Physical Stability of Caseinate-Stabilized Emulsions During Heating

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Physical Stability of Caseinate-Stabilized Emulsions During Heating

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stellingen

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1 Verscheidene factoren die van invloed zijn op hitte-geïnduceerde coagulatie van met caseïnaat gestabiliseerde emulsiedruppels zijn ook van invloed op coalescentie van emulsiedruppels.
Dit proefschrift

2 De stabiliteit van met caseïnaat gestabiliseerde emulsies gedurende verhitting hangt meer af van factoren waarbij lading een belangrijke rol speelt, zoals initiële calcium-ion-activiteit en pH, dan van sterische effecten door de caseïne, in vergelijking met emulsies gestabiliseerd door micellair caseïne.
Dit proefschrift

3 Als gevolg van defosforylering van caseïnaat zal de gevoeligheid voor, door calcium geïnduceerde aggregatie van caseïnaten afnemen.
D.G. Dalgleish & A.J.R. Law. *J. Soc. Dairy Tech.* 41(1) (1988) 1-4.
Dit proefschrift

4 Op basis van bepaling van de samenstelling van de fasen in een fase-gescheiden systeem van twee polymeren in een oplosmiddel kunnen geen conclusies getrokken worden t.a.v. attractie of repulsie tussen beide polymeren.
Picullel et al. In: Gums and Stabilizers for the Food Industry 7 (1994) pp. 309-322.

5 De toestand waarin de caseïne in melk vóórkomt vertoont overeenkomsten met die van een coacervaat.
H. Eilers. Colloïd-chemische studiën aan ondermelk. Algemene Landsdrukkerij, Den Haag (1945).

6 De stabiliteit van chocolademelk tijdens steriliseren wordt vooral bepaald door hitte-geïnduceerde dissociatie van caseïnes uit de micellen welke vooral afhankelijk is van de alkalyseringsgraad van de cacao.
T. van den Boomgaard et al. *Int. J. Food Sci. Techn.* 22 (1987) 279-291.

7 Het effect van de uitlaattemperatuur van de drooglucht op het ontstaan van "white flecks" bij de bereiding van melkpoeder is toe te schrijven aan aggregatie van uitgemergelde caseïnemicellen.
N. Parris et al. *J. Agric. Food Chem.* 38 (1990) 824-829.
H. de Ruyck. *Landbouwtijdschrift* 44(4) (1991) 751-761.

8 De consumptie van een beker melk geeft bij de meeste personen met veronderstelde lactose-intolerantie nauwelijks aanleiding tot darmklachten.
F.L. Suarez et al. *N. Engl. J. Med.* 333(1) (1995) 1-4.

9 Het mechanisme achter velvorming bij het koken van melk, dat beschreven is als een gevolg van de stijging van de calciumconcentratie door verdamping vlak onder het melkoppervlak, waardoor een aggregatie van de caseïne ontstaat en zich een gel van melkeiwit vormt, verklaart niet het achterwege blijven van een vel op vla door er direkt na het koken wat suiker op te strooien.
NRC Handelsblad, 30 maart 1995
De beste tips uit Libelle weet 't, 1976

10 Een hoge snelheid van ontwikkeling verhoogt de kwaliteit van een produkt.

11 Men mag het christelijk geloof algemeen betwijfelen, maar men kan niet die twijfel tot een algemeen geloof verheffen.
NRC Handelsblad, 10 april 1993

Stellingen behorende bij het proefschrift "Physical stability of caseinate-stabilized emulsions during heating" door J.M.M. Cruijsen.
Wageningen, 6 mei 1996.

voor mijn ouders
voor Maaike

Abstract

Cruijsen, J.M.M. (1996) Physical Stability of Caseinate Stabilized Emulsions During Heating. Ph.D. thesis, Wageningen Agricultural University (126 pp., English and Dutch summaries).

Keywords: Emulsions, caseinates, malto-dextrins, heat treatment, heat coagulation, phase separation, coalescence, lecithins, phospholipids

The physical stability of caseinate stabilized emulsions was studied during heating (80-120°C). Coagulation, coalescence and phase separation of the caseinate emulsions was studied using objective heat stability tests. The physical changes were characterized by light microscopy, particle size measurements and by determination of the solubility of coagula in various reagents. Additional information about physico-chemical changes in caseinate dispersions was obtained by determination of association of salts with caseinates, association of caseins with emulsion droplets, ^{1}H-NMR, ^{31}P-NMR and SDS-Page.

The susceptibility towards heat coagulation of caseinate emulsions was mainly determined by volume fraction of oil, decrease of pH during heating and initial Ca^{2+}-activity. The heat coagulation time could be related to association of calcium and magnesium and caseinate and to association of caseinate and emulsion droplets. Association of calcium and magnesium and caseinate in these conditions would diminish electrostatic repulsion between caseinate molecules, which would lead to aggregation of the caseinate. It was shown that the formation of chemical cross-links is not rate determining in heat coagulation.

During heating of caseinate emulsions containing malto-dextrins or a lactose/sucrose mixture, phase droplets were formed. The appearance of these phase droplets was accompanied by multi-layer formation of caseinate on the oil-droplets and a decreased stability to heating, often resulting in a highly viscous mass. Phase separation could be prevented in several ways; by using phosphates as stabilizing salt, by using malto-dextrins or glucose syrups with relatively high DE values or by using soya lecithin. The effect of lecithin was only found when lecithin was added prior to homogenization. It appeared that the association of caseinate in solution or with the emulsion droplet was modified and thereby prevented phase separation.

Although soya lecithin proved to be a very effective stabilizer, coalescence of the emulsion droplets could be observed when the concentration exceeded a certain value or when the phosphatidylcholine fraction of soya lecithin was used. The lecithins affected the coalescence behaviour, presumably by lowering the interfacial tension and lowering the caseinate surface load, and possibly by changing the structure of the caseinate layer on the emulsion droplets.

Contents

Chapter 1

Physical Stability of Caseinate Emulsions: Introduction

1.1 Introduction

Emulsions for dietetic use are complex food products, physically consisting of oil-globules dispersed in a serum phase. The oil globules are covered by a protein layer (e.g. caseinate, whey proteins, soya proteins). The serum phase may contain sugars (mostly malto-dextrins or glucose syrups), proteins, salts and minor components like vitamins and trace elements. These food products can be preserved in liquid (ready for use) form when heat sterilized by conventional sterilization or by UHT-treatment. The process steps during manufacturing also include homogenization, by which the oil-droplets are reduced to such a size that creaming is prevented, thereby extending shelf-life. Product composition and processing conditions are important factors affecting stability of caseinate emulsions during heat treatment and storage (McDermott, 1987; Fligner et al., 1990; Rowley & Richardson, 1985).

The major problems in the production of caseinate stabilized dietetic food products are coagulation or coalescence of emulsion droplets during the sterilization process and formation of a gel or sediment directly afterwards. Heating sodium-caseinate solutions at sterilization temperature (120°C) causes little changes (Guo et al., 1989), but if sugars like lactose or salts were present caseinate aggregated during heating (de Koning, 1982; Zittle, 1969b). Stability of the emulsions is primarily due to the adsorbed caseinate layer at the oil-water interface. It is therefore in particular necessary to understand the properties of the adsorbed caseinate layer in these complex systems and the relationship to the overall stability (Darling, 1982; Dalgleish, 1989; Mulvihill & Murphy, 1991; van Boekel & Walstra, 1995).

This chapter attempts to provide a survey on physical stability of emulsions with emphasis on caseinate systems. First, it describes formation of the emulsions and composition and properties of the surface layers. Second it describes the types of interactions which determine the stability of protein-stabilized emulsions or dispersions against aggregation, precipitation, or coalescence of the droplets. Finally phase behaviour and physico-chemical changes that occur in caseinate systems in different conditions and during heat treatment will be indicated.

A full review on these subjects is beyond the scope of this thesis. Physical background information relevant to dairy systems can be found in books by Mulder & Walstra (1974) and Walstra & Jenness (1984). Issues on stability of food colloids are discussed in Dickinson (1986); Walstra (1987a) and Walstra (1993b). Aspects of phase behaviour of mixtures of proteins and polysaccharides are treated by Tolstoguzov (1991) and Picullel et al. (1994).

1.2 Emulsion formation

A comprehensive review on the formation of emulsions has been given by Walstra (1993a). Only a very brief summary will be presented here. Emulsions can be formed when one liquid is dispersed in another by supplying external energy, as the free energy of an emulsion is higher than that of the separate liquid phases. During emulsification, droplets are deformed and possibly broken up. Simultaneously surfactant is adsorbed onto newly formed droplets. The role of surfactant is of great importance during and after emulsification. The surfactant lowers the interfacial tension, thereby facilitating or simplifying emulsion droplet break-up.
When a protein is adsorbed at an oil-water interface the protein-molecule is folded in such a way that the free energy is minimized (Phillips, 1981). The hydrophobic parts of the proteins tend to adopt a position in the oil phase, whereas hydrophillic parts are in a lower energy state in the aqueous phase (Figure 1.1).

The surfactant is also responsible for preventing recoalescence during emulsification via the Gibbs-Marangoni mechanism. Initially, newly formed droplets are insufficiently covered with surfactant, especially where the film between approaching droplets is thinnest. The uneven distribution of surfactant gives an interfacial tension gradient which causes surfactant in the interface to move in the direction of the highest tension. Movement of surfactant will drag liquid along the surface, which will keep droplets apart and stabilize them during emulsification (Walstra, 1993a).

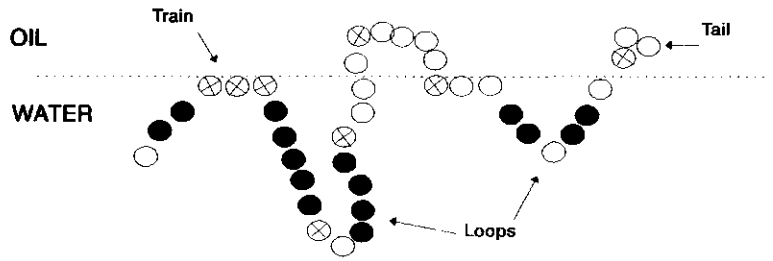

Figure 1.1 *Schematic representation of nonpolar (○) polar (●) and neutral (⊗) residues of protein.*

1.3 Caseinate at the interface

All proteins tend to adsorb at oil-water interfaces. Most food proteins including caseins have disordered random coil-like structures in solutions and at the interface. This is in contrast to the compact rigid globular proteins which show structural alteration when they adsorb, but remain compact. In any particular situation the conformation of the protein will depend on type, state of aggregation and the ratio of protein concentration and total interfacial area. This will certainly apply to (sodium) caseinate, which is a partially aggregated mixture of the four individual caseins α_{s1}-, α_{s2}-, κ- and β- in proportions 4:1:1:4 by weight (Phillips, 1981; Walstra, 1987; Dickinson, 1989b; Dalgleish, 1995).

