged earlier (Section 3.1) to be non-

bitter in normal aseptic cheeses (E8, AM1, AM2, Bos), did not produce bitter ARF cheeses either. The 'bitter' strains Wg2 and HP developed suprathreshold values of bitterness in ARF cheeses as well. Strain Z8 appeared to have less potency to produce bitter compounds on its own, bitterness being just noticeable in ripened ARF cheeses.

Applying a cooking temperature of 39.5 °C (15 min) in ARF cheesemaking with strain HP proved not to influence the development of bitterness in comparison with normal, low-cooking ARF cheeses. Starter populations were found to be normal in this cheese.

ARF control cheeses were made with all starter strains. In these cheesemakings a new portion of rennet was added to the treated cheese milk just before clotting. The development of bitterness in these cheeses proved to be essentially the same as described for normal aseptic cheeses. Strain Z8 also showed the characteristic slowly bitter-producing properties.

As was described earlier (23) the growth and survival of starter bacteria was similar to that in normal aseptic cheeses.

Table 7 also gives the results for rennet- and starter-free (ARSF) aseptic cheese. In this cheese only milk-proteolytic enzymes are active during ripening. As no bitterness could be detected, it can be stated that milk protease on its own is not capable of liberating significant amounts of bitter peptides under Gouda-cheese conditions.

3.1.4 Open-vat cheeses. We regularly made Gouda-type cheeses in open vats by using the starter strains E8, Wg2 and HP. Forty litres of mixed milk were made into cheese, approaching normal procedures for Gouda cheese manufacture.

Strain E8 never produced bitter-tasting cheese. HP nearly always gave bitter cheeses the intensity of the defect being comparable to that of normal aseptic HP cheeses. However, with the use of 'bitter' starter Wg2 we could not obtain consistent results as to bitterness. The open-vat Wg2 cheeses repeatedly showed lower levels of bitterness than did aseptic Wg2 cheeses, or bitter defects were even absent. Kleter (29) made the same observations. When, in a parallel experiment (open vats), two Wg2 cheeses were made from the same portion of milk with two different amounts of rennet, the bitterness proved to be positively correlated with the amount of enclosed rennet. But, comparing cheeses made on different dates from different milks, the occurrence and intensity of bitterness was far less dependent on the amount of rennet.

Lowrie et al. (19) found the presence of bacteriophages during Cheddar cheesemaking to influence markedly the bitterness characteristics of certain

starter strains. Strain Wg2, moreover, is known to be sensitive to attack by bacteriophages (J. Stadhouders, personal communication). Nevertheless, we regularly checked that the maximum populations reached by Wg2 in non-bitter open-vat cheeses were as high as in the aseptic cheeses. From non-starter counts in the cheeses it was thought that only lactobacilli could have been affecting bitterness development. From the experiment described in Section 3.1.1 we could not get any evidence for this.

Up to now we have no clear explanation for the phenomena observed in these Wg2 cheeses.

3.2 Development of cheese flavour

Apart from bitterness we evaluated the development of cheese flavour in the different aseptic cheeses. Table 8 gives the average results for cheeses ripened over 6 months, a period in which under normal conditions full flavour could have been developed in Gouda cheese.

All 'non-bitter' starter strains produced characteristic cheese flavour at satisfactory levels in normal aseptic cheeses. No differences in flavour levels could be observed between the individual 'non-bitter' strains. Distinct cheese flavour was observed in ARF cheeses made with the same starters.

Obviously the 'non-bitter' starters are capable of producing important amounts of compounds that contribute to the desirable cheese flavour without the help of rennet. The 'non-bitter' starters also produced good levels of cheese flavour in ARF control cheeses.

'Bitter' starter strains developed considerably less cheese flavour in the cheese types mentioned above, indicating that bitterness is accompanied by lack of cheese flavour. In parallel cheesemaking experiments the differences

Table 8. Cheese flavour as observed in different aseptic cheeses after 6 months of ripening.

Cheese type	Starter	Number of cheeses	Cheese flavour scores
Normal aseptic	non-bitter	8	3.6 - 4.4
	bitter	6	2.1 - 3.5
ARF	non-bitter	7	2.6 - 3.6
	bitter	5	2.0 - 3.0
ASF	—	6	< 2.0
ARSF	—	1	< 2.0

in cheese flavour intensity between 'bitter' and 'non-bitter' starters always proved to be significant ($P < 0.05$).

In ASF and ARSF cheeses hardly any cheese flavour could be detected. The taste of these cheeses was very bland, off-flavours not being clearly perceptible. Native milk protease and rennet do not contribute significantly to cheese flavour development on its own.

Neither in normal aseptic cheeses nor in ASF cheeses could any favourable effect on cheese flavour development be ascribed to rennet when parallel cheeses made with high and low rennet levels (2 n - 1/2 n) were compared. However, comparing the flavour intensities of normal aseptic and ARF control cheeses on the one hand with ARF cheeses on the other suggests that an auxiliary role for rennet could still be possible. In that case the concentration of rennet in our cheeses has always been high enough to induce this stimulatory effect on cheese flavour development.

3.3 Quantitative approach to the evaluation of bitter taste in cheese

We wanted to know somewhat more about the relation between the amount of bitter peptides present in a certain cheese and the bitter score given to it by our panel.

All the members of our taste panel showed threshold values for the detection of bitterness in quinine sulphate solutions between 20 and 19 D (Dove units), corresponding to 0.75-1.0 mg per litre. According to Dove (30), tasters have high, medium and low acuities for bitterness if they can detect bitter flavour in these solutions at 20, 17 and 14 D, respectively. Kouwenhoven (31) reports 0.75 mg per litre and Harper (32) found 6 mg per litre as a typical threshold value. In selecting our panel it was our experience that the individual sensitivity to quinine sulphate solutions gave only limited indications of the ability to detect bitterness in cheese.

The average bitter scores of our panel for supra-threshold values of quinine sulphate showed a narrow resemblance with the 'growing curve' as reported by Emmons et al. (24) for their panel. In Fig. 1 we have recorded this curve and the scores of our panel.

Normal-flavoured Gouda cheeses, previously judged to be non-bitter, were thoroughly mixed with varying amounts of quinine sulphate. We could estimate that about 16 times more quinine sulphate had to be added to these cheeses in order to obtain bitter scores equal to aqueous solutions of quinine sulphate. In Fig. 1 the growing curve for quinine sulphate in cheese, as observed by our panel, is reproduced.

Consistency, moisture content and numerous compounds interacting with

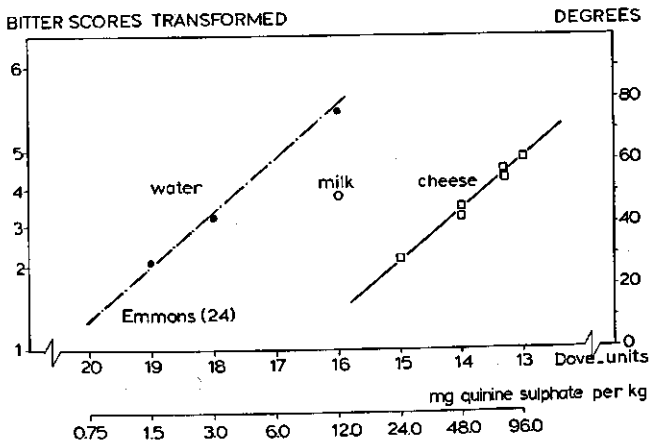

Fig. 1. Average bitter scores for different quinine sulphate concentrations in water, milk and cheese. Quinine sulphate concentrations are expressed as negative logarithms (base of 2) according to Dove (30). The bitterness scale is transformed (according to Emmons (24)) via proportions (1: p=0; 6: p=1) to degrees (x^0) according to x (radians) = arcsin p. The dotted line represents the 'growing curve' obtained by Emmons (24). All the points entered in the figure are from our own panel.

bitter perception (33, 34, 35, 36, 37, 38) can have been responsible for the observed difference between cheese and water. In Fig. 1 we have also pointed the average bitter score observed when quinine sulphate was added to milk, indicating that milk ranks in an intermediate position between water and cheese.

Because the bitter flavour of quinine sulphate was judged to be somewhat different from 'cheese bitter' – it lingered longer in the mouth – we also added a bitter-cheese extract both to water and to non-bitter cheese in different concentrations. About 20 times more bitter-cheese extract was needed (w/w) in cheese as compared with water in order to get the same bitter scores. The growing curve showed about the same slope as the quinine sulphate curve in water.

Starting from the above-mentioned relation between bitter scores and concentrations of quinine sulphate or bitter-cheese extract in water, we have transformed our bitter scores into bitter-stimulus concentrations. In Fig. 2 we have presented as a survey the bitterness of our aseptic cheeses in histograms, their altitude being related to this bitter-stimulus concentration. In this way an overall indication of relative bitter peptide concentrations in the different cheeses can be given.

The intensity of cheese flavour gradually increased during the six months of ripening of our cheeses. We were interested to know if the presence of

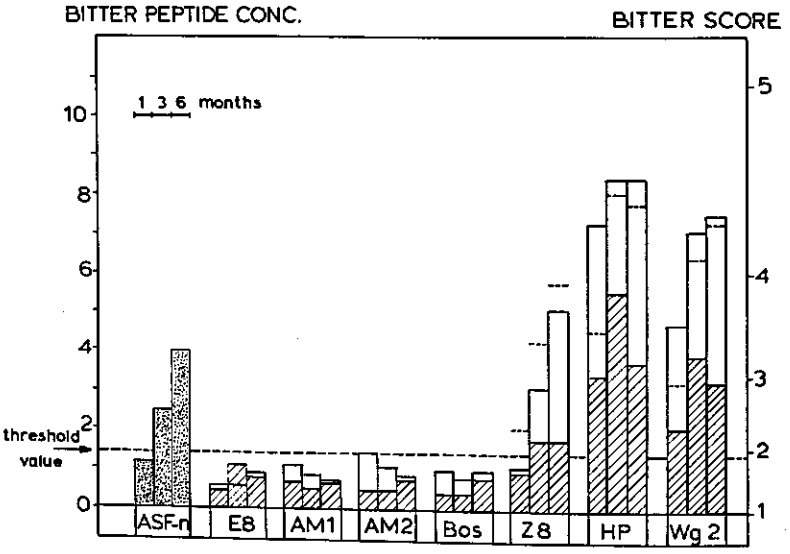

Fig. 2. Comparison of transformed bitterness in different aseptic cheese types at 1, 3 and 6 months of ripening. Bitter scores are converted into relative 'bitter-peptide concentrations' via the relation between bitter scores and quinine sulphate solutions outlined in Fig. 1. The histograms in each trio represent successively the bitterness at 1, 3 and 6 months of ripening. Bitterness in ARF cheeses (Table 7): level of hatched area. Bitterness in normal aseptic cheeses, containing normal rennet levels (Table 1): level of blank + hatched area. Bitterness in ASF-n cheese (Table 6): level of dotted area. For starters HP, Wg2 and Z8 the sum of ARF and ASF bitterness is indicated with marks (- -).

flavour substances in older cheeses could mask the bitterness in cheese to some degree, as was mentioned by Jago (39). Equal concentrations both of quinine sulphate and of bitter-cheese extract were mixed with portions of non-bitter cheese, one young cheese being mildly flavoured, the other much

Table 9. Comparison of bitter response to different additions of quinine sulphate or bitter cheese extracts to old and young cheese.

Age of cheese*	Cheese flavour	Bitter scores after addition of			
		quinine sulphate (mg/100 g)		bitter extract (% d.m.)	
		4.8	7.3	1.2	2.3
6 weeks	mild	3.6	4.4	2.3	3.3
10 months	strong	3.7	4.2	2.0	3.1

* E8 cheeses made with half the normal amount of rennet were used. These cheeses were judged to be not bitter before use in this experiment.

older and showing strong cheese flavour development. We were never able to observe significantly different bitter scores ($P < 0.025$) in the two cheese types on the addition of bitter substance, as can be seen in Table 9. This indicates that the bitter-masking effect of cheese flavour compounds during the ripening will not be very important. As a consequence a certain accumulation of bitter peptides would not need any considerable new development during the ripening to maintain a certain level of bitterness in the cheese.

4 Discussion

4.1 Formation of bitter peptides by rennet and milk protease

Various authors have demonstrated rennin to be capable of liberating bitter peptides from casein components in model systems (40, 41, 2). Other researchers have remarked casually on the occurrence of bitter off-flavours in sterile or non-sterile, starter-free cheeses (5, 29, 42, 43, 44). However, until now no determinations of rennet amounts in these cheeses have been made and, as was stated recently (23), the conditions for the manufacture of starter-free cheeses have often favoured very high rennet retentions in these cheeses as compared with normal cheeses.

Knowing the exact amounts of rennet in our ASF cheeses, we can now conclude that under cheese circumstances rennet alone has the potency to produce bitter peptides and to make the cheese very bitter if high concentrations are retained in the cheese. However, in amounts as normally present in Gouda cheeses, rennet produces bitter peptides slowly during the ripening, resulting in detectable bitterness only at 2-3 months and in distinctly bitter cheese at 6 months of age. Lower rennet contents, as reported to be present in Cheddar cheese (20), do not result in bitter ASF cheeses during the ripening, which could indicate that the contribution of rennet to bitterness development in Cheddar cheese is not as important as in Gouda cheese.

Lowrie et al. (17) suggested – in contradiction to Czulak's theory (15) – that in Cheddar cheese rennet would not contribute directly to the formation of bitter peptides but would provide predominantly non-bitter peptides of high molecular weight. Stadhouders (20) ascribed a more active role to rennet in the bitterness development in Gouda cheese.

Milk protease on its own does not noticeably contribute to bitterness in Gouda cheese conditions as was demonstrated with ARSF cheese and as could be expected from the absence of bitterness in low-rennet ASF cheeses. It may be interesting to report that we observed a bitter taste in pasteurized (20 s at 72 °C), aseptically drawn milk portions that had been incubated for 2 weeks at 30 °C without any bacterial growth. Noomen (45) reported that

milk protease develops considerable proteolytic activities under these circumstances, especially on β casein. Apparently milk proteolytic enzymes also have the power to liberate bitter peptides from casein. Under the conditions existing in cheese, however, this action is limited or altered for the greater part. Moreno & Kosikowski (46) observed extreme bitterness in peptide fractions from auto-hydrolysed β casein.

4.2 Formation of bitter peptides by starter bacteria

Several authors (47, 48, 49) found certain strains of *S. cremoris* to produce considerable amounts of bitter peptides in milk cultures without the addition of rennet. Stadhouders (49) reported that in model experiments bitter peptides were liberated from β casein by 'bitter' starters in contrast to 'non-bitter' strains.

Many external conditions can influence the synthesis of the complex enzyme system of starter bacteria, the autolysis of the cells and the activity of the enzymes. Therefore it is difficult to transpose the results on bitter production by bacterial cultures or enzymes in experimental systems to cheese conditions.

From our rennet-free cheesemaking experiments we now can conclude that certain starter strains of *S. cremoris* (HP, Wg2) are capable of producing bitter cheeses on their own, without any interaction with rennet. The same strains cause bitter flavour in normal Gouda cheese. Other strains are not able to produce detectable bitterness in cheese on their own; these strains are known to give no bitterness in normal cheeses either. These results indicate that certain differences must exist in the proteolytic properties of 'bitter' and 'non-bitter' starters grown under Gouda cheese manufacturing and keeping conditions. Exterkate (50, 51) studied the proteolytic enzyme system of *S. cremoris* strains and showed differences to exist in the composition of the cell-wall-associated proteolytic system. It was suggested that these cell wall enzymes are important for the liberation of bitter peptides (51). In the hypothesis of Lowry et al. (17) no specific differences in bitter-producing enzymes would occur in 'bitter' and 'non-bitter' strains since they showed that all starter strains were potentially able to produce bitter (or non-bitter) Cheddar cheese. The likelihood of bitterness development was exclusively determined by the maximum starter population reached in the cheeses. Production of bitter peptides, directly from paracasein, not involving rennet, was thought to be of lesser importance.

The maximum populations reached by all starters except AM1 in both the normal aseptic and the ARF cheeses were relatively high and would probably have caused bitterness in Cheddar cheese (19). However, the starters E8,

AM2 and Bos did not give bitter Gouda cheese. Strain HP, on the contrary, reaching comparable cell densities did produce bitter cheese. The application of a cooking temperature of 39.5 °C – never reached at Gouda cheesemaking in practice – did not appear to decrease the bitter-producing capacity of strain HP in our ARF cheese. Raising the maximum cell density of E8 artificially did not result in bitter Gouda cheese. It therefore seems justifiable to differentiate between 'bitter' and 'non-bitter' starters in Gouda cheesemaking.

The theory of Lowrie et al. (17) – developed for Cheddar and according to Martley (18) also applicable to Camembert – does not seem to fit the bitterness results in Gouda cheese. It should be borne in mind, however, that the manufacturing process, cheese composition and ripening conditions for Cheddar differ from those of Gouda cheese. High cooking temperatures and early salt addition to the curd in Cheddar could possibly influence the proteolytic properties of starter bacteria.

4.3 The degradation of bitter peptides by starter bacteria

Sullivan et al. (52, 53) incubated cell-free extracts both from 'bitter' and 'non-bitter' starter strains with a bitter-peptide extract from casein hydrolysate. All the starters appeared to possess the peptidases to hydrolyse the bitter peptides to non-bitter products at pH 7.0. At pH 5.0, however, only 'non-bitter' starters showed sufficient activity to do so. Exterkate (50) detected a cell-membrane-associated system of endo- and exopeptidase activities in *S. cremoris* that could be important in this degrading process. Differences in the composition of this enzyme system or in its accessibility to the hydrophobic bitter peptides might explain the different capacities of starter strains to degrade bitter peptides.

Czulak (15) and Stadhouders (16, 20) emphasized the possibility that 'non-bitter' starters are able to degrade bitter peptides in cheese. Lowrie (17) suggested this to be of minor importance in Cheddar.

In our opinion 'non-bitter' starters can degrade bitter peptides under the conditions existing in cheese to a considerable extent. Evidence for this is found in the observation (Fig. 2) that rennet produces bitter cheese on its own – especially in higher concentrations – whereas the normal aseptic cheeses made with 'non-bitter' strains do not show bitterness. In this connection we emphasize the absence of bitterness in E8 cheese made with double the amount of rennet and the decrease in bitterness during the ripening of E8 cheese made with threefold the normal amount of rennet.

It is tempting to conclude from the reduced bitterness on pairing bitter and non-bitter strains that breakdown of bitter peptides by the non-bitter starter had taken place. However, not knowing the exact populations of each

starter strain in the cheese, the possibility cannot be excluded that the non-bitter part of the starter has overgrown the bitter strain due to stimulatory effects.

It is quite possible that even bitter starter strains have a limited capacity to degrade bitter (rennet) peptides under cheese conditions. For strain Z8 this could be derived from the absence of bitterness at 1 month in normal aseptic Z8 cheese made with the double amount of rennet. However, more evidence for this will be given in a subsequent publication.

4.4 Mechanism proposed for bitterness development in Gouda-type cheese

Having assessed the capacities of rennet, starter bacteria and milk protease to accumulate bitter peptides under the conditions existing in cheese, some important paths – not investigated separately in cheese until now – have become more clear. With this knowledge a more realistic estimation of eventual interactions between enzymes can be made. The histograms given in Fig. 2 are helpful because bitterness is related to 'bitter-peptide concentration'. It must be noted that the bitter scores below the threshold values are not to be regarded quantitatively.

Any comparison of the bitter scores for normal aseptic cheeses and the addition of the separate scores in ASF and ARF cheeses must be done with the restriction in mind that average scores are compared of cheeses that often are not made in parallel experiments from the same portions of milk. Never-

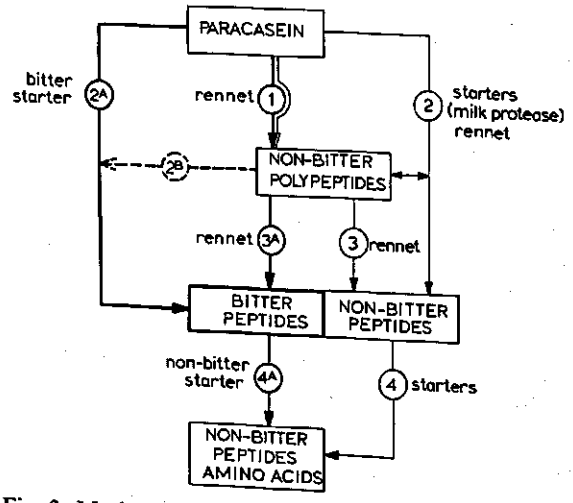

Fig. 3. Mechanism for the development of bitterness in Gouda-type cheese. Thin lines: paths of general proteolysis in cheese; thick lines: paths important for bitterness development.

theless it appears that, with 'bitter' starters strains, the separate actions of rennet and starter bacteria are responsible for the greater part of the observed bitterness in normal aseptic cheeses. Thus stimulatory effects between bitter starter and rennet in the production of bitter peptides seem to be of minor importance. Only in the first month of the ripening could a clear stimulatory effect be assumed with strain HP and to a lesser extent with Wg 2.

In Fig. 3 we have schematized the different actions that, in our opinion, are important for bitterness development in Gouda-type cheeses. On the right side of the scheme the normal proteolytic paths in cheese are drawn in thin lines (Nos 1, 2, 3 and 4). This general proteolysis is effected by the action of rennet, starter bacteria – both 'bitter' and 'non-bitter' – and milk protease. The thick lines represent the paths which have a determinative effect on bitterness development in our cheeses (1, 2A, 2B, 3A and 4A).
'Bitter' starters (such as HP and Wg2) liberate important amounts of bitter peptides directly from paracasein due to a highly specific proteolytic action (2A). The results of our studies on proteolysis in ARF cheeses support this view (to be published). The specificity of 'non-bitter' starters does not seem to be directed to the accumulation of bitter peptides. However, from our experiments it cannot be excluded that 'non-bitter' starters also liberate bitter peptides to some extent, but that their capacity to degrade them to non-bitter products prevents the cheese from becoming bitter. Before pronouncing upon this, more research is necessary on the proteolytic properties of 'bitter' and 'non-bitter' starters.

The formation of bitter peptides by rennet to supra-threshold values, in our opinion, proceeds far less specifically via intermediate polypeptides that are non-bitter (1 + 3A). This is concluded from the slowly accumulating bitterness during the ripening of ASF-n cheeses coupled with extensive proteolysis in these cheeses (to be published).

'Non-bitter' starters degrade the bitter peptides from rennet action to non-bitter peptides and amino acids (4A). This action probably prevents Gouda cheeses from always being bitter. Bitter peptides liberated by other enzyme systems in cheese – such as starter bacteria – are most likely to be degraded too. 'Bitter' starters show this capacity to degrade the hydrophobic bitter peptides to a far less extent.

Stimulatory action of rennet on the bitter-peptide formation by 'bitter' starter bacteria could proceed via the non-bitter polypeptides (2B). However, ARF experiments showed that the presence of these polypeptides is not a pre-condition at all for 'bitter' starters to produce bitterness in cheese. It was stated above that the stimulatory effect of rennet seems to be of restricted importance. We do not exclude the possibility that, during the ripening, bitter

peptides are liberated via pathway 2B from peptides of high molecular weight in the same way and at about the same rate as from paracasein directly.

Studies on proteolysis and bitter-peptide fractions in the different aseptic cheeses will be published in subsequent papers (54).

Acknowledgments

The author is indebted to all the members of the taste panel, for their continuing great readiness to evaluate the cheeses organoleptically. Thanks are due to Mrs T. van Pijkeren and Miss T. van der Haven for assistance in sensory experiments with quinine sulphate and bitter-cheese extracts. This study was made possible by the financial support of the Stichting J. Mesdagfonds, instituted by Kaascontrolestation Friesland, Leeuwarden.

