The adsorption of lysozyme and chymosin onto emulsion droplets and their association with caseins

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The adsorption of chymosin and lysozyme onto emulsion droplets and their association with casein.

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STELLINGEN NN08201, 2592

1. De retentie van chymosine in wrongel wordt veroorzaakt door associatie van chymosine met para-κ-caseïne.
Dit proefschrift: hoofdstuk 5,6 en 7

2. De inaktivering van enzymen ten gevolge van adsorptie aan een grensvlak wordt niet noodzakelijkerwijs veroorzaakt door ontvouwing en spreiding van de peptideketen.
Dit proefschrift: hoofdstuk 3

3. Olie-in-water emulsies kunnen fungeren als een vloeibaar en food-grade dragermateriaal voor immobilisering van biologisch actieve eiwitten met behoud van aktiviteit.
Dit proefschrift: hoofdstuk 4 en 5

4. Het bepalen van associatieconstanten van gezuiverde caseïnes in oplossing geeft geen informatie over het associatiegedrag van caseïnes in melk.
Dit proefschrift: hoofdstuk 7

5. De associatie van chymosine met geadsorbeerde caseïnes wordt primair bepaald door de aard van het adsorbens.
Dit proefschrift en K.I. Larsson & A. Andrèn. Int. Dairy J. (1997), 7, 615.

6. De berekening door Kumosinski et al. van de drie-dimensionale structuur van een caseïne-submicel met behulp van "energy minimized techniques" heeft een lage voorspellende waarde.
T.F. Kumosinski, G. King and H.M. Farrell jr., J. Protein Chemistry, 13, 681 (1994).

7. De inaktivering van lysozym in kaas wordt niet veroorzaakt door associatie van lysozym met caseïnes, maar door zout afkomstig uit de pekel.
Dit proefschrift: hoofdstuk 4

8. De invloed van adsorptie op de drie-dimensionale conformatie van biologisch actieve eiwitten is goed te constateren door verandering van hun activiteit vanwege die adsorptie.

9. Het verhogen van de stabiliteit van een enzym verhoogt niet altijd zijn commerciële waarde.

10. De neiging van industriën om onderzoek, behorend bij hun kerncompetentie, uit te besteden kan bedreigend zijn voor het voortbestaan van die industrie.

11. De oer-Hollandse gewoonte van het prakken van het eten kan gezien worden als een ernstige vorm van fysisch voedselbederf door verevening van de samenstelling.

12. De evolutie maakte de vrouw niet alleen meer multi-tasking maar vooral ook multi-asking.

13. De stroming Cobra in de schilderkunst begint eerder bij Jean Dubuffet dan bij Karel Appel.

Stellingen behorende bij het proefschrift "The adsorption of lysozyme and chymosin onto emulsion droplets and their association with caseines" door A.L. de Roos.
Wageningen, 12 maart 1999.

Contents

Abstract

De Roos, A.L. (1999) The Adsorption of Lysozyme and Chymosin onto emulsion droplets and their association with caseins. Ph.D. thesis, Wageningen Agricultural University, Wageningen, The Netherlands. (pp.116, English and Dutch summaries).

Keywords:

enzyme immobilisation, emulsion droplet, casein micelle, chymosin, lysozyme, adsorption, association.

Abstract:

In this study the enzymes chymosin and lysozyme were immobilised on soya-oil emulsion droplets by means of direct adsorption onto the oil droplets'surface during homogenisation. This emulsion was stabilised not only using the enzymes as the only emulsifier, but also in combination with bovine serum albumin in various ratios of concentration, simultaneously and successively. In every system the adsorbed enzyme lost all activity *in situ*, and also after desorption from the oil/water interface by means of a detergent.

Soya-oil emulsion droplets stabilized with casein proved to be a good adsorbant for the enzymes studied. Chymosin bound only with emulsion droplets stabilized with κ-casein and was strongly dependent on pH and ionic strength. The extents of association are given in adsorption isotherms. The enzyme immobilized in this way showed *in situ* activity. Lysozyme associated with every type of casein-stabilized emulsion droplet in the order α_s -casein > β-casein > κ-casein. The association is less dependent on pH and ionic strength within the ranges tested. Lysozyme also proved to be fully active while associated, indicating that the active site is not involved in the association.

The characteristics of association of the enzymes with casein were worked out using casein micelles of various casein composition and the individual caseins in solution. Lysozyme only associates with α_s- and β-casein and not with κ-casein, the latter in contrast with the binding capacity of adsorbed κ-casein. Consequently, casein micelles of high κ-casein content bind less lysozyme. The extent of association of lysozyme with α_s- and β-casein is about equimolar. The association did not result in loss of activity.

As with adsorbed caseins chymosin only associates with κ-casein. The characteristics for association are more or less the same. The association with κ-casein in solution, however, decreases strongly with contact time, which is not the case with adsorbed κ-casein. This dissociation seemed to be very much related to the proteolytic activity of the enzyme and thus related to temperature, pH and extent of association. The association of chymosin with κ-casein is one in competition with the other caseins for κ-casein and is determined by the association constants for casein-casein and chymosin-κ-casein interactions. A kinetic model for competitive association further explains the effect of casein micelle compostion and the low extent of chymosin association with mixtures of caseins and casein micelles in solution.

GENERAL INTRODUCTION

Chapter 1

Abstract

Immobilization of biologically active proteins is widely used in industry. The main advantage of immobilization is that the proteins can be easily regained after having played their role in the biochemical process. Immobilization is mostly carried out with various solid carriers. These carriers as well as the chemicals used in the immobilization procedure can mostly not be considered as food grade. For this reason, application of immobilized biologically active proteins to play a role in the realization of food stuffs or in pharmaceutics may not be allowed. The aim of this study is to immobilize enzymes, while retaining activity, on carriers that have a food grade status. We used soya-oil droplets and protein aggregates (casein micelles) as the carrier particles, and chymosin and lysozyme as the enzymes.

1.1 Immobilization of biologically active proteins.

Immobilization of enzymes can be defined as the transfer of enzymes from a water-soluble, mobile state into a water-insoluble, immobile state. Immobilization deprives the enzymes of their diffusional mobility and facilitates recycling by simple means such as centrifugation or filtration. Moreover immobilization often increases the conformational stability of the enzyme molecule to protect them against influences of pH, temperature, ionic strength and shear forces. The food processing industry is the largest consumer of industrial enzymes and many applications of immobilized enzymes can be found in this area [1]. Also in pharmaceutical and personal care products, biologically active agents are immobilized by encapsulation (e.g. in liposomes) to bring about specific targetting and controlled release.
Various techniques of immobilization are applied: adsorption, covalent binding, ionic binding, cross-linking, entrapment and encapsulation. The technique based on physical adsorption is carried out by simply bringing the adsorbate into contact with the adsorbing material.

Commonly used adsorbents are solid materials like clay, alumina, glass, carbon and siliceous materials. A disadvantage is that the adsorbed material can be desorbed on dilution according to process conditions. Moreover, considerable loss of biological activity may occur due to partial unfolding. In some cases good results have been obtained (amylase and phosphatase immobilized on tricalcium phosphate gel particles [2]). An improvement may be found in covalently attaching the adsorbates to the solid materials, making use of attachment reagents. Care must be taken to protect the active centre of the enzyme from coupling with the attachment material. This can be achieved by coupling the enzyme in the presence of a competitive inhibitor or of substrate. A well-known example of the covalent attachment technique is the cyanogen-bromide treatment of Sephadex©. Multipoint covalent attachment to the adsorbate prevents the enzyme from unfolding and makes the enzyme far more stable than in solution. Cross-linking of enzymes with glutaraldehyde is commonly used to achieve water insoluble network aggregates that can be adsorbed onto various materials. Enzymes can be entrapped in materials like silica, starch or polyacrylamide gels during gelation triggered by change in temperature or gel-inducing chemicals. Encapsulation of dissolved enzymes can be achieved by the formation of polymer capsules around enzyme-containing microdroplets that are dispersed in a water-immiscible solvent. The polymer can be chosen to be impermeable for the enzyme, but permeable for its substrates and the products formed.

The intrinsic kinetic constants for an enzyme, K_m and V_{max} may change greatly due to immobilization, as compared to those for the enzyme in solution. In the case of immobilization by means of physical adsorption this change may be the outcome of a conformational change of the enzyme due to partial unfolding on the surface. But also other parameters will affect the working mechanism of an enzyme immobilized on the surface of a particle in a given process [3]. In a bioreactor a stagnant film exists around the adsorbent particle in a flow stream containing the substrate. The thickness of this film depends on the turbulence (Reynolds

number) of the surrounding liquid. The mass flow is proportional to the concentration gradient over the stagnant film, created by the difference in substrate concentration of the bulk and around the interface of the particle. For the latter the substrate concentration will be lower due to conversion by the immobilized enzymes. As a consequence of the stagnant film, the substrate concentration in the immediate surroundings of the particle is lower than in the bulk solution and conversion of substrate for these reasons will be slower than would follow from Michaelis-Menten kinetics in solution. Measured kinetic parameters in the bioreactor should therefore be corrected, because of this external diffusion limitation.

If an enzyme is also immobilized in the interior of the particle (e.g. by adsorption, covalent attachment or entrapment) a correction should also be made for the internal diffusion limitation. A radial substrate concentration gradient will exist inside the particle as a consequence of substrate conversion. The substrate concentration will be highest at the outside of the particle and lowest in the inside. For the product formed this concentration gradient will be the other way around. These gradients, and also the particle radius and its specific surface area, will affect the substrate mass flow and thereby the kinetic parameters. In the literature one often speaks of the apparent K_m' and V_{max}'.

The techniques for immobilization described in this thesis are the physical adsorption of enzymes onto soya-oil emulsion droplets and the association of these enzymes with caseins adsorbed onto these emulsion droplets. The phenomena of internal and external diffusion limitation do not play a role, because the enzyme-substrate reaction can only occur at the surface of the particle and the reactions are carried out in static experiments without turbulent flow of the bulk solution. In the case of enzymes associated with caseins in casein micelles, which are to be considered as penetrable protein aggregates, effects of internal diffusion limitation may play a role.

1.2 The aim and outline of the research

The study originated from an attempt to induce acceleration of cheese ripening by adding a crude sample containing exo- and endoprotease activity, isolated from starter bacteria as used in the process of cheese-making, to the cheese milk. It was observed, however, that almost all of the added enzymes remained behind in the whey fraction, and were not transported into the curd. It was reasoned that these enzymes could in principle be transported into the curd by immobilizing them onto milk fat globules assuming that the enzyme activity would be retained.

To study this possibility of immobilization in a more general and fundamental way two enzymes, bovine chymosin and hen's egg lysozyme were taken as the enzymes to immobilize. These two enzymes are well defined as to their amino acid sequences and their three dimensional structures. Lysozyme can be sonsidered as the most exclusively studied enzymes.

Other reasons for choosing these enzymes are their differences in properties such as molecular weight, isoelectric pH, effective hydrophobicity, working mechanism, cost and last but not least their conformational stability. Moreover, both enzymes play a significant role in dairy technology, chymosin as being the most important renneting enzyme in cheese production, and lysozyme to prevent "late blowing " due to gas production of *Clostridium tyrobutyricum* during cheese ripening.

Both enzymes are known to be transported into the curd during cheese making, most probably due to association with the caseins, which is the main protein fraction in the milk. This association behaviour makes the casein micelle, i.e. the aggregated form of the casein molecules, suitable as an adsorbate for enzyme immobilization. Other biologically active proteins such as plasmin, lipase, bacteriocins, nisin and, most probably many others, can also associate with caseins. This could make the casein micelle a more or less universal adsorbate.

Many studies on the adsorption of proteins onto various interfaces have been performed over the last decades. Most of these studies deal with fundamentals of protein adsorption onto solid/water and air/water interfaces. Studies of protein adsorption onto the oil/water interface are mainly related to food emulsions. Very few of these studies, however, deal with the biological activity of the adsorbed proteins. Lysozyme is often used in adsorption studies on various interfaces, but mainly because of its properties as a protein and not for its role as an enzyme. To the authors' knowledge the immobilization of enzymes by means of adsorption directly onto the oil surface, that means, using the protein as emulsifier, has never been reported previously.

In this thesis the immobilization of chymosin and lysozyme with three different carrier systems is described. In the first system the enzymes are used as the emulsifiers to stabilize soya-oil emulsion droplets. This system can be regarded as a method of immobilization by physical adsorption. The enzymes adsorb onto the oil surface by means of hydrophobic interactions, which are for the main part entropically driven. In the second system the enzymes associate with casein molecules adsorbed onto the oil surface. This type of immobilization is for the greater part due to electrostatic interactions. The adsorbed casein molecule serves as an intermediate ligand. In the third system, the casein micelle, which is an aggregate of different casein molecules, is used as a carrier system that immobilizes the enzymes. We have to keep in mind that a casein molecule adsorbed onto the emulsion droplet may have changed its conformation due to partial unfolding; it may thus be exposed to the enzymes in a different manner compared to the casein molecules in casein micelles.

In chapter II a review of literature on the properties of the proteins (chymosin, lysozyme, the caseins and the casein micelle and bovine serum albumin) used in this study will be given. Furthermore theoretical aspects of adsorption of proteins in general and of enzymes in particular onto the various interfaces, of the adsorption isotherm and of emulsification will be derived. Protein-protein interactions and the stability of proteins in solution will also be

described. In this chapter general aspects of the methods used in the study will be outlined, although most methodology will be described in detail in the succeeding chapters, which are a compilation of journal publications.
Chapter III deals with the loss of enzyme activity due to adsorption onto soya-oil emulsion droplets.

The association of chymosin and lysozyme with adsorbed caseins and of lysozyme with caseins and artificially made casein micelles in solution is described in chapters IV and V. Less attention is paid to establish the kinetic or apparent kinetic parameters K_m' and V_{max}' of the enzymes immobilized in this way.

Dairy technologists have known about the transfer of chymosin into the curd during cheese-making for a long time; it was supposed to occur by means of association with the casein molecules in the casein micelle [4]. The mechanism of association, however, had not been worked out. Also influences of temperature, pH, ionic strength and of casein concentration on the transfer yield have been observed [5], without satisfactory explanations having been given. In chapters VI, VII attempts are made to answer these questions. To this end the association of chymosin with caseins in solution, resulting in a kinetic association model, and with synthetic casein micelles of various composition, was studied as a function of pH, temperature and casein concentration.

1.3 References

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THEORETICAL ASPECTS

Chapter 2

2.1 Interfacial phenomena

A surface is characterized by its surface tension γ. Surface tension and its equivalent surface free energy are often defined as the work required to increase the area of a surface [1]. The higher energy state of molecules in a surface arises because they have fewer nearest neighbour interactions than bulk molecules. This leads to a net attractive force that manifests itself as a two-dimensional tension ($N.m^{-1}$), which is numerically equal to the Gibbs free energy of the surface ($J.m^{-2}$). The value of γ is related to the nature of the attractive forces of the liquid. The surface tension of liquid helium, solely due to London forces, is very small. In organic liquids with contributions from dipole-dipole interactions, the surface tension is higher (20-30 $mN.m^{-1}$), and in water, with strong hydrogen bonds, the surface tension is 72 $mN.m^{-1}$. By and large, the stronger the cohesive forces, the greater the surface tension. In general, the surface tension increases as temperature is lowered. The interfacial tension between two immiscible liquids often lies between the individual surface tensions of these liquids.

When a solute, dissolved in either of two liquids in contact, accumulates in the interface between them, the interfacial tension decreases. The adsorbed substance is said to be surface active. The amount of interfacial tension decrease Π is called the surface pressure:

$$\Pi \simeq \gamma_0 - \gamma, \qquad (1)$$

in which γ_0 is the interfacial tension of the clean interface. The surface pressure can indeed be measured as a two-dimensional pressure between bars enclosing a certain interfacial area. The amount of adsorbed material, called the surface excess or surface load Γ (moles per unit interfacial area) is related to the activity a of the surface active material and the change in γ according to Gibbs:

$$-d\gamma = d\Pi = RT\Gamma d\ln a, \qquad (2)$$

in which R and T have their usual meaning. This relation only holds if one surfactant is present, and under equilibrium conditions. The decrease in interfacial tension continues until the solute activity does not increase further, either because the critical micelle concentration or the solubility limit is reached. At that point the interface is almost fully packed with surfactant.

An apolar molecule or a molecular group in an aqueous environment will cause rearrangements of the orientation of the adjacent water molecules, which exist as hydrogen bonded "aggregates". The accommodation of the apolar molecule will restrict the rotation of the water molecule along the O-H-O axis and thereby decrease entropy. Moreover, because of disturbed hydrogen bonds, lone pairs of electrons and protons will increase the potential

energy of water molecules. These two unfavourable thermodynamic factors increase the total free energy of the system. In order to restore the former situation the water molecules tend to eliminate the apolar molecule from the aqueous phase. This is called the hydrophobic effect. For an amphiphilic molecule, with a hydrophilic head and a hydrophobic tail, it will be smaller. The molecule will remain in solution when the decrease of the free energy for the water-hydrophilic head interaction outweighs the free energy increase due to the water-hydrophobic tail interaction. When, however, the amphiphilic molecule can reach an apolar interface, a restoration of rotational freedom and dipole interactions of the water molecules will increase their entropy and thereby lower the free energy of the whole system. Adsorption of surface active molecules from an aqueous phase therefore is predominantly entropy driven.

Detergents or soaps lower the surface tension, the more so for a higher concentration, until a critical micelle concentration (CMC) is reached. When from that point on the concentration is raised, the added amphiphilic molecules will form micelles and thus the bulk activity a and the surface tension will remain virtually constant. In micelles the detergent molecules will arrange in such a way that the hydrophobic tails group together, away from water contact, and the hydrophilic heads will be directed towards the water. The same will happen at an interface. The hydrophobic tails will protrude into a hydrophobic phase such as oil.

When the surface excess at the air/water interface is relatively high (condensed state), the hydrophilic heads will remain in the water phase and the hydrophobic tails protrude into the air to form a hydrophobic layer. When the surface excess is relatively small (expanded state) the apolar tails will lie flat on the surface to form a film, held together by the attractive forces of the apolar tails. Several detergents lower the surface tension of water to 30 - 40 $mN.m^{-1}$ before they reach their CMC.

2.2 Protein adsorption

Although most proteins are soluble in water, they can be spread as a monolayer on the surface. In early studies most research on monolayers was done with spread monolayers, because of problems in directly quantifying protein concentrations on the surface. An advantage of spreading a monolayer is the known amount of protein added to a given area of surface. On the other hand, spread monolayers would rarely be formed outside the laboratory. In most practical and biological systems monolayers are formed by adsorption in which the development in time is an important aspect. Moreover it is hard to avoid desorption of protein molecules into the bulk phase [2].

Nowadays, the study of adsorption or monolayer formation can be achieved by monitoring the change in protein concentration in the surface by methods such as ellipsometry or radioactive tracer measurement. Adsorption kinetics involves the following subprocesses:

a) the diffusion of the molecules of a subsurface region to the surface layer, b) the actual adsorption of the protein onto the surface and c) rearrangements of the adsorbed molecules with time with corresponding conformational changes [3]. Because of this last phenomenon kinetic research on monolayer formation cannot be carried out directly by surface tension measurements. A change in surface tension is not solely determined by a varying concentration of protein in the surface but also by a change in conformation of already adsorbed proteins with time. To obtain a conformation resulting in lowest free energy can take considerable time. Moreover a change in interfacial tension only becomes measurable when a considerable amount of protein molecules has been adsorbed already. Values of Π greater than 1 $mN.m^{-1}$ are to be expected only at surface coverages above some 80 % of monolayer coverage [4]. Proteins are far more surface active than small molecule surfactants. This means that a measurable adsorption is developed at bulk mass concentrations approximately two orders of magnitude smaller than for these surfactants On the other hand, most soaps have a higher capacity for lowering surface tension [5], presumably due to a higher packing density.

The time needed for a macromolecule to adsorb onto a clean surface can be estimated using the equation

$$(\Gamma / c)^2 = D\, t_{0.5} \qquad (3)$$

in which c is the surfactant bulk concentration, D the diffusion coefficient and $t_{0.5}$ the time needed to cover half the still uncovered part of the surface. Γ/c expresses the thickness of the liquid layer fom which the adsorbing surfactant is provided. The total adsorption time would be a low multiple of $t_{0.5}$, and for common parameter values this leads to a time of the order of 0.01 s [5].

The Gibbs equation for adsorption (eq. 2), which gives the relation between surface pressure and bulk concentration, is based on an equilibrium situation of adsorption and desorption of one species of molecules at an interface under constant conditions. However, a protein cannot be considered as one species, since it is accompanied by counterions and the number of counterions generally changes upon adsorption. Moreover, desorption of protein will occur very reluctantly when the bulk concentration is lowered by dilution. The reason for this "irreversibility" is the very high surface activity of the protein. Diluting needs to be done to very low bulk concentrations, resulting in a very small driving force for desorption. A very small desorption from the interface may already result in a bulk concentration that is in equilibrium with the surface. In an emulsion, with larger surface area, this will happen even sooner. Moreover, the desorption of a protein molecule generally takes a long time, the more so for larger molecules, and this may be longer than the duration of the experiment. This does not exclude exchange of one molecule for another. For β-casein an exchange of protein with the air/water interface and the bulk solution, measured with radio-active protein, was found. For lysozyme this was not the case [6].

An adsorption isotherm gives the relation between surface excess and the bulk concentration of the adsorbent in equilibrium with the adsorbate. The slope of the initial part of the curve gives an indication for the affinity of the adsorbent for the adsorbate. In the case of protein adsorption, the slope generally rises very steeply and is said to have high-affinity character. At low initial protein concentrations, most of the protein will be adsorbed onto the interface, leaving miniscule amounts in the bulk solution. When the protein concentration is raised the isotherm will reach a plateau value, indicating that full coverage (Γ_{plat}) of the available interfacial area is reached. Although a protein adsorption isotherm looks very much like the Langmuir adsorption isotherm for gas adsorption, one of the basic concepts does not apply, namely the absence of mutual interactions between adsorbed molecules. In particular, cross-linking reactions between protein molecules, which are very close to each other in the interface, may prevent desorption.

Desorption of protein from an air/water or oil/water interface can occur when a substance with a greater capacity for lowering interfacial tension (for instance a small-molecule surfactant) adsorbs onto the interface. Surfactants can attain a much higher surface coverage and in this manner push away the adsorbed protein. When the interfacial area is decreased, as can be done between the bars of a Langmuir trough [7], at constant surface pressure, desorption can also occur. This diffusion controlled desorption occurs faster for a smaller protein molecule and a higher surface pressure. Proteins adsorbed onto solid/water interfaces by means of electrostatic interactions with specific adsorption sites or ligands can be desorbed by changing the solvent conditions (pH, ionic strength) of the solution.

Often, different proteins do not displace each other, because they lower surface tension to more or less the same extent. But when an emulsion is made with a mixture of α_s-casein and β-casein, used as the emulsifying agent, β-casein proves to be adsorbed preferentially. Moreover it appears that α_s-casein will get exchanged by β-casein to a greater extent than when the experiment is carried out the other way round [8]. β-Casein can also displace α-lactalbumin but not β-lactoglobulin. In competition experiments, where gelatin and β-casein are adsorbed at the same time during emulsification, the proteins initially adsorb to the same extent. In the later stages gelatin is displaced by β-casein [9]. This behaviour can be explained by β-casein reducing the interfacial tension to a lower level (the difference in γ is about 13 $mN.m^{-1}$ between β-casein and gelatin for a paraffin oil/water interface).

The forces involved in adsorption of protein onto a clean air/water or oil/water interface are presumably for a large part due to the hydrophobic effect. Electrostatic intermolecular repulsion, in case of a solvent pH far away from the iso-electric pH of the protein involved, can hamper further adsorption onto the interface. It is said that an additional electrostatic potential barrier is built up [10]. It was shown that the adsorption rate of β-lactoglobulin was fastest near its iso-electric pH [11], where the protein has no net charge. Solvent conditions will strongly influence protein adsorption onto solid/liquid interfaces, because adsorption takes place at specific binding sites [12]. Special advantage of this

phenomenon is taken in ion-exchange and affinity chromatography, where adsorbed protein can be eluted by changing solvent conditions.

2.3 Protein conformation and its change on adsorption

After the initial segmental adsorption a protein can unfold to various extents. This is often depicted in a loop, train and tail model, but it is questionable as to whether this is always realistic. The extent of unfolding depends on the conformational stability of the protein. Due to the hydrophobic effect a protein in solution is folded into its so-called tertiairy structure, in which many of its hydrophobic parts are in the interior [13]. The polar amino acid side groups will be orientated, if possible, at the periphery of the molecule, shielding the hydrophobic interior from water exposure. In general it can be said that the larger the molecule the higher proportion of hydrophobic groups buried inside the molecule. In most proteins the hydrophobic parts are closely packed, resulting in a globular structure and relatively few internal water molecules. α-Helix, β-sheet and β-turn conformations are distinguishable. These conformations, due to internal hydrogen bonding, are generally found in the interior of the molecule and cause a large decrease in rotational and conformational freedom and consequently a loss in entropy. The water-entropy driven process of protein structure stabilization is counteracted by the loss of conformational freedom of the polypeptide chain. These antagonistic, large free energy contributions compensate each other almost entirely, resulting in marginal thermodynamical stability [12]. This means that in protein engineering only one strategic mutation, resulting in for instance an extra hydrogen bond formation, can lead to a significant change in thermal stability of a protein. Some thermophilic proteins differ from their mesophilic analogues by only a few salt bridges [13]. It also means that protein stability can easily be decreased due to changes in temperature, ionic strength or pH. The presence of an interface is another external circumstance that influences protein stability and, consequently, its conformation. On adsorption, the internal hydrophobic parts tend to become anchored onto or even to protrude for a small part into the hydrophobic phase, resulting in a change of tertiairy structure of the molecule. Depending on the conformational stability of the protein, the extent of unfolding ranges from almost full stretching (gelatin, casein) to almost complete retention of a globular shape (lysozyme).

The extent of unfolding is also determined by the time available. In a static system adsorption is diffusion controled and time required for adsorption is inversely proportional to the square of the bulk concentration (eq. 3). At high bulk concentration the interface may be fully packed before complete unfolding has occurred. In other words the extent of unfolding is very much influenced by the surface area available for the protein. Very little is known about

the conformation of a protein after having been adsorbed. HPLC analysis of peptide fragments split off from adsorbed α_s- casein [14] and β-casein [15] by means of tryptic digestion, has led to an impression of the protein conformation when adsorbed, assuming that only the protruding loop and tail parts could be attacked by the enzyme.

An equation of state, which relates surface pressure Π and surface excess Γ, has been derived by De Feijter and Benjamins [16]. They developed a "soft particle model" in which the radius of the protein molecule may change if the surface concentration is varied. They fitted experimental data found for Π and Γ for β-casein and ovalbumin at the air/water interface, with an equation put forward by Helfland [17] and developed the "soft particle" theory in order to explain the S-shaped equation of state curve.

$$\Pi = RT\,\Gamma/(1-\Theta)^2 \qquad (4)$$

with

$$\Theta = \pi r^2 N_{AV} \Gamma \qquad (4a)$$

where Γ is in mol.m^{-2}; R, T and N_{AV} have their usual meaning, r is the radius of a protein molecule in the interface and Θ is the surface fraction, which is the ratio of the surface occupied by protein to the available surface. It follows that the value of Θ is overriding in determining the magnitude of Π. For instance, for a value of Γ of 2 mg.m^{-2} as caused by a protein molecule of 40 kDa, $\Gamma \approx 5.10^{-8}$ mol.m^{-2}; then $RT\,\Gamma$ would equal about 0.1 mN.m^{-2}, whereas actually Π would equal about 25 mN.m^{-2}. Eq. (4) than would give $\Theta = 0.93$, and r = 3.1 nm. From the equation of state and the observed value of Π, the radius of the protein molecules adsorbed onto the interface can thus be estimated. The surface fraction will be high for a high value of Π and depending on the conformational stability of the protein, the radius of the adsorbed molecules may be larger than in solution, the more so for a lower value of Γ.

2.4 Adsorption of biologically active proteins

The study of the working mechanism and kinetics of enzymes is normally carries out in an aqueous phase under controlled environmental conditions. Conditions such as pH, temperature, ionic strength and substrate concentration will mostly be fairly optimal and may differ considerably from those in the physiological environment. In their actual cellular environment enzymes will often perform quite differently, under less ideal conditions. Surface phenomena in the living cell will alter protein conformation and hence specific enzyme activities. Since many enzymes operate at the interface between organelle membranes in aqueous surroundings, it is of importance to study enzymes at interfaces.