The properties of individual caseins in the surface layer of emulsions can be different from those in whole caseinate. Robson & Dalgleish (1987) found that all caseins were present at the interface, but the hydrophobic β-casein in somewhat larger proportions. Other experiments have shown that the two major caseins α_{s1}- and β-casein will displace each other fairly rapidly. Presence of individual caseins at the interface and exchange of them at the interface and with the caseins in the serum will depend on intermolecular interactions between the caseins. Possible interactions are calcium bridging, hydrophobic binding and disulfide bridging, the last one via cysteinyl residues on α_{s2}- and κ-casein. Conditions such as pH, ionic strength and the way the emulsion droplet membrane is formed will therefore largely determine the composition of the surface layer (Robson & Dalgleish, 1987; Dickinson, 1989; Dalgleish, 1993)

When a protein molecule like β-casein covers a surface in an unfolded conformation, the surface load Γ will be about 1 mg/m^2; below this value no stable emulsion can be made (Phillips, 1981; Fang & Dalgleish, 1993a). Only a fraction of the protein will be in direct contact with the interface (Figure 1.1). A part of the molecules protrude into the serum phase or into the oil. As a consequence, the interfacial protein membrane of unfolded protein molecules, but also of folded protein molecules like globular proteins, will be much thicker than the diameter of a protein chain. Two layers can be discerned for β-casein adsorbed at an oil-water interface: a dense inner layer with a thickness of about 2 nm, and a diffuse outer layer with a thickness of 5-7 nm, as could be concluded from neutron reflectivity measurements (Dickinson et al., 1993b; Horne & Leaver, 1995). These results are in reasonable accordance with measurements on adsorption of caseins and subsequent measurement of layer thickness after hydrolysis of these layers (Dalgleish, 1993). Mixtures of individual caseins or caseinate in oil-in-water emulsions showed that the thickness of a α_{s1}-casein layer was about 5 nm while the thickness of a β-casein layer was about 11 nm. The layer thickness of mixtures of individual caseins was variable and layer thickness of whole caseinate at the lowest adsorption of $\Gamma = 1$ mg/m^2 was about 5 nm while the thickness was about 10 nm at the maximum coverage of $\Gamma = 3$ mg/m^2 (Dalgleish, 1993; Fang & Dalgleish, 1993a). Any further adsorption is probably induced through molecular

interactions between caseins, such as electrostatic or hydrophobic forces. Adsorption isotherms of aggregates or mixtures of proteins can show surface loads $\Gamma > 5\text{-}10$ mg/m^2. For example, in homogenized milk the surface load Γ is about 10 mg/m^2 (Oortwijn & Walstra, 1979).

1.4 Caseinate stabilized emulsions containing low molecular weight surfactants.

Adsorption of caseinate in the presence of low molecular weight surfactants may have important implications for emulsion droplet interactions and therefore for emulsion stability. Composition and thickness of the adsorbed layer around emulsion droplets depend on the nature, the concentration and the interactions between the surfactants. In this section some relevant experimental information on competitive adsorption of natural surfactants such as mono-, diglycerides or phospholipids used in dietetic products is discussed (Fligner et al., 1990; Fang & Dalgleish, 1993b; Dickinson, 1993).

Low-molecular weight surfactants, such as monoglycerides and lecithins give in general a lower surface tension than proteins, so they will penetrate or even displace adsorbed protein. It is therefore likely that a low-molecular weight surfactant is directly associated with the emulsion droplet and that protein is adsorbed on top of the surfactant or only partly associated with the droplet. In this way a mixed protein/surfactant surface layer is formed. The surfactant-protein ratio will probably affect surface layer composition. Fang and Dalgleish (1993b) suggest that at low bulk protein concentration the adsorption free energy per protein segment is lowered more than for low molecular weight surfactants. At high protein concentration the opposite seems valid (Bergenståhl, 1988; Fang & Dalgleish, 1993b).

The stability with respect to creaming in cream liqueurs (30 g/kg sodium caseinate, 200 g/kg milk fat; 140 g/kg ethanol) was enhanced on addition of glycerol monostearate (GMS) or sodium stearyl lactylate (SSL). It was found that the caseinate at the surface was partly displaced above 1 % GMO, GMS or SSL and complexes were probably formed between caseinate and the surfactant (Dickinson et al., 1989; Heertje et al., 1990). Displacement of proteins by GMO, GMS or Tween 20 was also found in β-casein emulsions (Dickinson et al., 1993a) or recombined milk (Oortwijn & Walstra, 1979).

Fang & Dalgleish (1993b) studied the adsorption of egg-yolk lecithin in sodium caseinate emulsions containing 200 g oil/kg. The stability of the emulsion and structure of the adsorbed layer was affected by the presence of lecithin. They found that lecithin enhanced the stability towards aggregation and coalescence at low casein surface coverage $\Gamma < 1$ mg/m^2. It was found that the amount of caseinate adsorbed onto the droplets was a function of lecithin concentration (Courthaudon et al., 1991; Fang & Dalgleish, 1993b). When less than 10 percent of the surface area was occupied with lecithin at a concentration of 0.5 g/kg, no effect of the lecithin on

casein layer thickness could be detected. At relatively high level of lecithin (2-5 g/kg) and rather low casein concentration (< 10 g/kg), casein layer thickness increased. At comparable lecithin contents but at high caseinate concentration (>10 g/kg) the thickness decreased. Results on casein load measurements by a depletion method were in agreement with these trends from layer thickness measurements (Fang & Dalgleish, 1993b).

Summarizing, studies of addition of surfactants showed a considerable variation in effect on surface layer structure and stability. This effect predominantly depended on the type of surfactant and especially caseinate and surfactant concentration and their ratio. This variation probably arose from differences in association of the surfactant with the caseinate and with the interface.

1.5 Emulsion stability

Globules in an oil-in-water emulsion may be subject to various kinds of physical instability, namely coalescence, aggregation and creaming. Rapid creaming or sedimentation may be enhanced by coalescence or aggregation. These three major forms of instability are schematically shown in Figure 1.2. This figure includes the relationship between these phenomena. More types of instability in emulsions like bridging by particles, liquid neck formation, bridging flocculation, depletion flocculation, chemical cross-linking and Ostwald ripening are reviewed in: Mulder & Walstra (1974); Stainsby (1986); Walstra (1987) and Walstra (1993b).

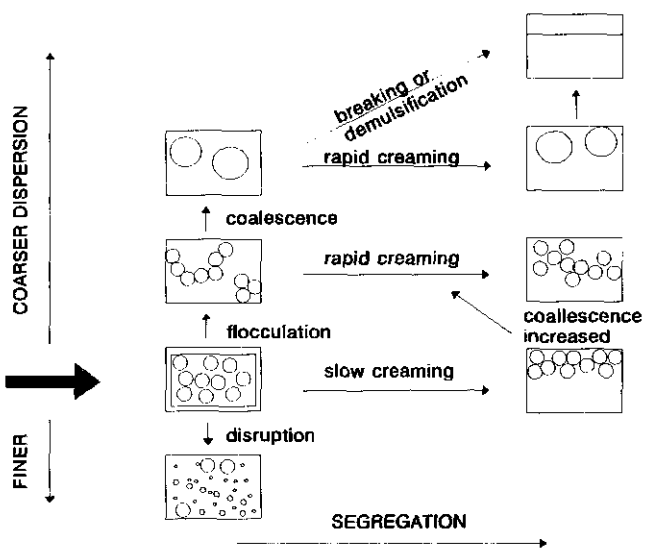

Figure 1.2 *Different types of instability of an O/W emulsion (schematic) (reproduced from Mulder & Walstra, 1974)*

Whether or not instability occurs depends primarily on the interacting forces between the droplets, and the surfactants or proteins on the droplets play a key role in these interaction forces. The types of forces involved are the field of colloid science and are useful in studying stability phenomena of protein stabilized emulsions.

1.5.1 Interaction forces

It is often sufficient to conceive the emulsion droplets as being covered with protein, of which parts protrude into the solution for some distance. When surfaces come very close, say <20 nm, attractive and repulsive forces affect the further approach of the droplets. Van der Waals attractions between them can become significant. Adsorbed protein allows the possibility of electrostatic repulsion of emulsion droplets. This repulsion is explained as a result of the local increase in osmotic pressure, when the ion clouds of charged residues of protein overlap. The effects of these two forces are accounted for in the DLVO theory (Israelachvili, 1992; Walstra, 1993b).

The forces between emulsion droplets are affected both by the particles themselves and the dispersing medium and depend on droplet separation distance h. For two equal-sized droplets in an o/w-emulsion at room temperature and for $h << a_d$, the London van der Waals interaction energy is given by:

$$G_a = -a_d A_h / 12\, h \quad \text{(J)} \qquad (1)$$

where a_d = radius of emulsion droplet (in m)
A_h = is the composite Hamaker constant (in J)
(for triglyceride oil in water $A_h = 5\ 10^{-21}$ J (Israelachvili, 1992)

The van der Waals forces are effective in the range of h = 0-20 nm for emulsions droplets with a radius of 0.5-2 μm. At greater distances, the forces are too small and are easily overcome by Brownian motion (Walstra, 1993b).

Electrostatic interaction energy G_r, is given in equation 2 for conditions in most emulsions ($\kappa a_d >> 1$; $h << a_d$; $|\psi_0| < 40$ mV) as a function of the separation distance h between the droplets.

$$G_r = 4.3*10^{-9}\, a_d\, \psi_0^2 \ln(1 + e^{-\kappa h}) \quad \text{(J)} \qquad (2)$$

where a_d = radius of emulsion droplet (in m)
ψ_0 = surface potential (in V)
$1/\kappa$ = the electric double layer thickness (in m)

To impart stability to emulsions, it is necessary that repulsive forces are stronger than attractive forces between the droplets. An example of the total interaction energy is given as function of the distance between two emulsion droplets in Figure 1.3 (after Walstra, 1993b).

Electrostatic repulsion can give rise to a substantial energy barrier at separations of a few nm at point B (Figure 1.3.a), near the primary minimum at point A, opposing close approach of droplets. A shallow secondary minimum can cause reversible flocculation at rather large droplet separations at point C.

The extent to which electrostatic interactions contribute to the stability of caseinate stabilized emulsions may only be marginal, because of ionic conditions and relatively low surface potential. The surface potential, as approximated by electrokinetic potential, was rather small, between -5 and -20 mV (Dalgleish, 1984). Even in an environment with a relatively low ionic strength, sufficient concentration of ions may minimise charge-interactions. Typically the thickness of the electric double layer $1/\kappa$, (which equals about $3.3\sqrt{I}$; Ionic strength I in mol/l) would fall from 10 nm at ionic strength 1 mmol/l to 1 nm at 100 mmol/l for an univalent electrolyte like NaCl. Hence, this distance is small compared to the distance at which emulsion droplets are thought to be stable (Walstra, 1993b; Bergenståhl, 1988).