Samenvatting

F. M. W. Visser, *De bijdrage van de enzymen van stremsel, zuurselbacteriën en melk aan de eiwitafbraak en smaakontwikkeling in Goudse kaas. 2. Ontwikkeling van het gebrek 'bitter' en van de kaassmaak*

In een voorgaande publikatie (23) werden verschillende soorten aseptische bereide kazen beschreven. Door onderzoek van deze kazen tijdens de rijping kon de afzonderlijke bijdrage van stremsel, zuursel en melkprotease aan het ontstaan van het gebrek 'bitter' en de ontwikkeling van de kaassmaak worden vastgesteld in Goudse kaas. Tevens werd o.a. de invloed van sterk variërende hoeveelheden stremsel onderzocht op de mate van bitterheid in op aseptische wijze bereide kazen, zowel met 'bittere' als 'niet-bittere' zuurselculturen. Het bleek onder meer dat:

– stremsel alléén in staat is om een kaas na enkele maanden rijping duidelijk bitter te maken. Gebruik van grotere stremselhoeveelheden maakt dat de kaas eerder en sterker bitter wordt;

– 'bittere' zuursels, in tegenstelling tot 'niet-bittere', het vermogen bezitten om zonder de hulp van stremsel een kaas duidelijk bitter te maken binnen een relatief korte rijpingsperiode;

– melkprotease alléén niet wezenlijk bijdraagt aan het ontstaan van bitterheid in Goudse kaas;

– onderlinge stimulatie bij de aanmaak van bittere peptiden door stremsel en zuurselbacteriën geen belangrijke rol hoeft te spelen bij het ontstaan van bitterheid in Goudse kaas;

– 'niet-bittere' zuursels het vermogen bezitten om onder de in kaas heersende omstandigheden grote hoeveelheden bittere peptiden, geproduceerd door stremsel, af te breken, dit in tegenstelling tot 'bittere' zuursels;

– de uitgroei van zuurselbacteriën tot hoge aantallen in Goudse kaas niet de bepalende factor lijkt te zijn voor het optreden van bitterheid;

– de intensiteit van de kaassmaak nauwelijks van invloed is op de waarneming van bitter;

– stremsel en melkprotease elk niet merkbaar bijdragen aan de ontwikkeling van kaassmaak tijdens de rijping;

– een 'niet bitter' zuursel in staat is om, zonder hulp van stremsel, een aanzienlijke kaassmaak te ontwikkelen. Dit vermogen bezitten 'bittere' zuursels in veel mindere mate.

De bitterheid van zuurselvrije en stremselvrije kazen werd vergeleken met die van normale aseptisch bereide kazen met behulp van o.a. figuur 2 waarin de bitterscores van de kazen zijn omgerekend naar relatieve concentraties aan bittere peptiden. Een mechanisme voor het ontstaan van het gebrek 'bitter' wordt bediscussieerd (figuur 3).

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Neth. Milk and Dairy J. 31 (1977): 210-239

Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 3. Protein breakdown: analysis of the soluble nitrogen and amino acid nitrogen fractions

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Received: 28 April 1977

Summary

Protein breakdown was studied in aseptically made Gouda cheeses in which the action of rennet or starter bacteria during the ripening could be eliminated. By comparing the results of the analyses of these cheeses with those of normal aseptic cheeses, it was possible to estimate the contributions of rennet, starter bacteria and milk protease to the production of soluble-N compounds and free amino acids. For a more quantitative approach the rennet contents of the cheeses were determined.

Rennet appears to be responsible for the greater part of the soluble-N production in Gouda cheese, but the starter bacteria and – to a lesser extent – milk protease also make contributions. The latter two were shown to be capable of producing soluble-N compounds in cheese without the help of rennet. In normal cheeses, no distinct mutually stimulatory or inhibitory effects on soluble-N liberation appear to occur between the enzyme systems.

Determinations of amino acid N and gel filtration of soluble-N compounds showed that rennet liberates peptides of high and low molecular weight (MW) in particular, but only very low amounts of amino acids. On the contrary, starter bacteria without rennet predominantly accumulate lower-MW (<1400) peptides and — dependent on the culture used – more or less significant levels of free amino acids. Milk protease on its own liberates amino acids and low-MW peptides but only in small amounts.

In normal cheeses, with the enzyme systems acting together, the action of rennet clearly stimulates the starter bacteria to accumulate amino acids and low-MW peptides. It could be shown that this was most likely due to the progressive degradation by starter peptidases of the higher-MW products of rennet action.

Free amino acid patterns of the different aseptic cheeses are presented and discussed.

Differences in the residual amounts of rennet proved to account completely for the differences in the rate of proteolysis observed in starter-free cheeses that showed different calcium levels due to the method of manufacture.

In this study both 'bitter' and 'non-bitter' starters were applied. Therefore the observed effects are also discussed in relation to (bitter) flavour development.

1 Introduction

Cheese proteolysis, primarily of casein components, is considered to result from several proteinase and peptidase activities. The main contributors to this protein breakdown are supposed to be residual rennet and the enzymes of starter bacteria. Organisms present as a result of random contamination can bring about adventitious contributions. Native milk protease was thought to be of minor importance but has received considerable attention recently (1, 2) as a possible agent in cheese proteolysis.

The relative importance and interactions of rennet and starter enzymes have not been fully understood until now, and are matter of some controversy (3, 4, 5, 6, 7). In general it is thought that rennet is mainly responsible for the breakdown of paracasein to the larger peptides while starter bacteria liberate smaller peptides and amino acids.

Many studies of casein hydrolysis in solution have been carried out, particularly with regard to the action of rennet on different casein components (8, 9, 10, 11). pH value, aggregation of protein, water and salt contents, and the temperature may all influence the rate and nature of hydrolysis of different caseins (12, 13, 14, 15). The proteolytic system as developed in cheese by starter bacteria is very complex, the effective activity being dependent on the manufacturing conditions of the cheese. As a consequence it is very difficult to introduce and study such a system in solution or in model systems (3, 16, 17, 18).

As was stated earlier (19), in our opinion an investigation of the contributions of rennet, starter bacteria and milk protease to cheese ripening in a more quantitative sense should be carried out by studying the separate and combined actions of these enzymes in aseptically made cheeses. The determination of residual rennet in the cheeses is essential for this work.

Several workers have studied the proteolysis in aseptic cheeses ripened by the combined action of rennet, starter bacteria and milk protease (4, 20, 21, 22). However, to unravel the actions of these enzyme systems, it is necessary to study their proteolysis separately in cheese. In previous papers (19, 23) we described methods for the aseptic manufacture of starter-free, rennet-free and rennet- and starter-free cheeses with normal Gouda cheese composition.

Gripon et al. (24) and O'Keeffe et al. (20) characterized the proteolysis in aseptic, chemically acidified model-curd and Cheddar cheese, respectively. Kleter (21) recently reported values for the soluble and amino-acid nitrogen in a sterile, starter-free Gouda cheese. The results on proteolysis from earlier starter-free cheese experiments were thought to be less reliable (25). All ex-

periments with starter-free cheeses which have been reported up to now lacked the estimation of residual rennet which would have been necessary for a more quantitative approach to the rennet action. We demonstrated earlier (19) that the starter-free cheesemaking procedures favoured high rennet retentions and low calcium contents in the cheese.

Until now no reports are known which deal with the protein breakdown of starter bacteria and milk protease in cheese without the interference of rennet.

This paper and the following one will report on the proteolysis as observed in aseptic Gouda-type cheeses that are ripened by the separate and by the combined actions of rennet, starter bacteria and milk protease.

As any conclusions on the relative importance and interactions of these enzyme systems are strongly dependent on the method used to measure proteolysis, we have characterized the protein breakdown using different techniques, such as fractionated precipitation, gel electrophoresis, gel filtration and amino-acid analysis. This paper deals with the results obtained on soluble and amino-acid nitrogen fractions.

2 Materials and methods

2.1 Aseptic cheeses

In earlier publications (19, 23) the methods used for the manufacture of normal aseptic, aseptic starter-free (ASF), aseptic rennet-free (ARF) and aseptic rennet- and starter-free (ARSF) cheeses were outlined.

Synchronous cheesemakings were often carried out, i.e. a pair of cheeses was made simultaneously in the two vats from the same portion of milk. If synchronous cheesemaking was not possible parallel cheeses were frequently made one immediately after the other but from the same portion of milk.

Seven different starter cultures were used. The *Streptococcus cremoris* strains E8, AM1, AM2 and mixed-strain (BD) starter Bos were shown earlier (26) to give well-flavoured Gouda cheese, whereas *S. cremoris* strains HP, Wg2 and Z8 appeared to produce bitter defects. In the histograms presented in this paper the starters will be indicated by numbers according to this key: 1 = E8; 2 = AM1; 3 = AM2; 4 = Bos; 5 = HP; 6 = Wg2; 7 = Z8. The 'bitter' starters will be marked with little circles (o) in the columns.

Dutch commercial calf-veal rennet with a clotting power of 10 000 was used in our cheese productions. The concentration of residual rennet in our cheeses was determined according to the method described by Stadhouders et al. (27) and reported on previously (19).

All aseptic cheeses approached the normal Gouda-cheese composition. The results on average compositions were presented earlier (19).

The cheeses were all ripened at 13-14 °C for a period of six months.

2.2 Determination of different nitrogen fractions in cheese

2.2.1 Total nitrogen (TN). A 1-gram portion of cheese was gently heated with 10 ml HCl (25 %). After transferring the solution quantitatively to a 100-ml volumetric flask and making up to this volume with distilled water, nitrogen was estimated in 2 ml of the solution.

2.2.2 Soluble nitrogen (SN). An amount of cheese corresponding to 10 g of dry matter was mixed at 30 °C for 5 min with an 'Ultra turrax' in 200 grams-less the moisture in the cheese-of an extraction solution containing 0.55 % calcium (as $CaCl_2$) and 4 % NaCl. After removing the fat, the pH of the homogenate (30 °C) was adjusted to 7.5. After centrifuging for 10 min at 40 000 *g* (30 °C) the clear supernatant was filtered and nitrogen estimated in 3 ml filtrate. Soluble nitrogen was calculated as a percentage of total nitrogen.

The above-mentioned method is described in detail by Noomen (28).

2.2.3 Amino acid nitrogen (AN). To 25 ml soluble-N filtrate 15 ml H_2SO_4 (25 %) and 6 ml phosphotungstic acid (50 % w/v) were added. After standing overnight the volume was made up to 50 ml with distilled water and filtered. Nitrogen was estimated in 4 ml or in 10 ml of the filtrate. Amino acid nitrogen was calculated as a percentage of total nitrogen.

All nitrogen contents were determinated in triplicate, using the micro-Kjeldahl method. An automatic titration unit was used (Radiometer, photo-titrator PMT 1 and autoburette ABU 12).

2.3 Fractionation of soluble N

2.3.1 Extraction of the cheeses. Extracts of cheeses were prepared in the same way as described for soluble-N determination. However, the solution for extraction now contained 0.15 % calcium (as $CaCl_2$) and no NaCl, in order to avoid high salt concentrations in the gel filtration samples. These extracts showed nitrogen amounts equal to the normal soluble-N extracts that contained 0.55 % calcium and 4 % NaCl. The extracts were freeze-dried and the total nitrogen content of the dry matter was determined according to the micro-Kjeldahl procedure.

For reasons of comparison we also extracted a cheese following the procedures described by O'Keeffe et al. (20) and Gripon et al. (24), both resulting in nitrogen fractions soluble at pH 4.6.

2.3.2 Gel filtration of the extracts. The peptides present in the different cheese extracts were fractionated on Sephadex G-50 (superfine) in a column of 100 × 2.6 cm (K26, Pharmacia). Gel filtration was performed at 3-4 °C in order to decrease hydrophobic interactions between products of casein hydrolysis. Elution was carried out with 0.01 *M* acetic acid at a flow rate of 29.9 ml/h.

The column was calibrated under the same conditions, with Bleu Dextran 2000 (Vo ~ 190 ml), pepsin (M = 35 500), chymotrypsinogen A (M = 25 000), trypsin-inhibitor (M = 21 500), cytochrome C (M = 13 000), insulin (M = 5730), protamin sulphate (M = 4000), bacitracin (M = 1410) and tryptophan.

In each run an amount of freeze-dried cheese extract, corresponding to 23.5 mg nitrogen, was dissolved in 9 ml 0.01 *M* acetic acid and applied to the column. During elution the absorbance of the column effluent was measured continuously at 280 nm (Gilson, UV 265/280) and monitored with a Honeywell FB 70 recorder.

The elution diagrams were subdivided into three molecular weight classes: fraction I, containing high-MW hydrolytic products (M > 14 000), fraction II, containing peptides between MW 14 000 and 1400 and fraction III consisting of lower-MW peptides and amino acids (M < 1400). The breakdown products present in each fraction were expressed as a percentage of the total absorbing material.

2.4 Electrophoresis of cheeses

Polyacrylamide gel electrophoresis was carried out according to de Jong (29).

2.5 Analysis of free amino acids

From a restricted number of cheeses, samples for amino acid analysis were prepared according to the method of Kosikowsky (30) with some modifications.

A 6 % (w/v) cheese homogenate in distilled water was held at 75 °C for 10 min with agitation, cooled, defatted and filtered successively. To 50 ml filtrate 150 ml 95 % ethanol were added and the mixture kept overnight at 7 °C for precipitation to take place. After filtration the clear solution was

dried in a rotary evaporator, taken up in 10 ml water and freeze-dried.

The amino acid profile of the powder was determined in a Jeol JLC-5AH automatic amino acid analyser with norleucine and AGPA (amino guanidine propionic acid) as internal standards in each sample. Depending on the amino acid-N content of the cheese, portions of 1.1-8.4 mg, dissolved in 0.02 *N* HCl, were used for analysis.

After hydrolysis of the powder in 11.3 *N* HCl at 110 °C (24 h) the amino acid profile was determined again.

3 Results

3.1 Formation of soluble nitrogen and amino-acid nitrogen during ripening

3.1.1 Normal aseptic cheeses. All the seven starters were studied for their proteolytic properties in aseptic cheeses containing normal amounts of rennet. It was reported previously (19) that these cheeses contained 280 μl rennet per kg cheese as an average, which is normal for Gouda-type cheeses. Over a period of 2½ years 25 cheesemaking trials of this type were carried out. In those series seven pairs of parallel cheeses were made in synchronous experiments to be able to compare two starter strains directly. All cheeses were analysed for fractions of soluble N and amino-acid N after 6 months of ripening. A number of cheeses was also analysed in earlier stages of ripening, from 9 days onwards.

In Fig. 1 the results of determinations of soluble N and amino-acid N are presented in histograms. In addition to the mean values for cheeses made with the same starter the observed extremes are also indicated. The values examined after 6 months of ripening in the cheeses made in synchronous experiments are given in Table 1.

Although the values varied somewhat within cheeses made with the same starter on different dates, there appear to be no important differences in the production of soluble-N compounds by our starters during the ripening of rennet-containing cheeses. Relatively small differences can be observed after 6 months of ripening. For some starters these differences seem to occur if they are compared in synchronous experiments. From Table 1 is can be seen that starter E8 produces somewhat more soluble N compared with Wg2; the same is true for strain AM1 in comparison with Z8. Strains HP and AM2 do not show any obvious difference. Our results give no indication of an overall difference between 'bitter' and 'non-bitter' starters with regard to soluble-N production in these cheeses.

As opposed to the above, the individual starters show relatively important

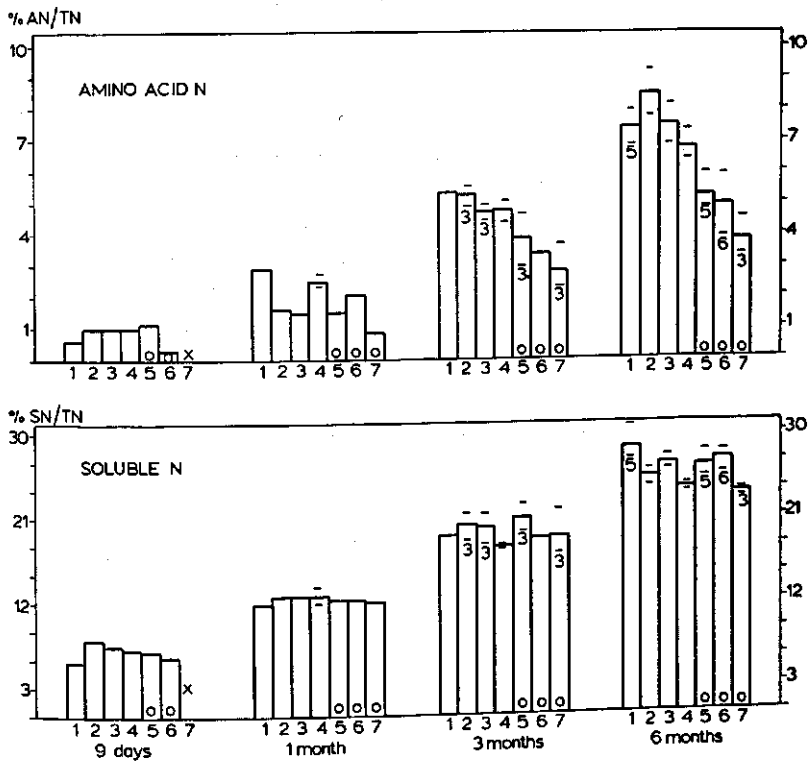

Fig. 1. The development of soluble N and amino-acid N during the ripening of normal aseptic cheeses made with 7 different starter cultures. All cheeses contained normal amounts of rennet. Extremes and number of cheesmakings – if more than two – are indicated in the columns. Starter numbers refer to Section 2.1. Bitter starters are marked by little circles (O).

differences in their capacity to liberate free amino acids during the ripening of these cheeses. In all cheeses a gradual increase in amino acids can be observed during the ripening period. In all the 'non-bitter'-starter cheeses considerably more amino-acid N is formed as compared with cheeses made with 'bitter'-starter strains. This is also obvious from the parallel experiments of Table 1. The mixed-strain starter Bos produces amounts of soluble N and amino-acid N that correspond to the values reached in cheeses made with 'non-bitter' *S. cremoris* strains. The values of amino-acid N in these cheeses are comparable with those observed in normal Gouda cheeses (31). Kleter (21, 32) obtained similar results in aseptic Gouda cheeses.

For a number of starter strains we varied the amount of rennet used in aseptic cheesemaking. With four different starters in total 10 pairs of parallel

Table 1. Proteolysis in normal aseptic cheeses after 6 months of ripening. Pairs of cheeses were made synchronously in parallel experiments with two different starter strains. Cheeses contained normal rennet amounts.

Parallel exp. No	Starter	Cheese at 9 days			Proteolysis at 6 months	
		pH	% moisture	% S/M*	% SN/TN	% AN/TN
30613	E8	5.09	45.2	4.6	30.9	7.5
	Wg2	5.10	45.2	4.4	27.2	5.5
30807	E8	5.06	44.0	3.4	28.8	7.8
	Wg2	5.08	44.1	3.6	27.6	5.0
60120	E8	5.08	44.3	5.5	28.6	6.8
	Wg2	5.11	44.1	5.1	27.9	3.8
50821	AM1	5.04	41.4	5.1	24.2	7.7
	Z8	5.02	40.0	5.4	23.0	3.6
51014	AM1	4.99	39.0	4.4	26.0	9.3
	Z8	5.01	39.6	4.5	23.8	4.5
50901	AM2	5.02	38.7	n.d.	27.4	8.2
	HP	5.04	39.2	n.d.	27.3	6.7
51126	AM2	4.93	43.2	3.1	26.0	6.8
	HP	4.90	42.9	3.1	26.0	5.2

* % salt in moisture.

cheeses were made (non-synchronously) showing different rennet concentrations within the pairs. We have reported previously (19) the rennet retentions in these cheeses. It was found that this amount was linearly related to the amount used for cheesemaking. The development of soluble N and amino-acid N, as influenced by the rennet concentration, is presented in Fig. 2A and 2B for two representative pairs of parallel cheeses. In the HP cheeses the rennet concentration was varied by a factor of 2, and in the Z8-cheeses by a factor of 4. In Fig. 2c we have outlined the development of soluble-N values as an average of the 20 parallel cheeses. No account is taken in this figure of the eventual variation between starters.

As can be seen from these results the production of soluble N is positively correlated to the amount of rennet in the cheeses. This is true for all starters tested. The production of amino-acid N, however, is not affected at all by the rennet concentrations used. From the results of the 10 parallel experiments we could conclude that no significant increase of amino acid formation occurred because of the use of much higher rennet concentrations. These results conform with the results of Stadhouders obtained in non-aseptic Gouda-cheeses (31).

3.1.2 Aseptic rennet-free (ARF) cheeses. Aseptic rennet-free cheeses were manufactured with the seven starter cultures mentioned before. Each starter was employed in at least two ARF cheesemaking experiments. In six parallel experiments the different starters could be compared directly. 12 ARF cheeses were made in individual experiments. The development of soluble N and amino-acid N was followed during the ripening of the cheeses. The average and extreme values of these fractions observed during the ripening are presented in Fig. 3. In Table 2 the results for proteolysis at 6 months of the parallel experiments are given. The development of soluble N and amino-acid N as observed in two representative pairs of cheeses is outlined in Fig. 4A and 4B.

A gradual increase in the soluble N and amino-acid N is observed during

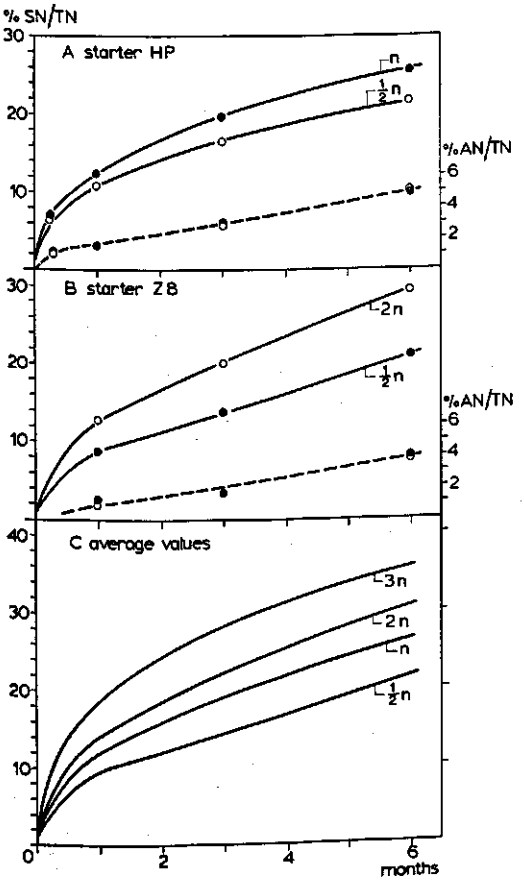

Fig. 2. The development of soluble N and amino-acid N during the ripening of normal aseptic cheeses containing different amounts of rennet.
——— soluble N, ---- amino-acid N
A. Results of a parallel experiment with HP as starter.
●: cheese containing a normal (n) amount of rennet; ○: cheese containing half the normal (½n) amount of rennet.
B. Results of a parallel experiment with Z8 as starter.
●: cheese containing half the normal (½n) amount of rennet; ○: cheese containing twice the normal (2n) amount of rennet.
C. Average results for soluble-N development in 20 normal aseptic cheeses containing various amounts of rennet.

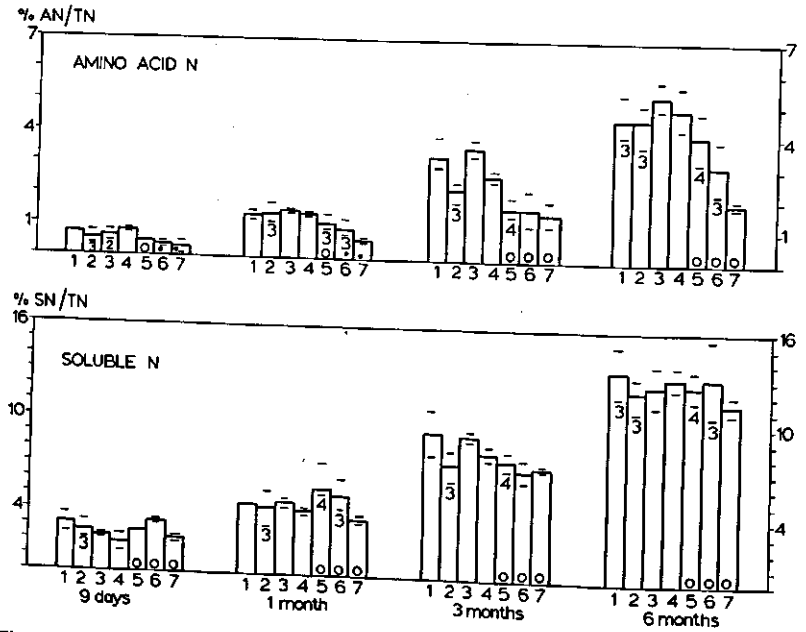

Fig. 3. The development of soluble N and amino-acid N during the ripening of aseptic rennet-free (ARF) cheeses made with 7 different starter cultures.

