CHEESE FROM ULTRAFILTERED MILK

– WHEY PROTEINS AND CHYMOSIN ACTIVITY –

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Proefschrift

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STELLINGEN

1. De produktie van half-harde kaas met behulp van membraantechnologie verdient een tweede kans.

2. Bereiding van half-harde kaas uit geültrafiltreerde melk, met als doel opbrengstverhoging door insluiting van natieve wei-eiwitten, heeft alleen zin voor een hoge UF-concentratiefactor in combinatie met een weinig intensieve wrongelbewerking.
   Dit proefschrift, hoofdstuk 2

3. De verlaagde activiteit van chymosine in aanwezigheid van wei-eiwitten wordt veroorzaakt door de (sterke) associatie van een hoogmoleculaire component uit de wei-eiwitfractie aan het actieve centrum van chymosine.
   Dit proefschrift, hoofdstukken 3 en 4

4. Bij de (directe) bepaling van de chymosineactiviteit in kaas is het van belang dat alle kaasbestanddelen die van invloed kunnen zijn op de activiteit tijdens de rijping ook aanwezig zijn in het opgewerkte, verdunde kaasmonster.
   Dit proefschrift, hoofdstuk 4

5. Verhoging van de chymosineactiviteit in traditioneel bereide kaas kan zowel bewerkstelligd worden door een verhoogde chymosinedosering als door voorzuring of snellere verzuring, terwijl bij kaas uit geültrafiltreerde melk alleen een verhoogde dosering daartoe kan dienen.
   Dit proefschrift, hoofdstuk 5

6. De belangstelling van de zuivelindustrie voor de bereiding van kaas uit geültrafiltreerde melk zou sterk toenemen als het vrijkomende permeaat gebruikt zou kunnen worden voor de standaardisatie van consumptiemelkprodukten op eiwitgehalte.

7. Bij veel onderzoek naar het achterblijven van de rijping van kaas bereid uit geültrafiltreerde melk is er aan voorbijgegaan dat chymosine niet alleen van belang is voor de stremming, maar evenzeer voor de rijping.

8. Toepassing van nieuwe bereidingsprocessen in de levensmiddelenindustrie vereist de bereidheid ook de traditionele receptuur aan te passen.

9. Gezien de herkomst van melkpermeaat zou het logisch zijn dat limonades bereid met melkpermeaat, overeenkomstig andere dranken met een zuivelkarakter, vrijgesteld worden van de heffingen die voor limonades gelden.
   Wet op de verbruiksbelastingen van alcoholvrije dranken en van enkele andere produkten (Staatsblad 683; 24-12-1992)

10. De sterische uitsluiting van langzaam verkeer door snelverkeer is sterk afhankelijk van de snelheid en omvang van de snelverkeersdeelnemers.

11. Door het milieuvriendelijke karakter van de vlasteelt en -verwerking en door de veelzijdige en duurzame mogelijkheden voor gebruik én hergebruik van de vezel verdient vlas het predikaat: groene grondstof voor textiel én allerlei andere produkten.

12. Stofbeheersing is vooral een kwestie van vochtregulering.

13. Het herstellen van de orde in een chaotische omgeving leidt veelal tot nieuwe wanorde, maar door toepassing van de zgn. dozenmethode* kan de chaos in principe op een effectieve manier geordend worden.
   *T. Jansen, Reflectie, de Volkskrant dd. 10-9-1994

14. Acceptatie van de (wedstrijd)ligfiets in de gevestigde professionele wielersport zou ook tot een doorbraak leiden voor de (toer)ligfiets in de recreatieve sfeer.

Stellingen behorende bij het proefschrift
CHEESE FROM ULTRAFILTERED MILK – WHEY PROTEINS AND CHYMOSIN ACTIVITY –
C.A.P. Buijsse – februari 1999

ABSTRACT

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Keywords: cheese, Gouda cheese, UF-cheese, whey proteins, ultrafiltration, chymosin activity, rennet, syneresis

The manufacture of (semi-)hard cheese from ultrafiltered milk (UF-cheese) enables the partial incorporation of whey proteins in the cheese, thereby increasing its yield. The transfer of whey proteins in curd from (UF-)milk was studied in relation to the degree of ultrafiltration of the milk and the degree of syneresis of the curd. In UF-cheese manufacture (from 5x concentrated UF-retentates, concentrated further by syneresis) approximately one-third of the whey protein fraction was enclosed.

Despite this yield increase, UF-cheese production trials in the last decennia have not been successful: the yield increase is limited and counteracted by retarded ripening. It was concluded that the latter is partly due to reduced chymosin activity in UF-cheese, as compared to traditional cheese. The lower chymosin activity was due to a reduced chymosin dosage to UF-retentates (because clotting occurs much faster at higher casein contents), and to enzyme inhibition.

The activity of chymosin in the first stage of cheese ripening was studied in cheese models, as well as in (semi-)hard Gouda type (UF-)cheese, with varying chymosin and whey protein contents. The chymosin activity was derived indirectly from the rate of release of degradation products from casein, or directly by application of a newly developed method that enables the direct estimation of the chymosin activity in gels, curd and cheese. Results of both methods corresponded very well.

Chymosin appeared to be inhibited in its activity by a component of the whey protein fraction. Because of this inhibition, the chymosin to casein ratio should be higher in whey protein-containing UF-cheese than in traditionally manufactured cheese. A higher chymosin activity in (UF-)cheese can be achieved by increasing the dosage of chymosin to the milk or retentate, as the enclosure in cheese is proportional to this dosage. The transfer of chymosin into cheese is mainly due to adsorption of chymosin onto casein, which is enhanced at lower pH. The enclosure of chymosin in cheese can thus also be increased by increasing the rate of acidification during cheese manufacture. Increasing the chymosin dosage to UF-milk and/or the rate of acidification would require drastic modifications of the manufacturing process.

CONTENTS

Chapter 1

GENERAL INTRODUCTION

Cheese

In cheese some important milk components, mainly caseins and fat, are concentrated. The moisture, consisting of water with soluble components, is largely removed with the whey. Cheese is conserved by fermentation, due to the conversion of lactose into lactic acid by lactic acid bacteria. Many cheese varieties have been developed worldwide, all with their own characteristics. Differences are due to variation in the process of manufacture and to (bio)chemical and physical transformations occurring during ripening, which determine flavour and textural characteristics.

Traditional cheese manufacture

The traditional cheesemaking process of semi-hard varieties starts with the addition of rennet enzymes and starter bacteria to the cheese milk. The rennet enzymes are responsible for splitting off the caseino-macropeptide-fraction from κ-casein. This enzymic reaction is followed by the aggregation of casein micelles, which leads fo the formation of a caseinate network, hence, a gel. In the caseinate network the fat globules and the starter bacteria are entrapped. The coagulum is then cut, to start syneresis. During syneresis whey is expelled from the coagulum, hereby concentrating the casein and the fat in the curd. Whey consists of water and dissolved milk components, i.e. whey proteins, lactose, salts and minerals. Syneresis is further enhanced by stirring the mixture of curd and whey. After a given time part of the whey is removed and replaced by water, to lower the concentration of lactose and lactic acid in the curd. In this manner, the final pH of the cheese is controlled. After sufficient syneresis has occurred, the curd is collected, drained and pressed. More whey is expelled from the curd block in these stages. Altogether, a considerable amount of whey (approximately 85-90% of the milk volume) is removed. The pressed curd blocks are then brined and stored under controlled conditions for ripening.

In this conventional cheesemaking process most of the soluble whey proteins are released in the whey. Both serum proteins (about 20% of proteins in milk) and the caseino-macropeptide, which is released from κ-casein during renneting, belong

to this whey protein fraction. In renneted milk, approximately 24% of the protein fraction consists of whey proteins. A considerable loss of proteineous material occurs thus in the traditional cheesemaking process.

These whey proteins can be applied in several food products. Although many applications are developed for the reuse of whey proteins, the direct enclosure in cheese would markedly enhance the yield of cheese. In general, the financial return of the whey proteins will be higher in case of incorporation in cheese, by means of yield increase, as compared to other applications of the whey proteins.

Several methods for the enclosure of whey proteins in cheese are available. The methods differ in the whey proteins being enclosed in the native state or, if an intense heat treatment is applied, in the denatured state.

Methods to enclose whey proteins in cheese

Preconcentration of the cheese milk by ultrafiltration (UF) allows much of the whey proteins to be incorporated in cheese in their native state. The milk is ultrafiltered prior to the addition of coagulant and starter bacteria. The degree of ultrafiltration is limited by the viscosity of the concentrated milk, i.e. the retentate. Therefore, in the manufacture of (semi-)hard cheeses from ultrafiltered milk, syneresis is required to further lower the water content of the coagulated UF-retentate, as it is not possible to ultrafilter the milk to the final dry matter content of the cheese. Some loss of soluble whey proteins is then inevitable. The whey volume will be strongly decreased, while the whey protein content in this whey will be higher, compared to the traditional process. Because of steric exclusion of whey proteins by (para)casein micelles, the whey protein content in the whey will even be stronger increased (Walstra, 1973; van Boekel and Walstra, 1989). The now released by-products, i.e. protein-free and fat-free permeate and protein-rich whey, are considered to be more useful for making by-products than is conventional whey (Lawrence, 1989). Concentrating all retentate components (including whey proteins) in the cheese can be achieved by evaporation of water from the retentate under low pressure (de Boer and Nooy, 1980; Ernstrom et al., 1980; Jameson et al., 1994ab). Rennet enzymes and starter bacteria are then added in-line to the concentrated retentate or pre-cheese. Covacevich and Kosikowski (1978) prepared pre-cheeses by mixing retentates with freeze-dried retentates. It was also tried to increase the recovery of (native) whey proteins in cheese by adding ultrafiltered whey to the cheesemilk of the next day. The result is expected to be minimal as the whey proteins will be largely excluded, again, during syneresis. Besides, also other

components are then added, such as chymosin, which may cause problems in the manufacturing process.

Other methods apply heat treatment to incorporate whey proteins. Heating of the cheese milk prior to renneting leads to the association of most whey proteins with κ-casein, and they are then, in a denatured state, accumulated in the curd. A severe heat treatment, however, also affects the rennetability of the milk (van Hooydonk et al., 1987), and it has some consequences for the later stages in the process. This impaired coagulation can be enhanced by lowering pH, increasing temperature, adding $CaCl_2$, or by higher rennet levels. Ultrafiltration, prior to or after a heat treatment, may also result in better coagulation properties (Maubois and Mocquot, 1975; Casiraghi et al., 1989; Green, 1990ab; Guinee et al.,1995). Whey proteins were also incorporated in the coagulum by addition of heat denatured whey protein concentrates or aggregates, or reconstituted whey protein concentrate, to the cheese milk (Abrahamsen, 1979, Brown and Ernstrom, 1982; Banks and Muir, 1985; Baldwin et al., 1986). These whey protein aggregates are entrapped in the coagulum, similar to the fat globules. In general, cheese from heat-treated milk is of inferior quality, meaning flavour and consistency (Zoon, 1993).

Henceforth, only the enclosure of whey proteins in cheese by means of ultrafiltration of the milk will be discussed. Any heat treatment of cheesemilk, other than thermising or pasteurization, with the aim to denature serum proteins is not considered, as severe heating is not common practice in Dutch type cheese manufacture.

Ultrafiltration in the cheesemaking process

The concept of cheese manufacture from milk concentrated by ultrafiltration was introduced by Maubois and Mocquot (1971). During ultrafiltration, a membrane separation process, proteins and fat are concentrated in the retentate, while water with lactose, salts, minerals and non-protein nitrogen components are selectively removed. At ongoing ultrafiltration, the concentration of high molar mass components in the retentate increases. These French workers applied ultrafiltration for concentrating the milk to the final dry matter content of soft cheeses (mainly Camembert), and added rennet and starter bacteria to the retentate. A considerable yield increase could thus be obtained for these high moisture cheeses.

In the manufacture of (semi-)hard cheeses from retentates, the increase in yield that can be achieved will be less, as whey drainage is required to reach the desired dry matter content in the curd. The whey protein content in the moisture is

higher in retentates and will therefore also be higher in the released whey, but the total loss of whey proteins may be minimized because of the lower volume of whey to be expelled. The possible yield increase, that can be achieved by application of ultrafiltration in the cheesemaking process, has lead to many studies, in which UF-cheese was compared with the traditionally prepared equivalent. Results from such yield studies were often contradictory, and strongly dependent on the method applied to compare cheese yields, milk savings and retention of components. The many different types of cheese that were subject of investigation made it even more confuse.

The increases in yield that were claimed by application of UF could not always be attributed to the inclusion of higher amounts of native whey proteins (with the corresponding additional water). Other components should also be considered. Increased yield may also be due to a higher fat or casein retention, or to aggregation of denatured whey proteins with caseins. For a good comparison, yields should be expressed unambiguously. Overviews of literature on cheese yield are published in documents of the International Dairy Federation (1993/1994). In these documents, methods of expressing yield and formulae are extensively discussed. In addition, Lucey and Kelly (1994) published a review on cheese yield. An extensive survey on transfer of milk components into Gouda cheese is given by van den Berg et al. (1996).

Besides the increase in cheese yield, the possible saving in rennet is reported as an advantage of UF-cheese manufacture. This saving arises from the decreased amount of retentate to be coagulated and the lower dosage required to coagulate the retentate. As rennet is of major importance for ripening as well, the saving should not result in a lower residual rennet activity in the cheese. A considerable saving can only be realized for cheeses prepared from retentate without release of whey. The reduction of the milk volume in an early stage of the process is also seen as an advantage, as it increases the capacity of the existing equipment (as far as it can be used at all for handling UF-retentates). In other situations specific equipment for the further processing of the retentate is needed instead (besides equipment for ultrafiltration). The use of UF-retentates may facilitate automation of the cheesemaking process. In the view of volume reduction, ultrafiltration of milk on the farm has been suggested. Costs of storage, refrigeration and transportation would be lower. The permeate could then be re-used as a cattle feed. Saving in the processing of whey from UF-cheese manufacture and the shorter manufacturing time are also put forward as advantages. Furthermore, ultrafiltration of milk may be

an opportunity for the development of new cheese varieties (Maubois and Mocquot, 1975; Goudedranche et al., 1981; Bush et al., 1983; Kealey and Kosikowski, 1985; Garrett, 1987; Guinee et al., 1994).

Ultrafiltration of milk to a very low CF (up to 2) is used as a method to standardize the protein content in the milk prior to cheese making (using normal equipment and conventional procedures with minor adaptations) in order to improve process control all over the year and to optimize efficiency (Chapman et al., 1974; Bush et al., 1983; Fernandez and Kosikowski, 1986; Sharma et al., 1989; Guinee et al., 1994, 1996). For this purpose normal milks were also supplemented by highly concentrated retentates (Kosikowski, 1983; Kosikowski and Masters, 1984; Kealey and Kosikowski, 1985; Kosikowski et al., 1985). The increased buffering capacity may enable a better control of the rate of acidification and the final pH (Kosikowski, 1983). In general, the yield increase at low degrees of ultrafiltration was reported to be negligible. Most whey proteins were still lost in the whey. Besides, when normal equipment is used for cheese manufacture from these retentates, the losses of fat and caseins may be increased due to curd shattering.

Whereas the concept of cheese manufacture from ultrafiltered milk is nowadays applied all over the world for the manufacture of several types of soft cheese, as UF Feta and Camembert, preconcentration of the cheese milk is hardly introduced for the manufacture of (semi-)hard cheeses, that undergo proteolysis for flavour and texture development. This can mainly be attributed to the atypical maturation and the changed consistency of the UF-cheese. Over the last decennia, many studies were performed on the manufacture of (semi-)hard cheese by ultrafiltration techniques. The investigations involved various aspects of manufacture, composition and ripening (flavour and texture development) of several cheese varieties. It was mostly tried to manufacture (semi-)hard UF-cheeses with properties similar to those of the traditional variants. It should be realized, however, that characteristics may be expected to be different from the traditionally prepared counterpart, due to the partial replacement of caseins by whey proteins, having different properties, and possibly other, minor changes in composition. According to Lawrence (1989), the whey proteins should not exceed 10% of total protein in cheese, for obtaining a cheese with similar properties.

In many trials a considerable yield increase was reported, but this advantage was mostly counteracted by the changed ripening characteristics and a poor consistency. Unfortunately, the relations between changes in the process, changes in the composition of the cheeses and the altered ripening characteristics were not

elucidated in many studies. Besides, comparison of results from various studies is difficult, due to differences in the process of production (procedures and type of equipment), additions to the cheese milk, methods of analysis and ripening conditions. Differences in the process of manufacture arose largely from the different types of cheese that were subject of research. Research groups in different countries have paid attention to the manufacture of their cheese varieties by application of ultrafiltration techniques. In the Netherlands research was focused on Gouda and Edam cheese, while in Australia, New Zealand, the United States and Great Brittain the investigations were aimed on the manufacture of Cheddar cheese from UF-retentates. In other European countries, ultrafiltration was introduced for several (semi-)hard cheese varieties (Havarti, Danbo, Saint-Paulin). About 20 years after the introduction of ultrafiltration in the cheesemaking process, some extensive overviews dealing with the various aspects of cheese manufacture from ultrafiltered milk have been published (Jensen et al., 1987; Lelievre and Lawrence, 1988; Bech, 1993). Also in the International Dairy Federation, the subject has received attention (IDF, 1993/1994; Lawrence, 1989; Jameson and Lelievre, 1994).

Ultrafiltration of cheese milk, prior to the addition of rennet enzymes and starter bacteria, changes the cheesemaking process considerably. Henceforth, various stages of the manufacture of UF-cheese are discussed. The consequences of ultrafiltration for the different stages in the process of manufacture, for the composition of the cheese as well as for the ripening are discussed.

Ultrafiltration of cheese milk

During ultrafiltration, the relative and absolute concentrations of components in the retentate change, as the membrane is only permeable for low molar mass molecules (lactose, salt, minerals, non-protein nitrogen). Proteins and fat are mainly concentrated by ultrafiltration, as well as components in the milk that are partly associated with protein (minerals like Ca, P and Mg). The distribution of small components between the retentate and the permeate depends on the cut-off of the membrane and may be influenced by factors as pH and temperature, as these affect the degree and equilibria of association or solubilization (Lawrence et al., 1983, 1984; Green et al., 1984). For components that do not interfere with the high molar mass components (such as lactose), an almost constant concentration is maintained in the water phase of the milk, retentate and permeate. The overall concentration of such components in the retentate will thus be decreased, compared to milk, because of the decreased water content. The 'overall' degree of concentration of the

milk can be expressed in several ways. Some authors give a concentration factor (CF) based on the volume reduction (milk versus retentate), while others define the concentration factor as the ratio of the concentration of a component in the retentate and its concentration in the milk. Also comparison of dry matter contents (in milk and retentate) is applied. In this thesis the CF of retentates is derived from the total protein contents in the retentate and the skim milk, from which the retentate is prepared.

The preparation of cheese from UF-retentates requires the components in the concentrated cheesemilk to be present in correct proportions, without an irreversible change in the physical state of the components (Sutherland and Jameson, 1981). Several tools, i.e. acidification and diafiltration, are available for the regulation of the content of minerals, like calcium and phosphate, and lactose in the retentate. The concentrations of these components in the retentate largely determine the rate of acidification during cheese manufacture and the pH of the cheese, by their impact on the buffer capacity (calcium, phosphate) and the production of lactic acid (lactose). The initial pH of the retentate and the rate of acidification affect the process of coagulation by rennet, as well as the composition and texture of the curd and the final cheese (Sutherland and Jameson, 1981; Lawrence et al., 1983, 1984; Qvist et al., 1987; Lucey and Fox, 1993; Guinee et al., 1994).

Preacidification of the milk results in the diffusion of micellar calcium phosphate into the serum, which is consequently removed with the permeate during ultrafiltration. This way the buffer capacity of the retentate is lowered. The concentrations of solubilized small components in the retentate can be further reduced by diafiltration. For this purpose the retentate is diluted with water and concentrated by ultrafiltration again. Diafiltration is mainly applied to regulate the lactose content in the retentate (de Boer and Nooy, 1980; Spangler et al., 1991). In fact, diafiltration replaces the curd washing step, that is performed in the traditional cheesemaking process. The omission of curd washing also prevents the additional loss of rennet enzymes with the second whey (Sutherland and Jameson, 1981; Jameson, 1987).

In general, ultrafiltration is applied to skim milk, instead of whole milk, and cream is added to the concentrated skim milk, prior to the addition of rennet and starter. A higher degree of concentration can be reached this way. Besides, it prevents the partial homogenization of fat globules in the valves of the ultrafiltration unit (de Boer and Nooy, 1980) and thereby the susceptibility to lipolysis (Green et al., 1984).

The degree of ultrafiltration is limited by the viscosity of the retentate, which increases with increasing concentration. At higher viscosity the pressure over the membrane increases, the membrane will readily be fouled and the flux will be decreased (Lawrence, 1989). To counteract the increase in viscosity, ultrafiltration is often applied at increased temperatures (up to 55°C). Also a decrease of the pH of the milk (to 6) results in a decreased viscosity, but this may affect later stages of manufacture and characteristics of the final cheese. At increased ultrafiltration temperatures whey proteins may denature and become aggregated with caseins. This can be checked by determination of the casein number, as applied by Guinee et al. (1996). In the literature, foaming of retentate and the passage through pumps of highly viscous retentate (shear denaturation) are also mentioned as causes for whey protein denaturation (Everett and Jameson, 1993). The extent of denaturation also depends on the extent of recycling and the residence time in the UF-plant. The retentate is recycled longer when diafiltration is applied (Sood and Kosikowski, 1979). The effect of ultrafiltration per se (to CF 5, at 50°C) on the suitability for cheese manufacture was investigated by Lelievre et al. (1986). Cheeses prepared from recombined UF-milk (from retentate CF 5 and permeate) appeared to be similar to cheeses from control milk. Also rennet coagulation was reported to be unaffected by ultrafiltration without concentration (Casiraghi et al., 1989).

The increased viscosity of retentate requires special attention concerning the uniform addition of rennet and starter. During mixing, the incorporation of air bubbles should be prevented, as these are not easily released (de Boer and Nooy, 1980; Jameson, 1987).

Coagulation of UF-retentates

The coagulation properties of retentates are considerably different from those of unconcentrated milks. Many studies are performed on the renneting of concentrated milks (Dalgleish, 1980, 1981; Garnot and Corre, 1980; Payens, 1984; van Hooydonk and van den Berg, 1988; Garnot, 1988; Sharma et al., 1993). The process of coagulation depends largely on the casein content, the rennet content, the pH and the coagulation temperature. In general, at higher casein content the degree of κ-casein hydrolysis is lower at the onset of gelation. A large degree of κ-casein hydrolysis and aggregation of casein micelles should be realized at cutting of the gel, while otherwise considerable amounts of casein micelles and fat may be lost in the whey (as observed by Green et al., 1981; Bush et al., 1983; Fernandez and Kosikowski, 1986; Guinee et al., 1994). Whereas in milk the stages of flocculation

and gelation can be distinguished clearly, these seem to occur as good as simultaneously in the case of retentates. At gelation of a retentate, however, possibly not all casein micelles are aggregated. At unchanged dosage of rennet enzymes (% based on the amount of milk before ultrafiltration), the gelation would occur within a few minutes. The coagulation is even enhanced in retentates of lowered pH.

To regulate the rate of coagulation, the coagulation temperature could be reduced (Green, 1985; Spangler et al., 1990; Guinee et al., 1996). At reduced temperature, however, the viscosity of the retentate is increased, which makes it possibly difficult to mix the additions uniformly through the retentate. The coagulation could also be controlled by regulation of the calcium content in the retentate. Reduction of the rennet dosage is another tool to maintain the cutting time at approximately 30 minutes (i.e. cutting time in the traditional process). In many studies the dosage was reduced in proportion to the concentration factor of the retentate, and was thus based on the volume of retentate to be coagulated. Such a reduction involves a strong decrease in the ratio of rennet to casein. As rennet enzymes are important in the breakdown of caseins during ripening, a reduction in dosage might have large consequences for the maturation processes, by means of a lowered enclosure in the cheese at unchanged conditions (Visser, 1977a). The enclosure of these enzymes in the curd, however, is also influenced by other factors (such as pH, syneresis and temperature).

Starter bacteria and acidification

On the growth of starter bacteria and the activity of the starter in retentates, contradictory and inconsistent results are reported (Mistry and Kosikowski, 1985; Meijer et al., 1995). This is partly attributed to the increased concentration of whey proteins in the substrate for these starter bacteria. The starter strain chosen, the amount added and its growth rate in the retentate influence the conversion of lactose to lactic acid during the cheese making process, as well as the development of characteristics of the cheese during ripening. In general, for UF-cheese manufacture higher amounts of starter bacteria are dosed to the retentate, because the total UF-cheesemaking process (until brining) lasts shorter, as water is removed faster by ultrafiltration than it is by syneresis. The total time of manufacture being shorter has consequences for the starter replication (Garrett, 1987; Jameson, 1987).

Starter culture can be either inoculated in heated milk or in heated retentate, which is added to fresh, unheated retentate shortly before renneting. Garrett (1987),

Jameson (1987) and van Leeuwen et al. (1984) reported the inoculation in a minor, heated portion (1-20%) of the retentate. By inoculation in milk, the problems with the growth of starter in retentate are partly overcome. Inoculated milk, however, dilutes the retentate to which it is added. Moreover, a higher volume is needed to obtain a sufficient number of bacteria in the cheese (Mistry and Kosikowski, 1986).

In retentates more acid production is required to achieve a given reduction in pH, as compared to fermentation of unconcentrated milk, because of the increased buffer capacity of protein-rich retentates. The effect of the higher buffer capacity can be counteracted by addition of higher amounts of starter, or starters with a higher activity. To enlarge the decrease in pH that can be realized in the conversion of lactose to lactic acid by the starter bacteria, preacidification of either the milk or the retentate can be applied. In the case of preacidification of milk, i.e. prior to ultrafiltration, the buffer capacity will be decreased. A further decrease can be achieved by diafiltration (removal of solubilized calcium and phosphate). Diafiltration, however, also lowers the lactose content in the retentate, which may limit the acid production and the decrease in pH (Sutherland and Jameson, 1981; Qvist et al., 1987; Lawrence, 1989; Spangler et al., 1991).

Preacidification (either of the milk or of the retentate) and the rate of acidification due to starter activity can also be used as tools to influence the retention of rennet enzymes (by means of adsorption onto casein in the curd) in the cheese. The inclusion of rennet in the curd depends largely on the pH at whey drainage. More rennet is retained at a lower pH (Green and Foster, 1974; Stadhouders and Hup, 1975; Holmes et al., 1977; Lawrence et al., 1983, 1984; Creamer et al., 1985; Qvist et al., 1987; Spangler et al., 1991).

Curd cutting and syneresis

The next stages of the UF-cheese manufacturing process depend largely on the CF of the retentate. At low CF (up to 2) the process resembles the traditional process, and the same cheese making equipment can be used, with some adaption of the cutting device (Lawrence, 1989; Guinee et al., 1994). At higher CF, however, special equipment is needed. The changed characteristics of the coagulated retentate requires adapted methods of curd cutting and enhancement of syneresis, to prevent the loss of fat and curd fines in the whey. The curd may be very firm and difficult to cut. The immediate release of whey as soon as the curd is cut does not take place in the case of retentates. The tendency towards whey expulsion is lower at higher protein contents in a retentate gel (Casiraghi et al., 1989). Stirring of the

mass of curd in its own whey to enhance syneresis, as in the conventional process, is therefore not possible. It would result in considerable shattering of the fragile curd, in which the degree of κ-casein hydrolysis may be lower at the start of cutting. Also the total whey volume is reduced considerably. For this purpose special coagulation and cutting equipment is designed. The patented APV-Siro-Curd process and equipment (van Leeuwen et al., 1984), developed in Australia, was described and evaluated by Jameson (1987) and Garett (1987). This continuous process was preceded by a batch pilot scale version, as used by Sutherland and Jameson (1981). Another type of equipment, the Alcurd Continuous Cheese Coagulator (Alfa Laval), was used by Qvist et al. (1987), by Spangler et al. (1991) and by Guinee et al. (1995). The Alcurd equipment is also used in the present study (see Chapter 5). Both types of equipment cut the gel by pushing it through a cutting grid, after which it is cut by a rotating blade into small cubes or other type of uniform particles. Depending on the dry matter content of the curd and the aimed content in the cheese, the freshly cut curd may be transferred to rotating drums to enhance syneresis (Siro-Curd) or it can be directly collected in cheese moulds and left standing for some time before pressing (Alcurd). The subsequent fusion of curd cubes from retentates may be different as well, because of the changed characteristics of the coagulated retentate and the curd grains, from which whey expulsion is relatively low. It may require adaptations in the stage of pressing.

Composition of UF-cheese

The composition of UF-cheese depends on the transfer of individual components of the retentate into the curd. As stated earlier, the main motive for UF-cheese manufacture is the expected yield increase. In general, yield increase refers to the enclosure of whey proteins that are almost totally lost with the whey in the traditional cheesemaking process. The partial incorporation of whey proteins will result in the protein fraction being different from that in traditionally manufactured cheese. Other water soluble components, that are normally lost with the whey, may be retained to a higher extent as well. The increased retention of whey protein also involves the enclosure of more moisture in the cheese, to keep the total protein to water ratio unchanged.

In this respect, however, also other components should be considered. The retention of fat in the curd will largely depend on the firmness of the coagulum, the moment of curd cutting, the curd size and the subsequent curd handling. It should

be prevented that the increased retention of whey proteins is offset by an increased loss of fat.

The mineral content in UF-cheese may be changed as well, especially the calcium phosphate to protein ratio, due to changes in the process of manufacture. In particular the stages in the process that influence the acidification will affect the mineral content, by means of the effect on the equilibrium of solubilization and subsequent loss during ultrafiltration and diafiltration in the removed permeate and in the released whey (Lawrence et al., 1983, 1984).

Proteolysis of UF-cheese

In the ripening of (semi-)hard cheese varieties, rennet enzymes, native milk proteinases and enzymes from starter bacteria are involved. Flavour and texture development largely depend on the proteolysis of caseins. Chymosin and milk proteinases are responsible for the primary degradation of α_{s1}-casein and β-casein, respectively, while secondary hydrolysis is due to the action of peptidases from starter bacteria. Their importance in Gouda cheese and related cheese types has been extensively studied by Stadhouders (1960), Stadhouders and Hup (1975), Visser (1977cd) and Noomen (1978ab), while for Cheddar cheese these aspects were investigated by several groups in various Cheddar cheese-producing countries. Several reviews on the ripening aspects of cheese were published (Grappin et al., 1985; Fox, 1989; Visser, 1993). Relations between cheese manufacture, composition, proteolysis and texture of ripened cheese are clearly elucidated by Lawrence et al. (1983, 1984, 1987). The role of rennet in cheese manufacture and ripening is reviewed by Guinee and Wilkinson (1992).

The retarded flavour development in ripened UF-cheese, compared with cheese prepared according to conventional procedures, is the main reason for the cheese manufacture from UF-retentates not being introduced on a large scale. A retarded breakdown of caseins was observed in several types of UF-cheese. The retarded proteolysis is mostly attributed to the incorporated whey proteins in general, as the presence of these whey proteins in UF-cheese is seen as the main difference with the traditionally prepared cheese. In many reports, a further specification or mechanism of the changes with regard to proteolysis was lacking. Apparently, the influence of whey proteins on cheese ripening processes is not fully understood. Several mechanisms of interaction between whey proteins and other components are mentioned in an attempt to explain the retarded maturation.

The incorporation of whey proteins in cheese involves a decrease in the casein content, as it is mostly aimed to maintain the total protein content in the cheese unchanged. Part of the casein fraction is thus replaced by the incorporated whey proteins. In fact, the caseins are diluted by the enclosed whey proteins. The whey proteins are reported to be resistant to hydrolysis by milk proteinases and the proteolytic enzymes of rennet and starter bacteria (Harper et al., 1989; de Koning et al., 1981; Qvist et al., 1987; O'Keeffe et al., 1978; Furtado and Partridge, 1988). The resistance to proteolytic activity by chymosin is exhibited by the caseino-macropeptide, released from κ-casein at renneting, which also belongs to the whey protein fraction in cheese (Shammet et al., 1992). Since purified whey protein concentrate has a neutral flavour (Wingerd, 1971), the whey proteins are considered as inert fillers, not contributing to the development of flavour (de Koning et al., 1981; de Boer and Nooy, 1980). The concentration of flavour forming breakdown products from caseins is thus lower as well. The decrease in the development of flavour compounds, due to this dilution, will be directly related to the degree of whey protein incorporation in the cheese.

Rennet enzymes, mainly chymosin, are of major importance for the proteolytic changes occuring in cheese during ripening. The amount and activity of these enzymes can be largely determinant for the rate of hydrolysis of caseins. The retarded flavour development is often attributed to the lower rennet to casein ratio in the UF-cheese than in traditionally manufactured cheese. The lower residual rennet level is due to the strongly reduced dosage, as is often applied for the coagulation of retentates (Covacevich and Kosikowski, 1978; Green et al., 1981; Green, 1985). Also other adaptations in the manufacturing process (acidification, whey expulsion) may influence the residual rennet level, by means of their influence on the rennet retention.

Whey proteins may affect ripening processes by specific inhibition of some of the enzymes involved. Inhibition of chymosin and alkaline milk proteinase (plasmin) have both been reported (Lawrence, 1989; Harper et al, 1989; Christensen et al., 1991; Bech 1993). Proteinase and peptidase inhibitors, if present in milk, may be concentrated in the retentate (Hickey et al., 1983). The inhibition of chymosin, reflected in the slower breakdown of α_{s1}-casein, is mentioned by Creamer et al. (1987). Lelievre et al. (1990) reported the inhibition of rennet action by high molar mass whey proteins, both in the milk clotting assay and in the proteolysis of casein. To counteract the partial inhibition it was suggested to increase the chymosin content in the cheese (Creamer et al., 1987). A lower plasmin activity is attributed to

the whey protein β-lactoglobulin (Lawrence et al., 1987; Qvist et al., 1987; Bech, 1993). Bastian et al. (1991) suggested to increase plasmin activity by activation of plasminogen.

Another explanation for the reduced release of flavour components is the limited accessibility of casein for hydrolyzing enzymes, due to specific blocking by whey proteins. Also the slower enzyme diffusion in the cheese, due to the increased viscosity of the aqueous phase in the cheese, containing whey proteins, was held responsible (Lelievre and Lawrence, 1988).

With regard to the ripening processes, also the starter bacteria play a very important role. Their growth can be affected in retentates during cheese manufacture and proteolytic activity may be changed in UF-cheese (Meijer, 1997).

Outline of this thesis

This thesis deals with many aspects of the manufacture and ripening of semi-hard cheese from ultrafiltered milk. One of the aims of this work was to unravel the problems encountered with proteolysis of UF-cheese. As these can not be seen independent of the protein composition of the cheese, the transfer of whey proteins into the (UF-)cheese has been subject of research. In particular the role of chymosin in the degradation of proteins is considered.

The role of micro-organisms in cheese ripening, of major importance for secondary proteolysis and flavour development, is not included here, but has been described by Meijer (1997). Also textural aspects of UF-cheese are not discussed in this study. The study is limited to (semi-)hard cheese types without any surface flora, like Gouda and Cheddar, in which ripening is realized by proteolytic activity of rennet, milk proteinases and starter bacteria.

As the UF-cheese manufacturing process is developed to improve the recovery of milk proteins in cheese, while the cheese characteristics are aimed to be similar to those of traditionally manufactured cheese, the various steps of preparation are compared with those in the conventional process. In this respect, the traditionally prepared cheese can be considered as a control.

Chapter 2 deals with the accumulation of whey proteins from milk and UF-retentate into the syneresed curd. For the manufacture of (semi-)hard cheese varieties from milk and retentates, additional syneresis is required to reach a sufficient dry matter content of the curd, and losses of whey proteins will occur. The losses of whey proteins during syneresis are quantified for retentates that were

concentrated to varying degrees (CF 1-4). The recovery of nitrogen (both casein and whey proteins) is determined for varying degrees of syneresis.

Proteolysis in cheeses from UF-retentates is reported to be retarded and atypical. In general, whey proteins are held responsible. Several mechanisms of the whey proteins influencing proteolysis were suggested. Because of the importance of chymosin in the ripening of cheese varieties like Gouda and Cheddar, especially the role of chymosin is studied. In Chapter 3 the effect of whey proteins on the activity of chymosin in UF-retentates and in cheese models is described. As highly concentrated retentates were prepared, the loss of whey, and thereby of chymosin, was prevented. Chymosin dosage was varied. In the cheese models, both chymosin contents and whey protein contents were varied.

In Chapter 4 the development of a method for the estimation of the activity of chymosin in chymosin-induced milk and retentate gels and curd is described. For a better understanding of ripening processes, knowledge about the activity of chymosin is required. A method for determining the chymosin activity in several substrates (chymosin-induced gels, curd, cheese) is developed. The method was evaluated at the same time, by application on gels with known chymosin contents.

Chapter 5 describes a pilot study on different aspects of UF-cheese manufacture. The effect of variations in amounts of ingredients (chymosin, starter bacteria) in the cheesemaking process was studied in UF-cheeses, as well as in traditionally manufactured Gouda type cheeses. Acidification, composition, chymosin activity and proteolysis were compared and some relations between these parameters were established.

Finally, in Chapter 6, results of the different subjects of this study are linked and discussed. That chapter includes general conclusions.

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Chapter 2

INCORPORATION OF WHEY PROTEINS IN (UF-)CURD

ABSTRACT

In the traditional cheesemaking process water-soluble whey proteins are largely lost in the whey during syneresis, when a large part of the moisture is removed from the curd. In the manufacture of UF-cheese the water content is already strongly decreased during ultrafiltration of the milk, prior to coagulation. The amount of whey to be expelled from the curd is lower, and, although its whey protein content is increased, the total loss of whey proteins is decreased. With the manufacture of cheese from ultrafiltered milk the retention of whey proteins, and thereby the total protein recovery in cheese, can be increased. Changes in the protein composition and the loss of whey proteins from curd during syneresis were studied (with cube-shaped curd grains) in relation to the degree of ultrafiltration of the cheesemilk and the degree of syneresis. In some cases extra whey proteins were added to the milk or retentate. During syneresis the paracasein content of curd increases, while the whey protein content decreases. Whey proteins are not accumulated in the curd by filtration in the network of paracasein micelles. In contrast, because of steric exclusion/negative adsorption of whey proteins by casein micelles (i.e. part of the water in the casein micelles is not available as a solvent for whey proteins), the loss of whey proteins in the whey is more than proportional. This is reflected in a decrease of the whey protein to water ratio in curd at ongoing syneresis. The steric exclusion factor (gram water per gram casein in which no whey protein was dissolved) appeared to be independent of the degree and the method of concentration of the caseins, either by ultrafiltration of the milk or by syneresis of the milk or retentate gel, and of the whey protein content and the pH. The total enclosure of whey proteins in cheese, however, does not only depend on the retention of whey proteins in syneresed curd, but also on other process steps as the moment and manner of curd collection and further curd handling.