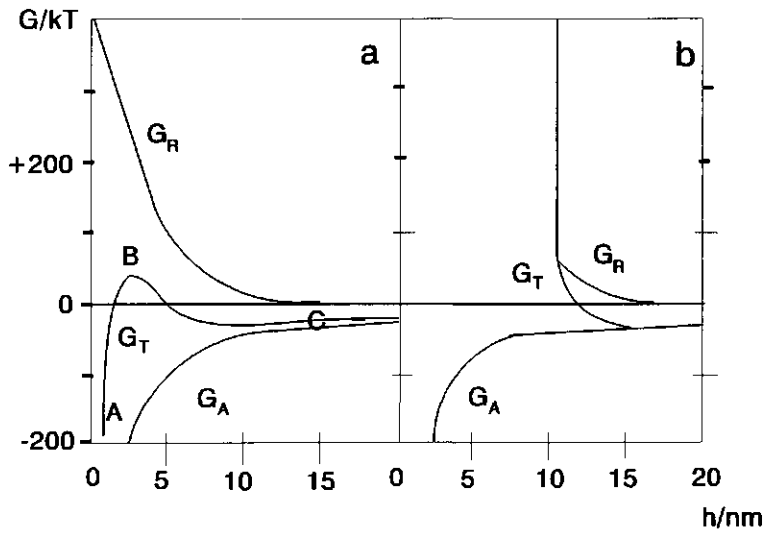

Figure 1.3 *Calculated total interaction energy (G_T) between two spheres as a function of surface separation distance h.* ***(a)*** *electrostatic repulsion and van der Waals attraction (DLVO);* ***(b)*** *steric repulsion and van der Waals attraction (reproduced from Walstra, 1993b)*

The theory can by extended by incorporating steric repulsion due to protruding molecular chains which can be of great importance in the case of proteins. The interaction free energy associated with steric repulsion is composed of two components, one arising from mixing of the protruding chains and, at small distance h, their elastic deformation. In Figure 1.3.b a strong steric repulsion at about 10 nm

is introduced. The interactions between segments of adsorbed polymers are generally repulsive, unless the solvent quality for the protruding chains is poor. However, quantitative models on steric stabilization are intricate, and models to account for steric stabilization by for proteins adsorbed at the interface do not exist. Mostly, steric repulsion will be strong enough to prevent droplets from approaching each other and thereby aggregation (Snowden et al., 1991; Israelachvili, 1992; Walstra, 1993b).

Non-adsorbed polymers like polysaccharides can have important effects on aggregation of particles or emulsion droplets (Vincent, 1987; Snowden et al., 1991; Luyten et al., 1993). The influence of polysaccharides on aggregation has been described by the exclusion of polysaccharide molecules from a layer around the particles giving a higher osmotic pressure. Aggregation of the particles then decreases the depletion volume, hence decreases osmotic pressure, hence decreases free energy. This depletion flocculation may only happen if the molar mass of the polysaccharide is high and the solvent quality is good (Walstra, 1993b).

Only recently, the interaction forces between proteins and other biological molecules adsorbed onto solid surfaces were measured directly. The various forces involved such as electrostatic forces are distinguished by adapting the experimental conditions such as ionic strength and pH. For example, electrostatic repulsion can be suppressed by raising the ionic strength, as a result of which the van der Waals attraction becomes more dominant in the force profile (Israelachvili, 1992). The measured force profiles were in remarkable agreement with theoretical predictions based mainly on DLVO-theory extended with a steric repulsion term and behaviour of the colloidal particles in solutions (Leckband & Israelachvili, 1993; Israelachvili, 1992). For example, behaviour of phospholipid bilayer membranes and protein films of ribonuclease and human serum albumin on mica interfaces were examined by this technique. Force profiles of ribonuclease and human serum albumin monolayers showed strong repulsive forces of steric nature at surface separations of 5-10 nm. This is correlated to the stability of these proteins in solution (Blomberg et al., 1991; Leckband & Israelachvili, 1993).

The overall interactions for caseinate emulsions are governed by a balance of electrostatic, van der Waals and steric repulsion forces between the surfactant covered emulsion droplets. It is very difficult to calculate the net interaction energy for a given situation, especially if the composition and structure of the interfacial layer is hardly known. Interfacial behaviour for caseinate stabilized emulsions is further complicated by protein surface heterogeneity and by time dependence of the structure. Nevertheless, these calculations are a useful tool in predicting trends, since a few studies showed that conditions of aggregation and subsequent coalescence of caseinate emulsion droplets were in reasonable agreement with predictions from theory (Dalgleish, 1984; Dickinson et al., 1987).

1.5.2 Aggregation

Colloidal aggregation may occur if attractive forces are larger than repulsive forces. The properties of the continuous and disperse phases and also the emulsion droplet surface layer determine to what extent aggregation occurs. Aggregation of protein stabilized emulsions may be induced in several ways: (i) by adjusting the pH towards the isoelectric point, which lowers the net surface charge density; (ii) by raising the ionic strength, to screen the charges; (iii) by adding divalent ions like calcium which may interact specifically with protein residues; thereby forming bridges between negative groups at the protein or reducing charge; (iv) by adding ethanol which reduces solvent quality (Dickinson, 1989a; Walstra, 1990; Walstra, 1993b).

The significance of surface potential or electrokinetic potential of caseinate stabilized emulsion droplets on stability was discussed by Dalgleish (1984). In pH region 5.5 to 7.5 a number of carboxyl and ester phosphate groups on caseinate will be dissociated and therefore provide net negative charge. The electrokinetic potential of sodium-caseinate covered droplets was -24 mV without calcium and about -12 mV at a calcium concentration of 10 mmol/l, measured in imidazole buffer pH 7.0 containing NaCl (50 mmol/l) at 25°C (Dalgleish (1984). If pH is lowered or Ca^{2+}-activity is increased the electrokinetic potential increases, i.e. it becomes less negative and this may cause caseinate chains to "curl up" (Walstra, 1990). Thus, in a qualitative sense, the possibility of interaction between protein segments and coagulation rate will increase, because of van der Waals attraction between the droplets (Walstra, 1993b). Emulsions prepared with sodium caseinate or with individual caseins (α_{s1}-casein or β-casein) aggregated after addition of 15 mmol/l $CaCl_2$, in a 20 mmol/l imidazole buffer, pH 7.5. Aggregation was reversible when the emulsion was diluted with buffer (Dickinson et al., 1987; Hunt et al., 1993). The effect was most pronounced when the protein load of the emulsion droplets was small (Dickinson et al., 1984). It seems that calcium plays a major role in this mechanism. When citrate was added to caseinate stabilized cream liqueurs, shelf life could be extended by some orders of magnitude, probably by preventing aggregation (Banks et al., 1981). This calcium-induced aggregation was particularly found when caseins were present which contained a number of phosphoserine residues. Emulsions made from reduced κ-casein were stable towards aggregation on addition of calcium-chloride (15 mmol/l) (Dickinson et al., 1987). Sodium chloride induced aggregation of sodium caseinate emulsions when the concentration exceeded 2 mol/l. At these high sodium chloride concentrations all charges at the caseinate were probably screened off and the caseinate precipitated (Dickinson et al., 1984; Dickinson et al., 1987). Furthermore, aggregation increased when the solvent quality was reduced by adding extra alcohol (140 g/kg) to caseinate emulsions (Banks et al., 1981).

These results from emulsions stabilized by caseinate or individual caseins were in agreement with behaviour of individual caseins in solution. The Ca^{2+}-concentration required to initiate precipitation of all individual caseins is roughly equivalent to the

number of phosphoserine residues. The calcium ions are probably very effective in decreasing net charge and electrostatic repulsion between the caseinate residues, hence promoting the possibility of hydrophobic interaction, leading to increased association and precipitation (Dalgleish & Parker, 1980; Parker & Dalgleish, 1981; Swaisgood, 1992). Aggregation of caseinate caused through charge neutralization by non-specific salts like sodium chloride seemed less important. It seems reasonable to assume that, also during heating, ionic composition and pH are important parameters in affecting aggregation of caseinate emulsions .

1.5.3 Coalescence

Coalescence of emulsion droplets results in emulsions with larger droplets and eventually in separation of oil. Coalescence rate is predominantly determined by the properties of the interfacial layer containing different types of protein or low molecular weight surfactants. Protein stabilized emulsions are mostly stable against coalescence. The most simple explanation for emulsion stabilisation is that the protein can form a "skin" around the surface of oil droplets and that the mechanical properties of the adsorbed protein film are responsible for the resistance to coalescence. Or more precisely: coalescence in an oil-in-water emulsion is a result of a number of successive processes: encounters of the droplets, drainage of the film of continuous phase between them and final rupture of the film due to some local fluctuation or disturbance. For stabilisation, the interfacial tension must not be too low and strong repulsive forces must act over a fairly long distance (Walstra, 1987a; Dickinson, 1989a).

Rate of coalescence mostly follows first-order kinetics, whereas aggregation is typically a second-order reaction. Semi-quantitative measurements of kinetics of (partial) coalescence were done with oil-in-water emulsions with caseinate, SDS and PVA in the presence of crystalline fat. The fraction of encounters leading to coalescence α for an unstable emulsion was 10^{-6}-10^{-7} whereas α for a "stable" emulsion will be $< 10^{-10}$ (van Boekel & Walstra, 1981; Darling, 1982; Mertens, 1989)

Caseinate was found to be very suitable to produce stable oil-in-water emulsions. Caseinate is highly surface active and gives rise to small stable emulsion droplets because it causes considerable repulsion between oil-droplets and gives not a very low interfacial tension (Walstra, 1987, Mertens, 1989; Fang & Dalgleish, 1993a). The minimum protein load for caseinate stabilized emulsions is about 1 mg/m^2 . Below this value droplets may aggregate and coalesce. Such a low caseinate load is obviously related to a very low caseinate to oil ratio in the emulsions. A caseinate concentration of 0.3 weight percent was enough to give a stable emulsion (droplets $< 1\mu$m) containing 20 weight percent of oil (Fang & Dalgleish, 1993a). When the four related but different individual caseins were studied separately in emulsion stabilization,

similar results were found. Stable emulsions could be formed above limiting amounts of the individual caseins (Dickinson et al., 1988). It was also found that the individual caseins formed surface layers with different structures and dimensions. Mixed layers of some of the individual caseins did not have intermediate composition and structure, but appeared to depend on interactions between the caseins at the conditions studied (Dalgleish, 1993).

The condition of limiting amounts of protein to stabilize emulsions was also found with whey protein stabilized emulsions during heating at 120°C. Stability of emulsion droplets fell markedly as surface coverage was reduced to about 1 mg/m^2 (Oortwijn & Walstra, 1982). Emulsions made from desalinated whey showed less coalescence on heating than at high ionic strength or calcium-ion-activity, indicating the importance of conditions like pH and ionic strength (Yamauchi, 1980; Melsen, 1987).