The amphipathic character of proteins (enzymes) will make them adsorb at air/water, oil/water and most solid/water interfaces. In this adsorption process, the reorientation of various parts of the enzyme molecule over the two different phases can interfere with the key-lock interaction of the enzyme-substrate complex, resulting in partial or total inactivation of the enzyme. The change in conformation mainly concerns the tertiary structure of the protein. The secondary structures, α-helix and β-sheet, are generally not greatly altered during adsorption [5].

It was concluded in an extensive review, at that time (1966), that little research on the behaviour of enzymes at interfaces had been carried out [18]. There are three ways of studying biological activity at liquid/liquid interfaces: 1) the interface is the substrate and the enzyme is in the bulk phase, 2) the interface holds the enzyme and the substrate is in the bulk phase (measurement *in situ*) and 3) transfer of enzyme spread on the interface onto, for instance,a wire loop, a silk net or onto glass or metal slides, followed by dissolution and activity determination.

Several studies were carried out in which an enzyme solution was injected under a spread monolayer of substrate. Generally it was found that enzyme-substrate reactions did not differ greatly from bulk situations. Normal dependencies on pH and enzyme concentration were found. At surface pressures of phosphatidylcholine of $\Pi < 3$ and $\Pi > 28$ mN.m^{-1}, no phospholipase activity was found, most probably because at low pressure the enzyme is irreversibly denatured at the air/water interface by (partial) unfolding and at too high a pressure the enzyme can not penetrate into the adsorption layer [19].

A main problem of measurement of enzyme activity *in situ* in monolayers is to maintain all enzyme molecules in the monolayer. The Trurnit method of spreading will never completely prevent dissolution of the molecules in the bulk phase. In a first study on the activity of spread acetylcholinesterase [20], a special four compartment trough was constructed in which a monolayer could be transported onto a new bulk phase. For a surface pressure of 10 mN.m^{-1}, an enzyme activity of 50% was found, compared to bulk situations. At lower and higher surface pressures enzyme activity was decreased. Zero activity was found at surface pressures < 2 mN.m^{-1}. In a comparable study [21], trypsin was observed to be fully inactivated. After desorption from the interface no recovery of enzyme activity was found. Full activity of lipase was found at the air/water interface, but not of lysozyme [22]. Hunter et al. [23] reported 30-60% residual activity for lysozyme after depositing and resuspension. A higher extent of denaturation was found for a lower surface pressure.

Several studies have been carried out on biological activity at interfaces after depositing the monolayers on glass or metal slides. The main problem of this technique is that it is hard to estimate the denaturing effect of adsorption on solid supports. No distinction can be made between the denaturation due to adsorption onto the interface studied or onto the support [18, 24]. Moreover, residual activities found on the supports do not reflect the actual

situation at the air/water interface. Even recovery of enzyme activity might have occurred. For several enzymes residual activities on the slides were found.

Apart from lipases and phospholipases there is hardly any literature that describes activity of enzymes adsorbed onto the oil/water interface. Gosh and Bull [25] studied the influence of a n-octadecane emulsion added to solutions of chymotrypsin in various mixing ratios. They found a strong inhibiting effect upon addition of the emulsion, going down to zero residual activity in the bulk solution, because of continuous chymotryptic digestion of unfolded enzymes at the interface. The peptides formed in this way were displaced by the intact enzymes that, in turn, became digested themselves. Electrophoretic mobility experiments showed a change in iso-electric pH of the emulsion droplets covered with the enzyme as compared to the enzyme itself, presumably because of a change of orientation of the enzyme molecule at the surface. At higher protein to emulsion ratios an increase of chymotrypsin activity *in situ* (up to 17 %) was found after washing the unadsorbed enzyme away. It was reasoned that at high concentrations of chymotrypsin, time of unfolding was shorter than time of interface coverage. On the other hand, the washing procedure was not very intensive.

In studies investigating the influence of biphasic systems, consisting of water and water immiscible organic solvents, on acid phosphatase, β-glucosidase and β-fructofuranosidase [26], complete inactivation of the enzymes was found after a few hours of intensive stirring. The stronger the immiscibility of paraffinic solvents like n-octane, n-tetradecane and n-hexadecane with water, the stronger the inactivation effect.

Sandwick [27] examined the interactions of several enzymes with solid, hydrophobic latex particles, by explicitly taking the enzyme activities as a probe for this interaction. The enzymes alkaline phosphatase, β-galactosidase, lysozyme, horseradish peroxidase, catalase and glucose-6-phosphate dehydrogenase were adsorbed in various concentrations to create inactivation isotherms, which were shown to be of a non-Langmuirian type. For most enzymes the extent of inactivation was less for higher initial bulk concentration. It was reasoned that at low concentrations the enzyme molecules had sufficient time to unfold at the hydrophobic surface, whereas at higher concentrations the rate of arrival became equal and then faster than the rate of unfolding. It was found also that the extent of conformational change was dependent on the mol mass, and thus the size, of the protein molecules, although lysozyme seemed to be an exception. Similar extents of inactivation were found for positively as well as negatively charged hydrophobic surfaces. Unfortunately, in this study, no enzyme activity was measured *in situ* after a washing procedure for the enzyme covered latex particles.

2.5 Protein adsorption onto emulsion droplets

An emulsion is a dispersion of one liquid in another with which it is immiscible. Thermodynamically seen such a dispersion is unstable and it needs a third component, a surfactant or emulsifier, to become more stable. In food emulsions the immiscible phase and

the emulsifier agents consist of many components. Milk can serve as a classical example of an oil/water food emulsion, in which the lipid phase is dispersed in the aqueous bulk phase. In a water/oil food emulsion, like butter, water is dispersed in the oily phase. The type of an emulsion is determined by the solubility of the emulsifier in either phases (Bancroft's rule). A protein, which is soluble in the aqueous phase, can only form O/W emulsions. Emulsions can also be classified as micro-, mini- and macro-emulsions by the size of the dispersed droplets, ranging from several nanometers to several micrometers.

The stability of an emulsion is, among other things, dependent on the size of the droplets. The smaller the droplets, the greater the stability of the emulsion against coalescence and creaming. It is easy to make coarse droplets, but to make them small enough to achieve stability needs a lot of energy input, most of which is dissipated in the form of heat [28]. The stress applied to deform and break up droplets is counteracted by the Laplace pressure, which is the difference in pressure between the concave and the convex side of the droplet. The smaller the droplet and the greater its interfacial tension the higher its Laplace pressure and the more energy is needed to cause deformation of the droplet. Addition of surfactants to lower the interfacial tension between the oil and water phase will facilitate droplet deformation. A Weber number (We) can be defined as the ratio of the external stress over the Laplace pressure. Beyond a critical Weber number, the value of which depends on the type of flow and the ratio of the drop and continuous phase viscosities, droplet burst can occur. The making of an emulsion is mostly done in high-speed stirrers or high-pressure homogenizers, which cause large external stresses on the droplets. The stress can be caused by a velocity gradient in laminar flow, and is then of shear type, or can be due to pressure fluctuations, of a size comparable to that of droplets, i.e. inertial forces, in turbulent flow caused by turbulent eddies. The latter mechanism is predominant in the process of industrial homogenization of oil/water emulsions. The turbulent field is fairly heterogeneous, causing a distribution of droplet sizes.

In the process of emulsification three main processes occur simultaneously:

1) Droplets are deformed and broken up.

2) Surfactant molecules are transported to and adsorbed onto the droplets, predominantly by convection.

3) Droplets encounter each other and may coalesce if surface coverage is small.

During emulsification the available area for the adsorbed protein will change, caused by droplet break up and coalescence. This means that the extent of unfolding of the protein adsorbed will vary with the changing surface area. Coalescence, in which two droplets fuse into a larger one, leads to a decrease in surface area, causing compression of the surfactant molecules. This compression produces an increase in surface pressure due to a higher packing density of the molecules and less space available for unfolding. Compression can also lead to multilayer formation, and even desorption seems possible. Desorption due to compression of an air/water interface area was demonstrated in slow macroscopic experiments [7]. The forces exerted on the adsorbed proteins may cause (some) denaturation, which may have a severe

impact on their biological activity if the adsorbed proteins are enzymes. During emulsification, in which homogenization valve passages may be repeated several times, these effects may proliferate.

As the literature does not provide studies of biological activity of directly adsorbed enzymes on emulsion droplets we can compare our results only with the few studies done in static conditions at the A/W and S/W interfaces. Although characteristics of protein adsorption in static conditions will resemble adsorption during emulsification, several aspects will be quite different. As mentioned before (eq. 3), adsorption in a static experiment will be diffusion controlled and relatively slow (order of a second), whereas during emulsification convection is the driving force leading to extremely fast adsorption. The approximate time needed to cover the droplets' surface is given by [28]:

$$t_{ads} = 10\,\Gamma\,\eta_c{}^{1/2}\,/\,d\,c\,\varepsilon^{1/3} \qquad (5)$$

where Γ is the surface load ($kg.m^{-2}$), η_c is the viscosity of the continuous phase (Pa.s), d is the diameter of the droplet (m), c is the concentration of the surfactant ($kg.m^{-3}$) and ε is the amount of energy dissipated per unit volume and per unit time ($W.m^{-3}$). Substituting reasonable values this will lead to an adsorption time of about 10^{-6} s or less. The very short time needed for adsorption may affect the extent of unfolding of the protein molecule. The extent of unfolding depends on the surface area available, thus on surface load, and full surface coverage may have been reached before unfolding could have occurred. This may raise possibilities for retention of biological activity in the case of emulsification with enzymes, especially for enzymes with considerable conformational stability. In contrast to static conditions, surface coverage due to convection may lead to preferential adsorption of the larger proteins in the case of emulsification with mixtures of proteins, although the preference will be marginal. Moreover, in static conditions the molecules with the greatest ability to lower the interfacial tension will preferentially adsorb, and it is uncertain whether this is also the case during emulsification. Another aspect is that in an emulsion the surface to volume ratio is higher, leading to different extents of depletion for the surface active components in the bulk phase. These aspects may cause different surface coverage for the two systems, i.e. the surface load as well as composition.

On solid surfaces a protein adsorbs at specific adsorption sites, whereas at the oil/water interface hydrophobic domains or amino acid residues may protrude to some extent into the oil phase. Since the air/water and oil/water interfaces are homogeneous and fluid they offer more possibilities for unfolding.

The suitability of a protein to stabilize emulsion droplets depends on intrinsic properties of the protein, like its effective hydrophobicity, its solubility, its flexibility and its ability to form intramolecular bonds during the interfacial film formation. These intrinsic factors are, in turn, influenced by external conditions such as pH, temperature, ionic strength

and protein concentration. The surface hydrophobicity, being a measure of the number of non polar amino acid groups at the periphery of the protein molecule, is alleged to correlate with its emulsifying ability [29]. The higher the surface hydrophobicity the greater the emulsifying activity would be, but clear exceptions to this rule have been observed e.g. for β-lactoglobulin [30].

The caseins are known for being hydrophobic and disordered. They contain a high proportion of proline residues, which hampers the formation of α-helix and β-sheet secondary structure. Moreover, most caseins do not contain cysteine residues and consequently no S-S bridges. Only κ- and α_{s2}-casein molecules contain two cysteine residues by which means they can polymerize. All caseins are phosphorylated to varying extents at the serine residues. In β- and κ-casein, the phosphorylated residues and the hydrophobic groups are not evenly distributed over the molecule, but are for the main part in separate regions along the chain. This will make these molecules behave like huge soap molecules with a hydrophilic and a hydrophobic part. These properties presumably make the caseins very suitable for stabilizing emulsions [31].

2.6 Milk proteins

Bovine milk contains about 33 g of protein per liter. About 80% of these proteins are caseins and the remaining 20% are the so-called serum proteins. Most of the latter leak away in the whey fraction during the normal process of cheese making and are called whey proteins for that reason. The whey proteins are, in order of decreasing concentrations, β-lactoglobulin (2-4 g/l), α-lactalbumin (1-1,5 g/l), immunoglobulins (0.6-1.0 g/l) and serum albumin (0.1-0.4 g/l), and all of them are globular proteins. Two genetic variants of α-lactalbumin and six of β-lactoglobulin are known [32].

The bovine caseins may be subdivided in four species of phosphoproteins that exist in agglomerates, called casein micelles. The four species α_{s1}-, α_{s2}-, β- and κ-casein occur in relative molar concentrations of about 4:1:4:1.6. The caseins become insoluble at a pH near their isoelectric point (pH ~ 4.6), of which property use is made in the production of some food stuffs (e.g. cottage cheese). The primary structures of the caseins are completely known, but as they cannot be crystallized little is known for sure about any secondary and tertiary structure. From circular dichroism, infrared spectroscopy and NMR studies [32] it is known that caseins in solution possess an open conformation with a high degree of conformational freedom of the side chains, having little α-helix structure. The latter is presumably due to the high content of proline residues, which hamper the formation of secondary structure. Because of this absence of organised structures the caseins are very insensitive for denaturation by heat. The amino acids are not randomly distributed over the molecule, but are grouped together in fairly hydrophilic and hydrophobic sequences. By means of molecular modelling via energy minimization techniques and molecular dynamics, based on results from Raman and Fourier

Transform Infrared spectroscopy, attempts have been made to predict the three dimensional structure of the individual caseins and even the casein submicelle, mimicking protein-salt-water interactions [34, 35, 36].

Isolation of the caseins can be done by various methods, making use of differences in solubility as a result of differing dependencies on pH, calcium ion and urea concentration. The identification of the types of caseins and their genetic variants was originally based on their mobility in various types of gel zone electrophoresis [32]. Further developments in isolating the caseins arose with ion-exchange chromatography by taking advantage of the knowledge gained from electrophoretic behaviour. Although separation techniques have greatly improved, it is still hard to achieve complete resolution of the caseins. Commercially available isolated fractions of caseins still contain impurities of other caseins. The presence of such impurities needs to be considered in experimental research.

The caseins can be divided into Ca^{2+}-sensitive and insensitive caseins. The α_{s1} -, α_{s2} - and β-caseins precipitate in solution in the presence of Ca^{2+}, whereas κ-casein will not. The sensitivity for Ca^{2+} is related to the extent of phosphorylation, which is mainly at seryl residues with an acidic glutamyl or a phosphoseryl residue in their vicinity. It is presumed that the sites of phosphorylation will be on flexible, external loops of the molecule, which are accessible for Ca^{2+}-ions [37]. The three-dimensional predictions of the casein submicelle by Kumosinski et al.[35] also show flexible loops, that would be accessible for Ca^{2+}-ion binding. α_{s1}-Casein has at least five genetic variants of which type B is predominant. The molecule consists of two fairly hydrophobic regions (residues 1-44 and 90-199) and a fairly hydrophilic region (residues 45-89) in which seven of the eight phosphorylated serine residues are located. The solubility is very much dependent on pH, ionic strength and Ca^{2+}activity and is considerably less than for the other caseins. Above pH 5, the solubility sharply increases and it is slightly higher at a lower temperature. At neutral pH the molecule binds 8 moles of Ca^{2+} and it is readily precipitated above a Ca^{2+}- ion concentration of 5 mM. The genetic variant type A is less sensitive to Ca^{2+}-ion concentration [36]. The self-association behaviour of α_{s1}-casein molecules depends on pH and ionic strength, but is not significantly affected by temperature [38]. The molecule contains 17 prolyl residues leading to a very open structure.

Four variants of α_{s2}-casein are known, as it is phosphorylated to various extents (10 - 13 mol P/mol). The casein molecule is very sensitive for Ca^{2+}- ion precipitation and readily self-associates to polymers of increasing size as concentration increases. It possesses one cystine and 10 prolyl residues resulting in a higher content of α-helix structure compared to α_{s1}-casein. Of all caseins α_{s2}-casein has been studied the least. In our study we used a mixture of α_{s1}- and α_{s2}-casein.

Bovine β-casein contains a hydrophilic, highly charged N-terminal part of about 47 residues, including all the phosphorylated serine residues, but only one of the 37 proline residues. Most hydrophobic groups are located in the C-terminal remainder of the molecule. The solubility decreases with increasing temperature. This effect is most pronounced between 5

and 15 °C. At a pH above the iso-electric pH solubility sharply increases. The molecule can be regarded as a huge surfactant molecule with a polar head and a non-polar tail. The self-association of β-casein molecules also resembles that of ionic surfactants, leading to micelle formation depending on concentration and on temperature. The critical micelle concentration decreases with increasing temperature and/or ionic strength [38]. At 4 °C, in the absence of Ca^{2+}, only monomers occur, but at room temperature micelles will form consisting of about 23 monomers and an average polymer radius of 17 nm [39]. The temperature dependence of micelle formation indicates that hydrophobic forces are mainly involved. β-Casein is a substrate for the proteolytic enzyme plasmin in milk, resulting in the casein fragments γ-caseins and proteose peptones. α_s-Caseins are also attacked by plasmin, but more slowly.

Two principal genetic variants, A and B, are known for κ-casein. In variant B Ile 136 is replaced by Thr and Asp 148 by Ala. The C-terminal end of the protein, called the caseino-macropeptide, has a high negative charge density. The N-terminal part of the molecule, called para-κ-casein, is slightly positively charged at neutral pH and is fairly hydrophobic, resulting in an amphiphilic nature for the molecule, like β-casein. In comparison with the other caseins, κ-casein almost lacks phosphoseryl groups (only one SerP at position 149) and it is, consequently, rather insensitive to Ca^{2+}. The solubility of κ-casein increases sharply above the iso-electric pH and increases, only in the restricted pH range of 4.7 - 5.2, as temperature is lowered [40]. In contrast to α_{s1}- and β-caseins, the κ-casein molecule contains two cysteine residues (Cys 11 and Cys 88). They are both positioned near the surface of the molecule [33, 34] in the hydrophobic part of the molecule, facilitating random interchain linking of different κ-casein molecules via S-S- bridging [41]. These disulfide bonds may lead to polymers ranging from 60 kD (trimers) to 150 kD (octamers) and probably even more. These polymers can further cluster to form particles of about 650 kD by noncovalent association [42].

Of all caseins, only κ-casein is a glycoprotein, glycosylated at the caseinomacropeptide part and making it highly charged and soluble. Glycosylation occurs at Thr 131, Thr-133, Thr-135 (or 136) and Ser-141 (or 142) residues [37]. The extent of glycosylation varies considerably from zero to five sialic acids, which are *N*-acyl derivatives of neuraminic acids. The carbohydrate moiety is normally composed of the monosaccharides *N*-acylneuraminic acid, galactose and *N*-acetylgalactosamine. The varying extent of glycosylation leads to various electronegative charge values and, hence, varying electrophoretic and chromatographic behaviour.

The junction of the caseinomacropeptide part of κ-casein to para-κ-casein is located at the Phe-105-Met-106 bond. At physiological pH, this bond is specifically split by aspartyl proteinases such as chymosin or pepsin. This cleavage is the basis of milk renneting and cheese production. Studies on the mechanism of this enzyme-substrate reaction and on κ-casein secondary structure predictions have relied on the use of synthetic peptide substrates, of varying sizes and amino acid composition, analogous to the cleavage region of

bovine κ-casein [43, 44]. The search for synthetic substrates derived from the amino acid sequence around the labile Phe-Met bond in κ-casein revealed that a minimum of at least five

His-Pro-His-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-
98 102 105 106 110 111

amino acids is necessary for cleavage by the enzyme. The Leu-Ser order is of importance (the reverse order leads to a much increased substrate turnover [45]). Also His 102 seems to be essential [46]. The His-Pro-His-Pro (98-102) on the N-terminal side and the Lys 111 in the C-terminal side seem to be electrostatically involved in the binding with the active centre cleft of chymosin [43]. Surprisingly the Phe-Met bond itself does not seem to be very essential for peptide cleavage [47]. The Pro residues play a role in the tertiary structure of the substrate making a good fit in the enzyme structure and promoting stabilization of the enzyme-substrate complex.

Several attempts have been made to predict the secondary and tertiary structure of the κ-casein molecule and especially the region around the Phe-Met cleavage side. The C-terminal part contains numerous negatively charged residues and few positive charges preventing the chain from folding into a compact structure. This part contains an α-helix at positions 136-148 (containing the residues specifying variants A and B) and a small β-structure at 151-154. The chymosin sensitive bond is believed to consist of a β-turn, β-strand, β-turn motif [43], although others predict the residues located between the β-turns to be of α-helical nature [34]. Whatever it is, the cleavage side must stand out on the molecular surface in order to be exposed to proteolytic attack by the chymosin molecule. The hydrophobic part of the κ-casein molecule is believed to contain more secondary structure with more evolutionary preservation among species. The estimated percentages of secondary structure diverge considerably depending on the methods used. Secondary structures in the individual caseins and submicelles vary with temperature, pH and ion concentration and identity [48]. All secondary and tertiary casein structure predictions have to be regarded as qualitative designations. Moreover, structure predictions lose relevancy, because under natural circumstances, the caseins are tangled with each other in casein micelle structures.

2.6.1 Casein-casein interactions and micelle structure

Besides the obvious self-associative behaviour of the caseins, studies of casein coassociation have been done to elucidate the mechanism and the order of casein assembly into submicelles and the even larger casein micelles from the moment of formation of the individual caseins in the mammary gland [49]. In these studies, α_{s2}-casein has been largely neglected. Interaction studies lose relevance if the composition of the solution diverges too far from milk.

Table 1. Physico-chemical characteristics of relevant proteins.

	α_{s1}-casein	β-casein	κ-casein	chymosin	lysozyme	BSA
m.w. (Da)	23600	24000	19500	35600	14600	66300
nr. of amino acids	199	209	169	323	129	582
glycosylation	-	-	+	-	-	-
S-S bonds	0	0	0	3	4	17
I.E.P.	4.1	4.5	4.1	4.7	11.1	4.3
tertiary structure	random	random	random	globular	globular	globular
net charge	-21 (pH 6.6)	-12 (pH 6.6)	- 4 (pH 6.6)	- 12 (pH 7.0)	+ 8 (pH 7.0)	

Casein associations are strongly dependent on ionic strength, Ca^{2+} activity, pH, temperature and concentration. Even at low concentration, caseins tend to self-associate and this will complicate association studies of individual caseins.

Varying mechanisms involving hydrophobic and electrostatic interactions between the caseins have been postulated. Payens [50] suggested hydrophobic forces to be mainly involved in the stabilization of the casein micelle, based on amino acid composition of the caseins, the temperature dependency and the established hydrophobic nature of α_{s1}-and β-casein interactions. In fact for all casein-casein interactions, positive entropy and enthalpy changes have been found, indicating that interactions increase with temperature and that hydrophobic forces are involved.

The interaction best studied is the one between α_{s1}- and κ-casein. This interaction is negligible at 0-6 °C and spontaneous above 37 °C [51]. The association constant ranges between 2 to 8 x 10^4 M^{-1}, depending on the methods applied and on pH, ionic strength and temperature. The association becomes less at increasing pH and ionic strength and decreasing temperature [52]. Several authors concluded that the interaction between α_{s1}- and κ-casein can be equimolar under ideal conditions, suggesting that only one hydrophobic site on either protein plays a part. On the other hand, the well-known stabilizing ability of κ-casein, to prevent precipitation reactions of self-associated α_{s1}-casein and β-casein in the presence of Ca^{2+} [53],

has been shown to be greatly influenced by some modifications of charged amino residues in the κ-casein molecule. Modification of lysine residues, using various techniques, and esterification of carboxylic groups, diminished this stabilizing ability to zero when five or more lysine residues had been modified [54]. κ- And α_{s1}-casein associations weaken at increasing pH and ionic strength, demonstrating that electrostatic interactions also play a role.

κ-Casein is able to stabilize α_s- and β-casein from precipitation against Ca^{2+} for about ten times its own mass. This stabilizing ability is pH dependent and increases with dilution of the system [55]. The stabilizing ability is lost after cleavage of the caseinomacropeptide by chymosin [46]. On the other hand, in the presence of α_s-casein, para-κ-casein, formed after cleavage of the caseinomacropeptide by chymosin, does not precipitate in the absence of divalent cations at pH 7 [56]. It was assumed that the carboxyl group of the peptide bond, split by chymosin, plays a role in the association of pure para-κ-casein. In the presence of α_s-casein this carboxyl group was no longer proton titratable, possibly due to an allosteric effect. It was found that the association accompanied conformational changes of the protein molecules and a positive change in entropy (ΔS = 21 J K^{-1} mol^{-1}). The rate of formation of the α_s-κ-casein complex was found to be faster than of κ-casein-chymosin formation. The dissociation constant was determined as 2.8 10^{-5} M and found to be independent of temperature. It was concluded that a reversible association occurred between κ-casein and α_s-casein on a site close to the peptide bond split by chymosin, but different from the site of fixation of chymosin during enzyme-substrate complex formation. The same effect was found for β-casein and total casein.

β-Casein is not able to protect α_s-casein against Ca^{2+} precipitation. The association of κ-casein and β-casein has not been studied extensively. A dissociation constant for the complex of $1.2.10^{-4}$ M was determined [57]. From moving boundary electrophoretic measurements, Payens [58] concluded that the interaction between α_s- and β-casein gives rise to an intricate assembly of complexes in which α_s-casein dominates. Qualitative agreement of experimental and computed Schlieren sedimentation patterns was only obtained if the simultaneous formation of a 2:1 complex was assumed. However, sedimentation patterns also have led to the conclusion that α_s-casein interacts with β-casein.

The caseins, as present in milk, are clustered into spherical spongelike aggregates, called casein micelles. These particles range in size from 20 to 400 nm and are responsible for the high turbidity of skimmed milk. The smallest particles are by far predominant in number but hardly contribute to total concentration of the casein in milk. The dry matter of the micelles consists of 93 % casein and the remainder is inorganic material, of which calcium and phosphate are predominant. The voluminosity of the micelles is estimated to be about 2.2 ml/g dry casein inside the casein micelle and distinctly larger for the micelle as a whole (taking a hairy layer of κ-caseins into account), but the values generated highly depend on methods used and experimental conditions [59]. The voluminosity increases at low temperature, partly due to β-casein molecules protruding. The elucidation of the structure and the composition has

been the subject of elaborate study over the last decades and several models have been proposed. The model most accepted is the one built up of casein submicelles, where the latter exist in varying casein molecule composition. The submicelles containing relatively high amounts of κ-casein are grouped on the periphery of the micelle. The hydrophilic, carbohydrated caseinomacropeptide parts of these κ-casein molecules protrude, like flexible "hairs", outside the micelle and in this way, due to electrostatic and entropic repulsion, guarantee the stability of the micelle in the milk dispersion. The hydrodynamic thickness of this hairy layer would be about 7 nm [60]. A crucial role in all concepts of the micelle structure is played by colloidal calcium phosphate (CCP). The submicelles may be linked together by means of CCP that bridges opposite serine-phosphate groups of the submicelles [60, 61]. Others believe that CCP fulfils a bridging function between casein molecules of the same kind, especially involving the α_{s1}-caseins [62, 63]. In this model, aggregation of submicelles into casein micelles is not due to permanent linking by the CCP, but due to a neutralisation of negative charges of the caseins [62]. Colloidal calcium phosphate consists for the largest part of calcium and phosphate, but also of minor components like magnesium, potassium, sodium and citrate [61]. The exact nature of CCP is under much debate and it is thought to be in a dynamic pseudo-equilibrium with the minerals in the milk serum. Lowering the pH and prolonged dialysis of milk result in removal of this CCP and in the breakdown of the micelle into its constituent submicelles [60, 61], or other less well-defined proteinaceous particles [63]. Submicelles would consist of 15-25 casein molecules and a diameter between 10-20 nm. The hydrophobic parts of the casein molecules would be buried in the interior of the submicelle and the hydrophilic, charged parts on the periphery, similar to soap micelles. The interaction forces between the casein molecules are believed to be of a hydrophobic and electrostatic nature.

Physical properties like size distribution and stability of submicelles have been studied systematically by Schmidt by means of electron microscopy of artificial casein micelles. The artificial casein micelles were made by mixing simultaneously appropriate volumes of solutions of calcium- and magnesium chloride, sodium- and potassium phosphate, potassium citrate and the various caseins, the latter in concentrations that could be varied. The mixing was done under vigorous stirring, with conditions such as pH and temperature kept constant [64]. The width of the size distribution and the average diameter decreases as the amount of κ-casein incorporated into the artificial micelle increases. A linear relationship between κ-casein concentration and surface to volume ratio of artificial and natural micelles was observed [65]. The relative β-casein concentration is also higher for the small micelles. The α_{s1}- and α_{s2}-casein concentrations are constant in the range of large to small micelles, but are relatively decreased in the very small particles [66].