Table 2. Proteolysis observed at 6 months in pairs of ARF cheeses made in parallel experiments.

Parallel exp. No	Starter	Cheese at 9 days				Proteolysis at 6 months	
		pH	% moisture	% salt in moisture	% Ca in dry matter	% SN/TN	% AN/TN
60303	AM1	5.08	49.8	5.3	1.53	13.1	5.0
	Z8	5.01	49.1	5.3	1.45	12.0	2.0
60310	AM2	5.10	49.2	3.4	1.57	13.9	5.8
	Wg2	5.15	48.6	4.3	1.58	12.0	3.2
60324	Bos	5.23	45.0	4.2	1.45	13.9	5.6
	HP	5.11	44.3	5.3	1.47	11.7	4.8
60331	E8	5.12	44.7	3.5	1.60	11.8	4.0
	Z8	5.14	45.5	3.5	1.56	10.9	1.8
60414	Bos	5.12	45.3	3.8	1.31	12.3	4.2
	AM2	5.09	43.0	3.6	1.39	11.3	4.8
60421	AM1	5.00	47.9	4.1	1.34	12.6	4.8
	Wg2	5.09	43.2	4.6	1.38	10.7	2.2

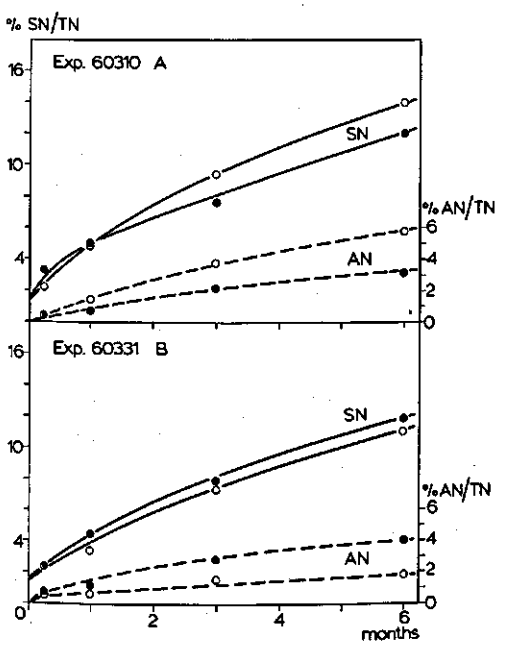

Fig. 4. The development of soluble N and amino-acid N during the ripening of two pairs of ARF cheeses made in parallel experiments with different starters.
—— soluble N; - - - amino-acid N.
A. Experiment with strain AM2 (○) and Wg2 (●).
B. Experiment with strain Z8 (○) and E8 (●).

the ripening period of ARF cheeses. Obviously the proteolytic enzyme system of the starter bacteria is capable of attacking paracasein in cheese without the help of rennet. Considerable amounts of soluble N, and particularly amino-acid N, are produced even after the death of most of the starter bacteria (19).

No important differences between the starters used can be detected with regard to the formation of soluble-N compounds. In fact the observed differences fall within the variation of values examined in cheeses made with the same starter but on different dates. However, in the parallel experiments all non-bitter cheeses show slightly more soluble N at six months than do bitter cheeses.

In conformity with the normal aseptic cheeses clear differences exist between certain starters as to their capacity to liberate amino acids in ARF cheeses. Relatively low levels are produced by the 'bitter' strains Wg2 and Z8 and to a lesser extent by HP. The 'non-bitter' starters all showed higher levels of amino-acid N.

The absolute levels of amino-acid N as observed in these cheeses for all the starters are lower than observed in normal aseptic cheeses, when combined with rennet. The same is true for the soluble-N values.

As was mentioned before, ARF control cheeses were made with the different starters to check the reliability of the ARF cheesemaking process. In

these cheeses rennet was re-introduced just before clotting of the ARF-treated milk (23). All the control cheeses showed essentially the same development of soluble N and amino-acid N as observed in normal aseptic cheeses containing comparable amounts of rennet.

Fig. 5 shows the results of a parallel experiment with starter strain AM1. From the same portion of 'ARF-treated' milk ARF cheese was manufactured in one vat while ARF control cheese was made in the other vat.

3.1.3 Aseptic starter-free (ASF) cheeses. 16 ASF cheeses were made with different amounts of rennet. Usually two rennet concentrations were compared in parallel cheesemakings.

The average results of soluble-N determinations during the ripening are comprised in the histograms of Fig. 6. The hatched column represents ASF cheeses containing amounts of rennet comparable to those in normal aseptic cheeses and Gouda cheese.

During the ripening of 6 months the soluble-N compounds gradually increase, indicating that the enclosed rennet stays active during this period. It can be seen that the production of soluble N is positively correlated with, but not proportional to, the amount of residual rennet in the cheese.

Very low levels of amino-acid N developed in the ripening ASF cheeses. A gradual increase from zero up to 0.80 % of TN (as an average) was observed during 6 months. The values at 6 months varied irregularly between

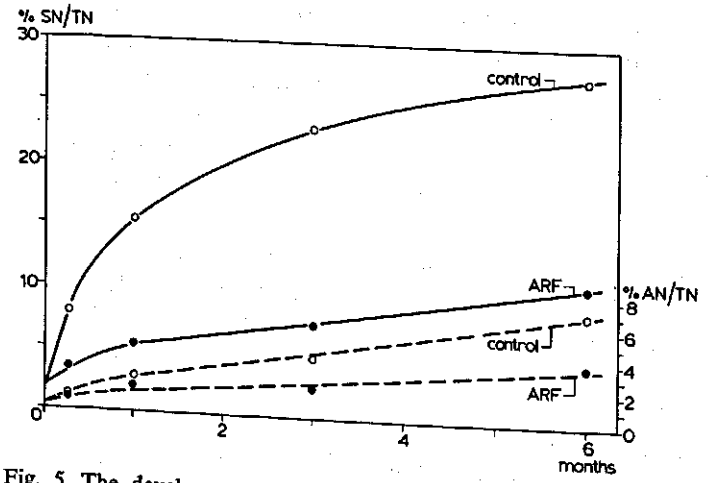

Fig. 5. The development of soluble N and amino-acid N during the ripening of an ARF and an ARF control cheese, made in a parallel experiment with strain AM1.
——— soluble N, - - - - amino-acid N.
●: ARF cheese; ○: ARF control cheese.

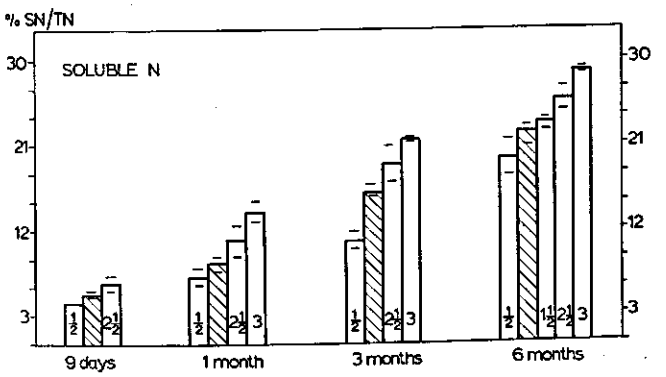

Fig. 6. The development of soluble N during the ripening of aseptic starter-free (ASF) cheeses containing different amounts of rennet. Rennet contents varied from half the normal amount (1/2) up to three times the normal amount (3) as indicated in the columns. The hatched columns represent ASF cheese containing a normal amount of rennet (average of 4 cheesemakings).

cheesemakings from 0.5 to 1.2 %. From parallell experiments it appeared that the amount of amino-acid N was independent of the level of residual rennet. The proteolytic activity of rennet appears to be directed largely to the liberation of soluble-N compounds.

O'Keeffe et al. (25) observed a very marked increase in the level of proteolysis in fresh ASF cheese when the 'Mabbit' method of acidification was used. This method induces an early and rapid decrease of the pH during manufacture. They suggested that the increased susceptibility of micellar caseins to proteolysis on the solubilization of the colloidal calcium phosphate could be the explanation for this phenomenon. However, we estimated such cheeses to contain about four times more rennet than usual (19).

To evaluate the effect of the ASF cheesemaking procedure on final proteolysis we made both ASF-lac and ASF-gal cheeses (19) from the same portion of milk. ASF-lac cheese was made by approximately the 'Mabbit' method

Table 3. The development of soluble N in two ASF cheeses made in a parallel experiment in different ways.

Cheese	Rennet conc.		% calcium in dry matter	pH	% moisture	% SN/TN	
	ml/40 l milk	µl/kg cheese				9 days	1 month
ASF-gal	14	500	1.10	4,99	42,2	5.6	10.3
ASF-lac	5	460	0.55	5,05	40,2	5.3	10.7

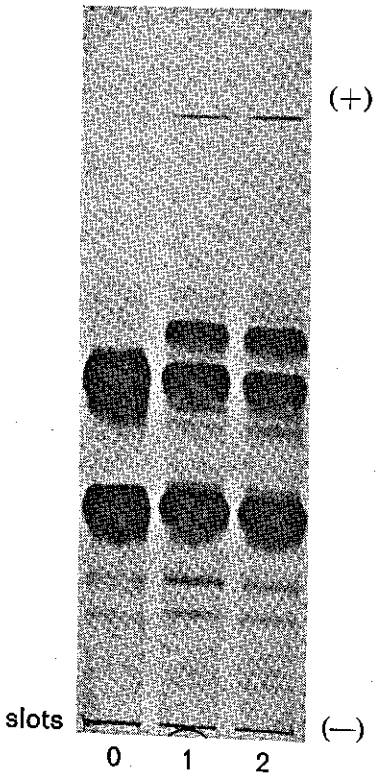

Fig. 7. Polyacrylamide gel electrophoretic pattern of two ASF cheeses at 9 days of ripening.
Slot 0: reference sample: cheese curd at 2 hours; Slot 1: ASF-lac cheese (made according to 'Mabbit' method); Slot 2: ASF-gal cheese.

in Gouda cheesemaking; ASF-gal cheese was made by the method normally employed for our ASF cheeses. Such amounts of rennet were added to the milk as to result in about equal concentrations of residual rennet in the two types of cheese. The results of the experiment are presented in Table 3. In Fig. 7 the gel electrophoretic patterns of both cheeses are compared. It can be seen that no increase in the level of proteolysis occurred in the ASF-lac cheese as compared to ASF-gal cheese. A second, identical experiment confirmed this. As a consequence it now can be concluded that the apparent difference in proteolysis between different ASF cheeses must be ascribed to the different concentrations of rennet present in these cheeses. The considerable reduction of calcium phosphate had only a limited – if any – influence on the proteolysis.

3.1.4 Aseptic rennet-plus starter-free (ARSF) cheeses. The only proteolytic enzyme system that could be active during the ripening of ARSF cheeses is milk protease, since this enzyme survives the pasteurization of the cheese

milk (1, 33) and since aseptically drawn milk with extremely low bacterial counts was used for aseptic cheesemaking.

Two ARSF cheeses were made which had a normal Gouda cheese composition, and followed as to development of soluble N and amino-acid N during the ripening. The results are presented in Fig. 8. As can be seen, the cheeses did indeed show a gradual increase in these nitrogen fractions, indicating that milk proteolytic enzymes do contribute to proteolysis in Gouda cheese.

Also after the ripening period of 6 months we could still detect increases in the values of soluble N and amino-acid N, showing the stability of the enzyme system under cheese conditions.

The accumulation of soluble N compounds and amino acids, however, is relatively very low as compared to the values found in normal aseptic cheeses. The low pH occurring in cheese will have been unfavourable for milk protease action (33).

3.2 Gel filtration of soluble-N fractions

Soluble-N extracts from a number of aseptic 'key' cheeses at different stages of ripening were fractionated by molecular sieving on Sephadex G-50. In this way the molecular-weight (MW) distributions of soluble products of hydrolysis in normal aseptic, ASF and ARF cheeses could be followed and compared during the ripening. An ARSF cheese was analysed after 6 months of ripening only. In Fig. 9 we have shown the elution patterns. In the case of starter-containing cheeses we have entered both the patterns of cheeses made with the 'non-bitter' starter E8 and the 'bitter' starter HP.

Clear differences appear to exist between the different types of cheese. All the starter-containing cheeses show patterns with relatively high proportions of material eluted in the lower-MW regions, as contrasted with the ASF cheeses. Also the patterns of normal aseptic cheeses made with starter

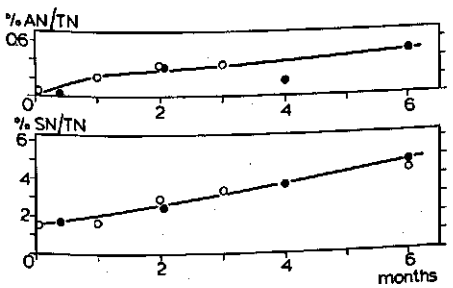

Fig. 8. The development of soluble N and amino-acid N during the ripening of ARSF cheeses.
●: cheesemaking experiment 50507;
○: cheesemaking experiment 50220.

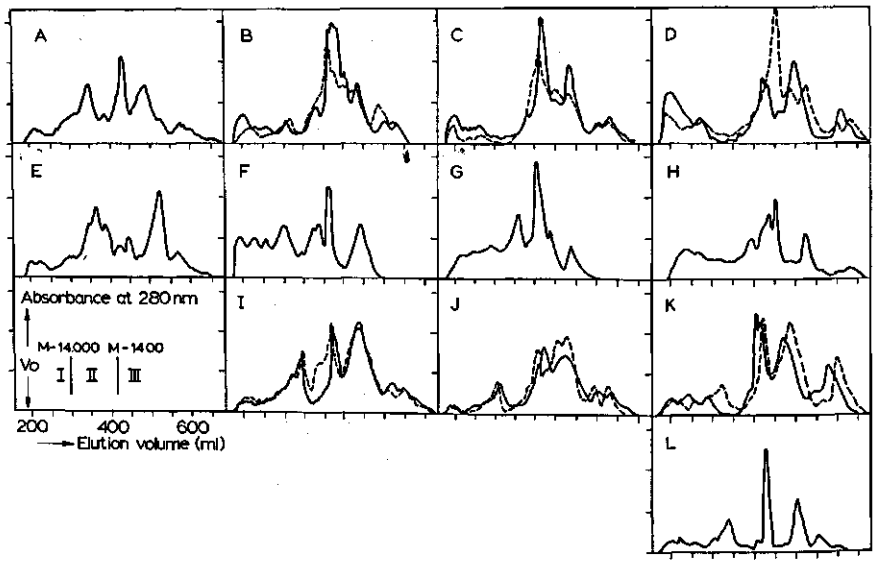

Fig. 9. Elution patterns of soluble-N extracts from aseptic cheeses at different stages of ripening. Gel filtration was performed on Sephadex G-50 with 0.01 *M* acetic acid at 3-4 °C.
Patterns A, B, C, D: normal aseptic cheese at 9 days, and 1, 3 and 6 months of ripening, respectively. ——— starter E8; - - - - starter HP.
Patterns E, F, G, H: ASF cheese at 9 days, and 1, 3 and 6 months of ripening, respectively.
Patterns I, J, K: ARF cheese at 1, 3 and 6 months of ripening, respectively. ——— starter E8; - - - - starter HP.
Pattern L: ARSF cheese at 6 months of ripening.
The normal aseptic and ASF cheeses contained comparable, normal amounts of rennet.

HP and E8 show differences. An important peak, representing small peptides, develops during the ripening of E8 cheese, accompanied by a decrease in the main peak of the pattern. With starter HP, however, a leading peak at 450 ml elution volume develops. The same differences between these starters were not recognized in the ARF cheese patterns, indicating that somehow the differences must originate from the interactions between rennet and starter bacteria. The gel filtration pattern of a 6-month old ARSF cheese, in which only milk protease has been active, shows three distinctly separated peaks, two of which represent lower-MW products.

The material eluted from the void volume up to about 260 ml – representing mostly a minor fraction of the soluble N – should have molecular weights above 20 000 and consequently should correspond to unaffected paracasein. Noomen (28), however, showed our soluble-N extracts to contain no unat-

tacked casein. It is possible that this fraction is composed of some smaller breakdown products in an aggregated state.

It is very difficult to compare our elution profiles directly with those obtained by other research workers (20, 22, 24, 34, 35) in various cheeses, because – apart from different cheeses – different extraction methods, materials and conditions for chromatography were used.

As far as our extraction method for soluble N is concerned, we found corresponding elution profiles for N extracts soluble at pH 4.6 made according to O'Keeffe et al. (20) and Gripon et al. (24) using the same cheese. Fig. 10 shows the patterns of the three different extracts.

For a more quantitative approach all patterns were subdivided into three molecular weight classes and the material eluted per class was expressed as a percentage of soluble N. Fig. 11 shows the results of this subdivision. Because in class III, with MW < 1400, the free amino acids are also included, we have introduced in this figure the percentages of amino-acid N as mentioned in Section 3.1.

It can be seen that, with the exception of ASF cheeses, the low-MW peptides and amino acids are dominant in the soluble N of the cheeses. During the ripening the relative importance of this fraction seems to increase somewhat. No clear difference in the percentages of the low-MW class could be observed when cheeses were compared – both ARF and normal aseptic – made with starters E8 and HP.

In ASF cheese, where rennet is the main proteolytic agent, the higher and medium MW classes of breakdown products are represented in distinctly higher percentages than in ARF cheese, indicating that the proteolysis of rennet is not as deep as that of starter bacteria.

Milk protease on its own appears to liberate preferentially low-MW peptides in cheese.

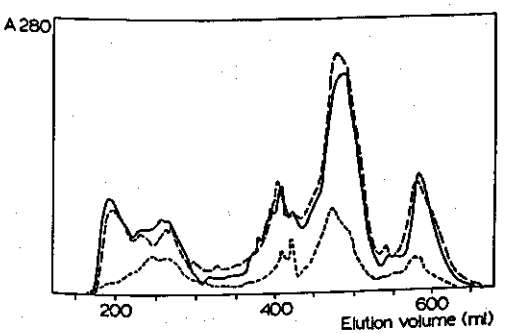

Fig. 10. Elution patterns of 3 different extracts from a 12 months old normal E8 cheese.
——— soluble-N extraction as desribed in this paper.
- - - - extraction of N soluble at pH 4.6, as described by O'Keeffe et al. (20).
..... extraction of N soluble at pH 4.6, as described by Gripon et al. (24).
Equal quantities of dry matter were applied to the column.

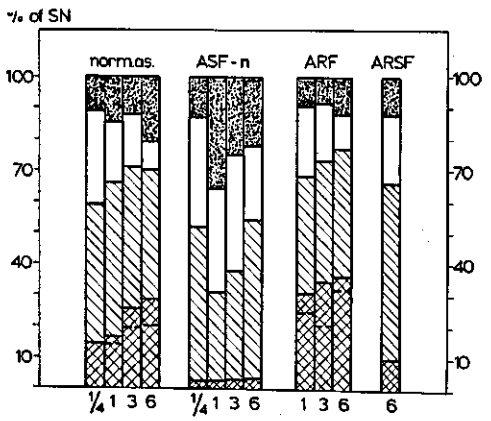

Fig. 11. Distribution of soluble-N compounds from different aseptic cheese types in molecular weight classes as deduced from the gel filtration patterns and determinations of amino-acid N.
Dotted area: products with MW $> 14\,000$; open area: products with $1400 < MW < 14\,000$; hatched + double-hatched area: products with $MW < 1400$; double-hatched area: amino acids.
The average percentage of amino acids in 'bitter'-starter cheeses is indicated by marks (- -).
Numbers under the columns refer to the ripening stage: ¼ (9 days), 1 (month), 3 (months), 6 (months).

3.3 Amino-acid profiles

One pair of normal aseptic cheeses, two pairs of ARF cheeses, one ASF-n and one ARSF cheese were analysed after 6 months of ripening for the concentration of individual free amino acids. Each pair of cheeses was made in a parallel experiment with a 'bitter' and a 'non-bitter' starter strain. Table 4 presents the results. As could be expected from the amino-acid N results, the quantity of amino acids varied strongly from one cheese to another. The ASF and ARSF cheeses both showed extremely low levels of amino acids as compared to the other, starter-containing, cheeses. Nevertheless the amino acids leucine, phenylalanine, arginine and methionine appear to be accumulated preferentially in the ASF cheese since these are present in far higher amounts than would be expected from the composition of casein. The alanine originates from the cheese-milk since this amino acid was added to the milk in this experiment as a spore-germination stimulant (19). The amino-acid pattern of the ARSF cheese is clearly different from that of the ASF cheese. Lysine, serine, glutamic acid and leucine have accumulated more than the other amino acids. In both cheeses hardly any free proline is present.

In Fig. 12 the quantities of the individual amino acids are expressed as a percentage of the total amount. For reasons of comparison the amino acid composition of whole casein is entered in the figures.

In Fig. 12A it can be seen that only very slight differences exist in the ratio of amino acids between the bitter and non-bitter, normal aseptic cheese. Others (34) have made the same observation for bitter and non-bitter Cheddar cheeses. For both cheeses the ratio is distinctly different from that in casein. Glutamic acid, isoleucine, aspartic acid, serine and particularly proline

Table 4. Concentration (mg/g) of individual free amino acids in normal aseptic, ARF, ASF and ARSF cheeses at 6 months of ripening.

Amino acid	Normal aseptic cheese		Aseptic rennet-free cheese				Aseptic starter-free cheese	ARSF cheese
	E8	Wg2	AM2	Wg2	E8	HP		
Lysine	2.02	1.04	2.57	1.05	1.52	0.64	0.09	0.03
Histidine	0.22	0.22	0.29	0.17	0.15	0.13	0.03	0.01
Arginine	1.18	0.60	0.68	0.30	0.46	0.21	0.14	0.01
Aspartic acid	0.45	0.19	0.32	0.22	0.21	0.10	0.01	0.005
Threonine	1.55	0.73	1.13	0.69	0.70	0.32	0.01	0.01
Serine	0.54	0.30	0.62	0.64	0.44	0.34	0.03	0.02
Glutamic acid	3.83	1.80	3.40	2.08	2.83	1.24	0.09	0.04
Proline	+	0.36	0.69	0.50	0.53	0.40	+	+
Glycine	0.37	0.13	0.29	0.13	0.19	0.07	0.01	0.01
Alanine	0.50	0.25	0.41	0.37	0.31	0.20	0.22*	0.01
Valine	1.13	0.64	1.04	0.80	0.74	0.39	0.05	0.01
Methionine	0.66	0.37	0.54	0.48	0.41	0.30	0.08	0.005
Isoleucine	0.65	0.17	0.93	0.34	0.60	0.16	0.02	0.01
Leucine	3.34	1.79	1.85	1.35	1.32	0.79	0.31	0.02
Tyrosine	1.00	0.46	0.50	0.39	0.31	0.28	0.02	0.01
Phenylalanine	1.67	0.99	0.90	0.70	0.70	0.53	0.26	0
Total	19.11	10.04	16.16	10.21	11.42	6.10	1.37*	0.20
Calculated from AN/TN:	16.90	9.30	16.60	9.10	11.60	6.40	2.20	1.20
Total in 'peptide material'	25.05	28.92	11.66	17.01	9.36	17.71	19.95	5.01

* Alanine was added to the cheese milk.