Low molecular weight surfactants added to caseinate or other protein-containing food emulsions can enhance, or more likely, decrease stability against coalescence. They are surface active, but cause repulsion of the droplets only at small distance (Bergenståhl, 1988). The addition of lecithin was found to enhance coalescence stability of emulsions at very low caseinate concentrations. Probably a stable mixed casein/lecithin surface layer was formed (Bergenståhl, 1988; Fang & Dalgleish, 1993b). When either caseinate or lecithin concentration was raised, stable emulsions were formed. However, addition of egg- or soya-lecithin (3 g/kg) to recombined milk fat globules, resulted in increased partial coalescence in a flow field (Melsen & Walstra, 1989), indicating that lecithin addition may increase susceptibility to destabilization in some conditions. The surfactant may displace the caseinate from the droplet interface, thereby lowering interfacial tension and diminish repulsion. Addition of SDS or Tween 20 to an emulsion containing either caseinate, whey or total milk protein caused desorption of the previously adsorbed protein and the oil-droplets coalesced (Oortwijn & Walstra, 1979; Mertens, 1989). When lecithin was added to whole milk before homogenization, the protein seemed not to be displaced by the lecithin (McCrae & Muir, 1991).

The properties of the surfactant such as the hydrophillic-lipophilic balance will partly determine behaviour of the surfactant when added to caseinate emulsions. The ratio of the solubilities of the surfactant in the oil and water phases will determine whether an oil-in-water or an water-in-oil emulsion will be formed, and also at what conditions phase-inversion takes place. The partition coefficient over both phases of the surfactant is about unity at the phase-inversion-temperature (PIT); at the PIT interfacial tension is very small, which strongly promotes coalescence of the emulsion droplets (Shinoda & Saito, 1969). Phase inversion temperatures in systems containing oil and water have been reported for monoglycerides (Wilton & Friberg, 1971) and fatty acid esters (Alander & Warnheim, 1989). As far as we know, such measurements have not been done for lecithin or fractions thereof. The sharp phase-inversion-temperature observed in systems of pure components will certainly not apply to surfactants like lecithins with a broad range of hydrophillic-lipophilic balance (Shinoda, 1989).

1.6 Phase behaviour

In this section we will shortly treat the types of phase behaviour expected for aqueous systems with either one polymer, two polymers and small particles. For the one polymer system, precipitation of the polymer will generally not occur if the solubility of the polymer is surpassed, but separation into two phases, one of a high and one of a low concentration of polymer. In this way, the polymer in the concentrated phase (that contains by far the greater proportion of polymer) does not lose its substantial conformational entropy (Lindman et al., 1993).

In mixed aqueous solutions of macromolecules (e.g. proteins and polysaccharides), liquid-liquid phase separation phenomena may be observed. The compatibility of macromolecules is usually small, except in dilute systems. The phase behaviour may be divided into two main categories, based on the distribution of the macromolecules between the separating phases. The associative type of phase separation (traditionally called complex coacervation), is characterized by a concentrated phase enriched in both macromolecules and a dilute phase. This type is primarily found for two oppositely charged macromolecules. The segregative type of phase separation (often called incompatibility) where the two macromolecules are enriched in separate phases is typical for most mixtures of macro-molecules (Lindman et al., 1993). Association and segregation are favoured by attractive and repulsive polymer-polymer interactions, respectively. However, polymer-solvent interactions are also important. In the association case, phase separation is always favoured under poor solvent conditions and may, under certain conditions, appear even without effective polymer-polymer attraction. Conversely, in the segregation case, differences in the polymer/solvent interactions may yield a segregation also in the absence of an effective polymer-polymer repulsion (Tolstoguzov, 1991; Picullel et al., 1994).

A dispersion of a high concentration of particles may show phase separation of the particles weakly attract each other (interaction energy of the order of -kT). One phase contains most of the particles at a very high concentration (volume fraction mostly > 0.5), whereas the other is depleted of particles. This may be comparable to the one polymer system described above. One cause of a weak interaction potential is depletion of a dissolved polymer near the particles surface; this case is comparable to the segregation type of phase separation. It is also possible that two kinds of particles show mutual attraction, which may lead to something like the attractive type of phase separation or coacervation. Phase separation of small particles does not occur if the attraction is strong: in such a case a precipitate or a gel is formed. Phase separation of small particles is more likely to occur if the particles are small, if they are fairly monodisperse, if they are anisometric, and when their volume fraction is high.

For caseinate-polysaccharide mixtures either associative or segregative interactions may occur, depending on type of polysaccharide, concentration, pH and ionic strength. Minimum concentrations for phase separation of casein-polysaccharide mixtures are about 2-4 weight percent. Segregation was found for mixtures of caseinate-dextran

(2 kDa) mixtures at pH 6.5 in 0.15 mol/l NaCl or mixtures of caseinate and sodium alginate or caseinate and gum arabic (Tolstoguzov et al., 1985). Sensitivity for incompatibility of caseinate-dextran mixtures increased with molecular weight of the dextran (M_w:10^5-10^6) and ionic strength (NaCl: 0.03-0.5 mol/l) (Tolstoguzov et al., 1985). The effect of pH and temperature in these caseinate-dextran mixtures appeared highly correlated with behaviour of caseinate in solution. Segregation of the caseinate-dextran (2 kDa) mixtures increased when pH was near the iso-electric point of caseinate and increased with increasing temperature (20-80°C) (Tolstoguzov et al., 1985).

Mixtures of caseinate and malto-dextrins have hardly been studied to date. Only recently, results of studies on properties of concentrated mixtures of caseinate (150 g/kg) and malto-dextrins ("dextrose equivalent" DE 2) were given. These concentrated systems showed a transition from a bicontinuous system at low malto-dextrin concentration to a continuous malto-dextrin phase and discontinuous caseinate phase, as measured at 85°C (Manoj et al., to be published). Mixtures of micellar casein (>1 w/w %) with potato malto-dextrins 16.5 % DE 2 at 20°C showed phase-droplets of casein. The size of the droplets increased slowly with time, which seemed to be due to aggregation of the micellar casein. Furthermore it was shown that the phase separation process was slowed down after a few hours, owing to gelation of the malto-dextrin (Abraham et al., to be published).

Although phase behaviour of casein-polysaccharide mixtures in quiescent conditions has been studied and reported in some detail, it is now becoming clear that the behaviour under high shear and during heating is of prime importance in controlling properties of such mixtures (Lindman et al., 1993; Harding et al., 1993; Donald et al., in press). Changes in the morphologies in those conditions and phase continuity issues have received little attention. Observed morphologies or microstructures are nearly always intermediate structures. To understand the microstructures, one needs to know the phase equilibria and the kinetics of phase separation. Various workers reported mainly on rheological measurements in rather concentrated systems. From such measurements it can be established whether the systems are bicontinuous and whether the macro-molecules are in the continuous or the dispersed phase (Lindman et al., 1993; Kasapis et al., 1993a,b; Abraham et al., to be published). Some new techniques like Fourier Transform Infrared (FTIR) spectroscopy and FTIR microscopy have been developed to describe kinetics of demixing and gelation quantitatively (Donald et al., in press).

1.7 Heat-induced changes in caseinate

During heat treatment of emulsions containing caseinate and sugar, aggregation of the caseinate in solution or adsorbed at the emulsion droplets, dephosphorylation of the caseinate and chemical cross-linking reactions may occur. These reactions may affect physical changes in the emulsions during heating.

1.7.1 Aggregation

Caseinate in solution is extremely stable to heat coagulation. Measurement of aggregation by turbidity (at room temperature) of sodium-caseinate solutions (10 g/kg) in distilled water (pH 7.0) showed relatively small changes during heating for 60 min between 120 and 130°C (Guo et al., 1989). Aggregation increased during heating between 130 and 140°C and turbidity was shown to increase sharply. Aggregation was also found in a solution of β-casein upon heating to 140°C. This type of aggregation in β-casein solution appeared to be reversible on cooling to 0-20°C (Guo et al., 1989). A κ-casein solution (2.5 g/kg), pH 7.2 was stable towards aggregation on heating (5 min 100°C). If however, sodium chloride (50 mmol/l) was added or the pH was decreased to 6.2, the stability of κ-casein was somewhat reduced (Zittle, 1969a,b).

1.7.2 Dephosphorylation

Heating of caseinate solutions at high temperatures (110-140°C) caused dephosphorylation of the caseinate (Belec and Jenness, 1962a,b; Guo et al., 1989). Dephosphorylation was measured as released phosphate during heating after precipitation of the caseinate with trichloro-acetic acid (TCA) (120 g/kg). About 25, 40 and 100 percent of phosphate groups was released after heating at 120°C for 0.5, 1 and 5 h, respectively, and appeared independent of pH in the range 6.0-7.0. Release of phosphate in calcium caseinate or micellar casein was slightly slower in the same conditions (Belec and Jenness, 1962a,b; Guo et al, 1989). The sensitivity towards aggregation (at 37°C) by calcium-ions was greatly reduced when phosphate-groups were enzymatically removed from the caseinate. The Ca^{2+} concentration leading to aggregation changed from about 4 to 6 to 8 mmol/l Ca^{2+} when dephosphorylation was 0, 40 and 93 percent of the sodium-caseinate in solution (2 g/kg), respectively. The aggregation of the caseinate induced by lowering of the pH in the direction of the isoelectric point seemed unchanged and remained at about pH 5.0-5.5 (Hekken & Strange, 1993).

1.7.3 Chemical cross-linking

Intra- or inter-molecular covalent bond formation between residues of caseinate, at temperatures ranging from 100°C to 140°C, can lead to irreversible changes of the caseinate (van Boekel et al., 1989; Singh, 1995). Estimation of protein polymerization reactions during processing has usually been done by measuring bands on urea or SDS-polyacrylamide gels or ion-exchange FPLC (Law et al., 1994; Guo et al., 1989; Zin El-Din & Aoki, 1993). Changes in pattern of caseins after heating have been examined by alkaline PAGE and ion-exchange FPLC at 5 min at 110°C (Law et al., 1994). SDS-PAGE of heat treated solutions of sodium-caseinate also showed remarkable changes with heating time between 0 and 60 minutes at 120°C. The intensity of all casein bands decreased, while intensity in the region of larger protein molecules increased, probably due to aggregates, and the region of smaller peptides also increased, probably due to degradation products of caseinate (Guo et al., 1989; Law et al., 1994). Zin El-Din & Aoki (1993) studied factors affecting polymerization of artificial casein micelles and sodium caseinate by high performance gel chromatography using a eluent containing 6 M urea, EDTA and 2-mercaptoethanol. The amount of polymerized casein formed at 140°C was relatively small in the initially unaggregated sodium-caseinate compared to artificial casein micelles. On addition of lactose to sodium caseinate, polymerization greatly increased during heating, probably because of Maillard type reactions (Zin El-Din & Aoki, 1993).