2.7 Chymosin

Chymosin (EC 3.4.23.4) is a member of a class of proteolytic enzymes characterized by two aspartic acid residues located in the active centre. This class of enzymes is now called aspartate proteinases and they are all inhibited, to various extents, by the microbial oligopeptide pepstatin. The three dimensional structures of the fungal endothiapepsin, rhizopepsin and penicillopepsin, the mammalian chymosin, pepsin (human, bovine, porcine), and human renin, all belonging to this class, have been elucidated by crystallographic studies [67]. These enzymes, except for renin, are used in the process of cheese-making because of their milk renneting property. Traditionally, calf rennet, which is an extract from the fourth stomach of the young calf and which comprises mainly chymosin as the renneting enzyme, has been used to clot milk. The scarcity of calf stomachs, due to the worldwide expansion of cheese production, has led to a search for chymosin replacers. The fungal proteases mentioned can be used as replacers for bovine chymosin for some cheese variaties. Biotechnology has led to production of identical bovine chymosin by means of recombinant DNA modified microorganisms.

The chymosin molecule has an irregular, kidney-like shape with approximate dimensions of 40 x 50 x 65 Å. The chymosin molecule contains a single chain of 323 amino acids, a mol mass of 35600 Da. It contains three cystine disulfide bridges. The secondary structure consists mainly of parallel and anti-parallel β-strands with little α-helix structure. Two isozymes, chymosin A (Asp-244) and B (Gly-244), are known which slightly differ in isoelectric pH (about pH 4.7), and are separable by isoelectric focusing [68]. Chymosin is secreted as the inactive zymogen prochymosin, which is activated in the stomach at pH 2 by removal of 42 N-terminal residues. The enzyme can readily be denatured in solution at temperatures above 40 °C, dependent on pH, ionic strength and buffer identity (stable at low pH and high I [69]).

The aspartate proteinases consist of two structurally similar lobes of about 160 residues. The lobes are separated by a deep cleft, forming the active centre, and each lobe contributes one aspartic acid. There is a small peptide chain (6 residues) connecting the two lobes and serving as a backbone to the active-site region of the molecule. The mammalian aspartate proteases are synthesized in precursor forms, whereas the fungal proteases are not. Zymogens like prochymosin and pepsinogen are inactive but, in acidic surroundings, the propeptides are split off to activate the enzymes. In the case of pepsin (bovine, porcine) the peptides (residues 1-16) released on acidic activation, are strong inhibitors of the enzymes above pH 4. This is not the case for chymosin [70].

The active site in aspartate proteases is very well conserved among the various proteases, which agrees with the mechanism of interaction of the essential aspartic residues with specific inhibitors being the same for pepsin and chymosin [71]. Also the three dimensional structures of the N-terminal lobes of the proteases are very similar, whereas larger differences exist for the C-terminal lobe structures. It is presumed that the two lobes, which have relatively little interaction with each other, can move as separate rigid bodies, thereby

altering the shape of the active centre [67]. This movement, which is largest for the C-teminal lobe, especially occurs on interaction with pepstatin, but has not been observed with substrates [72]. Crystallographic studies up to now did not provide good evidence for extensive conformational change of the aspartic proteases during catalysis. An exception was found for an extended loop projecting across the cleft to form a flexible "flap" (a β-hairpin; residues 73-82), which encloses substrates and inhibitors in the active site and which opens to allow access to the active site. This position of the flap region in the chymosin molecule is different from that found for other acid proteases. The mechanism of proteolysis by aspartate proteases, and chymosin in particular, has, in spite of crystallographic elucidation, still not been revealed in detail. The substrate binding cleft can accommodate seven amino acid residues. The two Asp residues 34 and 215 are located deep in the centre of the cleft and interact closely. In the active form they are connected to each other by the oxygen atoms of the two carboxyl groups sharing a proton. Due to this interaction the pK_a values of the two Asp residues differ considerably (in the case of pepsin being 1.5 for the one and 4.7 for the other). There is evidence that in the enzyme-substrate complex Asp 34 is negatively charged and Asp 215 is protonated [73]. The significantly higher activity of chymosin A for its natural substrate κ-casein compared to chymosin B, has been explained by an increased affinity, due to several negatively charged residues near the Asp-244 residue located on a loop on the surface of the molecule. These additional negative charges aid in the electrostatic stabilization of the enzyme-substrate complex. Some prerequisites, like chain length and amino acid identity, for the peptide to be suitable as a substrate, were established by Visser and others (42, 43) and form a so-called secondary specificity. Removal of the Leu in the hexapeptide sequence Leu-Ser-Phe-Met-Ala-Ile or the Ile considerably influence enzyme kinetic parameters. Several substrate analogs were found to act well as a substrate for determining chymosin and pepsin activity, although differences in turnover and affinity for both enzymes were found. The methylated synthetic hexapeptide H-Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe proved to be a useful substrate for determination of rennet activity.

An important part of the affinity between enzyme and substrate will be due to the opposite charges of the two molecules. In the case of chymosin and its natural substrate κ-casein, both being proteins, the opposite charges are formed by the constituent amino acids and depend on the pK_a values of the residues on the surface of the molecules. Chymosin has 54 charged residues, at neutral pH, resulting in a net charge of -12. The N-terminal lobe contains five amino acid residues within the sequence 48-62, forming a positively charged patch on the surface of the molecule. The C-terminal lobe, on the other hand, has nine negatively charged residues (Asp and Glu residues) within the sequence 208 - 290 that are located on the surface on the molecule (Figure 1). This polarization of the molecule may predispose the correct orientation of chymosin as it approaches the casein micelle surface. It may partly explain the strong dependence of enzyme activity on pH and ionic strength. It may also play a role in the association of chymosin with para-κ-casein during cheesemaking.

2.8 Lysozyme

Lysozyme (E.C. 3.2.1.17) is a glycosidase, more specifically a muramidase, that displays endo-*N*-acetylmuramoylhydrolase specificity. It is one of the best known and most amply studied proteins or enzymes and one of the first proteins of which the three dimensional structure was elucidated from X-ray diffraction of a protein crystal. Lysozyme is an antibacterial enzyme found in egg white and in saliva, tear secretions, and nasal mucus of mammals. It has also been isolated from fish, bacteria and bacteriophages. Treatment of gram-positive bacteria with the enzyme followed by osmotic shock can lead to lysis of the bacteria. The activity of the enzyme can be easily monitored spectrophotometrically by determining decreasing turbidity of *M. lysodeikticus* cell walls in suspension (74). The fluorescence increase with the substrate 4-methylumbelliferyl-*N,N',N''*-triacetyl-chitotrioside also may serve [75]. The lytic action of the enzyme is based on the attack of glycosidic bonds between the C-1 atom of *N*-acetylmuramate and the C-4 atom of *N*-acteylglucosamine of the murein skeleton of the bacterial cell wall. Lysozyme also has chitinase and transglycosidase activity [76].

Lysozyme from hen's egg white is a globular protein, consisting of 120 amino acids, a molar mas of 14.4 kD and an isoelectric pH of 11.1. It contains four S-S bridges and has a relatively high conformational stability in solutions of low pH. Lysozyme molecules tend to self-associate reversibly at high concentrations (20 g/l), at pH > 5.0 and dependent on temperature. In the head-to-tail self-association the Glu-35 and the His-15 residues are involved [77]. The self-association is accompanied by a reduction of activity. The pH optimum for activity is around 5, where two essential amino acid residues Glu 35 and Asp 52 in the active centre are protonated and deprotonated, respectively.

The structure shows two lobes, with a deep active site cleft in between (Figure 2). One lobe consists of the N- and C-terminal sequences, 1-36 and 85-129, containing four major α-helices and two short loops (orange coloured in Figure 2). The other lobe consists of the central region of the polypeptide chain, residues 37-84, containing a triple stranded antiparallel β-sheet. The two domains are linked by two crossings of the polypeptide chain in which two out of the four S-S bridges are involved.

The surface of the lysozyme molecule is characterized by a hydrophobic cluster of 8 residues at the back opposite to the active centre (Figure 2). At neutral pH the surface of the domain consisting of residues 37-84 has a net charge of +2, and the other domain of +6. The lysozyme molecule has striking similarity, structural as well as compositional, with bovine α-lactalbumin (40 % of amino acid residues corresponding). On the other hand α-lactalbumin does not show enzymic activity. It serves as a cofactor in the synthesis of the β (1→ 4) glycoside lactose, whereas lysozyme catalyses the cleavage of this bond [78]. In the cheese industry lysozyme has been applied as a means to control the outgrowth of *Clostridium tyrobutyricum*, which causes the defect late blowing [79].

Fig. 1. Chymosin.
Connoly surface model and a ribbon model of the enzyme molecule. Charged residues are coloured: Asp and Glu are negatively charged and coloured red, Lys and Arg are positively charged and blue[74]

Fig. 2 Lysozyme
Connoly surface model and a ribbon model of the enzyme molecule. Charged residues are coloured: Asp and Glu are negatively charged and red, Lys and Arg are positively charged and blue. Hydrophobic residues are yellow [81].

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LOSS OF ENZYME ACTIVITY DUE TO ADSORPTION ONTO EMULSION DROPLETS

Chapter 3

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Abstract

In order to study the possibility of immobilization of enzymes on a liquid carrier while retaining activity, we used hen's egg lysozyme (EC. 3.2.1.17) and bovine chymosin (EC. 3.4.23.4) as the emulsifying agents to make emulsions of soya-oil in water. The enzymes were applied in three different ways: (i) mixed with the soya-oil-water, then emulsified, (ii) immediately added to emulsions of soya-oil made with bovine serum albumin in a range of concentrations, and (iii) simultaneous emulsification with an enzyme and bovine serum albumin in various concentration ratios. The extent of adsorption was determined in depletion studies. Enzyme activity *in situ* was determined using flexible small-molecular weight substrates.

It was found that every mode of emulsification led to complete *in situ* inactivation of the enzymes. Even after desorption of the enzymes by the surfactant Tween© 20 the enzymes proved to be inactivated; the surfactant itself caused no inactivation of the enzymes in solution.

3.1 Introduction

Protein adsorption onto various interfaces has been the subject of many studies, relatively few of which deal with the change in biological activity of proteins due to their adsorption onto these interfaces. The enzyme lysozyme often has been used, mainly because of its specific properties as a protein rather than its properties as an enzyme.
James and Augenstein [1] reviewed the work done on biological activity at interfaces. Sandwick studied the conformational change of several enzymes due to adsorption onto hydrophobic S/L interfaces [2,3] in terms of enzyme activity. Unfortunately, the solid particles, which may have retained enzyme activity, were not separated from the bulk solution. Consequently, it was not established whether and to what extent the enzymes remained active when adsorbed.

The activity of enzymes at interfaces can be and has been studied in three ways:
(i) The substrate is at the interface and the enzyme is in solution. In these cases full or diminished enzyme activities were found.
(ii) Enzymes adsorbed onto the A/W interface are transferred by Langmuir-Blodgett like techniques and resuspended afterwards. Mostly, little or no activity was observed. Besides, any influence of deposition of enzyme on the materials used in the transfer was overlooked.
(iii) The interface holds the enzyme (activity *in situ*) and the substrate is in the bulk phase. This has mainly been done at the S/L interface [7] and at the A/W interface [4,5,6,7]. Mostly, partial to complete inactivation was observed, especially for low enzyme bulk concentrations. In all cases the inactivation was ascribed to (partial) unfolding of the enzyme molecule, presumably because contact of the internal hydrophobic protein residues with the surface results in a lower free energy. Activity *in situ* of enzymes adsorbed at the oil/water interface has not been studied before, except for lipases.

In our study we immobilized enzymes on a liquid carrier such as soya-oil emulsion droplets, by means of emulsification and by using the enzyme as the emulsifying agent. In this way immobilization would be achieved in an entirely food grade system, which could find application in specific targetting of enzymes in foodstuffs [8], providing that the adsorbed enzymes retain activity. Adsorption of enzymes onto the oil/water interface in the process of emulsification might offer an opportunity for retaining enzyme activity, because adsorption occurs on a very short time-scale, say 1 μs [9], which may be too short for substantial conformational change.

We studied the enzymes bovine chymosin (EC. 3.4.23.4) and hen's egg lysozyme (EC. 3.2.1.17), both globular proteins, but differing in properties such as isoelectric pH, molecular size and conformational stability [10, 11]. These enzymes were directly applied in the emulsification of soya-oil but also in coadsorption with bovine serum albumin in various ways.

3.2 Materials and methods

Recombinant-DNA bovine chymosin produced by *Kluyveromyces lactis* (Gist brocades) was dialysed against 25 mM K-phosphate buffer of pH 5.8 to lower salt content. The solution contained 44.5 μM of active chymosin and was electrophoretically pure, but still contained a rather high amount of peptide impurities (80 % of total protein content). Hen's egg lysozyme and bovine serum albumin were purchased from Sigma Chemical Company. Lysozyme was found to be electrophoretically pure on Coomassie-stained SDS-polyacrylamide gel. Commercially available soya-oil (Reddy) was used and made monoglyceride-free by stirring it with predried Kieselgel 60 (Merck) for two hours, followed by centrifugation and decantation. The interfacial tension between the soya-oil and water was 28 $mN.m^{-1}$, indicating absence of surface-active impurities. Emulsions (25 ml) were made with a lab-homogenizer (Condi), flow rate of 5.25 l/h, approximate pressure of 100 bar, for three minutes (about ten homogenization head passages) at room temperature. A soya-oil volume fraction of $\varphi = 0.02$ and a protein content of 2 g/l were used. The liquid fed into the homogenizer was vigorously stirred with a magnetic stirrer to ensure a homogeneous distribution of the initially large emulsion droplets in the system. Care was taken to avoid air inclusion.

Emulsion droplet size analysis was performed by means of a laser diffraction technique (Coulter LS130), yielding the volume/surface average diameter d_{VS}. The specific surface area (A) of the emulsion was calculated with the equation $A = 6\varphi/d_{VS}$, in which φ is the volume fraction of the soya-oil, determined by the method of Gerber [12], which was originally developed for milk fat analysis (a correction factor of 1.11 was applied to convert from mass to volume fraction).

Protein analysis was performed with the BCA protein assay (Pierce Ltd.) correlated to a bovine serum albumin standard curve. Spectrophotometric analysis was carried out with a Pharmacia LKB-Biochrom 4060 spectrophotometer. The protein surface load Γ (mg/m^2) was determined by measuring the protein concentration of the original solution and of the supernatant of the emulsion after centrifugation (Sorvall) for 20 min at 15000 g and dividing the difference value by the specific surface area A.

Chymosin activity was determined with the Berridge clotting test [13] in which the time needed for visually observable flocculation to occur is determined by pulling up substrate milk (100 g low heat milk powder per liter of water) against the glass wall of the reaction vessel. The measurement was performed at 30.4 °C. Chymosin concentration was calculated according to the relation of Storch and Segelcke [14], which states that the clotting time is inversely proportional to the chymosin concentration. The concentration of active chymosin could be calculated by taking as a reference that a 13.3 μmolar concentration would result in a clotting time of 300 s at a 4000-fold dilution [14].

This clotting test could not be applied to determine chymosin activity *in situ*, that means when adsorbed onto the emulsion droplets, because of the diffusion limitation of the

immobilized enzyme on the oil droplets and the κ-casein substrate, being in an aggregated form, i.e. in casein micelles. Therefore a flexible small-molecular synthetic hexapeptide substrate HLeu-Ser-Phe(NO_2)-NLe-Ala-Leu-Ome (Bachem) was used [15]. This substrate is split at the Phe(NO_2)-NLe linkage and the product can be monitored spectrophotometrically at 310 nm. The emulsion droplet layer was washed three times by means of centrifugation (2 ml Eppendorf vial, 12000 rpm for 1 min) and suspending in a 50 mmolar acetate buffer of pH 4.7, and finally suspended in a 0.5 mmolar hexapeptide solution in the same buffer. After 20 min of incubation the emulsion was filtered (pore size 0.2 μm) and the absorbance of the filtrate measured.

Lysozyme activities in solution and *in situ* were determined by measuring the intensity of fluorescence of the fluorescent label of methylumbelliferyl-4-N'-N''-N'''-triacetylchitotriose (5 μmolar) splitt off by lysozyme [16]. After 20, 40 and 80 min, samples were taken from a reaction mixture of a three times washed emulsion droplet layer suspended in a 5 μmolar substrate solution in 50 mM K-phosphate buffer of pH 5.1. The reaction took place at 40 °C. The reaction was stopped by adding 0.7 ml of sample to 2.5 ml of a solution of 1 M glycine, 1 M NaCl and NaOH to a pH of 12. Fluorescence was measured at an emission wavelength of 448 nm and at an excitation wavelength of 358 nm. The sensitivity of the method proved to be 13.3 DI (intensity units)/min for a lysozyme concentration of 1 g/l for the linear part of the curve.

It may be argued that the washing procedures employed could have caused desorption of proteins from the emulsion droplets. It is, however, well established [17] that proteins are extremely surface active, plateau values for adsorption at fluid interfaces being reached at bulk concentrations of about 1 mg of protein per liter. This makes the adsorption virtually irreversible (unless washing is repeated, say 100 times) and any desorption must have been negligible.

Deliberate desorption of the adsorbed enzymes was accomplished by adding 1% (v/v) of Tween© 20 (Merck) to the emulsion and letting it incubate for 16 hours, leading to virtually complete desorption. It was checked that Tween© 20 did not influence the enzyme activities and the enzyme and protein assays.

3.3 Results and discussion

3.3.1 Influence of homogenization

It was checked first whether the homogenization process itself did inactivate the enzymes in solution, since it has been observed [18] that various enzymes lose activity when a solution is brought under a high shear stress for a long time. Lysozyme in solution (2 g/l) did not lose any activity during 70 head passages with the lab homogenizer. Chymosin in solution lost activity rapidly if air bubbles were present during the process (Fig. 1).

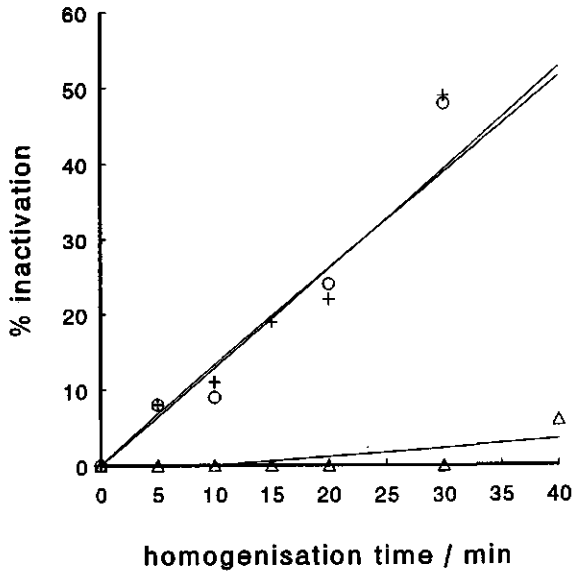

Fig. 1. The influence of number of homogenization head passages and foam formation during homogenization on the inactivation of chymosin (0.66 μmolar chymosin, pH 5.8, 50 mmolar K-phosphate buffer). Loss of chymosin activity in the solution during foam development (O) , in the foam itself (+) and in the solution when foam was not allowed to form (Δ) .

Chymosin activity measurements of samples taken from the foam formed and the corresponding bulk solution showed a gradual decrease in activity, indicating that the enzyme was denatured due to adsorption onto the expanding air/water interface. If no air was incorporated, the chymosin retained full activity. The loss in linearity of the curve in fig.1 for longer time of homogenization was probably due to deviation from the linear Storch and Segelcke relation at long clotting times. In a comparable study inactivation of various enzymes was observed to be related linearly to time of bubbling air through the liquid [19].

3.3.2 Enzymes as surfactants.

Soya-oil was emulsified (volume fraction $\varphi = 0.02$) in enzyme solutions of 2 g/l in 50 mmolar K-phosphate of pH 5.8. The emulsion made with chymosin had an emulsion droplet size $d_{vs} \approx 1.50$ μm and a surface load $\Gamma \approx 4.50$ mg/m^2 and for the lysozyme emulsion $d_{vs} \approx 3.67$ μm and $\Gamma \approx 7.9$ mg/m^2 was found. Since lysozyme shows a tendency for self-association above pH 5 at high concentration [20], another soya-oil emulsion was made with 2.8 g/l of

lysozyme in 50 mmolar of K-phosphate buffer at pH 5.1 (d_{vs} = 3.30 μm, $\Gamma \approx$ 10.9 mg.m^{-2}). Self-association of lysozyme was assumed then to be minimal. The high values of surface load are probably due to an overestimation of d_{vs} by the droplet size distribution laser diffraction analysis. Several size distributions were also measured by a spectroturbidimetric method [21], which generally resulted in a smaller average droplet size. The high values for the droplet diameter, however, do not affect our conclusions.

The emulsions showed no activity of lysozyme or chymosin *in situ*. Enzymes desorbed by adding Tween© 20 also did not show activity, indicating that the enzymes had been irreversibly inactivated. The surfactant did not affect the enzyme activity. Most probably, the inactivation is due to unfolding of the enzyme molecules during adsorption onto the oil surface. Much the same may possibly hold for adsorption onto the air/water interface, but for lysozyme a substantial residual activity has been observed [22]. We have to consider that adsorption of proteins onto a macroscopic air/water or solid/water interface is diffusion controlled and will, for low bulk concentrations, take place on a time scale of the order of a second. During emulsification adsorption takes place in an essentially isotropic turbulent field and time of adsorption can be estimated by using an equation [9], derived from Kolmogorov theory:

$$t_{ads} \approx 10\, \Gamma\, \eta_c^{1/2} / d\, m_c\, \varepsilon^{1/2} \qquad (s)$$

in which t_{ads} = time of adsorption (s), Γ = surface load (kg/m^2), η_c = viscosity of the continuous phase (l. 10^{-3} Pa.s), d = droplet diameter (m), m_c = protein concentration (kg/m^3) and ε = power density (about 10^{11} W.m^{-3}). For the systems used, adsorption times were estimated to be at most 10^{-6} s.

Because the unfolding of a protein at an interface also takes time, it may be possible that the interface becomes fully packed before appreciable unfolding of the protein occurs [17]. This was also suggested by Sandwick, who studied adsorption onto polystyrene (i.e. hydrophobic) particles [3], to explain the conservation of enzyme activity *in situ* at high bulk concentration, where the rate of arrival at the surface would become faster than the rate of unfolding. In the process of emulsification this possibility may well be real. Nevertheless, inactivation was complete. Any unfolding of the protein at the oil/water interface should therefore occur within a μs. Such a time scale for unfolding may be likely for chymosin, that apparantly has little conformational stability, but for lysozyme, which is known for its high stability, a greater reluctance to unfolding, and hence a longer time, would be expected.

As the extent of unfolding of a macromolecule is related to the surface area available per molecule, we used the equation of state applied by De Feijter and Benjamins [23] to estimate the extent of unfolding, expressed in the radius of the adsorbed protein molecule for lysozyme at the air/water and oil/water interfaces. We made use of data on surface pressure

(Π in mN.m^{-1}) versus surface area per molecule (A in m^2.mg^{-1}) by Graham & Phillips [24]. The equation reads

$$\Pi = \Gamma RT/(1-\Theta)^2$$

with

$$\Theta = \pi r^2 N_{AV} \Gamma$$

in which Γ = surface load (mol.m^{-2}), Θ = surface fraction and r = radius of an adsorbed protein molecule (m) in the plane of the interface. R, T and N_{AV} have their usual meaning. It follows (Fig. 2) that the radius of the adsorbed lysozyme molecule would be large at low surface load, especially at the oil/water interface. At $\Gamma > 2$ mg.m^{-2} the radii of lysozyme molecules at the air/water and oil/water interface seem to be hardly changed (the radius of end-on adsorbed lysozyme molecules at the air/water interface is 1.5 nm [21]).

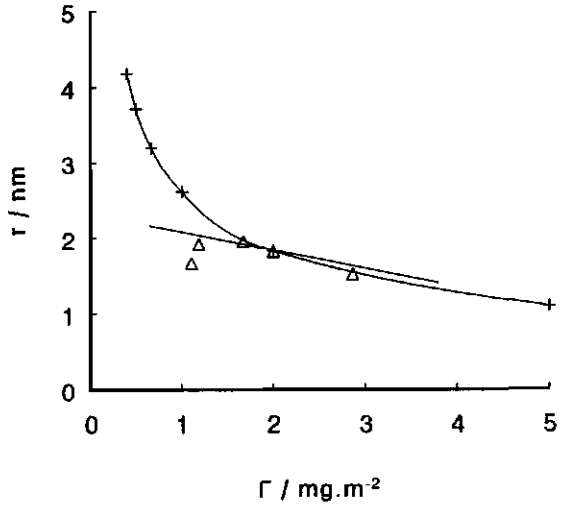

Fig. 2. Calculated radius of lysozyme molecules adsorbed onto an air/water (Δ) and an oil/water (+) interface as a function of surface load Γ. After refs. [22], [23].

Since the surface load of lysozyme in the emulsions made appeared to be high (observed Γ = 7.9 mg.m^{-2}), no unfolding and, consequently, no inactivation of the enzyme would be expected. Nevertheless complete inactivation, even after desorption, was observed. It thus appears that conformational changes in the enzyme molecule on adsorption do not necessarily become manifest in a larger radius of the molecule. Perhaps changes in protein conformation after adsorption may be more prominent at the oil-water interface as compared to air-liquid or solid-liquid interfaces. Hunter et al. [21] indeed found higher residual activities of lysozyme

adsorbed onto the air/water interface at higher surface loads. However, these were not enzyme activity measurements *in situ* but after redeposition on a Teflon bar and resuspension in solution. Norde et al. [25] found a decrease in α-helix structure of lysozyme due to adsorption onto hydrophilic, negatively charged latex particles at low surface loads. Complete recovery of α-helix content was found in bulk solution after desorption. At plateau surface coverage, no change in α-helix structure was established.

Since the conformational stability of a protein is highest at its isoelectric pH, soya-oil was emulsified with lysozyme at pH 11, but also here no activity *in situ* or after desorption was found. A single passage of a lysozyme solution with oil through the homogenizer valve sufficed to fully inactivate the lysozyme.

Emulsification with chymosin at its isoelectric pH was not studied, but it is very likely that no residual activity would result for this enzyme, because of its small conformational stability.

3.3.3 Enzymes added to soya-oil/BSA emulsions.

The extent of unfolding of a protein at an interface may, among other factors, depend on the surface area available; it would then be negatively correlated with the surface load obtained. Emulsions of soya-oil were made with various concentrations of bovine serum albumin in 50 mM of K-phosphate pH 5.8 (Table 1). By applying low concentrations of BSA we tried to create a low surface coverage, yielding fairly unstable emulsions.

Table 1. Effects of the addition of enzyme immediately after emulsification of soya-oil in BSA solutions of various concentrations. pH 5.8, 50 mmolar K-phosphate buffer, T = 20°C.

CHYMOSIN 0.17 g/l			**LYSOZYME** 2.7 g/l			
[BSA] (g/l)	chym. (% ads.)	Γ_{BSA} (mg/m^2)	[BSA] (g/l)	lysoz. (% ads.)	d_{vs} (μm)	Γ_{BSA} (mg/m^2)
0.03	45	coal[a].	0.008	0	coal[a]	-
0.06	25	0.7	0.04	0	coal[a]	-
0.13	0	2.5	0.20	0	3.63	1.06
0.62	0	2.9	1.0	0	1.90	2.74
1.25	0	3.6	2.5	0	1.55	3.66
5.0	0	6.9	5.0	0	1.52	6.75

[a]) Coalescence ; Γ could not be estimated

Immediately after emulsification, enzyme was added (up to 0.17 g/l for chymosin, based on activity, and 0.14 g/l for lysozyme, based on protein concentration).
It was reasoned that the enzyme could possibly fill the "gaps" of uncovered surface without being able to unfold, thereby retaining activity. Adsorption percentages were determined by depletion, by measuring residual enzyme activities in the first supernatant after centrifugation (20 min, 15000 *g*) after an incubation time of approximately one hour.
For chymosin activity measurements the hexapeptide assay was used and for lysozyme the fluorescence assay. Chymosin indeed was able to penetrate the oil-water interface partially covered by BSA and to further stabilize the emulsion, but lysozyme did not within the time of incubation.

The inability of lysozyme to coadsorb onto a partially covered interface was also shown by others [26, 27] who studied coadsorption of lysozyme and β-casein at the air-water interface. The emulsion droplets partially covered with chymosin showed no activity *in situ*. Chymosin desorbed by means of adding Tween© 20 proved to be fully inactivated.

3.3.4 Emulsification of soya-oil using BSA and the enzyme simultaneously.

Soya-oil was emulsified in solutions of BSA and enzyme in various concentration ratios at a constant total protein concentration of 2 g/l in 50 mM K-phosphate buffer of pH 5.8 (Table 2). Percentages of enzyme adsorbed were determined by measuring enzyme activities in the original solution and in the first supernatant after emulsification and centrifugation (20 min 15000 *g*). Enzyme activity was measured *in situ* after washing thrice to remove unbound enzyme. In the fourth supernatant no enzyme activity was measurable.