-Lactic acid (Merck): a 90% solution was diluted to 45% and sterilized before use to hydrolyse any esters present.
-Thiomersal (BDH Chemicals)
-Other chemicals of analytical grade (Merck): $CaCl_2$, sodium citrate, acetic acid, trichloroacetic acid (TCA)

Manufacture and syneresis of cube-shaped curd grains

Cube-shaped curd grains were prepared from chymosin induced skim milk gels and skim milk retentate gels. Skim milk was renneted with 0.03% (v/w) chymosin at 30°C. Prior to chymosin addition 1 mM $CaCl_2$ was added. Retentates were renneted without extra $CaCl_2$ at room temperature with 0.02% (v/w) chymosin. After addition of $CaCl_2$ and chymosin the milk or retentate was brought in a specially designed small square curd vat (see figure 2.1). The height of the milk layer was set at 6 mm. Part of the milk with additions was kept as a control sample. After approximately 30 to 90 minutes, as the milk or retentate was gelled, the gel layer was cut in squares with a frame of fine stainless steel wires (6x6 mm).

The fresh-cut cube-shaped curd grains were transferred, with a scoop designed to prevent curd shattering, into tubes (Greiner, volume 50ml, diameter 27 mm), partly filled with skim milk permeate (pH 6.7, 35°C). Per tube approximately 2 grams of curd grains were added. The tubes were then filled totally with permeate (35°C). By slowly rotating the tubes on a vertical wheel, syneresis of the curd was enhanced without the curd grains getting fractured of aggregated (see figure 2.2). After a certain time of stirring the syneresed curd grains and the permeate with whey, expelled from the curd, were separated by filtering. The amounts of permeate and freshly-cut curd (before rotating) and of syneresed curd (afterwards) were determined (in grams), in order to know the amount of whey that was expelled from the curd.

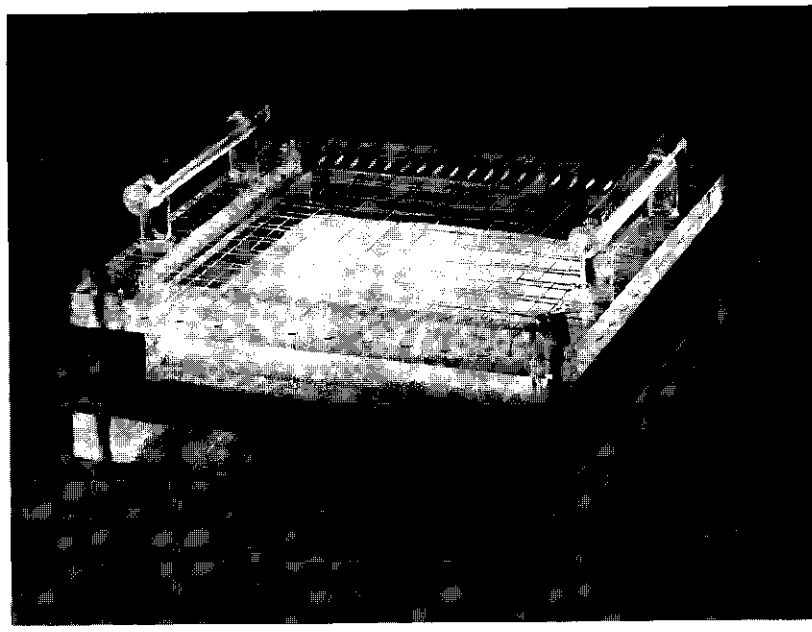

Figure 2.1 Cutting grid, for the preparation of cube-shaped curd

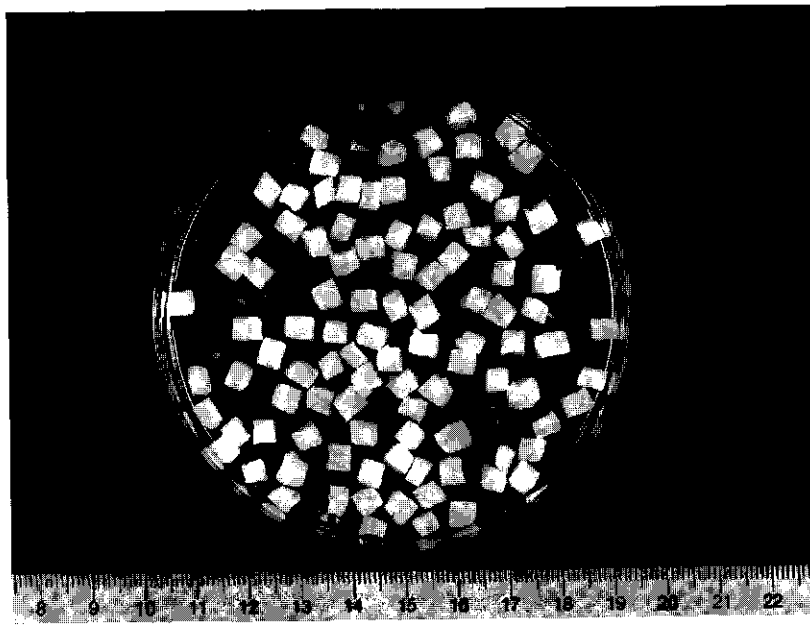

Figure 2.2 Syneresed curd cubes, prepared as described in text.

Most curd was prepared from skim milk and skim milk retentates at physiological pH (6.7). For some experiments the pH was decreased to 6.0 or 5.2 with lactic acid. A whey protein isolate (Bipro) was added to some batches of skim milk or skim milk retentate, in order to increase the whey protein content without increasing the casein content.

One-dimensional syneresis

In some preliminary experiments the gel layer was not cut, but a thin stainless steel plate (with many very small perforations) was put on top of the gel layer, as earlier applied by van Dijk (1982). Syneresis started due to the applied pressure. The external pressure could be varied by putting weights on top of the perforated plate. The expelled whey, which accumulated on top of the plate, was collected carefully with a pipette at certain time intervals. The amount of whey was weighed. Both the expelled whey and the syneresed gel were analysed.

Analyses

Dry matter contents from skim milk and skim milk retentates (without and with extra whey protein isolate), permeate and the permeate/whey mixtures were determined by the use of a Mojonnier apparatus.

The protein composition was determined in skim milk and skim milk retentates, before and after (in the control sample) gelling, in permeate, permeate/whey mixtures and in syneresed curd grains. To allow unbiassed sampling, the chymosin-induced gels and the syneresed curd grains were diluted with either a sodium citrate-solution or water and homogenized by the use of an Ultra-Turrax. The protein content and composition were determined by analysis of nitrogen in (partly precipitated) samples. All nitrogen determinations were performed by the macro-Kjeldahl method, according to IDF standard 20B (1993). The total nitrogen content (TN) was determined in some grams of the product. The non-casein-nitrogen content (NCN) was determined in the filtrate of a sample that had been diluted with water and acidified to pH 4.6 with 10% w/v acetic acid. For the determination of NCN in skim milk IDF procedure 29 (1964) was followed. The non-protein-nitrogen content (NPN) was determined in the filtrate of a sample in which all proteins had been precipitated with 15% TCA (final concentration 12% w/v), according to IDF standard 20B, part 4 (1993). The determined nitrogen contents in fractions of milk, retentates and other dissolved milk products were recalculated to nitrogen contents in the corresponding milk products, correcting for the volume of

the precipitated fraction and for non-solvent water according to the formula given by Walstra and Jenness (1984). In the correction for the amount of non-solvent water average steric exclusion factors were applied. In these calculations also considerations as mentioned by Karman et al. (1987) were taken into account. The steric exclusion factor for whey proteins, used for the correction of the NCN-content, was 2.6 (van Boekel and Walstra, 1989), while the applied factor for NPN-components was 0.2 (Walstra and Jenness, 1984).

With these corrected NCN- and NPN-contents the protein composition of the milk, retentate or dissolved milk product was calculated. The casein nitrogen content was derived from the difference between TN and NCN, while the serum protein nitrogen content was equal to the difference between NCN and NPN. The true protein nitrogen content could be derived from the difference between TN and NPN.

Due to the chymosin activity the NCN and the NPN contents in the gelled samples were increased, as compared to the skim milk and retentate samples. These increased contents were marked as NCN* and NPN*. With TN, NCN* and NPN* the paracasein and whey protein content was calculated. In the conversion of nitrogen concentrations to protein concentrations, Kjeldahl factors according to van Boekel and Ribadeau-Dumas (1987) were applied.

The (total) nitrogen content of the expelled whey was derived from the amounts and the nitrogen contents of permeate and the mixture of permeate with expelled whey. The composition of the used whey protein isolate (Bipro) was derived from the skim milk and skim milk retentate samples, to which known amounts of Bipro were added.

The gels and expelled whey samples from the preliminary syneresis experiments were analysed by HPLC. These gels were dispersed with 0.5 M sodium citrate solution. The HPLC-equipment consisted of an autosampler with a 20 µl fixed loop (Marathon), a Spectroflow 757 absorbance detector (Kratos Analytical Instruments), a Spectra Physics computing-integrator and an Epson personal computer with chromatography software (Spectra Physics), as was also used by van Boekel and Walstra (1989). A Superdex 75 HR 10/30 column with a volume of 24 ml was used. The eluent consisted of 0.125 M KH_2PO_4 and 0.125 M K_2HPO_4. The flow was 1 ml/min. Detection was performed at 280 nm.

RESULTS AND DISCUSSION

Skim milk and skim milk retentates, without and with extra whey proteins

Skim milks were concentrated up to 4 times by ultrafiltration. The corresponding dry matter contents varied between 9.1% (skim milk) and 22.6% (retentate 4 times concentrated). Concentration factors (CF) were based on the protein content of the retentate, relative to the protein content of the corresponding skim milk.

Addition of Bipro increased the serum protein content in skim milk batches from 0.56% to 1.1 or 4.9%. In retentates with extra whey protein isolate, the serum protein content was increased from 1.67 to 3.98% in one case, whereas it was raised from 2.77 to 4.81% in another retentate. Addition of Bipro increased TN and NCN, while NPN and caseinN remained unchanged.

Chymosin-induced skim milk gels and retentate gels

Chymosin is responsible for the hydrolysis of casein into paracasein and caseino-macropeptide (cmp). The latter is soluble and becomes part of the serum protein, which is then referred to as whey protein. The release of cmp leads to the increase of NCN and NPN (cmp partly dissolves in 12% TCA), and consequently to a decrease in the casein (now: paracasein) content. The NCN*-content and the whey protein content in chymosin-induced milk and retentate gels, prior to syneresis, is of major importance for monitoring changes due to syneresis. For the determination of NCN* in chymosin-induced skim milks and retentates the gels had to be dispersed or solubilized. In a sample from the resulting liquid the paracasein could be precipitated and NCN* determined in the filtrate.

Several methods for dispersing and fractionating cheese (mainly fractionation of nitrogen components by selective precipitation) were reviewed in IDF Bulletin 261 (1991). Fractionation at pH 4.6 is commonly applied for the precipitation of caseins. A well dispersed sample is required for good sampling. Based on the results of the comparisons in the IDF Bulletin, it was decided to disperse the chymosin-induced gels according to the method of Vakaleris and Price (1959), with some adaptions. Gels were dispersed with 0.5 M sodium citrate, thereby diluting them by 5 or 10 times, followed by precipitation at pH 4.6 with 10% acetic acid. Although a good pipettable dispersion was obtained with this method, it appeared that the determined paracasein content was by approximately 10% lower than the casein content of the corresponding milk or retentate. This would imply that the percentage of cmp,

released from the casein fraction by the action of chymosin, is very high. In the literature (Chapman, 1981; Phelan, 1981; Walstra and Jenness, 1984; Karman et al., 1987; van Boekel, 1994, derived from van Hooydonk and Olieman, 1982; Grappin and Lefier, 1994) percentages varying from 4 to 6% are given. Variation in this figure may be partly ascribed to the action of plasmin during renneting, as this enzyme, present in milk, shows maximum activity at 37°C (Walstra & Jenness, 1984). The high apparent level of hydrolysis as determined here, may have arisen from differences in the used solutions: the casein content was determined in the skim milk or retentate that was diluted with water, while the paracasein content was determined in a sample of the chymosin-induced gel diluted with sodium citrate. The pH of the dispersed gel was much higher (8 to 9) than the pH of the diluted milk or retentate. The relatively low paracasein content, as compared with the casein content in the milk or retentate, points to a relatively high NCN* content in the dispersed gel. The influence of using 0.5 M sodium citrate for the simultaneous dispersing and diluting of a gel, was studied by diluting the corresponding milk or retentate with sodium citrate as well. For these comparisons the same batches of skim milk and retentate were used again (after 15 days, conserved by thiomersal, stored at 4°C). To eliminate any time effects, skim milk and retentate samples were analysed again according to the initially applied methods, as well as by dispersing them with sodium citrate (diluted 5 or 10 times). Results are given in table 2.1.

Table 2.1 Nitrogen contents in (fractions of) skim milk and skim milk retentate, diluted with water or 0.5 M sodium citrate (by 5 or 10 times). Precipitation of casein by lowering of pH (in a sample diluted with water) to 4.6, in case of skim milk also according to IDF method 29 (1964).

	diluted with	day	TN%	NCN%	casein%	casein precip.
skim milk	water	1	0.567	0.124	2.814	IDF 29
	water	16		0.123		IDF 29
	water	16		0.128		pH 4.6
	sod.citr. (5x)	16	0.568	0.155	2.622	pH 4.6
	sod.citr. (10x)	16	0.563	0.171	2.491	pH 4.6
retentate	water	1	1.368	0.268	6.994	pH 4.6
	water	16		0.263		pH 4.6
	sod.citr. (5x)	16	1.371	0.332	6.608	pH 4.6
	sod.citr. (10x)	16	1.366	0.347	6.481	pH 4.6

The IDF 29 procedure (1964) was applied for the determination of the NCN% in milk. This method could not be applied as such for retentates, because of the higher protein content and corresponding stronger buffering. Retentates were partly diluted with water, prior to the lowering of the pH to 4.6 with 10% acetic acid.

Comparison of results of analysis of the milk and retentate after 1 day and after 16 days shows that the NCN% was not changed, as might have happened due to the action of native milk enzymes such as milk proteinase. In samples diluted with 0.5 M sodium citrate the NCN% was higher, with a lower derived casein content as a consequence. The increase was stronger as the samples were diluted more. Apparently part of the caseins in these sodium citrate solutions were not precipitated at pH 4.6, thus remaining in solution and determined as NCN-components. These results show that comparison of milk samples, diluted with water, and their chymosin induced gels, dispersed with 0.5 M sodium citrate, was not justified. This explains also that the derived percentage of cmp, released from casein, was estimated too high.

Since the NCN*-content of the gels and the protein contents that are derived from NCN* are very important for studying the changes due to renneting and syneresis, it was decided not to use anymore dispersing solutions like sodium citrate. Instead, the gels were dispersed with water while thoroughly mixing with an Ultra-Turrax. In this way the pH was unchanged and no substances that change the

dissolution of caseins were added. As the dispersion of a gel dispersed with water was not stable, thorough mixing during sampling (with a magnetic stirrer) was needed.

Changes in the (para)casein content of renneted gels due to release of cmp, as determined by analysis of these in water-dispersed mixtures, were now in line with expectations. Results are given in table 2.2

Table 2.2 Nitrogen contents in fresh (without *) and in chymosin-treated (with *) skim milk and retentates (concentration factor 2.5 and 4, respectively).

	TN	NCN	NCN*	NPN	NPN*
skim milk	0.560	0.120	0.150	0.029	0.037
retentate (CF 2.5)	1.354	0.255	0.302	0.029	0.046
retentate (CF 4)	2.164	0.395	0.453	0.027	0.052

From these data derived paracasein contents of the rennet-induced samples were 93.3%, 95.7% and 96.7% of the casein contents of the corresponding skim milk and retentates, respectively. In the retentates the release of cmp from casein was possibly not completed by the time of analysis. The incomplete hydrolysis in case of retentates must then be ascribed to the lower chymosin dosage (in relation to casein content) in combination with lower renneting temperatures. However, by the time of curd drainage, when curd and whey are separated, hydrolysis would be complete.

Syneresis

The preliminary one-dimensional syneresis experiments were not very successful, because syneresis was too slow, especially in the case of retentates. To reach sufficient degrees of syneresis, the experiment had to be continued for many hours. During these long lasting experiments proteolysis of caseins (due to the action of chymosin) was going on, as could be concluded from the decrease in the amount of caseins in syneresed gels that had been kept under pressure for many hours. This problem was not faced in the later experiments with cube-shaped curd grains, as syneresis then continued much faster. Most of the reported results are obtained from these enhanced syneresis experiments. Although the one-

dimensional experiments were not successful concerning the degree of syneresis that could be achieved, some results of analysis (of syneresed gels and expelled whey) gave useful additional information that could not be neglected. Therefore some additional results from the one-dimensional experiments are given as well further on.

The amount of whey expelled from the curd cubes and the rate of syneresis depended strongly on the initial moisture content of the curd and the pH of the milk or retentate. As the amounts of curd and permeate that were brought together in the tubes had to be weighed, the time that elapsed between cutting of the gel and the start of rotation of the tubes was approximately 10 to 15 minutes. Results from curd from skim milk and retentates, all at pH 6.7, are presented in figure 2.3A. The influence of the pH, in the case of curd from retentate with a casein content of 10.4%, is shown in figure 2.3B.

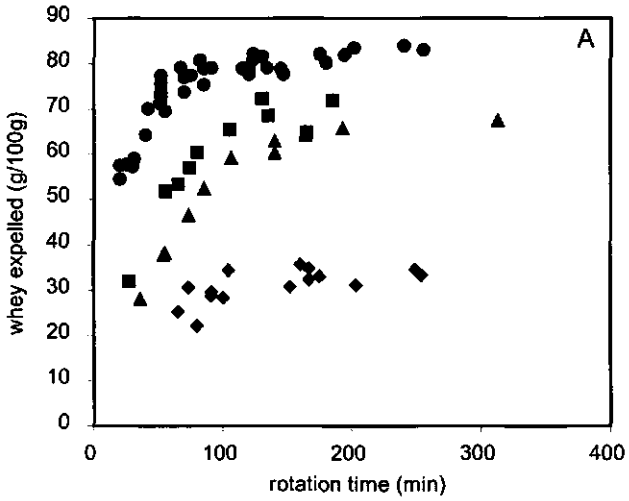

Figure 2.3A Syneresis of curd at pH 6.7. The expelled amount of whey in relation to the time of rotation for curds of various initial casein contents:
● skim milk 2.6-2.8% casein, ■ retentate 6.9% casein, ▲ retentate 7.8% casein, ◆ retentate 13.4% casein.

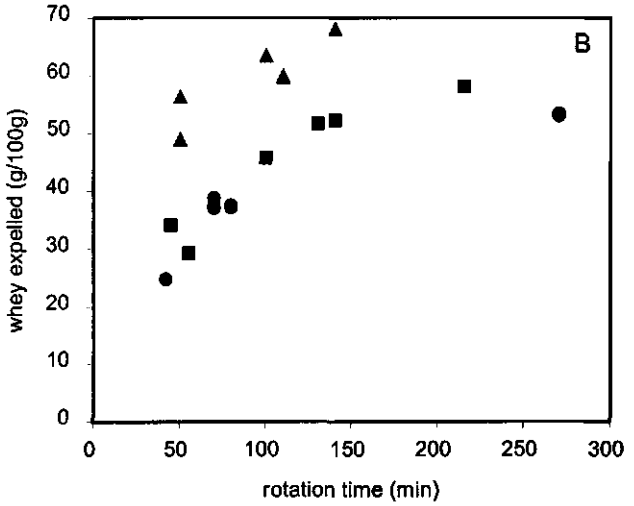

Figure 2.3B Syneresis of curd from retentate (10.4% casein) at various pH values: ● pH 6.7 ■ pH 6.0 ▲ pH 5.2.

Syneresis of skim milk gels started very soon after the gel was cut, even without stirring or rotating, while for retentate gels external forces (e.g., as caused by rotation) were required to enhance syneresis. This could also be noticed during performance of the experiment: the curd grains from retentate gels readily sticked together again after cutting the gel, while those from skim milk gels remained separated due to the thin layer of whey that was expelled immediately as the curd was cut. Therefore, it was decided to cut the retentate gels under a layer of permeate. The permeate, which was also used for stirring the curd in the rotating wheel, prevented the freshly cut cubes from sticking together again. Sutherland and Jameson (1981), who also prepared cubed curd (10x10x10 mm) with specially designed equipment, reported the tendency of curd cubes from retentates (CF 5) to fuse in the first few minutes after cutting as well. As they did not keep the curd grains in whey or permeate, they broke up fused aggregates of curd particles by gentle stirring, which resulted in more curd shattering. Syneresis of curd from skim milk continued very fast, which made it difficult to make curd with relatively small percentages of whey lost. This was easier for curd from retentates, as syneresis occurred far slower then.

Van Dijk (1982) and van den Bijgaart (1988) have extensively studied properties of rennet-induced skim milks and UF-concentrated retentates. Van Dijk

and Walstra (1986) reported a decreased rate of syneresis due to a decreased permeability at a higher concentration factor of the retentate. The lower permeability could be attributed to the paracasein network being denser. Also at increased shrinkage of the curd, after stirring for some time, the rate of syneresis was decreased, reflecting a smaller permeability as well. In our experiments syneresis of curd from retentate continued equally fast (or slow) at pH 6.7 and 6.0, while at pH 5.2 the loss of whey was faster (figure 2.3B). Van den Bijgaart (1988) found a higher permeability of the rennet-induced skim milk gels at lower pH values. The increased permeability in skim milk gels at pH 6.0, as compared with pH 6.7, seems to be counteracted in retentates, as no clear difference in syneresis could be observed in the experiments with cube-shaped curd grains. Although syneresis is in principle enhanced at lower pH, it may be slowed down because of the lower permeability of the outer layer of the curd particle, which is, in turn, caused by the rapid shrinkage (Walstra, 1993).

From these results the maximum amount of whey that could be expelled from the curd grains (pH 6.7) was derived (fig. 2.4). This maximum achieved loss is related to the initial whey content of the gel, which is derived from the paracasein content (including colloidal calcium phosphate) of the gel:

whey content = 100 - (1.08 × *paracasein content*)

in which the factor 1.08 represents the content of colloidal calcium phosphate in casein micelles.

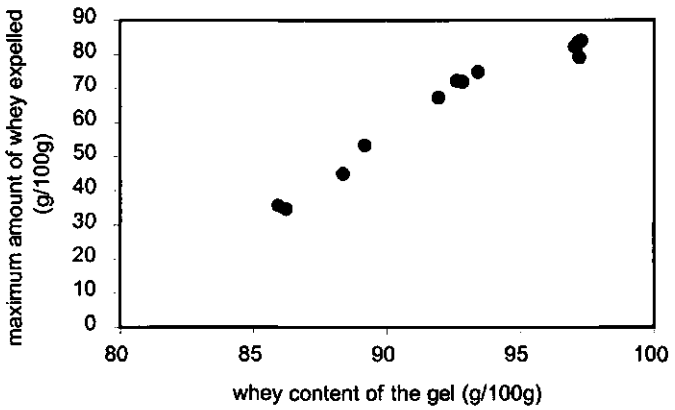

Figure 2.4 Syneresis of curd at pH 6.7: maximum amount of whey expelled after rotating as a function of the initial whey content of the gel.

The amount of whey that was expelled during rotation of the tubes with curd and permeate was strongly decreased at lower (initial) moisture contents of the curds (as in ultrafiltered milk). The ability to expel whey is very small for the retentate gels. Therefore, ultrafiltration of milk to a high protein content and subsequently concentrate this high-protein product further by stimulating syneresis is not necessarily the quickest method to reach a protein content as high as possible. As results presented later will show, a stronger concentration can be achieved by ultrafiltration to a concentration factor of approximately 2.5, followed by syneresis, compared to concentrating by ultrafiltration to CF 4 prior to syneresis.

Changes in curd composition due to syneresis

During syneresis the protein composition of the curd changes continuously. It was tried to follow these changes by making curd that had undergone syneresis to varying extents. With results from curd differing in the amount of whey expelled, all prepared from the same milk or retentate, it is possible to follow the change in protein composition in relation to the amount of whey expelled. Results are presented in figures 2.5 A - F. Both the nitrogen and protein composition of curds are given. All curves start with the composition of the chymosin-induced gel, in which the casein has been converted into paracasein. Possibly the initial contents (i.e. NCN%, NPN% and derived paracasein% and whey protein%) of the curd (at the time of cutting) deviated slightly from the contents as determined in the chymosin-induced gel, as the conversion of casein into paracasein in the gel was perhaps not yet complete at the time of cutting. These minor deviations, however, will not affect trends and can therefore be neglected for the aim of this study. In rennet-induced gels the content of soluble proteins, first referred to as serum protein content, is increased (due to the release of the cmp-fraction) and henceforth named the whey protein content. All results are given in relation to the extent of syneresis, i.e. the amount of whey (g) that was expelled per 100 gram freshly cut curd.

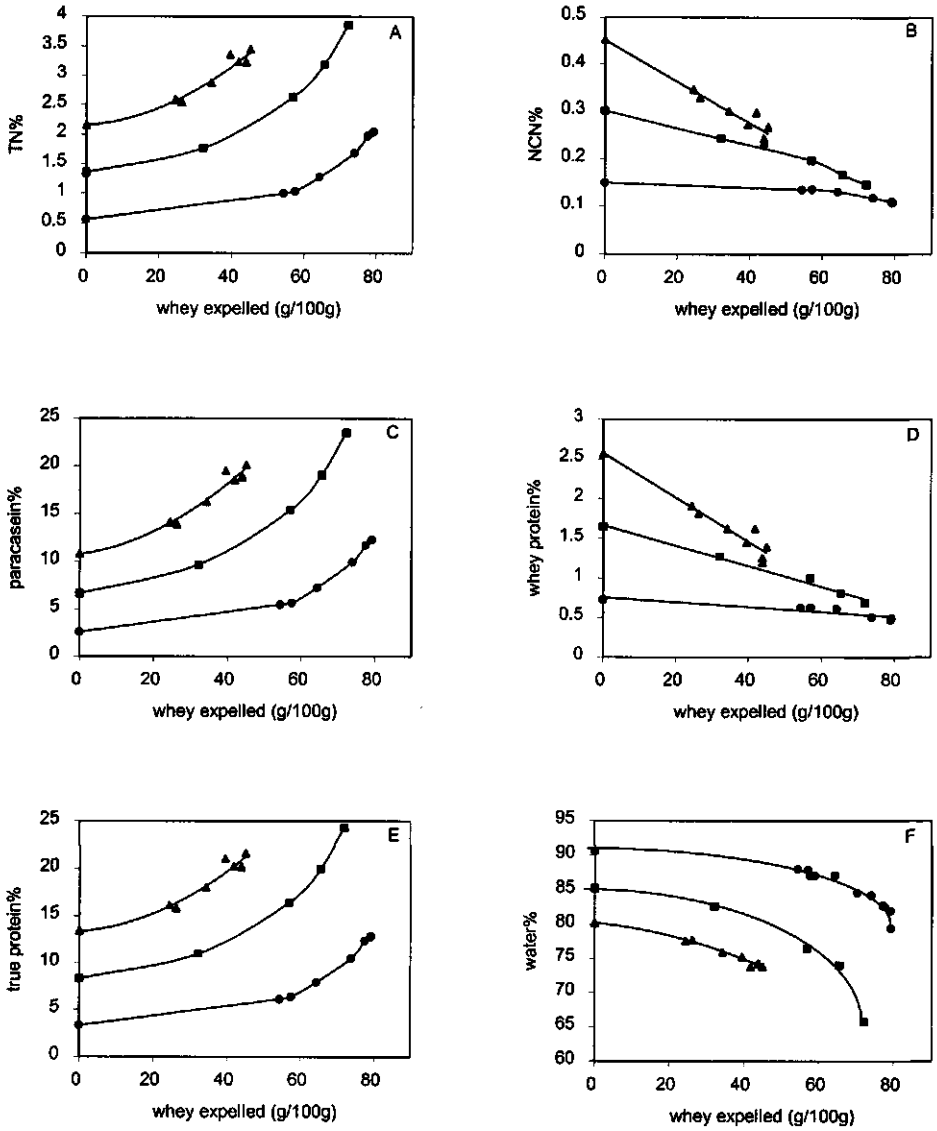

Figure 2.5 Changes in the nitrogen, protein and water contents (in %) of skim milk gels (● skim milk) and retentate gels (■ retentate CF 2.5; ▲ retentate CF 4), due to syneresis.
A: TN%, B: NCN%, C: paracasein%, D: whey protein%,
E: true protein%, F: water%.

It should be realized that in these experiments the curd was prepared from fat-free milk and retentate. Therefore the contents and percentages given in these figures can not be compared with curd from fat-containing milk as such. For a good comparison correcting for the fat content of the milk or curd is essential.

As syneresis continues, the paracasein content increases, while the NCN% decreases due to the loss of whey proteins in the expelled whey. Figures 2.5A, C, E and F show that the highest concentration factor of the ultrafiltered milk, followed by syneresis, did not necessarily result in the highest total nitrogen, paracasein, true protein content or dry matter content in the curd. The paracasein fraction could be further concentrated if, prior to syneresis, the casein content in the retentate was only increased by a factor of 2 to 3, as compared to the concentration in skim milk. The whey protein content, however, is higher in the curd from strongly concentrated retentate (figure 2.5D).

The decrease in the water content of curd due to syneresis depended strongly on the existing water content of the curd, i.e. the degree to which curd had already undergone syneresis. The calculated relation between percentage of whey removed and the water content in curd from whole milk was earlier given by Walstra et al. (1985). The amount of whey to be expelled to obtain similar water contents in the curd is very small in curd from retentates, compared to curd from skim milk. In order to reach a water content of 80% in the curd, the required syneresis percentage (amount of whey to be expelled per 100 gram freshly cut curd) is almost 80% in the case of a skim milk gel, whereas the percentages are 44% and 4%, for retentates with a CF of 2.5 and 4, respectively. Sutherland and Jameson (1981) reported whey production in cheesemaking from UF-retentate (CF 4.8) being 10% of the amount obtained in conventional Cheddar cheese manufacture. They succeeded in enhancing syneresis by hand-stirring in combination with a raise in temperature (to 38°C).

The paracasein content of the curd, after syneresis, could also be calculated, assuming that no casein was lost in the whey. This was checked in several batches of permeate/whey mixtures, by determination of TN% and NCN%. These appeared to be equal, which meant that the expelled whey contained no casein. Based on the (experimentally determined) paracasein concentration of the renneted gel, the paracasein concentration in the curd was calculated as a function of the percentage of syneresis. The experimentally determined paracasein contents of the syneresed curds agreed very well with the calculated curves (results not shown). Sutherland

and Jameson (1981), who also prepared cubed curd grains but stirred by hand, found that only 54% of the protein present in whey consisted of whey proteins. Apparently considerable amounts of caseins were lost with curd fines, caused by hand-stirring of the curd. Loss of caseinate-nitrogen in the whey may also be attributed to the action of proteolytic enzymes, especially from rennet, which causes the formation of soluble breakdown products from caseins. These losses, however, can be neglected if the duration of the process until curd collection is limited.

Loss of nitrogeneous milk components during syneresis

Also the absolute total amount of nitrogen and proteins in the syneresed curd changed during syneresis. These amounts could easily be calculated from the amount of syneresed curd and the corresponding nitrogen or protein concentrations in these curds, at varying degrees of syneresis. In order to get a view of the loss of nitrogeneous components during syneresis, these changing amounts are given in figure 2.6.

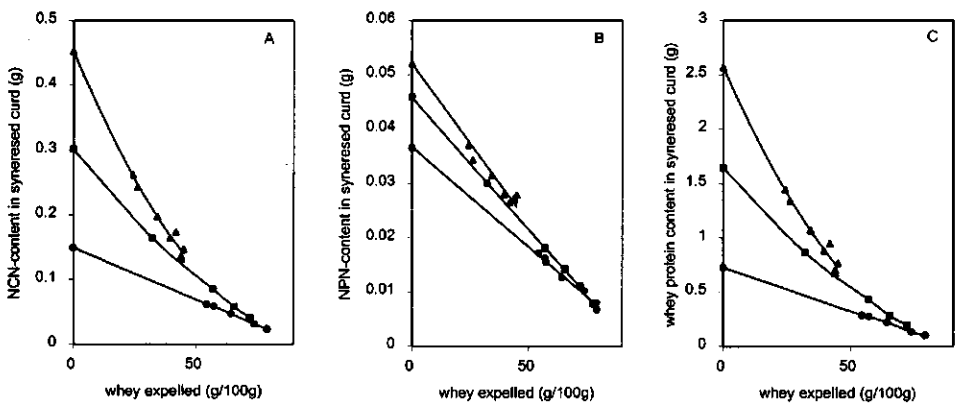

Figure 2.6 Loss of nitrogeneous components and proteins from skim milk gels (● skim milk) and retentate gels (■ retentate CF 2.5; ▲ retentate CF 4), based on an initial amount of 100g freshly cut curd, due to syneresis.
A: NCN, B: NPN, C: whey proteins.

At ongoing syneresis the amounts of NCN-components and NPN-components, which represents the amount of whey proteins in the curd, decreased continuously. In agreement with the loss of whey proteins also the total amount of

proteins in curd from 100 gram gel decreased, as measured by the content of TN. The absolute amounts of TN and NCN in the curd decreased equally fast, implying that only non-casein-nitrogen components were expelled. The total amount of casein in the curd from 100 gram gel remained constant during syneresis. These results confirm that no caseins were lost during syneresis, as was also concluded from the composition of the expelled whey and from the agreement between determined and calculated paracasein contents.

The decrease of the whey protein content during syneresis is faster in curd from retentate gels, due to the higher concentration of whey proteins in the aqueous phase of the gel (figure 2.6C). The ongoing loss of whey proteins as syneresis continues shows that the network of paracasein micelles does not act as a filter. Whey proteins will therefore not be accumulated in the curd in this way, at least up to the highest dry matter contents obtained in this study. This can also be concluded from the total nitrogen-to-water ratio in the expelled whey. This ratio was the same for all whey batches, obtained from syneresed curds that were prepared from the same batch of milk or retentate, independently of the extent to which syneresis was continued. This implies that the first whey that was expelled had the same nitrogen-to-water ratio as the whey expelled later. These results confirm that during syneresis the pores in the paracaseinmatrix do not become small enough for the whey proteins to be hindered in flowing out or diffusing.

Also in the preliminary one-dimensional experiments the composition of the expelled whey was constant during the experiment (the total nitrogen content was the same for the first and the later sampled whey fractions). The total nitrogen content of the whey was higher if the milk was concentrated stronger by ultrafiltration. The whey fractions were also analysed by HPLC. The amounts of the four major whey proteins in the whey, i.e. β-lactoglobulin, α-lactalbumin, immunoglobulins and bovine serum albumin, increased at the same rate with the total nitrogen content of the whey. The mutual ratios of these whey proteins in the whey was the same for the different whey fractions obtained from one gel. These observations, however, are not in agreement with most of the results of van Boekel and Walstra (1989). The ratio of the individual whey proteins was also the same in whey from retentates gels and in whey from skim milk gels. These observations imply that none of the whey proteins was accumulated in the gel during syneresis. This was confirmed by HPLC-analysis of dispersions of syneresed curd cubes (dispersed with sodium citrate). Although the determined concentrations deviated

due to the use of sodium citrate for dispersing the curd, trends in changes of amounts of individual whey proteins in the syneresed curd, could still be detected by HPLC-analysis. The amounts of β-lactoglobulin and α-lactalbumin in the curd decreased continuously at ongoing syneresis, indicating, again, that these whey proteins were not accumulated in the curd and not hindered in leaking from the curd as it became more concentrated and dense due to syneresis. The relative loss of these whey proteins was in good agreement with the decrease in the NCN-content.

Lawrence (1989) reported that the extra retained nitrogeneous components in UF-cheese consist for about 60% of the major whey proteins β-lactoglobulin and α-lactalbumin. In milk these whey proteins represent approximately 70% of the serum proteins (Walstra and Jenness, 1984). In renneted milk the total whey protein content is increased, due to the release of the caseino-macropeptide by rennet, which would lower the contribution of β-lactoglobulin and α-lactalbumin to approximately 58%. The fact that the share of these whey proteins is the same in the renneted milk and in cheese confirms our observation of unchanged mutual ratios.

This did not agree with results of Spangler et al. (1991). They found β-lactoglobulin and α-lactalbumin to be increased respectively 1.5 and 1.1 times in UF-cheese (from retentate CF 5), compared to conventional Gouda cheeses. However, these serum proteins were possibly only partly concentrated in the retentate, as ultrafiltration was performed with membranes of 50 kD molecular weight cut-off.

Paracasein, whey proteins and water: changes in ratios during syneresis

Varying the degree of ultrafiltration, in combination with the degree to which whey is expelled during syneresis, enables changing the mutual ratios of paracasein, whey protein, true protein and water in the curd. At ongoing syneresis, the water-to-paracasein ratio in the curd decreases (figure 2.7A). Simultaneously, the whey protein-to-total protein ratio, as well as the whey protein-to-water ratio in the curd decrease. The changing ratios, in relation to the decreasing water-to-paracasein ratio, are presented in figures 2.7B and 2.7C.

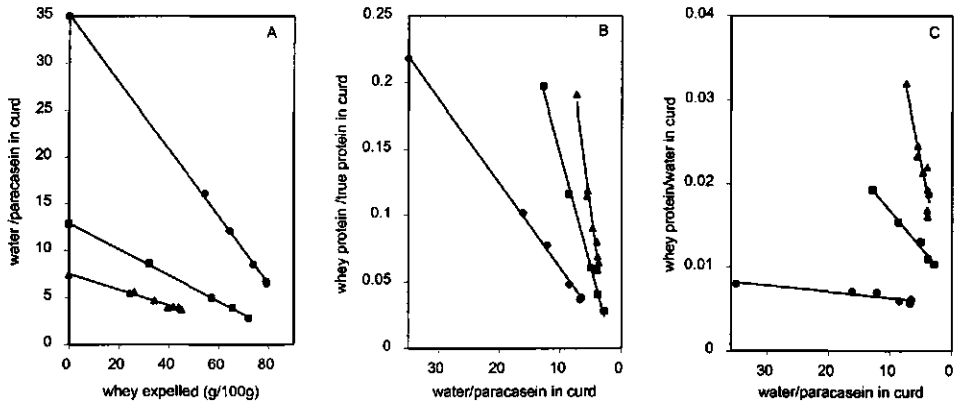

Figure 2.7 Changes in the water/paracasein ratio (A), the whey protein/total protein ratio (B) and whey protein to water ratio (C) in the curds of skim milk gels (● skim milk) and retentate gels (■ retentate CF 2.5; ▲ retentate CF 4), due to syneresis.

In practical cheesemaking (of Gouda cheese), the average water content of the curd at the moment of curd collection is about 69% (Straatsma and Heijnekamp, 1988). It can be derived that at this point the water to paracasein ratio is approximately 6. To obtain this ratio in the curd from skim milk or retentates as prepared in this study, the required degree of syneresis is approximately 80, 50 and 18 gram of whey per 100 gram gel, for skim milk, retentate CF 2.5 and retentate CF 4, respectively. The percentages of whey protein in true protein in these cases would then be approximately 3.8%, 7.4% and 14%, respectively (figure 2.7B). Extrapolation of the curves in figure 2.7B to lower water/paracasein ratios (2 to 3, as in the pressed cheese) would imply that almost no whey proteins are enclosed. As a further decrease in the water/paracasein ratio can not be achieved during syneresis, linear extrapolation may not be allowed to predict the protein composition of the cheese.