Quantification of polymerization using these methods remains a problem, because these methods do not distinguish between advanced Maillard reaction products and cross linked amino acids (lysinoalanine (LAL), histidinoalanine (HAL), lanthionine). The reactions studied in somewhat more detail in casein containing systems are: the Maillard reaction (between the ϵ-NH_2 group of proteins and reducing sugars), formation of reaction products of dehydroalanine (itself formed out of phosphoserine, cystein- or cystin-residues) and disulfide bridge formation. Formation of iso-peptides may be of limited importance because this takes place only during extreme heat treatments (e.g. 24 hours at 120°C; Singh, 1995).

Maillard reactions

The Maillard reaction involves a cascade of reactions starting with free amino groups and reducing sugars and leads to a very complex mixture of products. Generally, Maillard reactions are faster at higher temperature and higher pH. Considering the scope of this thesis and the overwhelming amount of literature available on this subject (Ledl, 1990; Berg, 1993; Rizzi, 1994), this review will be restricted to some general features possibly of importance for caseinate systems regarded in this study.

Major Maillard reactions pathways are shown in Figure 1.4 for aldehyde sugars. Similar reactions may occur for keto-sugars.

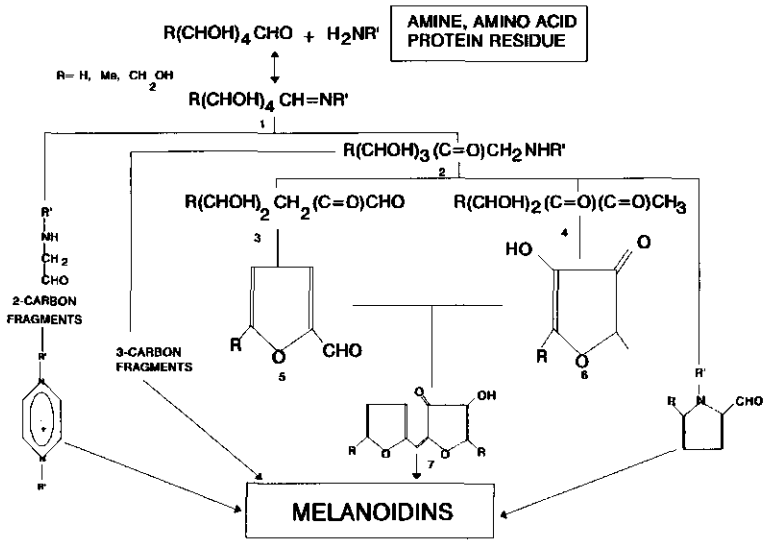

Figure 1.4 *Maillard reaction scheme, reproduced from Rizzi (1994) (Numbers are indicated in text)*

The initial condensation reaction between reducing sugars and amino acids and Amadori rearrangement of the Schiff base **(1)** lead to N-substituted 1-amino-1-deoxy-2-ketoses or Amadori compounds **(2)**. These rather stable Amadori compounds can react, via cyclization of 1-deoxyosones **(3)** further through 1,2-enolization (at low pH) to products like 5-hydroxymethyl-2-furaldehyde (HMF) **(5)**, which is recognized as a minor reaction product in milk (Berg & van Boekel, 1994). The Amadori compounds can also react through 2,3-enolization (at high pH) to flavour products like C-methyl-aldehydes, keto-aldehydes, dicarbonyls and reductones **(6)**. The third reaction is the Strecker degradation of amino acids by the conjugated dicarbonyl compounds during the above mentioned enolization reactions, which will be of less importance in caseinate systems. Chain fragmentation of compounds **(1)** and **(2)** to 2- and 3 carbon fragments will react further in which amino groups are again involved to form melanoidins. Polymerization of the many highly reactive compounds formed during these reactions is the final stage of Maillard reaction (Ledl, 1990; Rizzi, 1994). Knowledge about formation and structures of high molecular weight compounds such as melanoidins is limited. It appears that polymers formed have molecular weights above 1000 Daltons (Igaki et al., 1990).

It might reasonably be expected that Maillard reactions contribute to the coagulation of protein systems. To our knowledge, only few studies related Mailard reactions to possible changes in heat coagulation. None of them were with caseinate. Pyne (1958) found no differences in sensitivity towards heating on addition of either lactose or

sucrose (which is a non-reducing sugar) to lactose-free milk. Andrews (1975) found that the extent of polymerization of milk proteins because of Maillard reaction during UHT-treatment of milk was only 8%. Extended covalent binding leading to irreversible physical changes was found in a system with the globular protein BSA or soya-isolate with reducing sugars heated at 121°C (Armstrong et al., 1994). Cross-linking reactions of caseins may proceed at ambient temperature, after sugars have reacted with amino acid residues at high temperature, which will be of importance during shelf life of dairy based products (Andrews, 1975, de Koning & Kaper, 1985; de Koning et al., 1992).

Cross-linked amino acids

When caseinate solutions are heated at moderately high pH (6-8), reaction products of dehydroalanine such as lysinoalanine and histidinoalanine may be formed. In a number of milk protein containing products such as milk, concentrated milk and infant milks lysinoalanine contents were between 50 and 2200 (mg/kg protein) and histidinoalanine contents between 50 and 1800 (mg/kg protein) (Henle et al., 1993). The reaction starts with the formation of dehydroalanine (DHA) residues via β-elimination reaction from phosphoserine, glycosylserine, serine and cyst(e)ine residues (Figure 1.5). Due to addition of nucleophilic groups to the highly reactive DHA, the following cross-link amino acids may be produced : Lysinoalanine (from lysine), lanthionine (from cysteine), histidinoalanine (from histidine) and possibly ornithinoalanine (from ornithine) (Fujimoto et al., 1982; Henle et al., 1993).

Caseinates are likely to undergo these type of reactions during heating especially because of the relatively high phosphoserine content. Enzymatic dephosphorylation of caseinate before heating reduced possibility of LAL formation almost completely (Lorient, 1979; Kleyn & Klostermeyer, 1980). Furthermore, formation of lysinoalanine seemed very dependent on pH. Negligible amounts of LAL were formed out of α_{s1}-casein at pH 6.0 but increased at higher pH values (Lorient, 1979). Comparable results were found on heating of β-casein (Kleyn & Klostermeyer, 1980).

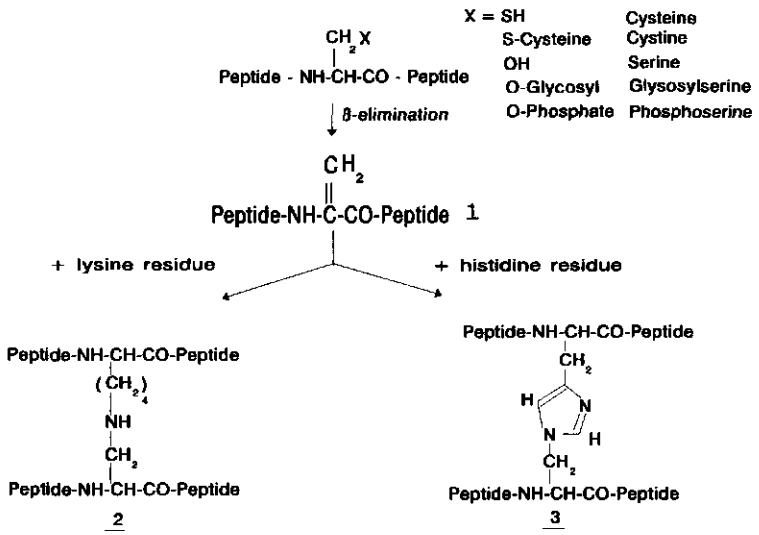

Figure 1.5 *Formation of protein bound dehydroalanine (DHA) (1) by β-elimination and of protein bound lysinoalanine (LAL) (2) and histidinoalanine (HAL) (3) by addition of nucleophilic groups to reactive DHA.*

Disulfide bonds

It is questionable whether interchain disulfide bond formation can play a role in caseinate systems, because of the low content of the amino acid cysteine in casein. Only α_{s2}- and κ-casein contain cysteine residues. Formation of disulfide bonds in relation to heat stability is, as far as we know, only studied in κ-casein/α_s containing systems in the presence of salts (sodium-chloride, 50 mmol/l) heated for 5 min at 100°C. κ-casein was able to stabilize α_s -casein against precipitation to calcium-ions, probably due to disulfide bond formation. When κ-casein was treated with reducing agents such as mercaptoethanol and dithiothreitol, κ-casein seemed not able to stabilize α_s-casein against precipitation to calcium-ions (Zittle, 1969b). Furthermore, a significant decrease in viscosity was found on addition of mercaptoethanol (0.3 g/kg) or other disulfide reducing agents to a caseinate solution (150 g/kg) at 60°C. No decrease was found when mercapto-ethanol was added to cooled caseinate solutions, indicating that aggregation of the caseinate at 60°C was partly due to disulfide bond formation (Towler et al., 1981).

Thus, heat treatments at 100-120°C under neutral or alkaline conditions may cause considerable polymerization of caseinate. The involvement of polymerization reactions in caseinate containing products may be an important factor in their heat coagulation. In the case of emulsions, it must be realized that only intermolecular reactions between caseinate molecules can lead to aggregation of the droplets (Walstra, 1993b).

1.8 Objectives of the study and outline of the thesis

The control of the stability of caseinate emulsions during heating is clearly multi-factorial. Most practical problems are solved by empirical, trial and error, methods. Further progress can be obtained in studying the contribution of the various heat-induced changes in the emulsions and relate these to sensitivity towards coagulation, coalescence or phase separation. The objectives of this study were to examine these heat-induced changes and to gain understanding of the mechanisms leading to instability and subsequent loss of homogeneity of the caseinate emulsions.

Heat treatment causes a number of physical and chemical changes in caseinate emulsions. These changes are summarized in Table 1.1. and investigated for caseinate stabilized emulsions, the results of which are described in chapter 3.

Table 1.1 *Changes in caseinate emulsions during heating*

Changes in caseinate
- dephosphorylation of caseinate
- changes in short-time mobility of caseinate chains
- physical association of individual caseinates
Adsorption of caseinate at the oil interface
Changes in ionization and partition of salts and pH
Chemical cross-linking of caseinate

A number of these changes may affect electrostatic and steric repulsion caused by adsorbed caseinate which are probably important for stability of the emulsions. Knowledge about the changes is required for interpreting the mechanisms involved in instability of the emulsions as described in subsequent chapters. Results on determination of sensitivity of caseinate stabilized emulsions and oil-free caseinate systems during heating are given in chapter 4. Four types of heat-induced instability may be distinguished: (i) salt-induced coagulation; (ii) coagulation due to covalent bond formation; (iii) heat-induced coalescence; (iv) phase separation. The heat-induced phase separation in caseinate emulsions may be very sensitive to small changes in ionic composition and type of sugars used. Therefore, the phase separation phenomena and consequences of this reaction were studied in somewhat more detail, as described in chapter 5. Effect of fractionation of malto-dextrin on heat-induced phase separation was determined. The consequence of phase separation for deposition of caseinate on emulsions droplets was determined by determining adsorption isotherms.