Lysozyme proved to be a poor emulsifier, yielding unstable emulsions containing large emulsion droplets. As the BSA/lysozyme ratio increased, the emulsion droplets obtained were smaller, and the emulsions were more stable. The various concentration mixtures of BSA and chymosin did not yield differences in emulsion stability or droplet size, suggesting that the emulsifying properties of these two proteins are more or less the same. The percentages of adsorption of the enzymes in both systems were consistent for all concentration ratios. However, activity *in situ* was not found for any system. Moreover, the enzymes proved to be fully inactivated after desorption by Tween© 20.

Table 2. Effects of the emulsification of soya-oil with solutions containing BSA and an enzyme in various concentration ratios. pH 5.8, 50 mmolar K-phosphate buffer, T = 20 °C.

[BSA] (g/l)	[Chymosin] (g/l)	d_{VS} (μm)	Chymosin (% ads.)	[BSA] (g/l)	[Lysozyme] (g/l)	d_{VS} (μm)	Lysozyme (% ads.)
0.33	1.67	2.04	21.1	0.5	1.5	4.90	18.4
0.67	1.33	2.00	16.4	1.0	1.0	4.27	15.3
1.00	1.00	1.97	16.4	1.5	0.5	2.56	12.5
1.33	0.67	1.96	17.7	1.75	0.25	1.96	17.0
2.00	0.00	1.94	0				

3.4 Conclusions

It may be concluded that adsorption of chymosin and lysozyme (and probably most other enzymes as well, apart from lipases) onto the triglyceride oil/water interface leads to complete inactivation of the enzymes. Of course there is the possibility that the active site of lysozyme or chymosin is involved in the adsorption onto the interface, thereby leaving no entrance for the substrate molecule to react. Furthermore, the adsorption of an enzyme onto the oil surface may reduce its average motion and flexibility, which is often an essential part in the mechanism of enzyme-substrate reactions. However, after desorption by Tween© 20, which, by itself, does not affect the activity of the enzyme, the enzyme brought back to its native environment did not show any activity. If the desorbed enzyme molecules would tend to carry any oil with them, the Tween 20 present would most likely have removed the oil, considering its strong detergent activity. This is strong, albeit indirect evidence for the process of adsorption of protein onto the oil/water interface leading to denaturation.

As we are not aware of other research been done in this way, comparisons can only be made with protein adsorption onto solid/liquid and air/liquid interfaces. These interfaces differ, however, in interfacial properties. Oil droplets have a homogeneous fluid surface, whereas solid particles, e.g. latex, would have specific adsorption sites for proteins. Hydrophobic amino acid residues may to some extent become buried in the oil phase, whereas this is impossible in solids and in air. The oil/water interface therefore may induce greater conformational changes in adsorbed proteins than the other interfaces. On the other hand, the short time needed for an emulsion droplet to obtain a fully packed surface in the process of emulsification (about a μs) may be supposed to prevent the protein from becoming unfolded. This would be more likely for a protein with a greater conformational stability. Nevertheless,

we always found the adsorbed enzymes to be fully inactivated, irrespective of the procedure of emulsification. This conclusion may not be surprising, considering the conformational stability of a globular protein to be actually rather small [28]. A globular protein is characterized by a small difference between two large terms that stabilize (mostly bond energy) and that destabilize the protein (for a large part conformational entropy). A small change in one of these energy contributions (for instance due to a higher temperature, addition of urea or the presence of an interface) may considerably alter conformational stability. It would, however, take elaborate and painstaking studies to establish what exactly will happen with the protein conformation upon adsorption onto an oil/water interface.

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THE ASSOCIATION OF LYSOZYME WITH CASEIN

Chapter 4

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Abstract

The association of hen's egg lysozyme with caseins was studied by using three casein substrates: (I) solutions of the various caseins, (II) artificially made casein micelles of various compositions and (III) caseins adsorbed onto soya-oil emulsion droplets. In solution, lysozyme associated most strongly with α_s- casein, less with β-casein and not with κ-casein. Accordingly, lysozyme associated less with casein micelles composed of β- and κ-casein (ratio 1:2) than with whole casein micelles, which contain α_s-casein as well. The extent of association with emulsified caseins was in the order α_s > β > κ, although the differences were not large. The effects of temperature and pH on the association appeared to be small.

After association with caseins, lysozyme was always found to be active, suggesting that the active site of the molecule was not involved in the association. The association of lysozyme with casein-stabilized oil droplets may provide a satisfactory technique for immobilization of the enzyme on a liquid carrier, since *in situ* activity is retained.

4.1 Introduction

In cheese making, the enzyme lysozyme (muramidase, EC. 3.2.1.17) can be used as an agent to prevent growth of *Clostridium tyrobutyricum,* which causes off-flavours and late blowing in some cheeses [1]. Another application of lysozyme may be the possible acceleration of cheese ripening, because lysis of starter bacteria would cause release of cytoplasmic enzymes, which play a key role in proteolysis during cheese ripening. The mechanism of transfer of lysozyme to the curd is believed to be due to association of the enzyme with the caseins, the main protein fraction in milk [2, 3]. The same may hold for other enzymes such as chymosin and plasmin.

In an attempt to immobilize enzymes on a liquid carrier while fully retaining their activity, we used lysozyme as the emulsifying agent in making oil/water emulsions [4]. The adsorption, however, caused lysozyme to lose all activity. After subsequent desorption by a surfactant (Tween™ 20) it also proved to be inactivated. As done earlier for the renneting enzyme chymosin [5], we therefore used caseins as intermediates to stabilize soya-oil emulsions, whereupon added lysozyme would associate with the droplets at various pH and temperatures. Concomitantly, synthetic casein micelles were used as a carrier for lysozyme immobilization. The suitability of these systems for immobilization purposes was checked by determination of *in situ* activity.

4.2 Materials and methods

4.2.1 Caseins in solution

Lysozyme (Sigma) and caseins (Sigma) were dissolved in about equimolar concentration (1.5 and 3.0 g/l respectively) in 0.05 M phosphate buffer, 0.05 M NaCl, pH 6.7. Equal amounts were mixed and the flocculated lysozyme-casein complexes were removed by means of filtration (0.45 μm). The proteins present in the clear filtrates were analysed by means of high performance size exclusion chromatography on a Bio-Sil TSK 125 column (column dimensions 600x75 mm, 150 μl sample, flow rate 1 ml/min, monitoring E_{280}). The original solutions of lysozyme and casein were diluted once with buffer, filtrated and used as blanks. The association of lysozyme with caseins was calculated from the decrease of the lysozyme and casein peak areas due to the complexation after mixing. The experiments were carried out twice. The relative standard deviation (RSD) for the peak area was 2 %.

The influence of the association (if occurring) on the activity of lysozyme was determined as follows. A lysozyme solution (500 μl) was mixed with a casein solution (500 μl) and, after a short incubation time, a suspension of *Micrococcus lysodeikticus* (500 μl, 0.6 g/l) was added. The caseins used were α_s-, β-, κ-casein, whole caseinate and para-κ-casein;

the latter was made by adding an appropriate amount of rennet (Maxiren™ ; Gist-brocades) to the κ-casein solution, followed by a 10 min incubation at 30 °C and addition of pepstatin to prevent further proteolytic activity during the assay. After mixing all reagents, the cuvette contained 400 U of lysozyme/ml (0.74 μM), 0.2 g/l *M. lysodeikticus*, and the molar concentrations of the various caseins being 10, 1, 0.1 or 0.01 times that of lysozyme. Immediately after addition of *M. lysodeikticus* the suspension was mixed and the decrease in turbidity was measured spectrophotometrically at 450 nm at 25 °C for 140 s in 20 s intervals. The activity measurements were done at pH 5.1 or 6.4 in 0.1 M potassium-phosphate buffer at 0, 0.5, 1 or 2% NaCl. In the blanks the casein solutions were replaced by buffer. The casein solutions themselves did not show any detectable lysozyme activity.

4.2.2 Caseinated emulsions

Emulsions of commercially available soya-oil (Reddy™; volume fraction φ = 0.02) were made with a Condi-lab homogenizer. The soya-oil used was first made monoglyceride-free by stirring the oil for two hours with predried Kieselgel 60 (10 % w/v, Merck) followed by centrifugation and decantation. The soya-oil volume fraction of the emulsion was determined according to the method of Gerber [6]. A factor of 1.11 was used for correction of mass to volume percentage. The emulsions were made after addition of the soya-oil fraction to 50 mM K-phosphate buffer pH 5.8 containing the casein (2 g/l).

Droplet size analysis was done by means of laser diffraction (Coulter LS130), yielding the volume/surface average diameter d_{VS} (m), normally used to characterize the emulsion droplet diameter. The specific surface area (A) of the emulsion ($m^2.m^{-3}$) was calculated by using the equation $A = 6\ \varphi / d_{VS}$.

The casein concentrations in the initial solution and in the emulsion supernatant (after centrifugation for 20 min at 15000 g) were determined using the BCA protein assay (Pierce) against a bovine serum albumin standard curve. The surface load (Γ_{casein} in $mg.m^{-2}$) was found by dividing the difference in protein concentrations (in $mg.m^{-3}$) by the specific surface area A (in m^{-1}).

The association of lysozyme and casein is shown in adsorption or association isotherms in which the surface excess Γ (mole lysozyme / mole casein adsorbed) is given as a function of the free lysozyme concentration. To this end various concentrations of lysozyme were added to the emulsion, after having removed unadsorbed casein by washing 3 times by means of centrifugation for 5 min at 11000 g (1 ml vials) with 50 mM K-phosphate buffer at the appropriate pH. After incubation for 30 min, the samples were centrifuged for 5 min at 11000 g (1 ml vials). The free lysozyme concentration in the supernatant was determined by determination of activity, compared to the original concentration of enzyme in the emulsion buffer, and the surface excess calculated.

Lysozyme activity was determined spectrophotometrically by following the lysis of *Micrococcus lysodeikticus* (0.2 g/l 50 mM K-phosphate buffer, pH 6.3) at 25 °C [7]. The slope of the initial decrease of turbidity was taken as a measure of enzyme activity.

To determine the effect of temperature on the association of lysozyme with the various adsorbed caseins, a depletion study was performed. The emulsions, made at pH 5.8 (50 mM K-phosphate buffer), were washed three times with the same buffer at various temperatures (5, 20 or 40 °C) to remove unadsorbed caseins. Lysozyme was added at these temperatures up to a concentration of 20 μM and the solutions were incubated for 30 min. Percentage association was determined by measuring the activity of lysozyme in the supernatant and in the original 20 μM solution. The experiment was repeated once for the same temperature range (average RSD = 7.8 %, n = 6). Activities were also determined by using a fluorescence method. In this assay, lysozyme activity was determined by measuring the intensity of the fluorescent label of methylumbelliferyl-4-N'-N''-N'''-triacetylchytotriose (Boehringer) split off by the enzyme [8]. Fluorescence was measured at an emission wavelength of 448 nm and an excitation wavelength of 358 nm. 750 μl of clear supernatant sample was mixed with an equal volume of emulsion buffer containing the fluorescent substrate (10 μM). The reaction was performed at 40°C and stopped by adding 0.7 ml of sample to 2.5 ml of a stopping solution of 1 M glycine, 1 M NaCl adjusted to pH 12 with NaOH. Samples were taken after 40 and 80 min of incubation. The activity was measured as the change in absorbance in time (ΔE/min).

Lysozyme activity *in situ* (i.e., while still being associated with the adsorbed casein) can only be measured by using a small molecular substrate. To this end the fluorescent substrate is suitable. The emulsion droplets were washed twice, by centrifugation and resuspending in phosphate buffer and finally by resuspending in the fluorescence substrate solution (5 μM in phosphate buffer; volume equal to the original emulsion sample). Before mixing with the stopping solution the sample was filtered (0.45 μm). Samples were taken after 40 and 80 min incubation.

4.2.3 Artificial Micelle Milk (AMM)

In artificial micelle milk the casein composition of the micelles can be readily varied; therefore, AMM is a suitable substrate to study lysozyme association with casein in relation to the casein micelle composition. AMM was made according to Schmidt [9]. The pH of the AMM was adjusted to 6.0 by slowly adding 0.3 M HCl at 30 °C, while stirring. To 4 ml of AMM, 1, 2, 3, 4 or 5% by volume of a lysozyme stock solution (varying concentrations) was added. After 10 and 50 min a 2 ml sample was removed and the Ca^{2+} concentration was raised to 1 mM. A small amount of chymosin (1 μl for undiluted AMM and 3 μl for three times diluted AMM; 0.12 g/l Rennin (Sigma)) was added to flocculate the casein micelles (whether or not associated with lysozyme). Subsequently, the sample was centrifuged (Eppendorf) at 11000 *g* for 1 min at room temperature.

The amount of lysozyme associated with the casein is expressed as mole of lysozyme per mole of casein and is plotted against the equilibrium concentration of the enzyme in adsorption isotherms. For the molar mass of casein in the whole milk, the mean molar mass of the various caseins was calculated to be 23400 Da, assuming a mass ratio of α_s-casein : β-casein :κ-casein = 3.8 : 3.0 : 1.3. For AMM of β-casein : κ-casein = 1 : 2, an average molar mass of 21030 Da was used. Dilution factors for the various amounts of lysozyme solution added were accounted for. No corrections were made for the volume occupied by casein or for steric exclusion. The amount of precipitated casein was calculated by subtracting the total casein concentration of the AMM by the free casein concentration in the supernatant (free $[\text{casein}]_{sup}$ = $[\text{protein}]_{sup}$ - $[\text{lysozyme}]_{sup}$). In this way the association of lysozyme with paracaseinate can be calculated. The lysozyme concentration was determined by the method of Smolelis [7] and the protein concentrations in the original solution and supernatant by the biuret method [10].

The effect of casein concentration on lysozyme association was established by diluting the AMM three times with its corresponding permeate, separately made by ultrafiltration in a (Filtron) stirred cell (pressure difference 1.5 bar, room temperature, molecular weight cut-off 100 kDa). Lysozyme was added to the diluted AMM and its association determined as described above for AMM.

The lysozyme activity *in situ*, i.e. while associated with casein micelles, was determined using the fluorescence assay. The AMM-lysozyme suspension (1.5 ml) was ultrafiltered in a stirred cell to prevent pellet formation, until 1 ml of permeate was collected. Then, 1.5 ml of potassium phosphate buffer (25 mM, pH 6.0) was added and ultrafiltration was repeated. The washing was repeated three times. The washing procedure applied has led to a total dilution by 20 times (by a factor of 4 by the first wash, a factor of 2.5 by the second and a factor of 2 by the third). Lysozyme activities were determined in the permeates and retentates. The lysozyme activity *in situ* was determined for three different lysozyme concentrations applied.

4.3 Results and discussion

4.3.1 Association of lysozyme with caseins in solution

Table 1 shows the percentages of lysozyme and of the caseins found in the filtrates made after removal of the casein-lysozyme complexes formed. Based on a calibration curve (retention time as a function of molecular weight) it was shown that the individual caseins in solution occurred mainly in di- and multimer forms and lysozyme in monomer form. Some 2/3 of β-casein and lysozyme were associated in an equimolar ratio and filtered out. One molecule of α_s-casein associated with more than one molecule of lysozyme, whereas κ-casein and lysozyme did not associate at all.

These results agree with those of Thapon and Brulé [3], who found 100% association with α_s-casein (even within a pH range of 7.0 - 4.8 and up to 4% NaCl). They observed that κ-casein did not show any affinity in the pH range mentioned, and β-casein and whole casein showed intermediate affinities, decreasing with decreasing pH and increasing ionic strength. The extent of association of lysozyme with caseins therefore appeared to be correlated with the degree of phosphorylation of the various caseins (α_{s1}-casein : 8 P, β-casein : 5 P and κ-casein : 1 P). Electrostatic interactions between the positively charged ε-amino groups of lysine residues of the lysozyme molecule with negatively charged phosphate groups were assumed to be responsible for the association [3].

In Fig. 1 the relative activities of lysozyme (the lysozyme activity of the casein free sample was taken as being 100%) are given in relation to the amount of casein added. The lysozyme was allowed to associate with the caseins for some time, before adding the cell suspension. Adding various concentrations of casein had only a small effect, if any, on lysozyme activity, as compared to a blank without casein. Similar results were found for higher NaCl concentrations and pH 6.4, although the absolute activity was significantly lower at higher ionic strength and pH (Fig. 2).

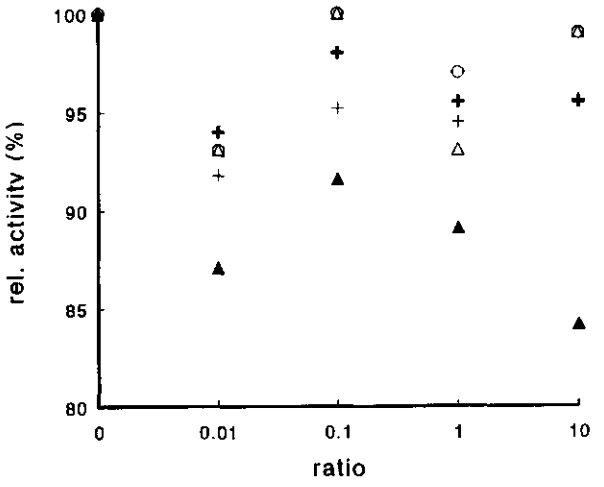

Fig. 1 The relative activity of lysozyme (0.74 μM) as a function of the molar ratio [casein]/[lysozyme] of various dissolved caseins added at pH 5.1. α_s-casein (+), β-casein (Δ), κ-casein (o), para-κ-casein (+) and whole caseinate (▲).

***Table 1** Amounts of lysozyme and casein in the filtrates, collected after having mixed equal volumes of lysozyme (1.5 g/l) and casein (3.0 g/l) solutions (50 mM phosphate buffer, 50 mM NaCl, pH 6.7), determined by measuring E_{280} after HPSEC. Percentage is relative to the amount in the original solution.*

Casein	% casein	% lysozyme
α_s-casein	38.9	2.0
β-casein	33.3	23.8
κ-casein	100.0	100.0

A relative standard deviation of 3.0 % and 4.3 % at pH 5.1 and pH 6.4 respectively were found for blank measurements in the absence of NaCl (n = 15).

A prerequisite for lysozyme activity is that the Glu 35 and Asp 52 residues in the active centre are protonated and deprotonated respectively [11]. This will occur around the optimum pH of 5.0.

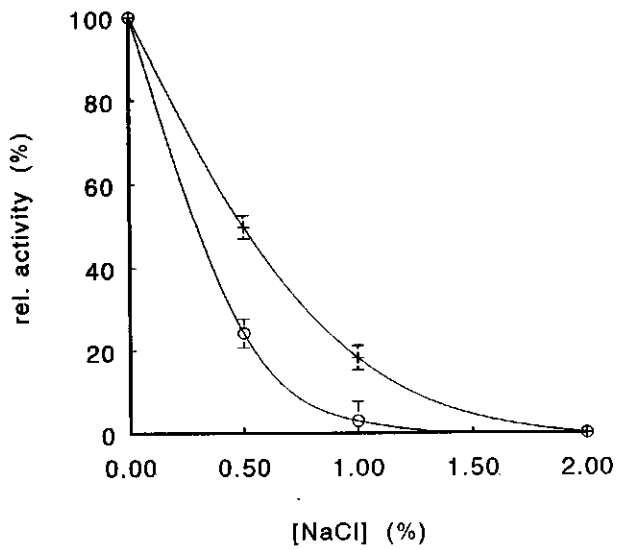

***Fig. 2** Influence of NaCl on the relative activity of lysozyme at pH 5.1 (+) and pH 6.4 (o) in 0.1 M K- phosphate buffer.*

The NaCl present in the experiments may well play a role in disturbing this delicate electrostatic interaction. Moreover, a high ionic strength will affect the secondary affinity, which defines the proper positioning of the strongly positively charged lysozyme molecule (isoelectric pH 11) and the bacterial cell wall. Since lysozyme did not or hardly lose activity when associated with α_s- and β-casein, its active site cannot be involved in this association. These results agree with those of Birkkjaer [12], but conflict with those found by others [3], who reported a thousandfold decrease in activity of lysozyme, due to association with whole casein at pH 6.2 (molar ratio lysozyme:casein = 1:4). Such a decrease seems to be at variance with the observed activity of fairly small amounts of lysozyme in cheese against *C. tyrobutyricum* [1]. Furthermore, it is important to note that we always found *in situ* activity of lysozyme in the other casein substrates applied (see below).

4.3.2 Association of lysozyme with casein micelles

The effect of association of lysozyme with casein micelles in milk on its activity cannot be studied by applying the experimental methods described above. The decrease in turbidity due to cell lysis cannot be determined in a milky, turbid medium. Moreover, an enzyme immobilized due to association with casein micelles, will exhibit a kinetic behaviour that differs from that of an enzyme in solution, the more so because the cell wall substrate itself is a particle [13]. To study lysozyme activity *in situ*, a better substrate would be a small molecule that can interact with the immobilized enzyme. A small-sized, fluorescent substrate was therefore selected. As shown in Fig. 3, the association with whole casein micelles is much stronger than with casein micelles composed of β-casein : κ-casein = 1:2 at the same (low) equilibrium concentration of lysozyme. This corresponds with the results on lysozyme association with caseins in solution. The lack of α_s- casein in the β/κ-casein micelle (a small amount of α_s- casein may be present as an impurity) and the presence of κ-casein not associating with lysozyme, could account for the weaker association.

The "association" isotherms in Fig. 3 do not reach a plateau value. The curve for undiluted AMM represents three separately made AMMs to which lysozyme in different ranges of concentration was added (coefficient of correlation = 0.99). Extrapolation of the curves for the whole casein micelle would lead to a plateau value of about 0.8 mole of lysozyme per mole of casein (the curve was mathematically described by $y = ax/(b + x)$, in which values for a and b were calculated by an iteration procedure). This would mean that caseins situated in the interior of the micelle may also bind lysozyme molecules. If, like in solution, κ-casein does not take part in the association with lysozyme, this would imply that an α_s-casein molecule (like in solution) can associate with more than one lysozyme molecule. It is also possible that lysozyme, due to its self-association will associate as a dimer or a higher aggregated form [14]. Self-association of lysozyme is most likely to have occurred in the original lysozyme stock solution, considering its concentration and the pH. On the other hand,

as self-association is a reversible reaction, dilution (which occurs by adding lysozyme to the casein solution) would cause monomeric forms of lysozyme to become exposed to the caseins. Whatever the mechanism, the casein molecules of casein micelles can bind considerable amounts of lysozyme. In other words, formation of casein-lysozyme micelles can readily occur[15, 16].

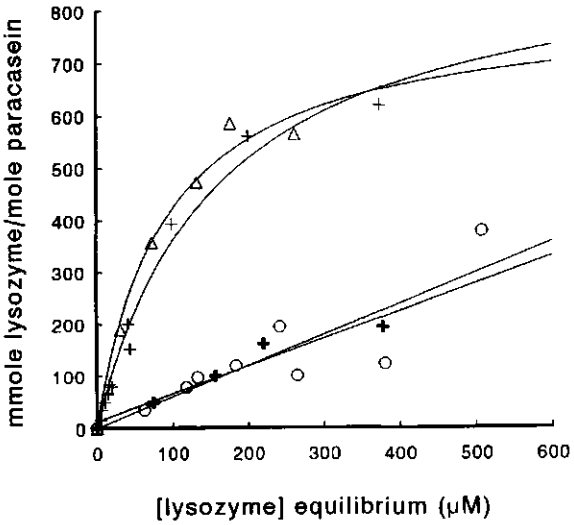

Fig. 3 Adsorption isotherms showing the lysozyme-para-casein association (mmol/mol) as a function of [lysozyme] in the supernatant of the milk at pH 6.0 and 30 °C. Artificial casein micelles: whole caseinate (+), whole caseinate three times diluted (Δ), β:κ-casein 1:2 (o) and β:κ-casein 1:2 three times diluted (✚).

Dilution of the system (i.e. decreasing the concentration of adsorbent) did not affect the lysozyme association (Fig. 3). This differs from the association of chymosin with caseins [17]. The increase of association with chymosin on lowering the casein concentration is partly explained by a mechanism of competitive adsorption (De Roos and Walstra, to be published), which means that "binding sites" for chymosin on the para-κ-casein molecule may be shielded by other caseins. On dilution of the system, a change in association equilibrium between the caseins would cause such a site to become exposed, leading to association of chymosin. In the case of lysozyme this mechanism will not hold, firstly because the lysozyme molecule does not associate with κ-casein; moreover, a change in casein micelle composition, due to α_s - or

β-casein leaving the micelle after dilution, would hardly be "sensed" by the lysozyme molecule, because its association with either of these caseins does not greatly differ. Table 2 shows lysozyme activity *in situ*, after association with whole casein micelles. By comparing the whole amount of lysozyme added with lysozyme remaining in the permeate (both based on activity measurement), the amount of lysozyme associated can be calculated. Percentages of association were roughly constant for the lysozyme concentrations involved. It means that the largest amount of lysozyme associated occurs in the system with the highest lysozyme concentration. However, the *in situ* activity in the retentates, although increasing, did not increase proportionally (Fig. 3 shows that the Γ_{plat} association value has not been reached at the free lysozyme concentrations of the permeates). The *in situ* activity measured was considerably lower than expected. Probably, in this system internal diffusion limitation for the substrate within the casein micelle plays a role [13].

Table 2. Association of lysozyme with whole casein micelles. In situ activity in the retentate was determined after three times washing with phosphate buffer pH 6.0 by means of stirring and filtration. % activity in situ is the amount of activity actually found compared to the amount expected.

sample	[lysozyme] μM		
original	92.6	198	304
in permeate	26.4 (71.4*)	56.3 (71.6)	66.3 (78.2)
in wash-1	14.6	18.7	20.3
in wash-2	8.0	8.3	5.4
in wash-3	5.9	9.0	4.2
in retentate	39.0	69.4	67.2
% act. *in situ*	93	68	38

* (% association)

In this system relatively more lysozyme would have been associated in the interior of the casein micelle. The decrease of lysozyme concentrations in the permeates due to dilution is less than calculated because partial desorption of lysozyme from the casein micelles occurred. The extent of desorption seems to be larger in the system of low lysozyme dosage. Nevertheles, all retentates contained much higher (immobilized) activity than the permeates from wash 3.

4.3.3 Association of lysozyme with adsorbed caseins

Fig. 4 shows association isotherms of lysozyme at pH 5.8 on soya-oil emulsion droplets stabilized by various caseins (coefficients of correlation for two separately made emulsions with α_s-, β- and κ-casein were 0.88, 0.95 and 0.94 respectively). Once again, lysozyme associated most strongly with α_s-casein. In contrast to its behaviour in solution, adsorbed κ-casein bound lysozyme in considerable amounts. In solution, however, κ-casein occurs in covalently linked multimers which associate into micelles, depending on concentration [18]. It is unlikely that κ-casein micelles would remain unaltered after adsorption onto the oil/water interface. Probably, due to adsorption, κ-casein changes its conformation by partial unfolding, thereby exposing amino acid residues which may subsequently associate with lysozyme molecules. However, such unfolding would be limited, because the caseins are known to have little secondary and tertiary structure. Another possibility is that lysozyme would coadsorb onto spots of uncovered oil/water surface area. Such anchoring of lysozyme would not result in extensive unfolding of the molecule and might result in preservation of activity. However, attempts to immobilize enzymes in such a system by coadsorption with bovine serum albumin failed [4].

Comparison of the lysozyme association isotherms of the casein micelle system (Fig. 3) with those of the caseinated emulsion droplets system (Fig. 4), shows that, for the latter

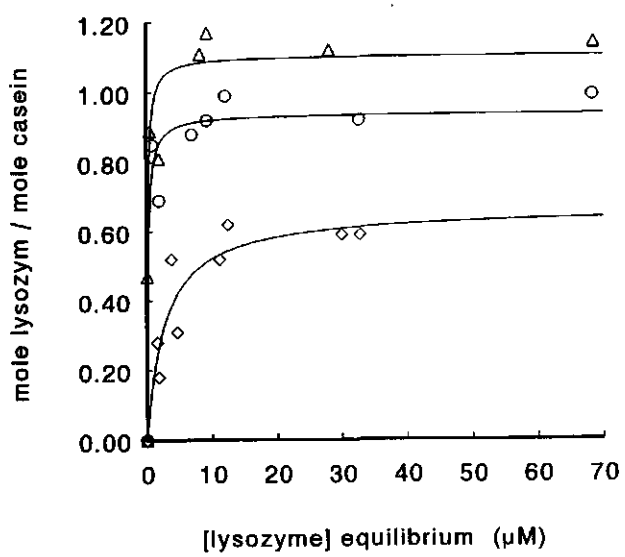

Fig. 4 Adsorption isotherms of lysozyme for various caseins adsorbed onto soya-oil emulsion droplets at pH 5.8 and 20 °C. α_s-casein (Δ), β-casein (o) and κ-casein (◊).

system, a surface excess plateau value (Γ_{plat}) is reached at a much lower lysozyme equilibrium concentration (about 5 μM) than for the casein micelle system (about 500 μM). Obviously, the accessibility of lysozyme for binding sites in a casein micelle is more limited than for caseins adsorbed onto an emulsion droplet. The hairy layer of flexible κ-casein molecules may sterically hamper accessibility of lysozyme molecules to penetrate the interior of the casein micelle. Moreover, κ-casein itself, as a part of the micelle, is presumably not a binding site for lysozyme. The greater accessibility to adsorbed caseins is reflected in a steeper slope (higher affinity) of the adsorption isotherm. The plateau adsorption levels reached for α_s- and β-casein corresponded with the values reached in solution (Table 1).