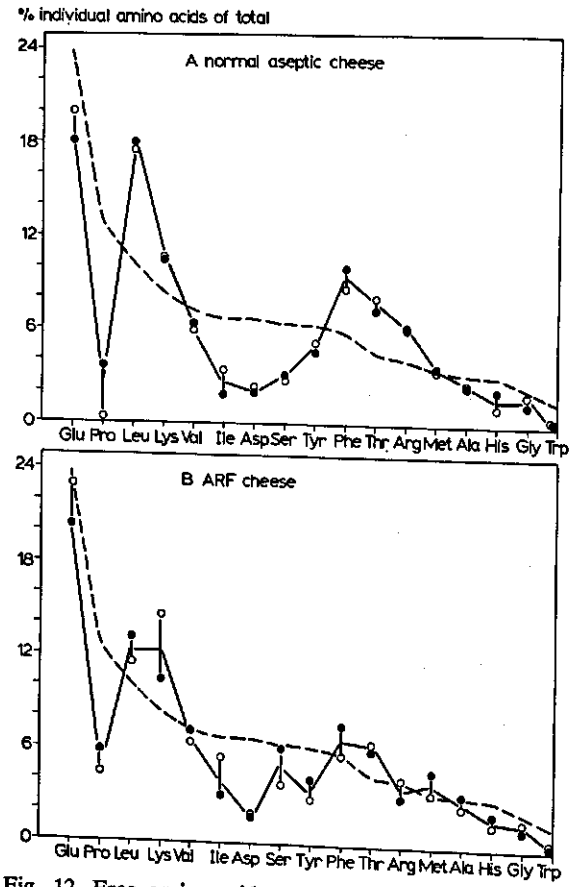

Fig. 12. Free amino-acid contents of different cheeses at 6 months of ripening. The results are expressed as g of the particular acid per 100 g of total free amino acids. The amino-acid composition of whole casein is shown for comparison (dotted line).
A. Normal aseptic cheeses; ○: starter E8 (non-bitter); ●: starter HP (bitter).
B. ARF cheeses; ○: 'non-bitter' starters); ●: 'bitter' starters.

are represented in far lower percentages. In contrast, phenylalanine, threonine, arginine and especially leucine appear to be over-represented.

As the amino acid ratios of the two bitter ARF cheeses (Wg2 and HP) harmonized very closely and as the same was true for the two non-bitter ARF cheeses (E8 and AM2), the average values for both the bitter and the non-bitter cheeses are given in Fig. 12B. Now – in the absence of rennet – somewhat more differences appear to occur between both pairs of cheeses. Lysine and isoleucine are accumulated in relatively higher amounts by the 'non-bitter' strains. Strain E8 in particular liberated somewhat more glutamic acid. Leucine, serine, phenylalanine and proline were represented in higher

percentages in the two bitter cheeses. However, the differences are still not very important. In the ARF cheeses proline and aspartic acid are liberated in low percentages in comparison to the composition of casein. The accumulation of leucine and phenylalanine is less pronounced as in the normal aseptic cheeses.

The amino acid tryptophan was not detected in our cheeses, neither were the products homocitrulline, 4-amino butyric acid or 2,4-diamino butyric acid.

In the HCl-hydrolysed extracts considerably higher amounts of amino acids were detected than as free amino acids. This indicates that most probably some peptides were not precipitated by the ethanol. From the amino acid profile of the hydrolysate and the above-mentioned free amino acid profile the amino acid composition of this peptide-material was calculated. Generally the different cheeses showed corresponding amino acid ratios, approaching the casein ratio. Proline was present in slightly higher percentages than in casein which contrasts considerably with the quantity of free proline mentioned above.

Obviously proline is difficult to attack in the peptides by the peptidases present in the cheeses. It was noticed casually – as can be seen at the bottom of Table 4 – that for the bitter-starter cheeses the amount of this peptide material was higher than for non-bitter starter cheeses.

4 Discussion

The experiments with normal aseptic cheeses, in which rennet, starter bacteria and milk protease act together, showed that the amount of residual rennet in the cheese has a distinct influence on accumulation of soluble-N compounds but not of amino acids. On the contrary, the strain of starter used strongly influences the liberation of amino-acid N and to only a minor degree the soluble-N production. Because the soluble-N values, as determined in our cheeses, give information on the 'extent' or 'width' of the protein breakdown and the values of amino-acid N on the 'depth', these results suggest that rennet influences the 'extent' of proteolysis and starter bacteria the 'depth'.

To evaluate more quantitatively the respective roles of rennet, starter bacteria and milk protease in cheese proteolysis and to estimate the possible interactions we have surveyed the most important results on development of soluble N and amino-acid N in Fig. 13A and 14, respectively. For the starter-containing cheeses we have presented the average results of 'non-bitter' starters.

As can be seen, the ASF cheeses, containing normal amounts of rennet,

show the development of high levels of soluble N, indicating the importance of rennet for soluble-N production. However, the soluble-N production in these cheeses is less than in normal aseptic cheeses, indicating that in addition to rennet (plus milk protease) the starter bacteria also contribute to soluble-N production. Fig. 13A indeed shows that in ARF cheeses distinct amounts of soluble N are produced, giving evidence that starter bacteria are capable of attacking paracasein in cheese and of converting it to soluble products, independently of the breakdown products of rennet action. Several authors thought that starter bacteria were scarcely capable of doing so (20, 36).

Some researchers working with ASF cheeses found these cheeses to develop more soluble N than did their starter cheeses (4, 37). According to our results (19), however, this will almost certainly have been due to the higher concentrations of rennet remaining in their ASF cheeses.

Before pronouncing upon possible interactions, the soluble-N values of the different cheeses need corrections. In the ASF and ARF cheeses the enzyme systems of rennet and starter bacteria act together with the milk proteolytic enzyme(s) during the ripening. As was demonstrated in ARSF cheeses, milk protease on its own contributes to a minor, but measurable, degree to soluble-N production.

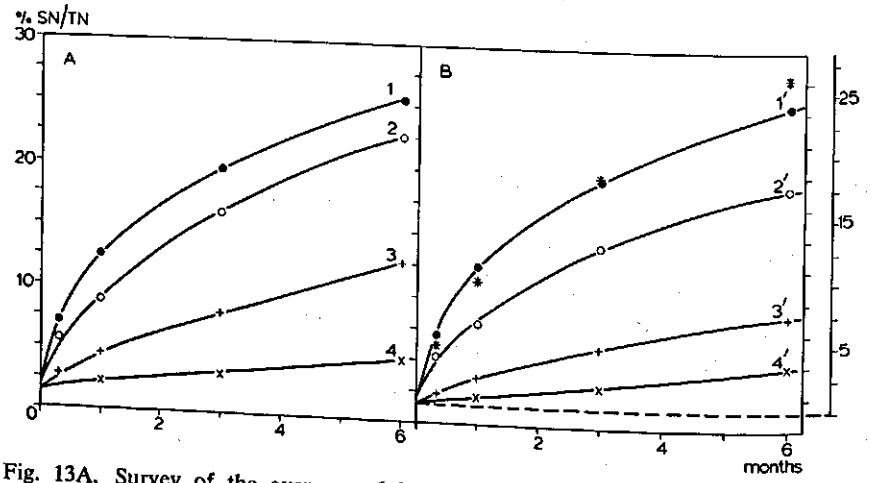

Fig. 13A. Survey of the average soluble-N values developed during the ripening of normal aseptic cheeses (1), ASF-n cheeses (2), ARF cheeses (3) and ARSF cheeses (4). Fig. 13B. Net contribution of rennet (2'), starter bacteria (3') and milk protease (4') to soluble-N production as compared with the net production in normal aseptic cheeses (1'). The values are derived from those in Fig. 13A correcting for milk protease action in ARF and ASF cheeses and for 1½ % SN/TN present in all cheeses at zero time. Key: 1' = 1 - (1½ %); 2' = 2 - 4; 3' = 3 - 4; 4' = 4 - (1½ %).
* The theoretical values of soluble-N production in normal aseptic cheeses as calculated from the separate productions, assuming no interactions.

To calculate the net contributions of rennet and starter bacteria we have subtracted the contribution of milk protease to soluble-N production from the values observed in the ASF and ARF cheeses. It is assumed in this that milk protease has no important stimulatory effect on the other enzymes. At the same time for all cheese types a correction has been made for the 1.5 % soluble N present in the cheese curd after 2 hours. In this way Fig. 13A has been transformed to Fig. 13B representing the net contributions of rennet, starter bacteria and milk protease to soluble-N development when acting separately in cheese. Their combined action is given by the curve of normal aseptic cheeses.

From Fig. 13B it is even more clear that in addition to rennet the enzymes of starter bacteria and milk protease must contribute to the production of soluble N in normal cheeses. Starting from the percentages of total N that are converted into soluble N by the separate actions, we have calculated (by multiplication of the fractions not attached) the theoretical soluble-N production that would result from the combined action, assuming no interactions to occur. These values are entered in Fig. 13B as asterisks (*) and appear to approach the values observed in normal aseptic cheeses. The addition of the separate actions of rennet, starter bacteria and milk protease thus covers for the greater part the width of proteolysis – as measured by soluble N – in normal cheeses. We have no reason to assume important mutual stimulatory effects between the enzyme systems with regard to soluble-N production, although we are aware of the restrictions of these calculations with the heterogeneous soluble-N fractions.

The fraction of amino-acid N contains well-defined end-products of proteolysis, and therefore conclusions as to interactions between enzymes can be drawn more easily.

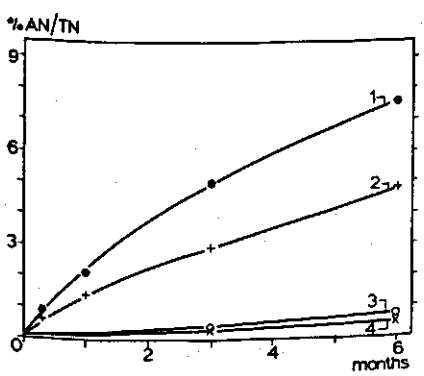

Fig. 14. Survey of the average values of amino-acid N developed during the ripening of normal aseptic cheeses (1), ARF cheeses (2), ASF-n cheeses (3) and ARSF cheeses (4). For starter-containing cheeses the average values of non-bitter cheeses are entered.

From Fig. 14 it is clear that the enzymes of starter bacteria are the only ones capable of liberating important amounts of amino acids. This agrees with the observations that *S. cremoris* strains possess several peptidase activities (38, 39) and that intracellular peptidases are released from the cells during cheese ripening (40).

Milk protease on its own appears to contribute significantly to the accumulation of amino acids in cheese, but at a low level. Moreno & Kosikowsky (41) reported amino acids to be liberated as products of β-casein hydrolysis by milk protease.

Since in the ASF cheeses, even at high rennet levels, low amounts of amino acids are accumulated, it is concluded that rennet is deficient in the ability to produce amino acids from paracasein. However, rennet has some function in the development of amino-acid N in ASF cheeses, since these cheeses always showed somewhat higher amounts of amino acids than did ARSF cheese. Reiter (4) suggested that the amino acids present in the ASF cheeses are derived principally from milk protease action.

Because in normal aseptic cheeses far higher levels of amino-acid N are accumulated than in corresponding ARF cheeses, the proteolytic action of rennet must stimulate the starter bacteria significantly to liberate amino acids. Apparently the concentration of rennet present in normal Gouda cheeses is high enough as to induce the maximum stimulatory effect (Fig. 2). More information as to the interactions between rennet and starter can be gained from the molecular weight distributions in the soluble-N extracts of the different cheeses.

As was demonstrated (Fig. 11), rennet converts the paracasein in Gouda cheese to soluble products of high, medium and low molecular weight, but hardly to amino acids. This generally agrees with the results of other workers (20, 24). As opposed to this, the action of starter bacteria in cheese is directed largely to the liberation of low-MW peptides and amino acids. Kikuchi et al. (17) found the same for the action of disintegrated cells of *S. lactis* on whole casein. Milk protease appears to liberate chiefly low-MW peptides.

In Table 5 the concentrations of the three soluble peptide fractions and the amino acid fraction resulting from the separate enzyme actions, are presented and compared with those resulting from their combined action in normal aseptic cheeses. These concentrations were calculated by combining the results on soluble-N production (Fig. 13) and the distributive patterns of Fig. 11.

It can be seen that rennet, in the absolute sense also, is the dominating agent for the production of peptides of high, medium and low molecular

Table 5. Concentration of soluble-N compounds as produced in cheese by the combined and separate actions of rennet, starter bacteria and milk protease and classified in molecular weight fractions.

Ripening time (months)	Fraction No ▶ Molecular weight ▶	I >14 000	II 14 000 - 1400	III* peptides <1400	IV amino acids	Total soluble N (Fig. 13)
1	cheese	1.8	2.3	6.1	2.0	12.2
	rennet	2.7	2.7	1.2	0.1	6.7
	starter	0.2	0.6	0.4	1.3	2.5
	milk protease (+ 1½ %)	(0.2)**	(0.4)	(1.3)	0.1	2.0
3	cheese	2.3	3.3	9.1	4.8	19.5
	rennet	3.6	5.2	3.7	0.2	12.7
	starter	0.3	0.7	1.4	2.3	4.7
	milk protease (+ 1½ %)	(0.4)	(0.7)	(1.9)	0.3	3.3
6	cheese	5.5	2.3	10.8	7.4	26.0
	rennet	4.4	4.1	8.4	0.3	17.3
	starter	0.9	0.3	2.4	4.0	7.6
	milk protease (+ 1½ %)	0.5	1.0	2.7	0.5	4.7

All values are expressed as percentages of total nitrogen. The values for rennet and starter bacteria represent the net contributions (curves 2' and 3', Fig. 13). The values for cheese and milk protease are not corrected for the 1½ % soluble-N (curves 1 and 4, Fig. 13).
* Obtained by subtraction of amino acid N from original fraction III.
** The values between brackets are calculated by the MW distributive pattern after 6 months.

weight during the ripening of cheese. Starter bacteria occupy the same place with regard to amino acid production.

During the ripening the normal aseptic cheese shows lower amounts of high- and medium-MW products than would have been expected to be present due to the separate enzyme actions. After six months this is also true for the low-MW peptides. On the other hand distinctly higher amounts of low-MW peptides and amino acids are present in the cheese.

Although the soluble-N compounds of only a restricted number of cheeses have been fractionated, these results indicate strongly that the proteolytic system of starter bacteria progressively converts high- and medium-MW soluble products of rennin action to low-MW peptides and amino acids. Possibly the peptidase system as released in the cheese by starter bacteria has a certain qualitative or quantitative over-capacity with respect to the starter-proteinase system.

The specific accumulation of phenylalanine, leucine and arginine found to occur in the ASF cheese is in general agreement with the results of Yamamoto (42), who used non-sterile starter-free Cheddar cheese, and those of Reiter et al. (4), who used ASF Cheddar cheese. The valine percentages found by the latter were considerably higher, however. The results of O'Keeffe et al. (20) could not be confirmed.

Although the total quantity of free amino acids differed considerably in ARF cheeses with different starters, the ratio of individual amino acids in these cheeses showed only relatively small differences. This indicates that no important differences in the specificity to liberate certain amino acids exist between our starter strains, whether 'bitter' or 'non-bitter'. Moreover no individual amino acids were liberated in appreciably higher percentages than would be expected from the composition of casein.

Zvyagintsev et al. (43) thought that the free glutamic acid produced in culture media would be a criterion for the selection of non-bitter starter strains. Although in our cheeses 'non-bitter' starters did indeed produce higher amounts of free glutamic acid, in the absolute sense, than did 'bitter' starters, this criterion seems to be related only to a minor degree with the specificity of the starters to liberate glutamic acid.

In the ARSF cheese only lysine was accumulated in higher amounts than would be expected from the casein composition. The cleavage of β-casein, known to occur in these cheeses (to be published), liberating products such as γ-, TS- and R-caseins (2), results in the preferential exposure of lysine residues in the terminal position.

The differences in free amino acids, qualitatively as well as quantitatively,

between the ASF and the ARSF cheese clearly suggest that rennet contributes to the formation of the low level of amino acids in ASF cheese. More research is needed on this aspect.

For the profile encountered in our normal aseptic cheeses there exists a good agreement with the patterns observed by Antila (44) in Gouda-type and Edam-type cheeses. The profiles found by Ali & Mulder (45) in open-vat Edam cheeses approach the casein ratio more closely than do ours. The profiles found in aseptic (4) and open-vat (34) Cheddar cheeses show some resemblance to our results.

The small amounts of free proline in the starter-containing cheeses are somewhat unexpected, since starter bacteria seem to possess the required peptidases to attack proline in the peptides (38, 39).

The higher percentages and absolute levels of leucine, phenylalanine and arginine in these normal aseptic cheeses as compared with ARF cheeses cannot be accounted for by the observed accumulation of just these amino acids by rennet, as observed in ASF cheeses. The accumulation is too low, in the absolute sense, in the latter. However, since it is known that rennet preferentially cleaves peptide bonds in hydrophobic regions, liberating products in which phenylalanine and leucine are progressively exposed in the terminal positions (8), it could be possible that starter exopeptidase action on these 'ready-made' peptides is responsible for the observed effect. This would be in agreement with the rennet-starter interaction discussed earlier.

As was shown previously (26), 'bitter' starter HP produces distinct bitterness in ARF cheeses from one month of ripening onwards. A normal amount of rennet is capable of doing this in ASF cheese only after longer ripening periods, from about 3 months onwards. It has been shown in this paper that in the corresponding periods in ASF cheeses far more soluble N is produced than in ARF cheeses. This also holds true for the low-MW peptide fraction in which most of the bitter peptides are contained in these cheeses (Visser, to be published). It can therefore be concluded that the liberation of bitter peptides from casein by 'bitter' starter bacteria proceeds far more specifically than by rennet. This is also supported by the gel electrophoretical studies of these cheeses, which will be published in the next paper.

Clear differences in soluble-N production could not be detected between 'bitter' and 'non-bitter' starters, either in the presence or absence of rennet. However, 'non-bitter' starters accumulate higher levels of free amino acids in ARF and normal aseptic cheeses than do 'bitter' starters. Lawrence et al. (46) and Emmons et al. (47) made similar observations in normal, open-vat Cheddar cheeses. As a consequence, 'non-bitter' starters could be thought

to possess a more powerful peptidase system and to degrade bitter peptides in cheese more intensively than 'bitter' starters. However, more qualitative differences in the peptidase systems also seem to play some part, since the starter Z8 – producing less bitterness than HP and Wg2 – has by far the least capacity to produce amino acids. No clear reasons for this are to be found in the profiles of free amino acids produced by different starters in the cheeses.

Qualitative differences in the low-MW fraction of soluble-N compounds exist between normal cheeses made with starter E8 and HP. Sinse these differences were not encountered in the ARF cheeses, they could result from the interaction with rennet. However, far more data are necessary, with other starters, to come to a decision in this respect.

In a previous paper (26) attention has been paid to the development of cheese flavour in our different aseptic cheeses. There appears to exist a striking relation between cheese-flavour scores and amino-acid-N values of the cheeses. This holds true both for the differences between 'bitter' and 'non-bitter' starters and for the role played by rennet, starter bacteria and milk protease. Even the stimulatory effect of rennet on amino acid production by starter bacteria corresponds to the effects on cheese flavour development. Although amino acids are thought to contribute only to the basic, background taste of cheese (45), these results emphasize the importance of deep proteolysis for the development of more specific cheese flavour constituents.

Acknowledgments

The author is indebted to Mr A. J. Vos, for his co-operation in gel filtration experiments, and to Mr K. J. Slangen of the Netherlands Institute for Dairy Research at Ede, for performing the amino acid analysis. The analytical assistance of Mr H. J. van der Stege and Aliza de Groot-Mostert was highly appreciated.

This study was made possible by the financial support of the Stichting J. Mesdagfonds, instituted by Kaascontrolestation Friesland, Leeuwarden.

Samenvatting

F. M. W. Visser, *De bijdrage van de enzymen van stremsel, zuurselbacteriën en melk aan de eitwitafbraak en smaakontwikkeling in Goudse kaas. 3. Eiwitafbraak: analyse van de oplosbare stikstofverbindingen en aminozuren*

In aseptisch bereide Goudse kazen werd de eiwitafbraak bestudeerd. In deze kazen kon de werking van stremsel en/of zuurselbacteriën tijdens de rijping worden uitgeschakeld. Op deze wijze kon de bijdrage van stremsel, zuurselbacteriën en melkprotease aan de produktie van oplosbare stikstofverbindingen en vrije aminozuren worden vastgesteld. Voor een kwantitatieve aanpak was het noodzakelijk de in kaas ingesloten hoeveelheden stremsel te bepalen.

Stremsel bleek verreweg de grootste bijdrage te leveren aan de produktie van oplosbare stikstofverbindingen in Goudse kaas. Echter ook de zuurselbacteriën en – in mindere mate — melkprotease maakten ze vrij uit paracaseine zonder de hulp van stremsel.

Door vergelijking van de afzonderlijke bijdragen aan de vorming van oplosbare stikstofverbindingen met het totale gehalte ervan in normale aseptisch bereide kazen, kon worden vastgesteld dat er geen belangrijke onderlinge stimulatie dan wel remming in de produktie van deze stoffen optreedt.

Aminozuurbepalingen en analyse van de oplosbare verbindingen met behulp van gelfiltratie toonden aan dat stremsel vooral peptiden, zowel grotere als kleinere, vrijmaakt en slechts in zeer geringe mate aminozuren. Zuurselbacteriën daarentegen bleken de paracaseïne in kaas hoofdzakelijk af te breken tot kleine peptiden (M <1400) en – afhankelijk van de gebruikte zuurselstam – grote dan wel minder grote hoeveelheden vrije aminozuren. Melkprotease bleek na zes maanden kaasrijping kleine hoeveelheden aminozuren en kleinere peptiden op te hopen.

In normale kazen, waarin de enzymsystemen dus gezamenlijk werken, bleek stremsel de zuurselbacteriën zeer duidelijk te stimuleren tot de aanmaak van grotere hoeveelheden aminozuren en kleinere peptiden. Klaarblijkelijk breken de zuurselpeptidasen de door stremsel gevormde grotere, oplosbare produkten versneld af.

De concentraties van individuele aminozuren in de verschillende kazen werden bepaald en bediscussieerd.

Verschillen in ingesloten hoeveelheden stremsel bleken volledig verantwoordelijk te zijn voor de verschillen in proteolyse-snelheid waargenomen in zuurselvrije kazen die een verschillend calciumgehalte vertoonden door verschillende methoden van bereiding.

Bij de aseptische kaasbereidingen werden tijdens dit onderzoek zowel 'bittere' als 'niet-bittere' zuursels toegepast. Daarom worden de resultaten ook met het oog op de smaakontwikkeling in kaas bediscussieerd.

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Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 4. Protein breakdown: a gel electrophoretical study

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Received: 13 June 1977

Summary

Quantitative polyacrylamide gel electrophoresis (PAE) was used to study the primary breakdown of α_{s1}- and β-casein in aseptically made Gouda-type cheeses. Any action of rennet and/or starter bacteria during the ripening could be eliminated in these cheeses.

In normal aseptic cheeses α_{s1}-casein is attacked rapidly, the degradation being nearly complete after one month of ripening. β-casein is more resistant to proteolysis; after 6 months ripening about 50 % is still intact.

From starter-free cheese experiments it was concluded that rennet – in the concentration as present in Gouda cheese – is completely responsible for the observed degradation of α_{s1}-casein and for the decomposition of β-casein during the first month of ripening. In the longer term starter bacterial enzymes also appear to contribute, especially in the further degradation of β-casein. The results of rennet-free cheese experiments are in agreement with this, since starter bacteria are able to degrade part of the α_{s1}- and β-casein in these cheeses after some months of ripening. This activity is relatively low during the first month of ripening.

No differences were observed in the PAE patterns of cheeses made with different starters – both 'bitter' and 'non-bitter' – neither in the presence nor in the absence of rennet.

From the rennet- and starter-free cheeses it appeared that milk protease is responsible for the formation of the 'minor caseins' from β-casein during the ripening of Gouda cheese. Its total contribution to the α_{s1}- and β-casein degradation, however, is low in relation to that of rennet and of starter bacteria.

The results are also discussed in combination with those of a previous paper (2) in which the soluble-N and amino acid-N fractions were characterized.

1 Introduction

To study proteolysis and (bitter) flavour development in Gouda cheese ripening, cheeses were made aseptically and were ripened by the separate

and combined actions of rennet, starter bacteria and milk protease (1). In the preceding paper (2) we reported on the contribution of these enzyme systems to the protein breakdown in characterizing the development and composition of soluble-N and amino acid-N fractions.