Stability was also determined as function of compositional factors such as oil concentration, type of sugar and legally permitted additives. Two categories of additives are usually incorporated into caseinate containing dietetic formulations:

stabilizing salts and surfactants (mostly lecithin). Each of these contribute to the stability of the caseinate emulsions during heating, but their mode of action may well be very different. Stabilizing citrates and phosphates were expected to influence salt equilibria and lecithins may affect heat coagulation by modifying the caseinate covered emulsion droplets. Lecithin may in some conditions also induce coalescence of the caseinate stabilized droplets (chapter 6). Therefore the use of different fractions of lecithin in caseinate emulsions was investigated through measurement of changes in particle size distribution during heat treatment and changes of the interfacial composition.

Chapter 2

Materials and Methods

2.1 Caseinate emulsions

The composition of the two model caseinate emulsions used in all experiments reported here is shown in Table 2.1. Model A was used in experiments on heat coagulation (chapter 4) and phase separation (chapter 5). Model B was used in experiments on coalescence (chapter 6).

Table 2.1 *Composition of caseinate emulsions*

	model A			model B		
oil	54 (g/kg)			37 (g/kg)		
protein	55 (g/kg)			38 (g/kg)		
carbohydrates	166 (g/kg)			114 (g/kg)		
Ca	520 (mg/kg)	14	(mmol/l)	75 (mg/kg)	2	(mmol/l)
Mg	160 (mg/kg)	7.4	(mmol/l)	190 (mg/kg)	8	(mmol/l)
Na	760 (mg/kg)	35	(mmol/l)	760 (mg/kg)	35	(mmol/l)
K	1250 (mg/kg)	35	(mmol/l)	1280 (mg/kg)	35	(mmol/l)
$P_{inorganic}$	0 (mg/kg)	0	(mmol/l)	110 (mg/kg)	4	(mmol/l)
citrate	870 (mg/kg)	5.0	(mmol/l)	1300 (mg/kg)	7.3	(mmol/l)
Cl	1760 (mg/kg)	54	(mmol/l)	1180 (mg/kg)	35	(mmol/l)
Total solids	280 (g/kg)			196 (g/kg)		
Density	1090 (kg/m^{-3})			1060 (kg/m^{-3})		

2.2 Preparation of emulsions

Model A

Emulsions were prepared from soya oil (54 g/kg) and a caseinate-carbohydrate solution. Equal amounts of sodium and calcium caseinate (mass ratio 1 to 1) (Campina Melkunie, Veghel, NL) were mixed with sugars and water to give a dispersion containing 55 g protein/kg and 166 g carbohydrate/kg. For the standard product, salts were added giving the following overall composition: tri-sodium citrate (4.0 mmol/l), tri-potassium citrate (1.0 mmol/l), magnesium chloride (7.4 mmol/l), potassium chloride (31 mmol/l). The pH was 6.9. Soybean-oil was coarsely dispersed in this mixture at a level of 54 g/kg using an Ultra Turrax. The emulsion was heated in a

plate heat exchanger (15 s, 75 °C) and subsequently homogenized in a Manton Gaulin two-stage laboratory homogenizer at 13 MPa in the first stage and 2 MPa in the second stage at 65°C. Finally the concentration of total solids was adjusted to 280 g/kg with tap water.

Model B

Equal amounts of sodium and potassium caseinate (Campina Melkunie, Veghel, NL) were mixed with water and sugars to give a dispersion containing 38 g protein/kg and 114 g carbohydrate/kg. For the standard product salts were added giving the following overall composition: tri-sodium citrate (2.6 mmol/l), tri-potassium citrate (4.7 mmol/l), magnesium chloride (8 mmol/l), calcium chloride (2 mmol/l), sodium chloride (12 mmol/l), di-potassiumhydrogenphosphate (4 mmol/l). The pH was 6.7. The dispersion was mixed with soybean-oil at a level of 37 g/kg, using an Ultra Turrax to coarsely disperse the mixture. Homogenization was done as described above. Finally the concentration of total solids was adjusted to 196 g/kg with tap water.

2.3 Variations in the procedure

2.3.1 Oil-free systems

Oil-free caseinate systems were prepared in exactly the same manner as described above except that the oil was omitted and the concentration of total solids was adjusted to 230 g/kg (model A).

Table 2.2 *Average composition of crude soya lecithin and derivatives (source Lucas Meyer, Hamburg, Germany)*

	fraction of component of total phospholipids (g/kg)			
	Topcithin (crude lecithin)	Epikuron 200	Epikuron 110	Emulfluid E
phosphatidylcholine	280	960	145	250
phosphatidylethanolamine	260	-	330	175
phosphatidylinositol	200	-	270	200
phosphatidic acid	220	-	165	75
lysolecithin	10	40	10	150
other	30	-	80	150
phospholipid (g/kg)	500	980	550	400

2.3.2 Addition of malto-dextrins

A number of malto-dextrin samples were used as supplied (Roquette Freres, Lestrem, Fr). These malto-dextrins had been produced from maize starch by partial hydrolysis and were characterized according to their "dextrose equivalent" (DE) , which expresses its reducing power, relative to glucose defined as 100. Thus a DE of 20 corresponds to an average degree of polymerization of 5.

2.3.2.1 Fractionation of malto-dextrin

In some experiments fractionated malto-dextrin was used. Fractionation was achieved by ultrafiltration (UF) of a malto-dextrin DE 12 solution (250 g/kg), through a spiral wound filtration system at 20°C (Koch, Germany) using 0.2 m^2 polysulfone membrane with a nominal molar mass cut-off of 2 kDa.

2.3.2.2 Gel Permeation Chromatography of malto-dextrins

Solutions of malto-dextrins were made up in tap water (70 g/kg).
In caseinate/malto-dextrin mixtures the caseinate was precipitated at pH 4.6, by addition of hydrochloric acid solution (1 mol/l) and successively filtered through S&S 595[1] filter paper and a 2 micron filter. Before injection of the filtrate the carbohydrate concentration was adjusted to about 70 g/kg.
A series of pullulans (M_W: 853, 380, 186, 100, 48, 23.7, 12.2, 5.8 kDa), of low polydispersity ($M_w/M_n < 1.14$) were used as standards to calibrate the GPC column (Polymer Laboratories, cat.no. SAC-8). Chromatography was carried out at 20°C on a series of acrylate columns: HEMA 40 (6000 x 7.5 mm, 10 micron) and HEMA BIO sec 1000 (6000 x 7.5 mm, 10 micron) (Alltech). The eluent was a buffer solution of 0.4 M acetic acid/sodium acetate (pH 3.0) (Schols et al., 1991). The flow rate was 25 ml/h. Detection was based on refractive index.

2.3.3 Addition of lecithin

In this study, four commercial soya lecithin preparations were used. The concentration of phospholipids in the fractions varied from 40-98 percent. The composition of the four fractions is given in Table 2.2. The lecithins were added to the soya oil and dissolved at 60-70°C.

2.3.4 Adjustment of pH and stabilizing salts

The pH was adjusted just before heat treatment by slowly adding amounts of hydrochloric acid solution (0.1 mmol/l) or sodium hydroxide solution (1 mol/l), while stirring vigorously.
Salts necessary to reach the desired calcium, citrate and phosphate levels were added to the caseinate-carbohydrate solution prior to homogenization. When citrates or phosphates were added, the amounts of corresponding chlorides in the recipe was proportionally decreased leaving the pH and ionic strength almost constant.

2.4 Simulated filtrate

To prepare a salt solution which simulates the serum phase of the caseinate stabilized emulsions, emulsions were filtered at 20°C, using a ceramic membrane module (Ceramen) with pore diameter of about 0.2 μm. Caseinate emulsions were prepared with varying citrate concentration (5; 10; 15 and 20 mmol/l) or varying phosphate concentration (5, 10, 15 and 20 mmol/l). The composition of the filtrates was analyzed. Calcium, magnesium, sodium and potassium in emulsions and filtrates were determined by atomic absorption spectrometry at 422.7; 285.2; 589.0 and 766.5 nm using an Unicam type 939 AA spectrometer. Phosphorus was determined by a colorimetric method at 660 nm with Fiske Subba Row Reagent as phosphomolybdate (AOAC method, 98624, 1990), after ashing at 550°C. Inorganic phosphorus content was obtained as phosphorus-level in the filtrate after precipitation of the protein on addition of trichloroacetic acid (TCA) in a final concentration of 12 percent and filtering through S&S 595' filter paper.

Table 2.3 *Formulation of simulated filtrates of caseinate emulsions containing 5-20 mmol/l citrate and 5-20 mmol/l phosphate.*

		citrate				phosphate			
		5	10	15	20	5	10	15	20
$Na_2HPO_4.2H_2O$	(g/kg)	0.36	0.36	0.36	0.36	-	-	-	-
$Na_2HPO_4.12H_2O$	(g/kg)					1.60	3.36	4.96	6.34
$MgCl_2.6H_2O$	(g/kg)	0.81	0.89	1.13	1.27	0.60	0.56	0.55	0.49
$CaCl_2.6H_2O$	(g/kg)	1.09	1.53	1.97	2.41	0.59	0.46	0.37	0.28
KCl	(g/kg)	2.31	1.85	0.16	0.23	2.51	2.44	2.49	2.79
NaCl	(g/kg)	0.88	-	-	-	1.81	1.20	0.71	-
Na_3citrate.$2H_2O$	(g/kg)	1.47	2.82	2.46	2.86	-	-	-	-
K_3citrate.H_2O	(g/kg)	-	-	2.51	3.01	-	-	-	-

Chloride was determined with a potentiometric titration method, using a Metrohm 686 Titroprocessor, equipped with a combined silver electrode (Metrohm, type 6.0401.100). Citrate was determined by enzymatic analysis, using the Boehringer Mannheim kit (cat.no. 139076).

The composition of the filtrates was simulated by dissolving combinations of the following salts in demineralized water: di-sodium hydrogen phosphate, magnesium chloride, calcium chloride, sodium chloride, potassium chloride, tri-sodium citrate and tri-potassium citrate (Table 2.3). The pH in the simulated filtrates was adjusted to 6.8 with sodium hydroxide solution (1 mol/l).

2.5 Heat treatments

2.5.1 Sterilization in glass bottles

Sterilizations were carried out in 200 ml glass bottles. The products were heat sterilized during 20 or 60 minutes at 120°C. The heating up temperature profile of the product in the bottles is shown in Fig 2.1.