Table 3. The influence of pH on the association of lysozyme with various caseins adsorbed onto soya-oil emulsion droplets. Plateau values found by extrapolation of adsorption isotherms

pH	$\Gamma_{plateau}$ (mole lysozyme / mole casein)		
	α_s-casein	β-casein	κ-casein
5.4	0.97	0.99	0.91
5.8	1.03	0.99	0.63
6.2	1.20	0.99	0.57

The influence of pH on the association (Table 3) is relatively pronounced for adsorbed κ-casein, and limited and absent for α_s- and β-casein, respectively. In solution, association of lysozyme with α_s- and β-casein was independent of pH, within the range chosen. α_s- and β-casein are able to bind lysozyme in about equimolar amounts; this result corresponds with those found for dissolved caseins.

Table 4. The influence of temperature on the association of lysozyme with various caseins adsorbed onto soya-oil emulsion droplets at pH 5.8 (mole lysozyme/mole adsorbed casein at $[lysozyme]_{equilibrium}$ = 1 μM). Total concentration of lysozyme added to the emulsion 20 μM.

casein	5 °C	20 °C	40 °C
α_s-casein	0.80	0.67	0.69
β-casein	0.44	0.48	0.51
κ-casein	0.31	0.22	0.26

The influence of temperature on the association is given in Table 4. The extents of association of lysozyme with the caseins is given for a comparable lysozyme equilibrium concentration (1 μM). Within the temperature range chosen (5 - 20 - 40 °C) no significant influence of temperature was found, indicating that hydrophobic interactions do not play an essential role in the association.

Lysozyme activity *in situ* was measured after a threefold washing procedure by means of centrifugation and resuspension (the fourth supernatant did not contain any lysozyme activity within the detection limit of the test). Reliable measurements could only be done with lysozyme associated with adsorbed κ-casein, because only in this system could the droplet layer, formed after centrifugation, be resuspended. Table 5 shows that the total activity of the lysozyme present is recovered. Emulsions containing α_s- and β-casein as the emulsifiers, exhibited considerable lysozyme activity *in situ*, but not all activity was recovered, but this could have been caused by the poor resuspending ability.

***Table 5.** Partitioning of lysozyme activity (fluorescence DE/min) over the subphase and while associated with adsorbed κ-casein (in situ) in the cream layer at pH 5.8 after centrifugation of an emulsion. 20 μM added to the emulsion.*

added	**in 1st subphase**	***in situ***
0.40	0.26	0.16
0.47	0.28	0.21
1.52	1.24	0.39

4.4 Concluding remarks

Lysozyme readily associated with α_s- and β-caseins (α_s > β) in solution, but no association with dissolved κ-casein was observed. This difference in association of lysozyme with the various caseins is reflected in the associations with casein micelles of various compositions. In contrast to results obtained by others, no significant loss in activity due to the association was observed, indicating that the active centre of lysozyme is not involved in the association. It appeared that immobilization of lysozyme by association with whole casein micelles resulted in a loss of activity, probably due to association of lysozyme with casein molecules inside the casein micelles, resulting in internal diffusion limitation.

The association properties of κ-casein for lysozyme were drastically changed after the casein had been adsorbed onto an oil/water interface (compare Fig. 4 and Table 1). A change in aggregation or conformation of the κ-casein molecules due to adsorption may result in

exposure of an accessible part of the molecule, followed by association with lysozyme. Another possibility is that casein-coated emulsion droplets can coadsorb lysozyme with preservation of activity. The association of lysozyme with caseins is dominated by electrostatic interactions, as shown by the influence of ionic strength. The small effect of temperature on the association suggests that hydrophobic interactions do not play a substantial role.

The lytic activity of lysozyme towards *M. lysodeikticus* strongly depends on pH and ionic strength. Probably, in applying lysozyme to prevent "late blowing" in Gouda and Emmentaler-type cheese ripening, the enzyme will only be active during the first manufacturing stage, i.e. before the salting of the cheese. In salted cheese, where the salt content may be 5 % in the water, no activity is expected. However, at high salt content, the clostridia cannot germinate anyway.

Casein micelles and casein-stabilized emulsion droplets can be regarded as suitable adsorbants for the immobilization of lysozyme. The adsorption of lysozyme onto the carriers implies that the activity of lysozyme towards substrates that cannot diffuse to the enzyme, i.e. in many cases bacteria, will be diminished. In every instance, it would be necessary to estimate this effect.

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ASSOCIATION OF CHYMOSIN WITH ADSORBED CASEINS

Chapter 5

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Abstract

We studied the association behaviour of chymosin with caseins, adsorbed onto soya-oil emulsion droplets, under various conditions by making up adsorption isotherms. It was found that chymosin associated solely with adsorbed κ-casein and not with α_s- or β-casein. This association was stronger for lower values of pH, ionic strength, temperature and κ-casein concentration, indicating that electrostatic interactions are involved.

Associated chymosin was found to be still active. The system described can be conceived as a new technique for immobilization of biologically active proteins on a liquid carrier.

5.1. INTRODUCTION

In studies aimed at the accelleration of cheese ripening by means of adding exo- and endoproteases, isolated from starter bacteria, to the cheese milk, it is often observed that, during the cheese making process, almost all off the extra added enzymes go to the whey fraction and very little to the curd. It was reasoned that these enzymes may be transported into the curd by immobilizing them onto milk fat globules on the condition that the enzyme activity is retained.

In a more general study we used the renneting enzyme chymosin as the emulsifying agent in order to stabilize a soya-oil/water emulsion. In this study it was found that the enzyme had lost its activity and even proved to be denaturated after having been desorbed from the oil/water interface by means of adding a small-molecule surfactant.

It is well established that chymosin is transported partly (depending on production conditions) into the curd during cheese-making, playing a key role in cheese ripening. The mechanism of transport of chymosin into the curd is believed to be one of adsorption onto (or better association with) casein micelles. This mechanism of chymosin association with caseins, however, is not well understood, nor is it known which casein is primarily involved. We used caseins as an emulsifier with which chymosin could be associated. In this paper aspects of the mechanism of chymosin association with adsorbed caseins are described. The influences of various conditions such as pH, ionic strength, temperature and substrate concentration are shown in adsorption isotherms. We have to keep in mind, however, that these adsorption isotherms reflect a protein/protein interaction between the adsorbed casein and chymosin and not the adsorption of proteins onto solid/liquid or fluid/liquid interfaces.

5.2 Materials and methods

Emulsions were made with a Condi lab homogenizer using purified soya-oil (volume fraction $\varphi = 0.02$) and various caseins (w/v 2 %, Sigma) as the emulsifying agents. The emulsions were made in 25 mM K-phosphate buffer, pH 6.7, repeating the homogenization ten times. Commercial soya-oil (Reddy™) was made monoglyceride-free by stirring it for one hour with predried Kieselgel 60 (Merck) followed by centrifugation. The soya-oil volume fraction was determined by the Gerber method [1] (a correction factor of 1.11 was used for the soya-oil density).

In a preliminary experiment, meant to establish the casein with which chymosin associates, the cream layers of soya-oil emulsions made with α_s-, ß- or κ-casein (2 g/l) were, after having washed away the unbound caseins twice by centrifugation (20 min, 12000 *g*) and resuspending with 25 mM K-phosphate buffer solutions of pH 5.0 - 5.4 - 5.8 - 6.2 or 6.8, resuspended in K-phosphate buffers with 0.7 µmolar chymosin.

In the following experiment the cream layers of a κ-casein/soya-oil emulsion were washed two times with K-phosphate buffers of different concentration, pH, temperature and ionic strength (by adding KCl), and finally resuspended in the same buffers with chymosin included at concentrations of 10 - 5 - 4 - 3 - 2 - 1 and 0.5 μmolar. The chymosin (Chymax, Pfizer) was lyophilized after having been made salt-free by means of dialysis against water, in order to minimize the influence of salts on the adsorption conditions due to addition of the enzyme. The emulsion was incubated with the chymosin for 20 min and then centrifugated in 1 ml vials (Eppendorf, 1 min - 12000 *g*). The residual chymosin activity in the supernatant was determined with the Berridge flocculation test [2]. Chymosin adsorption percentage could be calculated according to the relation of Storch and Segelcke, which states that the flocculation time is inversely proportional to the chymosin concentration [3].

Chymosin solutions corresponding with the experimental conditions, in a concentration to give a flocculation time of about 300 s (on 4000 times dilution) were used as the blank.

Emulsion droplet size analysis was performed by means of laser diffraction (Coulter LS130) yielding the volume/surface average diameter (d_{vs}). The specific surface area (A) of the emulsion was calculated with the equation $A = 6\ \varphi / d_{vs}$.

The casein concentrations in the starting solution and the emulsion supernatant were determined with the BCA protein assay (Pierce). With the difference between these two concentrations the casein surface load (Γ_{cas} in $mg.m^{-2}$) could be calculated.

The chymosin surface load on adsorbed casein (mmol chymosin/mol casein) was determined considering that a 13.3 μmolar chymosin concentration [3] leads to a rennet clotting time of about 300 s (4000 fold dilution).

Chymosin activity *in situ*, i.e. while being associated with the casein-coated droplets, was determined with a small synthetic hexapeptide substrate HLeu-Ser-Phe(NO_2)-Nle-Ala-Leu-Ome (Bachem), that is split at the Phe(NO_2)-NLe linkage [4]. The emulsion droplet layer was washed three times in a 0.05 molar acetate buffer of pH 4.7 and resuspended in a 0.5 mmolar hexapeptide solution in the same buffer. After 20 min of incubation the emulsion was filtered (pore size 0.2 μm) and the absorbance of the filtrate measured at 310 nm spectrophotometrically.

Alternatively, chymosin activity *in situ* was measured by following caseinomacropeptide release by means of HPLC chromatography [5] after various times of incubation (0 - 0.5 - 1 - 2 - 5 - 15 and 30 min) of washed emulsion droplets with a κ-casein solution (2 g/l at room temperature).

5.3 Results and discussion

The first determination to be made was with which casein was the chymosin associated [6]. In figure 1 the association of chymosin with emulsions made with the three different

caseins is shown. Chymosin was associated strongly and exclusively with adsorbed κ-casein; the association was higher at lower pH. Association with α_s- or ß-casein was weak or absent.

Fast creaming of the κ-casein emulsion was observed after addition of the chymosin. This must be due to flocculation of the emulsion droplets, caused by loss of steric repulsion after splitting off the caseinomacropeptide of the κ-casein molecule. This would imply that the caseinomacropeptide part is not attached to the soya-oil/water interface. The association of chymosin probably takes place at the remaining para-κ-casein part, most likely after splitting off the caseinomacropeptide. Emulsions made with α_s- and ß-casein were far more stable after addition of chymosin. In the supernatant of these emulsions with added chymosin, casein fragments were found by means of HPLC gel permeation measurements. Identification of these fragments was not carried out.

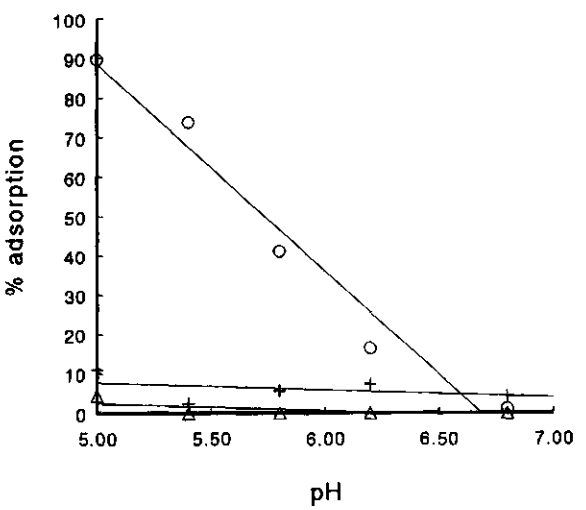

Fig. 1 The association of chymosin in % of initial amount of chymosin (0.7 μmolar) with adsorbed α_s-casein (+), ß-casein (△) and κ-casein (o) as a function of pH, after 20 min of incubation at room temperature in 25 mM K-phosphate buffer.

Chymosin adsorption isotherms under various conditions were made starting from one κ-casein/soya-oil emulsion with the following characterictics: $\varphi_{soya} = 0.0185$; volume/surface

average weighed diameter d_{vs}= 1.63 10^{-6} m ; specific surface area A = 0.068 m^2/m^3; κ-casein surface load Γ = 8.97 mg/m^2 corresponding with a κ-casein concentration of 31 μM for the emulsion. The chymosin surface load on this emulsion is expressed as mmol chymosin/mol κ-casein. The results were fitted to a Langmuir-type equation.

Figure 2 shows the influence of the pH on chymosin association. Chymosin association increased with decreasing pH. This pH dependence points to electrostatic interactions being involved. At pH 5.0 the chymosin molecule will have a small net negative charge (i.e.p. = 4.7) that will increase at increasing pH.

In the pH-range mentioned para-κ-casein will be positively charged (the i.e.p. is unknown but electrophoretic mobility towards the cathode is observed at pH 7 (7)). As we do not know the mechanism the enzyme-substrate complex formation and the pK-values of the amino acids involved, it is difficult to explain this result. The three histidine groups (amino acids 98 - 100 - 102) are believed to play an essential role in the formation of the enzym-substrate complex that precedes the cleavage of the Phe-Met bond of κ-casein [8]. At pH values above pH = 6.0 the histidine groups become less positive and more and more the negatively charged chymosin is not able to associate. This speculation on the mechanism of association can only be valid if it would be coupled to the active centre of the enzyme.

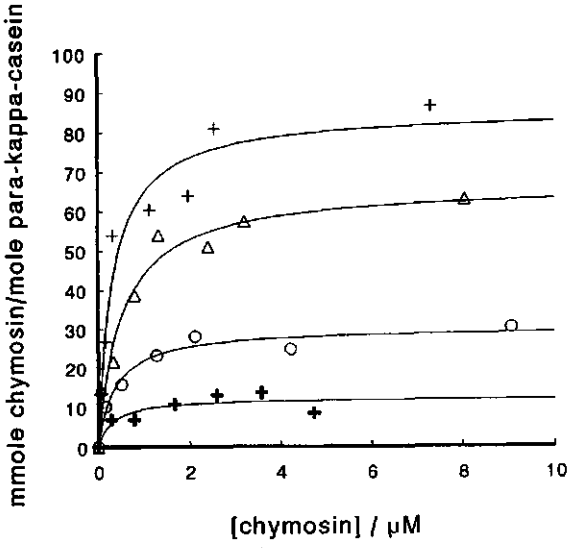

Fig. 2 The influence of pH on the association of chymosin with κ-casein, adsorbed onto soya-oil emulsion droplets, after 20 min of incubation in 25 mM K-phosphate buffer at room temperature. pH 5.0 (+), 5.4 (△), 5.8 (o) and 6.2 (+).

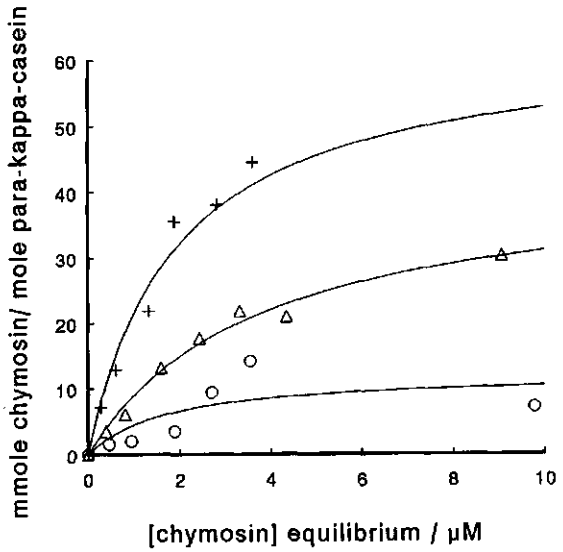

Fig. 3 The influence of ionic strength on the association of chymosin with κ-casein, adsorbed onto emulsion droplets, after 20 min of incubation in 25 mM K-phosphate buffer pH 5.5 at room temperature. [KCl] 0 mM (+), 75 mM (Δ) and 150 mM (o).

If the protein/protein interaction is of an electrostatic nature, ionic strength should also play a part. We varied the ionic strength at constant pH by adding KCl at various amounts. As can be seen in figure 3, chymosin association becomes less at increasing ionic strength. Although the Debye length is expected to become shorter [7] (for 0 mM KCl ≈ 0.95 nm and for 150 mM ≈ 0.63 nm) and closer approach of the two proteins would thus be possible, association becomes less. This may be due to association of the ions with the charged groups, which play an essential role in the electrostatic protein interaction. Other salts, such as NaCl, LiCl and varying concentrations of the K-phosphate buffer showed comparable effects.

In cheese making the transfer of chymosin into the curd is highly affected by temperature [9]. A similar effect was found in the system used here (figure 4). Chymosin association became much stronger at decreasing temperature. For this reason the interaction of chymosin and para-κ-casein is not believed to be a hydrophobic one.

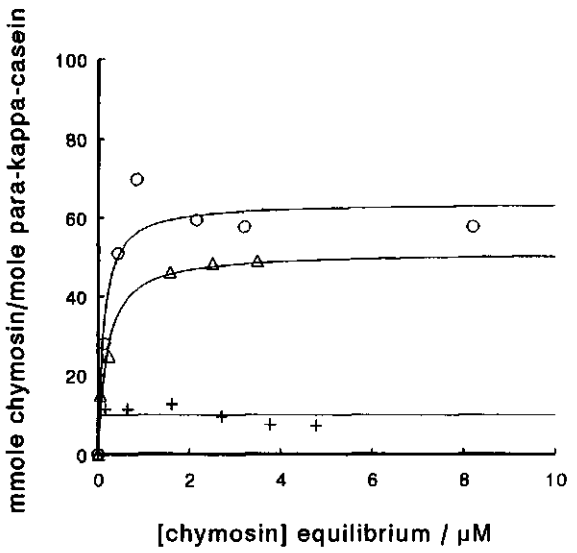

Fig. 4 The influence of temperature on the association of chymosin with κ-casein adsorbed onto soya-oil emulsion droplets, after 20 min of incubation in 25 mM K-phosphate buffer at pH 5.5. 4 °C (o), 20 °C (△) and 40 °C (+).

When chymosin was added to dilutions of the emulsion with adsorbed κ-casein the absolute percentage of association became less. At first sight this looks quite obvious. However, if we express the association in values of surface load, i.e. mmol of chymosin associated per mol casein, we find, surprisingly, at comparable chymosin equilibrium concentration a higher surface load for the diluted system. Dilution of the emulsion results in a decreased number of fat globules, hence, an increased water/adsorbed protein ratio. In other words, at a higher water to protein ratio of the system, chymosin association becomes higher.

In a very concentrated system like cheese, a surface load of 0.1 mmol chymosin/mol κ-casein was found and for milk (pH 5.0, I = 120 mM, [κ-casein] = 130 μM), the diluted form, 1.4 mmole chymosin/mole κ-casein [10]. By comparing the surface load for the emulsion system (under the same experimental conditions as in milk and a chymosin equilibrium concentration of 0.1 μM), we find the same surface load of 1.4 mmole chymosin/mole κ-casein. An explanation for this phenomenon of chymosin surface load being dependent on the κ-casein concentration has not been worked out completely yet, but we feel it must be due to a shift in association equilibrium situations with competitive associating molecules, presumably other caseins, upon dilution. The effect of temperature on the chymosin surface load may also be the consequence

of the shift in the association equilibrium of the competitive associating molecules. Hydrophobic interactions may play a role here.

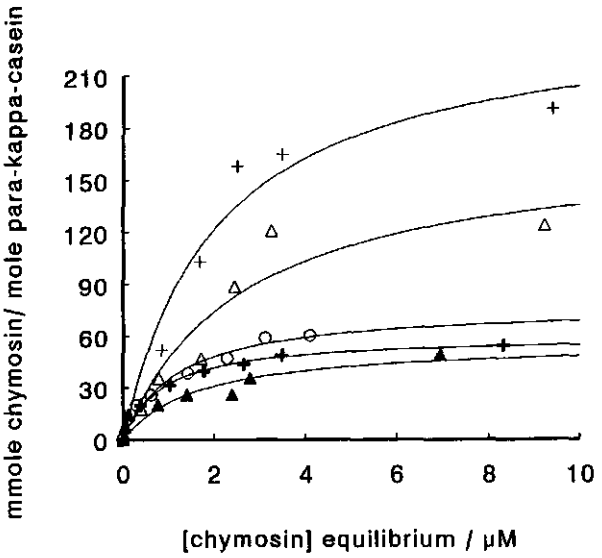

Fig. 5 The influence of the κ-casein concentration, in various dilutions of the soya-oil/κ-casein emulsions, on the association with chymosin after 20 min of incubation in 25 mM K-phosphate buffer pH 5.5 at room temperature. Emulsion dilution: 10 × (+), 5 × (△), 2 × (○), 0 × (✚) and 0.5 × (▲).

As the association of chymosin is so delicately dependent on environmental conditions like pH and ionic strength, the reverse process of release of already associated chymosin may be induced by altering these conditions. If emulsion droplets loaded with κ-casein and chymosin (made, for instance, at pH 5.5) are added to milk in the Berridge flocculation test (experimental pH 6.3), a chymosin activity is found, corresponding to the differences in association between pH 5.5 and 6.3. If emulsion droplets, made at pH 6.3 and at an ionic strength comparable with that in the flocculation milk [11], were used in the flocculation test, no chymosin activity was found, presumably because the associated chymosin had not been released. This means that association of chymosin with adsorbed κ-casein does not inactivate the enzyme. It also means that the concentration of associated chymosin *in situ* cannot be determined by the flocculation assay, because diffusion limitation and steric hindrance may effect their affinity. In another chymosin activity assay in which a highly mobile low-molecular synthetic hexapeptide substrate was used, complete conversion of the substrate by the

associated chymosin *in situ* was found. No further quantification of this finding was made, but it was shown that chymosin associated with κ-casein remains active. In the experiment where κ-casein was used as the substrate, complete conversion of the substrate was found. If the association interaction is restricted to the active centre of the enzyme, as was speculated before, it is hard to imagine that the enzyme can be associated to para-κ-casein and be able to be active at the same time. The amino acids involved in the association interaction are thus most likely different from those involved in the enzyme-substrate reaction.

It is known that other biologically active proteins, like lysozyme, lipase, plasmin, bacteriocins and nisin also have an associating affinity for caseins. All these proteins can be transfered into the curd during the cheese making process. Although we have to keep in mind that the process of emulsification may well cause a conformational change of the casein molecules and thereby exposure of different functional groups for association, there is still a fair chance that adsorbed caseins will associate with the proteins mentioned above. Moreover other proteins may possess binding affinity for caseins. In this way emulsion droplets can serve as a liquid carrier for immobilizing proteins with caseins as the intermediate ligand.

5.4 References

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ASSOCIATION OF CHYMOSIN WITH CASEINS IN SOLUTION

CHAPTER 6

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Abstract

The association of chymosin with para-κ-casein was studied in a solution of κ-casein in imidazole buffer, with or without α_s- or β-casein being added. Standard conditions were pH 6.3, 30°C and ionic strength 0.04 *M*. Association was determined by removing the coagulum by centrifugation and determining the activity of chymosin in the supernatant.

The association of chymosin with para-κ-casein was decreased by adding α_s- or β-casein and was increased upon dilution with imidazole buffer. Competition between chymosin and α_s- or β-casein for association with para-κ-casein is offered as an explanation for these results. The association was slightly lower at 10°C than at 40°C and decreased with increasing ionic strength or pH. Presumably, hydrophobic interactions as well as electrostatic forces are involved.

6.1 Introduction

During cheese making, part of the chymosin is retained in the drained curd. During cheese ripening, this chymosin hydrolyses the caseins in the cheese. The peptides formed include bitter peptides. Subsequently, the proteases and peptidases of starter bacteria may hydrolyse the peptides into smaller non-bitter peptides and amino acids. These processes contribute to the taste of cheese. Chymosin is therefore an essential factor in cheese ripening [1].

An even distribution of chymosin over the aqueous phases in milk and curd during cheese manufacture would lead to about 5% of the added chymosin being retained in the curd. However, Stadhouders and Hup [1] found this to be about 15% in Gouda cheese. They also showed that by decreasing the pH of the cheese milk or the scalding temperature, the amount of retained chymosin increased.

By using artificial micelle milk, the composition of the casein fraction was found to be an important factor in chymosin retention (De Roos et al., unpublished results). The retention increased when the content of κ-casein in casein fractions increased, and chymosin retention was stronger in case of micelles composed of β- and κ-casein than in case of micelles composed of α_s- and κ-casein.

Chymosin may be retained in the curd by association with paracasein. The aim of this study was to achieve a better understanding of the mechanism of this association. For that purpose, the interaction between chymosin and several caseins was investigated in a model system. To make this model as simple as possible, caseins were in a dissolved state. Factors investigated were contact time between chymosin and casein, casein and chymosin concentrations, composition of the casein mixture, temperature, ionic strength and pH.

6.2 Materials and methods

Caseins were dissolved in 0.05 *M* imidazole-HCl buffer of pH 6.3 and ionic strength 0.04 *M* [2]. pH 6.3 is a common pH at the start of pressing of curd in Gouda cheese manufacture [3]. The concentration of the individual caseins was calculated from the casein content (87% w/w by Kjeldahl analysis) and the composition of the casein preparations used (Sigma Chemical Company, St. Louis, USA). The casein in the κ-casein preparation contained 94.6% κ- and 5.4% α_s-casein, the α_s-casein preparation 88.5% α_s- and 11.5% β-casein and the β-casein preparation 91.2% β- and 8.8% α_s-casein (w/w; estimated from polyacrylamide gel electrophoresis and densitometry of the resulting gels). Chymosin (lyophilized powder, 94 units/mg; Sigma Chemical Company) was dissolved in the same buffer as casein. In this buffer, the chymosin activity did not decrease during the experiments.

A 1 ml volume of κ-casein solution (imidazole-HCl buffer for the blank) was mixed with 2 ml of chymosin solution. Concentrations of caseins and chymosin are mentioned later.

The mixture was kept stirred at 30°C. High concentrations of chymosin were used (by a factor 300 to 3000 times higher than in cheesemaking), and para-κ-casein coagulated within a few seconds. At 0.5, 2 and 4 min after mixing (contact time), a sample was centrifuged for 1 min at 11000 *g* in an Eppendorf centrifuge to remove the coagulum containing the associated chymosin. In the supernatant, the chymosin activity was determined by the Berridge flocculation test [4]: *x* ml was added to a polystyrene conical tube containing 0.15 ml reconstituted whey (no residual chymosin activity; Borculo Whey Products, Borculo, The Netherlands) and 0.5 ml 0.2 *M* $CaCl_2$; water was added to give 5 ml. The whey was used to prevent inactivation of chymosin during storage of the sample at about -22°C (Geurts, unpublished results). A solution of 20 g low-heat skim milk powder in 100 g water (double-concentration reconstituted skim milk [5], stirred at 45°C for 1 h and stored at 4°C overnight), the conical tube containing the sample, and a 25 ml beaker with a glass rod were warmed to 30°C. Then, 5 ml of the milk was added to the sample, mixed, poured into the beaker and kept at 30°C. The time after which the first flocs became visible was taken as the flocculation time (t_f).

The percentage of associated chymosin (P) was calculated using the rule of Storch and Segelcke [6], which states that the flocculation time is inversely proportional to the chymosin concentration: $P = [(t_{fs} - t_{fb}) / t_{fs}] \cdot 100$, where t_{fs} and t_{fb} are the flocculation times for supernatant and blank, respectively. The maximum chymosin association at a certain chymosin concentration, reached immediately after hydrolysis of the Phe_{105}-Met_{106} bond of κ-casein to form para-κ-casein, was determined by extrapolating the percentages of association after 0.5, 2 and 4 min of contact to reach time zero, using linear regression.