Gel electrophoretical methods are capable of high resolution and are increasingly used in studies of casein hydrolysis, both in cheese and in solution. They give information on the nature and extent of casein attack.

Up to now these methods have rarely been used to study proteolysis in Gouda cheese (3, 4, 5, 6), and far more reports exist on Cheddar cheese proteolysis (5, 7, 8, 9 and others). De Jong (10) recently showed that the degradation of α_{s1}- and β-casein in cheese can be followed quantitatively by polyacrylamide gel electrophoresis, and described in this way the protein breakdown in a Dutch soft cheese type (11).

The specificity of proteolytic enzymes – particularly of rennet – towards α_{s1}- and β-caseins has been investigated rather extensively in solutions (12, 13, 14, 15, 16, 17), and some of the primary breakdown products identified have also been found to occur in cheese (5, 18). It is generally accepted that in hard cheese varieties such as Gouda and Cheddar α_{s1}-casein is degraded more easily than is β-casein (19). Several workers have reported that para-κ-casein is not degraded during cheese ripening (14, 20, 21).

As to aseptically made cheeses, gel electrophoretic results on the types of proteolysis — occurring in the presence and absence of starter bacteria – were reported by three research groups. French workers (22, 23) reported on aseptically made model-curd, O'Keeffe et al. (24, 25) on Cheddar cheese and Ohmiya (26) on 'rennet-curd'.

In the present paper the breakdown of α_{s1}- and β-casein will be described quantitatively for aseptically made Gouda type cheeses, ripened by the separate and combined action of rennet, milk protease and starter bacteria, both 'bitter' and 'non-bitter'.

2 Materials and methods

2.1 Cheeses

Normal aseptic, aseptic starter-free (ASF), aseptic rennet-free (ARF) and aseptic rennet- and starter-free (ARSF) cheeses were made as described previously (1). They had the normal composition of Gouda cheese (1).

Seven different starter cultures were used, 6 of which were *S. cremoris* strains (E8, AM1, AM2, Wg2, HP and Z8) and one was a mixed strain starter (Bos). Strains Wg2, HP and Z8 were shown to produce bitter cheeses (27).

2.2 Gel electrophoresis

Quantitative polyacrylamide gel electrophoresis (PAE), including densitometry, was carried out as described by de Jong (10). The cheeses were analysed at 9 days, 1, 3 and 6 months of ripening.

Total protein was estimated in the cheeses as described previously (2). For PAE-sample preparation as much cheese was dissolved in 25 ml Tris-HCl buffer pH 8.5, containing 8 *M* urea, as to get exactly 2.0 % protein in solution. After centrifugation at 12 000 *g* at 6 °C the fat was removed. After filtration the solution was diluted to a protein content of 0.5 % and 50 μl of this was used for PAE.

From the densitograms the proportions of α_{s1}- and β-casein were calculated in comparison with the proportions present in reference samples from fresh cheese (2 hours old).

3 Results

3.1 Normal aseptic cheeses

Using all the seven starter cultures, normal aseptic cheeses were made containing amounts of rennet comparable with those in Gouda cheese (280 μl/kg). In Fig. 1 an example is given of the breakdown of α_{s1}- and β-casein during the ripening of such a cheese. The PAE patterns of all the cheeses proved to be identical, irrespective of the strain of starter used. Consequently

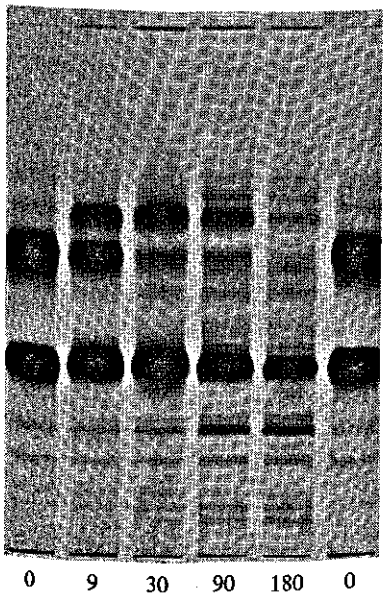

Fig. 1. The PAE patterns of a normal aseptic Gouda-type cheese. The numbers refer to the age of the cheese in days.

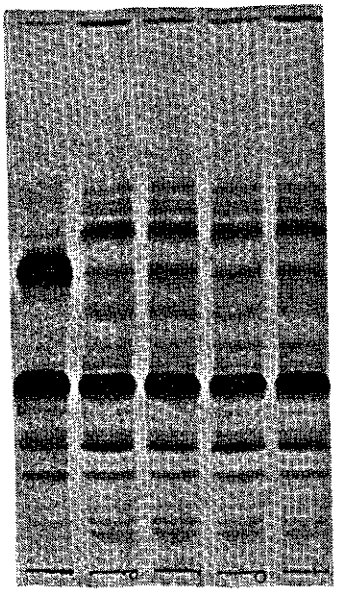

Fig. 2. The PAE patterns of 4 normal aseptic cheeses made with different starter strains. The cheeses were ripened for 3 months. Slot No 1 and 2: cheeses made with strain AM2 and HP, respectively, in a parallel experiment. Slot No 3 and 4: cheeses made with strains AM1 and Z8, respectively, in another parallel experiment.

no differences could be observed between cheeses made with 'bitter' and 'non-bitter' starters, which is in agreement with the results of Richardson & Creamer for open-vat Cheddar cheese (21). To demonstrate this in Fig. 2

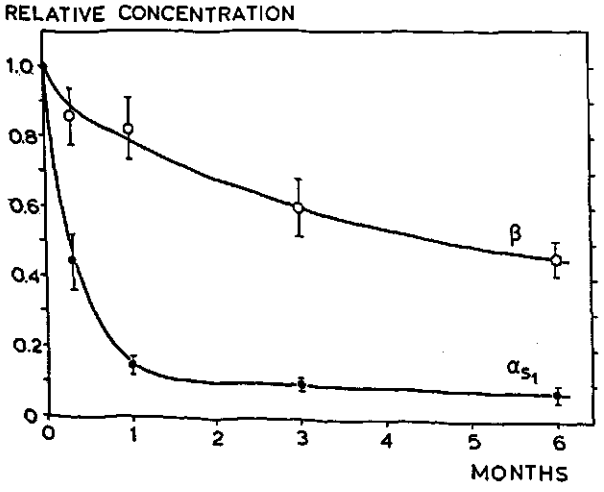

Fig. 3. The decomposition of α_{s1}- and β-casein during the ripening of normal aseptic cheeses containing normal amounts of rennet (280 μl/kg cheese). Averages and extreme values of 8 different cheeses are shown.

the 3-month PAE patterns of two pairs of cheeses are given, made in parallel experiments with different starters.

In Fig. 3 the residual proportions of α_{s1}- and β-casein during the ripening are presented as calculated from the densitograms. The average and extreme values of 8 different cheeses are given.

It can be seen that the α_{s1}-casein fraction is degraded very intensively early in the ripening. Within a period of 1 month almost all the α_{s1}-casein has disappeared and only a faint zone, representing 7 - 10 % of the original quantity, remains detectable during further ripening. α_{s1}-casein is decomposed primarily into a product with a slightly higher mobility, most probably identical with α_{s1}-I (18). From about 1 month of ripening onwards this product is broken down further to smaller compounds, at least three of which move in front of α_{s1}-I on the gel, the others being too small to be detected anymore.

β-casein degradation proceeds much more slowly and less extensively than degradation of α_{s1}-casein. Its proportion decreases gradually during the ripening resulting in about 50 % being unattacked at 6 months.

From 1 month of ripening some zones between α_{s1}- and β-casein are visible. They show no regular increase during further ripening but could originate from β-casein.

During the ripening, increasing amounts of some low-mobility products

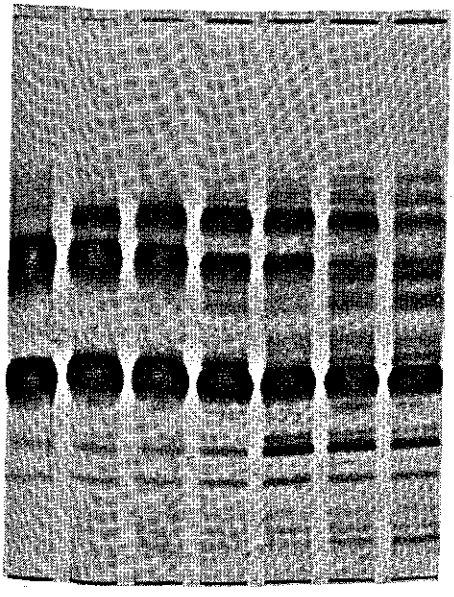

Fig. 4. The PAE patterns of 3 normal aseptic cheeses made with the same starter (E8) but with different amounts of rennet. Rennet amounts used: ½ n, n and 2 n (n is normal).

Table 1. Relative proportions of unattacked α_{s1}- and β-casein present during the ripening of two aseptic cheeses made with different amounts of rennet.

Age of cheese	α_{s1}-casein		β-casein	
	½ n	2 n	½ n	2 n
0 days	1.0	1.0	1.0	1.0
9 days	0.70	0.28	0.91	0.78
1 month	0.31	0.09	0.80	0.69
3 months	0.21	0.08	0.66	0.49
6 months	0.09	0.04	0.47	0.38

The cheeses were made in a parallel experiment, with Z8 as starter. The pH, moisture and salt contents of both cheeses were identical. The rennet contents were 130 μl/kg (½ n) and 540 μl/kg (2 n), respectively.

are detected in the γ-casein region, probably representing the γ-, TS- and R-caseins. Several authors assumed that these products resulted from milk protease action on β-casein (5, 28, 29).

Finally three bands with very low mobility, just above the slots, are visible in the cheese patterns, showing a slightly increased intensity on ripening. When larger or smaller amounts of rennet were used for the manufacture of these cheeses the velocity of α_{s1}- and β-casein degradation was distinctly affected. In Fig. 4 the PAE patterns of three comparable cheeses, showing a low, normal and high rennet content, are presented at two stages of ripening. With regard to the breakdown products it can be seen that the appearance and intensity of the zones between α_{s1}- and β-casein is dependent on the rennet concentration in the cheese. In connection with the residual proportions of α_{s1}- and β-casein, Table 1 gives the results of a parallel experiment with very different amounts of rennet. It can be seen that there is a positive correlation between the amount of rennet and the extent of α_{s1}- and β-casein degradation in the first part of the ripening period. At 6 months however the differences between the cheeses are leveled down considerably. For β-casein this could indicate the presence of a certain fraction that is more resistant to attack. The results of this single parallel experiment were confirmed by those with other cheeses containing different levels of rennet.

3.2 Aseptic starter-free (ASF) cheeses

As an example, in Fig. 5 the PAE patterns are presented of an ASF cheese containing a normal amount of rennet. In Fig. 6 the relative concentrations of α_{s1}- and β-casein are given as an average of cheeses from three different experiments.

Comparison of these results with those of normal aseptic cheeses presented

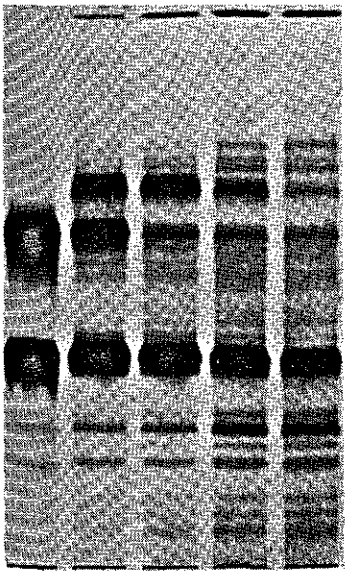

Fig. 5. The PAE patterns of an ASF cheese containing a normal amount of rennet. The numbers refer to the age of the cheese in days.

earlier (Section 3.1) shows that the casein breakdown proceeds in a very similar manner in both cheese types. The degradation of α_{s1}-casein is accomplished in the same way and at the same rate in the ASF cheeses as in

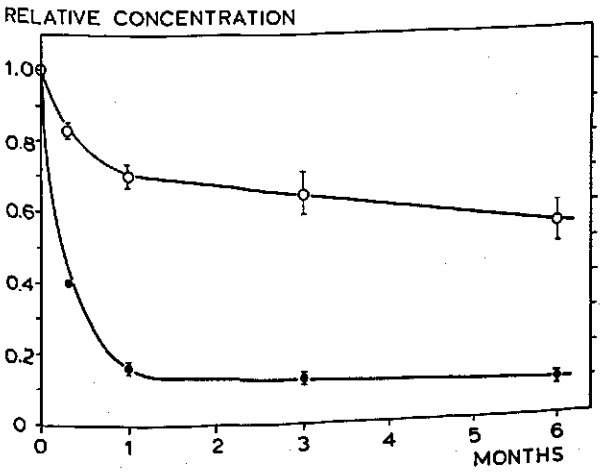

Fig. 6. The decomposition of α_{s1}- and β-casein during the ripening of ASF cheeses with normal rennet amounts (300 μl/kg cheese). Averages and standard deviations of 3 different cheeses are shown.

the presence of starter bacteria. As far as can be detected by PAE the α_{s1}-I product is broken down further in the same way too, in spite of the absence of starter bacteria. It is known that the phe 23 – phe 24 bond in α_{s1}-casein is particularly susceptible to rennet action in solutions (16), the cleavage resulting in the product α_{s1}-I.

In the first month of ripening of the ASF cheeses the β-casein decreases somewhat more than in the comparable normal cheeses. During further ripening, however, the β-casein is degraded less intensively. As a result, about 10 % more residual β-casein is present in the ASF cheeses after 6 months ripening. Breakdown products such as βI, βII and βIII were accumulated in our cheeses only to a minor degree. Several authors (10, 13), studying rennet action on β-casein in solution, showed that these products were liberated primarily and often accumulated to some extent. Only when very high amounts of rennet were enclosed in the ASF cheeses we could observe clear zones in the region of βI and βII. The bands in the γ-casein region develop essentially in the same way as in a normal aseptic cheese.

We observed a clear zone of stainable products with high mobility at the top of the gels. This zone probably represents relatively large breakdown products of rennet action which are not easily converted to lower-MW products in the absence of starter bacteria. In starter-containing cheeses these products are represented far less. The concentration of these compounds

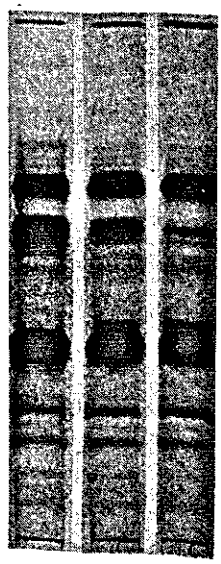

Fig. 7. The PAE patterns of 3 ASF cheeses after 1 month of ripening. The cheeses showed 3 different levels of enclosed rennet: slot 1: 180 μl; slot 2: 300 μl and slot 3: 830 μl (per kg cheese). The cheeses of slots 2 and 3 were made in a parallel experiment.

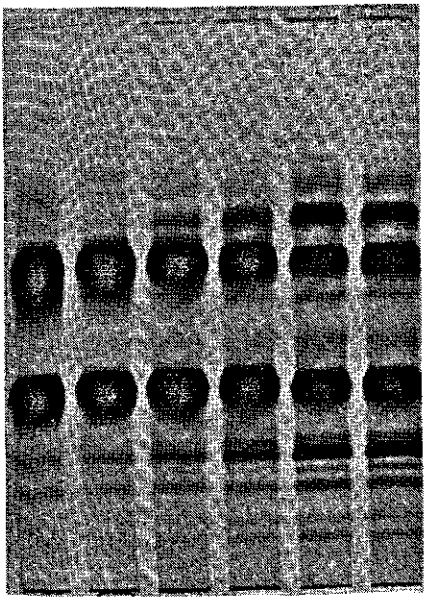

Fig. 8. The PAE patterns of an ARF cheese. Starter culture Bos was used during manufacture. The numbers refer to the age of the cheese in days.
* ARF cheese (Bos) made in another cheesemaking experiment.

seems to increase during ripening and to be dependent on the amount of rennet in the cheeses (see also Fig. 7).

In Fig. 7 the patterns at 1 month of ripening of three ASF cheeses containing different amounts of rennet can be compared. Two of the cheeses were made in a parallel experiment. It can be seen that the degradation of α_{s1}-casein is distinctly increased by the higher amount of enclosed rennet, and the same is true for β-casein but here the effect is much smaller. The PAE results with varying rennet concentrations generally conform to the results obtained for normal aseptic cheeses (see e.g. Fig. 4).

3.3 Aseptic rennet-free (ARF) cheeses

ARF cheeses were used to study the contribution of starter bacteria to the degradation of paracasein during cheese ripening.

Fig. 8 presents the PAE patterns observed during the ripening of a representative ARF cheese. In this particular cheese starter Bos was used. However, on the application of the other 6 starter cultures we obtained essentially the same patterns. As is demonstrated in Fig. 9 we could not detect any substantial difference between ARF cheeses made with different starters by means of the PAE techniques used. For this the starters were also compared in parallel experiments.

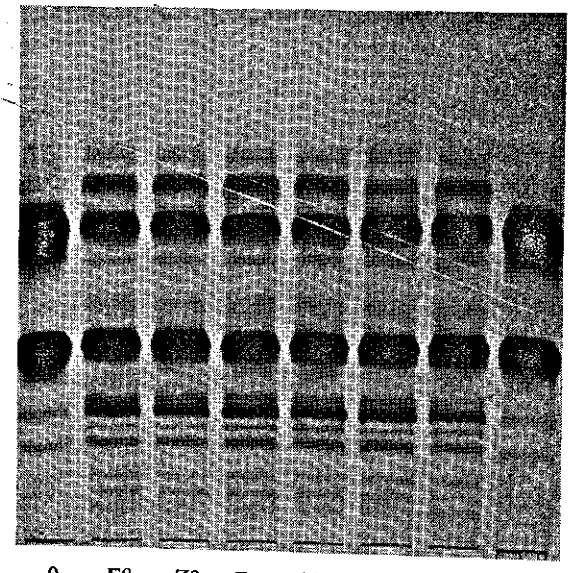

Fig. 9. The PAE patterns of 6 different ARF cheeses after 6 months of ripening. The different starters used are indicated. Cheeses E8 and Z8, Bos and AM_2 were made in two parallel experiments.

In Fig. 10 the degradation of α_{s1}- and β-casein in these cheeses is outlined as an average of 13 different cheeses.

As can be seen in the presented figures, α_{s1}- and β-casein are both attacked during the ripening, although to a lesser extent than in the normal aseptic and ASF cheeses. Particularly during the first month of ripening only a weak activity is manifested. During further ripening α_{s1}-casein is definitely degraded, and a primary breakdown product showing the same mobility as α_{s1}-I is accumulated. In front of this zone another product with a somewhat higher mobility gradually becomes visible. Finally, after 6 months of ripening, 40 - 50 % of the original α_{s1}-casein is degraded. In the same period the proportion of unattacked β-casein decreases by about 30 %. No higher-mobility breakdown products of β-casein are detected in the patterns. In the γ-casein region 4 separate bands develop during the ripening. As was also observed in the other cheeses, 3 faint zones with very low mobility, just above the slots, become visible during the ripening.

3.4 Aseptic rennet- and starter-free (ARSF) cheeses

The endogenous milk protease, surviving the cheese-milk pasteurization (28),

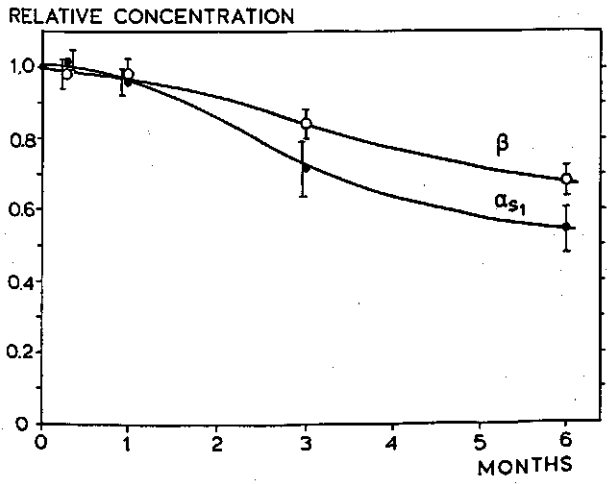

Fig. 10. The decomposition of α_{s1}- and β-casein during the ripening of ARF cheeses. Average and extreme values of 13 cheeses are indicated.

is the only proteolytic enzyme system that can act during the ripening of ARSF cheeses. From Fig. 11 the contribution of milk protease to the degradation of paracasein can be assessed. The PAE patterns after 6 months ripening of three different ARSF cheeses are presented. For purposes of comparison the pattern of one of these cheeses after 9 days ripening only is also shown.

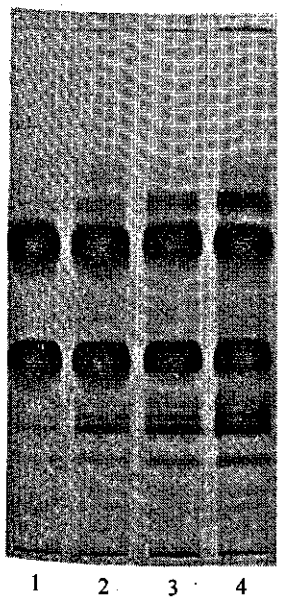

Fig. 11. The PAE patterns of ARSF cheeses. Slot 1 and 2: Exp. 50507, age of cheeses 9 days and 6 months, respectively. Slot 3: Exp. 50220, age of cheese 6 months. Slot 4: Exp. 50227, age of cheese 6 months.

It can be seen that the bands in the γ-casein region are developed distinctly in these cheeses after 6 months ripening. This indicates that milk protease is indeed responsible for the production of these typical caseins from β-casein during the ripening of Gouda-type cheeses. A slight attack on α_{s1}-casein is registered too, since a band with about the same mobility as α_{s1}-I becomes visible after 6 months. On the basis of the intensity of the bands of these breakdown products, two of the cheeses show about the same degree of milk protease action while the other cheese was subject of a somewhat greater activity. Although we have no exact densitometer readings of this restricted number of patterns we did not observe any considerable decreases in the α_{s1}- and β-casein proportions. For the cheese of slot 4 the decrease for both caseins will have been at the most 15 %.

The portions of milk used for these ARSF-cheesemaking experiments were drawn from only a limited number of cows (4 - 5). Noomen (28) observed a considerable variation in milk protease activity among these individual cows. Possibly the higher activity in one of our cheeses can be explained in this way.

4 Discussion

In a previous paper (2) we reported rennet to be the most important enzyme system for the production of soluble-N compounds in Gouda-type cheeses. Starter bacteria and milk protease appeared to contribute too, but to a minor extent. From this it was concluded indirectly that rennet was the determinative agent for the extent or 'width' of proteolysis in Gouda cheese.

In this paper we have studied the degradation of casein more directly by following the decrease of α_{s1}- and β-casein in our cheeses quantitatively by means of gel electrophoresis.

In the normal aseptic cheeses a very quick and almost complete degradation of α_{s1}-casein was observed in one month, while β-casein was degraded more gradually, after 6 months ripening about 50 % still being unaffected. For normal Gouda cheeses Creamer (5, 6) found the same tendencies.

Surveying the results of the cheeses in which rennet, starter bacteria and milk protease acted separately, it can be concluded that rennet indeed is of the utmost importance for the primary attack of casein in Gouda cheese. Rennet in a normal concentration, without the presence of starter bacteria, was able to accomplish the same degradation of α_{s1}-casein and nearly the same of β-casein as found in normal aseptic cheeses. Only in the longer term – from about 2 months of ripening onwards — did the β-casein degradation lag somewhat behind that found in the presence of starter bacteria.

From the ARF cheeses it appeared that starter bacterial enzymes were able to achieve a slow but significant degradation of both α_{s1}- and β-casein without any interference from rennet. This process only proceeds however from 1 month of ripening onwards at a considerable rate. In a normal cheese, rennet will already have decomposed most of the α_{s1}-casein in this period, unaffected β-casein being still available at that time. Obviously the proteinases of starter bacteria in cheese need some time to become effective. Possibly this is related to the autolysis of the bacteria. Anyhow, the starter bacterial enzymes were able to contribute to the direct casein breakdown in cheese. This observation fits well with the somewhat more extended breakdown of β-casein in aged normal aseptic cheeses as compared to ASF cheeses. Strictly speaking the same is true for the α_{s1}-casein, since after 1 month in normal aseptic cheeses a slightly lower residual amount was always found than in the ASF cheeses.