2.5.2 Determination of heat stability

The Klaro-Graph used in this study is essentially a falling-ball viscometer (de Wit et al., 1986). The falling time of the glass ball in the 20 ml glass tubes (inner diameter 9.30 mm) is indicative for changes in viscosity during heating. The time required to induce visible coagulation at a fixed temperature of 120°C was chosen as a measure of heat stability. The reported heating times do not include the heating-up period, which was measured to be 4 minutes. The temperature profile of heat treatment in glass tubes in the Klaro-Graph is also shown in Fig. 2.1.

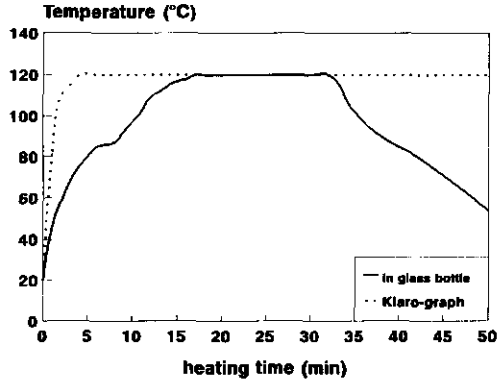

Figure 2.1 *Heating profiles of caseinate systems in glass tube (20 ml) of Klaro-Graph and glass bottle (200 ml) (20 min heating at 120°C).*

2.5.3 Determination of coalescence

The coalescence versus heating time profiles at 80-140°C were obtained by either heating in 20 ml glass tubes in the Klaro-Graph while rotating or in 3 ml stainless steel tubes in an oil-bath while rotating. No differences were observed between the results of these two methods.
Changes in droplet-size distributions of the caseinate emulsions were determined by a spectroturbidimetric method (Walstra, 1968) and expressed as d_{vs} or relative change during heating ($d_{vs,t}/d_{vs,0}$), where t is the heating time. Accelerated creaming tests were done according to the centrifuge method (30 min 200 g; 40°C) of Mol (1963) and expressed as relative change of fraction of oil creamed before and after heating (ϕ_t/ϕ_0). The oil content was determined by the Röse Gottlieb method (NEN 3197, 1961).

2.6 Measurement of pH, acid formation and Ca^{2+}-activity

The pH was measured using a Metrohm 686 Titroprocessor, equipped with a combined glass electrode (Metrohm model 60233100) at 20°C.
Acid formation was determined by titration of oil-free systems before and after heat treatment. For this determination 20 g sample was titrated with standardized sodium-hydroxide solution (0.1 mol/l) to pH 8.3. Titratable acidity was calculated from the difference between acidity before and after heat treatment and expressed as mmol/l.

Ca^{2+}- activity
Calcium ion activity was determined using an ion meter (Orion model 720 A) with a calcium selective electrode (Orion, model 93-20) and reference electrode (Orion, model 90-02) at 20°C. Calibration was performed with solutions of calcium-carbonate dissolved in the minimum amount of hydrochloric acid and potassium-chloride (80 mmol/l) (calcium concentration 0.5 and 5 mmol/l). Calcium ion activity was calculated by multiplying the concentration obtained from the ion meter with the activity coefficient of Ca^{2+} (0.42) as calculated by the Debye-Hűckel limiting law for this ionic strength (Chang, 1981). Contrary to milk, it was observed that the Ca^{2+}-activity remained constant after heat treatment of the caseinate systems.

2.7 Measurement of association of ions with caseinate

To determine the association of calcium, magnesium, sodium, potassium, inorganic phosphate, citrate and chloride with caseinate at 20, 40, 60 and 80°C, respectively, the caseinate emulsions were filtered using microfiltration (Pouliot et al., 1989). A ceramic membrane module (Ceramen) with pore diameter 0.2 μm was used. The maximum pressure difference was 0.5 bar. Maximum time for the process of heating

the emulsion to the filtering temperature and collection of the filtrate was about 45 minutes. The volume of filtrate never exceeded 5 percent of the total volume. Protein was reflected completely by the membrane. All ions passed the membrane completely when salt solutions were applied to the filter. The composition of the filtrates was analyzed as described before. Protein (N* 6.38) was analyzed according to the Kjeldahl method.

2.8 Determination of association of caseinate

Dissolved caseinate was defined as the fraction which did not sediment from caseinate dispersions during 2 h centrifugation at 200 000 g at 20°C. A Beckman XL-90 centrifuge with a Ti-75 rotor was used for this purpose. Caseinate compositions of the caseinate dispersions and supernatant after centrifugation were determined with reversed-phase HPLC by gradient elution on HiPore RP-318 column (Bio Rad) and detected with UV-adsorbance at 220 nm (Visser et al., 1991). Eluents were prepared by mixing 900 ml water, 100 ml acetonitril and 1 ml tri-fluoroacetic acid (TFA) (eluent **A**), or 100 ml water, 900 ml acetonitril and 1 ml TFA (eluent **B**). A linear gradient of 27 to 70 % of **B** in **A** in 60 min was used at a flow rate 0.8 ml/min. All solutions analyzed were 1:1 diluted with a solution containing Tris(hydroxymethyl)aminoethane (0.2 mol/l), 4 mol/l urea and 3 g/kg dithiothreitol. After 1 h these diluted solutions were again 1:1 diluted with eluent **A** containing urea (6 mol/l). 20 μl of this solution was injected. Peak area was taken for quantification purposes. Concentrations of individual caseinates were estimated using bovine serum albumin (BSA) as internal standard and applying 1.41. 1.67 and 1.91 as relative response factors for α_s-, β- and κ-casein, respectively (Visser et al., 1991). A typical chromatogram is shown in Fig. 2.2.

2.9 Determination of amounts of caseinate and phospholipids associated with emulsion droplets

The method of Oortwijn & Walstra (1979) was followed. Protein and phospholipid loads were calculated from protein and phospholipids depletion in the serum of the emulsions. Serum was separated from caseinate emulsions by centrifugation (44 000 g for 60 min) at 10-20°C using a Sorvall RC-5 centrifuge[1]. Average globule size and oil and caseinate contents were analyzed as described before. The phospholipid concentration was determined as phosphorus in the oil phase after addition of extra sodium chloride (10 g/kg) to the sample (Walstra & De Graaf, 1962) and extraction according to the Röse Gottlieb method. Phospholipid concentration was calculated taking into account the average molar mass of the phospholipids (760 g/mol).

[1] We thank the Technical University Delft, section Enzymology cordially for the use of the centrifuge and Mr. B. Groen for his assistance.

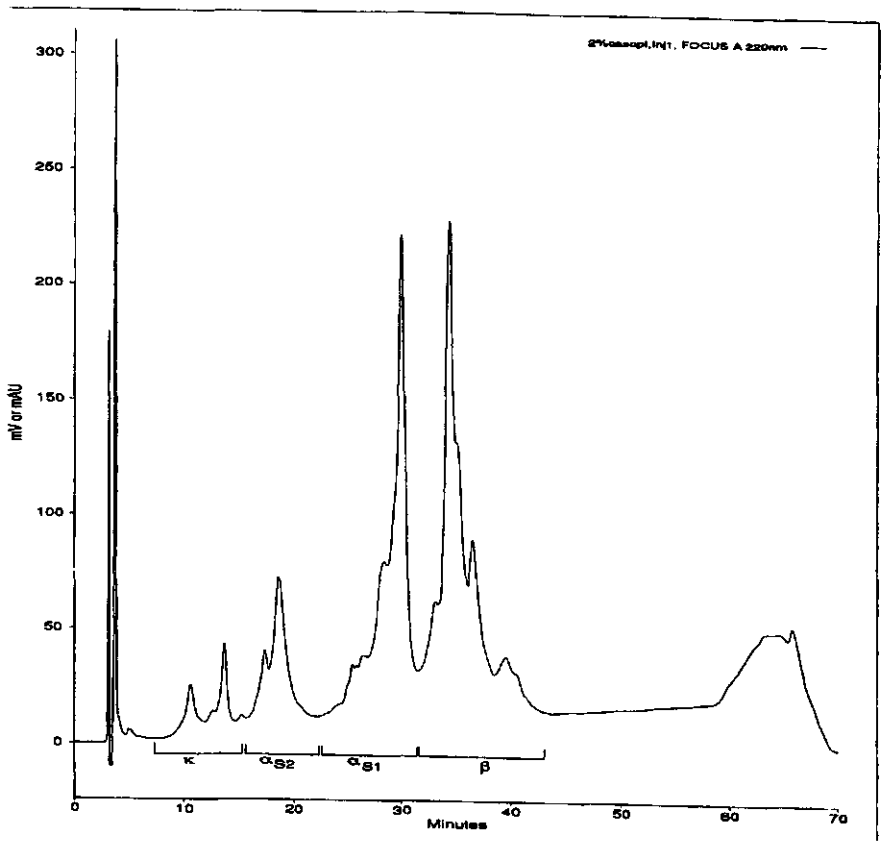

Figure 2.2 *Typical chromatograph of whole sodium caseinate (κ-casein, α_{s2}-casein, α_{s1}-casein, β-casein).*

2.10 Determination of individual caseins associated with emulsion droplets

The method for qualitative determination of the individual caseins was closely related to that of Sharma & Dalgleish (1993). Emulsion droplets and associated caseinate were separated from the emulsions by centrifugation at 44 000 g during 60 min at 10-20°C using a Sorvall RC-5 centrifuge. The cream layer was washed twice with a simulated filtrate to preserve the original composition of the caseinate interfacial layer (about 2 g cream layer in 35 ml simulated filtrate) and centrifuged during 30 min 6000 g at 20°C. Coalescence of droplets was avoided. Afterwards the cream layer was drained on S&S 595' filter paper. To liberate the individual caseins completely from the emulsion droplets, aliquots (85 mg) of the washed and drained cream layer were

mixed with 0.3 ml of pH 8.0 buffer (10 mmol/l Tris and 1 mmol/l EDTA) and 20 µl of a freshly prepared solution of bovine serum albumin (BSA)(3 g/kg) as internal standard. Caseinate was desorbed during 2 h at 45°C after addition of 500 µl of a 20% solution of sodium dodecyl sulfate (SDS). S-S bridges were reduced with 110 mg dithiothreitol by heating for 5 min in a boiling water bath (Nijhuis & Klostermeyer, 1975). The mixtures were centrifuged for 5 min to isolate the subnatant, containing all of the caseinates which were desorbed from the emulsion droplets. An aliquot of the subnatant was loaded onto a 20% homogeneous Phastgel (Pharmacia) and subjected to electrophoresis at 250 V for 195 Vh at 15°C. The gels were then silver stained (silver nitrate 0.4%) in the PhastSystem® and destained (10% ethanol and 5% acetic acid) according to SDS-PAGE development technique no. 210 (Pharmacia) with some slight modifications.