The whey protein to water ratio in the curd decreases during syneresis (figure 2.7C), which means that the relative loss of whey proteins is disproportionate with the relative loss of water. The whey protein to water ratio is higher in the expelled whey than in the aqueous phase of the syneresed curd. This implies that part of the water in the curd does not contain whey proteins, so that the content in the other part is increased. In the following it is shown that this can also be concluded from

comparison of the nitrogen content of the aqueous phase of the renneted gel and the nitrogen content of the expelled whey.

The nitrogen-to-water ratio of the expelled whey was compared with this ratio in the aqueous phase of the corresponding gel. In the gel the content of soluble nitrogen components was determined as the NCN*-content. In figure 2.8 both the NCN*/water ratio in the gel and the TN/water ratio of the expelled whey are presented. In this figure also the relation $y = x$ is plotted, which makes comparison of both ratios easier.

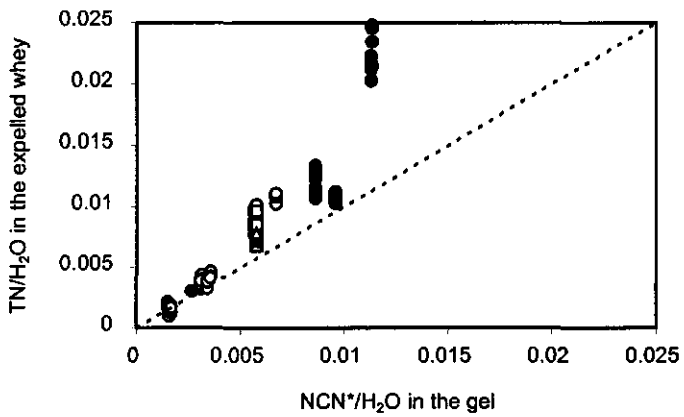

Figure 2.8 Total nitrogen/water ratio in the expelled whey (y) in relation to the non-casein-nitrogen/water ratio in the gel (x); the broken line is for $y = x$. Results from skim milks and skim milk retentates pH 6.7 (○), retentates pH 6.0 (□), retentates pH 5.2 (△), skim milks and skim milk retentates with added whey proteins pH 6.7 (●).

Figure 2.8 shows that the whey protein concentration in the expelled whey was higher than that in the aqueous phase of the gel. This means that part of the water in the gel is non-solvent for whey proteins. This confirms the steric exclusion phenomenon as earlier reported by van Boekel and Walstra (1989). From the retentate gels with strongly increased casein contents and whey protein to water ratios, caused by ultrafiltration of the milk (and in some cases by the addition of extra whey proteins), a considerable amount of these whey proteins is lost during syneresis. At increased paracasein contents of the gel, the overall exclusion of whey proteins will be larger.

From these results steric exclusion factors *h* (gram water/gram casein), which represents the amount of water that is not available as a solvent for whey proteins, can be calculated:

$$h = \frac{W_g}{P_g} - \frac{N_g W_w}{N_w P_g}$$

where

W_g = water content in milk or retentate gel
P_g = paracasein content in renneted milk gel or retentate gel
N_g = NCN-content in renneted milk or retentate gel
N_w = NCN-content in whey expelled from milk or retentate gel
W_w = water content in whey expelled from milk or retentate gel

The steric exclusion factors, derived from these experiments, are given in figure 2.9, in relation to the NCN*/water ratio in the gel.

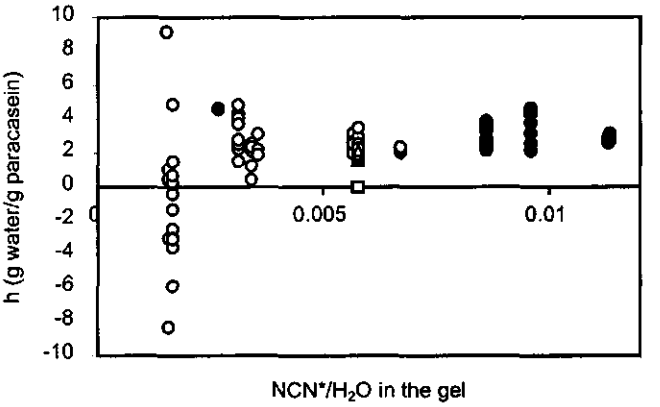

Figure 2.9 Steric exclusion factors h for skim milk gels and retentate gels, in relation to the NCN*/water ratio in the gel. Results from skim milks and skim milk retentates pH 6.7 (O), retentates pH 6.0 (□), retentates pH 5.2 (Δ), skim milks and skim milk retentates with extra whey proteins pH 6.7 (●).

The steric exclusion factor was found to be of the same order of magnitude for the different gels. The skim milk gels are an exception. The large scatter in *h*

values for skim milk gels is due to the very small differences in composition of the aqueous phase of the gel and of the expelled whey. The *h* value appeared to be roughly between 2 and 4. This is in agreement with the value of 2.6, as given by van Boekel and Walstra (1989). The experimentally derived values of *h* seem to be independent of the degree of syneresis the gel had undergone, indicating that steric exclusion is independent of the degree to which the casein fraction is concentrated by syneresis. Also the concentration factor of the retentate does not change the value of *h* appreciably. Steric exclusion would thus be independent of the method and the degree of concentration of the caseins, either by ultrafiltration of the milk or by syneresis of the milk or retentate gel. As in these experiments an 'overall' exclusion factor (for the total casein network) is derived, no statements about the factor being the same for the interior and the exterior of the micelles and about the accessibility of the micelles for solutes can be made.

The soluble whey proteins that were added to increase the soluble nitrogen to casein nitrogen ratio in the gel were to a large extent lost with the whey during syneresis (figure 2.9). Addition of soluble components to cheese milk, with the aim to increase the amount of these components in cheese, will not be very successful, as these will only partly be transferred into the cheese. Better yields would be obtained with non-soluble components (such as denatured whey proteins aggregates) that can be entrapped in the curd.

Within the accuracy of the determinations, the derived *h* values were independent of the pH value of the retentates. Apparently, the conformation of the casein micelles does not sufficiently change at lower pH to significantly affect the amount of water non-solvent for whey proteins. The same conclusion, i.e. the amount of non-solvent water being independent of pH, was drawn by Geurts et al. (1974), who studied the effect for several sugars. Van Boekel and Walstra (1989) had determined that also renneting did not materially influence the exclusion of serum proteins. Apparently, the magnitude of the steric exclusion effect is more or less independent of changes occuring in the casein micelles during the first cheesemaking steps.

The significance of steric exclusion of whey proteins in traditionally manufactured Gouda cheese manufacture was illustrated by van den Berg (1994). Regarding the final composition of cheese before brining, with an average water to paracasein ratio of approximately 2, the water content in pressed cheese is supposed to be too low for whey proteins to be dissolved. All whey proteins will thus be excluded from curd. In these calculations, however, an average steric exclusion factor for whey proteins of 2.6 gram water per gram paracasein was applied, which

leads to a very rough estimate of the total whey protein content in cheese. Besides, as the factor *h* may significantly vary among the different whey proteins (van Boekel and Walstra, 1989), one must be very careful with such 'overall' calculations.

The importance of steric exclusion of whey proteins for the final content of whey proteins in cheese will largely depend on the method of cheese manufacture. Therefore the steps in cheese manufacture must each be considered, especially those in which moisture is removed from the curd mass.

Accumulation and loss of whey proteins in different cheesemaking steps

In the manufacture of traditional Gouda cheese, the loss of moisture that contains whey proteins largely depends on the process parameters curd handling, applied temperatures and pressures during the various process steps. The first step in the fabrication process aimed at release of moisture is the cutting of the gel, which also induces syneresis. Whereas in the experiments with cube-shaped curd grains the size and shape of the curd grains was very uniform, in curd prepared during Gouda cheese manufacture, the size and shape of the curd grains varies widely. The ratio of water to paracasein in syneresed curd grains, prior to drainage of the curd, appears to vary between 10.4 and 1.8 (data derived from Akkerman, 1992; curd was prepared in commercially used cheesevats at NIZO, Ede, the Netherlands). The strong decrease in this ratio (the original ratio in the rennet-induced milkgel was approximately 35) was established during stirring of the curd, due to syneresis. These data show that a decrease to a water/paracasein ratio of 1.8 (and thus lower than the ratio of 2.6, where all whey protein dissolving water would be expelled) by syneresis due to stirring only, is well possible. Very low ratios exist mainly in small curd grains. The lowest ratio reached in the experiments with cube-shaped curd grains was approximately 2.8. However, lower ratios could have been reached if lower pH values had been applied (Walstra et al., 1985).

Syneresis, i.e. expelling of whey with whey proteins, may be enhanced if curd grains collide and get fractured during stirring of the curd/whey mixture. In the experiments with cube-shaped curd grains the possibility of this to happen was very small, as the grains were present in an excess of permeate. During rotation of the vessel the grains hardly collided with other grains. In large cheesevats, as used in conventional cheesemaking, the cutting knives or collisions between curd grains may lead to fracture of curd grains, thus forming fresh surfaces from which whey proteins probably are readily expelled.

Also the composition of the liquid surrounding the curd grains during syneresis influences the expelling of whey and its composition. In the experiments with cube-shaped curd grains, syneresis occurred in an excess of permeate. In the traditional curd preparation process, the curd will initially be surrounded by its own whey and later, in the manufacture of Gouda cheese, with diluted whey (so called second whey), as part of the whey is drained off and partly replaced by water (for dilution of lactose). A lower concentration of a component in the surrounding medium than in the curd enhances diffusion. Therefore, in these experiments with curd in permeate, diffusion will contribute more to the loss of whey proteins from curd than in the case of curd that is kept in its own whey.

The different composition of the moisture in the curd, compared with the moisture in which the curd grains are kept (the so-called washing effect), and the diffusion of molecules were studied by van den Berg and de Vries (1975). They reported a slow increase in the whey protein content of second whey let off (after dilution of the system with curd washing water). The increase was especially measurable in the whey that was leaking from the moulds shortly before and during pressing. The increase is partly caused by the ongoing syneresis of the curd, which is even enhanced by the higher temperature of the curd/whey mixture (as the washing water was higher in temperature than the original curd/whey mixture). The whey protein content of the whey that now was expelled from the curd was higher than the initial whey protein content in the second whey, thereby increasing the content in the second whey. Although the system was diluted to decrease the lactose content, the 'washing effect' would also affect the whey proteins. Due to dilution, the whey protein content in the medium surrounding the curd grains was decreased, resulting in more diffusion of whey proteins from the curd grains. Diffusion of whey proteins is therefore also partly responsible for the increase in the whey protein content of the second whey, under the condition that the contact time is long enough. Because of the slower diffusion of whey proteins, however, they will be washed out to a much lower extent than lactose molecules.

Although a considerable part of the moisture is released from the curd shortly after cutting the gel and during stirring, an important amount of moisture is lost from the curd or curdblock after the step of stirring is finished. Data from Straatsma and Heijnekamp (1988) show that in Gouda cheese manufacture the moisture content of the curd, prior to curd collection, is approximately 69%. After drainage of curd this content is decreased to 57% and after leaking to 54%. Due to pressing the moisture content in the curd block is lowered further to 47%.

At drainage the curd grains are collected. The rate of drainage will markedly depend on the size and the size distribution of curd grains. In the case of curd prepared in conventional cheese vats, the size of the curd grains varies widely, resulting in a wide range of the ratio water/paracasein among individual grains. Continuing loss of moisture after drainage will mainly be accounted for by the curd grains with a high water/paracasein ratio. After curd collection, the whey expelled from the curd grains initially fills the holes between the curd grains. The ability of whey to leak from the curd block largely depends on the dimensions and the porosity of the curd block, which, in turn depends on the size, the size distribution, the deformation and the fusion of curd grains. Due to this size distribution and the resulting varying tendency for deformation of the grains, the stacking will be very different from that of uniform squared particles. Small particles may fill holes and be responsible for the curd block becoming silted up (Akkerman et al., 1996). Also the size and the filling degree of the curd vessel play important roles in this respect. Part of the whey, either entrapped in the curd block during curd collection or expelled from the curd grains in the curd block after curd collection, will be enclosed in the curd block. The total amount of whey, enclosed in the curd block will largely depend on the moment and the method of drainage and pressing. The accumulation of whey proteins will depend on the amount and composition of the whey that is surrounding the curd grains (and in this way entrapped in the curd block) and on the amount of whey proteins present in the curd grains. Thus, for the moisture content of cheese and for enclosure of whey proteins, the moment of drainage is very important. However, also for other properties (such as curd structure and final pH), this step is a key stage in cheese manufacture, as stressed by Lawrence et al. (1984).

The enclosure of whey proteins in a pressed curd block has not been studied with cube-shaped curd grains. However, the factors that play a role in the formation of a curd block in conventional cheese manufacture, are also expected to determine the accumulation of whey proteins in a curd block of cubed curd. The relation between the loss of whey from individual curd grains and the expulsion of whey from a curd block, consisting of these individual curd grains, was extensively studied by Akkerman (1992). Unfortunately, no data for the expulsion of the amount of whey proteins were collected.

Based on these considerations, enclosure of whey proteins in a curd block, consisting of curd grains that have a water/paracasein ratio below 2.6, is not impossible. In the traditional concept of Gouda cheesemaking, it is well possible that

whey proteins are incorporated. Therefore, results as found by G. van den Berg (NIZO, the Netherlands, in van den Berg et al., 1996) are not surprising and even confirm this line of thought. They determined whey proteins in the moisture phase of fresh, unbrined cheese (moisture released from cheese by centrifugation) and found the same relative proportion of all whey proteins in the cheese as in the expelled whey. This result, however, can not be extrapolated to derive the total whey protein content in cheese, as the moisture that is released by centrifugation possibly represents the whey enclosed between curd particles at curd collection. Therefore, this observation does not give a decisive answer on the total enclosure of whey proteins in cheese. The presence of (native) whey proteins in traditional cheese was reported earlier. O'Keeffe et al. (1978) and de Koning et al. (1981) derived that whey proteins represent approximately 1% of the total cheese protein, in Cheddar and Gouda cheese, respectively. A whey protein content of approximately 0.35% in traditional Cheddar cheese was calculated by Lawrence (1989). These derivations, however, were fairly rough. In some cases the calculations were based on assumptions that were not justified.

Most factors that influence the enclosure of whey proteins in curd and in cheese in the traditional concept of cheesemaking, are also determinant for the accumulation of whey proteins in curd and cheese from UF-concentrated retentates. But, as the production process of UF-cheese is very different, some aspects may now be more important, compared to conventional cheese manufacture. In the UF-cheese manufacture the degree to which the milk is preconcentrated, strongly determines the further processing. At low concentration factors, conventional cheese preparation equipment can be used, while for strongly concentrated milk specially designed equipment, such as Siro-Curd (Garrett, 1987; Jameson, 1987; Everett and Jameson, 1993) or Alcurd (Qvist et al., 1987; Spangler et al., 1990; Spangler et al., 1991; Guinee et al., 1992) is required. The latter was also used in another part of this study (see Chapter 5). With this equipment retentates of higher viscosity can be handled and the coagulated curd can be cut with limited curd shattering. In this way losses of fat can also be limited. UF-cheesemaking equipment, used for coagulating and cutting of the curd, was often applied in combination with conventional equipment, for later steps as syneresis and drainage. Usually curd particles were collected in the same cheese moulds as used in conventional cheese manufacture.

In the first case, when conventional equipment is used for low-concentrated retentates (up to approximately CF 2), factors as discussed for the conventional cheesemaking process are determining the enclosure of whey proteins. Curd handling now requires special attention because of the slower syneresis; measures

aimed at enhancing the loss of whey may readily cause increased curd shattering. The greater tendency of curd shattering could in many cases be ascribed to changes during the coagulation of retentate, as compared to renneting of milk. Depending on other factors (like pH, chymosin dosage, coagulation temperature and calcium content) as well, a higher casein content generally results in an increased curd firming rate. As cutting of the gel is usually started at a certain firmness of the coagulum, the degree of κ-casein-hydrolysis at that moment is lower in a retentate gel than in a milk gel (van Hooydonk and van den Berg, 1988). This is possibly the reason why a retentate gel is more susceptible to curd shattering during curd cutting and curd handling. Curd shattering results in a high content of curd fines which may be lost with the whey. They may also pose problems in the drainage of the curd (blocking of pores). Along with the loss of curd fines, fat losses will be increased in the case of curd shattering. Further, the high total solids content of the curd grains at drainage may prevent the curd grains from deformation and growing together (as observed by Green et al., 1981), thus forming a porous curd block from which more whey is easily lost.

At higher concentration factors of the milk, specially designed equipment mostly cuts uniformly sized curd grains. Also for this study special cutting and curd handling equipment, that could be used for making uniformly sized curd grains from retentates with different concentration factors, was designed. Sutherland and Jameson (1981) also stressed the need of specially designed equipment, in order to be able to cut and handle the curd without losing too much fat and curd fines.

Curd particles can be collected in the cheese moulds directly after cutting, followed by pressing. This method, however, can result in body openness, which can be prevented by collecting the curd in permeate, followed by drainage and pressing (Zoon, 1994). In some cases curd grains were transferred into an excess of permeate of elevated temperature (as was done in our experiments), with the aim of further lowering the water content of the curd by stimulating syneresis; the curd is then stirred as in the traditional cheesemaking process. Besides syneresis, also the loss of whey proteins from these curd grains by diffusion is then enhanced. Although the amount of whey lost in this step may be limited, the amount of whey proteins lost can be considerable, because of the high whey protein content of the expelled whey. However, this loss might be counteracted by a decrease in curd shattering, possibly even resulting in an increased (overall) recovery of proteins in cheese.

The development of a continuous UF-cheesemaking process with its equipment, named Siro-Curd, was described in detail by Jameson (1987). In this process, a relative small amount of whey, i.e. approximately 8% of the original milk volume, with a strongly increased protein content, is released. Garrett (1987) came to the same figures, using this process.

Protein recovery in (UF-)cheesemaking

In practical cheesemaking it is tried to precisely attain a given water content in combination with a given protein to fat ratio. The protein fraction of curd from skim milk and skim milk retentates may consist partly of whey proteins, depending on the extent of syneresis. For a constant loss of curd fines, an increased retention of whey proteins leads to an increased total protein recovery. Increased protein recovery should be accompanied by an increased retention of moisture in the cheese. Fat contents in the dry matter of the final curd can be kept on level by adapting the fat content of the cheesemilk or the retentate.

In our experiments, the overall inclusion of proteins in curd depends on the degree of syneresis. The recovery of proteins (casein and whey proteins) in curd is high at low degrees of syneresis, but decreases with ongoing syneresis as whey proteins are lost with the expel of whey. At higher CF of the retentate, the recovery of whey proteins in its curd decreases faster as syneresis continues, because of the higher whey protein to water ratio in the expelled whey. In our experiments, protein recoveries in the syneresed curd (syneresed until a water/paracasein ratio of about 6) were approximately 79% (CF 1), 84% (CF 2.5) and 90% (CF 4), respectively. The recovery of nitrogen in the pressed cheese will deviate from the recovery as determined in the syneresed curd, depending on the moment and the manner of curd collection and on further curd handling. However, trends in relations between water contents and recoveries would be similar if these steps are comparable.

Protein recoveries as determined in these experiments are in good agreement with data in the literature. Unfortunately, in most studies no distinction is made between the recovery of caseins and the recovery of whey proteins. In the traditional process nearly all whey protein is lost, and protein recovery mainly involves the caseins. In the conventional cheese manufacture the overall protein recovery was estimated at 74-77% by Phelan (1981), with a recovery of caseins between 94% and 96% (due to the loss of the caseino-macropeptide-fraction and possibly curd fines with the whey). In the UF-cheese manufacture higher recoveries are determined. In general the increase is attributed to the whey proteins. However,

the changed process often has consequences for the recovery of caseins. Due to the changed curd structure, the method of curd cutting and further curd handling, more curd fines can be lost, resulting in a decreased casein recovery. In an attempt to increase the total protein recovery in cheese by applying ultrafiltration, it is important not to counteract this gain in whey proteins by higher casein losses. With the development of special equipment (Siro-Curd, Alcurd) it was tried to minimize the loss of curd fines.

Jameson (1987) reported protein recoveries in the range 78 to 81% with the Siro-Curd process (from retentate CF 5), 7-8% more than in conventionally made cheese. In this process a relatively small amount of whey (7-8% of milk volume) with high amounts of protein (40-50% of non-fat solids) is released. Garrett (1987) also described the Siro-Curd process (CF 5) and reported 5.05% protein (50% of non-fat solids) in the whey (whey volume also 8% of milk volume). Sutherland and Jameson (1981) estimated a total protein loss of 13.9% (before salting) to 16.5 (after salting) in UF-Cheddar cheese from retentate (CF 4.8). Qvist et al. (1987) reported 76% and 84% nitrogen recovery in Havarti cheese made according to the traditional process and with Alcurd-equipment from retentate (CF 5), respectively. The percentage of whey protein nitrogen in total nitrogen was determined at 4% for UF-cheeses and 1.4% for traditional Havarti cheeses. The higher protein recovery in UF-cheese can thus only partly be attributed to extra whey proteins, but is mainly due to a reduced loss of casein fines (this could be derived from the increased fat recovery in the UF-cheese, i.e. 98-99% against 92% in the traditional process). In a study by Everett and Jameson (1993), protein soluble at pH 4.6 as a percentage of total nitrogen was determined. The initial value in UF-cheese (from retentate CF 5, with Siro-Curd) was 2.6%, only 0.8% higher than in their conventionally prepared cheeses.

In studies from Lelievre et al. (1986) and Iyer and Lelievre (1987), the loss of milk solids to wheys from milk and UF-retentates was determined. Consequently, the retention of proteins in cheese was derived from the composition of the milk or retentate and the whey. Approximately 2 to 3% of the paracasein was lost as fines in conventional cheesemaking. In UF-cheese manufacture from retentate (CF 5) this loss was slightly increased (3%). The recoveries of whey proteins were 5.1% for control cheeses and 35.6% for UF-cheeses. The total nitrogen recoveries were 72.5% and 80.2%, respectively. They also calculated the whey protein to water ratio for retentates (CF 3 and CF 5) and for the resultant UF-cheeses. The ratio in the cheese was derived from the whey protein content in the retentate and in the

expelled whey. In both cases (CF 3 and CF 5) the ratio in the UF-cheese was approximately 1.4 times the ratio in the retentate. The whey protein to water ratio in the expelled whey must thus have been lower than in the milk or retentate. The whey proteins were thus concentrated in the cheese. Although comparison of these results with determined whey protein to water ratios in our study (fig. 2.7C) is only partly possible (in our study the syneresed curd particles were not collected to form cheese) their data seem to be in contrast with our results.

Similar conclusions with respect to accumulation of whey proteins in the cheese can be drawn from experiments by Kosikowski and Masters (1984), Kealy and Kosikowski (1985) and Kosikowski et al. (1985). They studied the composition of Cheddar cheeses and corresponding wheys from milk and retentates (up to CF 1.9). Comparison of the protein contents in wheys from retentates and control milks showed that this content in the whey from retentate (in which the total protein content was increased by 1.9 times) was only 1.83 times higher than in the whey from unconcentrated milk. Also in other cases, where Cheddar cheese was made from retentates with concentration factors between 1.1 and 1.9, the protein content of the whey was increased with a smaller factor than the concentration factor of the retentate. The increase in the protein content in the wheys was on average 96% of the increase of this content in the UF-concentrated milk. These results indicate an accumulation of (soluble) whey proteins in curd. They reported a total loss of proteins with the whey of 8 to 9% for control Cheddar cheeses (from unconcentrated milk) and approximately 7% for the UF-cheeses (from retentate CF 1.9). In all cases 0.5 to 1% of these protein losses could be attributed to casein, the rest was whey protein. The total protein recovery was thus only slightly (1%) increased in the cheese at higher concentration factors of the retentates (up to 1.9). Besides, the increase was of limited importance since the protein recovery in control cheese exceeded already 90%. These high protein recoveries might reflect the (partial) association of whey proteins with caseins, possibly caused by heat treatment of the milk or retentate. These associated whey proteins are, of course, concentrated in the curd with the caseins. Possibly this changed casein network caused the entrapment of dissolved whey proteins.

On the other hand, in a study by Qvist et al. (1987), the protein content in the whey from retentate CF 5 was increased by a factor 5.9, as compared to whey from unconcentrated milk. Unfortunately, no distinction was made between caseins and whey proteins in the whey, but, as the loss of casein fines was strongly reduced in the manufacture of UF-cheese, the increased protein content in the whey is mainly due to whey proteins. The amount of whey obtained per kg cheese was strongly

decreased: 9.9 kg for conventional cheese versus 0.57 kg for UF-cheese. Rao and Renner (1988) reported the protein content in whey from UF-cheese, manufactured from retentate CF 4, being 4.9 times higher than in whey from traditional Cheddar cheese (protein contents in the whey respectively 3.56% and 0.72%). Again, no casein contents were reported. Overall protein recoveries of 80% for traditional cheese and 91% for cheese from retentate (CF 4) were given. If the losses of casein fines would be comparable for curds from milk and retentate, these results also point to a more than proportionate exclusion of soluble whey proteins. This is then in agreement with results from our study: the TN contents in the wheys from retentates CF 2.5 and CF 4 were increased by a factor of 2.68 and 5.22, respectively, compared with the whey from skim milk. Rao and Renner (1989) determined nitrogen soluble at pH 4.6 as a fraction of total nitrogen in cheese. The initial values in the fresh cheeses, that give an indication of the amount of whey proteins enclosed, were about 4% and 7% for traditional cheese and UF-cheese, respectively. However, the first breakdown products from caseins might have been included in these values as well.

In several studies, cheesemaking yield was predicted, based on known contents of several components in cheesemilk and in whey. In these calculations, contents of soluble components were often assumed to be the same in the whey, in the moisture in the milk and/or in the moisture of the cheese. In agreement with this theory, the whey protein to water ratio in curd was supposed to be constant during syneresis. Micketts and Olson (1974) assumed the amount of whey protein in cheese serum being the same as in milk serum. Based on this, they derived the casein content of Mozzarella cheese from total cheese protein corrected for whey protein in the cheese, which was in turn calculated from the cheese water content. Also Dejmek (1986) calculated yields and possible milk savings in cheesemaking by ultrafiltration, based on similar assumptions. Lelievre and Lawrence (1988) founded their calculations on this assumption of equal whey protein to water ratios in retentate and cheese as well. According to these calculations 30% of the whey proteins in the milk should be recovered in hard UF-cheese, which they saw confirmed in mass balance studies from Iyer and Lelievre (1987). In our experiments it is shown that these whey protein to water ratios are not the same and that they do change in the syneresing curd. The assumptions made in the yield calculations just mentioned were thus not justified. However, in rough yield estimates, where results

also depend on the accuracy of amounts and contents of other components, as well as on other assumptions, it may not lead to large deviations.

According to Green et al. (1981), protein retention in Cheddar cheese increased (from 83% to 90%) with the concentration factor of the retentate (CF 1 to CF 4); this could be attributed to the retention of an increasing proportion of whey, containing progressively higher levels of protein. At higher CF both moisture and protein content of the cheeses were increased, while the ratio of both was unchanged. In the calculations of the expected percentage of proteins, accumulated in the curd, the whey was assumed to contain only non-casein proteins at the same concentrations as present in the retentates (CF 1-4). This assumption implies that steric exclusion was not taken into account. The observed recovery of protein in the curd was lower than calculated, implying that caseins were lost in the whey. Most probably curd shattering was considerable, because also fat losses in the whey were found to be high.

A linear relationship between the milk protein content of the cheesemilk and the protein content in the whey was observed by Guinee et al. (1992). As the whey was filtered (through a 1 mm sieve), most curd fines were removed and the protein fraction in the whey mainly consisted of whey proteins. Fat contents in the whey were increased at higher milk protein contents, which points to considerable curd shattering in the retentates. The loss of casein fines and fat is greatly affected by the method and moment of cutting the gel, as well as by the method of curd handling and curd collection. Guinee et al. (1992) used conventional equipment up to retentate protein contents of 8.2%, and gels from retentates of different CF were cut at similar 'curd strength'. In other experiments (Guinee et al., 1996), an increasing amount of curd fines in the whey was detected at higher protein concentrations of the retentate (applied protein contents 3 to 4.5). Approximately 2 to 4% of the protein content in the filtered whey originated from very small (not sieved) curd fines. Our results for (casein-free) whey from skim milk and from retentate CF 2.5 corresponded fairly well with their data. The slope of their curve (protein level in whey versus protein level in retentate, up to retentate protein levels of 8.2%) was approximately 0.3. This points to the occurrence of exclusion of whey proteins by caseins. Exact calculation of the exclusion factor was not possible due to a lack of additional information.

According to our results with syneresing curd particles the whey protein/water ratio in the expelled whey was higher than this ratio in the milk or retentate gel, resulting in a decrease of the 'overall' ratio in the curd. Different conclusions, as reported in several earlier referred studies, may, for instance, originate from the

larger size of the curd particles in the study from Iyer and Lelievre (1987). Possibly the whey proteins were physically hindered to be expelled from the curd and filtered in the casein network. Besides, in most studies the expelled whey was not diluted so that at collecting the curd the whey proteins in the whey surrounding the particles were entrapped as well. Further, association of whey proteins with caseins, due to denaturation, may lead to the enclosure of whey proteins in the curd.

Inclusion of other solubilized components in UF-cheese

Results and conclusions from this research, together with considerations derived from other studies, enable prediction of the transfer of other (partly) in serum solubilized components into cheese. Such a component, with a comparable size as whey proteins have, is the enzyme chymosin, added to cheesemilk to form a coagulum. Chymosin also plays a very important role in the ripening of cheese. In cheese chymosin is responsible for the breakdown of α_{s1}-casein to peptides, that can then be hydrolysed further to smaller peptides and amino acids by the enzymes of lactic acid bacteria. Therefore, inclusion of active chymosin in cheese is of major importance for the ripening of cheese. The concentration of chymosin molecules in cheese does, however, not only depend on factors that are determinant in the inclusion of solutes (as whey proteins) of equal size and conformation, but also on the adsorption onto paracasein. The inclusion will for the greater part be determined by adsorption if circumstances for adsorption are ideal (especially determined by pH) and if inclusion with moisture is expected to be limited (due to steric exclusion of chymosin). The concentration of soluble chymosin in the moisture that is surrounding curd particles will be determined by steric exclusion, as well as by processing factors that also partly determine the inclusion of whey proteins in cheese. In its turn, adsorption of chymosin onto paracasein depends on the concentration in the moisture. The enclosure of chymosin, and its activity in (UF)cheese, is discussed extensively in Chapter 5 of this thesis.

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Chapter 3

CHYMOSIN-INDUCED PROTEOLYSIS IN UF-CHEESE

ABSTRACT

In UF-cheese the protein fraction consists besides caseins partly of whey proteins (up to 24%). In most cheeses initial hydrolysis of caseins during ripening is due to rennet enzymes, predominantly chymosin. In this study the influence of native whey proteins on chymosin activity was investigated in different cheese models with known chymosin and whey protein contents. Ripening conditions as in Gouda cheese were simulated: the pH was set at 5.2, the salt-in-water content at 4%. The chymosin activity was derived from the formation of breakdown products (peptides). High-moisture UF-cheeses (whey protein/total protein ≈ 24%) were prepared from skim milk retentates (up to CF 5.8), to which chymosin was added. From these UF-cheeses no whey proteins or chymosin were lost anymore, as syneresis did not occur. Below a certain chymosin concentration, no chymosin activity was detected. The minimum amount of chymosin needed to detect any chymosin activity was of the order of n, the normal chymosin to (para)casein ratio that exists in traditionally manufactured Gouda cheese. The decreased proteolytic activity of chymosin could be ascribed to the whey proteins present in the aqueous phase of these UF-cheese models. The inhibiting influence of whey proteins on the chymosin activity was also observed in another type of cheese models. These models consisted of paracaseinate to which various amounts of whey proteins (whey protein/total protein ≈ 0 to 20%) and chymosin were added. At similar chymosin contents, the chymosin activity was lower at a higher whey protein content of the cheese models. The partial inhibition of chymosin could be counteracted by increasing the chymosin dosage.

INTRODUCTION

In cheese ripening flavour development is the result of the activity of several enzymes. Proteolytic enzymes are responsible for the breakdown of milk proteins to components that eventually contribute to the specific flavour and taste of ripened cheese. For a balanced ripening the combined action of various enzyme systems is needed. Some enzymes are of importance in the initial stages of proteolysis, while others are essential in a latter phase for the further breakdown of intermediately formed ripening products.

In the ripening of semi-hard Gouda type cheeses, the main enzymes that can hydrolyse caseins into large peptides are chymosin and milk proteinases (i.e. alkaline milk proteinase, mostly referred to as plasmin, and acid milk proteinase). Chymosin is largely responsible for the degradation of α_{s1}-casein, while the breakdown of β-casein is mainly due to the activity of plasmin (Grappin et al., 1985). Chymosin and milk proteinases do not greatly contribute to the further hydrolysis of caseins into small peptides, amino acids and other flavour compounds. The first breakdown products from casein can be hydrolysed into smaller peptides and amino acids by the enzymes originating from starter bacteria. As enzymes from starter bacteria are hardly able to hydrolyse intact caseins, the predigestion of caseins into large peptides by chymosin and milk proteinases is of major importance for the development of taste and flavour (Visser, 1977c; O'Keeffe et al., 1978). In Gouda type cheeses the contribution of chymosin to the formation of peptides from caseins is considerably greater than that of milk proteinases (Visser, 1977cd; Noomen, 1978ab). In Gouda and Cheddar cheese a considerable degradation of α_{s1}-casein was accompanied by β-casein being hydrolysed only to a small extent (Visser, 1977d; O'Keeffe et al., 1978; Lawrence et al., 1987). The importance of chymosin activity in cheese ripening has been established in several studies (Stadhouders, 1960; Visser, 1977cd).

The retention of rennet enzymes and milk proteinases in curd depends largely on the pH at drainage. Also their activity, and thus their relative importance during cheese ripening, is determined by pH, cheese composition and ripening conditions (Lawrence et al., 1984). Therefore, in evaluating the activity of chymosin and milk proteinases in cheese not only the enzyme concentration must be considered, but also the cheese composition and the conditions during ripening.

The composition of semi-hard cheeses made according to the traditional cheesemaking process is fairly constant, due to requirements for fat and dry matter contents. The protein fraction in these cheeses consists mainly of caseins, because

most whey proteins are lost in the whey. Over the last decennia, it has been tried to increase cheese yield by enclosing native whey proteins in the cheese, in particular by ultrafiltering the cheese milk prior to addition of rennet and starter enzymes. The manufacture of cheeses by ultrafiltration techniques (UF-cheeses) introduces major changes in the composition of the cheese, especially in the protein fraction. In UF-cheeses the protein fraction does not only consist of caseins, but also whey proteins are partially included.

Flavour development in whey protein containing cheese appears to be retarded and deviating from that in traditionally manufactured cheese. Several causes were mentioned in literature. In some studies the native whey proteins have been reported to affect the activity of chymosin (de Koning et al., 1981; Creamer et al., 1987; Harper et al., 1989). This assumption is investigated further in this study. It is tried to determine the influence of native whey proteins on the activity of chymosin in UF-cheese and UF-cheese models. For this purpose the chymosin activity is derived from the formation of breakdown products from a caseinate substrate. Determination of the rate of formation of breakdown products is in fact an indirect method of measuring the activity of both chymosin and milk proteinases. The contribution of milk proteinases can be determined by comparison with chymosin-free cheeses, in which milk proteinases are not eliminated. The so derived chymosin activity can then be related to the chymosin content of the studied cheese, in combination with the composition of the cheese, especially the protein composition, and other parameters, such as pH, salt content and external ripening conditions. In order to be able to relate the chymosin activity to the chymosin content, the chymosin concentration should not be decreased (due to loss of chymosin containing whey in case of syneresis of chymosin-induced caseinate gels) and chymosin should not be inactivated (due to pH-shifts or heat-treatments). In the cheeses made for the purpose of this study, it was tried to fulfil these conditions.

In this study two methods were applied to include whey protein in the cheese. In the first method the whey proteins were enclosed in highly ultrafiltered milk, to which chymosin, salt and lactic acid were then added. The casein to whey protein ratio is the same as in milk. This product could be considered as a high moisture UF-cheese, in which varying chymosin concentrations could be applied. Besides chymosin, milk proteinases may also contribute to proteolysis, as these native milk enzymes are not inactivated. The retentates were concentrated by ultrafiltration to a fairly high extent, to prevent the spontaneous occurrence of syneresis and the subsequent loss of

chymosin with the whey. In the second method a concentrate of whey proteins was added to a caseinate concentrate. All ingredients could be added independently. The advantage of these cheese models was that varying concentrations of whey proteins as well as various amounts of chymosin could be obtained. In our laboratory, these cheese models, based on a caseinate concentrate with ingredients added independently, were earlier succesfully used by Noomen (1978ab) and Youssef (1992) for studying the effect of certain treatments or additions on ripening aspects. By applying both methods their advantages and disadvantages can be compared.

MATERIALS AND METHODS

Materials

-Chymosin: Maxiren 15, strength 10800 Soxhlet Units (Gist-brocades)
-Calf rennet, strength 10 000 Soxhlet Units (CSK)
-Lactic acid, 90% solution (Merck), diluted to a 45% solution (w/v) and heat-treated (15 min at 121°C) to hydrolyse any lactic acid esters present
-Thiomersal (BDH Chemicals)
-Acetonitril (Labscan)
-Trifluoroacetic acid (Pierce)
-Other chemicals of analytical grade (Merck):
acetic acid, trichloroacetic acid (TCA), NaOH, NaCl, $CaCl_2$

Skim milk and skim milk retentate

Skim milk was prepared from milk that was either obtained from the Netherlands Institute for Dairy Research (NIZO) or from the University herd. Skim milk retentate (SMR) was prepared by ultrafiltration of skim milk at 30°C, using a membrane with a molecular weight cut-off of 8 kD (Filtron). To prevent microbial growth in the retentates thiomersal was added (100 ppm).

Micellar paracaseinate

Rennet-free micellar paracaseinate was prepared as described by Noomen (1978a). Rennet-induced skim milk was stirred during curd formation, to obtain small paracasein particles. These were washed in a large amount of water. The wet paracasein particles were submitted to a heat-treatment (121°C for 15 minutes) aimed

at inactivating milk proteinases, and subsequently freeze-dried and stored at room temperature. Hence, the powder will be referred to as paracaseinate.

Rennet whey, acid whey, whey retentates and whey permeates

Rennet whey and acid whey were prepared from skim milk. For the preparation of rennet whey skim milk was clotted with calf rennet (0.02% v/w). The gel was cut and the syneresed whey was collected and centrifuged in order to remove all curd fines. This clarified whey was then heated at 63°C for 20 seconds to inactivate the rennet enzymes in the whey. Acid whey was prepared by acidifying skim milk to pH 4.6 using glacial acetic acid. After separating it from the coagulated casein, the acid whey was clarified by centrifugation. Both the rennet and the acid whey were ultrafiltered at 30°C (membrane cut-off 8 kD, Filtron). The whey was concentrated until a high as possible protein content in the retentate was achieved (whey retentate B). Part of each retentate was diluted with its permeate (1:1 v/v) to prepare a retentate with a lower concentration factor (whey retentate A). The products were cooled to 4°C and thiomersal (100 ppm) was added to prevent microbial growth. Rennet whey products are referred to as RWP (permeate), RW (whey), RWRA (retentate A) and RWRB (retentate B). Acid whey products are analogues coded as AWP, AW, AWRA and AWRB.