For the calculation of the concentration of chymosin (C), it was assumed that the chymosin concentration in a commercial calf rennet of 10800 Soxhlet units was 13.3 μ*M* [6]. 4000-Fold dilution of this rennet was found to lead to a flocculation time of about 300 s. As a standard throughout the experiments a rennet solution guaranteed to contain 9660 Soxhlet units (i.e. 11.9 μ*M* chymosin by using the above-mentioned assumption) (RIKILT-DLO, Wageningen, The Netherlands) was diluted 3578-fold to achieve a flocculation time (t_{fg}) of about 300 s. C was then calculated on the basis of the rule of Storch and Segelcke: $C = (11.9/3578) \cdot (t_{fg}/t_{fb}) \cdot D$, where C is in μ*M*, and D is the dilution factor for the samples. From P, C and the concentration of casein, the association and the chymosin equilibrium concentration can then easily be calculated. Subsequently, isotherms can be made. No correction was made for the volume occupied by caseins; for the highest κ-casein concentration applied (i.e., 15 g/l) the calculated association therefore might be up to 3% too low. Throughout this publication, the association is expressed relative to para-κ-casein, because chymosin appeared to associate mainly with para-κ-casein [7].

To estimate proteolysis, the protein concentration in the supernatants was determined by the Biuret method [2]. Absorbance was measured at 20°C and 540 nm. Bovine serum albumin (Sigma Chemical Company) was used as a reference.

Unless stated otherwise, the experimental conditions were pH 6.3, 30°C and ionic strength 0.04 *M*.

6.3 Results and discussion

6.3.1 Contact time

Figure 1 shows the association of chymosin with para-κ-casein as a function of time, for some chymosin/κ-casein ratios. The association immediately reached a maximum, followed by dissociation. The higher the ratio of chymosin to casein, the faster the dissociation. The dissociation and the increase in protein content of the supernatant were faster at 40°C than at 10°C (Figure 4C). A decreasing stability of the para-κ-casein precipitate during some prolonged incubations was also noticed (i.e., the precipitate formed a weaker pellet and was hard to fully separate from the supernatant).

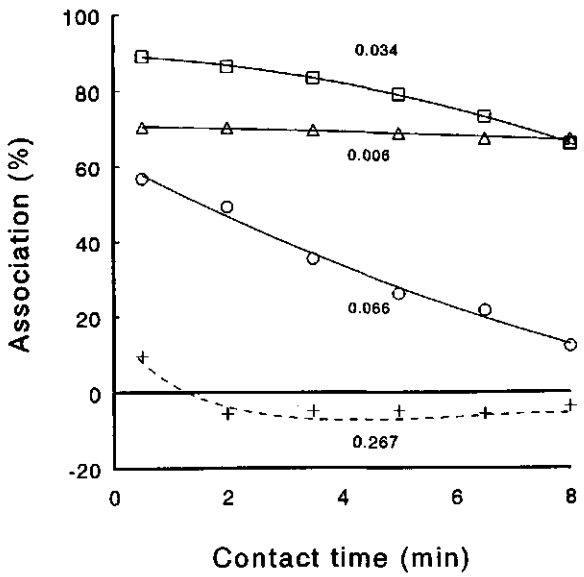


Figure 1. Association of chymosin with para-κ-casein (in % of total chymosin) as a function of time after addition of chymosin to solutions of κ-casein. pH 6.3, 30°C, ionic strength 0.06 M (20 mM NaCl added). Concentrations of κ-casein (g/l) and chymosin (μM): 3.3 and 5.71 (□); 1.0 and 0.31 (Δ); 1.0 and 3.37 (o); 0.15 and 2.05 (+), respectively. The ratio of chymosin to κ-casein (mol/mol) is indicated.

Obviously, proteolysis is involved, and this is a complicating factor in determining association. Very high ratios of chymosin to casein and/or optimal conditions for proteolysis may even result in excessive dissociation, so that any determination of association becomes impossible. In the experiments discussed in the following sections, care was taken to avoid such excessive dissociation The association of chymosin with para-κ-casein had to be determined immediately after hydrolysis of the Phe_{105}-Met_{106} bond of κ-casein to form para-κ-casein. The above mentioned dissociation and varying rates of proteolysis were, therefore, the reasons to estimate the maximum association by extrapolating the percentages of association to time zero (see also Section 2).

6.3.2 Casein composition and casein concentration

To further investigate the effect of casein composition as mentioned in the introduction, small amounts of α_s- or β-casein were added to κ-casein solutions. α_s- And β-casein each clearly suppressed the association, α_s-casein having the stronger effect (Figures 2A and 3). Additional α_s-casein in the κ-casein solution further decreased the association, though to a decreasing extent (Figure 2B). Similar results were found at 10 and 40°C (Figures 4A and 4B).

Because of the impurity of the κ- and β-casein preparations, the solutions with added β-casein contained more α_s- (5.6% w/w) than β-casein (4.5% w/w). Therefore, the effect of added β-casein may be (partly) through the action of α_s-casein rather than β-casein. These results may explain the results with artificial micelle milk as mentioned in the introduction and the low chymosin association found in cheese (i.e., 0.1 mmol/mol) and in milk (i.e., 1.4 mmol/mol; α_{s1}:α_{s2}:β:κ= 4:1:4:1.6 on a molar basis, 25°C, pH 5.0, ionic strength 120 m*M* and κ-casein concentration 130 μ*M* or 2.5 g/l) (Geurts, unpublished results).

At 11.3 mol % α_s-casein it took more than 0.5 min for the association to reach a maximum. Above 17 mol% α_s-casein, the maximum association was not reached within 4 min. Therefore, the true values of the maximum association at these α_s-casein concentrations may be slightly higher than shown in Figure 2B. This effect may be due to inhibited aggregation of para-κ-casein as a result of the presence of α_s-casein [8].

Diluting a solution of κ-casein (with or without added α_s-casein) with imidazole-HCl buffer resulted in a higher association (Figure 3). In the case of added α_s-casein about 13% α_s-casein was present; again, as mentioned in the discussion of Figure 2B, no maximum in association was found within 4 min. An increase in association as a result of dilution has also been found in artificial micelle milk (de Roos, unpublished results). Furthermore, in a very concentrated casein system like cheese an association of 0.1 mmol/mol was found and for milk, the diluted form, 1.4 mmol/mol (see above). Also in soya oil emulsions with κ-casein as an emulsifier, the association of chymosin with para-κ-casein increased by diluting the emulsion [7].

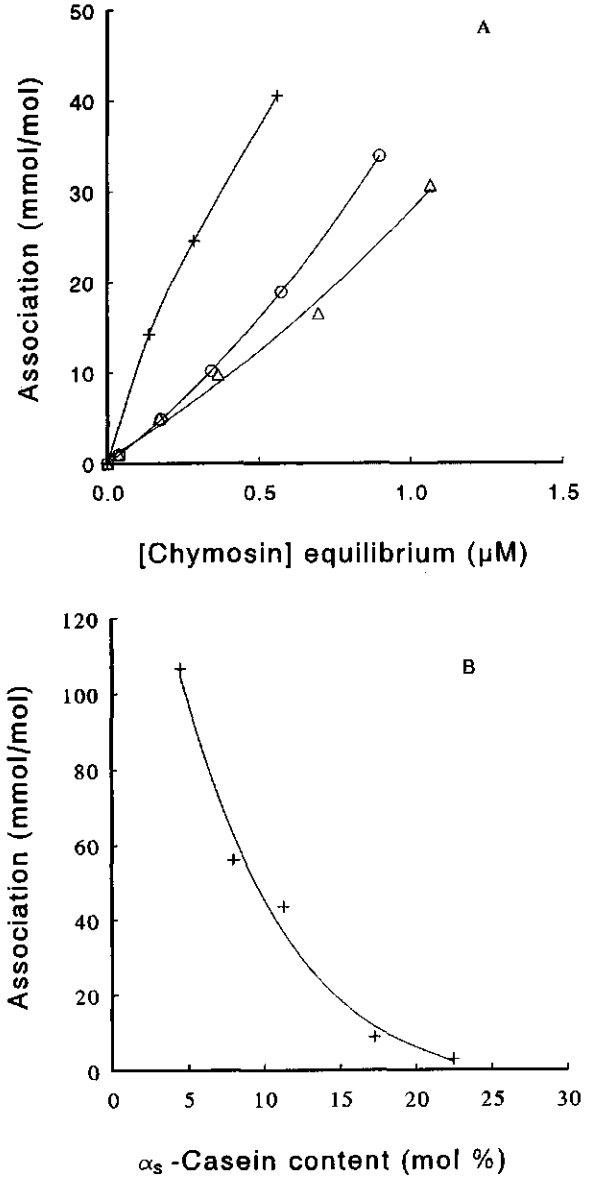


Figure 2. *Effect of α_s- or β-casein addition on the association of chymosin with para-κ-casein (mmol chymosin/mol para-κ-casein) in a solution of 1.0 g κ-casein/l. pH 6.3, 30°C, ionic strength 0.04 M. (A) κ-casein (+); 0.05 g α_s-casein/l added (Δ); 0.05 g β-casein/l added (o). (B) Association at 3 μM chymosin equilibrium concentration, estimated by linear regression of association curves, as a function of the α_s-casein content of the casein fraction.*

κ-Casein forms complexes with α_s- and β-casein [9, 10, 11]. Obviously, such complex formation upon addition of α_s- or β-casein to a κ-casein solution resulted in a weaker chymosin association. The negative effect of complex formation, including self-association of κ-casein [6, 12], is also clear from the higher association in diluted casein solutions. As determined by the association constant [9, 10, 11], dilution of a casein solution will cause κ-casein to be less (self-)associated. This means that upon dilution, chymosin can increasingly associate with para-κ-casein.

Garnier et al. [13, 14] found upon addition of α_s-casein or β-casein to a κ-casein solution no change in the enzymatic activity of chymosin on the Phe_{105}-Met_{106}-bond of κ-casein, although fewer protons were released. They concluded that α_s- and β-caseins associate with κ-casein on a site different from the site of enzyme binding, but close to the ester bond hydrolysed by chymosin. Binding close to this ester bond would decrease the dissociation constant of the carboxyl group of Phe_{105} of para-κ-casein, leaving it in a more protonated form. Other possible explanations are an increased buffering effect from added α_s- or β-casein and a totally different conformation of the C-terminal end of para-κ-casein, thereby interacting with α_s- or β-casein molecules.

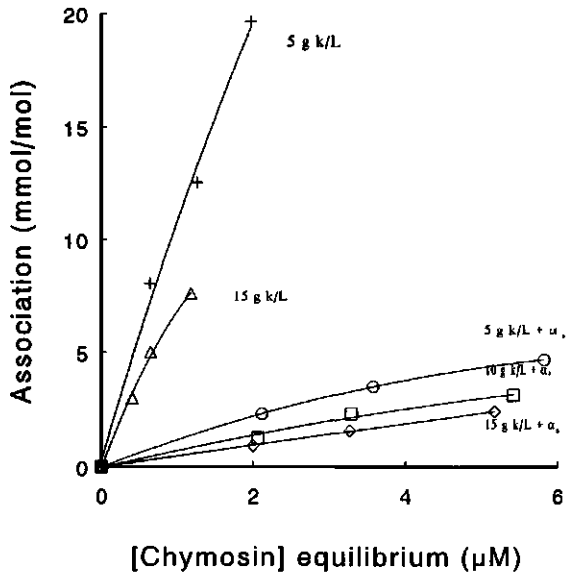


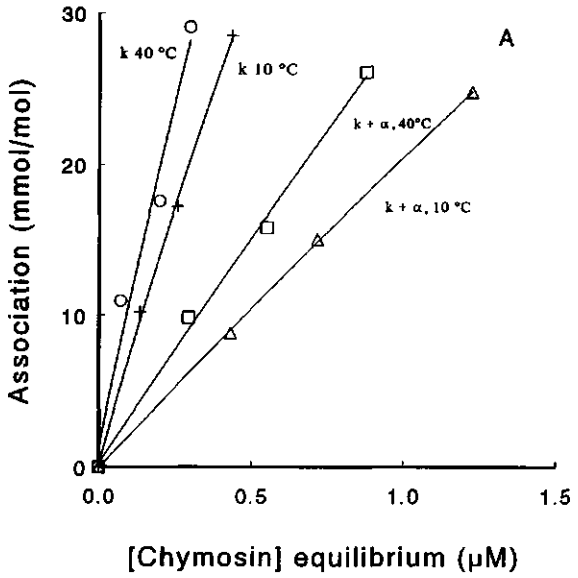
Figure 3. *Effect of casein concentration on the association of chymosin with para-κ-casein (mmol chymosin/mol para-κ-casein) in solutions of κ-casein, with or without α_s-casein (κ:α_s 10:1 mass ratio) added. pH 6.7, 30°C, ionic strength 0.04 M.*

Therefore, complex formation between caseins may have caused steric hindrance or different electrostatic interactions, resulting in decreased association of chymosin with the C-terminal end of para-κ-casein. Kumosinski et al. used molecular modelling to construct three-dimensional models for a casein submicelle consisting of one κ-casein, four α_{s1}-casein B and four β-casein molecules in the absence [15] and presence [16] of water molecules.

In their model, two α_{s1}-casein B dimers would interact with a κ-casein molecule and β-casein molecules would be docked into massive hydrophobic areas of the α_s-κ-casein complex. These proposed structures would suggest that interaction of one α_{s1}-casein dimer with the so-called 'front-leg' (residues 20-34) of κ-casein can sterically interfere with the binding of chymosin. However, this in itself cannot explain the enormous effect of α_s-casein on the chymosin association with κ-casein; only 20 mol % α_s-casein sufficed to reduce the association to nearly zero (Figure 2B). Since κ-casein molecules in solution can associate to form structures resembling detergent micelles [12], interaction of one α_s-casein molecule with one κ-casein molecule may also have caused other κ-casein molecules of the same κ-casein micelle to be sterically hindered from associating with chymosin.

6.3.3 Temperature

Chymosin association in solutions containing κ-casein, with or without α_s- or β-casein being added, was determined at 10 and 40 °C. Because of the impurity of the preparations used, the final α_s-casein concentration in the β-κ-casein solution was 0.05 g/l higher than the β-casein concentration. The casein and chymosin solutions were kept for at least 30 min at the measuring temperature before mixing. Slower or inhibited precipitation of para-κ-casein as a result of weaker hydrophobic interactions at 10 °C did not appear to affect the results, since no additional coagulum was formed in the 10 °C supernatant after warming it to 40 °C. At 40 °C the association was higher than at 10 °C for all solutions (Figures 4A and 4B). This seems to contradict the effects of a varying scalding temperature in cheese manufacture, reported by Stadhouders and Hup [1]. De Roos *et al.* [7] also found increasing chymosin association with decreasing temperature in soya oil emulsions with κ-casein as an emulsifier. The discrepancy between this earlier work and the present results may be ascribed to differences in contact times applied, i.e., about 1 h and 20 min for Stadhouders and Hup, and De Roos *et al.*, respectively, and extrapolated to 0 min in the present experiments. The importance of this aspect is illustrated in Figure 4C. At 40 °C, the initial chymosin association was higher than at 10 °C. However, at 40 °C the dissociation was much faster than at 10 °C, probably due to higher proteolytic activity [6, 17]; after about 3 min the association at 40 °C became less than that at 10 °C. Obviously, contact time (or rather the degree of proteolysis) is an essential factor in chymosin association with caseins, especially at the very high chymosin concentrations used in the present study.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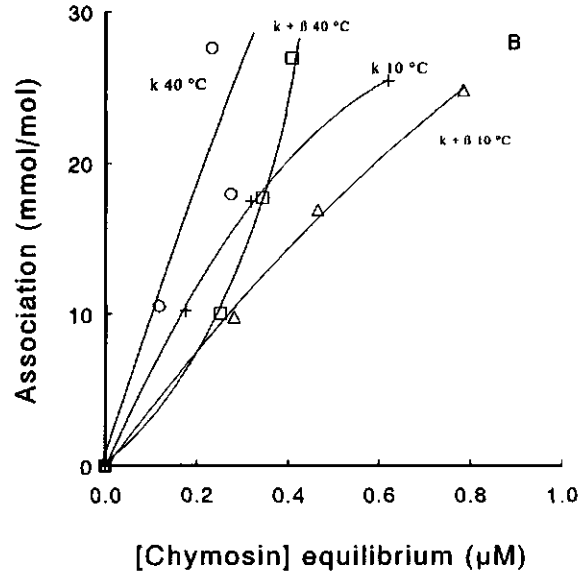
B
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Association (mmol/mol)
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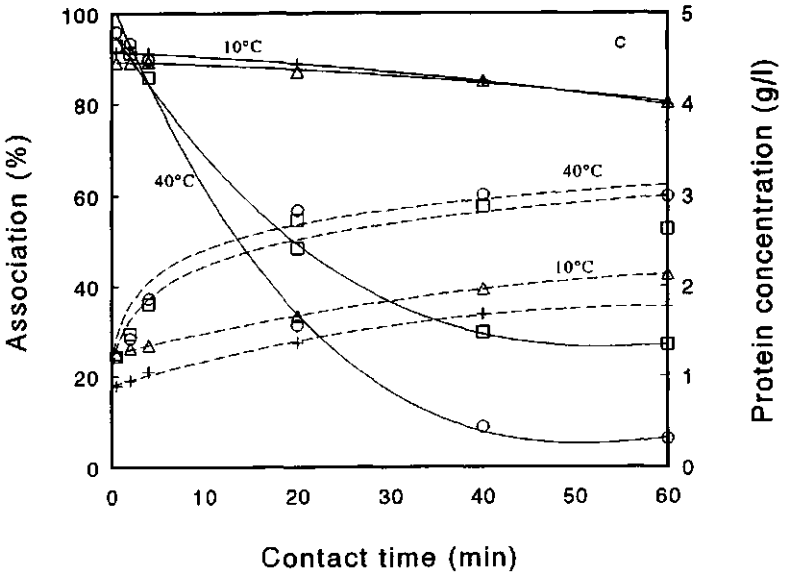

Figure 4. *Association of chymosin with para-κ-casein (mmol chymosin/mol para-κ-casein) at 10 and 40 °C in solutions of 5 g κ-casein/l, with or without 0.25 g/l added α_s-casein (A) or β-casein (B). (C) Association of chymosin with para-κ-casein (% of total chymosin) and protein concentration in supernatants (broken lines) as a function of time after addition of chymosin to a solution of 5 g κ-casein/l with or without 0.25 g β-casein/l added.*
pH 6.3, ionic strength 0.04 M. κ-casein at 10 °C (+) and 40 °C (o), respectively; κ- and α_s- or β-casein at 10 °C (Δ) and 40 °C (□), respectively.

At 10 °C, association was less than at 40 °C, though the differences were small as compared to the effect of addition of α_s- or β-casein to κ-casein solutions (Section 3.2). Chymosin preferentially cleaves between amino acid residues with non-polar side-chains [18], so this temperature effect may be ascribed to hydrophobic interactions.

The association between α_s- and κ-casein [19, 20], and probably also between β- and κ-casein [21, 22, 23], decreases with decreasing temperature. With κ-casein becoming less (self-)associated, chymosin can increasingly associate with para-κ-casein. This may partly have compensated for the tempœrature effect on chymosin association (see also effect of dilution, Section 3.2). Hydrophobic interactions may thus have played a more important role than one would conclude by considering only the data presented in Figure 4.

6.3.4 Ionic strength and pH

Ionic strength was varied by adding NaCl to the casein and chymosin solutions. To determine the association at low ionic strengths, in this experiment a 0.01 *M* imidazole-HCl buffer (I = 8 m*M*) was used. The association decreased with increasing ionic strength (Figure 5). This shows that charged groups were involved in chymosin association.

This can also be concluded from the influence of pH. Increasing the pH from 4.7 (isoelectric pH of chymosin) to 7 (where para-κ-casein is still positively charged [24])) will render chymosin more negative and para-κ-casein less positive. Association was estimated in solutions of 5 g κ-casein/l with or without 0.25 g α_s-casein/l added, at pH 6.3 and 6.7. At 0.5 μ*M* chymosin equilibrium concentration and 30 °C, the chymosin associations were about 13 and 44 mmol chymosin/mol para-κ-casein, respectively, at pH 6.3 (Figure 4A), and 0.4 and 5.0 mmol/mol, respectively, at pH 6.7 (Figure 3). Obviously, chymosin association decreased with increasing pH. Ionic strength and pH seemed to affect chymosin association more strongly than temperature, in the ranges studied. Similar effects of ionic strength and/or pH were found in emulsions containing κ-casein adsorbed onto soya oil droplets [7], in casein suspensions [25] and in artificial micelle milk [26].

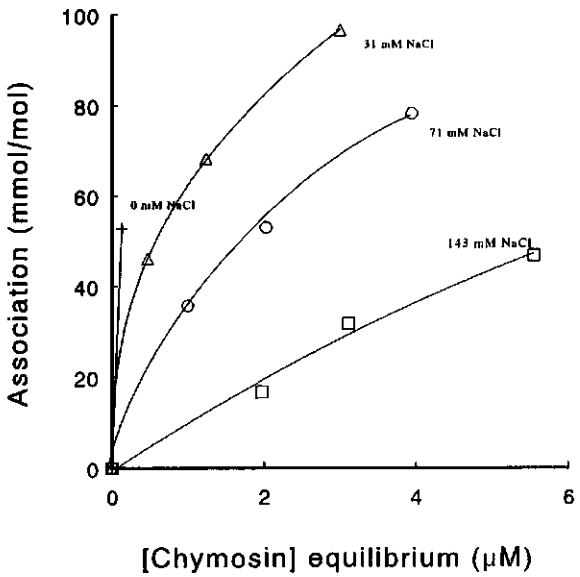

Figure 5. *Effect of ionic strength on the association of chymosin with para-κ-casein (mmol chymosin/mol para-κ-casein) in a solution of 1.0 g κ-casein/l in 0.01 M imidazole-HCl buffer. pH 6.3, 30 °C. Amount of NaCl added is indicated . I=8 plus amount of NaCl added (mM).*

The ionic strength and the pH may have affected, among others, the interactions between chymosin and the C-terminal end of para-κ-casein. Besides being affected by the pH, the dissociation of the carboxyl group of Phe-105 of para-κ-casein may be decreased by addition of α_s- or β-casein to a κ-casein solution [13, 14; see also Section 3.2]. Furthermore, Dalgleish [24] concluded that amino acid residues 98-102 of κ-casein (His-Pro-His-Pro-His) are important for chymosin binding. This fragment contains the only three histidine residues in κ-casein. Histidine is the only amino acid of which the pK_a of the side group (6.00) is between pH 5 and 7 [27]. The strong association at low ionic strength, i.e. at a thick electrical double layer ($1/k \approx 3.4$ nm at $I = 8$ mM), suggests that the three histidine residues are all involved in chymosin association, possibly acting as one positive charge. At higher ionic strength, hence at a thinner electrical double layer (0.8 nm at $I = 151$ mM), the histidine residues will increasingly act as separate charges. His-102 is then probably essential [28]. Electrostatic interaction between κ-casein and negatively charged κ-carrageenan was also suggested to involve positive charges (including the three histidine residues) on κ-casein. However, at pH 6.7, the latter interaction increased with increasing ionic strength up to 0.2 M [29].

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THE ASSOCIATION OF CHYMOSIN WITH CASEIN MICELLES

Chapter 7

Abstract

The influence of casein micelle composition on the extent of chymosin association is described. The association of chymosin with artificial micelles, made with combinations of α_s- or β-casein with κ-casein, in a range of α_s or β : κ of 12:1 to 1:5 at varying pH has been studied. Isotherms for chymosin association with caseins were constructed. The micelles composed of β- and κ-casein retained more chymosin than micelles composed of α_s- and κ-casein. The chymosin association increased with higher κ-casein content and due to dilution of the system. Furthermore the association depended on contact time, on casein and chymosin concentration, on temperature and on pH. It is hypothesized that chymosin associates mainly with para-κ-casein and that the mechanism of association is one in competition with the other casein molecules. A model for this competitive adsorption is presented. The model can explain several observations on rennet retention in curd.

7.1 Introduction

In the process of cheese making the renneting enzyme chymosin is partly retained in the curd. This retention is not due to an even distribution over whey and curd, but is controlled by a delicate mechanism of association, influenced by pH [1, 2, 3, 4]. The lower the pH the more rennet is found in the curd. Recently the association of chymosin with caseins has been investigated in various ways [5, 6, 7]. The casein concentration and the time of contact seem to play an important role, as do properties of the proteins involved such as isoelectric pH, occurrence of specific adsorption sites and the three-dimensional structure.

As was demonstrated recently [6], the presence of α_s- and/or β-casein in a solution containing κ-casein and chymosin greatly affects the mutual association of the latter two proteins. For this reason, the composition of the constituent caseins in the casein micelle will, most probably, determine the extent of possible association of chymosin with para-κ casein. This was partly worked out by Larsson *et al.* [7]. In order to achieve a better understanding of the mechanism of chymosin association with casein micelles, a broad range of ratios of the various caseins was applied.

This investigation is part of a trial to find a food-grade carrier for the immobilisation of enzymes.

7.2 Materials and methods

Artificial micelle milk (AMM) was made according to Schmidt *et al.* [8]. Artificial micelles, composed of α_s- and κ-caseins or β- and κ-caseins (Sigma), were made in various concentration ratios of 12:1, 3:1, 3:2, 1:2 and 1:5 (α_s-casein or β-casein : κ-casein) or from whole caseinate. In composing the AMMs, the impurity of the caseins [6] was not taken into consideration. It was determined (Kjeldahl protein assay) that 87 % of weighed material consisted of protein and this was accounted for, such that the solution contained 2.6 % of casein (like in bovine milk). The AMM was brought to pH with 0.5 M HCl during cooling on ice to prevent casein flocculation.

Chymosin was obtained from Sigma (Rennin). A solution of 1.2 g/l 0.02 M acetic acid buffer was made, corresponding in activity to a standard calf rennet. Various amounts (1 to 5 % w/v) of this solution were added to 6 ml of AMM, while stirring, at 30 °C. Chymosin activity in the "whey" supernatant was determined by means of the Berridge flocculation test [9]. This chymosin concentration is the chymosin *equilibrium* concentration in the aqueous phase of the system. The difference between the total concentration of chymosin originally applied and the equilibrium concentration in solution, is considered to be the amount of chymosin that has associated with the casein in the AMM. The concentration of chymosin in the "whey" supernatant was determined using the rule of Storch and Segelcke, which states that the flocculation time is inversely proportional to the chymosin concentration [10]. It was

assumed that a 4000-fold dilution of a commercial calf rennet in milk, resulting in a flocculation time of about 300 s, had a 13.3 μM concentration [11, 6]. The casein participating in the association with chymosin, was precipitated by means of centrifugation. Its amount is derived from determination of the protein concentration in the whey. In this manner the surface load Γ (mole chymosin / mole casein) can be calculated. No volume corrections for solute exclusion by the casein were made [12]. For the undiluted AMM this would lead to an underestimation of the association by at most 10 %. This underestimation will be less in the cases where the milk had been diluted. The various chymosin equilibrium concentrations found in the system can be given in an adsorption (association) isotherm. As was demonstrated earlier [6], the extent of association is dependent on contact time between the chymosin and the casein. For that reason, at three different times, within an hour, 2 ml samples were taken and centrifuged for 1 min (Eppendorf; 11000 *g*). The value for association found after extrapolation to zero contact time was used for the association isotherms. It was found experimentally that extrapolation of the (apparent) amounts of chymosin associated was the most reliable method (extrapolation of flocculation time is an alternative). The protein content in the whey samples was determined by the Kjeldahl and later by the biuret method [13].

In the experiments in which the casein concentration was varied, the AMM had to be diluted with a whey-like solvent. It was found that this solvent could best be made by addition of a small amount of chymosin to a stirred AMM, followed by centrifugation after clotting has occurred [6]. Alternatively, the AMM can be ultracentrifuged, but then not all casein will be separated; or a Jenness-Koops buffer, mimicking the ionic composition and strength of whey may be used, or the AMM may be ultrafiltered. However, all of these methods result in a somewhat changed protein composition. None of the mentioned dilution solvents can be considered ideal but mutual differences were small. Experimental errors were minimized by choosing the appropriate blanks. The association isotherms shown were made once; the experiments needed to determine a value within the isotherm were carried out twice.

7.3 Results and discussion

7.3.1 Casein composition

The synthesis of a casein complex in micellar form is only possible if κ-casein is present [8]. The relative amount of the κ-casein needed for making AMM with respect to the amounts of the other casein types does not seem to be very critical, although for α_s-/κ-casein micelles the ratio should not exceed 10:1. β-Casein appears to have a weaker micelle forming capacity than α_s-casein. The relative amount of κ-casein in the micelle strongly affects micellar size [8].