That we could detect no differences in the PAE patterns of normal aseptic cheeses made with different starters is not surprising in the light of the observed dominating role of rennet. However, by gel electrophoresis, there was also no difference between starters when used in ARF cheeses, in the absence of rennet. Although Exterkate (29) showed differences to exist in the proteinase system of our *S. cremoris* starters, these are not reflected in the primary attack of α_{s1}- and β-casein in cheese.

As to the contribution of milk protease to the casein breakdown in Gouda cheese we could show that this enzyme system was indeed responsible for the production of the typical 'minor caseins', such as γ, R, TS and S, from β-casein. Creamer (5) had already suggested that milk protease had this property. However, the total contribution of milk protease to the degradation of α_{s1}- and β-casein was estimated to be small in relation to those of rennet and starter bacteria.

The results for our ASF cheeses agree generally with the work of Gripon (23) and O'Keeffe (24). However in their studies on aseptic model-curd and ASF Cheddar cheese, respectively, the amounts of rennet acting in the cheese were not known. Moreover the PAE patterns of O'Keeffe could be regarded as only qualitative. Gripon (23) reported the accumulation of considerable amounts of three products with an intermediate mobility between that of α_{s1}- and of β-casein, one of which was βI. This is in contrast to our observations. Creamer (15) showed that the appearance of βI, βII and βIII was strongly dependent on the environmental conditions in the β-casein solutions. Probably in our cheeses these products – if formed — have been degraded further at a relatively high rate.

We casually observed that the consistency of our ARF cheeses was less

smooth during ripening than that of comparable ARF 'control' cheeses, normal aseptic and ASF cheeses, in all of which rennet was active during the ripening. De Jong (11, 31) reported in detail on the relation between the (α_{s1}-)casein breakdown and the consistency of 'Meshanger' cheese, emphasizing the importance of rennet in this respect. Since rennet is determinative for the breakdown of α_{s1}-casein in our Gouda-type cheeses the same relation could be true for Gouda cheese.

The total decrease of original casein in cheese is a good measure of the extent of the proteolysis. In Fig. 12 we have presented the decrease of α_{s1}- plus β-casein together in normal aseptic, ASF and ARF cheese. In calculating these values we have taken into account that α_{s1}-casein is present in higher amounts than is β-casein (5 : 3). Both caseins together constitute about 90 % of the cheese protein.

It can be seen that rennet is indeed completely responsible for the extent of the casein degradation during the first month of Gouda cheese ripening. As a matter of fact the ASF cheese showed a slightly larger decrease in the casein during this period than did the normal aseptic cheese. The rennet content of the ASF cheeses in this study averaged 300 μl/kg, and 280 μl for the normal aseptic cheeses. After some months of ripening the starter bacterial enzymes also appear to contribute, which agrees with the observed casein attack in ARF cheeses. After 6 months ripening only 20 - 30 % of the original casein in normal cheese is present, representing predominantly unattacked β-casein.

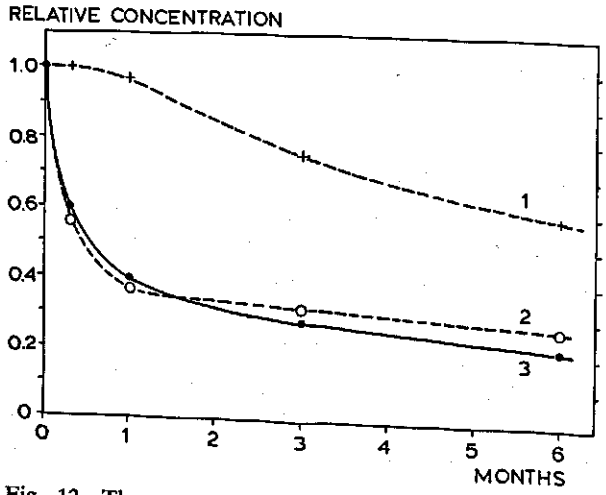

Fig. 12. The average decomposition of casein (α_{s1}- plus β-casein) in ARF cheese (curve 1), ASF cheese (curve 2) and normal aseptic cheese (curve 3).

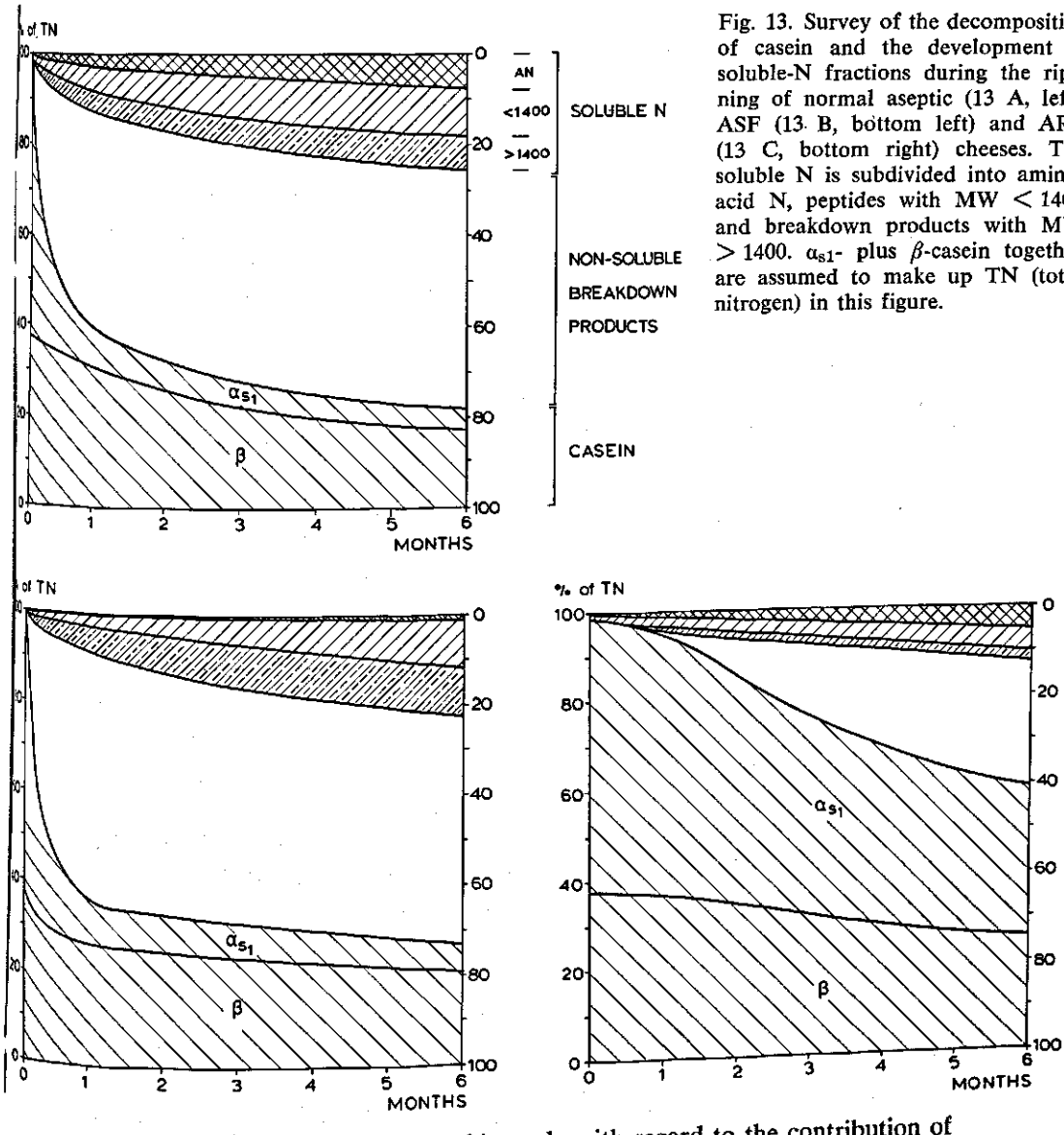

Fig. 13. Survey of the decomposition of casein and the development of soluble-N fractions during the ripening of normal aseptic (13 A, left), ASF (13 B, bottom left) and ARF (13 C, bottom right) cheeses. The soluble N is subdivided into amino-acid N, peptides with MW < 1400 and breakdown products with MW > 1400. α_{s1}- plus β-casein together are assumed to make up TN (total nitrogen) in this figure.

The conclusions drawn from this study with regard to the contribution of rennet, starter bacteria and milk protease to the protein breakdown are in general agreement with the results of soluble-N determinations, reported previously (2). Only the extent of proteolysis by starter bacteria during the first months of ripening was somewhat over-estimated on the basis of soluble-N values. Obviously these soluble-N values can indeed be regarded as a reflection of the extent or 'width' of the proteolysis.

Combining the results of this study with those of the previous work on soluble N and amino-acid N, makes it possible to characterize the protein breakdown in the different aseptic cheeses more completely. In Fig. 13 this is done for the normal aseptic, ASF and ARF cheese. For ease of survey we have subdivided the soluble-N fraction into amino acids, lower MW (<1400) peptides and higher MW (>1400) breakdown products. For the same reason it is assumed that the cheese protein is composed only of α_{s1}- and β-casein. We did not obtain any data on the breakdown of para-κ-casein in our cheeses.

It can be seen that important proportions of non-soluble breakdown products are produced in the cheeses that are not represented in the soluble-N values. Although we have not involved this insoluble fraction in our analyses, we can deduce its proportion from the other results. This fraction can be expected to contain chiefly larger breakdown products (e.g. α_{s1}-I).

The sharp decrease in the rate of casein attack, observed at 1 month in normal aseptic and ASF cheese and due to the completeness of α_{s1}-casein degradation, is not reflected in the production of total or individual soluble-N compounds. This indicates that the proteinase activity is gradually directed to the larger (insoluble) breakdown products of rennet action. In the ASF cheese PAE patterns, for example, rennet started the attack on α_{s1}-I. The proteinases of the starter bacteria also will attack degradation products of rennet action. In the gel patterns of normal aseptic cheeses evidence for this is found in the absence of the (rennet) products at the top of the gels. At the time that starter proteinase is beginning to become active, most of the casein in cheese has already been decomposed by rennet.

It can be seen that during the first month of ripening of ARF cheeses the small proportion of casein that is attacked is largely converted to (small) soluble products. In contrast, rennet predominantly produces larger and insoluble products in this period.

Apart from the 'extent' of protein breakdown, the 'depth' of it can now be described by a value expressing the ratio between the smaller breakdown products (e.g. amino acids, products with MW <1400) and the total quantity of degradation products.

From this it is clear that proteolysis, as manifested in normal aseptic Gouda-type cheeses, bears the stamp of rennet as to the 'extent' and of starter bacteria with regard to the 'depth'.

Acknowledgments

This study was made possible by the financial support of the Stichting J. Mesdagfonds, instituted by Kaascontrolestation Friesland, Leeuwarden.

Samenvatting

F. M. W. Visser en A. E. A. de Groot-Mostert, De bijdrage van de enzymen van stremsel, zuurselbacteriën en melk aan de eiwitafbraak en smaakontwikkeling in Goudse kaas. IV. De eiwitafbraak, bestudeerd met behulp van gelelectroforese

Met behulp van kwantitatieve polyacrylamide-gelelectroforese (PAE) werd de afbraak van α_{s1}- en β-caseïne bestudeerd in aseptisch bereide Goudse kazen. De werking van stremsel en/of zuurselbacteriën tijdens de rijping kon in deze kazen worden uitgeschakeld.

In de gewone aseptische kazen wordt α_{s1}-caseïne zeer snel aangetast, binnen een maand is de afbraak bijna volledig. β-caseïne is minder onderhevig aan proteolyse; na 6 maanden rijping is ongeveer de helft nog intact.

Uit experimenten met zuurselvrije, aseptische kazen werd geconcludeerd dat het stremsel gedurende de eerste maand van de rijping volledig verantwoordelijk is voor de waargenomen afbraak van α_{s1}-caseïne en voor de hydrolyse van β-caseïne. Op wat langere termijn dragen de enzymen van zuurselbacteriën ook bij, met name aan de wat verdere afbraak van β-caseïne. De in de stremselvrije kazen waargenomen eiwitafbraak was hiermee in overeenstemming. De zuurselbacteriën bleken namelijk in enige maanden tijd in staat α_{s1}- en β-caseïne gedeeltelijk af te breken. Gedurende de eerste maand van de rijping is deze activiteit nog zeer gering.

Het gebruik van verschillende zuursels had geen verschillen in de PAE-patronen tot gevolg, noch in stremselvrije, noch in normale aseptische kazen.

Uit de rijping van stremsel- én zuurselvrije kaas bleek duidelijk dat melkprotease verantwoordelijk is voor de vorming van de 'minor caseins' uit β-caseïne, tijdens de rijping van Goudse kaas. De totale bijdrage van melkprotease aan de afbraak van α_{s1}- en β-caseïne is gering in verhouding tot die van stremsel en zuurselbacteriën.

De resultaten worden ook besproken in verband met de gegevens over oplosbaar stikstof en aminozuur-stikstof uit een vorige publikatie (2).

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Neth. Milk Dairy J. 31 (1977): 265-276

Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 5. Some observations on bitter extracts from aseptically made cheeses

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Received: 30 June 1977

Summary

A number of aseptically made cheeses, ripened by the combined or separate action of rennet and/or 'bitter' and 'non-bitter' starter bacteria, was extracted with a chloroform/methanol mixture. The extracts were tasted for bitterness and fractionated by molecular sieving on Sephadex LH-20. The bitter fractions were localized in the different elution profiles.

The quantities of extractable peptide material increased during the ripening of the cheeses. From normal aseptic cheese higher amounts were obtained than from rennet-free or starter-free cheeses. The extracted material of bitter cheeses showed a far more intensive bitterness than did that of non-bitter cheeses.

The molecular weight of the greater part of the extracted compounds was less than 4000. Intensive bitterness was detected mainly in the fractions with a molecular weight less than about 1400.

As opposed to normal aseptic and rennet-free cheeses, starter-free cheeses appeared to contain considerable quantities of bitter-tasting oligopeptides. Some bitter-tasting fractions were also detected in the extracts of non-bitter cheeses.

The elution profiles of rennet-free cheese extracts differed from those of starter-free cheeses. Some tentative differences were observed between the profiles of cheeses made with a 'bitter' and a 'non-bitter' starter.

Astringent compounds were isolated from all of the cheeses.

1 Introduction

The bitter flavour, frequently observed when proteins are hydrolysed enzymatically, can be attributed to the accumulation of bitter-tasting peptides. Among other proteins, casein appears to be extremely sensitive to bitter flavour development on proteolysis (1, 2, 3).

In the past decade a considerable number of bitter peptides from casein

hydrolysates has been isolated and identified (4, 5, 6, 7 and others). The occurrence of the 'bitter' defect in cheese is dependent on the accumulation of bitter peptides from casein. Some workers succeeded in the identification of bitter peptides from cheese (8, 9, 10). These peptides, both from whole casein and from cheese, were all shown to originate from α_{s1}- or β-casein. Until now no bitter peptides have been isolated originating from para-$\varkappa$-casein. Several workers have tried to designate α_{s1}- or β-casein as the most important source of bitter peptides in cheese (11, 8).

Ney (12, 13) suggested that the degree of hydrophobicity of a peptide was the most important factor in determining whether or not it would be bitter. Indeed the characterized bitter peptides from casein all show a high content of hydrophobic amino-acid residues. Recently Wieser & Belitz (14, 15) reported on the relation between the structure and bitter taste of amino acids and peptides. The presence of hydrophobic groups in the molecule appeared to be an essential requirement for bitterness. The taste thresholds of a great number of di- and tri-peptides were determined and corresponded to their calculated hydrophobicity (16).

From this it seems acceptable that α_{s1}-, β- and para-$\varkappa$-casein, with the high average hydrophobicities of 1.17, 1.33 and 1.31 kcal/residue, are potential sources of bitter peptides on hydrolysis.

In previous papers we have reported on the development of bitter flavour and the proteolysis in aseptically made Gouda cheeses, ripened by the combined and separate action of rennet, starter bacteria and milk protease (17, 18, 19). It was shown that rennet and 'bitter' starter bacteria, each on their own, were able to produce bitterness in cheese. The starter bacteria appeared to do this far more specifically than did rennet. The use of 'non-bitter' starters did not result in bitter cheeses. However, only minor differences in the protein breakdown processes could be found between cheeses made with 'bitter' and 'non-bitter' starters. In the present study we have tried to detect differences in the bitter-peptide pools of our cheeses.

2 Materials and methods

2.1 Cheeses

Normal aseptic, aseptic starter-free (ASF), aseptic rennet-free (ARF) and aseptic rennet- and starter-free (ARSF) cheeses were manufactured as described previously (20) and had a normal Gouda cheese composition. *Streptococcus cremoris* strains HP and Wg2 were used as 'bitter' starters, and strain E8 as 'non-bitter' starter.

Before extraction the cheeses were grated and freeze-dried.

2.2 Extraction

With chloroform/methanol (2/1; v/v) 60 g of freeze-dried cheese were extracted according to Harwalkar & Elliot (21). The extract was made biphasic with 0.2 volume of water and separated overnight into two layers. A white precipitate was formed at the interface (fraction A_1). The aqueous methanolic layer was dried in a rotary evaporator under reduced pressure and taken up in demineralized water. Further separation of astringent fractions was achieved by adjusting the pH of the solution to pH 6-7. A white precipitate was formed that was separated by centrifugation (fraction A_2) (21). The supernatant (bitter extract) was freeze-dried. Total nitrogen was estimated in the dry material according to a semi-micro-Kjeldahl method.

2.3 Gel filtration of bitter extracts

The bitter extracts were fractionated on Sephadex LH-20 in a column of 100 cm × 2.5 cm (KR 25, Pharmacia). Elution was carried out at 20-25 °C with a mixture of n-propanol and water 7:3 (v/v) to decrease hydrophobic interactions known to occur between the hydrophobic bitter peptides (4). In each run an amount of bitter extract, corresponding to 16 mg nitrogen, was dissolved in 3 ml eluent and applied to the column. The flow-rate was 30 ml/h.

The column was calibrated using Bleu Dextran 2000 (Vo ~145 ml), protamin sulphate (M = 4000), glucagon (M = 3865), insulin chain A (M = 2450), bacitracin (M = 1410) and tryptophan.

During elution the absorbance of the column effluent was measured continously at 280 nm (Gilson, UV 265/280) and monitored with a Honeywell FB 70 recorder. 5-ml fractions were collected by means of a Gilson automatic fraction collector.

The absorbance of each fraction was measured, after dilution, at 220 nm in a Seitz spectrophotometer.

2.4 Gel filtration of astringent fractions

An astringent fraction was fractionated on Sephadex G50 with 0.01 *N* acetic acid as eluent at 3 °C. For further conditions see (18).

2.5 Evaluation of bitterness

Extracted material was tasted for bitterness by members of the taste-panel used for cheese grading. Sufficient freeze-dried extract was dissolved in 25 ml demineralized water to provide 40 mg peptide material in solution (N × 6.38), and this was used for tasting.

The fractions leaving the column were pooled according to the pattern

Table 1. Cheeses used for the extraction with chloroform/methanol; survey of the production of bitter and astringent extract fractions.

Normal aseptic cheese[1]			Chloroform/methanol extract		Astringent fractions (yield)[4]
starter	age (months)	bitter score	yield[2]	bitter score[3]	
HP	1/4	—	0.53	2.3	0.24
	1	4.6	0.78	3.7	0.37
	3	4.1	0.87	4.2	0.79
	6	4.8	1.03	4.0	0.81
Wg2	3	4.2	0.89	4.2	0.80
E8	3	1.5	0.92	2.2	0.77
Aseptic rennet-free cheese					
starter	age	bitter score			
HP	1	3.4	0.34	4.3	~0.44
	3	3.5	0.44	4.3	0.50
	6	3.4	0.69	2.7	0.81
Wg2	1	2.7	0.32	3.5	~0.48
	3	3.4	0.39	5.3	0.64
	6	3.9	0.80	4.2	0.96
E8	3	1.4	0.31	2.2	0.72
Aseptic starter-free cheese					
rennet[5]	age	bitter score			
n	1	1.6	0.23	2.2	0.68
	3	2.5	0.44	3.5	0.88
	6	3.3	0.51	4.7	0.90
1/3n	3	1.3	0.27	3.7	0.72
2n	3	4.6	0.84	4.3	1.11
ARSF	1	1.2	0.11	—	0.36

[1] The normal aseptic cheeses contained about 280 μl rennet per kg cheese.
[2] Expressed in grams peptide material (N×6.38) per 60 grams of dry cheese (excluding the astringent material).
[3] Freeze-dried extract standardized at 40 mg peptide material (N×6.38), was dissolved in 25 ml demineralized water.
[4] Expressed in grams dry substance per 100 grams of cheese (fractions A_1+A_2).
[5] n: about 300 μl rennet/kg cheese.

(2-5 fractions together), freeze-dried and dissolved in 2 ml demineralized water for taste evaluation.

3 Results

In Table 1 some data are presented concerning the material extracted with chloroform/methanol from different cheeses. Normal aseptic and ARF cheeses, made with 'bitter' starter strains, and ASF cheeses, containing a normal amount of rennet, were extracted at various stages of ripening. For purposes of comparison two cheeses made with the 'non-bitter' starter E8 and ASF cheeses made with a high and a low amount of rennet, were also extracted. Finally, extracted material was obtained from a rennet- and starter-free cheese.

It can be seen from the table that for all cheese types the amount of extracted material increased during the ripening. Normal aseptic cheeses yielded far more material than did ARF or ASF cheeses. The amount of material obtained from the non-bitter E8 cheeses was of the same magnitude as that from the comparable bitter-starter cheeses. However, on tasting the extracted material of these E8 cheeses, hardly any bitterness could be detected. By contrast, the extracts from bitter cheeses all were judged to be clearly bitter. In general, the extracted material of the non-bitter cheeses showed less intensive bitterness than did that of bitter cheeses. The ASF-1/3n cheese was an exception as to this. In relating the bitterness of the extracted material to the bitter flavour of the cheese both the yield and the bitter score of the extract should be taken into account.

The yield of proteinaceous material extracted from the different cheeses was not a reflection of protein breakdown as measured by means of soluble-N values. The ASF cheeses showed somewhat lower amounts of extracted ma-

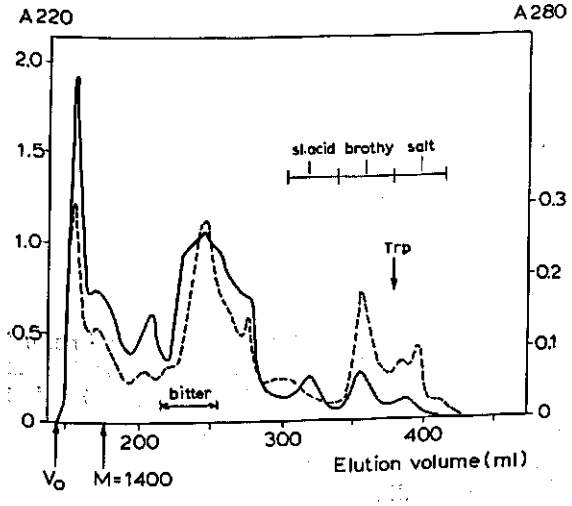

Fig. 1. Fractionation of a chloroform/methanol cheese extract by gel filtration on Sephadex LH-20 with n-propanol/water (7:3, v/v) as eluent. Detection at 220 nm (——) and at 280 nm (. . . .). A non-bitter, normal aseptic cheese of 3 months old was used for extraction. Starter E8 was used for cheesemaking.