Cheeses from skim milk retentates (UF-cheeses)

High moisture UF-cheeses were prepared from skim milk retentates. Two different batches of skim milk were concentrated by ultrafiltration. The dry matter contents of the resulting retentates were 25 and 22%. Each retentate was divided into two batches. In each of the 4 batches of retentate, NaCl was added to a salt-in-water content of 4%. The pH was lowered to 5.2 by addition of lactic acid. Each batch was then divided in several portions to which chymosin was added. Several dosages of chymosin were applied for the ripening of these UF-cheeses. The standard amount, *n*, was set at 1.5 ml of chymosin per kg paracasein in the cheese. This dosage corresponds with the amount of chymosin in Gouda cheese (Visser, 1977a; Stadhouders *et al.*, 1977). Other dosages were 3*n* and 6*n*. The blanks were the salted and acidified retentates to which no chymosin was added. All samples (with and without chymosin) were divided in samples of about 10 to 15 g, which were stored in glass bottles under anaerobic conditions at 13°C.

Cheese models

Cheese models were prepared by mixing paracaseinate with one of the whey products, NaCl and lactic acid. This resulted in 8 cheese models of varying serum or whey protein contents. Except for the serum or whey protein content, the composition of the 8 cheese models was comparable: the paracasein to water ratio was set at 1:2.5 and the concentration of NaCl was adjusted to 4% in the water. The pH was adjusted to 5.2 with lactic acid. Additional thiomersal was added to a total of 0.01% in the cheese model. Preceding the chymosin addition, each of the 8 cheese models was divided into 5 portions, of which one was the control (blank). Chymosin was added to the 4 other portions of each cheese model and mixed thoroughly. The applied chymosin dosages were: 0.25*n*, 0.5*n*, *n* and 3*n*, in which *n*, the normal chymosin dosage to the cheese model, was 1.5 ml chymosin per kg paracasein. The cheese models were divided into portions, and kept in glass bottles that were stored at 13°C under anaerobic conditions. Henceforth, the cheese models prepared with a rennet whey product will be coded as RC (Rennet-whey-containing Cheese models), while the cheese models prepared with an acid whey product will be referred to as AC (Acid-whey-containing Cheese models).

Ripening index: soluble nitrogen and non-casein nitrogen

The soluble nitrogen content (SN) of UF-cheeses and cheese models was determined according to the method of Noomen (1977), with some minor adjustments. For this purpose samples of UF-cheeses were diluted with 0.037 M $CaCl_2$-solution (4.25 g per g cheese). Shortly before analysis, the blank UF-cheese was renneted with 0.02% (v/w) chymosin during 3 hours, in order to convert the casein fraction into precipitable paracasein. Subsequently, this sample was diluted with $CaCl_2$-solution and analyzed like other samples. For the SN determination in cheese models a weighed amount was diluted (w/w) with 0.037 M $CaCl_2$-solution. Samples of cheese models with the low whey protein content (cheese models prepared with whey or whey permeate) were 7 times diluted with $CaCl_2$-solution, while the samples of cheese models with high whey protein contents (cheese models prepared with a whey retentate) were 10 times diluted. These different dilution factors were chosen to attain acceptable soluble nitrogen levels in the extracts. The diluted UF cheese and cheese model mixtures were stirred at 30°C for some hours while the pH was raised to at least 7.5 with 1 N NaOH. After pH adjustment the mixtures were centrifuged and/or filtered.

In all UF cheeses (chymosin-containing cheeses and blanks) also the non-casein-nitrogen content (NCN) was determined. The NCN extract was obtained after precipitation of the casein fraction (in chymosin-free blanks) or the paracasein fraction in a cheese sample homogenized with water. The (para)casein fraction was precipitated by acidification to pH 4.6 with 10%-acetic acid. The mixtures were then filtered.

The soluble nitrogen content (SN) or non-casein-nitrogen content (NCN) of all filtrates was determined in some grams of the filtrates, by performance of the macro-Kjeldahl method. In the conversion of the SN and NCN contents in the filtrates to the SN or NCN of the corresponding cheeses not only the dilution with the $CaCl_2$-solution, but also the dilution due to NaOH addition was taken into account. In this conversion, also the volume of the precipitate (casein) and the related amount of non-solvent water were accounted for, as was done for NCN contents of other products.

Standard analyses - composition

The dry matter content of skim milk, skim milk retentates and whey products was determined using a Mojonnier apparatus. The dry matter content of the paracaseinate was determined by drying the powder until constant weight at 102°C.

The protein content and the protein composition of skim milks, retentates, paracaseinate, whey products and prepared UF-cheeses and cheese models was determined by analysis of the nitrogen content in these products and in filtrates from partly precipitated products. All nitrogen determinations were performed by the macro-Kjeldahl method, according to IDF standard 20B (1993). The total-nitrogen content (TN) of milks, retentates and whey products was determined in some grams of the product. The total-nitrogen content of paracaseinate was determined by dissolving some powder in 10 ml HCl (25%) under gently heating. After cooling, this solution was diluted to 100 ml with demineralized water and nitrogen was then determined in 25 ml. For the determination of the non-casein-nitrogen content (NCN), a sample of the product was diluted with water and the pH was lowered to 4.6 with 0.1 N acetic acid, in order to precipitate all casein. After filtration of this mixture the nitrogen content was determined in some grams of the filtrate. Non-protein-nitrogen (NPN) was determined by precipitating all proteins with 12% (w/w) trichloroacetic acid (final concentration) and determining nitrogen in the filtrate. The determined NCN and NPN contents in the filtrates were recalculated to nitrogen contents in the corresponding milk products, correcting for the volume of the

precipitated fraction and for non-solvent water according to the formula given by Walstra and Jenness (1984). The steric exclusion factor for serum proteins, used for the correction of the NCN content, was 2.6 (van Boekel and Walstra, 1989), while the applied factor for NPN components was 0.2 (Walstra and Jenness, 1984). The casein-nitrogen content of a product was derived from the difference between the TN content and the NCN content. For the determination of the paracasein-content of acidified and salted retentates the NCN content was determined in these retentates after incubation with 0.03% chymosin for 2 hours (further referred to as NCN*). The serum protein nitrogen content of skim milk and skim milk retentates was calculated from the difference between NCN and NPN. The total true protein content was derived from the difference between TN and NPN. The whey protein content of the whey products was calculated from the difference between TN and NPN. For the conversion of nitrogen contents to protein contents, the factors given by van Boekel and Ribadeau-Dumas (1987) were used.

HPLC analyses

The SN extracts of the cheese models, prepared for the determination of SN, were also analysed with reversed phase (RP) HPLC, derived from methods described by Visser et al. (1991) and Exterkate et al. (1991). A 250 x 4.6 mm Biorad Hi-Pore RP-318 analytical column (C_{18}-alkylated, 5 mm particle size) was used. The HPLC equipment consisted of a P2000 Binary Gradient Pump, an AS3000 Variable Volume Injector and a Spectra Physics Focus Detector. Solvent A consisted of acetonitril-water-trifluoroacetic acid in the proportions 100:900:1 (v/v/v). Solvent B consisted of the same components in the proportions 900:100:0.7 (v/v/v). For all eluents Millipore water was used. Acetonitril and trifluoroacetic acid (TFA) were both HPLC grade. The injection volume was 20 µl. The flow rate was 0.8 ml/min. Analysis started with 100% solvent A. Over the first 30 minutes the percentage A in the eluent was linearly decreased to 60%, over the next 20 minutes this was decreased further to 50%. Over the following 5 minutes the eluent was switched back to 100% solvent A, after which the column was equilibrated for another 5 minutes before the next sample was injected. Detection was performed at 220 nm. The α_{s1}-casein-(1-23)-fragment eluted after approximately 27 minutes.

RESULTS AND DISCUSSION

Composition of cheese components

The composition of skim milk retentates (SMR), used for the preparation of UF-cheese, is given in table 3.1, while the composition of the rennet and acid whey products, as were used in the cheese models, is given in table 3.2. The paracaseinate, prepared for the manufacture of cheese models, had a water content of 10.0%, a total nitrogen content of 12.93%, corresponding to a paracasein content of 81.6%.

Table 3.1 Composition (in %) of skim milk retentates

	water	TN	NCN	NPN	casein	serum protein
SMR 1	75.1	2.90	0.504	0.031	15.17	3.04
SMR 2	78.0	2.45	0.439	0.033	12.79	2.55

Table 3.2 Composition (in %) of rennet and acid whey products

	TN	NPN	whey protein	dry matter
rennet whey permeate	0.031	ND*	0	5.69
rennet whey	0.139	0.031	0.68	6.32
rennet whey retentate A	0.686	0.053	3.98	10.34
rennet whey retentate B	1.330	0.078	7.86	14.98
acid whey permeate	0.027	ND*	0	6.15
acid whey	0.132	0.027	0.66	7.04
acid whey retentate A	0.725	0.034	4.34	11.52
acid whey retentate B	1.423	0.040	8.69	16.88

ND*: not determined

Composition of the cheeses

Skim milk retentates are slightly diluted due to salt addition and adjustment of the pH to 5.2. The addition of chymosin to these UF-cheeses (referred to as UFC 1 and UFC 2, prepared from respectively SMR 1 and SMR 2) led to an increase in the NCN-content and the serum protein content (now referred to as whey protein), due to the release of caseino-macropeptide (cmp) from κ-casein, and a decrease in the casein content (now referred to as paracasein). This increased NCN-content, in which cmp is included, is marked as NCN*. The composition of UF-cheeses is given in table 3.3.

Table 3.3 Composition of UF-cheeses (in %)

	water	TN	NCN	casein	serum protein	NCN*	para-casein	water/para-casein
UFC 1	72.3	2.730	0.474	14.34	2.87	0.543	13.85	5.22
UFC 2	74.6	2.356	0.422	12.30	2.45	0.488	11.81	6.32

The paracasein content of the UF-cheeses (determined after chymosin addition) was slightly lower than the calculated paracasein content on which the applied amount of chymosin was based. The applied chymosin to paracasein ratios were therefore slightly higher than was planned. Chymosin dosage *n* was calculated at 1.588 and 1.524 ml chymosin per kg paracasein for UFC 1 and UFC 2, respectively. The water/paracasein ratio was higher in UFC 2. The pH of UFC 1 appeared to have decreased further after preparation to 5.0. The pH of UFC 2 remained unchanged at 5.2.

The composition of the cheese models before addition of chymosin could be derived from the amounts and composition of its components. The soluble nitrogen content before chymosin addition was determined by analysis as well. The SN-content appeared to be slightly higher than calculated. Presumably, the paracaseinate still contained a small amount of soluble nitrogen. The composition of the cheese models, prepared with rennet or acid whey products, is given in table 3.4.

Table 3.4 Composition (in %) of cheese models at t = 0, before addition of chymosin

cheese code	whey product	paracasein	whey protein	SN/caseinN
RC1	RWP	25.90	0	1.53
RC2	RW	25.73	0.42	3.14
RC3	RWRA	25.13	2.52	11.45
RC4	RWRB	24.20	5.08	22.40
AC1	AWP	25.87	0	1.24
AC2	AW	25.73	0.42	2.72
AC3	AWRA	24.88	2.81	11.44
AC4	AWRB	23.95	5.75	21.90

Breakdown of (para)casein by chymosin and milk proteinases in UF-cheeses

In the initial stage of ripening, the release of breakdown products is directly related to the paracasein-nitrogen content of the cheese. Therefore the increase in the SN-content of all UF-cheeses and the NCN-content of chymosin-containing UF-cheeses is given in relation to the initial paracasein content of the cheese. In the blank cheeses, in which casein was not yet converted into paracasein, the NCN-content is related to the initial casein content.

UF-cheeses were analysed up to 11 weeks. Results from SN- and NCN-analyses are given in figures 3.1A and 3.1B. Results from the two batches of UFC 1 are averaged, as are the two batches of UFC 2.

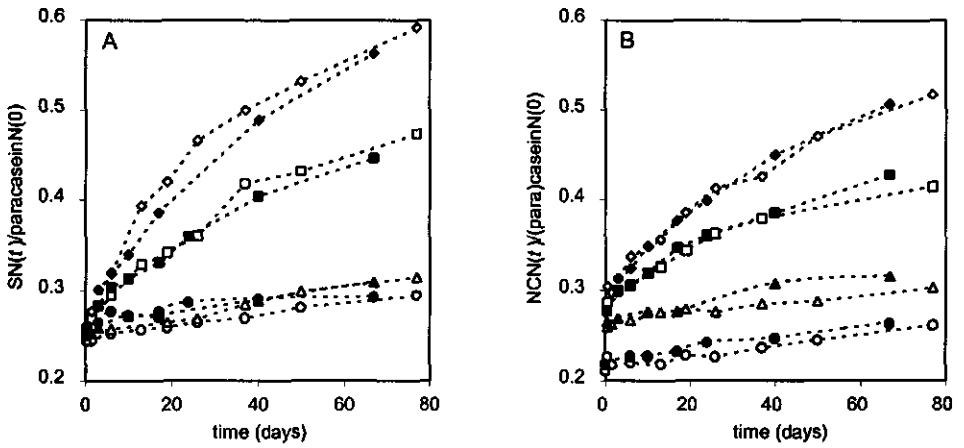

Figure 3.1 The soluble nitrogen content (SN) (fig. A) and the non-casein-nitrogen content (NCN) (fig. B) in UF-cheeses, relative to the initial (para)casein-nitrogen content (see text), in UFC 1 (open symbols) and UFC 2 (closed symbols), for various chymosin dosages: ● blanks, no chymosin during ripening, chymosin added shortly before analysis to allow SN-analysis, ▲ *n*, ■ 3*n*, ◆ 6*n*.

In the SN-content of the blank cheeses the caseino-macropeptide-fraction is included, due to the conversion of non-coagulated casein into precipitable paracasein shortly before analysis. Therefore the SN-content of the blanks was similar to the content of other UF-cheeses at t = 0 (i.e. a few hours after chymosin addition). The NCN-content of the blanks could be determined without this conversion. The difference between the NCN content in the cheese with dosage *n*, shortly after chymosin addition, and in the blank cheese represents the content of the caseino-macropeptide-fraction, released from casein by chymosin.

The SN-content increased faster than the NCN-content, as can be clearly seen in figure 3.1 at higher chymosin contents. The difference must be ascribed to the different extraction methods, used for the release of the breakdown products from the ripened cheeses. During extraction, non-hydrolysed caseins are precipitated, but also some breakdown products (mainly large peptides). Apparently, more of these breakdown products remain solubilized at pH 7.5 than at pH 4.6, as is used for the determination of NCN. Also the extraction temperature, the time of extraction or the $CaCl_2$ might have caused the SN-content being higher than the NCN-content. Several extraction procedures were compared in IDF-bulletin 261 (1991). An advantage of the extraction with $CaCl_2$ at pH 7.5 is the inactivation of the chymosin in the cheese

sample. Although not further investigated in this study, more information about the extraction and the components in the extract could be obtained by HPLC-analyses of the SN- and NCN-extracts or by gel electrophoretic analysis of the non-soluble fractions (as is done by Noomen, 1977).

In the blank cheeses the SN- and NCN-contents increased during ripening. This increase must be ascribed to the action of alkaline or acid milk proteinase. These enzymes are responsible for the proteolysis of β-casein and, although to a lower degree, α_s-casein. Some activity of milk proteinases could be expected, because these enzymes were totally enclosed in the UF-cheese and not inactivated by heating. These results show that the contribution of milk proteinases to the formation of SN-components can not be neglected, as is also reported by Visser (1977c), Noomen (1978a) and O'Keeffe et al. (1978).

The formation of breakdown products appeared to be only slightly higher in the cheeses with the normal chymosin dosage (*n*), compared to the blank cheeses. During the whole period of ripening both the SN-content and the NCN-content did increase at approximately the same rate in the blank cheeses and in cheeses with dosage *n*. The absence of further formation of breakdown products in cheeses with chymosin dosage *n*, compared to the blanks, implies that this amount of chymosin was hardly able to hydrolyse paracasein. The negligible increase suggests that chymosin was (partly) inhibited or inactivated. These results, however, do not give evidence about the particular component in these UF-cheeses that is responsible for this inhibition or inactivation.

At strongly increased chymosin concentrations (3*n* and 6*n*) the increase in the SN- and NCN-content was considerable. The formation of breakdown products from paracasein in relation to the chymosin dosage is given in figure 3.2. Such a figure could be given for any ripening time, and a ripening period of 56 days was chosen to compare results from this study with data in the literature (i.e., de Koning et al., 1981).

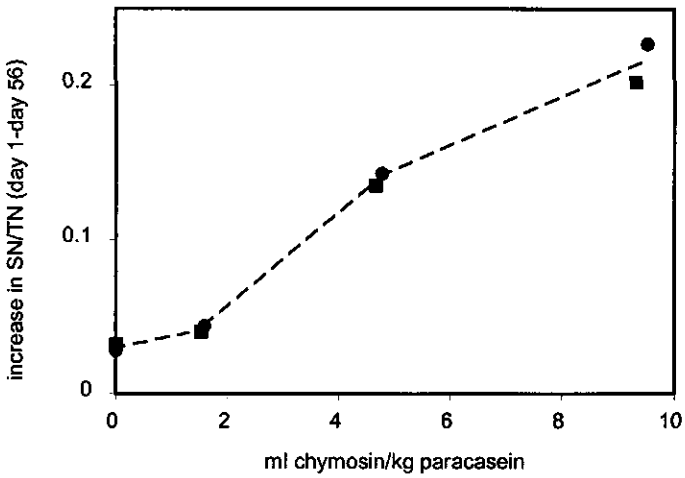

Figure 3.2 The increase of the soluble nitrogen content (after 56 days of ripening) relative to the total nitrogen content, as a function of the chymosin dosage to UF-cheeses: ● = UFC 1; ■ = UFC 2

Figure 3.2 illustrates that below a minimum concentration of chymosin hardly any SN-components were released from paracasein. At low dosages the increase in the SN-content is negligible compared to the control UF-cheese to which no chymosin had been added. To obtain significant breakdown of paracasein by chymosin, the chymosin content should thus be higher than this minimum, the so-called threshold-value, below which no or hardly any chymosin activity could be detected.

A study from de Koning et al. (1981) is to some extent comparable. It can be derived that in their UF-cheeses the protein content was approximately 25%. The fat-in-dry-matter content was 20%. The applied rennet dosages were 0.25 and 0.5 ml undiluted (strenght 1:10 000 SU) calf rennet per kg pre-cheese, which corresponds to approximately 1.3 and 2.6 ml rennet per kg paracasein, respectively. It is assumed that the used clotting agents (chymosin versus calf rennet) could be compared without conversion for the chymosin content, as the strength was differing only slightly. As in our study, the SN-extraction method of Noomen (1977) was applied. They gave the soluble nitrogen content as a percentage of the total nitrogen content of the cheeses. Also in their study, no whey was expelled and thus all whey proteins were accumulated in the cheese. They determined an increase in SN/TN of 0.055 and 0.095 after 56 days of ripening, at 1.3 and 2.6 ml rennet per kg paracasein, respectively. These values correspond fairly well with increases in %SN/TN as found

for the UF-cheeses prepared in this study (see figure 3.2), despite some differences in composition of the UF-cheeses (as water content, fat, starter bacteria, rennet enzymes).

Results from our chymosin-free cheeses show that simple extrapolation of results from chymosin-containing cheeses to lower chymosin dosages would not have been justified, as also in the blank cheeses soluble nitrogen components were formed during the period of ripening, most likely due to milk proteinase activity. Absence of results on blank cheeses may lead to wrong conclusions on the relation between chymosin content and breakdown of paracasein. Moreover, when extrapolating it must be realised that the release of SN-components is not linear in the time during ripening (Visser, 1977c).

In comparing the chymosin-free and chymosin-containing cheeses, besides the activity of the proteolytic enzymes other differences must be considered. The composition of the moisture phase is not the same, in particular the whey protein fraction in the moisture. In the chymosin containing cheese this fraction does not only contain serum proteins, but also caseino-macropeptide released from casein shortly after addition of chymosin. Approximately 18% of the whey protein fraction in the UF-cheese consisted of caseino-macropeptide. Hence, the substrate from which breakdown products were to be released was not the same for the blank UF-cheese and that with chymosin. In the blank the casein fraction was unchanged, while in the chymosin containing cheese it was converted into paracasein. This conversion, together with the changed composition of the whey protein fraction in the water phase, may have consequences for the accessibility of the substrate for chymosin and/or milk proteinases and for the activity of the enzymes as well.

The rate of proteolysis was similar in UFC 1 and UFC 2, despite the small differences in the water/protein ratio (5.22 and 6.32, respectively) and the pH (5.0 and 5.2, respectively).

Proteolysis in standard and UF-cheese: similarities and differences

Results from this study on high moisture UF-cheeses can to some extent be compared with ripening aspects of traditionally manufactured cheese. Although the latter were not prepared and investigated in this study, ripening aspects of these conventional cheeses were studied extensively by several researchers (Stadhouders, 1960; Visser, 1977abcd; O'Keeffe et al., 1978). Whereas in the UF-cheeses in our

study all whey proteins are enclosed, the amount of whey proteins in traditionally manufactured cheese is very small or negligible (see Chapter 2).

Due to the removal of whey during the conventional cheesemaking process, part of the rennet enzymes are lost as well. Visser (1977a) has determined rennet contents in several types of standard cheese, using the method of Stadhouders et al. (1977). The standard chymosin dosage (n), used in our study, was based on the amount detected in traditionally manufactured cheese (Stadhouders et al., 1977; Visser, 1977a). The amount of chymosin in relation to the paracaseinate substrate was therefore similar for traditionally manufactured cheeses and UF-cheeses with dosage *n* in this study. In conventional cheeses the standard dosage results in considerable release of SN-components, mainly consisting of large peptides (Visser, 1977c).

The milk proteinase activity in any cheese will primarily depend on the content of milk proteinases in the cheesemilk. Milk proteinases, associated with the casein micelle in milk, will be fully enclosed in the casein matrix of high moisture UF-cheese as prepared in this study. In traditionally manufactured cheeses, on the other hand, milk proteinases will be partly lost with the whey, as these enzymes dissociate from casein at lowered pH (Lawrence et al., 1983). However, the total milk proteinase activity in cheese depends not only on the content, but also on the conversion of plasminogen to plasmin, the cheese composition and ripening conditions. A higher pH at drainage of the curd, as well as a higher pH of the cheese during ripening, will result in a higher plasmin activity. In this study the pH of the UF-cheeses was set at 5.2, as in traditional Gouda cheese, to eliminate differences in specific activity due to the pH during ripening.

Also the composition of cheese is of importance for the rate of proteolysis, as it affects the activity of the enzymes. In particular the pH, the water/paracasein ratio and the salt/water ratio must be considered (Noomen, 1978ab; Lawrence et al., 1984). In the UF-cheeses the pH and the salt-in-water content were adjusted similar to the values in traditionally manufactured cheese; the water content was much higher in the UF-cheeses. A further decrease in the water content can hardly be achieved, because of the increasing viscosity of the retentate during ultrafiltration. The water content of these cheeses was not further lowered by syneresis, because this would also have resulted in the loss of chymosin. The water/paracasein ratios in UFC 1 and UFC 2 were 5.22 and 6.32, respectively. In traditionally manufactured cheese moisture is removed by syneresis of curd; smaller water/paracasein ratios can be reached this way. In traditional Gouda cheese this ratio is approximately 1.7. As stated earlier, the

rate of proteolysis was not affected by the value of this ratio. This was concluded from comparison of UF-cheeses with various ratios in this study and in the study by de Koning et al. (1981).

In the UF-cheese manufacture it was thus tried to adjust the most important factors that influence the rate of proteolytic activity by chymosin to those in traditionally manufactured cheese. The main difference between traditionally prepared cheeses and UF-cheeses with chymosin dosage *n* in this study is then the whey protein content in the aqueous phase. This leads to the conclusion that the relatively low proteolytic activity in the UF-cheeses with dosage *n* must be ascribed to the whey proteins present in the aqueous phase of the cheese. Assuming the retarded proteolysis to be caused by enzyme inhibition, it is thus very probable that one or more whey proteins are responsible for the inhibition.

Visser (1977c) and de Koning et al. (1981) had also determined the formation of breakdown products in standard, traditionally-prepared, cheeses. Results from both studies can be used to compare proteolysis in UF-cheeses with that in traditionally prepared cheeses. Results for whey protein-containing cheeses could be extrapolated to cheeses with lower or negligible whey protein contents. Visser (1977c) followed the formation of breakdown products (SN/TN) up to 6 months for aseptically manufactured cheeses (no UF), at varying amounts of rennet. The applied amounts of rennet were 0.5*n*, *n*, 2*n* and 3*n*, in which *n* corresponds to approximately 1.5 ml rennet per kg paracasein in the cheese. The %SN/TN values after almost two months were about 12, 15.5, 18 and 23.5, respectively, which is clearly more than in the case of UF-cheeses. De Koning et al. (1981) reported a similar degree of proteolysis in traditionally prepared (standard) cheeses. For these cheeses a normal rennet dosage was applied (22 ml per 100 kg milk), which results in an amount of approximately 1.5 ml per kg paracasein in traditionally prepared Gouda cheese (Visser, 1977a; Stadhouders et al., 1977). In the study by de Koning et al. (1981), the rate of proteolysis (expressed as formation of SN-components) was initially similar for these standard cheeses and for UF-cheeses with a dosage of 2.6 ml rennet per kg paracasein. After 56 days the release of SN-components in these UF-cheeses appeared to be somewhat retarded. In their UF-cheeses with a halved dosage (1.3 ml rennet/kg paracasein) the formation of breakdown products was almost proportionally lower. The degradation of α_{s1}-casein was almost similar for standard cheeses and UF-cheeses with a dosage of 2.6 ml/kg paracasein. The retarded formation of SN-components in UF-cheese (dosage 2.6 ml/kg paracasein) was probably due to slower

degradation of β-casein (degradation was about 30, 10 and 0%, for standard cheeses and UF-cheeses with dosage 2.6 and 1.3 ml/kg paracasein, respectively. Qvist et al. (1987) also reported the degradation of β-casein in UF-cheese being lower.

Creamer et al. (1987) also compared proteolysis in traditionally manufactured cheeses and UF-cheeses from retentate (CF 5). They determined both the residual rennet content and the rate of breakdown of α_{s1}-casein. At similar residual rennet concentration in the conventional and UF-cheeses, the rate of proteolysis appeared to be slower in the UF-cheese.

In these few studies it is shown that, at a similar chymosin/paracasein ratio, the degree of proteolysis in the UF-cheeses is much less, or, to obtain a similar rate of proteolysis, the amount of chymosin per kg paracasein must be considerably higher in whey protein containing UF-cheese than in traditional cheese. Lawrence (1989) also stressed the importance of sufficient chymosin activity in UF-cheeses, but advised to ensure that the chymosin/casein ratio in UF-cheese be similar to that in traditional cheese. Based on our results, as well as the results from de Koning et al. (1981) and Creamer et al. (1987), the ratio in UF-cheese should be higher, the increase depending on the whey protein content, due to inhibition of chymosin activity by these whey proteins.

The action of the whey protein fraction on the casein hydrolysis by chymosin might be clarified by investigating the influence of separate whey proteins or by varying the concentration of whey proteins in the cheese. Application of various whey protein to (para)casein ratios in UF-cheese requires the selective removal of whey proteins from the retentate, prior to addition of ingredients, for instance by membrane filtration techniques. A lowered whey protein concentration, compared to the casein content, could also be obtained by syneresis, but then part of the added chymosin will be lost as well, while for this purpose it is very important to know the chymosin concentration in the cheese.

Therefore this study was extended with cheese models, in which more factors could be varied, both independently and simultaneously. These models enabled further research on the effect of the composition of the aqueous phase of cheese on the hydrolysis of (para)casein by chymosin and milk proteinases.

Breakdown of (para)casein by chymosin in cheese models

The cheese models were analysed during 21 days. Also for these models the increase in the soluble nitrogen content is related to the initial paracasein-nitrogen

content. Since the initial SN-content varies for the cheese models prepared with various whey products, the rate of hydrolysis of paracasein by chymosin is followed by comparison of the increase in SN, relative to the initial paracaseinN content (SN/paracaseinN). The formation of breakdown products was for most cheese models also monitored by HPLC-analysis of SN-extracts. The (1-23)-fragment of α_{s1}-casein, released during proteolysis of α_{s1}-casein by chymosin, eluted after approximately 27 minutes and could be detected as a sharp peak on the chromatogram. The height of this peak was multiplied by a dilution factor, which represents the dilution of the cheese model in the extraction procedure. Both the formation of SN-components and the increase in the peak of the (1-23)-peptide in the chromatogram was determined for cheese models of various whey protein contents and chymosin dosages. The changes in the SN-content are presented in figure 3.3, whereas formation of the (1-23)-peptide is given in figure 3.4. All results are given as a function of ripening time (in days).

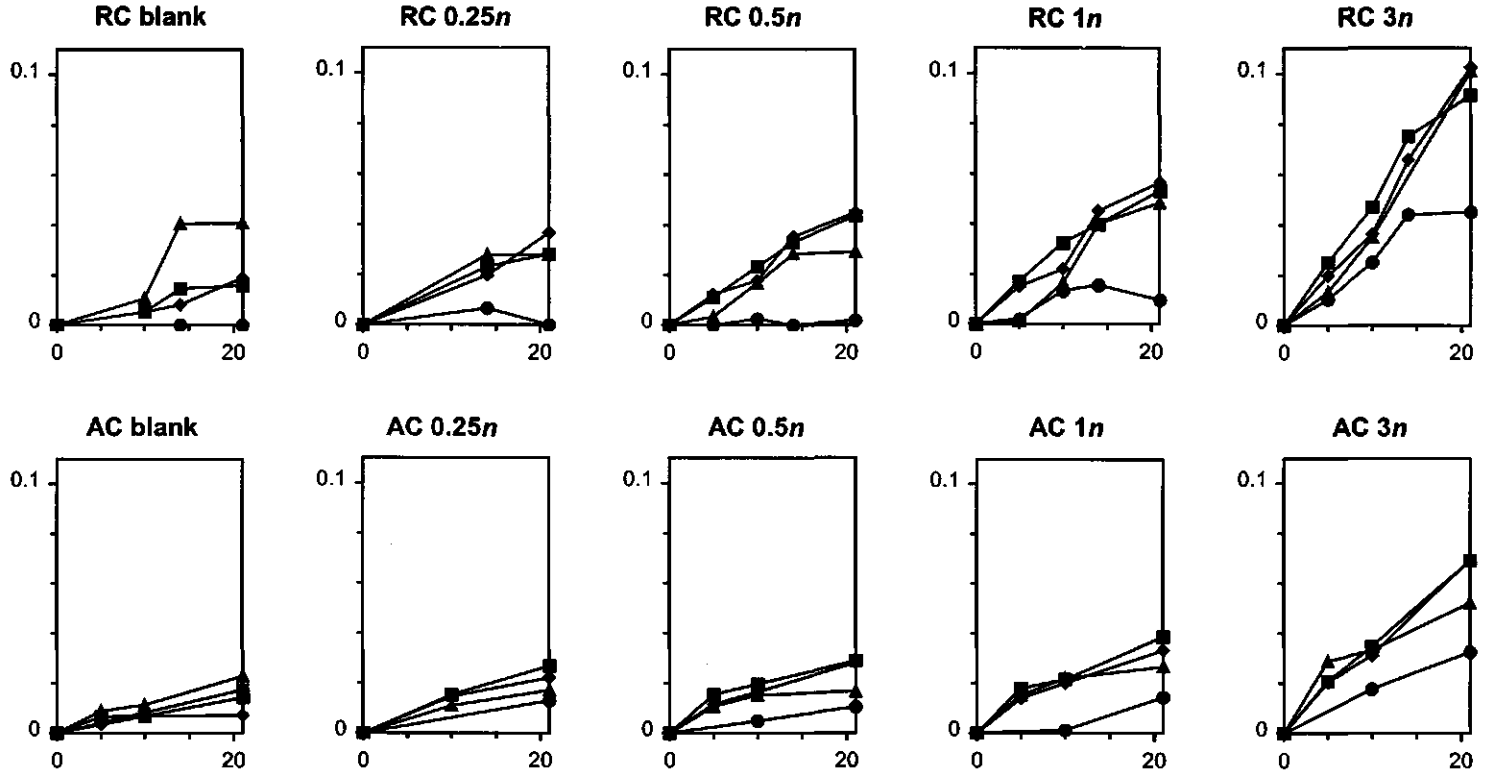

Figure 3.3 The increase in SN/paracaseinN in cheese models, as a function of storage time (days), at various chymosin dosages

upper figures: cheese models containing rennet-whey products: ◆ RC1, ■ RC2, ▲ RC3, ● RC4

lower figures: cheese models containing acid-whey products: ◆ AC1, ■ AC2, ▲ AC3, ● AC4

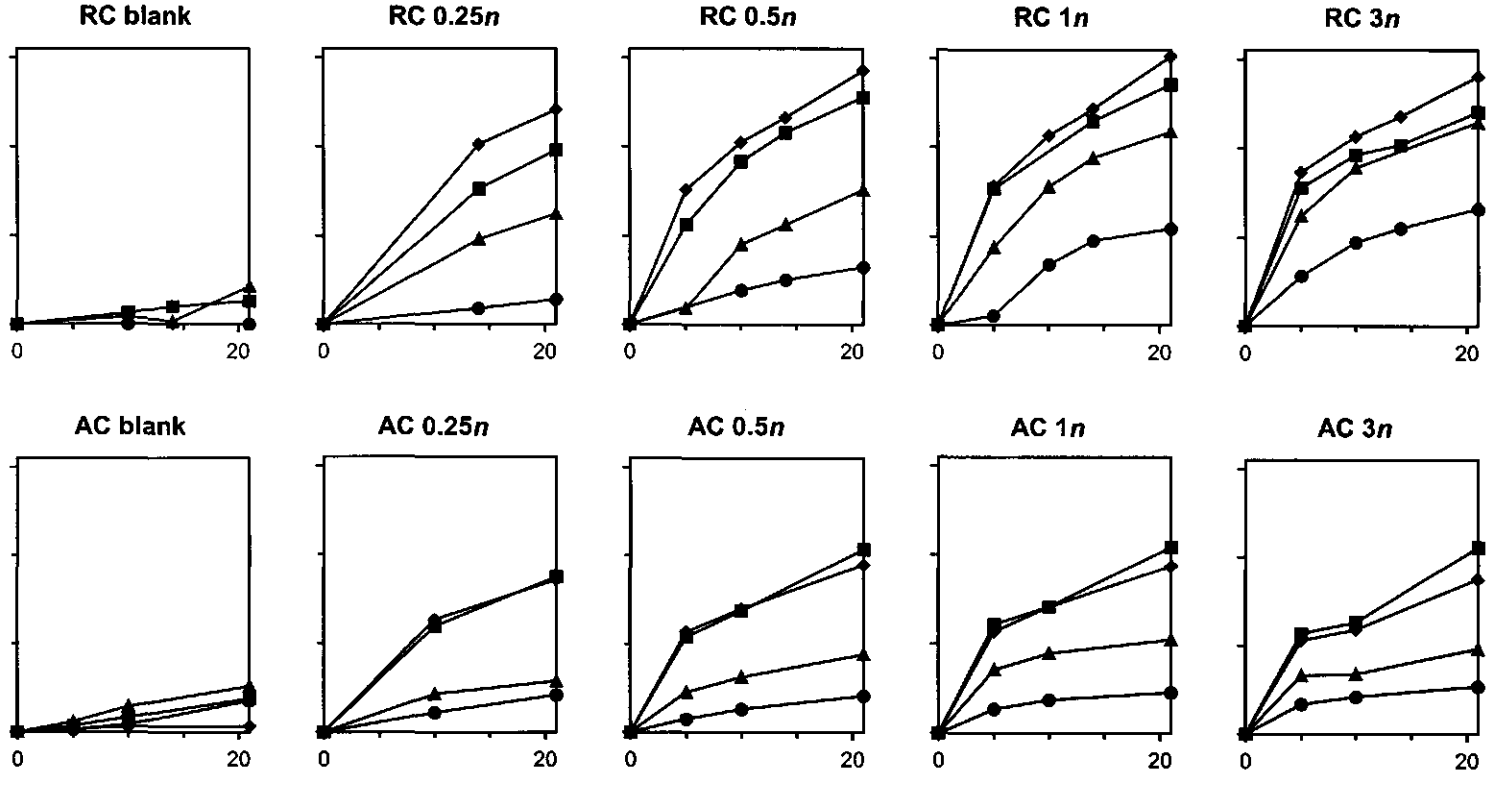

Figure 3.4 The release of the (1-23)-peptide in cheese models (given in arbitrary units), as a function of storage time, at various chymosin dosages

upper figures: cheese models containing rennet-whey products: ◆ RC1, ■ RC2, ▲ RC3, ● RC4

lower figures: cheese models containing acid-whey products: ◆ AC1, ■ AC2, ▲ AC3, ● AC4

In general, a higher chymosin dosage results in an increased rate of formation of SN-components (figure 3.3). This could be clearly observed by comparison of dosages *n* and 3*n*, but differences between cheeses with 0.5*n* and *n* are very small. At higher whey protein contents of the cheese, the increase in SN/paracaseinN was in most cases lower. The formation of SN-components was almost similar in the cheese models with permeate and with unconcentrated whey, but this could be expected, as differences in the serum or whey protein contents of these cheeses were very small as well. Compared with these cheeses of very low whey protein contents, the release of SN-components in the cheeses RC3 and AC3 were a little lower, whereas the formation of breakdown products in the cheeses with the highest whey or serum protein contents (RC4 and AC4) was clearly retarded. After longer times of ripening, differences caused by variation in chymosin dosage or in whey protein content became smaller. In the models with the highest whey protein content (RC4 and AC4), however, the SN levels remained lower than in the cheeses with lower whey protein contents.