Fig. 1 shows typical examples of adsorption isotherms found for chymosin association with the α_s/κ-casein and β/κ-casein systems in ratios 1:2 at pH 5.0. The adsorption isotherms did not reach a plateau value. Within the range of chymosin concentrations applied (0.11 -

0.56 μM) percentages of chymosin associated remained approximately constant (about 84% for the α_s/κ-casein system and about 95 % for the β/κ-casein system). The extent of association was extrapolated back to contact time t = 0. Due to the association, the equilibrium chymosin concentration in solution was at most 0.08 μM. Comparison of various systems (for instance variation in casein composition, pH, temperature or ionic strength) is only useful at the same equilibrium concentration of chymosin.

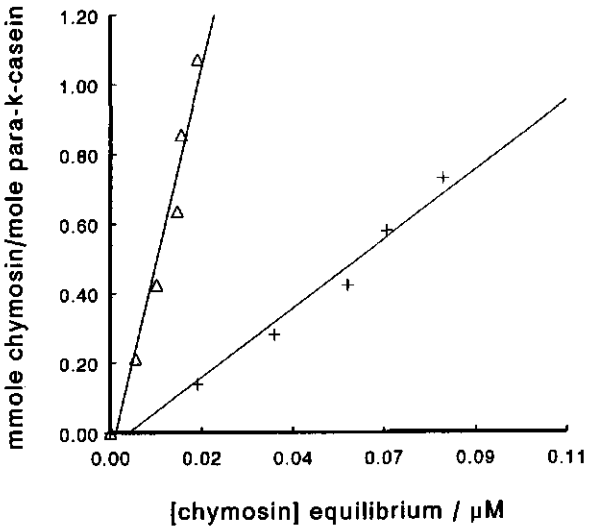

Figure 1 *Adsorption isotherms of chymosin for casein micelles composed of α_s- or β- casein and κ-casein. Ratio α_s/κ-casein = 0.5 or β/κ = 0.5 at pH 5 and room temperature. β:κ (Δ) (r = 0.98) and α_s:κ (+) (r = 0.96).*

Fig. 2a shows the effect of the relative amount of κ-casein in casein micelles, made of α_s/κ- or β/κ-casein, on the extent of chymosin association with the paracasein at a chymosin equilibrium concentration of 0.07 μM at pH 5.0. Curves for α_s/κ-casein micelles and β/κ-casein micelles are given. The association of chymosin in the AMM with β/κ-casein micelles is 2-3 times larger than with the α_s/κ-casein AMM. Furthermore, a slight increase of association is observed in the AMM systems with higher content of κ-casein. In Figure 2a the association of chymosin has been expressed relative to the total concentration of casein applied (the mean of molar masses of the various casein compositions were used in the calculations). Alternatively, the association may be expressed in moles of chymosin per mole

of κ-casein used (Fig. 2b). There is evidence that chymosin associates (almost) exclusively with κ-casein [5, 7, 14]. In that case a higher surface excess Γ will be calculated for κ-casein alone as compared to total casein content. Under the experimental conditions (pH, temperature, casein and chymosin concentrations) used, percentages of association were all rather high (75 - 95 %) and about constant over the range of κ-casein incorporated, resulting in a higher surface excess per mole of κ-casein for a lower concentration of κ-casein. A rough estimate of chymosin association with pure dissolved κ-casein, based on previous results [6] and accounting for pH and concentration differences, would result in an association of 10-20 mmole chymosin per mole κ-casein. This would mean a very steep increase in association when the κ-casein content is nearing 100 %, which agrees with the finding [6] that addition of a small amount of α_s- or β-casein to a κ-casein solution strongly decreased the extent of chymosin association with the para-κ-casein. The effect of α_s-casein addition in such an experiment was by about two times stronger than addition of β-casein.
At the lower percentage of κ-casein applied the values found for association are becoming very inaccurate. Moreover, at very low, but also at very high percentages of κ-casein content, the casein aggregates become of a different nature, ranging from casein dimers to whole casein micellar structures to κ-casein "submicelles". If the assumption of chymosin associating only with κ-casein would be correct, then the curve would go down to zero association when nearing 0 % content of κ-casein. In conclusion it may be stated that chymosin association markedly depends on the composition of the micelle.

7.3.2 pH

The dependency of the extent of rennet retention in cheese on the pH during curd making has long been known [1, 4]. This retention behaviour varies among types of renneting enzyme [2, 3, 4]. For chymosin, the lower the pH of the curd, the higher the amount of chymosin retained. In model systems [5, 6, 7] simulating the cheese making process, similar behaviour has been observed. Fig. 3 gives another example of this trend.

As κ-casein is mainly involved in the association with chymosin, the composition of the caseins in the casein micelle will determine the binding affinity for chymosin. The effect of pH on the association of chymosin with the micelle may also vary with this composition. It was indeed shown that the influence of pH on chymosin association was stronger for micelles with lower content of κ-casein [7].

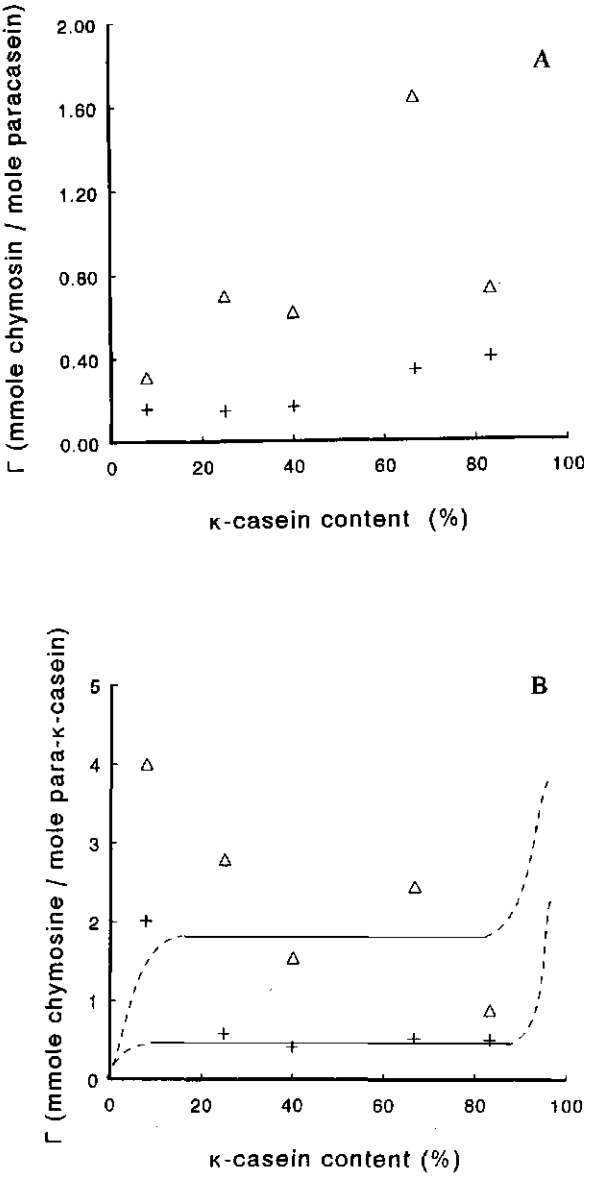

Figure 2 *Effect of the relative amounts of κ-casein in α_s/κ and β/κ artificial casein micelles on the association of chymosin with paracasein at pH 5.0 and a chymosin equilibrium concentration of 0.07 μM. α_s/κ-casein (+) and β/κ-casein (Δ). (A) Association expressed per mole paracasein, and (B) per mole para-κ-casein. (----) Hypothetical extrapolations*

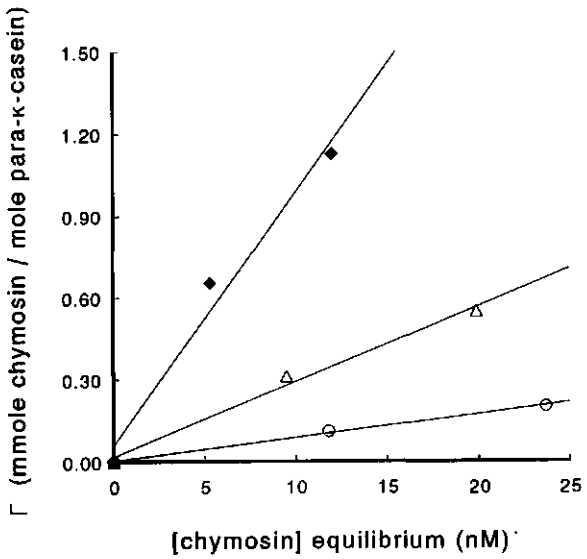

Figure 3 *Effect of pH on the association of chymosin with casein micelles made of whole caseinate. pH 5.2 (◆), pH 5.8 (Δ) and pH 6.5 (o).*

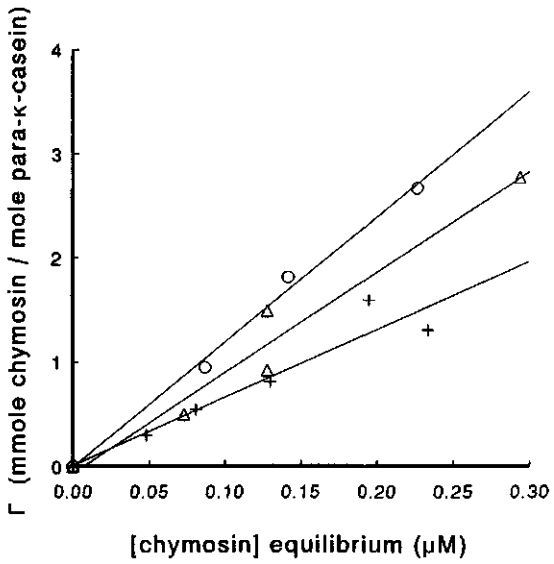

Figure 4 *Effect of casein concentration on the association with chymosin at pH 6.0. Casein micelles composed of β- and κ-casein, ratio β/κ = 0.5. Undiluted (+), diluted twice (Δ) and diluted 5 times (o).*

7.3.3 Casein concentration

In previous work [4, 5, 6] the influence of casein concentration on the chymosin-casein association has been mentioned. The association of chymosin with casein in milk has also been found to be stronger than in cheese, which may, in a way, be considered as a concentrate of milk (Geurts, to be published). In other words, chymosin-casein association depends on the ratio of water to protein. In figure 4 the effect of dilution for chymosin association in a β/κ-casein (1:2) AMM is given.

7.3.4 Contact time

The association of chymosin with para-κ-casein is dependent on the time of contact of the associated chymosin [6]. Most probably due to proteolysis, chymosin association decreases with contact time; the more so for a lower pH and a higher temperature. On the other hand at extrapolated contact time $t = 0$ the association of chymosin appeared to be somewhat larger at higher temperatures. In the cheese making process there may be an inactivating effect of high temperature on chymosin, during scalding of the curd, that would diminish the amount of remaining activity in the curd.

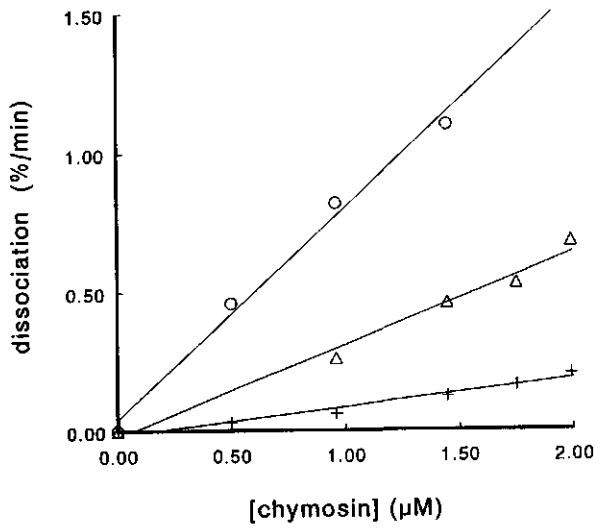

Figure 5a *Effect of the chymosin concentration on the dissociation of chymosin at pH 6.0 at various casein concentrations. Casein micelles composed of β- and κ-casein, ratio β/κ = 0:5. Casein concentration = 0.34 mM (o); 0.85 mM (Δ); and 1.70 mM (+).*

Figure 5a shows how the rate of decrease of the amount of chymosin associated with casein micelles, for convenience called dissociation rate, is related to the casein concentration of the system and the concentration of chymosin added (note that it is not the equilibrium chymosin concentrations that are plotted). In the more diluted system chymosin dissociated faster than in the concentrated system. As expected, at higher concentrations of chymosin applied,

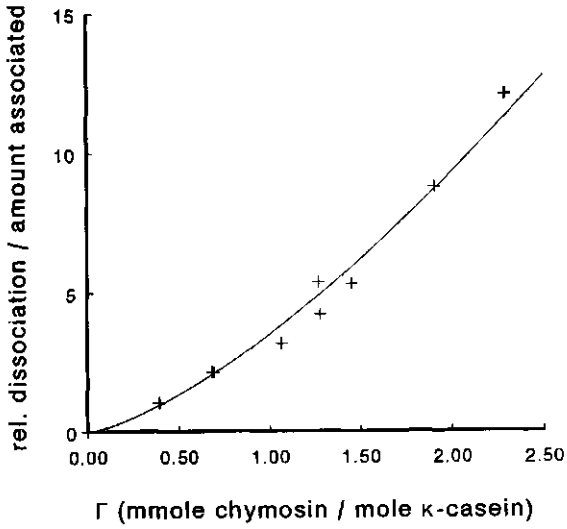

Figure 5b *Effect of the surface excess Γ on the proportion of chymosin dissociating per min at pH = 6.0, between 20 and 40 min of contact time. Casein micelles composed of β- and κ-casein, ratio β/κ = 0.2.*

dissociation, determined at 20 - 40 and 60 min of contact time, proceeded relatively faster than at low chymosin concentration applied. Figure 5b shows the correlation (r = 0.96) between the surface excess of chymosin on casein and the rate of dissociation. The values comprise all surface excess values (Γ) determined over three dilutions of β/κ-casein = 1:5 AMM (undiluted, 2 times and 4 times diluted). The higher the surface excess the larger the rate of dissociation. In the range of high surface excess this dissociation is no longer linear with time, meaning that during the first 20 minutes much more chymosin dissociated than between 40 and 60 min.

The same trends were found for AMM of other casein composition, although the extent of dissociation may be different. Figure 6 gives an example. Chymosin dissociation from the β-/κ-casein micelle proceeded more readily than from the α_s-/κ-casein system. For these

reasons it is essential that values used in the association isotherms are determined through extrapolation to $t = 0$. Another consequence of this dissociation behaviour is that the casein micelle is not a suitable carrier to immobilize a protease, unless the contact time is quite short. The protease will digest its own association site. When a non-proteolytic enzyme, like lysozyme, has become associated with a casein micelle the extent of association will remain constant in time [15]. Within half an hour of contact time chymosin, associated with caseins adsorbed onto soya-oil emulsion droplets, was not found to dissociate [5].

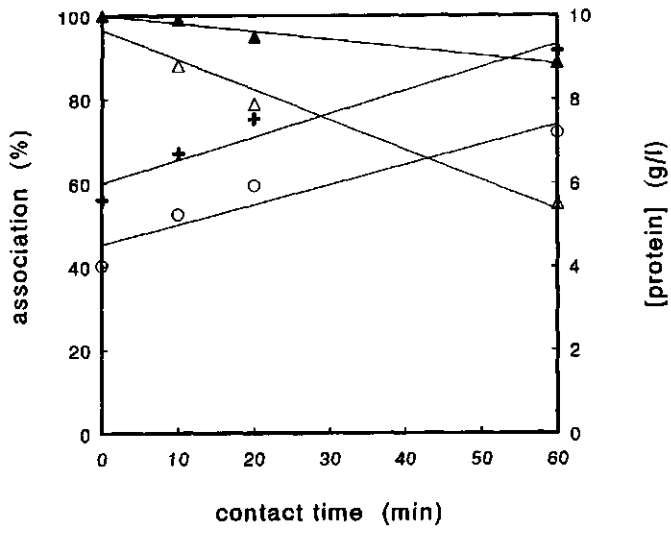

Figure 6 *Effect of the casein micelle composition on the dissociation of chymosin (surface load Γ was 0.42 mmol chymosin/mole casein at t = 0, both for the α_s/κ- as well as the β/κ-casein micelles) as a function of contact time. Chymosin activity in the whey fraction as a function of contact time: α_s/κ-AMM (▲) and β/κ-AMM (Δ). Amount of protein in the whey fraction as a function of contact time: β/κ-AMM (+) and α_s/κ-AMM (o).*

The protein concentration in the supernatant increased with time (Fig. 6). Most of this protein fraction consisted of the caseinomacropeptide, but, as time passed, the protein concentration exceeded the theoretical concentration of CMP available in the system. The released chymosin itself contributed to a negligible extent to this protein increase; hence, it must originate from the paracaseinate. It was found that the increase in protein in the wheys of β/κ-AMM and α_s/κ-AMM was the same. For the whole series of AMMs (α_s, or β/κ ratios from 1:2 to 12:1) at pH 5 the β/κ-AMM contained more non-micellar casein than the α_s/κ-AMM; percentages of total casein were 35.6 (RSD 17.3 %; $n = 9$) and 13.7 (RSD 14.1 %; $n = 9$), respectively.

7.3.5 Competitive association

One of the most obvious results from this study on the extent of chymosin association with casein micelles of various casein composition, is that the α_s-/κ-casein micelles bind less chymosin than the β-/κ-casein system. This result might lead to the conclusions that β-casein offers a more suitable binding site than the α_s-casein and that κ-casein is *not* involved in the association (since otherwise both types of micelles would show the same association per mole of κ-casein). Former work [5, 6, 7] has generated strong evidence that all of these conclusions are incorrect. Chymosin does not associate with α_s- or β-casein adsorbed onto oil emulsion droplets, and only to a minor extent when these caseins were adsorbed onto a microtiter plate. On the other hand, chymosin associates markedly with κ-casein adsorbed on these systems. Of course, we have to keep in mind that adsorbed caseins may expose different amino acid residues to chymosin as micellar caseins do in an aqueous phase. However, in studies on dissolved casein it was also found that chymosin adsorbed exclusively with κ-casein in the form of precipitating complexes. Addition of small amounts of α_s- or β-casein to the κ-casein/chymosin solution resulted in a strong decrease of chymosin association. So in fact, κ-casein (or rather para-κ-casein) appears to be the one and only casein to bind the chymosin molecule, and α_s- and β-casein hinder this association. This ability to hinder the association of chymosin with para-κ-casein in solution would then be stronger for α_s-casein than for β-casein. Similar behaviour may occur in casein micelles. A suspension of casein micelles consisting of α_s-/κ-caseins binds less chymosin than one consisting of β-/κ-casein micelles. Apparently, α_s- and β-casein compete with chymosin for one or more binding sites located on para-κ-casein, or a potential binding site for chymosin on the para-κ-casein molecule is shielded by the other caseins.

7.4 Discussion

Besides the observation that β- and α_s-casein decrease the association of chymosin with para-κ-casein, some other aspects mentioned above need explanation: (i) chymosin association with κ-casein is extremely small considering the excessive amount of κ-casein present, and (ii) association of chymosin increases upon dilution of the system. These aspects can in a simplified form be approached using the following concept of "competitive association", which will be discussed below in its most simple form.

The adsorbate molecule A (chymosin) will associate with the adsorbant molecule B (κ-casein) to form a complex AB. However κ-casein is known to have a strong tendency for self-association. So, chymosin has to compete for an association site on a para-κ-casein molecule. The following reactions are assumed to take place in the system (the subscript 0 refers to the equilibrium concentration in solution, and t to the total concentration):

(a) $A + B \rightleftarrows AB$ and $K_1 = [AB]/[A]_0.[B]_0$

(b) $B + B \rightleftarrows B_2$ and $K_2 = [B_2]/[B_0]^2$

The following relations would then be valid:

$$[AB] = K_1 [A]_0.[B]_0 \quad (1)$$

$$[B_2] = K_2 [B_0]^2 \quad (2)$$

$$[A]_t = [AB] + [A]_0 \quad (3)$$

$$[B]_t = [B]_0 + [B_2] + [AB] \quad (4)$$

If [A] << [B] then [AB] in relation (4) will be negligible. In cheese making conditions this will normally be the case. Consequently,

$$[B]_t \approx [B]_0 + [B_2] \quad (4a)$$

The adsorption isotherm for chymosin is a relation between the surface load Γ (mole of adsorbate/mole of adsorbent) and the equilibrium concentration of adsorbate in solution:

$$\Gamma \approx [AB]/[B]_t = f([A]_0) \quad (5)$$

Substitution of (1) and (4a) gives:

$$\Gamma = K_1 [B]_0.[A]_0/([B]_0 + [B_2]) = K_1.[A]_0/(1 + [B_2]/[B]_0) \quad (6)$$

In Table 1 the effect of dilution has been calculated based on experimental results by Dunnewind et al. [6] (see Fig. 3) on association of chymosin in solutions of κ-casein (5 and 15 g/l). In this calculation the equilibrium constant K_1 was taken as 10^4 $m^3.mol^{-1}$. Values for $[B]_0$, $[B]_2$ and K_2 were calculated using eqs. (2) and (4a). Γ of chymosin in the diluted system was calculated using (6). The calculated value of 13.7 x 10^{-3} mole/mole corresponds well with the experimental value of 12.0 x 10^{-3} mole/mole found. In reality things are more complicated because κ-casein does not only simply dimerize but may polymerize to up to 20-40 κ-casein molecules. The model explains, however, why the association of chymosin is so small despite the large amount of κ-casein present and the effect of dilution of casein on the association.

Table 1. Sample calculation of the effect of dilution of a κ-casein solution on the association of chymosin. See text.

Variable	unit	"normal"	diluted
$[A]_0$ (chymosin)	$nmol.m^{-3}$	1.	1.
$[B]_t$ (κ-casein)	$mol.m^{-3}$	0.77	0.26
K_1	$m^3.mol^{-1}$	$1.\ 10^4$	$1.\ 10^4$
$[B_2]/[B]_0$	_	713.8	713.8
Γ	mol/ mol	0.007	0.0137 (0.012)
$[B_2]$	$mol.m^{-3}$	0.414	0.130
$[B]_0$	$mol.m^{-3}$	0.0020	0.0011
K_2	$m^3.mol^{-1}$	$1.\ 10^5$	$1.\ 10^5$

Casein micelles consisting of β- and κ-casein will associate better with chymosin than micelles consisting of α_s- and κ-casein. In former experiments with caseins in solution [6] the α_s-casein was more effective at preventing chymosin association with κ-casein than β-casein, and much more than κ-casein itself. Apparently the association constants for association with κ-casein is in the order of $\alpha_s > \beta >> \kappa$. In other words, the purer the κ-casein, the stronger its association with chymosin. These effects may be approached as follows. The adsorbate molecule A (chymosin) will compete with a competitive adsorbate C (for instance β-casein) for an adsorption site on para-κ-casein B.

(a) $A + B \rightleftarrows AB$, and $K_1 = [AB]/[A]_0.[B]_0$

(b) $C + B \rightleftarrows CB$, and $K_2 = [CB]/[C]_0.[B]_0$

The following relations are valid:

$$[AB] = K_1\,[A]_0.[B]_0 \quad (1)$$
$$[CB] = K_2\,[C]_0.[B]_0 \quad (7)$$
$$[A]_t = [AB] + [A]_0 \quad (3)$$
$$[C]_t = [CB] + [C]_0 \quad (8)$$
$$[B]_t = [AB] + [CB] + [B]_0 \quad (9)$$

If $[A] << [B]$ then $[AB]$ in relation (9) will be negligible. In cheese making conditions this will normally be the case, hence:

$$[B]_t \approx [CB] + [B]_0 \qquad (9a)$$

However, since C associates also with B (competitively), [C] will not be negligible. The concentration of C (and thus of CB) may vary with process conditions like pH, temperature and calcium and phosphate concentration.

Our experiments do not reveal any information on the quantities of C and B associated. On the other hand we do know the added amount $[A]_t$ in reaction (a) and $[A]_0$ has been determined; consequently, the contribution of A in the decrease of [B] to free $[B]_0$ is known. Reaction (b) also contributes to $[B]_0$, but to an unknown extent.

By substitution of (8) and (9a) in (7) we can express $[B]_0$ in a quadratic equation:

$$K_2[B]_0^2 + (K_2[C]_t - K_2[B]_t + 1)[B]_0 - [B]_t = 0 \qquad (10)$$

which yields

$$[B]_0 = [\![K_2[B]_t - K_2[C]_t - 1 + \sqrt{\{(K_2[C]_t - K_2[B]_t + 1)^2 + 4K_2[B]_t\}}]\!] / 2K_2 \qquad (11)$$

The adsorption isotherm for chymosin is a relation between the surface load Γ (mole of adsorbate / mole of adsorbent) and the equilibrium concentration of adsorbate in solution:

$$\Gamma \approx [AB]/[B]_t = f([A]_0) \qquad (5)$$

When $[B]_0$ in (1) is substituted by (11), and the left and right hand terms are divided by $[B]_t$, we have an equation like (5) in which both K_1 and K_2 are expressed. By means of an iteration procedure values for these equilibrium constants can be estimated.

In Table 2 the effects of dilution and casein compositional change have been calculated making use of arbitrary values for K. It shows that the calculations lead to an increase in association upon dilution and also upon increasing κ-casein content (comparable to "purification" of κ-caseins), which has also been found experimentally. Of course, also this approach is an oversimplification. In milk numerous interactions (of different nature) between the various caseins (including self-association) will take place, each of them has its own association constant and each affects the extent of chymosin association. It is not feasible to take all these values into account in a trial to support experimental results with kinetic calculations.

The value of the model is that it supports the idea of competitive association between chymosin and casein molecules for interacting with κ-casein and that the low extent of association, the influence of dilution and compositional variation can be semi-quantitively explained by it.

Table 2. Sample calculation of the effects of concentration and compositional change of casein micelles (κ+β) on the association of chymosin. See text.

Variable	unit	normal	diluted	other ratio κ/β
$[A]_t$ (chymosin)	$nmol.^{m-3}$	3.0	3.0	3.0
$[B]_t$ (κ-casein)	$mol.^{m-3}$	0.20	0.10	0.20
$[C]_t$ (β-casein)	$mol.^{m-3}$	0.40	0.20	0.10
K_1	$m^3.mol^{-1}$	20	20	20
K_2	$m^3.mol^{-1}$	40	40	40
$[B]_0$	$mol.m^{-3}$	0.0204	0.0175	0.118
$[AB]/[B]_t = \Gamma$	mol/mol	2.04 $[A]_0$	3.5 $[A]_0$	11.8 $[A]_0$

The renneting of milk by chymosin is accompanied by virtually complete cleavage of κ-casein, releasing caseinomacropeptides (CMP). Coagulation of casein micelles starts after release of some 70 % of the available CMP, and cleavage still continues after flocculation. Consequently, association of caseins with κ-casein does not prevent the proteolytic attack of chymosin of the Phe-Met bond (as is the case for β-lactoglobulin associated to κ-casein by heat treatment [16]. This was already postulated by Garnier *et al.* [17]. The kinetic parameters of the reaction, however, are slightly changed due to casein molecules associated with κ-casein. Association of the κ-casein substrate with itself increases the K_m -value by a factor of two (from 10 μM to 20 μM) under physiological conditions, but the proteolytic coefficient (K_m/K_{cat}) is changed even less [18]. However, the association of a chymosin molecule with a casein-associated para-κ-casein molecule is strongly affected, even impeded by the associated casein. Also self-associated κ-casein prevents the association of chymosin. The effectiveness of the prevention of chymosin binding would be determined by the association constants of the caseins with each other, causing the extent of association of chymosin with para-κ-casein to be inversely related to the extent of association of other caseins with para-κ-casein. The chymosin molecule binds exclusively to the para-κ-casein molecule after having split the Phe-Met bond. The amount of chymosin association will increase with decreasing pH, increasing concentration of chymosin added and increasing water-protein ratio.

Association constants were $3.2 \cdot 10^4$ M^{-1} (pH 6.8; I = 0.02; according to Nakai and Kason [19]) for α_{s1}-/κ-casein and $8.3 \cdot 10^3$ M^{-1} for β-/κ-casein (pH 6.9 ; [17]). The association constants increase with increasing pH, ionic strength and temperature. The lower value of the $K_{ass.}$ for β-/κ-casein means that more κ-casein is available for association with chymosin. It also explains why supernatants of these AMMs contain more protein. These values are valid for the simple systems in which they were determined and will lose significance when the caseins are considered in the complex casein micelle system in milk, where interactions

between all caseins (in various ratios of concentration) and with the colloidal calcium phopsphate will be far more complicated. Moreover, the history of the treatment of the milk will also have an effect on these interactions [20]. It does not appear feasible to take all these relations into account in an attempt to support our experimental results with a full kinetic calculation.