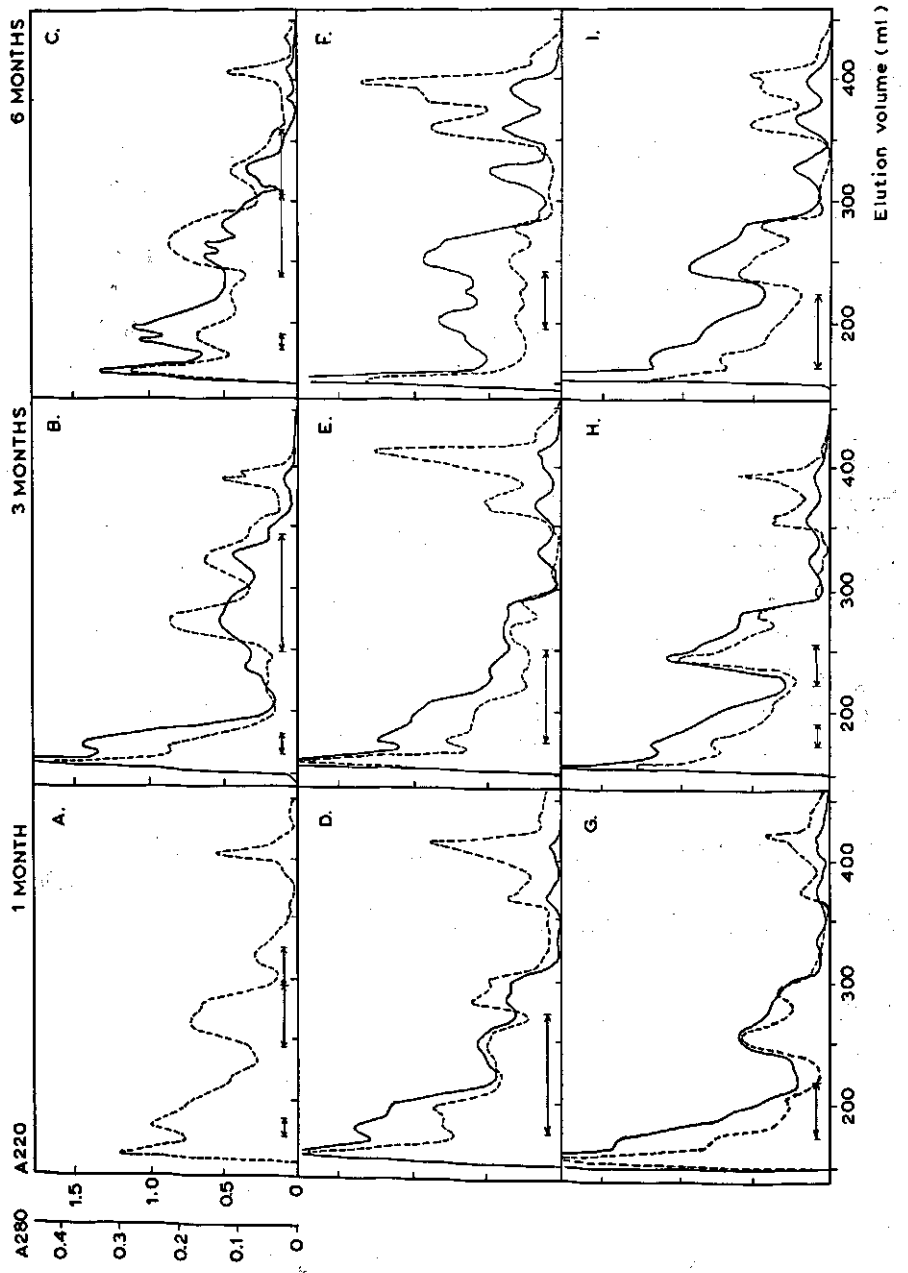

Fig. 2. Survey of the gel filtration patterns of the extracts of ARF, ASF and normal aseptic cheeses. The cheeses were extracted at 1 month (first column), 3 months (second column) and 6 months (third column) of ripening. Detection at 220 nm (——) and at 280 nm (. . . .).
2A-2C: ASF cheese, containing a normal amount of rennet.
2D-2F: ARF cheese, with 'bitter' starter HP.
2G-2I: normal aseptic cheese with 'bitter' starter HP.
The intensive bitter fractions are indicated by horizontal lines.

terial than did the ARF cheeses, which is not in line with the soluble-N results (18). In Fig. 1 we have outlined as an example the gel chromatographic pattern of the chloroform/methanol extracted material of a normal, 3-month-old, aseptic E8 cheese. As can be seen, most of the extracted compounds have molecular weights lower than 4000, which were shown (18) to be preferentially liberated by starter bacteria.

As can be seen from Fig. 1, some strongly bitter-tasting fractions were observed in this extract, although the cheese and total extract showed hardly any bitterness. Apart from the bitterness, other taste sensations were observed in all cheese extracts after being eluted from the column. The first few fractions eluted after the void volume often showed some astringent taste sensation. The pooled fractions eluted from 300-340 ml often showed a slightly acid taste, from 340-375 ml they always tasted like broth and from 375 ml onwards they had a salty taste.

In Fig. 2 the patterns of extracts from some 'key' cheeses at different stages of ripening are presented together. The regions in which intensive bitterness was observed are indicated.

In the extracts of all the cheeses investigated regions showing bitterness were detected after separation on the column. As can be seen, the bitter fractions in the HP-ARF cheese were eluted within the volumes of 175-275 ml. We found the same to be true for the ARF cheese made with the 'bitter' Wg2 starter. With the use of 'non-bitter' starter E8 some bitterness was observed in the 220-240 ml region, representing a more narrow range than in the case of HP and Wg2. However this E8 cheese only was investigated after 3 months ripening. In Fig. 3 we have given the 3-month patterns of the ARF cheeses made with Wg2 and E8, respectively. They can be compared with the pattern in Fig. 2E where HP was used as starter. Particularly in the 175-250 ml region the extract of the non-bitter E8 cheese appears to possess far less material than the other two cheeses.

From Fig. 2A-2C it can be seen that the bitter peptides in the ASF cheese are detected in a more extended range of elution volumes, from 170 up to even 360 ml. In the 190-240 ml region the bitterness was mostly less intensive or even absent. The same was observed in the extracts of the ASF cheeses made with a large and a small amount of rennet. Obviously rennet produces relatively important amounts of small bitter peptides. In general the elution patterns of the ASF cheeses are different from those of the ARF cheeses.

In Fig. 2G-2I the patterns are presented of the extracts of normal aseptic cheeses, in which rennet and starter bacteria (HP) act together. The bitter peptides in these cheeses are detected in the region of 175-255 ml. It was somewhat unexpected that the bitter peptides in the larger elution volumes

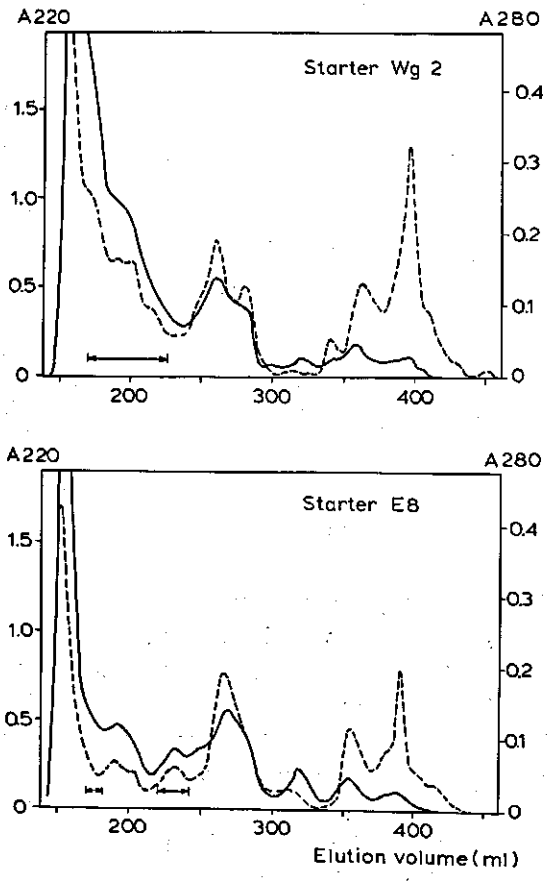

Fig. 3. Gel filtration patterns of 3-month-old ARF cheeses with the 'non-bitter' starter E8 and the 'bitter' starter Wg2. Detection at 220 nm (——) and at 280 nm (. . . .). Bitter fractions are indicated by horizontal line.

– produced by rennet – are not present. The normal aseptic cheese made with the 'bitter' Wg2 starter showed approximately a similar pattern. Comparison of these bitter-cheese patterns with the pattern presented in Fig. 1, concerning a non-bitter E8 cheese, shows that in the extract of the E8 cheese less absorbing material is present in the 160-240 ml region and somewhat more in the larger elution volumes (>300 ml).

The ARSF cheese, extracted at 1-month of ripening and showing a very low level of proteolysis, showed some bitterness in the 240-340 ml region.

Harwalkar (21) described the isolation of 2 fractions of astringent peptides during the chloroform/methanol extraction of cheeses. He showed that a normal Cheddar cheese contained an appreciable amount of these astringent compounds and characterized them as hydrophobic polypeptides with an average molecular weight of 9 000-12 000 (22)

We have also isolated the two astringent fractions during the extraction of

our cheeses, and in Table 1 the total dry weight of the two fractions is given. It can be seen that the weight of the astringent fractions increases during the ripening of the cheeses. In normal aseptic, ARF- and ASF cheeses about the same amount of astringent compunds is produced after 6 months.

In Fig. 4 we have presented the elution profile of the astringent fraction (A_2) of a normal aseptic cheese after molecular sieving on Sephadex G50. This fraction did not appear to be homogeneous, and consisted of a major fraction with a molecular weight of approximately 7000 and a second fraction of about 3000. Gel chromotagraphy of astringent fractions (both A_1 and A_2) on Sephadex LH-20 showed that by far the most of the isolated material was eluted at the void volume. Possibly the astringency that often accompanied the first fractions of our bitter cheese-extracts was caused by this astringent polypeptide fraction not being sedimented completely.

4 Discussion

The aim of this work was to see if, by means of relatively simple separation techniques, differences could be observed in the pool of bitter peptides pro-

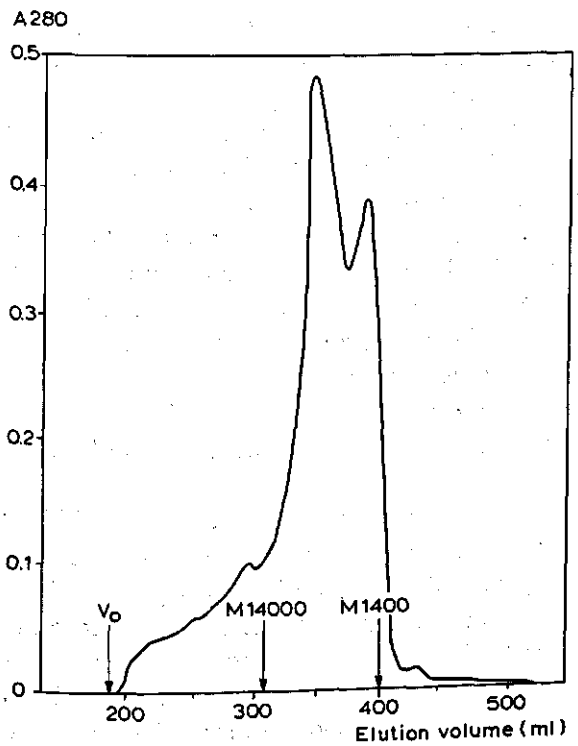

Fig. 4. Gel filtration pattern of astringent fraction (A_2) from a normal aseptic E8 cheese on Sephadex G50.

duced in cheese by the separate and combined action of rennet and starter bacteria. Moreover it was interesting to check if a variation could be detected in the amount and composition of bitter peptides present in a bitter and a non-bitter cheese.

In the chloroform/methanol extracts of our cheeses bitter peptide fractions could always be detected. However, in the extracts of non-bitter cheeses the proportion and the bitterness of these fractions was considerably less than in those of bitter-tasting cheeses. These results are in agreement with those on bitter and non-bitter Cheddar cheeses, reported by other workers (8, 23). Obviously the concentration of bitter peptides in the non-bitter cheeses was not so high as to pass the threshold value for bitter detection in cheese. It is unlikely from this that bitter-masking compounds, such as glutamic acid-rich oligopeptides (24), are of importance in non-bitter cheeses. We reported earlier (17) that 16-20 times more bitter peptides were necessary to make a cheese as bitter as aqueous solutions.

The bitter peptide fractions found in our cheese all showed molecular weights of less than about 1400. In the extracts of the ASF cheeses only, significant contents of bitter-tasting oligopeptides showing high elution volumes on Sephadex LH-20, were detected. These small bitter peptides were not found in the normal aseptic cheeses and the ARF cheeses, nor in those made with a 'bitter' or with a 'non-bitter' starter strain. This could indicate that both 'bitter' and 'non-bitter' starters have the capacity to degrade these small bitter peptides to non-bitter products. However further research is needed into this aspect before definite conclusions can be drawn.

The presence of some bitter peptide fractions in the normal aseptic and ARF cheeses made with E8 need not to indicate necessarily that the 'non-bitter' starter E8 was responsible for the production of these bitter peptides. In the normal aseptic cheese they also could originate from rennet action and in the ARF cheese to some degree from milk protease action. However, inspection of the accumulation of hydrophobic amino-acid residues in the casein molecules makes it seem rather unlikely that absolutely no bitter peptides would be liberated on hydrolysis by the proteinases of 'non-bitter' starter bacteria.

Anyhow, it was also clear from these results that the total pool of bitter peptides normally produced by rennet – and extracted from the ASF cheeses – must have been degraded by the E8 starter during the ripening of the normal aseptic cheeses. The preliminary observation that the peptide material in the (bitter) 160-220 ml elution region was under-represented in the normal aseptic E8 cheese as compared to the 'bitter' starter cheeses supported this view. In the ARF cheese made with starter E8 about the same fractions were

under-represented.

However, the possibility still cannot be excluded at this particular time that 'non-bitter' starter bacteria do produce bitter peptides during cheese ripening to a considerable extent, but that their peptidase system has the potential or is sufficiently specific to prevent the accumulation of a supra-threshold pool of these peptides.

In an earlier publication (18) we concluded that 'bitter' starter bacteria liberate bitter peptides far more specifically during cheese ripening than does rennet, since bitterness in the ARF cheeses is accompanied by a relatively low degree of proteolysis. From the elution profile of the extract of the one-month-old ARF cheese made with HP as starter, it can be seen that a rather wide range of bitter peptides is involved. The further identification of these particular peptides would be very interesting since this could elucidate the mechanism of bitter peptide liberation by starter bacteria in cheese.

Harwalkar (21) suggested that the astringent flavour components could contribute to the Cheddar flavour. However, since considerable quantities of the astringent fraction were also isolated from our ASF cheeses, and since these cheeses were observed to lack cheese flavour (17) completely, the contribution of astringency to the cheese flavour should not be overestimated.

Acknowledgments

The skilful technical assistance of Aliza de Groot-Mostert is highly appreciated.

This study was made possible by the financial support of the Stichting J. Mesdagfonds, instituted by Kaascontrolestation Friesland, Leeuwarden.

Samenvatting

F. M. W. Visser, *De bijdrage van de enzymen van stremsel, zuurselbacteriën en melk aan de eiwitafbraak en smaakontwikkeling in Goudse kaas. 5. Enkele waarnemingen met betrekking tot bittere extracten van aseptisch bereide kazen*

Een aantal op aseptische wijze bereide kazen werd volgens Harwalkar (21) geëxtraheerd met een mengsel van chloroform en methanol. Nadat de bitterheid van de extracten was beoordeeld, werden ze gefractioneerd met behulp van gelfiltratie over Sephadex LH-20. Na de gelfiltratie werden de bittere peptidefracties gelokaliseerd. Zowel stremsel- vrije als zuurselvrije en normale aseptische kazen met 'bittere' en 'niet-bittere' zuursels werden in het onderzoek betrokken.

De opbrengst aan geëxtraheerd materiaal nam toe naarmate de rijping van de kazen vorderde. Normale aseptische kazen leverden grotere hoeveelheden extract dan zuursel-

vrije of stremselvrije kazen. Het geëxtraheerde materiaal afkomstig van bittere kazen bleek veel bitterder dan dat van niet-bittere kazen.

Een sterk bittere smaak werd hoofdzakelijk waargenomen in de fracties met een molecuulgewicht beneden 1400. In tegenstelling tot normale aseptische en stremselvrije kazen vertoonden de extracten van zuurselvrije kazen steeds aanzienlijke hoeveelheden bittere peptiden van zeer laag molecuulgewicht. In de extracten van niet-bittere kazen werden toch enige bittere fracties aangetroffen.

De gelfiltratiepatronen van extracten van stremselvrije kaas vertoonden een ander beeld dan die van extracten van zuurselvrije kaas. In een oriënterend experiment werden verschillen geconstateerd in de patronen van extracten van kazen gemaakt met een 'bitter' en een 'niet-bitter' zuursel.

Adstringerende verbindingen konden uit alle kaasextracten worden afgescheiden.

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Discussion

1. Cheeses and cheesemaking techniques

We succeeded in developing a method for the small-scale manufacture of a rennet-free milk coagulum that can be used for the production of different varieties of rennet-free cheese. In this process, rennet is used normally to convert the casein into paracasein, but is completely inactivated before coagulation is allowed to proceed. Consequently the protein substrate in the rennet-free cheese is the same paracasein as in normal cheese. Because the complete process is suitable for use under controlled bacteriological conditions, the influence of unwanted bacteria or enzymes is eliminated from the ripening too.

The growth and survival of each of the different starter cultures in the rennet-free cheese was the same as in normal cheeses. If a new portion of rennet was introduced during manufacture, the resulting cheese showed a protein breakdown and flavour development on ripening similar to that in normal cheeses, so confirming the suitability of rennet-free cheeses for ripening studies. Thus one can now study the action of starter bacteria in the ripening cheese itself without the influence of rennet.

During the work on proteolysis and bitterness, we made a considerable number of rennet-free Gouda-type cheeses aseptically and of normal composition. De Jong (1) recently reported on rennet-free soft cheeses made by our method and used in consistency studies.

To study the action of rennet in cheese ripening without the interference of starter bacteria, a method of chemical acidification with δ'-gluconic acid lactone was used during aseptic manufacture of the cheeses. Since this acidification was done as late as possible in the Gouda cheesemaking process, our starter-free cheeses showed only a slightly decreased calcium content and a slightly increased rennet content. By altering the amount of rennet used, starter-free cheeses could be obtained with contents of enclosed rennet similar to those in the normal aseptic cheeses (280–300μl/kg cheese).

All previous reports on rennet action in starter-free cheeses have lacked an estimate of the amount of residual rennet, which is anyhow necessary to evaluate its role in the ripening of cheese. Cheeses made by the 'Mabbit'-method (2) of acidification – used by several investigators – had extremely high contents of rennet and low contents of calcium, which has consequences for the interpretation, for instance, of their results on proteolysis.

Our aseptic starter-free cheeses did not contain antibiotic substances, and so required very high standards of asepsis during the milking and cheesemaking.

Chemical acidification during the manufacture of a rennet-free cheese resulted in a cheese in which only the proteolytic system of milk is active during ripening. We manufactured a few such cheeses and so for the first time could study the action of

the native milk protease during cheese ripening on its own. The developed method for the preparation of a rennet-free milk coagulum was also used by Noomen (3) to produce curd and a calcium paracaseinate- calcium phosphate complex, both free from rennet and starter, which were used to study the action of milk protease under various conditions.

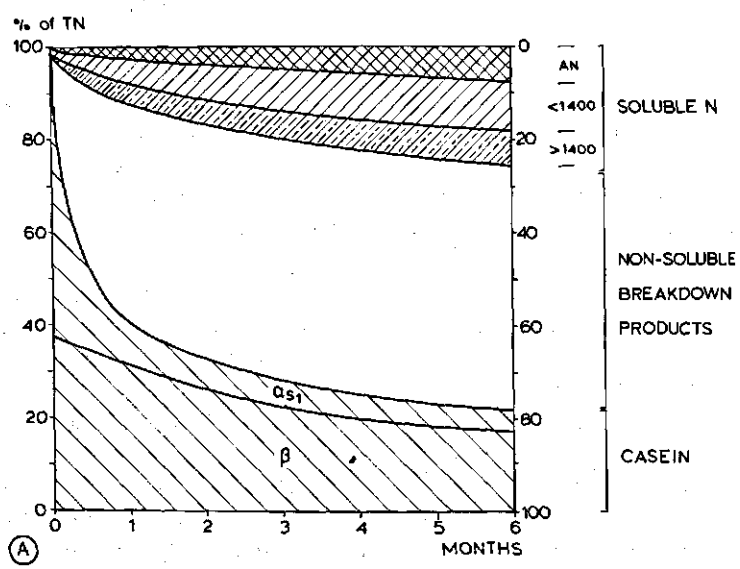
All the cheeses made, both normal aseptic, aseptic rennet-free, aseptic starter-free and cheeses free from rennet and starter, fulfilled the requirement of the absence of unwanted bacteria.

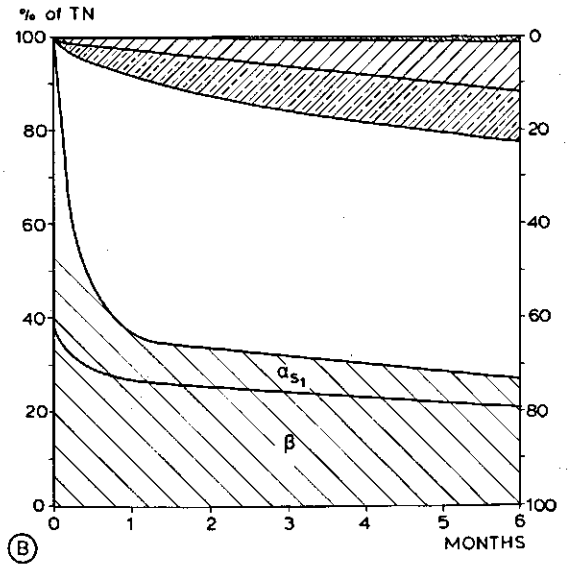
2. Rennet, starter and milk protease in protein breakdown

The experiments with normal aseptic cheeses, using different starter strains and amounts of rennet showed that the enclosed amount of rennet in the cheese distinctly influences the rate of casein attack and the accumulation of soluble-N compounds but not the accumulation of free amino acids. However, the strain of starter strongly influences liberation of amino acid N but only to a minor exte the production of soluble N. The pathway and rate of the primary casein attack is not at all affected by using different starters.

Both the primary degradation of casein – as determined by the quantitative polyacrylamide gel electrophoresis – and the production of soluble N can be regarded as characterizing the 'extent' of protein breakdown, and the accumulation of free amino acids and small peptides the 'depth' of it. So these results suggest that rennet influences the 'extent' of proteolysis in cheese and starter bacteria the 'depth'.

In a normal aseptic Gouda cheese, in which rennet, starter bacteria and milk protease act together, and that contains a normal amount of rennet, α_{s1}-casein is quickly and intensively broken down. Within the first month of ripening, most of the original α_{s1}-casein has vanished. β-Casein is attacked more slowly, after 6 months of ripening about half still being intact. In Figure 1a this breakdown and the accompa-





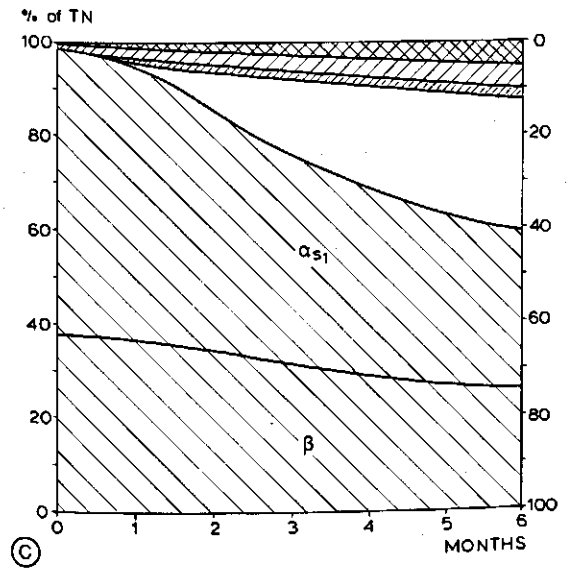


Fig 1. Breakdown of casein and development of soluble-N fractions during the ripening of different aseptically made cheeses. A: normal aseptic cheeses. B: aseptic starter-free (ASF) cheeses. C: aseptic rennet-free (ARF) cheeses. Soluble N is subdivided into amino acid N (AN), peptides with mol.wt. < 1400 and breakdown products with mol.wt. > 1400 α_{s1} -Casein plus β-casein together are supposed to make up total nitrogen (TN). For A and C, the average values obtained in cheeses made with 'non-bitter' starter are entered. The normal aseptic and ASF cheeses both contained normal amounts of rennet.

nying accumulation of free amino acids, smaller peptides and larger soluble products is summarized. The soluble-N fraction in this cheese is constituted mainly of smaller products and represents about a third or a quarter of the total amount of breakdown products during ripening. Large amounts of insoluble breakdown products must be produced in the cheese. Although we did not estimate this insoluble fraction directly, including mostly products of high molecular weight, its proportion is calculable from the other data. The small fraction of para-κ-casein was not measured in our cheeses; others (4,5) reported that it is not likely to be broken down during ripening. For ease, it is assumed that the protein in cheese is constituted only of α_{s1}-and β-casein.