In cheeses with chymosin the concentration of the (1-23)-peptide increased during ripening (figure 3.4), but the increase was smaller as the whey protein content of the cheese was higher. By this method, the increasingly limited chymosin activity at increasing whey protein content is very clear. Differences between AC1 and AC2 are nil, while the concentration of the released peptide was a little less in RC2 than in RC1. These figures also show that increasing the chymosin dosage hardly leads to a higher concentration of the (1-23)-peptide in the cheese after 21 days of ripening. Whereas an increase from 0.25*n* to 0.5*n* resulted in a higher degree of proteolysis, a further increase to dosage *n* or 3*n* did not cause a further increase of the concentration of the breakdown product. In the acid whey containing cheeses, the final peak levels (after 21 days) for the 3*n* chymosin dosage were equal to or even lower than those for the *n* dosage. The absence of a further increase in peptide concentration must be ascribed to further breakdown of the (1-23)-fragment itself. If breakdown of the (1-23)-peptide is indeed the explanation for the peak being smaller for 3*n* than for *n* chymosin dosage, it points to a clear disadvantage of this method for monitoring the proteolytic activity, since the height of the peak cannot be seen as a measure for the release of the (1-23)-fragment, but is the resultant of formation and breakdown of this component. A similar observation was reported by Christensen et al. (1991) and by Hansen (1993). They determined a maximum in the concentration of α_{s1}-I-casein after 3 weeks of ripening. After an initial increase in the concentration, this breakdown product was hydrolysed further. During the 3 weeks of ripening, the SN-contents, as well as the levels of the (1-23)-peptide, did not only increase in the

chymosin containing cheeses, but also in the blanks. Obviously, the blank cheese models contained hydrolysing enzymes, that were able to form soluble nitrogen-containing breakdown products. These enzymes may originate from the paracaseinate or from the rennet or acid whey product, although this is not very probable. Rennet enzymes in the paracaseinate were inactivitated by sterilization, whereas rennet whey was thermalized to inactivate these enzymes. Also milk proteinases in the paracaseinate would be inactivated by sterilization. The increase of SN-components in the blanks may point to the slow solubilization of the paracaseinate. This was also suggested after determination of the composition of the cheese models, by comparing results of analysis of the models with the calculated composition as derived from the composition of the individual components. However, solubilization cannot be the only explanation because then all blanks would give the same increase. As the concentration of the (1-23)-peptide in these blanks was also increased, it must be concluded that proteolytic enzymes have been (somewhat) active in these blanks. On the other hand, the increased SN-contents in the blanks may also be caused by the whey proteins not being transferred quantitatively to the SN-extracts. The transfer may be influenced by the whey protein content of the model.

The described trends were observed for models with rennet whey products and those with acid whey products. However, some differences could be observed between these two types of cheese models. In models containing rennet-whey products the SN-content and the concentration of the (1-23)-peptide increased faster than in those prepared with acid-whey products, at corresponding chymosin dosage. The levels were not only higher in the models containing whey proteins (RC2, RC3 and RC4) but also in those without whey proteins, which were composed with permeate (RC1). The reached levels of the SN-content and the content of the released (1-23)-peptide in rennet-whey containing cheeses were up to 1.5 times higher, after 21 days of ripening. As the paracaseinate was the same for both types of cheese, the differences must be ascribed to the used whey products, different chymosin activity or other factors that can influence proteolysis.

The total protein contents, as well as the composition of the rennet and acid whey products, were slightly different. Whereas acid whey contains mainly serum proteins, rennet whey also contains caseino-macropeptides (cmp). The presence of this extra component in rennet whey products does not explain the higher rate of proteolysis in the corresponding cheeses. This peptide cannot be held responsible for differences between the two types of cheese models.

The most probable explanation for the higher rate of proteolysis in RC-models is the higher activity of chymosin in these, as the formation of the (1-23)-peptide, which is released from α_{s1}-casein by chymosin, is also enhanced. As both type of cheeses were not prepared on the same day (AC was prepared later), the specific activity of the added chymosin might have been reduced in the case of AC, compared to RC.

Chymosin activity in whey protein containing cheese

Although not all results of the experiments with cheese models can be explained unambiguously, one main conclusion can be drawn. The retarding effect of serum or whey proteins on the rate of proteolysis of casein by chymosin is clearly shown in these cheese models. A similar conclusion was drawn from comparison of proteolysis in the UF-cheeses in this study with proteolysis in traditionally manufactured cheese, reported in other studies (Visser, 1977c; de Koning et al., 1981). The whey protein fraction in cheese can thus be held responsible for a decreased activity of chymosin. The inhibition of chymosin by whey proteins explains the retarded proteolysis of α_{s1}-casein in UF-cheese.

Our conclusions with respect to the retarding effect of whey proteins on proteolysis of caseins are in agreement with results from other studies with different types of cheese models. Hickey et al. (1983), who had observed a strongly retarded further breakdown of peptides (measured as the accumulation of amino acids), referred to the existence of proteinase and/or peptidase inhibitors in milk, that may be concentrated by ultrafiltration. Later, Harper et al. (1989) composed Cheddar cheese slurries (40% total solids), with various amounts of whey proteins. These cheese slurries were in fact also cheese models. They determined a slower release of SN-components and a smaller degree of proteolysis of α_{s1}-casein and β-casein at higher native whey protein contents as well. The inhibiting effect of denatured whey proteins, on the other hand, was smaller. Based on the facts that the inhibiting component was partially inactivated by heat and concentrated by ultrafiltration, they concluded that the inhibitor was a protein. These results are in agreement with findings of Lelievre et al. (1990). They also detected the inhibition of chymosin by whey proteins, both in the first stage of cheesemaking during milk coagulation as in the second stage during proteolysis of α_{s1}-casein. The effect on the rate of the first reaction (cleavage of κ-casein by chymosin) was studied using the milk clotting assay, while the second reaction was followed in the rate of hydrolysis of α_{s1}-casein in solutions (by gel electrophoresis). By comparing the influence of various whey protein fractions, they concluded that a high molar mass protein was responsible for the

inhibition. Both reactions were not inhibited in the presence of denatured whey proteins. Results from these studies are in good agreement and consistent with the α_2-macroglobulin being responsible for the inhibition of chymosin, as suggested by McLean and Ellis (1975). This globulin is present in milk serum, will be accumulated during ultrafiltration and will most probably be inactivated by heat treatment, as is concluded by Harper et al. (1989) and Lelievre et al. (1990). Besides, it retains its inhibiting capacity in both acid and rennet casein whey, which were used in our cheese models. The latter was also used in the study from Lelievre et al. (1990).

In the ripening of most cheese varieties, chymosin contributes more to the formation of peptides from caseins than milk proteinases. For the further breakdown to flavour compounds, a sufficient quantity peptides should be formed. Therefore, chymosin activity must be sufficient. If chymosin is partly inhibited in its activity by whey proteins, this inactivation can be counteracted by increasing the amount of active chymosin in the cheese. This can be realised by increasing the chymosin dosage to UF-retentate. Visser (1977a) observed a linear relation between rennet dosage to cheesemilk and the rennet content in the cheese (at similar cheesemaking procedures). A constant percentage of rennet retention was also reported by Dulley (1974), who found this for a normal and a double rennet dosage. Also other adaptions in the cheesemaking process that influence the retention of rennet can be used to increase the chymosin content in the cheese. The pH strongly affects the retention of rennet in cheese (see also Chapter 5). In this respect, the moment of drainage is of major importance, not only for structural properties of cheese, but also for the proteolytic processes. The pH and the water/paracasein ratio at drainage determine largely the enclosure of chymosin and milk proteinases (Lawrence et al., 1983). In general, a lower pH at drainage results in a higher chymosin content in the cheese. Increasing the residual rennet content for obtaining a higher rate of proteolysis was also suggested by Creamer et al. (1987). In general, however, changes in chymosin dosage, pH or temperature, with the aim to increase the residual rennet content also have their impact on other aspects of the cheesemaking process and on properties of cheese. Therefore, changed process parameters usually require more adaptions in the cheesemaking process.

Whereas from this study it is concluded that at higher whey protein contents of the cheese, the chymosin to casein ratio should be increased as well, it is remarkable that in many studies about UF-cheese manufacture strongly reduced chymosin

dosages (chymosin/casein ratios) were applied. Chapman et al. (1974) reduced the amount of rennet for Cheddar and Cheshire cheese from retentates (CF 2) to approximately 50% of the normal quantity. Sutherland and Jameson (1981) added 22% of the amount that would be added to the milk, from which the retentate (CF 4.8) was prepared. UF-cheese was prepared in a similar way by Hickey et al. (1983). They measured a residual rennet activity of 0.2 μl/g UF-cheese, while a level of 0.3 μl/g cheese was detected in conventional Cheddar cheese (rennet activity measured according to Dulley, 1974). Green et al. (1981) reduced the rennet dosage (per liter retentate) at increased concentration factors, with as a consequence a strongly reduced rennet dosage in relation to the casein content. In agreement with the lower dosage at higher CF of the retentate, also lower concentrations of active rennet enzymes in the curd were determined, with as a result the retarded degradation of caseins. Rao and Renner (1988, 1989) reduced the amount of rennet for UF-cheese manufacture from retentates (CF 4.1) with 80%, in relation to the traditional process. Guinee et al. (1994) applied dosages of 0.22 ml rennet per liter retentate, independent of the protein concentration in the retentate.

Although in most studies the dosages were reduced in relation to the casein content of the retentate, further comparison is hardly possible, due to differences in cheese manufacture. During cheese manufacture from retentates, whey was expelled to reach a sufficiently low water content in the cheese. Consequently, part of the whey proteins from the retentate, as well as part of the rennet was lost. Although applied rennet dosages were mostly given, the retention level of rennet enzymes in the cheese was not given or cannot be derived. Besides, various types of rennet of various strength were used. Neither were the whey protein contents of the cheeses given in most studies (although they can in some cases be derived from initial soluble nitrogen content as a percentage of total nitrogen). In most of these studies with a reduced dosage also a lower rate of proteolysis in the UF-cheeses was determined. The lower rate of formation of breakdown products in cheeses from retentate can in many cases be explained by the rennet/casein ratio being lower in these cheeses. The lack of knowledge concerning the rennet retention and whey protein contents, hampered the interpretation of results of ripening.

In general, the chymosin dosages in UF-cheese manufacture were kept relatively low to keep the clotting time more or less the same as in traditional cheese manufacture. Because of the strongly increased casein content coagulation occurs relatively faster after release of the cmp-fraction from κ-casein. Moreover, in concentrated milks coagulation starts already at a lower degree of κ-casein hydrolysis (van Hooydonk & van den Berg, 1988). The faster gelation may be

counteracted by reducing the coagulation temperature, but this may give problems because of the higher viscosity of retentates. Although rennet concentrations can be decreased for κ-casein hydrolysis and subsequent curd formation, the reduced amounts used are in general too small for obtaining a normal rate of ripening, as was earlier suggested by Covacevich and Kosikowski (1978). Especially in these cases of relatively low chymosin dosage, considerable adsorption is required for achieving a sufficient chymosin/casein ratio in the cheese. This can be achieved by lowering the pH at which curd drainage occurs, but possibilities to use this tool for increasing chymosin retention are limited due to other parameters in the cheesemaking process.

In this study it is confirmed that chymosin is a key factor in proteolysis of (semi-) hard cheeses; moreover, it is an essential factor in the retardation of flavour development in UF-cheese. For the understanding of the process of proteolysis, knowing the content and the activity of the rennet in cheese is essential. In these experiments the content was similar to the dosage, as no whey was expelled from the high moisture UF-cheeses and the cheese models. In the manufacture of hard cheese, however, the required moisture content can only be reached if some whey is released from the curd, with as a consequence a decreased chymosin content as well. The chymosin activity in the cheese can be derived from the rate of proteolysis, although this is an indirect method (also depending on ripening conditions), for which some ripening time is needed before results can be obtained. A direct method for estimating the chymosin activity would be helpful in the attempts to explain the ripening of cheese. In Chapter 4 several methods for the estimation of the chymosin activity are evaluated, and an improved method is developed and tested.

Activity of milk proteinases in (UF-)cheese

The role of milk proteinases in cheese proteolysis can not be neglected, although its contribution to ripening of Gouda and Cheddar type cheeses is less than that of chymosin. Whereas chymosin mainly breaks down α_{s1}-casein, milk proteinases are mainly responsible for the proteolysis of β-casein, forming γ-casein and proteose-peptones (Grappin et al., 1985). In the cheesemaking process, the retention of milk proteinases in the curd depends on the pH at draining; at lower pH the inclusion is limited. The activity of milk proteinases in the final cheese depends largely on the pH during ripening, which depends on the type of cheese. In acid cheeses the activity is lower and it then plays a less important role in proteolysis (Lawrence et al., 1983).

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Chapter 4

ESTIMATION OF THE CHYMOSIN ACTIVITY

ABSTRACT

In the first stage of ripening of many cheese varieties, degradation of caseins is mainly due to chymosin activity. The total chymosin activity during ripening depends on the amount of chymosin that is enclosed in the cheese and its specific activity. A method for estimating the chymosin activity in gels, curd and cheese was developed and evaluated. The chymosin-containing sample was 'dissolved' completely in JK-buffer. Dispersing was most successful at pH 5.2, at low temperature (4°C) and with 1% NaCl in the dispersion. During homogenization of the dispersion there was no loss of chymosin activity. This dispersion was incubated with κ-casein. The chymosin activity in this dispersion was derived from the rate of release of the caseino-macropeptide-fraction from κ-casein, hereby also introducing internal standards (i.e. known amounts of chymosin added to the dispersion). The described method was evaluated by determination of the chymosin activity in chymosin-induced skim milk and retentate gels, with known chymosin contents. These tests showed that a certain amount of chymosin in the sample was not active under test conditions, independent of the moment of chymosin addition (to the milk/retentate or later to the gel dispersion as an internal standard). Components originating from the gel (most probably one or more whey proteins) can be held responsible for this inhibition, since the threshold value was higher for retentate gels than for skim milk gels, while in the absence of milk components the enzyme was not inhibited. A similar restraining influence of whey proteins on the activity of chymosin was also observed during cheese ripening towards casein degradation (Chapter 3). Therefore, the presence of all cheese components in the assay mixture justifies the interpretation of the chymosin activity, as determined according to this method, being equal (or at least comparable) to the activity in the original cheese during ripening.

INTRODUCTION

The maturation of cheese is greatly affected by the amount and activity of ripening enzymes in the cheese. In most varieties without a specific internal or external (surface) flora, rennet and starter enzymes are most importantly involved in cheese ripening. The main enzyme in calf rennet is chymosin. Chymosin is responsible for the breakdown of casein into predominantly larger peptides. These can then be further degraded into smaller peptides and amino acids by the enzymes of lactic acid bacteria.

The rate of the breakdown of casein by chymosin depends on the amount of chymosin that is enclosed in the cheese, and on its specific activity in the cheese during storage. The inclusion of active chymosin in the cheese is predominantly determined by the conditions during the cheese making process, while its specific activity during ripening depends on the physico-chemical composition (pH, ionic strength) of the particular variety of cheese and on storage temperature. In this respect it must be realised that the concentration and specific activity of chymosin are not necessarily related, and need to be distinguished. During the latter steps of cheese manufacture enclosed chymosin may be denatured (and thereby inactivated) as, for instance, during scalding of Emmentaler cheese. Moreover the specific activity can in principle be affected.

Ripening processes in cheese, especially those in the initial stages, would be better to interpret if the concentration of active chymosin could be determined directly. In many studies the activity of chymosin is derived from the rate of proteolysis, but this is an indirect method that measures the total proteolytic activity from all enzymes present in the cheese. In the literature several methods for the estimation of the chymosin concentration in cheese are described. An overview of methods was given by Baer and Collin (1993). Since then methods that were partly derived from these, have been published by Zoon et al. (1994) and by Boudjellab et al. (1994).

Most methods are based on the extraction of chymosin from cheese, followed by the estimation of the chymosin concentration or chymosin activity in the extract. A major drawback of this procedure is that the part of chymosin molecules, associated with the proteineous substance that sediment upon centrifugation of the crude extract, will not be detected, with a relative low recovery as a consequence. To overcome this disadvantage, in several studies desorption of chymosin from casein was promoted by extraction at an increased pH (Holmes et al., 1977; Zoon et al.,

1994). Many different extraction buffers have been used, as is shown by Baer and Collin (1993). Suitable extraction buffers proved to be limited, due to the properties of chymosin. Chymosin is easily inactivated at pH values higher than 6.8 and at temperatures above 35°C (Mickelsen and Ernstrom, 1967). Besides, components in the buffer (especially salts) may influence the results from the subsequently performed chymosin activity measurement in the extracts. For the removal of salts, peptides and other components, possibly disturbing the chymosin activity test (clotting test), Stadhouders et al. (1977) dialysed the extracts. They then concentrated the dialysed extract by freeze-drying to increase the chymosin concentration in it. These steps, however, are very time consuming, which delays the analysis considerably.

Chymosin activity in an extract is often derived from the clotting time of a milk standard after addition of the chymosin-containing extract (Dulley, 1974; Stadhouders et al., 1977; Carlson et al., 1985; Qvist et al., 1987; Singh and Creamer, 1990). Instead of determining the clotting time of a milk standard, the release of caseino-macropeptide (cmp) from κ-casein in the milk standard (as measured by HPLC), can be taken as a criterion of chymosin activity in the extract. This procedure for estimating the chymosin activity in an extract was applied by Zoon et al. (1994). Several other methods (agar diffusion assay, the use of an enzyme specific synthetic peptide, immunological methods) are available to determine chymosin in a cheese extract, but most of them have drawbacks, as being very laborious, not being very sensitive, detecting both active and inactived chymosin or being very expensive.

The disadvantages of existing methods led us to approach the determination of the amount of active chymosin in cheese along a partly different way. The problems related to the quantitative extraction of chymosin from cheese prompted us to search for a method to dissolve the cheese completely, to prevent partial loss of chymosin with the sediment of the crude extract. Therefore, a method to prepare a milky (fat-free) dispersion from the cheese was developed. An assay for the determination of the chymosin activity in the dispersion was partly based on previously published methods. Instead of a milk standard for the incubation of a sample of the dispersed cheese, as is often applied, a pure κ-casein solution was used as a substrate. Released cmp was measured by HPLC. Chymosin activity was derived from the rate of cmp-release by chymosin (in the dispersion) from κ-casein.

The presented method has been the result from studies on the estimation of active chymosin in samples derived from non-syneresed chymosin-induced milk gels

with different physico-chemical composition. During that research procedures during particular experiments varied because of necessary adaptations. In fact, the described method was developed and tested for its utility at the same time, by application on chymosin-induced milk gels, with known concentrations of chymosin.

MATERIALS AND METHODS

Materials

-Skim milk was prepared from raw milk, obtained from the university herd. Skim milk retentate was prepared by ultrafiltration of skim milk at 30°C, using a membrane with a molecular weight cut-off of 8 kD (Filtron). The permeate was collected as well. Skim milk, retentate and permeate were preserved with 0.01% thiomersal (BDH Chemicals). The concentration factor of the retentate was approximately 4.5 (on a protein basis).
-JK-buffer, at different pH values, according to Jenness and Koops (1962).
-Lactic acid (Merck) was used for the adjustment of the pH of milk and retentate samples and for the adjustment of the pH of JK-buffer. Diluted lactic acid (45%) was heated (10 min at 121°C) before use to hydrolyse any esters present.
-Chymosin was obtained from Gist-brocades, under the trade name Maxiren 15. All chymosin solutions were prepared shortly before use, with JK-buffer at pH 6.7, and kept on melting ice.
-κ-casein-solutions were prepared by solubilizing κ-casein (Sigma) in JK-buffer of pH 6.7 (3 mg κ-casein/ml JK-buffer), shortly before use. The pH of the solution was 6.8.
-Other chemicals were of analytical grade (Merck):
NaOH (0.1 N), acetic acid (10%), NaCl.

Preparation of curd

Cube-shaped curd grains were cut from chymosin-induced skim milk and retentate gels (pH 6.7) about 70 minutes after chymosin addition. The applied chymosin dosage was 0.03% (v/w). The freshly cut curd grains from a gel were transferred to an excess of skim milk permeate (pH 6.7, 30°C). This mixture was stirred, to enhance syneresis. The manufacture and syneresis of curd is described in Chapter 2.

Dispersing chymosin-induced milkgels and syneresed curds

Chymosin-induced milk- or retentate gels and syneresed curds were homogenized by thoroughly mixing the particular samples with 3 parts (w/w) of JK-buffer of pH 5.2 by the use of an Ultra Turrax. The NaCl-content in this mixture was adjusted to 1% of the fat-free mass, taking the NaCl-content of the sample into account. The mixture was stirred overnight at low temperature (4°C).

Chymosin activity assay

The activity of chymosin in solutions or dispersions was tested by incubation of 0.1 ml of a chymosin-containing sample with 0.9 ml κ-casein solution at 30°C. The test tubes with κ-casein solution were equilibrated in a waterbath at 30°C for at least 10 minutes before incubation was started. After 20, 30 or 60 minutes the reaction was stopped by addition of 0.15 ml 0.1 N NaOH to this mixture. For the blanks 0.15 ml NaOH was added to the 0.9 ml κ-casein solution a few minutes before the 0.1 ml chymosin-containing sample was added. Ten minutes after the reaction was stopped, the pH of the samples was lowered to 4.6 by addition of 45 µl 10% acetic acid. The mixtures were then centrifuged and the supernatant, containing released cmp, was analysed by HPLC. For each tested chymosin-containing sample at least 2 incubation times were applied (in some tests 20 and 30 minutes, in others 30 and 60 minutes), both in duplicate. For every κ-casein solution that was made its $[cmp]_{max}$ was determined by incubating it with a more concentrated chymosin-solution (1 µl chymosin per ml JK-buffer) for 30 and 60 minutes. The chymosin activity in a sample was derived from the rate of cmp-release from the κ-casein substrate, using first-order kinetics.

HPLC-analysis

Analysis of cmp by HPLC was based on methods described by van Hooydonk and Olieman (1982) and Sharma et al. (1993). Centrifuged samples, obtained from the chymosin-activity test, were analysed on a Superdex 75 HR 10/30 column (gel filtration chromatography) with a volume of 24 ml. The HPLC-equipment consisted of an autosampler (Marathon) with a 20 µl fixed loop, a Spectroflow 757 absorbance detector (Kratos Analytical Instruments), a Spectra-Physics integrator and a personal computer with TSP software. The eluent consisted of K_2HPO_4 (1.74 g/l), KH_2PO_4 (12.37 g/l) and Na_2SO_4 (21.41 g/l) in Millipore water. The flow was 1 ml/min. Detection was performed at 215 nm. Cmp elutes after 11 to 12 minutes.

Chymosin activity in JK-buffer

Solutions of chymosin were prepared in JK-buffer of pH 5.2, with concentrations varying from 0 to 1 µl chymosin per ml solution. The NaCl-content in each solution was adjusted to 0.01 g/ml. The activities in the solutions were then determined according to the method described before.

Chymosin activity in skim milk and retentate gels

Chymosin was added to samples of skim milk and skim milk retentate of pH 5.2 at 0.01%, 0.02% or 0.03% (v/w). After 1 hour at room temperature the obtained gels were homogenized with 3 gram JK-buffer (pH 5.2, containing 1.33% NaCl (g/ml)) per gram skim milk or retentate gel. After overnight stirring (at 4°C) each dispersion was divided into 4 parts. Extra chymosin was added to 3 parts of each dispersion. The added amounts were respectively 0.05, 0.1 and 0.15 µl chymosin per gram dispersion.

Skim milk and retentate samples, to which no chymosin was added initially, were treated the same as the samples with chymosin. After a fourfold dilution, additional chymosin was added to 3 of the 4 samples. These samples with chymosin added later were then stirred at room temperature for 1 hour before chymosin activity was determined.

In the obtained 16 dispersions the chymosin concentration now varied from 0 to 0.225 µl chymosin per gram dispersion. The chymosin activities in all dispersions were measured by application of the method described earlier.

This procedure was also followed for determination of the activity in other chymosin-induced (non-syneresed) gels (such as those from which curd is prepared). This way chymosin activity was measured in 4 samples per gel.

Chymosin activity in syneresed curd from skim milk and retentate gels

For the determination of the chymosin activity in curd, samples of the syneresed curds were also dispersed with 3 grams JK-buffer (pH 5.2, with 1.33% NaCl) per gram curd. Corresponding to the treatment of chymosin-induced gels, each dispersion was divided into 4 parts and additional chymosin (0.05, 0.1 and 0.15 µl chymosin per gram dispersion) was added to 3 parts. Chymosin activity was then measured in all 4 dispersions from curd.

RESULTS AND DISCUSSION

Dispersing milk or retentate gels and curds

It was tried to dissolve samples of the chymosin-induced gels and syneresed curds completely, with the aim to determine chymosin activity in these dispersions, that contain all components of the gel or curd. For being able to take a representative sample (100 µl) for the chymosin activity test, a homogeneous dispersion had to be prepared from the gel or curd. The optimal conditions for dispersing were based on findings of Noomen and Geurts (personal communication).

Milk or retentate gels and curd samples were homogenized at low temperature with JK-buffer of pH 5.2, containing NaCl. These conditions are optimal for the solubilization of calcium in the paracaseinate gel, resulting in a weaker network. The homogeneity of a dispersion turned out to be strongly depending on the pH of the mixture. Homogenization proved to be most effective at pH 5.2. For mixtures of milk gels of pH 5.2, with 3 parts JK-buffer of pH 5.2, this condition was easily fulfilled, while milk gels of pH higher than 5.2, mixed with JK-buffer of pH 5.2, needed adjustment with lactic acid. The pH of dispersions of concentrated samples (retentate gels and syneresed curd) kept increasing during homogenization, even for samples of pH 5.2. This pH increase must be due to the release of buffering components. Therefore the pH should be controlled several times during homogenization (especially shortly after mixing) and, if needed, the pH should be adjusted to 5.2. Further, for an optimal homogenization it appeared to be important to keep the mixture, while stirring, at low temperature. Temperature increase resulted in demixing. In this respect also the addition of some NaCl (to a total content of 1% in the dispersion) was important. Both a low temperature and an increased ion strength enhanced the solubilization of calcium in the paracaseinate gel (Walstra and Jenness, 1984). According to Noomen (personal communication) homogenization occurs more readily in the case of ripened cheese.

Well homogenized dispersions, from which a 100 µl sample could be taken easily, were obtained faster if more of the homogenizing buffer was used per unit mass of gel or curd. On the other hand, a higher buffer to gel or curd ratio results in a lower chymosin concentration in the dispersion. In the case of samples with a low chymosin activity, dilution with buffer should be minimized in order to be able to detect the low activity. Therefore the samples from gels and curds were diluted only four times. For samples with a relatively high water content (such as milk gels), this dilution factor was sufficient. From dispersions of more concentrated samples (such

as retentates or syneresed curd samples), which were also four times diluted, it was sometimes difficult to take a representative 100 µl sample, due to the presence of very small particles in the dispersion.

These optimal conditions for homogenizing a sample, at pH 5.2, with some NaCl, at low temperature and sufficiently diluted with buffer, were gradually established in the course of the work. Initially, the required pH of 5.2 was not maintained for several samples, which were therefore not always fully homogeneous by the time the chymosin activity test was performed. This concerned especially the more concentrated samples (such as retentates and syneresed curd). For these, the reproducibility in the chymosin activity assay was not good. Therefore, the chymosin activity is only given for dispersions that were homogeneous and that resulted in acceptable replicates in the assay.

In some preliminary experiments, these conditions appeared to be satisfactory for dispersing full-cream cheese samples as well. After homogenizing at low temperatures, the fat was clumped, and could be easily separated from the dispersion, in which subsequently the chymosin activity could be determined.

The chymosin activity assay

A pure κ-casein solution (in JK-buffer) was used as a substrate for the incubation of a sample of the dispersion. The incubation reaction was stopped by increasing the pH to approximately 9 (with 0.15 ml 0.1 N NaOH), which inactivates chymosin. Léonil and Mollé (1991) also inactivated chymosin (in a milk gel) by addition of NaOH to pH 9, in combination with a heat-treatment at 60°C for 15 minutes. They reported that the heating step was not essential, as chymosin was irreversibly and completely inactivated at pH 9. Also in our samples the pH increase proved to be sufficient, the release of cmp stopped immediately at NaOH addition and no cmp-formation was detected in the blanks. Residual non-hydrolysed κ-casein, as well as para-κ-casein, were precipitated by lowering the pH to 4.6 and removed by centrifugation.

The partly clarified sample, containing released cmp, contains also any non-sedimentable components originating from the dispersion. In this study this concerns native whey proteins, present in the dispersions of skim milk and retentate gels. Syneresed curd may also contain whey proteins, depending on the degree of syneresis and the method of manufacture (see further Chapter 2). With the gel or curd dispersion, these native whey proteins are introduced in the activity assay as well. Caseino-macropeptide, released from micellar κ-casein during renneting of the

milk or retentate, also belongs to this whey protein fraction of the gel. In the case of ripened cheese, the sample may also contain peptides that are released from casein. Since they will not be precipitated and removed in any of the steps of the activity assay, they will end up in the clarified HPLC-sample.

Due to the comparable molecular sizes of certain serum proteins, caseinomacropeptides and possible other peptides, elution times may be similar and their peaks may overlap in the size-exclusion-chromatography as applied here. The peak in a chromatogram of a chymosin-containing sample that has been incubated for some time with κ-casein, thus comprises these whey proteins, together with the cmp released during the chymosin-activity test. For these whey proteins and any other comparable components originating from the dispersion, the blank sample provides a correction. The blank contained the same amount of whey proteins but no cmp was released in this sample during incubation. The difference in peak areas can then be used to derive the activity of the chymosin in the dispersion.

In this respect, before testing the chymosin activity in a dispersion, it is important to be sure that the hydrolysis of the micellar κ-casein (to para-κ-casein and cmp) in a dispersion of a chymosin-induced gel is complete. If this primary hydrolysis of the native milk micellar κ-casein in the dispersion would not be complete, the results of the activity assay would be unreliable. The blank, representing the content of pH 4.6 soluble components of a sample, including the cmp released during primary hydrolysis of κ-casein from the gel or curd, would then be estimated too low. The amount of micellar κ-casein introduced with the 0.1 ml dispersion would result in an increased substrate concentration in the incubated mixture. The changed substrate concentration and the incorrect blank would then lead to a miscalculated chymosin activity in such a sample. This also involves the initially chymosin-free dispersions to which chymosin is added later. The micellar κ-casein of the milk sample must be split by the chymosin added later before chymosin activity is determined in these samples. One hour after chymosin addition the cmp concentration in this initially chymosin-free (and cmp-free) dispersion did correspond to the concentration in dispersions from the gels. Chymosin activity could then be determined.

In other studies (Dulley, 1974; van Hooydonk and Olieman, 1982; Carlson et al., 1985; Qvist et al., 1987; Singh and Creamer, 1990; Sharma et al., 1993; Zoon et al., 1994) a milk standard (reconstituted milk) was used as a substrate for chymosin in the assay. After incubation, they selectively removed the whey proteins, as well as the casein, from the samples by precipitating them with trichloroacetic acid (TCA). At

the same time, upon addition of TCA, chymosin was inactivated. A high TCA-concentration (8% w/w) was required to precipitate all of the whey proteins (mainly originating from the milk standard), because otherwise these whey proteins would still interfere with the peak of cmp in HPLC-analyses. A disadvantage of applying such a high concentration of TCA to clarify the sample, however, is the partial degradation of cmp in TCA upon storage of the sample (van Hooydonk and Olieman, 1982). Cmp is also partly precipitated in TCA, depending on the degree of glycosylation of the κ-casein. According to Léonil and Mollé (1991) only 50 to 75% of the cmp was recovered in 8% TCA. When using a milk standard, its components in the cmp-containing sample should be removed anyway before HPLC-analyses, to clarify the sample, and it is therefore not possible to correct for these components with a blank sample. The use of a pure κ-casein solution overcomes the use of TCA. The assay mixture then only contains the whey proteins from the dispersion, introduced in the assay with the chymosin-containing dispersion. This amount, however, is, depending on the type of cheese or curd, very low (as it is strongly diluted) and will not disturb the HPLC-measurement of cmp in the assay. The blank provides a correction for this low amount. According to Léonil and Mollé (1991) cmp hardly precipitates at pH 4.6 during storage (5% loss during 8 days). In our study the samples were analysed within 2 days.

In most of the mentioned studies that used reconstituted milk as a substrate for chymosin, the chymosin activity was derived from the clotting time of the substrate. The homogeneity of the dispersions of curd or cheese as applied in our study, depended largely on pH and temperature. When a homogeneous sample of a dispersion (pH 5.2, 4°C) was added to κ-casein solution (pH 6.7, 30°C), small flocks were sometimes formed in the assay mixture because of the changed conditions. If the chymosin activity would have been derived from the clotting time of the substrate, these flocks, originating from the dispersion, might disturb this determination and lead to false observations concerning the clotting time. The turbidity of the test mixture, after addition of a sample of the dispersion, does not interfere with the measurement of released cmp for the estimation of the chymosin activity in a dispersion.

Derivation of the chymosin activity

The chymosin activity in a dispersion can be derived from the rate of release of cmp from κ-casein. The kinetics of this enzymic conversion in milk were extensively described by van Hooydonk et al. (1984). The release of cmp from

micellar casein by chymosin at pH 6.7 can be approximately described with first order kinetics. At lower pH the rate was higher, and slightly deviating from first-order kinetics (van Hooydonk et al., 1984 and 1986). In our study the pH during the incubation of a test sample with κ-casein varied between 6.4 and 6.7. Although the conditions during the chymosin assay in this study differed slightly, thereby possibly introducing minor deviations in the results, chymosin activity was derived using first-order rate equations:

$$-d[S]/dt = k \,.\, [S], \text{ with } k = k' \,.\, [E]$$

in which [S] is the substrate concentration (i.e. κ-casein), [E] is the chymosin concentration, k is the rate constant and t is the reaction time. The decrease in amount of substrate can be derived from the release of caseino-macropeptide. Conversion and integration of this relation then gives:

$$-\ln x = -\ln (([cmp]_{max}-[cmp]_t)/[cmp]_{max}) = [\text{chymosin}] \,.\, k' \,.\, t$$

In this relation, $[cmp]_{max}$ is the maximum concentration of cmp that can be released from the κ-casein (at complete hydrolysis of κ-casein), while $[cmp]_t$ is the amount that is released after t minutes of reaction.

Chymosin activity in a dispersion may be derived from a calibration curve, in which a relation is given between the concentration of active chymosin in a buffered system and its activity, expressed as $d(-\ln x)/dt$. The activity of chymosin is strongly dependent on factors like pH, temperature and NaCl-content. Derivation of chymosin activity by comparing $d(-\ln x)/dt$ of a sample with $d(-\ln x)/dt$ from a calibration curve is therefore only allowed if the conditions during the activity test of the unknown sample are comparable with those of the buffered system of the calibration. Only if the effect of changed conditions on the activity of chymosin is known well, a calibration curve obtained for certain circumstances may be used to determine activity at other conditions.

In this study a calibration curve was made for the buffered system of chymosin in JK-buffer. In figure 4.1A the release of cmp during the activity test is presented (i.e. $d[cmp]/dt$, in which [cmp] refers to the content in the mixture that is analysed by HPLC). In figure 4.1B the converted results, $d(-\ln x)/dt$, as a function of [chymosin], are given. In this case the pH of the test mixture of κ-casein with the chymosin solution was 6.6.

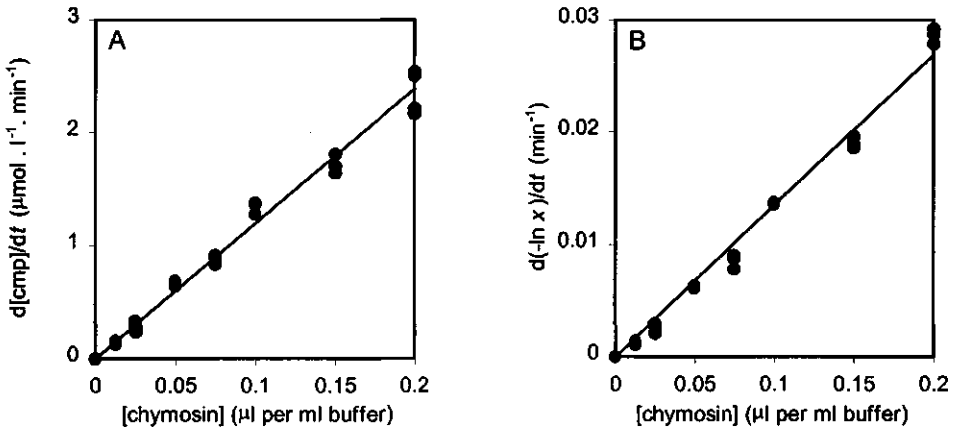

Figure 4.1 Activity of chymosin in JK-buffer.
A. Rate of release of cmp as a function of [chymosin]
B. d(-ln *x*)/d*t* as a function of [chymosin]

At low chymosin activities the relation between chymosin concentration and release of cmp is virtually linear, which would justify application of first-order kinetics to derive the chymosin-activity. At high activities, however, the amount of substrate may be limiting. Then, the reaction should be described by Michaelis-Menten kinetics. Although the activity test, as applied in this study, is used for samples of different origin, with either low or high activity, first-order kinetics was applied for all samples to derive chymosin activities. Depletion of substrate was prevented by application of shorter incubation times in case of high chymosin activities.

For the calculation of chymosin activities in the dispersions, no use was made of a calibration curve, since the conditions (like pH) were in most cases different from that in the calibration. In these experiments the chymosin activity in a dispersion was derived from the activities of the corresponding dispersions to which additional chymosin was added. The activities of these dispersions, expressed as d(-ln *x*)/d*t*, were plotted against the added amount of chymosin (0 to 0.1 µl/ml dispersion): see figure 4.2.

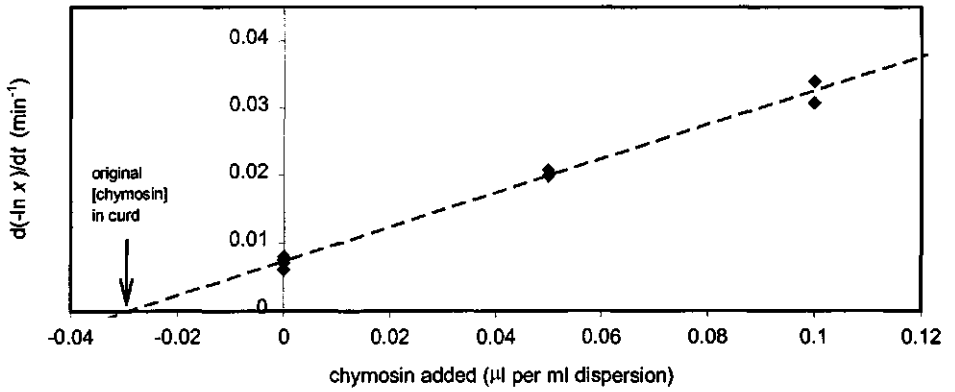

Figure 4.2 Chymosin activity in dispersions of a curd sample (from skim milk, whey syneresis 69 gram per 100 gram curd, pH of dispersion 6.4) with extra dosed chymosin, as a function of the amount of chymosin added to the original dispersion.

The slope of such a plot is a measure of the activity of the chymosin under the test conditions (pH of the mixture, temperature, NaCl-content, presence of other solubilized components). The intercept of the plot is a measure of the amount of the chymosin enclosed in the curd, with an activity as derived from the slope (see figure 4.2). The amount of active chymosin was calculated from dividing the intercept by the slope of the plot. In this derivation, the specific activities of the chymosin in the dispersion, which is originating from the gel or curd, and of the chymosin that is added to its dispersion, are supposed to be the same, since the conditions (pH, temperature, NaCl-content, other components present) were equal for all chymosin molecules in a dispersion.