7.5 Concluding remarks

We propose that retention of chymosin in curd during cheese making is based on association of chymosin exclusively with para-κ-casein. The very small relative association is determined by the association constants that exist between the caseins. This model of competitive association further explains the influence of dilution of the system and casein compositional change in solution and casein micelles. The effects of contact time and temperature severely complicate the mechanisms involved. The casein micelle is not suitable as a carrier for immobilisation of a protease due to the enzyme digesting its adsorbent.

7.6 References

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SUMMARY

The proteolytic action of proteases present in cheese plays a major role in the ripening of cheese. These proteases originate from the rennet, the starter cultures and from the milk itself. The proteolysis in cheese results in the degradation of the casein proteins into smaller peptides and free amino acids, which act as flavour precursors. The ripening of cheese under conditioned storage is time consuming and costly. Addition of specific enzymes to the cheese milk is one of several options to accelerate ripening. A major problem then is that hardly any of these proteases end up in the cheese and most disappear with the whey stream. Entrapment of bacteria and milk fat globules into the casein matrix of the curd is due to their particle character. Immobilisation of proteases onto particles would thus result in retention of these proteins in the curd. Ideally, for reasons of acceptance, these particles should originate from the milk itself or at least be edible.

In this study, in a more general approach, soya oil emulsion droplets and casein micelles, being protein aggregates, were tested as the carrier system. Chymosin and lysozyme were taken as the enzymes to be immobilized, because of their relevance to the dairy industry and because they are scientifically well-known. Moreover, their biochemical divergence make them suitable models for study.

The literature provides several studies on adsorption of proteins onto interfaces. Few of these proteins are enzymes. In cases where lysozyme was studied, it was included because of its extraordinary properties as a protein and not because of its enzymatic activity. Apart from (phospho)lipases there is scarcely any literature that describes activity of enzymes adsorbed onto the oil/water or air/water interfaces. Proteins tend strongly to accumulate in interfaces and for that reason are said to be very surface active. This adsorption is accompanied by a conformational change of the three-dimensional structure of the protein that results in some unfolding, ranging from almost full stretching of the peptide chain to a more conserved conformation. Hydrophobic residues or patches of the protein, mostly buried inside the molecule, will tend to position themselves next to or even protrude partly into, the hydrophobic phase of an oil/water interface. The extent of conformational change depends on the conformational stability of the protein, which, in turn, depends on pH, temperature, ionic strength etc. Furthermore, the extent of unfolding will be dependent on the surface area available and on the time scale, and hence, on protein concentration. In a static condition, adsorption at the interface will be diffusion driven, whereas during emulsification the time of adsorption will be determined by convection and will be very much shorter. Consequently, conformational changes of proteins during emulsification should be smaller because full surface coverage may be reached before unfolding can occur. In the case of enzymes being adsorbed the emulsification process would therefore offer an opportunity to retain activity.

The relation between the surface pressure (Π) i.e. the extent of surface tension decrease and the amount of protein adsorbed per unit surface area available (Γ), provides a

possibility to relate the size of the adsorbed protein molecules, and thereby the extent of unfolding, to the surface load. As mentioned earlier the extent of unfolding should be less at a higher Γ value. It has been calculated that lysozyme, an enzyme of high conformational stability, hardly unfolds at the air/water interface, even at low surface coverage. For the oil/water interface, however, a considerable increase in the radius of the protein molecules was observed at low surface load (see Chapter 3). At surface coverage of > 1.5 $mg.m^{-2}$, the radius remained more or less constant, indicating that substantial unfolding did not occur. Despite this rigidity, the enzyme had lost all of its enzymatic activity *in situ* and it even remained inactive after desorption (Chapter 3). Apparently, conformational changes in the enzyme molecule do not necessarily become manifest in a larger size for the molecule. Chymosin, being an enzyme of smaller conformational stability, naturally lost all of its activity due to adsorption onto the oil/water interface. In experiments with the enzymes coadsorbed simultaneously with bovine serum albumin, or the one after the other, there was no retention of *in situ* activity. Chymosin also proved to be inactivated at the expanding air/water interface due to air incorporation, if this occurred e.g. during homogenization.

During the cheese-making process enzymes like chymosin and lysozyme are retained in the curd. This retention must be due to association of the enzymes with the casein from milk. In order to adsorb the enzymes with retention of activity, the various casein fractions were used to make and stabilize a soya-oil emulsion, and the enzyme was subsequently allowed to associate with the casein. The extent of association of lysozyme with the casein fractions was in the order α_s-casein > β-casein > κ-casein. Only for the κ-casein stabilized emulsion, was lysozyme association dependent on pH within the range of pH 5.2 - 6.4 (greater for a lower pH). Furthermore, the association with the caseins was not dependent on temperature, indicating that hydrophobic interactions were not predominant. The same trends were found with the various caseins in solution, albeit that association with κ-casein hardly occurred. It should be kept in mind that casein adsorbed at an interface will expose other amino acid residues compared to its behaviour when free in solution. For that reason the association behaviour in the two systems may differ. Because the association varies between caseins the extent of association with lysozyme depended on the composition of the casein micelles (aggregates of many casein molecules and calcium phosphate, as occurring especially in milk). As expected, casein micelles containing a higher proportion of κ-casein associated less with lysozyme. It was found that lysozyme did not lose activity due to association with casein adsorbed on soya oil droplets or free in solution. However, lysozyme activity was markedly reduced when the enzyme was associated with casein micelles. In this system lysozyme also associated with casein in the interior of the casein micelle. The apparent loss of activity was most probably due to internal diffusion limitation. The difference of association for the various systems was also reflected in the free equilibrium concentration at which the surface excess

plateau value was reached. In the system of adsorbed caseins this value was reached at a free lysozyme concentration of about 3 μM, whereas for the micellar system this value was about 100 times higher.

The association of chymosin with casein has been studied in the same three systems of casein adsorbed onto soya-oil emulsion droplets, caseins in solution and caseins aggregated in casein micelles. Chapter 5 describes the association of chymosin with adsorbed caseins. It appears that chymosin only associated with adsorbed κ-casein and not with adsorbed α_s- or β-casein. Preceding the association, the caseinomacropeptide part of κ-casein is split off, followed immediately by the aggregation of the soya-oil emulsion droplets containing the remaining para-κ-casein. This coagulation behaviour is identical to the renneting of milk during the cheese-making process. The association characteristics for chymosin are also comparable. The association was strongly dependent on pH and ionic strength, and on chymosin and casein concentration. In Fig.1 these relations are schematically given. The κ-casein stabilized emulsion has proven to be a good model system for studying chymosin retention in curd. The chymosin associated with para-κ-casein was shown to be still active on added κ-casein or on a fluorescent small hexapeptide substrate. Consequently, the active centre of the enzyme is presumably not involved in the association with casein.

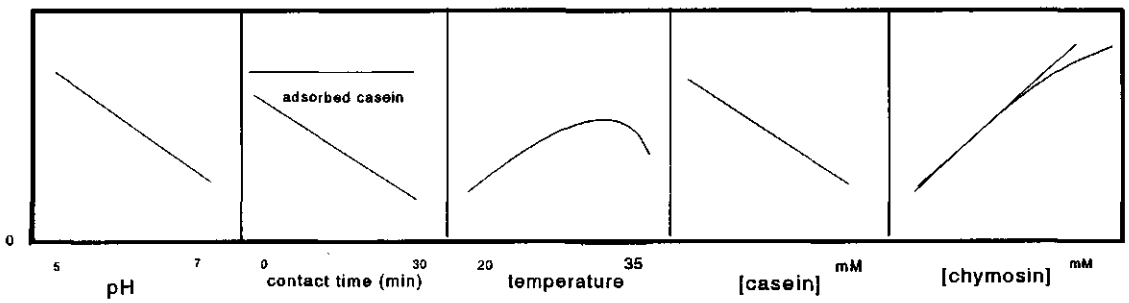


Figure 1. % of association of chymosin with casein

The association of chymosin with caseins free in solution is considered in chapter 6. Only in a solution containing κ-casein will addition of chymosin result in protein flocculation and precipitation. This flocculation is due to splitting off the caseino-macropeptide part of κ-casein and the consecutive aggregation of the fairly hydrophobic and almost electrically neutral para-κ-casein molecules. The precipitated protein fraction also contains associated chymosin, to an extent depending on conditions like pH, ionic strength and casein and

chymosin concentrations. Similar relations were found as shown in Fig.1. In this system time and temperature also affected the extent of chymosin association. The association decreased with increased contact time and was stronger at higher temperatures. The protein content in the supernatant after centrifugation increased not only due to dissociation of chymosin but also due to the presence of casein fragments. Apparently, the dissociation of chymosin was related to its proteolytic action. The dissociation rate increased with decreasing pH where chymosin becomes more active and less specific. The dissociation also increased with temperature for a given time of contact. However, when extrapolated to a contact time of t = 0 (i.e. when dissociation due to proteolysis has not occurred yet) the association was observed to be somewhat stronger for a higher temperature. The effect of temperature on the proteolysis-dependent dissociation, apparently was stronger than its effect on the increase of the association. Since chymosin association depends on mutual association of caseins (see below), it will also depend on the temperature dependence of the latter. Dissociation of chymosin was not found in the system of caseins adsorbed onto emulsion droplets. Relations for the dissociation behaviour are shown schematically in Fig.2.

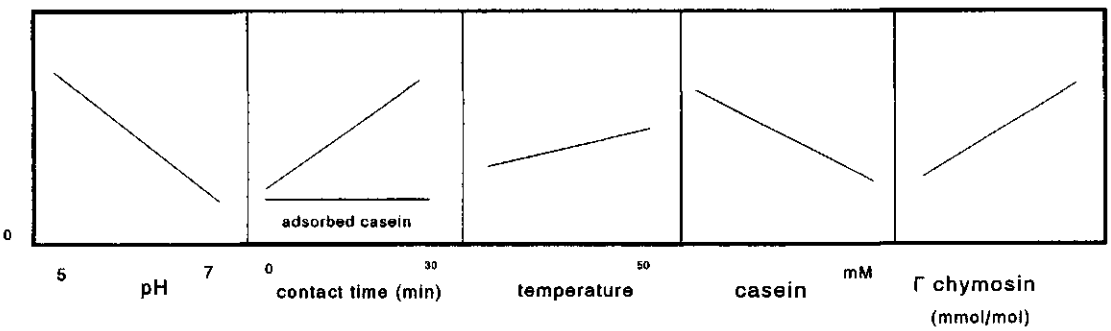


Figure 2. *% of dissociation of chymosin from casein*

The addition of small amounts of α_s- or β-casein strongly decreased the extent of association of chymosin with para-κ-casein. This effect was stronger for α_s-casein than for β-casein. It was also found that the extent of chymosin association (moles of chymosin per mole of para-κ-casein) was larger when the system was diluted or, in other words, when the casein concentration was reduced. Both phenomena can be explained by assuming that competitive association occurs between the caseins and chymosin for interaction with a para-κ-casein molecule. Chymosin is only able to associate with a para-κ-casein molecule when that is not associated with other casein molecules. Thermodynamically speaking, the extent of

association of chymosin is determined by the association constants that exist between all caseins under conditions as in the system. These association constants vary with pH, ionic strength, casein concentration and temperature.

In chapter 7 the model of competitive association is further developed and applied to the association of chymosin with casein micelles of various composition. It follows that chymosin will associate less with casein micelles composed of α_s- and κ-casein than with micelles composed of β- and κ-caseins. Again, this behaviour can be explained by competitive association, since different association constants exist for the caseins and chymosin for association with para-κ-casein. The relations for association and dissociation found in this casein micelle system are comparable with those found with caseins in solution. The kinetic model for competitive association is only a crude approximation. It does not provide possibilities of calculating all the association constants occurring in milk from the relations found from retention of chymosin in curd.

SAMENVATTING

De lange duur van rijping van kaas in geconditioneerde ruimten bepaalt voor een aanzienlijk deel de kostprijs van kaas. Er is de industrie veel aan gelegen deze rijpingstijd te verkorten. De rijping van kaas is in hoofdzaak het gevolg van proteolytische splitsing van caseïne, het belangrijkste eiwitbestanddeel in koemelk. Deze eiwitafbraak wordt veroorzaakt door proteasen afkomstig uit de melk zelf, uit het stremsel en uit de melkzuurbacteriën van het zuursel. Om de rijping van kaas te versnellen is een voor de hand liggende optie om extra proteasen toe te voegen aan de kaasmelk . Het probleem dat zich hierbij tijdens de procedure van kaasbereiding voordoet, is dat deze enzymen met de weistroom kunnen verdwijnen en derhalve niet in de wrongel terecht komen, dit in tegenstelling tot grote deeltjes zoals bacteriën, oliedruppels en eiwitaggregaten - zoals de caseïnemicellen - die zich in de wrongel laten insluiten. Indien de gewenste proteasen kunnen worden geïmmobiliseerd aan grotere deeltjes dan zouden ze wel in de wrongel terecht kunnen komen. Methoden om dit te bewerkstelligen zijn vaak te duur gebleken of kunnen niet met "kaas eigen" grondstoffen worden uitgevoerd. In dit onderzoek is geprobeerd enzymen, met behoud van activiteit, te immobiliseren aan food-grade deeltjes, zoals oliedruppeltjes en eiwitaggregaten. Hiervoor is gebruikt gemaakt van soja olie en caseïnemicellen. De enzymen chymosine en lysozym zijn gekozen als modelenzymen om de haalbaarheid te toetsen.

De adsorptie van eiwitten aan grensvlakken is uitvoerig beschreven in de wetenschappelijke literatuur en nog steeds is er op dit terrein veel onderzoek gaande. De meest bestudeerde grensvlakken zijn lucht/water, vaste stof/water en, zoals in emulsies, olie/water. Eiwitten hebben sterk de neiging zich in grensvlakken op te hopen. Ze hebben een hoge oppervlakteactiviteit, hetgeen inhoudt dat ze al in geringe concentraties de grensvlakspanning kunnen verlagen. Adsorptie van een eiwit aan een grensvlak gaat veelal gepaard met veranderingen van de ruimtelijke conformatie van het betreffende eiwit. Gedeelten van het eiwit zullen zich ontvouwen, afhankelijk o.a. van de conformatiestabiliteit van het eiwit en van de hoeveelheid beschikbaar oppervlak. De biologische activiteit van een eiwit is in sterke mate afhankelijk van zijn conformatie. Voor biologisch actieve eiwitten zal adsorptie grote invloed kunnen hebben op die activiteit. Als het adsorptie aan oliedruppels betreft, zal de bedekking van het olie/water grensvlak tijdens het proces van emulsievorming zo snel verlopen dat ontvouwing nog niet of nauwelijks heeft kunnen plaatsvinden. Het grensvlak is dan al geheel bedekt voordat ontvouwing van het eiwit plaats heeft kunnen vinden. Dit zou kunnen betekenen dat een enzym met behoud van activiteit aan het olie/water grensvlak wordt geadsorbeerd. Merkwaardigerwijs is er op dit gebied nauwelijks onderzoek bekend (uitgezonderd voor lipases). Hoofdstuk 2 beschrijft theoretische aspecten van eiwitadsorptie, vooral ook met betrekking tot enzymen.

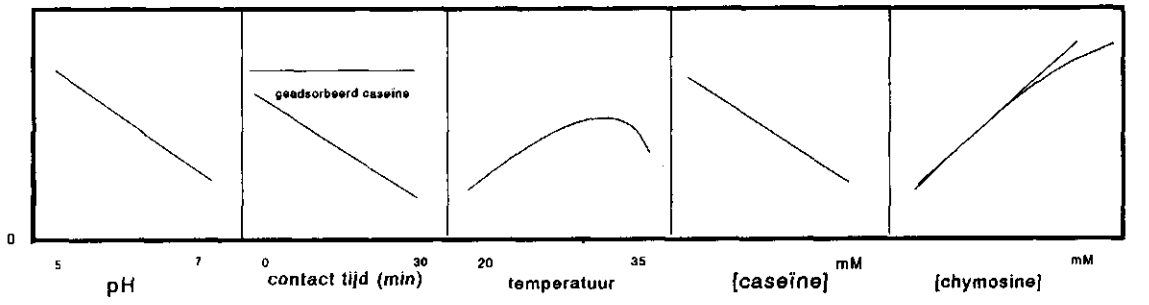
In hoofdstuk 3 is de invloed van adsorptie van lysozym en chymosine aan het soja-olie/water grensvlak beschreven. De enzymen zijn als zodanig als emulgator gebruikt,

maar ook in verschillende samenstellingen van concentratie met boviene serum albumine. Ook zijn de eiwitten in verschillende volgorde via homogenisatie aan het grensvlak olie/water gebracht. In geen der gevallen is *in situ* enzymactiviteit gemeten. Vastgesteld werd dat een geringe conformatieverandering van het enzym, niet leidend tot meetbare spreiding over het grensvlak, al voldoende was voor volledige inactivering en zelfs tot denaturatie van het enzym. Na desorptie van het enzym door middel van verdringing van het grensvlak door laag-moleculaire oppervlakte-actieve stoffen , blijkt geen herstel van enzymactiviteit op te treden. Als conclusie komt naar voren dat adsorptie van enzymen aan emulsiedruppels gepaard gaat met irreversibele denaturatie.

Lysozym en chymosine associëren met caseïne. Verkazing van melk waaraan deze enzymen zijn toegevoegd leidt ertoe dat beide enzymen in de wrongel terecht komen. Van deze eigenschap is gebruik gemaakt in de poging tot immobilisatie aan emulsiedruppels. Caseïnes zijn bij uitstek geschikt om olie/water emulsies te stabiliseren vanwege hun hydrofobiciteit en hun niet-globulaire structuur. Emulsies van soja olie worden met de individuele caseïnes gestabiliseerd. Na toevoegen van lysozym aan elk van deze emulsies trad onmiddellijke associatie op. De beladingsgraad van lysozym aan geadsorbeerde caseïnes is in de volgorde α_s-caseïn > β-caseine > κ-caseïne. De associatie aan geadsorbeerde κ-caseïne is van de pH afhankelijk (hoger bij lagere pH). Voor de andere geadsorbeerde caseïnes is pH afhankelijkheid in het pH-traject van 5,4 - 6,2 niet of minder duidelijk aanwezig. De associatie bleek nauwelijks temperatuursafhankelijk, hetgeen erop wijst dat hydrofobe interacties niet de oorzaak van de associatie zijn. In hoofdstuk 4 worden naast de associatie van lysozym aan geadsorbeerde caseïnes ook associatie aan opgeloste, vrije caseïnes en caseïnemicellen beschreven. Met vrij opgeloste caseïnes wordt dezelfde voorkeursvolgorde van associatie gevonden als voor geadsorbeerde caseïnes, met dien verstande dat associatie met κ-caseïne nauwelijks optreedt. Dit komt ook tot uiting in de verschillende mate van associatie aan caseïnemicellen van verschillende composities. Caseïnemicellen die meer κ-caseïne bevatten associëren minder met lysozym. Lysozym geassocieerd aan geadsorbeerde caseïne bleek de volledige enzymactiviteit te behouden. Dit was ook het geval voor associatie met vrije caseïnes. Associatie aan caseïnemicellen had een aanzienlijk (schijnbaar) activiteitsverlies tot gevolg, vermoedelijk door diffusielimitatie.

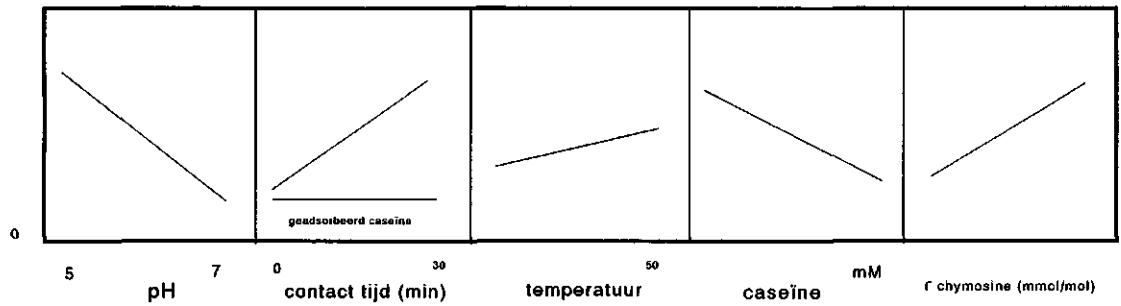
Op vergelijkbare wijze werd de associatie bestudeerd van chymosine met caseïnes die geadsorbeerd waren aan emulsiedruppels van soja olie. Chymosine associeert uitsluitend met geadsorbeerde κ-caseïne en niet met door α_s- of β-caseïne gestabiliseerde emulsiedruppels (hoofdstuk 5). De karakteristieken van adsorptie aan door κ-caseïne gestabiliseerde oliedruppels zijn gelijk aan die zoals bekend van het kaasmaakproces. De door κ-caseïne gestabiliseerde emulsie is een goed modelsysteem gebleken om retentie van chymosine in kaas te bestuderen. Toevoeging van chymosine resulteerde in de afsplitsing van het caseïnomacropeptide van κ-caseïne, waarna een onmiddellijke aggregatie van de door

para-κ-caseïne gestabiliseerde emulsiedruppels volgde. Dit coagulatiegedrag is analoog aan het stremmen van melk. De associatie is in sterke mate afhankelijk van de pH en ionsterkte, maar ook van de chymosineconcentratie en de caseïneconcentratie. Deze afhankelijkheden zijn schematisch weergegeven in Fig. 1. De geassocieerde chymosine bleek in staat om extra toegevoegde, vrije κ-caseïne te splitsen, evenals een fluorescerend synthetisch hexapeptide. Het aktieve centrum van het enzym is dus niet betrokken bij de associatie met geadsorbeerde κ-caseïne.



Figuur 1. *% associatie van chymosine met caseïne*

In hoofdstuk 6 wordt de associatie van chymosine met opgeloste caseïnes beschreven. Toevoegen van chymosine aan de afzonderlijke caseïnes leidde alleen met κ-caseïne tot een waarneembare precipitatiereactie. De associatie met para-κ-caseïne bleek tijdsafhankelijk. Na een korte contacttijd van chymosine met κ-caseïne werd in het supernatant na centrifugeren minder chymosine gevonden dan na een langere contacttijd. Met andere woorden, geassocieerde chymosine desorbeerde in de tijd. Deze desorptie bleek afhankelijk van de chymosine- en de caseïneconcentratie, van de pH en van de temperatuur. De gevonden relaties (schematisch weergegeven in Fig.2) doen vermoeden dat dissociatie van chymosine het gevolg is van proteolytische afbraak. De associatie van chymosine aan para-κ-caseïne wordt sterk verminderd door toevoeging van andere caseïnes. α_s-caseïne heeft een sterker reducerend effect op de associatie dan β-caseïne. De associatie van chymosine met para-κ-caseïne wordt sterker bij lagere concentraties van caseïne. Dit is ook het geval bij verdunnen van mengsels van κ-caseïne met α_s- en β-caseïne. Dit verschijnsel kan worden verklaard door competitieve associatie. Chymosine kan alleen associëren met para-κ-caseïne als deze niet reeds met een ander caseïnemolecuul is geassocierd. De mate van associatie van chymosine wordt bepaald door de associatieconstanten voor de associaties tussen de caseïnes onderling. Deze constanten variëren met pH, ionsterkte en temperatuur.



Figuur 2. % dissociatie van chymosine van caseïne

De temperatuur heeft op een ingewikkelde manier invloed op de mate van associatie. Extrapolatie van associatie naar tijdstip $t = 0$ laat een sterkere associatie zien bij een hogere temperatuur. Maar na een korte contacttijd blijkt deze associatie zo sterk te zijn verminderd dat juist bij lagere temperatuur de associatie groter is. Dit verschijnsel wordt verklaard doordat de dissociatie, die berust op temperatuursafhankelijke proteolyse, sterker is bij hogere temperatuur. In hoofdstuk 7, waarin de associatie van chymosine met caseïnemicellen van verschillende caseïnecompositie wordt beschreven, worden dezelfde relaties gevonden als in hoofdstuk 6 met opgeloste caseïnes. Caseïnemicellen bestaande uit β- en κ-caseïne associëren sterker met chymosine dan caseïnemicellen bestaande uit α_s- en κ-caseïne. Het principe van het model van competitieve associatie werd verder uitgewerkt. Het model is slechts een benadering van de complexe werkelijkheid en voorziet niet in de mogelijkheid om associatieconstanten tussen de caseïnes, zoals die in melk voorkomen, te berekenen aan de hand van de gemeten chymosineretentie.

NAWOORD

De schrijver Godfried Bomans heeft ooit over zichzelf geschertst dat hij een van die zeldzame Nederlanders was die op middelbare leeftijd het middelbare schooldiploma haalde. Iets daarvan voel ik mee met het voltooien van dit proefschrift. Het onderstreept de (om)weg die gegaan is om tot hier te komen. Als echter vooraf was zichtbaar geweest dat de voltooiing van dit "project" deze tijdsspanne zou omvatten en deze inspanning zou vergen, zou ik me flink achter de oren hebben gekrabd. Desalniettemin ben ik blij deze weg te hebben bewandeld en is het gevoel van destijds dat deze kans tot promoveren een buitenkans was altijd gebleven.

De totstandkoming van dit proefschrift is het resultaat van bijdragen door meerdere mensen geleverd en de koppeling van alleen mijn naam met dit proefschrift is derhalve niet geheel gerechtvaardigd en vanzelfsprekend. Ik wil Pieter Walstra bedanken voor de prettige samenwerking en de zeer stimulerende begeleiding. Pieter, ik beschouw het als een grote eer onder jouw supervisie dit werkstukje te hebben afgeleverd. Tom Geurts wil ik zeer bedanken voor zijn onbaatzuchtige inbreng. Veel van hetgeen beschreven is, kan gezien worden als een afronding van werkzaamheden en gedachtegoed die door jou al was ingezet en aangegeven. Via jou bedank ik tevens de studenten die voor mijn tijd al een bijdrage hadden geleverd en wier resultaten ik deels heb verwerkt. Hugo, Henk & Henk bedank ik voor hun ondersteuning.

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Mijn collega-promovendi hebben er zeer toe bijgedragen dat mijn Wageningse intermezzo zeer plezierig was (de low-budget tour door industrieel Groot-Brittannië beschouw ik als hoogtepunt). Frans den Ouden wordt bedankt voor drie jaar gezellig hokken.

Aad en Joke Barendse, bij wie ik de zolderetage heb mogen bewonen, hebben een niet te onderschatten rol gespeeld. Op een of andere manier kwam ik, na nachtelijke Wageningse dwalingen, steeds weer bij jullie huis uit.

Marijke, wat mij betreft mag jouw naam ook op de omslag. Dit project hebben we tenslotte samen uitgevoerd.

Curriculum Vitae

Andreas Leonardus de Roos werd geboren op 13 mei 1954 te Den Haag. In 1970 behaalde hij het MULO diploma aan de St. Tarcisius MAVO te Den Haag en in 1972 het HAVO diploma aan het Thomas More College te Den Haag. In 1975 rondde hij een studie Chemisch Analytisch analist HBO-A af aan de Delftse Analisten Cursus. In de periode 1975 -1981 werkte hij als chemisch analist bij het RVO/TNO te Rijswijk. Hij werkte aan de ontwikkeling van biochemische detektiereakties voor het aantonen van toxische stoffen in water en lucht en de applicatie hiervan tot detektieapparatuur. Tijdens die periode werd de avondstudie Biochemie HBO-B afgerond aan het Van Leeuwenhoek Instituut te Delft.

In de periode 1981-1988 werkte hij als biochemisch analist bij de Stichting Samenwerkende Delftse Ziekenhuizen aan de isolatie van humane eiwitten ter bereiding van antisera en hiervan afgeleide producten ten behoeve van de ontwikkeling van diagnostische tests van immunologische en histochemische aard. Vervolgens werd gewerkt aan de ontwikkeling van een diagnostische test op DNA-niveau waarmee in een vroegtijdig stadium virale (HPV) infectie kan worden aangetoond.

In de periode 1982-1988 volgde hij de avondopleiding voor Beeldend kunstenaar aan de Koninklijke Academie der Beeldende Kunsten te Den Haag.

In 1988 werd hij te werk gesteld bij Gist-Brocades BV te Delft. Na twee jaar werkzaam aan de ontwikkeling van farmaceutische eiwitten werkt hij op het gebied van biochemie en applicatie van enzymatische ingrediënten ten behoeve van de zuivel. In 1990 werd een post HBO avondcursus Grensvlak - en Kolloïd chemie gevolgd.

In de periode 1991-1994 is hij gedetacheerd geweest aan de Landbouwuniversiteit Wageningen aan de toenmalige Sectie Zuivel en Levensmiddelennatuurkunde. Gedurende deze periode is gewerkt aan het onderzoek waarvan de resultaten zijn vastgelegd in dit proefschrift.