To estimate the respective roles of rennet, starter bacteria and milk protease in cheese proteolysis, breakdown of protein during ripening of aseptic starter-free (ASF), aseptic rennet-free (ARF) and aseptic rennet- and starter-free (ARSF) cheeses was monitored. A broad survey is given for ASF and ARF cheeses in the Figures 1b and 1c.

In the ARSF cheeses a rather small but detectable amount of free amino acids and other soluble products is formed on ripening. By gel electrophoresis some attack of α_{s1} - and β-casein can be recorded after extended ripening times. Since the 'minor'caseins (γ,R,TS) – always detectable in ripened Gouda cheeses (6) – are also normally produced in the ARSF cheeses, milk protease was directly responsible. Milk protease apparently contributes to protein breakdown in Gouda cheese to a minor degree. Consequently, any clear proteolytic action observed in the ASF or ARF cheeses can be largely attributed to the action of rennet and starter bacteria, respectively.

Figure 1b shows that in ASF cheeses, containing a normal amount of rennet, the same breakdown of α_{s1}-casein and nearly the same breakdown of β-casein is observed as in normal aseptic cheeses. The primary breakdown products are also identical. Considerable but somewhat smaller amounts of soluble N are formed in the ASF cheeses. The composition of this soluble fraction, however, differs from that in normal cheeses. Only small amounts of free amino acids were detected and the larger products (mol. wt. $>$ 1400) are relatively more important in the ASF cheeses.

Figure 1c shows that the rate of casein attack in the ARF cheeses is far less. Only after 1 month of ripening are α_{s1} - and β-casein degraded to some extent. Nevertheless considerable amounts of soluble N, especially amino acids and smaller peptides, are liberated. Starter bacteria can apparently degrade paracasein in cheese without rennet. Different starter strains ('bitter' and 'non-bitter') cannot be distinguished by casein attack or soluble-N production but can be by amount of amino acid N produced. In general the 'non-bitter' starters produce considerable larger amounts of free amino acids than the 'bitter'starters. In the patterns of individual amino acids, however, there are only slight differences.

In conclusion, rennet is completely responsible for the primary degradation of α_{s1} - and β-casein in Gouda cheese during the first one or two months of ripening. Thereafter the proteinases of starter bacteria and to a lesser extent milk protease begin to contribute too. Since rennet will break down most of the α_{s1}-casein in the mean time, their contribution is, however, restricted to the further attack of β-casein. On the disappearance of the original α_{s1}-casein the action of the enzymes will also be directed to the degradation of the larger, predominantly insoluble, products.

Although rennet produces the greater amount of the soluble N in normal cheese,

liberating especially peptides of low and high molecular weight, starter bacteria and, to some extent, milk protease contribute too, producing especially amino acids and smaller peptides. From the net contributions of rennet, starter and milk protease, mutual stimulation or inhibition cannot be considerable in total production of soluble N in normal cheese. The combined action of the three enzyme systems in normal cheese however does influence the composition of soluble-N compounds. Far larger amounts of free amino acids and smaller peptides are liberated than can be accounted for by separate action. The starter bacterial peptidases progressively degrade the (soluble) products of rennet action of higher molecular weight. Obviously the peptidase system of starter bacteria has a certain qualitative or quantitative overcapacity relative to the proteinase system. Differences in amino acid patterns of ARF cheeses from those of normal aseptic cheeses also indicate some rennet/starter interaction.

The production of free amino acids in cheese is almost exclusively due to starter bacteria. Rennet only seems to have an indirect, stimulating capacity, while milk protease contributes only slightly.

Generally speaking proteolysis in Gouda cheese bears the stamp of rennet action for the 'extent' of breakdown and of starter bacterial enzymes for the 'depth'.

3. Rennet, starter and milk protease in development of bitterness

Normal aseptic, ASF, ARF and ARSF cheeses were made with different amounts of rennet and different starters. In normal aseptic cheeses, four starters (E8, AM1, AM2 and Bos) always produced a good flavour without bitterness and three other starter strains (HP, Wg2 and Z8) consistently produced bitter cheeses. The starters were strains of *Streptococcus cremoris,* except Bos.

From all cheeses – both bitter and non-bitter – bitter tasting peptide substances were isolated by extraction. On fractionating these peptides, the bitterness was always detected in a fraction with molecular weights less than 1400. From the non-bitter cheeses, less bitter material was however extracted than from bitter cheeses. In cheese, 16 – 20 times as much bitter substances are needed as in water to achieve an equal bitterness. Obviously a considerable amount of bitter peptides can be formed in cheese before they can be detected sensorially. The presence of cheese flavour did not interfere with the sensation of bitterness.

Figure 2 summarizes some results on the development of bitterness during the ripening of the different cheeses.

The results with ASF cheeses show that rennet contributes directly to the production of bitter peptides in cheese. During ripening, the amount of bitter peptides steadily increases. The moment when and the degree to which the cheese becomes bitter only depends on the amount of rennet enclosed in the cheese. For the amount normally present in Gouda cheese, about 2 – 3 months ripening are necessary before bitterness reaches the threshold value. On further ripening the cheese becomes increasingly bitter. The bitter peptide pool produced by rennet in ASF cheese contains both larger peptides and – in contrast to other cheeses – considerable amounts of oligopeptides.

Since Cheddar cheese contains smaller amounts of enclosed rennet than Gouda

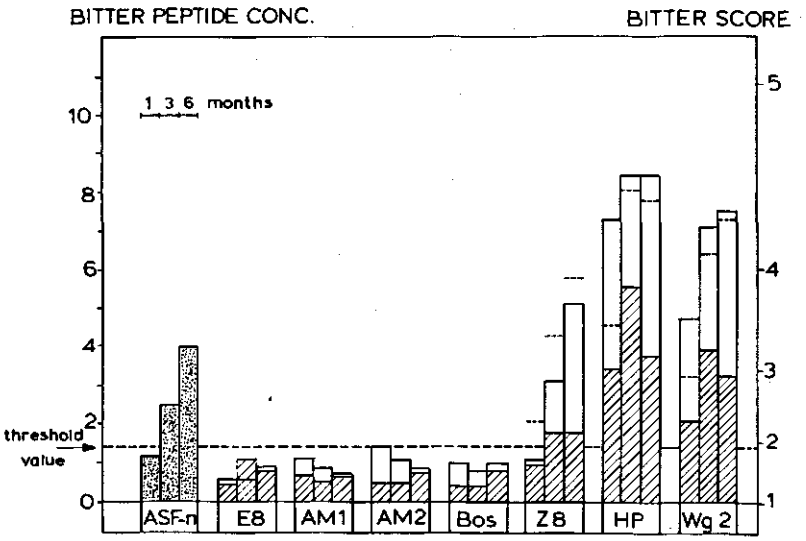


Fig. 2. Bitterness observed in different aseptic cheese types after 1, 3 and 6 months of ripening. Normal aseptic cheeses: blank + hatched area. ASF cheeses: dotted area. ARF cheeses: hatched area. The normal aseptic and ASF cheeses both contained normal amounts of rennet. For the 'bitter' starter strains HP, Wg2 and Z8 the sum of ARF and ASF bitterness is indicated with marks (–). Bitter scores of the panel are transformed into relative concentrations of bitter peptides.

cheese (7), the contribution of rennet to the bitterness in this cheese type will be less, possibly sub-threshold.

The experiments with ARSF cheeses showed that the proteolytic action of milk protease during the ripening of Gouda cheese is not intensive nor specific enough to cause detectable bitterness.

To what extent starter bacteria cause accumulation of bitter peptides during cheese ripening in the absence of rennet depends closely on the starter culture used. The 'bitter' starters HP and Wg2 liberated bitter peptides to such an extent as to cause early and persisting bitterness in ARF cheeses. Since bitterness appeared in these cheeses too. It is a mute point, whether these starters hydrolyse casein specifically obviously produced more directly and specifically from casein than by rennet. When the ASF cheeses had become bitter, far more casein had been attacked and much larger amounts of smaller peptides, the class to which the bitter peptides belong, had been liberated.

The 'non-bitter' starters (E8, AM1, AM2 and Bos) all did not produce detectable bitterness in ARF cheeses, although some bitter fractions were extracted from those cheeses too. It is a mute point, whether these starters hydrolyse casein specifically without the liberation of bitter peptides or the absence of bitterness is only the balance of a normal production of bitter peptides and an intensive immediate breakdown to non-bitter products. The truth may lie between these two ideas.

That 'non-bitter' starters indeed degrade bitter peptides in cheese to substances like amino acids, that are less bitter, if at all, can be deduced from the absence of bitterness in the normal aseptic cheeses. Even the addition of twice the normal amount of

rennet did not lead to bitterness, indicating the capacity of the peptidase system. Obviously the 'bitter' starter bacteria have far less capacity to degrade the bitter peptides from rennet action, since the level of bitterness found in normal aseptic cheeses with 'bitter' starter can be largely explained by the separate productions of bitter peptides by rennet and 'bitter' starters. Mutual stimulatory effects need not play an important role. The decrease in the amount of enclosed rennet in these cheeses indeed results in less bitterness.

Multiplication of starter bacteria to high counts in the cheese is not decisive for the appearance of bitterness in our aseptic Gouda cheeses, though it was for Cheddar cheese (8). In this respect our results agree with those of Stadhouders (7) for normal open-vat Gouda cheese. The selection of reliable 'non-bitter' starter cultures is essential for the production of non-bitter Gouda cheese. Although rennet actively contributes to bitter-peptide formation, the decrease in the retained amount of rennet by adapting the procedure of cheesemaking only comes into play when the starter culture is not optimum in breaking down bitter peptides. It is difficult to relate differences in the protein breakdown of cheeses made with 'bitter' and 'non-bitter' starters to the appearance of bitterness in these cheeses. No differences at all can be detected in the pathway or extent of primary casein attack as analysed by gel electrophoresis. No relation was found in the total production of soluble N, although, in the presence of rennet, there were some differences in composition of the peptide fraction of lower molecular weight. However far more research is needed, especially to identify the (bitter) peptides present in young rennet-free cheeses.

The only consistent and distinct difference observed between 'bitter' and 'non-bitter' starters was a higher production of free amino acids in 'non-bitter' starter cheeses, both in the presence and absence of rennet. 'Non-bitter' starters in cheese are obviously equipped with a more active exopeptidase system than 'bitter' starters, hence, perhaps, their greater capacity to degrade bitter peptides. Indications that also specific differences in the quality of the peptidase system exist between starters were not evident from comparisons of the amino acid patterns in the different cheeses.

4. Rennet, starter and milk protease in development of cheese flavour

By aseptic vat techniques Kleter (9) and others (10) demonstrated that starter bacteria play the central role in the development of flavour in cheeses like Gouda and Cheddar.

Since hardly any cheese flavour developed in our ASF and ARSF cheeses during ripening, milk protease and rennet – even at high concentrations – do not directly contribute to the formation of cheese flavour compounds. The presence of cheese flavour, to varying degree, in normal aseptic cheeses suggested that starter bacteria do produce these compounds, but direct evidence was the presence of cheese flavour in ARF cheeses. Although 'non-bitter' starters in both types of cheese produced distinctly more cheese flavour than 'bitter' starters, starter bacteria are directly involved and determine cheese flavour production. However, the amount of cheese flavour in normal aseptic cheeses is considerably more than in comparable ARF cheeses, so rennet must stimulate flavour production by starter bacteria. A relatively small amount of rennet is obviously sufficient.

The most widely accepted theory on cheese flavour is the Component Balance

Theory, which supposes cheese flavour to be produced by a blend of different compounds which must be present in the proper balance (11, 12). Numerous primary and secondary breakdown products of lactose, fat and protein may contribute. Among the products of protein breakdown, amino acids are thought to contribute to the basic background flavour of cheese. Peptides, that can cause bitter, acid or astringent flavour, may also contribute. During our work we extracted considerable amounts of astringent peptides from all types of aseptically made cheeses. These compounds – as was suggested by Harwalkar (13) – may contribute to the cheese flavour. Proteins in themselves have no taste, but they contribute to flavour indirectly with tactile and similar effects.

From the results on cheese flavour and proteolysis of all our cheeses one can conclude that there seems to be a striking relation between observed cheese flavour and amount of amino acid N. The contributions of rennet, starter and milk protease to the production of free amino acids in cheese show the same tendencies as those to cheese flavour production. Even the stimulatory effect of rennet on starter bacteria runs parallel. The observed difference between 'bitter' and 'non-bitter' starters in cheese flavour production and amino acid liberation also corresponds. The peptidase system of the starter bacteria must link the two flavour effects by degrading bitter peptides to amino acids that give cheese flavour or act as precursors.

Deep proteolysis and therefore starter bacteria are essential in the development of a well-flavoured cheese.

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Summary

To unravel the contributions of rennet, starter bacteria and milk protease to proteolysis and development of (bitter) flavour in Gouda cheese, cheeses were made aseptically in which the separate and combined action of these three enzyme systems could be studied during the ripening.

The effect of their combined action was determined in normal aseptically made cheeses with different amounts of rennet and seven starter cultures, some 'bitter' and some 'non-bitter'.

The action of rennet, without the influence of starter bacteria, was studied in aseptic starter-free cheeses with δ'-gluconic acid lactone as an artificial acidifying agent. These cheeses were normal in composition. To assess the role of rennet in quantitative terms, the amount of rennet retained in cheese, and in aseptic starter-free cheese in particular, must be measured.

To study the action of starter bacteria and their enzymes in cheese, without the influence of rennet, a method for the aseptic manufacture of rennet-free cheese was developed. By manipulating the calcium content and the temperature of the cheese-milk, the rennet could be inactivated after the completion of its enzymic action on κ-casein but before coagulation of the milk. In this way a normal gel was obtained for cheesemaking. The action of several 'bitter' and 'non-bitter' starters was studied during the ripening of these rennet-free cheeses.

With the artificial acidification and the rennet-free cheesemaking process, aseptic cheeses were made in which only milk protease was active during ripening. In this way a reliable estimate could be made of the contribution of this enzyme to ripening of Gouda cheese.

During ripening for 6 months, the cheeses were judged periodically for bitterness and cheese flavour by a taste panel. Breakdown of α_{s1} - and β-casein was characterized during ripening by quantitative polyacrylamide gel electrophoresis. Soluble breakdown products were estimated as soluble N and were characterized by gel filtration on Sephadex G-50. The amount of free amino acids and the patterns of individual amino acids were also determined. Bitter peptides were extracted from the cheeses with chloroform/methanol and were fractionated by molecular sieving on Sephadex LH-20.

Rennet was largely responsible for the primary degradation of α_{s1} - and β-casein in Gouda cheese, producing large insoluble fragments and soluble peptides of a range of molecular weights. α_{s1} -Casein in particular was broken down quickly and completely by rennet.

The proteolytic enzyme system of starter bacteria in cheese broke down casein slowly in the absence of rennet, producing especially free amino acids and soluble peptides of low molecular weight. Distinct differences in the protein breakdown

between 'bitter' and 'non-bitter' starters were detected in the amount of total free amino acids produced and not in the pattern of individual free amino acids. Milk protease contributed only slightly to proteolysis in Gouda cheese, being responsible for the formation of 'minor' caseins from β-casein and producing small amounts of free amino acids and soluble peptides.

During ripening of Gouda cheese the rennet clearly determined the 'extent' of protein breakdown whereas enzymes from starter bacteria determined the 'depth'. No stimulatory interactions were observed between rennet and starter bacteria in breakdown of paracasein nor in total production of soluble N. However, a clear shift towards smaller soluble products was detected. Peptidases from starter bacteria produced clearly larger amounts of free amino acids and smaller peptides by the presence of breakdown products of rennet action.

The separate actions of rennet and starter bacteria were responsible for the development of the 'bitter' defect in Gouda cheese. During ripening, rennet gradually liberated an increasing amount of bitter peptides, resulting in a distinctly bitter starter-free cheese after 3 months. 'Non-bitter' starters hardly accumulated any bitter peptides in cheese, but they did degrade the bitter peptides produced by rennet. By doing so they prevented matured Gouda cheese from becoming always bitter. 'Bitter' starters could in a highly specific way liberate such amounts of bitter peptides as to cause early bitterness in rennet-free cheese. They had far less capacity to degrade bitter peptides to non-bitter products than 'non-bitter' starters.

Cheeses made with 'bitter' starters always had less intensive cheese flavour than those with 'non-bitter' starters, either in the presence or absence of rennet. Although neither rennet nor milk protease contributed significantly to the production of cheese flavour components, rennet appeared to play an indirect auxiliary role.

Samenvatting

De bijdrage van de enzymen van stremsel, zuurselbacteriën en melk aan de eiwitafbraak en smaakontwikkeling in Goudse kaas

Stremsel, zuurselbacteriën en in mindere mate melkprotease bepalen tesamen grotendeels de afbraak van eiwit tijdens de rijping van kazen van het Goudse type. Deze proteolyse bepaalt in belangrijke mate de structuur en smaak van een kaas. Zowel de ontwikkeling van de gewenste kaassmaak en -geur als het ontstaan van het gebrek 'bitter' ten gevolge van de aanwezigheid van bittere peptiden, hangen ten nauwste samen met de eiwitafbraak.

Om inzicht te krijgen in de rol die elk der genoemde enzymsystemen hierbij speelt, werd tijdens het onderzoek gebruik gemaakt van aseptische kaasbereidingstechnieken die het mogelijk maakten de gescheiden en gecombineerde werking ervan in kaas te bestuderen.

In de 'normale' aseptische kazen is de eiwitafbraak tijdens de rijping het gevolg van de gecombineerde werking van de enzymen van stremsel, zuursel en melkprotease. Om de bijdrage van stremsel vast te stellen, zonder aanwezigheid van zuurselbacteriën, werden kazen bestudeerd waarin tijdens de bereiding de verzuring op chemische wijze werd gesimuleerd. Om de rol van stremsel enigszins kwantitatief te kunnen benaderen, bleek het noodzakelijk de hoeveelheden stremsel in de kaas te bepalen. Het bepalen van de afzonderlijke bijdrage van zuurselbacteriën, zonder de interactie met het stremsel, werd mogelijk door de ontwikkeling van een methode voor de aseptische bereiding van stremselvrije kaas. Door regeling van het calciumgehalte en de temperatuur van de kaasmelk bleek het mogelijk het stremsel, dat werd gebruikt voor de paracaseinevorming, te inactiveren alvorens coagulatie tot een gel optrad. Deze kazen bleken zeer geschikt om de werking van de proteolytische enzymen van het zuursel tijdens de rijping te bestuderen. Door de stremselvrije en zuurselvrije kaasbereidingstechniek te combineren werden kazen verkregen waarin tijdens de rijping nog slechts melkprotease actief was. Daarmee was de mogelijkheid geschapen om de bijdrage van dit enzym tijdens de kaasrijping vast te stellen. Voor de bereiding van de kazen die zuursel bevatten, werden verschillende zuurselcultures gebruikt met het doel de werking van zowel 'niet-bittere' als 'bittere' zuursels te vergelijken. Voor de bereiding van stremselbevattende kazen werden de gebruikte hoeveelheden stremsel sterk gevarieerd.

Tijdens de rijpingsperiode van zes maanden werden de kazen organoleptisch gevolgd om de bitterheid en de kaassmaak vast te stellen. De eiwitafbraak in de verschillende kazen werd gekarakteriseerd met behulp van verschillende analysetechnieken. Kwantitatieve polyacrylamide gelelectroforese werd gebruikt om de afbraak van α_{s1} - en β-caseïne en het ontstaan van de grotere afbraakprodukten te volgen. De hoeveelheid oplosbaar stikstof werd bepaald door extractie en de peptidesamenstelling ervan onderzocht met behulp van gelfiltratie. De hoeveelheid vrije aminozuren en de samenstelling ervan werden eveneens onderzocht. Bittere en adstringerende peptiden

werden uit de verschillende kazen geëxtraheerd en gefractioneerd met behulp van gelfiltratie.

Er kon worden vastgesteld dat stremsel in Goudse kaas grotendeels verantwoordelijk is voor de primaire afbraak van α_{s1}- en β-caseïne tot grote, onoplosbare brokstukken en oplosbare peptiden met een van hoog tot laag variërend molecuulgewicht. Vooral α_{s1}-caseïne wordt zeer snel en volledig afgebroken. Aminozuren worden echter door stremsel nauwelijks gevormd.

Het proteolytisch enzymsysteem van zuurselbacteriën bleek in kaas op een wat langere termijn caseïne te kunnen afbreken onder de gelijktijdige vorming van aminozuren en grotendeels kleine oplosbare peptiden. Duidelijke verschillen wat betreft de eiwitafbraak tussen 'bittere' en 'niet-bittere' zuursels werden slechts gevonden in de totaal geproduceerde hoeveelheid vrije aminozuren.

Aangetoond werd dat melkprotease verantwoordelijk is voor de vorming van de 'minor'caseins uit β-caseïne tijdens de rijping. Bovendien wordt er een geringe hoeveelheid vrije aminozuren en peptiden gevormd.

Wanneer de enzymsystemen tesamen werken, zoals in een normale Goudse kaas, wordt de breedte van de afbraak duidelijk bepaald door de stremselwerking en de diepte door het zuursel. In de primaire afbraak van paracaseïne en de totale aanmaak van oplosbare afbraakprodukten vindt onderling geen duidelijke stimulatie plaats. Er treedt echter wel een verschuiving naar kleinere oplosbare verbindingen op. Zuurselpeptidasen produceerden duidelijk grotere hoeveelheden vrije aminozuren en kleine peptiden door de aanwezigheid van door stremsel gevormde afbraakprodukten.

Tijdens het onderzoek werden de afbraak van de caseïnes en de vorming van de verschillende afbraakprodukten steeds zo kwantitatief mogelijk beschreven.

Bij de ontwikkeling van het gebrek 'bitter' in Goudse kaas blijken de afzonderlijke werkingen van zowel het stremsel als de zuurselbacteriën een belangrijke rol te spelen. Het stremsel produceert tijdens de rijping een gestaag toenemende hoeveelheid bittere peptiden die na drie maanden een duidelijk bittere smaak veroorzaken in de zuurselvrije kaas. 'Niet-bittere' zuursels hopen niet of nauwelijks bittere peptiden op in kaas; ze breken daarentegen juist de door stremsel gevormde grotendeels af. Daarmee verhinderen ze dat een Goudse kaas steeds bitter zou smaken ten gevolge van de stremselwerking. 'Bittere' zuursels kunnen snel en op een zeer specifieke wijze zodanige hoeveelheden bittere peptiden vrijmaken, dat de stremselvrije kaas bitter wordt. Ze bezitten klaarblijkelijk veel minder vermogen tot afbraak van bittere peptiden dan de 'niet-bittere' zuursels.

Zowel in aanwezigheid als in afwezigheid van stremsel blijken kazen bereid met 'bittere' zuursels steeds minder kaassmaak te bezitten dan die gemaakt met 'niet-bittere' zuursels. Hoewel kon worden vastgesteld dat stremsel en melkprotease elk niet bijdragen tot de directe aanmaak van kaassmaak-componenten, blijkt de aanwezigheid van stremsel de kaassmaakvorming door zuursels wel te bevorderen.