This procedure with internal standards, in which extra enzyme was added to the test mixture (of substrate and rennet-containing sample) or to samples of a dispersion or extract, was earlier applied by Stadhouders et al. (1977), Carlson et al. (1985), Qvist et al. (1987), Singh and Creamer (1990) and Zoon et al. (1994). Known amounts of rennet were added to unheated and heated (to inactivate chymosin) cheese samples (Stadhouders et al., 1977) to determine the recovery with the applied method. Zoon et al. (1994) derived the recovery of their method by comparing chymosin activities in cheese-buffer mixtures and in ultrafiltered, chymosin-free cheese extracts, both with extra rennet added. In both studies a chymosin-free sample was prepared for this purpose, either by heating the sample

or by removing chymosin in the sample by ultrafiltration. This method was not applied in our study, as these treatments may not only inactivate or remove chymosin, but also result in other changes in the sample (for instance the denaturation or removal of whey proteins). As specific chymosin activity in a sample is related to the composition of the sample, any changes affecting the composition should be prevented. In our study the internal standards are added to the samples, without any treatments prior to the addition of extra chymosin. As these conditions were the same for samples without and with extra chymosin, it is justified to assume the specific activity of chymosin in these samples to be similar.

Chymosin activity in skim milk and retentate gels; recovery of extra chymosin

In figure 4.3 the estimated chymosin activity of the homogenized skim milk and skim milk gels, expressed as $d(-\ln x)/dt$, is given as a function of the applied chymosin concentration. The total chymosin concentration in the dispersions varied between 0 and 0.225 μl per gram dispersion. This chymosin concentration in the dispersion is either originating from the initial dosage to the milk sample (0 to 0.3 μl per gram milk, which gives 0 to 0.075 μl chymosin per gram dispersion), or it includes chymosin that is added to the dispersion shortly before testing (0.05 to 0.15 μl per gram dispersion). The pH of the skim milk and the dispersions was 5.2. During incubation the pH was 6.4.

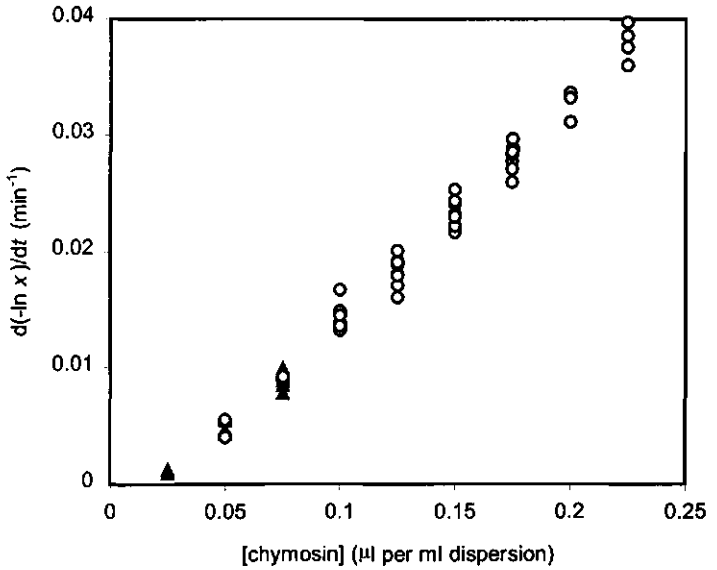

Figure 4.3 Chymosin activity in homogenized skim milk gels as a function of the total chymosin concentration in the diluted homogenized mixtures. Dispersions without (▲) and with (O) chymosin added after homogenization.

In the dispersed milk sample with a chymosin concentration in the dispersion lower than about 0.025 µl chymosin per ml dispersion no cmp is produced during the incubation of the dispersion with the κ-casein-solution. This means that a certain amount of chymosin is not active or not detectable under the test conditions. The amount of non-active chymosin can be derived by extrapolating the curve in figure 4.3 to the X-axis, where the activity is negligible. For these dispersions of skim milk the derived amount is approximately 0.025 µl chymosin per ml dispersion. This value holds for all samples from this experiment, both without and with additional chymosin added. It thus appears as if under the prevailing conditions this amount of chymosin is not active.

However, since the chymosin in the milk sample (with dosage 0.01%) caused the skim milk to clot (within approximately 1 to 1.5 hour), it can be concluded that the cleavage of cmp from micellar κ-casein was not totally hindered, as the used chymosin still exhibited activity at that stage. In the assay, on the other hand, partial inhibition is not to be denied. The different observations might be explained by the conditions during the clotting of milk and during the assay being very different. The strong dilution during the assay (40x, i.e. 4x during dispersing and 10x in the assay)

may prevent detection of very low activities. The process of milk clotting, on the other hand, does not only depend on the chymosin activity, but is also influenced by other factors (as casein and calcium content). Most probably, the curve that could be drawn through the data in figure 4.3 bends away towards the origin, when extrapolating to lower chymosin concentrations, indicating a strong decrease of the specific activity.

Figure 4.3 also shows no significant difference between the activities of chymosin added to the skim milk samples and of chymosin added to the homogenized dispersions. Comparing the activity in the initially chymosin-free dispersed milk sample, to which then chymosin was added, with the activity of the non-dispersed milk sample with an initial dosage of 0.02% chymosin without any further chymosin added, each containing 0.05 μl chymosin per ml dispersion, shows that in both samples the same activity is observed. This behaviour was repeatedly and consistently found. The lack of activity is thus independent of the moment that the chymosin is added, to the milk sample or to a dispersion of the milk or milk gel. This means that chymosin is not being inactivated during homogenization of the milk gel.

The fact that no discrepancy between activity of chymosin enclosed in a skim milk gel and chymosin added to the dispersion of the gel was observed (figure 4.3), also justifies the method of extrapolating the activities from samples to which chymosin was added to determine the activity of the chymosin in the sample without added chymosin.

In other experiments, skim milk of pH 6.7 was incubated with 0.03% chymosin, to prepare curd from the obtained gels. Chymosin activity was determined in these gels as well. Also in these gels chymosin was not fully recovered (figure 4.4). By testing these dispersions, it appeared that almost no cmp was produced at concentrations lower than approximately 0.023 μl chymosin per gram dispersion. The pH of the dispersions was 6.3 (the gels were dispersed with JK-buffer pH 5.2, without further adjustment of the pH with lactic acid, dispersions were well homogenized), the pH during incubation was about 6.6.

Several batches of retentate (pH 6.0 and 5.2) were incubated with chymosin (0.03%). After dispersing the gels with JK-buffer (pH 5.2) the pH values were 6.2 and 5.8, respectively. During incubation with κ-casein (pH 6.8) the pH values were 6.6 and 6.5, respectively. The activity test of these dispersions showed that no activity was found below 0.066 μl chymosin per gram dispersion of the retentate gel of pH 6, while for the gel of pH 5.2 a non-active amount of 0.052 μl chymosin per

gram dispersion was derived (figure 4.4). Except for the slightly smaller amount of chymosin not exhibiting activity in the retentate dispersion at the lower pH, the specific activity seemed a little larger (the slope of the plot was slightly higher). This can most probably be ascribed to the slightly lower pH during incubation, which results in an increased activity during the assay. Different slopes, however, were not observed for the skim milk gels of different pH.

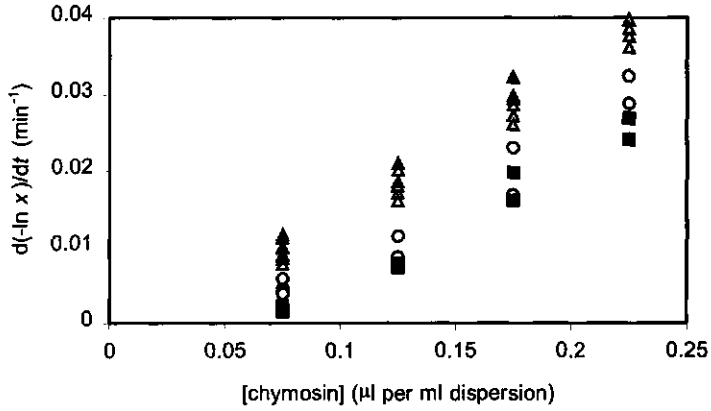

Figure 4.4 Chymosin activity in skim milk gels of pH 5.2 (Δ) and pH 6.7 (▲) and retentate gels of pH 5.2 (O) and pH 6.0 (■), with chymosin added to samples of these dispersions. Initial chymosin dosage was 0.3 ml chymosin per kg skim milk or retentate (i.e. 0.075 µl chymosin per ml dispersion).
The pH values of the dispersions were: 5.2 (Δ), 6.3 (▲), 5.8 (O), 6.2 (■).
During the assay the pH values were: 6.4 (Δ), 6.6 (▲), 6.5 (O), 6.6 (■).

These non-active amounts were not detected in systems of chymosin in JK-buffer. Here a linear relationship was found between the chymosin concentration and the release of cmp, represented as d(-ln *x*)/d*t* (see figure 4.1). This shows that components originating from the dispersion should be held responsible for the partial inactivation of chymosin. This is confirmed by the higher activity loss at a higher concentration of components (i.e. in retentates) in the activity test mixture, as shown in figure 4.4. Other results in our studies, as well as results reported in the literature, show that components from the whey protein fraction are responsible for the lower activity of chymosin (see also Chapter 3). Lelievre et al. (1990) reported the inhibition of chymosin by whey proteins both in the milk coagulation stage and in the degradation of casein.

Chymosin activity in syneresed curd from skim milk and retentates

Several batches of curd were prepared from skim milk gels of pH 6.7. The initial chymosin dosage to the skim milk was 0.03%. Syneresis of the freshly cut curd was enhanced by stirring. For 5 batches, the loss of whey varied from 69 to 75 gram per 100 gram gel (after stirring 75 to 90 minutes).

The curds from skim milk gave homogeneous dispersions by diluting them 4 times with JK-buffer of pH 5.2, addition of NaCl (1%) and stirring at 4°C. The pH of the dispersions was in all cases approximately 6.4. Plotting d(-ln *x*)/d*t* (derived from 2 different incubation times) versus the added amounts of chymosin (0 to 0.01 μl chymosin added per ml dispersion), as shown in figure 4.2, gave linear relations with regression coefficients close to 1. The derived chymosin activities for these dispersions are given in figure 4.5.

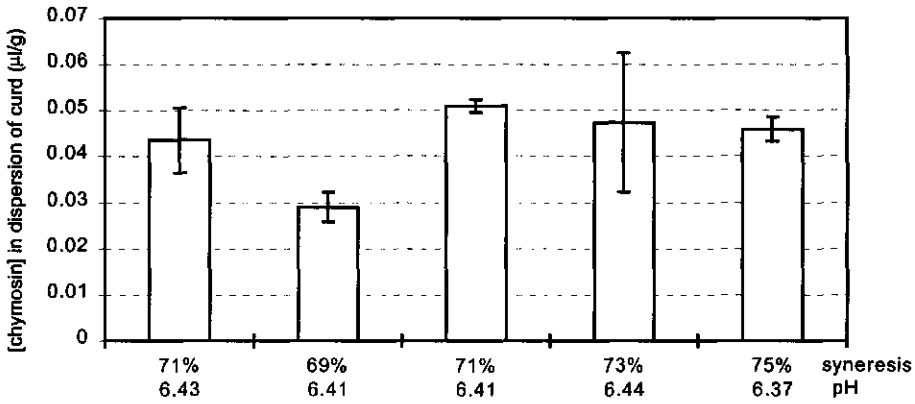

Figure 4.5 Chymosin activity in dispersions of curd (dilution factor 4) from skim milk. Degrees of syneresis: 69 to 75%, pH of dispersions: 6.37-6.43, pH during the assay: 6.6-6.7

The average chymosin activity in the dispersions was 0.043 μl per gram dispersion, the activity for the curds was thus 0.172 μl (± 0.022 μl) chymosin per gram syneresed curd. From this chymosin activity, the percentage of chymosin added to the milk that is enclosed in the curd can be calculated, taking the concentration of the gel, due to syneresis, into account. Apart from the loss of chymosin with the whey, however, the chymosin activity in the syneresed curd may be further lowered due to interference of components in the dispersion with enclosed

chymosin. The assay applied does not allow to make a distinction between a decrease in chymosin activity due to the loss of chymosin with the whey or due to inhibition of enclosed chymosin. From activity measurements in milk and retentate gels, it was suggested that the whey proteins in the dispersion will be responsible for the inhibition. During syneresis of curd from skim milk these whey proteins are largely lost with the whey (see Chapter 2). Therefore, in skim milk curd that is syneresed to a large extent, the enclosed chymosin will then not be inhibited any more by these whey proteins. Together with the whey proteins, however, also solubilized chymosin, that is not adsorbed onto casein, is then largely lost. Therefore, in this case of strongly syneresed curd from skim milk, the recovery of chymosin is derived from the chymosin dosage to the milk (0.03%), the average chymosin activity in the curd (0.172 μl per gram curd) and from the average degree of concentration of the curd (72 gram whey lost from 100 gram gelled milk), without correcting for the possible inhibition of enclosed chymosin by whey proteins. The recovered amount of active chymosin for these curds will then be 16% (on average).

In the case of curd from retentate, with significant amounts of whey proteins enclosed, this simple derivation of the recovery may not be allowable, due to the possible inhibition of chymosin. Then a distinction should be made between total amount of chymosin enclosed in curd and the specific activity under the prevailing conditions (with whey proteins present). In Chapter 5 such a derivation is given for UF-cheese, in which whey proteins are enclosed.

Results of the chymosin activity determination in curds from retentates were far worse. This can be partly ascribed to the homogeneity of the dispersion, that was sometimes fairly poor, especially at an increase of its temperature. Reproducibility was poor, and duplicates of blanks and of dispersions without additions, were often not acceptable. In many cases, the amount of cmp that was produced by incubation of the dispersion to which no extra chymosin was added appeared to be negligible (there was no difference between the incubated dispersion and its blanks). This absence of chymosin activity may either be due to complete loss of chymosin with the whey, or to inhibition of chymosin included in the curd by components present in curd (which also inhibited chymosin activity in dispersions of the gels). Moreover, for most curds the relation between the activity and the amount of chymosin added was not linear. This may be expected if the total concentration of chymosin (including chymosin added later) in dispersed samples with added chymosin is still lower than the amount that would be inactive under the test conditions. In these cases it was not possible to derive the chymosin activity in the dispersion.

Chymosin enclosure and chymosin activity in curd

In evaluating chymosin activities and chymosin retention in curd, as well as in explaining differences between curds, several mechanisms of chymosin enclosure in curd should be considered. In this respect also the inhibition of enclosed chymosin should be taken into account.

Chymosin may be enclosed in the curd by adsorption onto paracasein. The pH at drainage of curd largely determines the adsorbed amount of chymosin. Adsorption is stronger at lower pH (Stadhouders and Hup, 1975; Creamer et al., 1985; Qvist et al., 1987). The pH was shown to be a strong tool for regulation of the inclusion of chymosin in curd (see also Chapter 5).

In addition, chymosin, solubilized in the moisture of the curd, will be enclosed, in as far as it is not expelled from the curd during syneresis. This solubilized chymosin can be considered as being a whey protein, which is expelled at ongoing syneresis. The loss of whey proteins during syneresis is enhanced due to steric exclusion of whey proteins by casein, resulting in a higher whey protein content in the expelled whey (see Chapter 2). Because of these whey protein losses, the enclosure of solubilized chymosin, may be negligible. The total losses with the whey during syneresis will depend largely on such factors as the degree of concentration of the milk prior to curd preparation, the degree of syneresis and other manufacturing conditions. These factors are extensively discussed in Chapter 2.

For a good comparison of results from chymosin activity measurements, more information on the quantitative effect of the individual mechanisms would be needed. The relative importance of the mechanisms is largely determined by processing parameters during curd manufacture. The loss or inhibition of chymosin can be counteracted by an increased enclosure at optimal conditions for the adsorption of chymosin onto paracasein.

In comparing curd from retentate with curd from skim milk, the loss of chymosin per gram of whey expelled is higher at higher concentration factors of the milk, because of the higher whey protein to water ratio in curd from retentate and the further increased ratio in the expelled whey (due to steric exclusion of whey proteins). The degree of syneresis needed to reach a certain protein content in the curd, is lower for curd from retentate than for curd from skim milk. Therefore, at similar total protein contents, the whey protein content was higher in curd from retentate than in curd from skim milk, where practically no whey proteins were present (see Chapter 2). Chymosin dosage, based on ml per kg (concentrated) milk, was similar for skim milk and retentate. The fact that absence of activity is not

observed in the experiments on skim milk curd implies that the very low activity in curd from retentate must be ascribed to one or more whey proteins that most probably inhibit chymosin activity in curd from retentate. These results confirm that the chymosin activity in such curds depends on a balance between enclosed chymosin and components that inhibit its activity. Due to this balance, the chymosin activity may be very small in dispersions with considerable amounts of whey proteins.

Usefulness of the chymosin activity assay

The method of incubating a dispersed cheese sample with a substrate involves the introduction of other cheese components, besides chymosin, in the assay mixture. The presence of other cheese components in the assay mixture further justifies the interpretation that the results from the assay with dispersed cheese are a measure of the activity of chymosin in cheese. The observed inhibition of chymosin in the dispersion, probably caused by some particular whey proteins, can be extrapolated to the situation in a cheese that contains those whey proteins. Also in cheese, the enclosed chymosin will then be inhibited in activity. This will result, for instance, in a lowered degree of degradation of α_{s1}-casein during ripening. This correspondence between the direct chymosin activity assay and the derived chymosin activity in cheese during ripening is reflected in the observed inhibition in UF-retentate gels and the high-moisture UF-cheese, as prepared in Chapter 3, which was of the same order.

Extrapolation of the results of an assay to the situation as in the tested cheese may not be allowable if it concerns a method that determines the chymosin activity in an extract of cheese. Many cheese components then are excluded, whereas they may possibly influence chymosin activity in the cheese. In this respect also the activity of chymosin that is adsorbed onto paracasein must be considered. In the dispersing step of our method, the conditions are optimal for adsorption (de Roos et al., 1995). But, as a sample of the dispersion comprises all components of the cheese (except fat), also chymosin that is adsorbed onto paracasein can exhibit activity in the assay, especially under conditions as during the assay (in solution: good accessibility of the substrate).

Due to the dependence of the result of a chymosin activity assay on the composition of the gel, the curd or the cheese, a recovery percentage for chymosin activity, as obtained with this assay, cannot be given. This precludes the comparison of this assay with other methods. A mutual comparison of samples, all analysed with

this assay, is very well possible. The method can in principle be applied for many cheese varieties. In this study it is applied for traditionally manufactured cheese and cheese from ultrafiltered milk (see Chapter 5).

With the method described in this study, detection of very small activities posed problems, especially when the dispersions were not quite homogeneous, which resulted in poor reproducibility. The chymosin activity could then hardly be determined, because of the very small differences between the blanks and the incubated samples in which cmp was released. The determination of small activities may be more accurate if the reproducibility were better. This may be achieved with better homogenized dispersions, especially those of concentrated samples, such as retentates and curd. In some preliminary experiments, stronger dilution with JK-buffer appeared to improve homogeneity. Also maintaining the pH of a dispersion at 5.2 resulted in a better homogeneity of the samples. In the course of the work (Chapter 5), the pH was controlled at 5.2. Problems with the detection of very small activities may be solved by extending incubation times. These considerations indicate how an improved accuracy may be obtained with this method of determination of chymosin activity.

It can be concluded that a better understanding has been gained on the interference of components in cheese, in all probability certain whey proteins, with chymosin. The method developed can also be applied to cheese, for instance to study to what extent hydrolysis of caseins into large peptides by enclosed chymosin is hindered in the presence of whey proteins. This is discussed in Chapter 5.

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Chapter 5

CHYMOSIN ACTIVITY IN STANDARD AND UF-CHEESES

ABSTRACT

In this study Gouda type cheeses were manufactured from milk according to the traditional method, as well as from milk that was preconcentrated (5x) by ultrafiltration. For the manufacture of UF-cheese from concentrated retentate, a specially designed coagulator/curd preparator (Alcurd) was used. In several trials the dosages of chymosin and starter culture to milk or retentate were varied. In UF-cheese approximately one third of the whey proteins in the retentate was enclosed, whereas the retention of whey proteins in the standard cheese was negligible. The enclosure of the chymosin, added to milk or retentate, was determined by measurement of the chymosin activity in the cheese (after 1 day and after 2 weeks). The chymosin activity in the cheeses remained unchanged in the first two weeks of ripening. The inclusion of chymosin in cheese is to a high degree (90-100%) determined by the adsorption of chymosin onto casein, which depends largely on the pH in the initial steps of cheese manufacture. In standard cheese manufacture, an increased rate of acidification, due to a higher dosage of starter culture, resulted in an increased enclosure of chymosin. In the UF-cheesemaking process variation in the dosage of starter culture hardly lead to differences in the retention of chymosin in the cheese, as differences in pH until curd collection were small, due to the slower acidification in retentates and the shorter time until curd collection. The chymosin activity in the final cheese can be increased by adding more chymosin to the milk or retentate, as the relative enclosure in cheese was independent of the chymosin dosage. The chymosin activity was related to the rate of breakdown of caseins during the first 2 weeks of ripening. A good correlation between the chymosin activity and casein degradation was found for both standard cheeses and UF-cheeses. Similar chymosin activities in standard cheeses and UF-cheeses also resulted in a corresponding degree of proteolysis (after 2 weeks). This confirms the usefulness of chymosin activity determination for the prediction of proteolytic processes during ripening of the cheese, independent of the whey protein content of the cheese.

INTRODUCTION

In the manufacture of semi-hard cheese by ultrafiltration techniques (UF-cheese) many processing aspects are changed as compared to the conventional cheesemaking process. The preconcentration of the milk by ultrafiltration requires adjustment of the amounts of coagulant and starter bacteria added to the retentate. All adjustments in quantities of these ingredients needed for the manufacture of UF-cheese will, together with changes due to the use of UF-concentrated cheesemilk, cause further changes in the process, e.g. in evolution of pH and in coagulation. These changes can greatly affect the composition and the ripening of the resulting cheese. It is therefore very important to realise the consequences of the changes applied to the cheesemaking process when making UF-cheese.

Addition of starter culture to cheese milk or retentate leads to a lowering of pH. The rate of pH decrease depends not only on the applied initial starter culture concentration and on the ability of the starter bacteria to multiply and to produce lactic acid from lactose, but also on the buffering capacity. The latter is greater in milk concentrated further, primarily due to the increased contents of protein and calcium phosphate. In turn, the pH during the initial steps of the cheesemaking proces is determinant for the content of these minerals in cheese. A slower pH decline in retentates leads to more calcium phosphate in the UF-cheeses, which results in a higher final pH of the cheese (Sutherland and Jameson, 1981; Creamer et al., 1987; Green et al., 1981). The development of the pH can be influenced by preacidification of the milk before ultrafiltration or acidification of the retentate, which lowers the buffering capacity (Creamer et al., 1985; Qvist et al., 1987; Spangler et al., 1991).

Rennet is added to the milk or retentate to form a coagulum. The rennet enzymes are partly enclosed in the cheese. The inclusion of chymosin in cheese strongly depends on the evolution of the pH during curd manufacture. At lower pH, the adsorption of chymosin onto paracasein, and thereby its inclusion in cheese, is increased (Stadhouders and Hup, 1975). The amount of chymosin enclosed is also related to the quantity added to cheese milk (Dulley, 1974; Visser, 1977a; Creamer et al., 1987). The proportion of the added chymosin that will be lost with the whey will not only depend on the pH, but also on the amount of whey that still has to be drained from the gelled milk or retentate to obtain the desired moisture content in the cheese.

During cheese ripening chymosin is mainly responsible for the breakdown of α_{s1}-casein. The rate of hydrolysis of α_{s1}-casein is directly related to the amount of

chymosin in the cheese (Visser, 1977c). At least as important for proteolysis is its specific activity in the cheese during ripening. The proteolytic activity of chymosin may not only be influenced by the external conditions during ripening, but also by the cheese composition. Especially the presence of whey proteins is reported to affect chymosin activity (de Koning et al., 1981; Creamer et al., 1987; Harper et al., 1989). This could be confirmed in the ripening studies, described in Chapter 3. An inhibitory effect of whey proteins on the activity of chymosin was also found during application of a method in which the chymosin activity in chymosin-induced milk and retentate gels was estimated (see Chapter 4). In the protein fraction of cheese manufactured from ultrafiltered milk the caseins are partly replaced by whey proteins, whereas the amount of whey proteins in traditionally manufactured cheese is negligible. The enclosed whey proteins have a considerable effect on the quality of the ripened UF-cheeses, partly due to inhibition of chymosin. Besides, due to the presence of whey proteins the caseins are diluted, which will also decrease the concentration of breakdown products of caseins (de Koning et al., 1981; Creamer et al., 1987). Several studies have shown that native whey proteins are resistant to hydrolysis by rennet enzymes (O'Keeffe et al., 1978; de Koning et al., 1981; Qvist et al., 1987; Furtado and Partridge, 1988; Harper et al., 1989).

In this study the effect of variations in amounts of ingredients in the cheesemaking process was both studied in standard, conventionally prepared cheese (from milk) and in UF-cheese (from ultrafiltered milk). This would not only broaden knowledge of the traditional cheesemaking process, it may also enable to distinguish between changes caused by the changed dosage of an ingredient (chymosin and/or starter culture) and changes that must be attributed to the use of preconcentrated milk in the UF-cheesemaking process. It was tried to make standard cheeses and UF-cheeses of about the same pH, dry matter, fat, salt and total protein contents (Gouda type cheeses: with at most 42% water and at least 48% fat in dry matter). In this study semi-hard UF-cheese was prepared from pasteurized milk that was 5 times concentrated by ultrafiltration. Proteolysis during the first two weeks of ripening was related to the protein composition and the chymosin activity in the cheese. The inclusion of active chymosin was, in turn, related to the applied concentrations of chymosin and starter culture. The effect of preconcentration of the cheesemilk by ultrafiltration was studied by comparison of the observed relations for the standard, traditionally manufactured cheese and the UF-cheese.

MATERIALS AND METHODS

Ingredients

-Cheese milk: thermalized and bactofuged bulk tank milk was standardized to a fat/protein ratio of 1, as is used for the manufacture of Gouda-type full-fat cheese, and pasteurized at 74°C for 10 seconds.
-Chymosin: Maxiren 15 (Gist-brocades), with a strength of 10800 Soxhlet Units.
-Starter culture: SK110/C17-concentrate, containing $2.5x10^{10}$ CFU/ml (NIZO).
-$CaCl_2$ solution (33% w/w)
-$NaNO_3$ solution (35% w/w)

Traditionally manufactured cheese (standard cheese)

After pasteurization the milk was cooled to 30.5°C, the renneting temperature. Then $NaNO_3$ solution (37.5 g/100 l milk), $CaCl_2$, starter concentrate (0.025, 0.1 or 0.4%) and chymosin (0.0168 or 0.021%) were added. Several combinations of dosages of starter and chymosin were chosen. The dosage of $CaCl_2$ varied from 30 to 50 g solution per 100 l milk, depending on the amount of chymosin used, in order to standardize the clotting time. In table 5.1 the codes used for the standard cheeses with the applied percentages of starter culture and chymosin are given. Variant S1 was manufactured 4 times (coded a/b/c/d), while variant S4 was prepared twice (a/b).

Table 5.1 *Standard cheese manufacture: dosages of starter concentrate and chymosin* to milk (w/w)

cheese code	S1 a/b/c/d	S2	S3	S4 a/b
starter (%)	0.025	0.1	0.4	0.4
chymosin (%)	0.021	0.021	0.0168	0.021

After renneting the gel was cut and stirred with the released whey for 20 minutes. Then part of the whey (40% of the original milk volume) was removed and water was added (35% of the volume of the remaining curd/whey mixture). This mixture, in which the temperature was increased to 35°C, was stirred for 20 more minutes. The curd was collected (approximately 4 kg per mould) and 45 minutes later the curd

was pressed for 90 minutes. The cheeses were salted in a brine bath for 20 hours after pH 5.5 was reached.

Cheese manufactured from ultrafiltered milk (UF-cheese)

Standardized and pasteurized milk was ultrafiltered at 50°C using a three-stage UF system with spiral-wound Abcor S4-HFK 131 modules (Koch). It was tried to achieve a concentration factor of 5 (based on protein content). In the final stage the retentate was diafiltered. In a preliminary experiment retentate was diafiltered by 100%, i.e. an equal amount of water was added to the retentate (retentate P), while retentates A and B (from different days) were diafiltered by 50%. Retentates were cooled to 33°C prior to the addition of ingredients: $NaNO_3$ (17 g solution per 100 kg retentate), $CaCl_2$ (0 to 42 g solution per 100 kg retentate), starter culture (0.125, 0.5 or 2%) and chymosin (21, 33 or 45 g per 100 kg retentate). The applied amounts of starter culture and chymosin, in combination with the used cheese codes, are given in table 5.2.

Table 5.2 UF-cheese manufacture: dosage of starter concentrate and chymosin to retentate (w/w).
Cheeses pU 1/2/3 were made from retentate P; a and b are duplicates. Cheeses U1-U5 A/B were manufactured in duplicate on different days from retentates A and B, respectively.

cheese code	pU 1 a/b	pU 2	pU 3	U1 A/B	U2 A/B	U3 A/B	U4 A/B	U5 A/B
starter (%)	0.125	0.125	0.125	0.125	0.5	0.5	0.5	2
chymosin (%)	0.021	0.045	0.07	0.033	0.021	0.033	0.045	0.021

Cheese from ultrafiltered milk was prepared by the use of an Alcurd Continuous Cheese Coagulator (Alfa Laval). Retentate, mixed with additions was pumped into a pipe (internal diameter 4 cm) that was placed in a waterbath thermostatted at 33°C. The firmness of the retentate was monitored during renneting with a Gelograph in a control sample (van Hooydonk and van den Berg, 1988). At a certain firmness, the gel in the pipe was displaced with water. When leaving the pipe, the gel passes through a grid and is then cut in particles by a rotating knife.

This way small particles were formed (retentates P and A: particles of approximately 1 cm^3, retentate B: approximately 0.65 cm^3). These were collected in the same cheese moulds as were used for the standard cheeses. After draining for 30 minutes the curd blocks were pressed for 90 minutes. After reaching pH 5.5 the cheeses were brined for 20 hours.

Storage of cheese

Cheeses were stored at 13°C and 88% relative humidity. After two weeks the cheeses were wrapped in foil.

Standard analyses

During manufacture of the cheeses the pH was measured frequently. The pH of the cheeses was also determined after 1 day and after 2 weeks. The composition (fat, protein, lactose, dry matter content) of the milk was determined by the use of a Milkoscan (Foss Electric), while the composition of the retentate and the cheese was determined using Netherlands standard methods (NEN).

Chymosin activity in cheese

Chymosin activity was determined in cheeses just after pressing, before brining (approximately 8 hours after the beginning of cheesemaking) and/or in cheeses of 2 weeks old. Samples were diluted 10 times with JK-buffer, containing NaCl and the pH was adjusted to 5.2, as described earlier (Chapter 4 of this thesis). The NaCl-content was adjusted to 1% in the dispersion, taking the NaCl-content of the cheese sample into account. After stirring overnight at low temperature, the fat could readily be removed from the homogenized dispersion. Chymosin activity in the dispersion was derived by incubating samples of the dispersion with κ-casein (3 blanks, 2 samples for 30 minutes, 2 samples for 60 minutes), as described in Chapter 4. From most cheeses 2 samples were homogenized and analysed.

The activity of the pure chymosin was determined in JK-buffer (pH 5.2) with 1% NaCl. A sequence with concentrations varying from 0 to 0.2 μl chymosin per ml buffer was prepared and chymosin activities were estimated, according to the same method. With this calibration the amount of active chymosin in the cheese samples was derived from the chymosin activity as determined in the dispersions of the cheeses.

Proteolysis

The proteolysis in the cheeses was monitored by determination of the change in the ratio of soluble nitrogen (SN) to total nitrogen (TN) during the ripening period. This ratio was determined after 1 day and after 2 weeks. The total nitrogen content of cheese was determined according to IDF method 20B (1993). The determination of the soluble nitrogen content in cheese was based on the method described by Noomen (1977). SN was mostly expressed as a percentage of TN, or otherwise in relation to the casein-nitrogen content. The whey protein content of cheeses was derived from the SN/TN-ratio at day 1.

RESULTS AND DISCUSSION

Composition of the milk and retentate

The pH and fat, protein, lactose and dry matter contents for milk and different retentate batches are given in table 5.3.

Table 5.3 Composition of standardized milk and retentates as used for manufacture of standard and UF-cheeses. The concentration factor (CF) of protein in retentates is given between brackets.

	fat	total protein (CF)	lactose	dry matter	pH
milk	3.44	3.43 (1)	4.57	12.13	6.67
retentate P	17.30	16.02 (4.67)	1.42	36.10	6.75
retentate A	17.95	17.35 (5.06)	1.84	39.48	6.70
retentate B	17.80	16.89 (4.92)	1.89	38.77	6.72

The water content of the retentates (≈ 60%) is similar to the water content of a curd block after curd collection in the conventional cheese manufacturing process (Straatsma and Heijnekamp, 1988). Traditionally prepared curd from unconcentrated milk and freshly-cut curd from UF-retentate gels are thus collected in the cheese moulds at approximately the same (average) water content.

Development of pH

The initial pH of the milk or retentate was equal for all cheeses (approximately 6.7); no preacidification was applied. The pH values during the day of preparation are given in figures 5.1A and 5.1B.

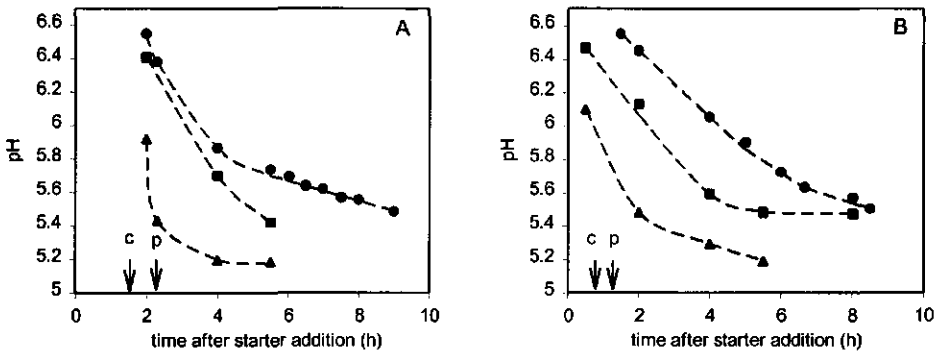

Figure 5.1 Evolution of the pH during cheesemaking, for various dosages of starter concentrate. Results from cheeses with corresponding dosages of starter culture are averaged.
c = curd collection, p = start pressing
A: standard cheeses: ● 0.025% (S1) ■ 0.1% (S2) ▲ 0.4% (S3,S4)
B: UF-cheeses: ● 0.125% (U1) ■ 0.5% (U2,U3,U4) ▲ 2% (U5)

After 1 day, the pH of traditionally manufactured cheeses was approximately 5.3 for the cheeses with 0.025% and 0.1% starter culture added, while a pH of 5.13 was reached for the cheeses with the higher dosage (0.4%). At higher amounts of starter the pH decreased faster. A lower pH during manufacture will enhance solubilization of micellar calcium phospate, which then is lost with the whey. A decreased calcium phospate content in the cheese, which implies a reduced buffering capacity, results in a lower pH of the cheese. This also means that the calcium phosphate content of cheese gives information about the rate of acidification during manufacture (Lawrence et al., 1984). Reduction of the buffering capacity of the cheese can also be achieved by lengthening the time between starter addition and whey drainage, in order to have a lower pH at the stage of whey removal.

Also during manufacture of UF-cheeses, the rate of pH decrease depended on the amount of starter added to the retentate and on starter growth. The quantity

of starter added to the 5x concentrated UF-retentate was increased by 5 times (compared to standard cheese), in order to achieve a comparable rate of acidification and a sufficient number of starter bacteria. The time between addition of starter and cheese pressing is approximately 1 hour shorter in UF-cheese manufacture than in the standard cheesemaking process. At low starter dosage the pH decrease until pressing of the UF-cheese (75 minutes after addition of ingredients) was almost negligible. Since the pH must have decreased enough (till 5.5) before brining can start, the cheesemaking process would take longer if pH decrease is slow. Only the UF-cheeses with the high starter dosage (2%) had reached pH 5.2 (as measured after 1 day). The pH of the other UF-cheeses was stabilized at values between 5.3 and 5.4 (as measured after 1 day). In the cheeses manufactured from retentate P, in which the lactose content was decreased more when diafiltered, the pH did not decrease below 5.45. Because of the relatively high final pH of these cheeses, the later prepared retentates A and B were diafiltered to a lesser degree. More lactose was then maintained in the retentate (1.8% against 1.4%). This lactose concentration, in combination with the applied percentages of starter culture, was still not sufficient to reach a pH of 5.2. Sutherland and Jameson (1981) clearly demonstrated that the final cheese pH is strongly related to both the retentate lactose content and the pH at ultrafiltration. A higher final cheese pH at higher diafiltration levels (lower lactose content) was reported by Spangler et al. (1991) as well. They also mentioned a significantly higher pH in UF-cheeses with 1% starter than in those with 3% starter.

The stronger buffering was most likely also the cause of the slower pH-decrease and the higher final pH. A slower decrease of pH in retentates and a higher pH in UF-cheeses than in conventional cheeses was also reported by Green et al. (1981), who attributed this to the higher buffer capacity as well. The slow decrease of the pH and high final cheese pH may be partly solved by preacidification of the cheese milk, as applied by Qvist et al. (1987) and Spangler et al. (1991). At lower pH, calcium phosphate will be solubilized and it will subsequently, during ultrafiltration, be removed with the permeate. This results in a reduced buffer capacity of the retentate and a lower cheese pH can be reached. The retention of calcium was strongly influenced by the pH at ultrafiltration. According to Qvist et al. (1987) the retention of calcium in milk that had been preacidified to pH 6.3 was 62-72%, versus 77-84% in milk that had not been acidified. These percentages are in good agreement with data for calcium from Sutherland and Jameson (1981), who reported that retention of phosphate was also influenced,

although to a lesser extent, by the pH at ultrafiltration. They also found that calcium and phosphate levels in cheese were highly correlated with the levels in retentate, which emphasizes the possibility to control the mineral content in cheese by adjustment of the milk pH. Creamer et al. (1987) determined the ratios of calcium to solids-not-fat-not-salt and found these to be higher in UF-cheeses than in control cheeses (no preacidification was applied). In another study Creamer et al. (1985) showed that calcium and phosphate concentrations in cheese decreased steadily with increasing acidification. According to Sutherland and Jameson (1981), buffering was mainly accounted for by protein and phosphate. Since it is mostly tried to keep the total protein content in UF-cheese at the same level as in conventional cheese, the increased buffering must be due to the increased phosphate content in UF-cheese, assuming equal buffering capacities per unit mass of protein for caseins and whey proteins. Everett and Jameson (1993) suggested later that the increased buffering might be caused by a difference in buffering capacity between casein and whey proteins.

Jameson (1987) considered the high buffering capacity of retentate an advantage for the ongoing starter growth, not hindered by acid-induced cell damage, so that sufficient numbers of starter bacteria in the final cheese can be obtained. The number of starter bacteria is not only important to achieve conversion of lactose in lactic acid. During cheese ripening the starter bacteria and their enzymes are essential for the conversion of peptides (from caseins) to amino acids and flavour compounds. In both standard and UF-cheeses the lactic acid bacteria grew out to equal cfu per gram cheese (on average $5x10^8$). Concerning the growth of lactic acid bacteria in ultrafiltered milk and the rate of acidification, however, rather variable results were obtained by Meijer et al. (1995) and the average effect of ultrafiltration was small.

Composition of the cheeses

The composition (water, fat, total protein and salt content) of the cheeses after 1 day and/or 2 weeks is given in table 5.4.

Table 5.4 Average composition (with standard deviations between brackets) of standard cheeses (S1-S4) and UF-cheeses (U1-U5 A/B) after 1 day and/or 2 weeks (in %)

	water	water	protein	protein	fat	salt
	1day	2 weeks	1day	2 weeks	2 weeks	2 weeks
standard cheeses	44.2 (1.24)	40.3 (1.00)	23.2 (0.59)	24.6 (0.65)	29.9 (0.63)	1.5 (0.11)
UF-cheeses A	50.8 (0.38)	45.4 (0.23)	20.4 (0.19)	22.5 (0.13)	26.5 (0.22)	2.1 (0.13)
UF-cheeses B	49.3 (0.67)	43.8 (0.37)	21.1 (0.3)	23.3 (0.37)	27.4 (0.17)	2.0 (0.08)

The water content of the standard cheeses with the high starter dosage (S3 and S4) was considerably lower than the other standard cheeses (after 1 day on average 45.3% in S1 and S2, against 43% in S3 and S4). The faster acidification did enhance syneresis, resulting in lower water contents in the fresh cheeses. The difference had decreased to approximately 1.4% after 2 weeks.

The higher water content of UF-cheeses must be attributed to the slow syneresis of the curd from retentate, which is due to the changed casein matrix and the relatively high pH, as compared to conventional curd. In the manufacture of UF-cheese B the curd particles were initially about 35% smaller (in volume). This resulted in a somewhat lower water content than in UF-cheese A.

During ripening (before the cheeses were coated in foil after 2 weeks) the loss of water was somewhat stronger in UF-cheeses than in standard cheeses. This may possibly be explained by the curd particles not being well fused in the UF-cheeses. The curd particles from retentate were fairly uniform in size and shape, in contrast to curd prepared from unconcentrated milk. The particles from retentate were also firmer. For these reasons the particles did not readily fuse. After pressing the rind was not fully closed and curd grains were easily distinguished). This would promote transfer of salt into the UF-cheeses during brining, which is reflected in the higher salt-to-water content in UF-cheeses. Moreover, the higher water content of UF-cheeses would also promote the transfer of salt (Geurts et al., 1974).

Cheese yield

The cheese yield can, in principle, be calculated from the fat content in the milk and in the cheese and the relative transfer of milk fat into the cheese. In standard cheese manufacture the transfer of milk fat into the cheese is approximately 93%. For UF-cheese manufacture various retention percentages are reported. Considerable fat losses are often reported, although it depends largely on the applied chymosin concentrations, the moment of curd cutting and the used equipment. According to Qvist et al. (1987), however, the recovery of fat in UF-cheeses, made by the use of the same equipment as in our study (Alcurd), is 98 to 99%. During UF-cheese manufacture in our study, however, a milky solution was lost from the cheese moulds shortly after curd cutting, indicating a considerable loss of fat. Therefore, yield calculations for our UF-cheeses are also based on a 93% transfer of fat, as in standard cheesemaking.

The yield can be calculated for cheeses of 1 day as well as for cheeses of 2 weeks, by correcting for the loss of weight during these 2 weeks. The percentage shrinkage was derived from the total nitrogen content of the cheese, determined both after 1 day and after 2 weeks. For standard cheeses the shrinkage percentage was on average 6%, for UF-cheeses the loss of weight was approximately 10%. The higher percentage for UF-cheeses was also reflected in the stronger decrease of the water content in the first two weeks (see table 5.4).

The calculated yields, in grams of cheese per 100 grams of milk (for standard cheese) or per 100 grams of retentate (for UF-cheese) are given in table 5.5. These yields are not corrected to a standardized cheese composition, with, for instance, equal water contents or fat/protein ratios in standard and UF-cheeses. It concerns the yields of the cheeses of the composition given in table 5.4. Calculations based on a higher transfer of fat from retentate into UF-cheese would lead to a higher estimated yield for the UF-cheeses.

Table 5.5 Cheese yield (expressed in gram per 100 gram milk or retentate, respectively) for standard and UF-cheese, based on cheese composition after 1 day and after 2 weeks, as given in table 5.4

	yield 1 day	yield 2 weeks
standard cheeses	11.4	10.7
UF-cheeses A	69.3	63.0
UF-cheeses B	66.7	60.4

The somewhat lower yield of UF-cheeses B, compared to A, is related to the lower water content in these cheeses.

As mentioned before, it was observed that during the manufacture of UF-cheese, after cutting the retentate gels, a milky liquid flowed out of the cheese moulds. Excessive losses during cutting have also been reported earlier. Sutherland and Jameson (1981) found that 54% of the protein in the expelled whey was whey protein, the rest was casein. Besides, cutting of fragile curd might give a large amount of curd fines, which are readily lost during curd collection. Adequate equipment can diminish curd fines. The losses of fat and casein may be partly caused by low chymosin concentration combined with high casein concentration (Dalgleish, 1981). As the protein concentration increases, the degree of splitting of κ-casein at gelation decreases (Garnot, 1988). At cutting and during syneresis some of the casein micelles that are not incorporated in the gelled casein network may be expelled from the curd. Higher amounts of chymosin added to retentate lead to faster gelation (at constant temperature). Although the enzymic reaction is somewhat slower in retentate, the caseino-macropeptide-fraction (cmp) is split off faster at increased chymosin concentrations. Because gelation occurs at a smaller degree of κ-casein hydrolysis for a higher protein content, the firming will be much faster in retentate (van Hooydonk and van den Berg, 1988; Garnot, 1988). Cutting the retentate gel at a certain firmness (as done by Green et al., 1981), i.e. after a short renneting time, would then result in higher losses of casein micelles that are not retained in the paracasein network. At high chymosin dosage, gelation can be slowed down by lowering the renneting temperature. At lower temperature, however, the viscosity of the retentate is higher, and this may give problems in the uniform mixing of ingredients. Losses of casein at low chymosin concentrations can be limited by lengthening the time before cutting the curd (Dalgleish, 1981). The final degree of κ-casein hydrolysis (after more than 90 minutes) is 100%; it is indepen-

dent of the protein content of the substrate, the enzyme concentration and the pH (Garnot and Corre, 1980).

Protein recovery

The recovery of milk protein can be derived from the protein content in the milk or retentate, the protein content in the cheese and from the cheese yield. In this study, however, protein contents were derived from total nitrogen content. Therefore, only values for total nitrogen recovery can be given. For the standard cheeses the recovery of nitrogen was on average 76.8%. The recovery for UF-cheeses A and B was 81.6% and 83.5%, respectively. These recoveries are in agreement with values given in literature. Qvist et al. (1987) reported a 84% recovery for nitrogen in UF-Havarti. Jameson (1987) reported 78-81% protein recovery in UF-Cheddar cheese (cheese prepared according to the Siro-Curd process).

These derivations show that a significant increase in total nitrogen recovery can be obtained by preconcentrating the cheesemilk by ultrafiltration. For a justified comparison of recoveries in standard and UF-cheeses, the results should be corrected to a similar water content and fat/protein ratio in both types of cheeses.

From these total nitrogen recoveries it cannot be derived whether the increased nitrogen recovery in UF-cheese, as compared to standard cheese, is totally due to whey protein, or also to a somewhat higher transfer of paracasein. Also an increased loss of paracasein and a greater enclosure of whey proteins may cause a net increase in nitrogen recovery.

The amount of whey protein in cheese can be derived from the SN/TN-ratio, as determined in one day old cheeses. The SN-fraction consists then mainly of whey proteins (including caseino-macropeptide), but also includes some peptides released from paracasein by chymosin during this first day. Distinction between whey proteins and released peptides can be made by determination of an initial SN/TN-ratio of the freshly prepared cheese. Because of ongoing changes in protein content (loss of moisture during pressing and brining of cheeses) during this first day and the heterogeneity of the cheese (which makes sampling difficult), an accurate determination of soluble nitrogen and total nitrogen is not easy in freshly prepared cheese. After 1 day a good sample can be taken from the pressed and brined cheese.

In this study, the enclosed amount of whey protein was estimated by correction of the SN/TN-ratio (determined after 1 day) for the amount of soluble nitrogen components released from paracasein (due to proteolysis) on the first day.

The amount of peptides that are released from paracasein during the first day can be derived from the increase in SN/TN in the following days. Further separation of the components of the SN-fraction, by means of precipitation of whey proteins, as was done by de Koning et al. (1981), is another method to estimate the whey protein content of cheese. For standard cheeses, SN/TN after one day varied from 0.02 to 0.04, with higher values for the variations with increased amounts of starter. De Koning et al. (1981) reported a SN/TN-ratio of 0.04 in standard cheeses (after 1 day), of which 0.03 was non-coagulable-N (peptides, released from paracasein); it was assumed that the rest (0.01) consisted of whey proteins. In this study this distinction was not made, but the SN-fraction in standard cheeses is expected to consist mainly of peptides, since the enclosure of whey protein will be negligible (see Chapter 2). The SN/TN-ratio for the UF-cheeses after 1 day was much higher, between 0.09 and 0.12. Assuming that the amount of peptides (released from paracasein) in this fraction is more or less equal to the amount in standard cheeses after 1 day (this assumption is based on the chymosin activities being similar in the standard cheeses and the UF-cheeses, see figure 5.2), these results show that a considerable amount of native whey protein (including caseino-macropeptide) was enclosed in the UF-cheeses. Approximately one third of the total amount of whey protein present in the retentate gel (≈ 22% of total protein) was thus transferred into the UF-cheese. Comparable levels were found in an earlier study, performed at the Netherlands Institute for Dairy Research (NIZO: Escher, 1988). Higher SN/TN percentages in 1 day old cheeses at increased starter culture dosage, as found for standard cheeses in this study, were also found in the NIZO-study for 1 day old UF-cheeses, when higher amounts of starter culture were applied. This higher initial SN/TN ratio can be ascribed to the stronger inclusion of rennet enzymes in the cheeses at higher percentages of starter culture, due to a lower pH at whey drainage, as is shown further on. The increased rennet retention would have enhanced proteolysis during the first day.

Chymosin activity measurements

For each cheese the average chymosin activity was determined after incubation of samples from a dispersion of the cheese with added κ-casein. Some cheese varieties were produced more than once. These batches are replicates with respect to the variables chymosin and starter dosage, but because they were not manufactured on the same day (i.e. different batches of milk or retentate), analytical results of these replicates were not averaged. Concentrations of active chymosin were

measured in fresh cheese samples (taken 8 hours after addition of ingredients to the milk or retentate) and/or in 2 weeks old cheeses.

Since initial breakdown of casein is primarily due to action of chymosin, the degree of proteolysis, measured as the increase of the soluble nitrogen content in the first two weeks of ripening, was related to the chymosin activity in cheese. Both chymosin activity and the degree of proteolysis were measured, which makes it possible to determine the relation between these parameters (see figure 5.6, further on). In figure 5.2 both the measured chymosin activities of all cheeses (standard cheeses and UF-cheeses) and the corresponding results of proteolysis in these cheeses are given. Proteolysis is presented as the increase in the amount of soluble nitrogen between day 1 and day 14, relative to the initial casein-nitrogen content (SN/caseinN). The initial casein-nitrogen content was for each cheese derived from the difference between the total nitrogen content and the soluble nitrogen content in the 1 day old cheeses.

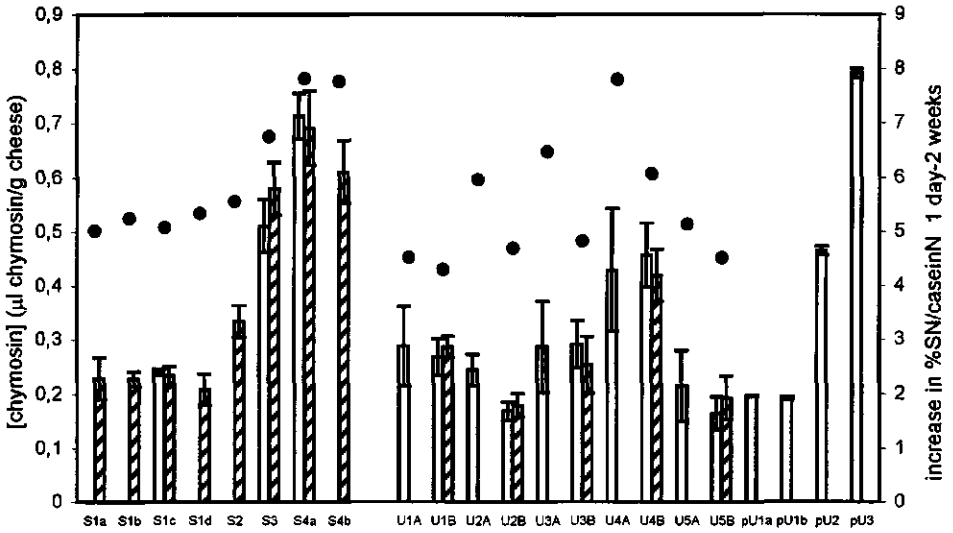

Figure 5.2 Chymosin activities (left-hand axis), measured after 8 hours (white bars) and/or 2 weeks (hatched bars) and increases in %SN/caseinN (right-hand axis: ●), for standard cheeses and UF-cheeses. For cheese codes see table 5.1 and 5.2

Chymosin activities in fresh cheeses (samples taken 8 hours after starting cheesemaking) and in those ripened for 2 weeks showed no significant differences. This indicates that the chymosin activity is constant during storage of the cheese, at

least for 2 weeks. According to Zoon et al. (1994) rennet remained also active in the next 4 weeks of ripening. Because of this, results from chymosin activity determinations in fresh and ripened cheeses are not distinguished anymore in the following (but presented as duplicate measurements, not averaged).

Standard deviations for chymosin activities determined in UF-cheeses were often fairly high, but not, for instance, for cheeses from retentate P. No explanation could be found for these somewhat inconsistent results.

The chosen chymosin additions, in combination with the acidification, resulted in comparable chymosin levels in cheeses prepared from milk and from milk retentate. The correspondence of chymosin activities justifies comparison of the cheeses with respect to other parameters, such as proteolysis, flavour development and texture.

The chymosin activity as determined in conventional cheese with standard additions (S1) corresponds on average with 250 μl chymosin per kg cheese, and is close to the activity in standard Gouda cheese (280 μl per kg cheese), as determined by Stadhouders et al. (1977) and Visser (1977a), and in standard Cheddar cheese (300 μl per kg cheese) by Hickey et al. (1983). The latter reported a rennet activity in UF-cheese of 200 μl per kg (UF-cheese was prepared according to Sutherland and Jameson, 1981: retentate CF 5, rennet dosage 20% of the amount added in conventional cheese). This result is in agreement with chymosin activities in UF-cheeses with similar chymosin dosages (0.021%) to the retentate, as prepared in our study (variants pU1a/b, U2 A/B, U5 A/B).

Chymosin activity in relation to chymosin dosage

The inclusion of chymosin depends strongly on the added amount to the milk or retentate. The results for standard cheeses and UF-cheeses are given in figures 5.3A and 5.3B.

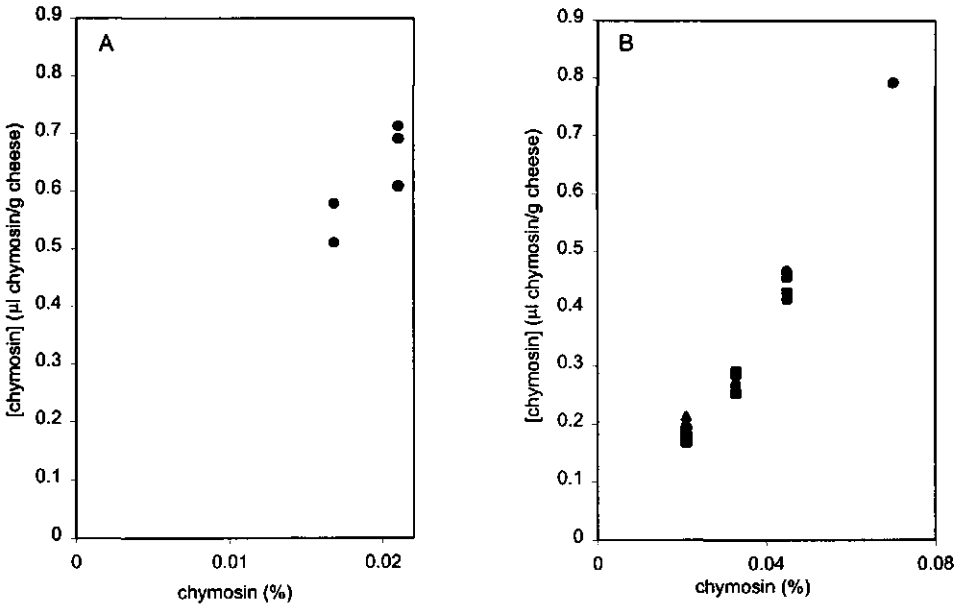

Figure 5.3 Chymosin activities in cheese as a function of the applied chymosin percentages.
A: standard cheeses, 0.4% starter culture added to milk (S3 and S4)
B: UF-cheeses, with starter added: ● 0.125% (U1, pU1, pU2, pU3)
▲ 0.5% (U2, U3, U4) ■ 2% (U5)

In standard cheeses only two cheeses, one with 0.021% chymosin (w/w) and the other with a 20% lower percentage, 0.0168% chymosin can be compared. The pH profiles for both cheeses were about the same (0.4% starter culture was added). The measured chymosin activities are about proportional to the chymosin additions.

With respect to the cheeses manufactured from retentate, four different chymosin dosages can be compared. Variation in the amount of starter culture applied, which had not yet led to significant differences in pH in the first hour of UF-cheese manufacture, had no effect on the enclosed chymosin activity. Therefore the measured chymosin activities in cheeses, with the same dosage of chymosin to the retentate, are strongly clustered in figure 5.3B. An increased amount of chymosin added to the retentate resulted in a proportionally higher enclosed activity in the cheese, or even more.

Chymosin activity in relation to the rate of acidification

The inclusion of chymosin in cheese is largely determined by the adsorption of chymosin onto paracasein. The adsorption depends strongly on the pH. The pH is therefore a major variable for chymosin inclusion. In this respect the first hour(s) of manufacture, before the cheese is pressed, are determinant. It is, however, not exactly known which moment in the curd making process determines the adsorption of chymosin. It may be anywhere between aggregation of the paracasein micelles and the stages of curd collection or pressing. During these steps in the process, the pH is continuously decreasing. For standard cheeses chymosin inclusion is related to the pH at the start of pressing, since this pH is known in most cases, but plotting versus the pH at another process step can also be done and it would likely give a comparable relation. Results for standard cheeses are shown in figure 5.4.

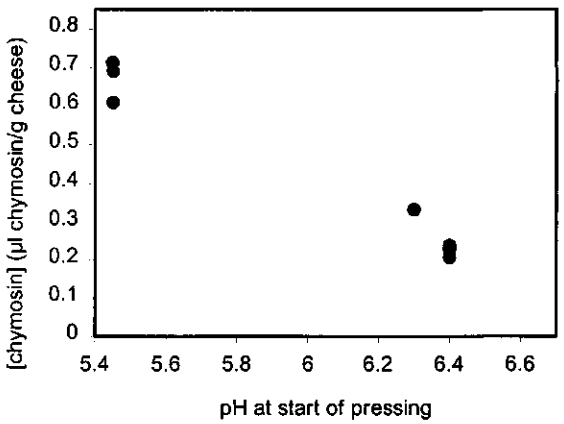

Figure 5.4 Chymosin activity in standard cheese, with 0.021% chymosin added to milk (S1, S2, S4) as a function of the pH at the start of pressing.

In the case of traditionally manufactured cheeses, the curd is collected about 90 minutes after addition of ingredients to the milk and the curd blocks are pressed 45 minutes later. The differences in chymosin inclusion in cheese varieties with similar initial chymosin additions can be ascribed to the variations in pH that have arisen in these first hours of the cheesemaking process. A fourfold increase in amount of starter culture (0.1% against 0.025%) did not lead to large changes in pH during the first hours (see figure 5.1A), and, therefore, neither in chymosin activity in the pressed cheese. A further increase to 0.4% gave a stronger decrease in pH in

the beginning of the manufacture. This led also to a considerable increase of the amount of active chymosin enclosed (see figure 5.4).

The different way of manufacture of the cheeses from ultrafiltered milk implies a shorter period between addition of ingredients to the retentate and collection of the curd (45 minutes) or pressing of the curd blocks (75 minutes). In this relatively short period until curd collection the pH values decreased to approximately 6.7, 6.4 and 6.0, for starter dosages of 0.125%, 0.5% and 2%, respectively (see figure 5.1B). Despite these differences in pH at this stage of the process, the measured chymosin activities appear to be independent of the applied starter culture percentages and corresponding pH values. In figure 5.3B it can be seen that the chymosin activities of UF-cheeses with similar chymosin dosage and varying starter dosage are similar. A relationship as for standard cheeses could not be established. This may indicate that the adsorption of chymosin onto paracasein in retentates, which leads to transfer into the cheese, occurs in an earlier stage of the process, when differences in pH values are smaller or even absent (for instance soon after chymosin addition). Apparently, the decreasing pH does not introduce major changes in the retention of chymosin. Besides, since the amount of whey released from retentate curd is very small, loss of solubilized chymosin will also limited.

The importance of the amount of starter added to the retentate as a tool for regulation of the inclusion of chymosin now seems to be negligible, in contrast to its importance for the conventionally prepared cheeses in this study. In standard cheese manufacture, on the other hand, the curd undergoes more treatments until curd collection. Possibly this enables a better exchange of chymosin between the curd and the whey, especially when the conditions for adsorption of chymosin onto paracasein (as pH) change.

The amount of starter may be used to control the inclusion of chymosin if the starter is added some time before renneting, in order to reach a reasonable conversion of lactose into lactic acid, with a corresponding decrease in pH. Differences in pH can also be induced by chemical acidification, which is better controllable and therefore easier in production planning. Stadhouders and Hup (1975) reported a higher rennet retention in cheese at lower initial pH of the milk (measured according to Stadhouders et al., 1977). They showed that differences in the rate of acidification during the pressing of curd do not result in variations in the amounts of rennet retained in curd. This emphasizes the importance of the initial pH of the milk or retentate and the reduction in pH until whey drainage for enclosure of rennet in the

cheese. Later, Creamer et al. (1985) came to the same conclusion, using the method of Holmes et al. (1977) to estimate rennet retention in cheese. They also showed that the rate of breakdown of α_{s1}-casein, as determined by gel electrophoresis, was greater in cheese from more strongly acidified milk, due to the increased rennet retention. The breakdown of β-casein was not affected by milk acidification. Qvist et al. (1987) showed that the level of rennet in UF-cheese from milk that was not preacidified and to which 0.05% rennet was added, was the same as in UF-cheese from milk preacidified to pH 6.3 before ultrafiltration and with only 0.03% rennet added. They reported a rennet retention in cheese from preacidified milk of 36-50%, whereas a value of 22-28% was given for not preacidified milk.

It must be realised that renneting at a lower pH (down to pH 6) also leads to a faster gel formation, if other variables, such as percentage of rennet and renneting temperature, are kept constant. Adjustments may thus be required to control the renneting.

Relative retention of chymosin in cheese

The percentages of chymosin enclosed in the cheeses were calculated from the chymosin dosages (ml chymosin added per kg milk or retentate), the determined chymosin activities in cheese (ml chymosin per kg cheese), and the calculated cheese yield (kg cheese per kg milk or retentate). In this calculation only the amount of active chymosin is taken into account, and not the amount that is enclosed but that does not show activity (because of inhibition by whey proteins present in the UF-cheese). For standard cheese such correction for decreased activity is not necessary as hardly any whey proteins, that can inhibit chymosin in its action, are enclosed. In the case of UF-cheese, this 'inactive amount' is not exactly known (it depends largely on the composition of the cheese). If it was assumed that the 'inactive amount' is approximately 0.1 ml chymosin per kg UF-cheese (see the next section for the derivation), correction would increase the amounts transferred to the UF-cheese by 20-50%. Further it was assumed that no chymosin was inactivated during cheese manufacture. The percentages of chymosin, added to milk or retentate, transferred into the cheese are given in figures 5.5A en 5.5B.

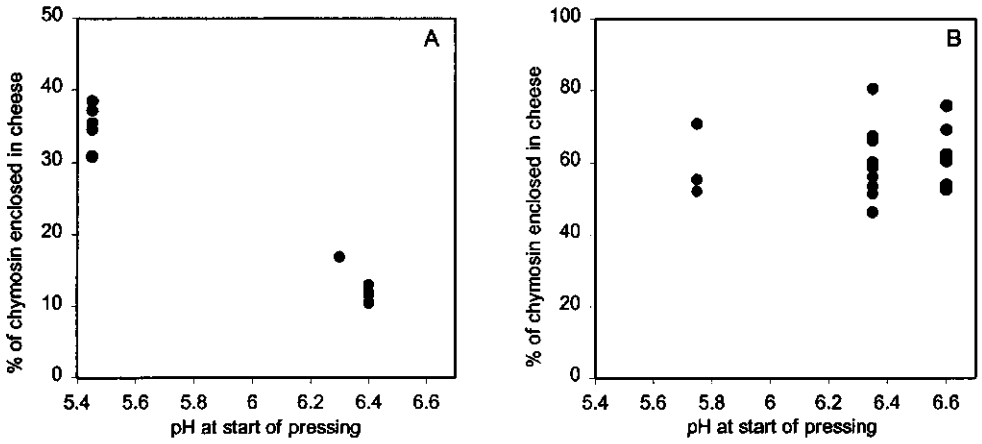

Figure 5.5 Percentages of 'active' chymosin enclosed in cheese, as a function of the pH at the start of pressing. A: standard cheeses B: UF-cheeses

In standard cheeses the percentage of added rennet retained in the cheese is strongly influenced by the pH in the first stages of cheesemaking, as shown before (figure 5.4). This implies a stronger adsorption of chymosin onto casein at a lower pH. The chymosin retention was increased by 2 to 3 times at pH 5.4, compared to a pH of 6.4 (at start pressing). The stronger association of chymosin with paracasein at lower pH was also observed by de Roos et al. (1995).

As discussed in the previous section, a relationship between pH and chymosin enclosure in the cheese, as determined for standard cheeses, was not observed in UF-cheeses. For the UF-cheeses the chymosin retention varied for the most part between 50 and 80%, independent of the pH values at the stage of curd collection, which varied from 6.0 to 6.7.

Transfer of chymosin into cheese - mechanisms of inclusion

For a better understanding of the mechanism of inclusion of chymosin in the cheese, it is tried to distinguish between the inclusion due to adsorption of chymosin onto casein and the amount enclosed in cheese with the water phase (in solution). For calculation of the proportion of enclosure of chymosin due to both mechanisms several assumptions have to be made. Concerning the transfer of solubilized chymosin into the cheese, there is supposed to be an analogy with the transfer of whey proteins. The molecular weight of chymosin molecules is in the same range as

whey proteins. Therefore, solubilized chymosin can be considered as a whey protein, and the transfer of solubilized chymosin into the cheese will be comparable with the transfer of other whey proteins.

Due to steric exclusion of whey proteins by casein, their enclosure in standard cheese is limited. This has been discussed extensively in Chapter 2. The enclosure of whey proteins was derived from the amount of soluble nitrogen, in relation to the total nitrogen content, in the freshly prepared cheese (after 1 day). In traditionally manufactured (semi-)hard cheese, the proportion of whey proteins in the protein fraction is negligible. Therefore, the enclosure of solubilized chymosin would also be negligible. This implies that chymosin inclusion in traditionally manufactured cheese can largely be attributed to adsorption onto paracasein.

The derivation of the mechanism of chymosin inclusion in UF-cheese is more complicated because of the enclosed whey proteins. The derivation and the assumptions, as well as an example, are given below.

In the UF-cheeses, approximately one third of the whey protein present in the gelled retentate is enclosed. These whey proteins in the UF-cheese are also responsible for a partial inhibition of chymosin in its activity (see Chapter 3). This inhibition is reflected in the chymosin activity as determined with the applied assay as well (see also Chapter 4). For the determination of the total amount of chymosin in cheese, the amount that is present but inactive (due to inhibition by whey proteins) should be added to the amount as determined in the assay. While one third of the whey proteins from the gel is transferred into the UF-cheese, it is assumed that the amount of chymosin-not-active (the so-called threshold value) in these UF-cheeses is also one third of the amount that does not show activity in chymosin-induced gels with all whey proteins present (as studied in Chapter 3 and 4). In these gels, with a whey protein/total protein ratio of about 0.22, approximately 1.5 ml chymosin per kg paracasein is not active (derived in Chapter 3). In the UF-cheeses as manufactured here, with a whey protein/total protein ratio of about 0.075, the inactive amount would be 0.5 ml chymosin per kg paracasein. With a total protein content of 23%, i.e. 21.3% paracasein and 1.73% whey protein, the inactive amount would be approximately 0.1 ml chymosin per kg UF-cheese. The total amount of chymosin in UF-cheese is then the amount that is determined in the assay, enlarged with the inactive amount. The derived total chymosin content in the cheese can then be divided in a part that is enclosed due to adsorption onto casein, and solubilized chymosin (not adsorbed).

The amount of solubilized chymosin is derived from the chymosin content in the whey, and the whey content of cheese (i.e. moisture in cheese containing solubilized chymosin and whey proteins). The chymosin content in the whey was derived from the total chymosin dosage to the retentate (ml chymosin per 100 gram retentate), the total chymosin content in the UF-cheese and the cheese yield. The UF-cheese yield (after 1 day) is approximately 68 gram per 100 gram retentate, and 32 gram whey is released. It was assumed that the chymosin content in the moisture of the cheese was equal to the chymosin content in the released whey.

The amount of whey (i.e. moisture with solubilized chymosin and whey proteins) enclosed in the UF-cheese was derived from the enclosure of whey proteins in the UF-cheese. In these calculations it is taken into account that, due to steric exclusion of whey proteins, the moisture in the retentate is partly not accessible for whey proteins (this is dealt with in detail in Chapter 2). For the derivation of the amount of moisture in the gelled retentate in which the solubilized whey proteins are concentrated, an average steric exclusion factor of 2.6 ml water per gram paracasein is applied (as determined by van Boekel and Walstra, 1989; see also Chapter 2 of this thesis). Since approximately 1/3 of the whey proteins in the gelled retentate is enclosed in UF-cheese, it is assumed that also 1/3 of the moisture in retentate that can contain whey proteins is transferred into the cheese.

It appeared that only 8% of the chymosin in UF-cheese was solubilized (average for UF-cheeses with different chymosin and starter dosages to the retentate). The larger part (92%) of the chymosin in UF-cheese was thus enclosed by means of adsorption.

Example:

UF-cheese with 0.033% chymosin added to retentate of CF 5 (cheeses U1 and U3)

The average 'active' chymosin content for these cheeses is 0.28 ml chymosin/kg cheese.

The 'inactive' amount is 0.1 ml chymosin/kg cheese.

The total amount is then 0.28+0.1 = 0.38 ml chymosin/kg cheese.

Per 100 kg retentate, 68 kg cheese and 32 kg whey are obtained.

The chymosin content in the whey is then 0.22 ml chymosin per kg whey.

[chymosin] in cheese moisture = [chymosin] in released whey = 0.22 ml/kg

The composition of the 100 kg chymosin-induced retentate:

17 kg total protein, in which 13.2 kg paracasein and 3.8 kg whey proteins

61 kg water, in which 34 kg is not available for dissolving whey proteins

(i.e. 2.6 kg water per kg paracasein x 13.2 kg paracasein)

The rest, 27 kg, is 'free water', in which whey proteins can be solubilized

1/3 of this 'free water' containing whey proteins (9 kg) is enclosed in UF-cheese

The amount of chymosin in the moisture of the cheese is then:

9 kg x 0.22 ml chymosin/kg moisture = 1.98 ml

The total amount of chymosin in 68 kg UF-cheese: 25.8 ml

The percentage of chymosin that is enclosed in UF-cheese by adsorption onto paracasein is then 92%.

The inclusion of chymosin in cheese is thus largely determined by adsorption onto casein, both in the traditional process of manufacture and in the UF-cheesemaking process. Therefore, factors that influence the adsorption can be used as tools for controlling the transfer of chymosin into cheese. De Roos et al. (1995) showed that chymosin becomes associated mainly to para-κ-casein, the association being stronger (i.e. more chymosin per gram κ-casein) at lower pH. In diluted systems the association was observed to be stronger, as compared to systems with a higher content of para-κ-casein. Furthermore, the amount associated is proportional to the chymosin concentration in solution, with which an equilibrium is maintained. According to Lawrence et al. (1984), the water added to the curd/whey mixture to lower the lactose content, as in Gouda, washes out some of the chymosin. Differences in the conditions with respect to the pH, the κ-casein-content and the chymosin concentration in solution may lead to differences in the amount adsorbed in the case of cheese manufacture from milk and from retentate. In the case of corresponding amounts of chymosin per gram κ-casein, the total adsorption will be 5 times higher for retentate (with a fivefold increased casein content) than for milk. In the conventional cheese manufacture, with pH 6.4 at the start of pressing, approximately 11% of the chymosin is enclosed (see figure 5.5A). In the UF-cheese, from retentate CF 5, with a similar rate of acidification in the first stages of the process, the inclusion of chymosin is much higher, about 60% (this figure is derived from the 5x increased casein content in retentate compared to milk, the retention of 11% in standard cheese manufacture, and ratio of the total amount and the adsorbed amount of chymosin in UF-cheese (5 x 11% x (100/92)). This is in agreement with retention percentages as shown in figure 5.5B. These results confirm the importance of adsorption onto casein for the inclusion of chymosin in cheese.

The percentages of rennet retention in Cheddar cheeses from retentates (concentrated fivefold), given by Creamer et al. (1987) varied between 20% and 38%, with increased retention percentages at higher amounts of rennet added to the retentate. The relatively small retention may have been due to a different method of manufacture (especially higher cooking temperatures) for Cheddar cheese, as compared to the Gouda-type cheeses made in this study.

Proteolysis in relation to chymosin activity

Proteolysis was estimated from the increase of soluble nitrogen during storage of the cheeses relative to the initial casein nitrogen content (as determined in one day old cheeses). The increase of SN/caseinN gives information about the breakdown of caseins into predominantly large peptides, mainly by the action of chymosin. The rate of formation of peptides that are soluble in the extraction buffer (0.037 molar $CaCl_2$), is therefore a measure of the chymosin activity. This chymosin activity is the product of the amount of chymosin, included in the cheese, and its specific activity under ripening conditions. The increase of SN/caseinN is not only due to the action of chymosin. Also milk proteinases are partly responsible for the proteolysis of caseins. The action of milk proteinases in Gouda cheese is, however, mainly restricted to the breakdown of β-caseins and is small compared to the chymosin activity (Visser, 1977c).

Comparison of the degrees of proteolysis in the cheeses is in fact only justified if the pH of the compared cheeses does not vary too much, because the proteolytic activity of chymosin during ripening also depends on the pH (Noomen, 1978). Standard cheeses and UF-cheeses (A and B) have comparable pH values after 1 day (5.1-5.3). The pH in UF-cheeses from retentate P had not sufficiently decreased, and proteolysis in these cheeses was therefore not determined.

The increases in the %SN/caseinN between day 1 and day 14 are given in figure 5.2, in which also the corresponding chymosin activities are presented. In figure 5.6 the increase in the %SN/caseinN-ratio between the one day and two weeks old cheeses is given in relation to the measured chymosin activities in the cheeses. For this purpose the chymosin activities as measured in cheese samples from 8 hours and from 2 weeks are averaged.

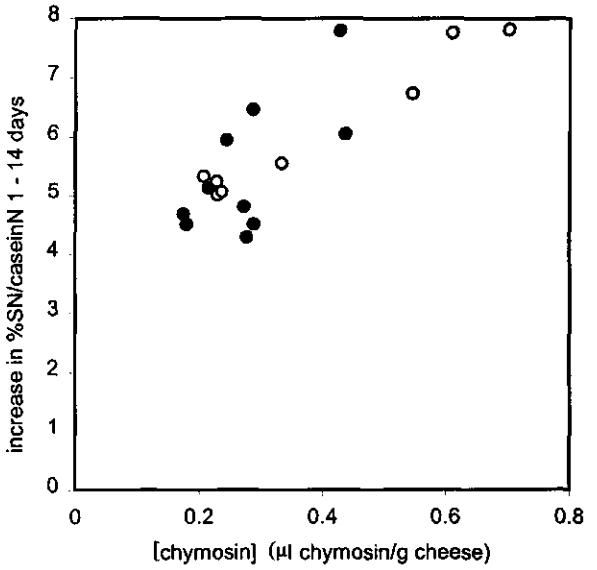

Figure 5.6 Rate of proteolysis, given as the increase in the SN/caseinN-ratio over the first 2 weeks of ripening, in relation to the measured chymosin activities in standard cheeses (O) and UF-cheeses (●)

A good correlation between the concentration of active chymosin and the breakdown of casein is found for both standard cheeses and UF-cheeses, although the results for the UF-cheeses show more variation. As the aim was to make UF-cheeses with chymosin activities corresponding to those in standard cheese, many results are clustered around the chymosin activity as determined in conventional Gouda cheese, i.e. approximately 280 µl per kg cheese (Stadhouders et al., 1977; Visser, 1977a). The released peptides (SN-components) are mainly originating from α_{s1}-casein, which is most readily attacked by chymosin. In cheese with a normal amount of chymosin most of the α_{s1}-casein is degraded in the first month of ripening (Visser and de Groot-Mostert, 1977). At higher chymosin contents this hydrolysis of α_{s1}-casein is completed faster. This explains that at higher chymosin activities, the formation of breakdown products does not increase proportionally to the chymosin activity anymore. In the cheeses with the highest chymosin activities, the formation of SN-components, originating from α_{s1}-casein, levelled off after 2 weeks of ripening. After the initial breakdown of caseins, the peptidases of starter bacteria are responsible for the further breakdown of the peptides that are released from the caseins by chymosin and milk proteinases. This 'ripening-in-depth', however, does

not increase the SN-content further, as both the substrate (large peptides) and its breakdown products (smaller peptides) belong to the SN-fraction of the ripened cheese. This slower increase at higher chymosin activities and/or after longer ripening times was also shown by Micketts and Olson (1974) and Visser (1977c), as well as in Chapter 3 of this thesis.

The correspondence between standard cheeses and UF-cheeses, as shown in figure 5.6, stresses the value of a determination of the chymosin activity to predict the breakdown of casein. The chymosin activity, as determined in a dispersion of a cheese sample, may be influenced by the composition of the cheese sample (for instance the concentration of the whey proteins), but then the breakdown of caseins will be influenced (in this case: retarded) as well by those components, since in both determinations all cheese components are present (during the chymosin activity test as well as during ripening of the cheese).

In this study, standard cheeses and UF-cheeses with corresponding chymosin activities, gave a more or less corresponding degree of proteolysis. Creamer et al. (1987) however, found that in standard Cheddar cheeses and in UF-Cheddar cheeses, with corresponding residual rennet concentrations, the rate of proteolysis was slower in the UF-cheeses. On the one hand, they attributed this to the inhibition of the rennet activity (during proteolysis) by whey proteins. On the other hand they suggested that this might be due to the dilution of the casein substrate by whey proteins (and thus less substrate). From their results none of these explanations can be rejected, since proteolysis (as measured by the decrease of α_{s1}-casein and the formation of α_{s1}-I-casein) was not related to the initial substrate (α_{s1}-casein) concentration. The inhibition of rennet activity in the presence of whey proteins was also determined in our study (see Chapter 3), and in other studies (Hickey et al., 1983; Harper et al., 1989). The dilution of caseins in UF-cheese by whey proteins, which were considered as being inert fillers, was earlier mentioned by de Koning et al. (1981). The discrepancy between results of Creamer et al. (1987) and ours has probably arisen from the different methods used for the estimation of the rennet activity. It seems most likely that the whey proteins, present in the UF-cheese, were not transferred into the rennet-containing filtrate in which they determined the rennet activity. In the other, indirect, method of chymosin activity determination (via rate of proteolysis), these whey proteins are still present. As a component of the whey protein fraction is supposed to be responsible for the inhibition of chymosin activity, the absence of this component in the direct chymosin activity measurement would result in a higher estimated activity in the UF-cheese, compared to the activity

derived from the breakdown of caseins in this UF-cheese during ripening. This discrepancy between the measured chymosin activity in a cheese extract and the observed breakdown of α_{s1}-casein is also reflected in the conclusion of Creamer et al. (1987) that about 66% more residual rennet was required in UF-cheese than in standard Cheddar cheese to achieve a similar rate of α_{s1}-casein-breakdown. This extra added rennet then would represent the amount that, although present, does not participate in proteolysis, due to the inhibition by a component of the whey protein fraction.

In the direct chymosin activity measurement, applied in this study, the whey proteins were not removed in the preparation of the sample (the chymosin-containing cheese dispersion). Therefore, the results of this chymosin activity determination would show good correlation with the formation of breakdown products from α_{s1}-casein in the cheeses, independent of the whey protein content of the cheese, as in both methods all whey proteins are still present. The chymosin to whey protein ratio in the dispersed cheese sample was equal to this ratio in the cheese.

In the assay of the direct chymosin activity determination all cheese components were diluted 40 times. The contents of whey proteins and chymosin are thus 40 times lower in the direct assay than in the cheese. There is no indication that the result, i.e. the derived chymosin activity, is significantly influenced by the dilution of the cheese. At similar ratio of chymosin to whey protein, the inhibition of chymosin by a component of the whey protein fraction would not depend on the concentration of both components. In Chapters 3 and 4 it is shown that the inhibition of chymosin is of the same order in the direct and the indirect determination.

That inhibition of chymosin by whey proteins was not observed here, must be due to the similar conditions during the direct and the indirect determination of chymosin activity. In another part of this study (Chapter 3), however, the inhibition in the presence of whey proteins was already determined, reason why it was tried to cope with this inhibition by taking measures that resulted in an adequate level of chymosin activity in UF-cheese. It is shown that the acidification, together with adjustments in the dosage of chymosin to retentate, are strong tools to increase the chymosin activity in UF-cheese, hereby counteracting the inhibiting effect of whey proteins on chymosin activity.

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

Preconcentration of the cheesemilk by ultrafiltration, prior to coagulation, offers opportunities to increase the cheese yield, by means of incorporation of the whey proteins in the cheese. The yield increase holds especially for cheese varieties with a high water content, as the milk can be concentrated by ultrafiltration to a dry matter content that is close to that of the final product. Nowadays, ultrafiltration is introduced on a large scale for the production of high moisture, soft cheeses, while the technology is hardly applied for the manufacture of (semi)hard cheeses, where additional syneresis is essential for achieving the required dry matter content. The main reason is that the potential advantages, as mentioned in the general introduction (Chapter 1), are counteracted by the changed (retarded) development of flavour and texture during ripening of the UF-cheese, compared to cheese prepared according to traditional methods. In most studies so far, these problems were observed and attributed to the enclosed whey proteins, but not further unraveled or related to other parameters. In this project several aspects on the manufacture and ripening of semi-hard cheese from ultrafiltered milk (UF-cheese) were studied. Especially the roles of the native whey proteins and of the enzyme chymosin were investigated. Results were compared with those of the traditionally prepared Gouda cheese, which was considered as a control.

The ripening of cheese is strongly related to its protein composition. Therefore, possible changes in the protein composition of the cheese due to preconcentration of the cheesemilk were studied. The transfer of whey proteins into the UF-cheese was studied in relation to the degree of ultrafiltration of the milk and the degree of syneresis of the curd (Chapter 2). In the traditional cheesemaking process, the enclosure of whey proteins is negligible, as almost all whey protein is expelled with the whey during syneresis of the curd. In the manufacture of (semi)hard cheese varieties from preconcentrated UF-retentates, additional syneresis is essential for further lowering the water content to the desired dry matter content of the cheese. Although the volume of whey to be expelled from the curd from retentate is decreased, compared to curd from unconcentrated milk, the loss of whey proteins from the curd during syneresis still is considerable, because of the higher whey protein content in the whey. The whey protein content in the expelled

whey is increased even more than the whey protein content in the serum of the gelled milk or retentate, because of steric exclusion (also called negative adsorption) of whey proteins by paracasein micelles. Water in and close to the casein (sub)micelles cannot contain whey protein, resulting in a higher whey protein content in the rest of the serum, and thus also in the expelled whey. The difference between the 'overall' whey protein to water ratio in the gel and this ratio in the expelled whey is higher in the case of UF-retentates, because of their increased casein and whey protein content. The more than proportional loss of whey protein during syneresis was reflected in the decrease of the whey protein to water ratio in the curd at ongoing syneresis. During syneresis, the whey protein to total protein ratio in the curd decreased faster at a higher concentration factor of the retentate. The steric exclusion factor for whey protein (i.e. the amount of water per gram casein in which no whey protein is dissolved) appeared to be independent of the initial casein and whey protein content, the pH and the degree of syneresis. Besides, it was also shown that whey proteins did not accumulate in the paracaseinate network by filtration. Therefore, the total enclosure of whey proteins in syneresed curd grains may be relatively small, also in curd from UF-retentates. The total transfer of whey proteins into the cheese, however, depends not only on factors that determine the whey protein content in the syneresed curd grains, but also on the enclosure of whey (with whey proteins) during drainage of the curd, and on losses during leaking and pressing of the curd blocks. In a pilot study, in which Gouda cheese was made according to the traditional process, the total enclosure of whey proteins was negligible, while approximately one third of the whey protein fraction in the gelled retentate (5x concentrated milk) was recovered in the fresh UF-cheese. The protein fraction in these UF-cheeses consisted for approximately 8% of whey proteins.

In comparing the ripening of traditionally prepared cheese and cheese from ultrafiltered milk, especially the role of chymosin was studied. Chymosin, added to coagulate the cheesemilk, plays also an important role in cheese ripening, especially in semi-hard cheeses like Gouda and Cheddar. In the first stage of ripening, chymosin is responsible for the degradation of caseins to peptides, which are precursors for further flavour development. Therefore, an adequate level of chymosin activity in the fresh cheese is essential in the first stage of ripening. It was tried to explain the retarded ripening of UF-cheese by studying the activity of chymosin in the degradation of caseins, in relation to the protein composition of the cheese, in the first weeks of ripening.

The total chymosin activity depends on the chymosin content in the cheese, and on its specific activity during ripening. Initially, research was focused on the specific activity of chymosin towards paracasein during ripening, because a good method for the determination of the chymosin content was not available. The influence of whey proteins on the proteolytic activity of chymosin was investigated in various types of cheese models with known amounts of whey proteins and chymosin (see Chapter 3). It was clearly shown that chymosin was inhibited in its activity by whey proteins. Chymosin-treated UF-retentates (pH 5.2 and 4% salt-in-water) with a normal chymosin content (i.e. ≈ 1.5 ml chymosin per kg paracasein), showed hardly any activity during ripening. In cheese models that were based on a paracaseinate substrate, containing varying amounts of whey proteins and chymosin, it was shown that the inhibition of chymosin was about proportional to the whey protein content. The observed trends were similar for cheese models with serum proteins and for models with whey proteins also containing the caseino-macropeptide-fraction. The inhibition was thus not caused by the cmp-fraction.

In this part of the study, the chymosin activity was derived from the degradation of paracasein (corrected for the degradation by milk proteinases), during the first weeks of ripening. Unfortunately, it takes some time (days or even weeks) before reliable results of this indirect determination of the chymosin activity are available. Besides, results may be influenced by conditions (such as temperature) during ripening. These disadvantages stress the need for a method to determine the chymosin activity directly in unripened cheese. Such an assay would also enable estimation of the percentage of the added chymosin that is enclosed in (semi-)hard cheeses. With a direct method the effect of changes in the process of cheese manufacture on the enclosure of chymosin and the degradation of casein can be clarified in an early stage of the ripening.

The development and evaluation of such a method is described in Chapter 4. For the derivation of the chymosin activity, the cheese sample was first dispersed under controlled conditions (to prevent inactivation of chymosin) with JK-buffer. The optimal conditions for dispersing were a low temperature (4°C), pH 5.2 and 1% NaCl in the dispersion. As the cheese sample was 'dissolved' completely, no extraction step was needed in the preparation of a homogeneous, pipettable dispersion or solution. This way the loss of any chymosin from the cheese sample was prevented. Consequently a sample of the cheese dispersion could be incubated with κ-casein. The chymosin activity in the cheese dispersion was derived from the rate of release of the caseino-macropeptide-fraction from κ-casein. This assay was developed and

tested at the same time, by estimating the chymosin activity in chymosin-induced milk gels, as well as in dispersions from such gels with extra added chymosin (added after dispersing), all with known amounts of chymosin. These tests showed that no chymosin activity was lost during preparation of the dispersion.

During these control experiments, it also appeared that a certain amount of chymosin in the dispersion showed virtually no activity under test conditions. The loss of activity was higher in case of dispersions from gelled retentates than in dispersions from chymosin-induced milk gels, whereas chymosin in JK-buffer (without milk proteins) was not inhibited. From these results it could be derived that components originating from the dispersion, most probably components of the whey protein fraction, are responsible for the inhibition. The amount of chymosin that was not active (the threshold value) appeared to be of the same order in the direct chymosin activity assay on (unripened) chymosin-induced retentate gels and in the indirect determination (via degradation of caseins) in the ripened UF-cheeses. In both cases approximately 24% of the protein fraction consisted of whey proteins (as in milk/retentate after hydrolysis of κ-casein). For cheese from unconcentrated milk, with a negligible enclosure of whey proteins, the threshold value for chymosin activity during ripening is very small.

The restraining influence of whey proteins on the activity of chymosin would thus not only be manifest in the degradation of caseins during ripening (see Chapter 3), but also in the hydrolysis of κ-casein during coagulation (Chapter 4). This dual effect had also been observed by Lelievre et al. (1990). In general, the restrained chymosin activity during coagulation of concentrated milk will hardly be noticed. In retentates, with an increased inhibition of chymosin, the hydrolysis of κ-casein would be slower because of the decreased chymosin activity, but this would be counteracted by the strongly increased rate of curd firming. In this stage a decreased chymosin activity does not cause problems, because several tools are available to control the process of coagulation (van Hooydonk, 1987). During ripening, however, a decreased chymosin activity cannot easily be counteracted by other enzymes, as the contribution of each enzyme to the proteolytic processes is very specific. Changes in mutual activities will disturb a balanced proteolysis of caseins, and also the further degradation of peptides by enzymes of starter bacteria.

The mechanism of lowering the chymosin activity in the presence of whey proteins was not unraveled. As the amount of chymosin that exhibited no activity was proportional to the whey protein content, it appears likely that the active centre of chymosin molecules was occupied by a molecule of the whey protein fraction. The

inhibition being in the same order in a retentate gel and in a strongly diluted (40x) sample of the retentate gel, indicates that the association (between the inhibiting molecule and chymosin) is very strong. Otherwise, the inhibition would be strongly decreased in the diluted system. It also points to a very low concentration of the inhibitor in milk, smaller than, but of the same order as the chymosin content. In this study, the effect of individual whey proteins was not investigated. The components of the whey protein fraction were present in the same mutual ratios as in the milk from which this fraction was isolated after removal of the casein fraction. It is obvious that the component responsible for the inactivation is concentrated in the retentate during ultrafiltration, and partly accumulated in UF-cheese. It thus concerns a high-molecular component in the whey protein fraction, or a component released from it during renneting. In the literature, α_2-macroglobulin, a blood serum protein in milk that is concentrated by ultrafiltration, was suggested (but not confirmed) to be responsible for the inactivation (McLean and Ellis, 1975; mentioned by Harper et al., 1989, and by Lelievre et al., 1990). Selective removal of such a molecule from the milk or the retentate will presumably prevent the lowering of the activity of the chymosin in the cheese. In this respect, (ultra)filtration, in combination with other separation techniques might offer opportunities. However, more information would be needed on the molecule(s) responsible for the inhibition and on the mechanism involved.

Other mechanisms causing a slower release of peptides in UF-cheese have been suggested in the literature (see Chapter 1), such as a the slower enzyme diffusion in retentates and the limited accessibility of casein; they were not investigated further in this study, since they are considered quite unlikely.

The fact that this inhibition of chymosin by components in the UF-retentate gel (or its dispersion) was observed both in the chymosin activity assay (Chapter 4) and during the ripening of the chymosin-induced UF-retentate (Chapter 3), justifies the extrapolation of the results of the assay to the proteolysis in UF-cheese. In principle, the assay is expected to be applicable for the derivation of the chymosin activity in most types of cheese, irrespective of the (protein) composition of the cheese. However, more experiments would be needed to confirm this. In this study, the assay was optimized further in a later stage, when used for semi-hard cheeses with unknown chymosin activities (Chapter 5).

In a pilot study, described in Chapter 5, the various aspects of (UF-)cheese manufacture and ripening, which had been investigated independently under controlled conditions (Chapters 2, 3 and 4), were integrated by making both

traditional Gouda cheese and UF-cheeses (from 5x concentrated retentates). It was tried to make the latter with characteristics (i.e. pH, water content, salt-to-water content) similar to those of conventionally prepared Gouda cheese, to ascertain an unbiassed comparison. The influence of the dosages of chymosin and starter culture (to the milk or retentate) on the chymosin activity and proteolysis in cheese during the first two weeks was established.

For the (direct) determination of the chymosin activity the assay was applied on fresh and ripened cheeses (2 weeks). The rate of proteolysis was determined by measuring the degradation of casein in these cheeses in the first two weeks (indirect method). The results of the direct and the indirect determination of the chymosin activity corresponded fairly well, both for traditionally manufactured cheeses and UF-cheeses, thereby confirming the practicability of the direct assay. The chymosin activities in the fresh and ripened cheeses were similar, indicating that there was no loss of activity during these first weeks of ripening. This showed that the assay can be used for cheese of various age.

The starter culture has, like chymosin, a dual role in the manufacture and ripening of cheese. On the day of curd making, lactose is converted into lactic acid. During ripening, the enzymes of the starter culture are responsible for the further degradation of peptides released from caseins by chymosin or milk proteinases. In this study, only their role in the acidification was considered.

The rate of acidification is strongly determined by the applied dosage of starter culture, and the acid buffering in the growth medium. The increased protein content in retentates is responsible for the higher buffer capacity, and the slower acidification. The slower acidification can be counteracted by preacidifying the milk (before ultrafiltration) or retentate. Preacidification of the milk also results in a smaller buffer capacity of the retentate, because of the removal of calcium phosphate. The acidification largely determines the enclosure of chymosin in the cheese, because of the pH-dependence of the adsorption of chymosin onto casein. It was shown that, for a similar course of the pH values, the enclosure of chymosin was proportionally increased with its dosage to the milk. A lower pH during curd making (either established by a higher rate of acidification during the first hours or by preacidification), however, resulted in a relative higher inclusion of chymosin in the cheese.

From these results it could be calculated that most (i.e., 90-100%) of the transfer of chymosin into the cheese, was due to the adsorption onto casein. The amount of chymosin enclosed with the whey was very small (UF-cheese) or

negligible (standard cheese). This is due to steric exclusion of solutes by casein micelles, resulting in the loss of dissolved chymosin in the whey during syneresis. These results on chymosin enclosure are thus consistent with those on transfer of whey proteins into cheese. By taking measures that enhance adsorption of chymosin onto casein, like acidification, the relative transfer of chymosin into cheese will be increased, implying that the dosage can be lowered to arrive at a given chymosin activity in the cheese.

To achieve a comparable rate of casein degradation in UF-cheese and in standard cheese, the chymosin activity in relation to the casein content should be similar in both cheeses. The chymosin activity depends on the chymosin content and its specific activity under the prevailing conditions (pH, temperature, etc.). Besides, it is shown that (part of) the chymosin is inhibited in its activity by whey proteins. Because of this, the total chymosin content has to be higher in UF-cheese than in standard cheese to achieve a given proteolysis. A sufficient chymosin activity in UF-cheese should thus be realized by choosing a suitable combination of chymosin dosage and acidification. The development of the pH can be regulated by means of the starter culture dosage or by chemical acidification. Whereas in retentates a lower chymosin dosage (in relation to the casein content) can be applied to achieve a given rate of coagulation, i.e. a similar cutting time as compared to standard cheese manufacture, such a dosage may be too low for realizing a certain chymosin activity and rate of proteolysis in the cheese. A reduction of the chymosin dosage by a factor similar to the concentration factor of the retentate, as is often applied, is sufficient to cause coagulation but it results in a lower chymosin activity in the UF-cheese (at an unchanged rate of acidification) and a lower rate of proteolysis. The tools to achieve an increased chymosin activity in the cheese, a higher chymosin dosage to the milk or a lower pH, however, also affect the process of coagulation. Especially in the case of retentate, these measures may give problems, as coagulation is already enhanced due to the increased casein content. Lowering the coagulation temperature slows down the coagulation, but this also results in an increased viscosity of the retentate. To cope with these problems, special equipment should be used, that can handle the retentate gel and curd without shattering it during handling. If traditional cheesemaking equipment were used for handling gels and curd from retentate, the increased loss of curd fines and fat would undo the yield increase realized by the increased retention of whey proteins.

The rate of the degradation of caseins during ripening can thus be increased by taking measures that enhance the chymosin activity in UF-cheese. Although not

studied here, the role of milk proteinases, which are of importance for the degradation of β-casein, should also be taken into account in evaluating the process of UF-cheese ripening. A decreased chymosin activity can possibly be partly counteracted by an increased proteolytic activity of milk proteinases. However, this may well have consequences for further flavour development, because different type of peptides, that are the source for further breakdown by peptidases from starter bacteria, are released by both enzymes during the initial stages of proteolysis.

Whenever comparing the degradation of caseins with other parameters of cheese ripening, it should be realized that in UF-cheese the casein fraction is diluted by the whey proteins enclosed. The total casein content is decreased compared to traditionally prepared cheese, in which the protein fraction consists almost totally of caseins. With a similar chymosin activity, in relation to the casein content, the content of breakdown products in the ripened cheese will also be lower, as whey proteins are not degraded during ripening. Besides, for a good development of flavour, the breakdown of caseins by chymosin and milk proteinases should be well-balanced with the degradation of peptides to smaller components by the enzymes of starter bacteria. In UF-cheese, a high chymosin activity may result in bitterness, and peptidase activity is essential for degradation of these bitter peptides. However, the growth and the release and expression of enzymes by starter bacteria in retentates and UF-cheese is different from that in unconcentrated milk and standard cheese (Meijer, 1997). This is probably the main cause of the changed flavour development in UF-cheese.

In this study, the relation between the retarded casein breakdown in UF-cheese, and the parameters in the process of manufacture (preconcentration milk, chymosin dosage, acidification) affecting it, are unraveled. These parameters influence the protein composition (caseins/whey proteins) and the enclosure of chymosin in the cheese. It is shown that the whey proteins are partly responsible for the changed ripening, due to the dilution of the casein fraction, and especially because of the reduced activity of chymosin onto casein.

Because of the changed protein composition and the changes in the amount and activity of ripening enzymes, the characteristics of UF-cheese will always differ from its traditionally manufactured equivalent. Therefore, preconcentration of the cheesemilk is not very advantageous for the manufacture of the current Dutch type semi-hard cheeses, but it may offer opportunities for the development of new cheese types, with other characteristics.

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SUMMARY

In traditional cheese manufacture, most of the whey proteins are released in the whey. Transfer of these whey proteins into the cheese would increase the cheese yield. Whey proteins can be incorporated in the cheese by preconcentrating the milk by ultrafiltration (UF). The increased yield is, however, less than might be expected, and its advantage is offset by the changed ripening characteristics of UF-cheese. This concerns especially semi-hard cheese varieties (like Gouda and Cheddar) in which ripening is largely due to the proteolytic activity of rennet enzymes (mainly chymosin), milk proteinases and starter bacteria. The work described in this thesis deals with several aspects on the manufacture and ripening of UF-cheese. Especially the transfer of whey proteins and chymosin into (UF-) cheese, and the role of chymosin in proteolysis was investigated. Gouda cheese manufactured by conventional methods was taken as a reference.

The transfer of whey proteins to cheese strongly depends on the desired dry matter content of the cheese. For the manufacture of semi-hard cheese from ultrafiltered milk, additional syneresis is required to achieve this dry matter content, since the degree of ultrafiltration is limited by the viscosity of the concentrated milk (the retentate). The transfer of whey proteins to (UF-)cheese was studied in relation to the degree of ultrafiltration and the degree of syneresis. It was shown that, due to steric exclusion of whey proteins by casein micelles (i.e. part of the water in and around the casein micelles is not available as a solvent for whey proteins), the loss of whey protein in the expelled whey is more than proportional. At ongoing syneresis, the whey protein to water ratio in curd decreases. Therefore, the yield increase that can be obtained by preconcentrating the milk, will be limited, depending on other manufacturing parameters and on the required dry matter content. The steric exclusion factor (gram water per gram casein in which no whey protein was dissolved) appeared to be independent of the degree and the method of concentration of the caseins, either by ultrafiltration of the milk or by syneresis of the milk or retentate gel, and of the whey protein content and the pH (Chapter 2).

In the first stage of ripening, chymosin is responsible for the degradation of caseins to, predominantly large, peptides, which are precursors for further flavour development. The influence of whey proteins on the proteolytic activity of chymosin was studied with various types of cheese models, with known amounts of whey proteins and chymosin (Chapter 3). It was clearly shown that chymosin is inhibited in its activity by a (yet unknown) component of the whey protein fraction, the inhibition being stronger at a higher whey protein content. At this part of the study, chymosin activity was derived from the rate of release of degradation products from casein in the first weeks of ripening, which is, in fact, an indirect determination.

A direct method for the determination of the chymosin activity in gels, curd and cheese of various age was developed (Chapter 4). This method comprises a dispersing step, in which the sample was 'dissolved' completely under controlled conditions, and an assay, in which the dispersed sample was incubated with κ-casein. Chymosin activity in the sample was derived from the rate of release of the caseino-macropeptide-fraction from κ-casein. This method was tested by estimating the chymosin activity in chymosin-induced milk and retentate gels. It appeared that under test conditions a certain amount of chymosin in the dispersion was inhibited in its activity, most probably by whey protein in the gel. In this diluted system, the inhibition, i.e. the amount of chymosin that was not active, was of the same order as in the indirect determination in ripened, undiluted cheese models (with a similar whey protein to total protein content). This points to a strong association between the inhibiting component and the active centre of chymosin. The inhibitor, present in very low concentrations, is most probably a high-molecular component of the whey protein fraction of milk.

The various aspects on UF-cheese manufacture and ripening were integrated in a pilot study (Chapter 5). Gouda type cheese was prepared according to conventional methods (standard cheese), as well as from UF-retentates (5x concentrated). Whereas in standard cheese the enclosure of whey proteins was negligible, approximately one third of the whey protein fraction in the retentate was transferred into UF-cheese. The protein fraction in UF-cheese consisted for about 8% of whey proteins; this would correspond to an increase in yield by nearly 5%. Chymosin activity in the cheeses was determined both directly, by application of the recently developed assay, and indirectly, by measuring the degradation of caseins in the first two weeks. The results of both determinations corresponded very well. This justified the interpretation of the result of the determination to the chymosin activity during ripening. An increased rate of acidification during cheesemaking (when more starter was added) resulted in an increased chymosin activity in cheese. The enclosure of chymosin was proportionally increased with its dosage to the milk or retentate. Transfer of chymosin into the cheese was mainly (for 90-100%) due to adsorption onto casein. The amount of solubilized chymosin enclosed with the whey was very small (UF-cheese) or negligible (standard cheese).

Most of the relations between the protein composition, chymosin activity and proteolysis, that were determined in this study, are valid both for cheeses prepared from (unconcentrated) milk and for UF-cheeses prepared from retentates. It can be concluded that this study has contributed to a better understanding of the process of cheese manufacture and ripening in general.

SAMENVATTING

Bij de bereiding van kaas worden enkele belangrijke bestanddelen uit de melk, voornamelijk de vetten en de eiwitten, geconcentreerd. De eiwitfractie van melk bestaat voor ongeveer 80% uit caseïne en voor 20% uit serumeiwitten. Door toevoeging van stremsel (dat grotendeels uit chymosine bestaat) aan de melk wordt de caseïno-macropeptidefractie (ca. 5%) van de caseïne afgesplitst. De resterende paracaseïne (ca. 95%) vormt een gel, waarin het vet ingesloten wordt. Door dit paracaseïnegel te snijden start de synerese, waarbij het vocht (de wei) uittreedt. De gesynereerde paracaseïne met het vet (de wrongel) wordt verzameld en tot kaas gevormd. Kaas wordt voornamelijk verduurzaamd door de omzetting van de melksuiker lactose tot melkzuur door de aan de kaasmelk toegevoegde melkzuurbacteriën. Tijdens de rijping van kaas vinden allerlei (bio)chemische en fysische omzettingen plaats, die bepalend zijn voor de ontwikkeling van de smaak en de consistentie.

De wei, met daarin de opgeloste wei-eiwitten (serumeiwitten inclusief het caseïno-macropeptide), is een bijprodukt van de kaasbereiding. Het eveneens insluiten van deze wei-eiwitten in de kaas zou tot een verhoging van de opbrengst kunnen leiden. De wei-eiwitten kunnen gedeeltelijk in de kaas worden ingesloten door de melk voorafgaand aan de stremming te concentreren door middel van ultrafiltratie (UF). Hierdoor is na de stremming minder wei-uittreding nodig om het vereiste vochtgehalte van de kaas te bereiken, en kan het totale verlies van wei-eiwitten beperkt worden. Tegenover het voordeel van de mogelijke opbrengstverhoging staat echter de achterblijvende rijping van UF-kaas. Dit betreft vooral de half-harde kaassoorten, zoals Goudse en Cheddar, waarin de rijping vooral bepaald wordt door de proteolytische activiteit van het stremenzym chymosine, de melkproteïnase en de enzymen van de melkzuurbacteriën. In dit proefschrift worden de verschillende aspecten van de bereiding en rijping van UF-kaas behandeld. Het onderzoek is toegespitst op de insluiting van de wei-eiwitten en chymosine in (UF-)kaas, en op de rol van chymosine in de eiwitafbraak tijdens de kaasrijping. Goudse kaas, bereid volgens de conventionele methode, met een verwaarloosbare insluiting van wei-eiwitten, is hierbij als referentie genomen.

De insluiting van wei-eiwitten in de kaas is sterk afhankelijk van het eiwitgehalte in de (UF-)melk, de mate van wei-uittreding tijdens synerese, de wrongelbewerking en het gewenste droge-stofgehalte van de kaas. Het relatief hoge droge-stofgehalte van half-harde kazen kan niet bereikt worden door ultrafiltratie alleen. Door de verhoogde viscositeit van de geconcentreerde melk (het retentaat) is het concentreren van de melk door ultrafiltratie beperkt tot ca. 6x (op eiwitbasis). Om het vochtgehalte verder te verlagen is aanvullende synerese vereist. De insluiting

van wei-eiwitten in (UF-)wrongel is onderzocht in relatie tot het concentreren van de melk door ultrafiltratie en het verder concentreren van de wrongel door synerese (Hoofdstuk 2). Door sterische uitsluiting van wei-eiwitten door caseïnemicellen (een deel van het water in en rond de caseïnemicellen is daardoor niet beschikbaar als oplosmiddel voor wei-eiwitten) is het verlies van wei-eiwitten in de wei relatief hoog. Bij verdergaande synerese neemt de wei-eiwit/water-verhouding in de resterende wrongel af. Deze afname is sterker bij synerese van UF-geconcentreerde melkgelen. De wei-eiwitinsluiting in UF-wrongel is hierdoor beperkt. De sterische uitsluitingsfactor (de hoeveelheid water per gram caseïne waarin geen wei-eiwitten opgelost kunnen zijn) bleek onafhankelijk te zijn van de methode van concentreren (ultrafiltratie van de melk of synerese van de wrongel), de concentratiegraad, het wei-eiwitgehalte en de pH.

In de eerste fase van de kaasrijping is in de kaas ingesloten chymosine verantwoordelijk voor de afbraak van caseïnes tot vooral grotere peptiden, die van belang zijn voor de verdere smaakontwikkeling. De invloed van de wei-eiwitten op de proteolytische activiteit van chymosine werd onderzocht in verschillende kaasmodellen, met ingestelde concentraties aan wei-eiwitten en chymosine (Hoofdstuk 3). De chymosineactiviteit werd (indirect) afgeleid van de snelheid van het vrijkomen van afbraakprodukten van de caseïne (in de eerste weken van de rijping). Chymosine bleek in zijn activiteit te worden geremd door een (vooralsnog onbekende) component van de wei-eiwitfractie. De schijnbare hoeveelheid chymosine die geen activiteit vertoonde was hoger bij een verhoogd wei-eiwitgehalte in de kaasmodellen.

In Hoofdstuk 4 is de ontwikkeling van een methode voor de directe bepaling van de chymosineactiviteit in melkgelen, wrongel en kaas beschreven. Deze methode omvat een dispergeerstap waarin het monster werd 'opgelost' onder gestandaardiseerde condities, en een analysestap waarin de chymosineactiviteit in het gedispergeerde monster werd bepaald. De chymosineactiviteit in het monster werd afgeleid van de snelheid van vrijkomen van de caseïno-macropeptidefractie na toevoeging aan een κ-caseïne-oplossing. In de ontwikkelingsfase werd deze methode toegepast om de chymosineactiviteit in gestremde melk- en retentaatgelen met bekende concentraties chymosine te bepalen. Hieruit bleek dat onder de proefomstandigheden een deel van de chymosine in een dispersie van het gel geen activiteit vertoonde.

De remming (de schijnbare hoeveelheid chymosine die niet actief is) was in de onverdunde, gerijpte kaasmodellen en in de verdunde geldispersie (met een overeenkomstige wei-eiwit/totaal eiwit-verhouding) van dezelfde orde van grootte.

Dit wijst op een sterke associatie tussen de remmende component en het actieve centrum van chymosine. De remmer is waarschijnlijk een in zeer lage concentratie aanwezige hoog-moleculaire component van de serumeiwitfractie van de melk.

Voor de stremming van UF-retentaat kan de chymosinedosering (ml chymosine per gram caseïne) gereduceerd worden, omdat bij een verhoogde caseïneconcentratie de gelering veel sneller verloopt. Om tijdens de rijping voldoende afbraak van caseïne te bereiken, moet de verlaagde specifieke activiteit van chymosine in aanwezigheid van wei-eiwitten gecompenseerd worden door een verhoogde chymosine-insluiting (in relatie tot het caseïnegehalte) in de kaas. Toch zal door de gedeeltelijke vervanging van caseïne door wei-eiwitten (die niet aangetast worden tijdens de rijping), de concentratie aan peptiden waaruit smaakcomponenten kunnen worden gevormd, in UF-kaas verlaagd zijn.

De verschillende aspecten van de bereiding en rijping van UF-kaas zijn geïntegreerd in een opschalingsstudie (Hoofdstuk 5). Kaas (type Goudse) werd bereid volgens de conventionele methode (standaard kaas) en uit 5x geconcentreerd UF-retentaat. De insluiting van wei-eiwitten in de standaardkazen was verwaarloosbaar. Ongeveer één derde van de wei-eiwitfractie in het UF-retentaat werd ingesloten in UF-kaas. De eiwitfractie in UF-kaas bestond voor ongeveer 8% uit wei-eiwitten; dit komt overeen met een opbrengstverhoging van bijna 5%. De chymosineactiviteit in de kazen was bepaald, zowel direct door toepassing van de hiertoe ontwikkelde methode, als indirect door de afbraak van caseïnes in de eerste 2 weken van de rijping te meten. De resultaten van beide bepalingen kwamen goed overeen. Dit rechtvaardigt de interpretatie van het resultaat van de directe activiteitsbepaling naar de chymosineactiviteit tijdens de rijping. De insluiting van chymosine in de kaas nam evenredig toe met de chymosinedosering aan de melk of aan het UF-retentaat. De insluiting van chymosine in de kaas was grotendeels (90-100%) toe te schrijven aan de adsorptie aan paracaseïne. De hoeveelheid opgeloste chymosine die met de wei ingesloten wordt in de kaas, is zeer laag (UF-kaas) of verwaarloosbaar (standaard-kaas). Een versnelde verzuring tijdens het kaasmaken (door een verhoogde zuurseldosering aan de melk) resulteerde in een verhoogde chymosineactiviteit in de kaas, door de toegenomen adsorptie van chymosine aan paracaseïne.

De meeste relaties tussen de eiwitsamenstelling, de chymosineactiviteit en de eiwitafbraak die in dit onderzoek zijn bepaald, gelden zowel voor kaas bereid uit (ongeconcentreerde) melk als voor UF-kaas bereid uit door ultrafiltratie geconcentreerde melk. Dit onderzoek heeft daarom bijgedragen aan een beter begrip van het kaasbereidingsproces en de kaasrijping in het algemeen.

NAWOORD

De afgelopen jaren hebben in het teken gestaan van het onderzoek naar de bereiding en rijping van (UF-)kaas, hetgeen met de publicatie van dit proefschrift afgerond wordt. De veelzijdigheid van de produktgroep zuivel, en in het bijzonder van kaas, maakt dat ik met plezier aan dit onderzoek gewerkt heb. Het vereeuwigen van dit werk in een proefschrift bleek nog een hele klus (en uitdaging!), maar deze is dan nu toch geklaard. Dit is tevens het moment even stil te staan bij de voorbije jaren, en bij de personen die hierbij een rol hebben gespeeld.

Mijn promotor Pieter Walstra, en co-promotor Ad Noomen, wil ik bedanken voor het vertrouwen dat ze in mij gesteld hebben om deze klus aan te pakken. Als begeleiders van het eerste tot en met het laatste uur hebben ze, ieder op hun eigen wijze en voor hun eigen specialismen, de lijn van het onderzoek en de vastlegging op papier bewaakt. Een belangrijke rol in deze was ook weggelegd voor Tom Geurts, die altijd tijd maakte om nog even verder te filosoferen over kaasvraagstukken.

De werkbesprekingen werden opgeluisterd door leden van de UF-groep, waaronder mijn 'microbiologische counterpart' Wilco Meijer, vertegenwoordigers van het Mesdagfonds, collega's en andere geïnteresseerden. Allen wil ik bedanken voor hun bijdragen aan de discussies.

Diverse studenten (Gert, Maaike, Jacques, Wieke, Anko, Carline, Marjo, Erwin, Elske) hebben zich in het kader van hun afstudeeronderzoek beziggehouden met één van de vele facetten van (UF-)kaas of met de ontwikkeling van de chymosine-activiteitsbepaling. Voor deze methode heeft Barbara Hart na haar studie nog belangrijk pionierswerk verricht. Het was leuk en leerzaam om met jullie samen te werken.

Mede dankzij mijn collega's van de voormalige sectie Zuivel en Levensmiddelennatuurkunde, die mij veelal met raad en daad hebben bijgestaan, heb ik het verlengde verblijf op het Biotechnion, en in Wageningen, als een aangename tijd ervaren. Ook de contacten na mijn vertrek uit Wageningen, tijdens het schrijven van dit proefschrift, heb ik zeer gewaardeerd. Ik wil hierbij met name Henk Jansen, die vele printopdrachten voor mij uitvoerde, bedanken.

Maria Baltussen, je hebt me vaak geholpen bij HPLC-problemen, ik vind het erg leuk dat jij mij (ons!) nu als paranimf wil bijstaan tijdens de promotie. Dit laatste geldt ook voor Ronald Buijsse, die een belangrijke rol in mijn opvoeding opeist (maar die naar mijn mening toch discutabel is). Bij voorbaat dank voor jullie assistentie bij de voorbereidingen voor 26 februari.

Ook de rol van het 'thuisfront' (in de ruime betekenis van het woord) in de afgelopen jaren valt niet te bagatelliseren. Afstuderen, promoveren, melk, kaas, kaas en nog meer kaas..... het lijkt allemaal één pot nat (maar als jullie dit boekje gelezen hebben, praten we daar nog wel eens verder over). Jullie zorgden direct of indirect voor de broodnodige afleiding en ontspanning.

De laatste jaren heb ik me ook bezig mogen houden met de voedingskundige aspecten van melk/zuivel. Nienke, zo klein en levendig als je was is het je gelukt me zowel van het werk als aan het werk gehouden. Ik kan terug kijken op twee unieke jaren, het was en is hardstikke leuk om met en voor jou bezig te zijn! Binnenkort 2x zo leuk en levendig?

Coen, het rustgevende getik vanuit de computerhoek zit er op. Het zal vervangen worden door getok, plannen te over! Het schrijven van het proefschrift deed me vaak denken aan een lange fietstocht, met bergen en dalen, en met de laatste kilometers vals plat. Jij zorgde, nadat je de route ook al eens gefietst had en dus goed kende, als één der supporters langs de zijlijn voor de energievoorziening. Met de finish in zicht kan ik zeggen dat jij één van de trouwste supporters was. Voordat we de volgende route gaan verkennen, mag je je verdiende bonus in ontvangst nemen!

CURRICULUM VITAE

Carla Angèle Paula Buijsse is geboren op 31 januari 1966 te IJzendijke. In 1984 behaalde zij het diploma van het Voorbereidend Wetenschappelijk Onderwijs aan de Scholengemeenschap 'St. Eloy' te Oostburg. Aansluitend begon ze aan de studie Chemische Technologie aan de Hogere Technische School te Breda. Na de propadeuse begon zij in 1985 aan de studie Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. Aan het einde van 1991 studeerde zij af in de Zuivelkunde. Aansluitend was zij tot en met 1995 werkzaam als Assistent In Opleiding bij de toenmalige Sectie Zuivel en Levensmiddelennatuurkunde van de Landbouwuniversiteit Wageningen. Het in deze periode uitgevoerde promotie-onderzoek is beschreven in dit proefschrift. Dit promotie onderzoek werd uitgebreid met een opschalingsonderzoek (1996), waarvan de resultaten eveneens in dit proefschrift beschreven zijn. Sinds oktober 1998 is zij werkzaam bij de afdeling Technology Development and Quality van Numico Research te Wageningen.