2.11 ^{1}H-NMR and ^{31}P-NMR measurements

^{1}H-NMR and ^{31}P-NMR spectra were obtained at 25, 90 and 120°C, using a Bruker AM 400 instrument. In all experiments 10 percent D_2O was added as an internal lock. Temperature was measured using tetramethylammonium chloride (TMA). The response of the probe at varying temperatures was calibrated with a solution of sodium salt of 3-(trimethylsilyl)-propionic 2,2,3,3,-d_4 acid (TSP) reference for ^{1}H-NMR spectra and a phosphate reference solution for ^{31}P-NMR spectra.
^{1}H-NMR spectra were recorded at 400.13 MHz , using a 90° observation pulse and a delay of 3.1 s between consecutive pulses to ensure complete relaxation. Residual solvent resonance was suppressed by low power preirradiation. ^{1}H-NMR spectra were referenced to external TSP.
^{31}P-NMR spectra were obtained at 161.98 MHz using a 90° observation pulse and an inter pulse delay of 5 s. Longitudinal relaxation times (T_1) of ^{31}P resonances were estimated to be 1 s for Ser-P and 2.2 s for inorganic P. Because of its rather long relaxation time, compared to the inter pulse delay, quantification in these conditions was not possible for inorganic P. Quantification was possible for Ser-P. Chemical shifts were not determined in ^{31}P-NMR spectra.

2.12 Determination of types of bonds formed during heating

2.12.1 Dissolution of coagulates

The solubilities of coagulated products were determined using the following test conditions: addition of sodium hydroxide solution (1 mol/l) to pH 8.0; addition of 10 volumes simulated filtrate; addition of 10 volumes demineralized water; addition of urea to final concentration of 6 mol/l; addition of 1 volume of a solution containing 0.5 percent Tween 20 and EDTA (0.04 mol/l); addition of 1 volume of a sodium

dodecylsulfate (SDS) solution (200 g/kg); addition of dithiothreitol (DTT) to a final concentration of 110 g/kg and subsequently heating for 5 min at 100°C and finally addition of 1 volume of a solution of SDS, DTT and urea in concentrations given above. The degree of dissolution was examined by microscope (Olympus BH-2 polarising microscope, with a 40 x objective, total magnification x400).

2.12.2 SDS-PAGE

Chemical cross-linking was estimated by measuring the decrease in monomeric caseinate on SDS-PAGE. Sodium-dodecylsulfate (SDS) (200 g/kg) and dithiothreitol DTT (110 g/kg) were added to the samples, to disrupt all non-covalent and disulfide bonds. Gel-electrophoresis was performed as described.

2.12.3 Determination of lysinoalanine

The methods of Fritsch and Klostermeyer (1981) and Henle et al. (1991) were followed with some minor modifications. After acid hydrolysis during 22 h at 110°C in a hydrochloric acid solution (6 mol/l), the amino acids were separated by means of an amino-acid analyzer using Spherisorb S5SCX (Phase Separations) as a cation exchange resin and detected fluorometrically (λ_{ex} = 340 nm and λ_{em} = 455 nm), using an o-phthalicaldehyde as a post-column reagent. The detection limit was 50 mg/kg protein and the accuracy was 10 mg/kg protein.

Chapter 3

Physico-chemical changes

3.1 Introduction

During emulsification and heat treatment of caseinate emulsions physical and chemical changes may occur in the caseinate and the environment of the caseinate. It must be realized that the changes occurring in the present type of caseinate solutions and emulsions have hardly been studied so far. Therefore, changes similar for stability in related systems such as milk and concentrated milk were studied. This implied that we had to study many aspects. Complex formation may occur between individual caseinates and also with salts at high temperatures. Changes are probably in some way related to the mobility of caseinate chains during heating and to deposition of individual caseins or aggregates on emulsion droplets (Lieske & Konrad, 1994; Mulvihill & Murphy, 1991). Of particular interest were changes of caseinate systems on pH and addition of stabilizing salts (citrates and phosphates). The possible importance of these variables for the stability of caseinate emulsions and solutions during heating will be discussed in subsequent chapters. Ideally, we should have measured changes in caseinate and environment at the temperature of heat treatment. However, this was only possible with the NMR-technique, and to some extent, for salt partitioning by use of a ceramic membrane. The other changes were measured after cooling to room temperature.

3.2 Dephosphorylation of caseinate

Dephosphorylation of caseinate is probably an important change during heating as it affects charge interactions (e.g., calcium sensitivity of caseinate) and association of caseinate and metal ions (Hekken & Strange, 1993). Phosphate can be cleaved from phosphoserine groups of the caseinate at temperatures above 100°C. The rate of dephosphorylation can be examined by determination of the trichloro-acetic (TCA) (120 g/kg) soluble phosphorus or quantitative measurement of organic phosphorus with ^{31}P-NMR.

Formation of TCA-soluble phosphorus from organic phosphate groups of caseinate emulsions during heating for 60 min at 120°C was somewhat dependent on addition of citrate. Dephosphorylation was between 26 and 34 percent at citrate concentrations of 5 and 20 mmol/l, respectively. In this emulsion, malto-dextrin DE 20 was used and the pH decreased from pH 6.9 to pH 6.0. For a system without sugar about 36 % had become soluble in TCA (120 g/kg) after 60 min at 120°C, while the pH was rather constant (Table 3.1). From P-NMR measurement at 25°C intensity of Ser-P resonance had decreased by about 40 % in caseinate

emulsions with citrate (5 and 15 mmol/l) after heating for 40 minutes at 120°C. Thus, the rate of dephosphorylation in ^{31}P-NMR was found somewhat higher for unknown reasons.

Table 3.1 *Formation of TCA soluble phosphate during heat treatment at 120 °C in a standard caseinate emulsion and in a system without oil and sugar (calcium 14 mmol/l; citrate 20 mmol/l; initial pH 6.9). Initial organic phosphorus 15 mmol/kg.*

	caseinate emulsion containing malto-dextrin			**caseinate solution**		
heating time (min)	**TCA soluble phosphorus (mmol/kg)**	**dephos-phorylation (%)**	**pH**	**TCA soluble phosphorus (mmol/kg)**	**dephos-phorylation (%)**	**pH**
0	1.6	0	6.9	1.6	0	6.9
20	3.9	15	6.5	4.7	21	6.8
60	6.8	34	6.0	7.0	36	6.7

These observations on dephosphorylation of different caseinate systems comply with findings of other workers on heat induced changes in sodium caseinate solutions (Belec & Jenness, 1962a,b; Guo et al., 1989). They found cleavage of approximately 40 percent of phosphoserine groups at 120°C after 60 min in sodium caseinate solutions (20-30 g/kg). Dephosphorylation in whole caseinate systems was not found to depend on pH but slightly on calcium concentration. Two possible mechanisms of dephosphorylation are: **1)** hydrolysis expected at acid or neutral conditions, or **2)** β-elimination, expected at alkaline conditions. This indicates that phosphate was probably released via both mechanisms from the caseinate in our systems.

3.3 Changes in pH and salt partitions

Adjustment of pH or addition of stabilizing salts like citrate and phosphate may be used to improve heat stability of caseinate solutions and emulsions. The effect of pH and stabilizing salts on ion equilibria and partitioning of salts can be described, taking into account underlying physico-chemical principles. Also heat-induced changes of pH and Ca^{2+}-activity and partitioning of salts are probably important for stability of caseinate emulsions. We have studied both increase in titratable acidity in caseinate systems and resulting pH-decline upon addition of reducing and non-reducing sugars to caseinate emulsions.

pH and Ca^{2+}activity.

Changes in Ca^{2+}activity on addition of stabilizing salts and due to heat treatment were determined using an ion selective electrode. The effect of citrate and phosphate addition on Ca^{2+}activity of caseinate emulsions is shown in Figure 3.1. Citrate strongly affected Ca^{2+}activity. At 5 mmol/l citrate a Ca^{2+}activity of about 1.5 was found at pH 6.9. Raising citrate to 20 mmol/l gave a Ca^{2+}activity of about 0.4, while the Ca^{2+}activity was hardly dependent on pH. A similar trend was found upon addition of phosphate, but the Ca^{2+}activity was clearly more pH dependent.

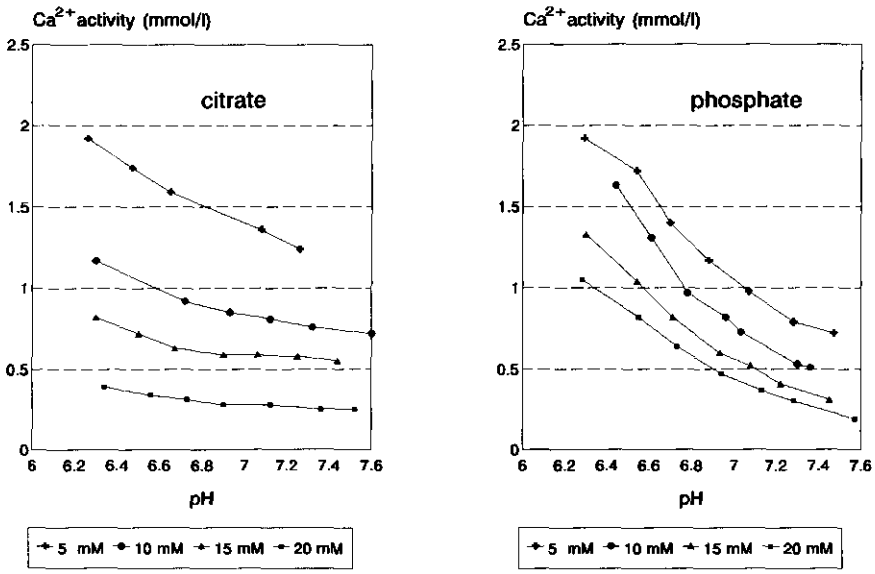

Figure 3.1 *Ca^{2+}-activity as a function of pH of unheated caseinate emulsions (50 g oil/kg) with sucrose/lactose ratio (1.5) and various amounts added of citrate (left) or phosphate (right) (5-20 mmol/l) at 20°C.*

Notable changes in Ca^{2+}-activity in oil-free caseinate systems were observed on heating during 60 minutes at 120°C (Figure 3.2). The pH decreased on heating and at the same time Ca^{2+}-activity increased. This can probably be explained by the high affinity of protons for caseinate compared to Ca^{2+} as counterion (Swaisgood, 1992), resulting in higher Ca^{2+}activity of the dispersion. But also from dephosphorylation of caseinate, which may also result in an increase in Ca^{2+}-activity. Effects of citrate and phosphate on Ca^{2+}activity were comparable in these systems. Also at higher citrate and phosphate concentration (10-20 mmol/l), Ca^{2+}activity increased significantly on heating, but less pronounced.

In all our systems, Ca^{2+}activity increased on heating, implying that formation of

calcium phosphate was of lesser importance as compared to native casein micellar systems in milk products. In milk and concentrated milk a fast decrease in Ca^{2+}activity was observed during the first minutes of heat treatment because of formation of calcium phosphate (van Boekel et al., 1989; Nieuwenhuijse et al., 1988).

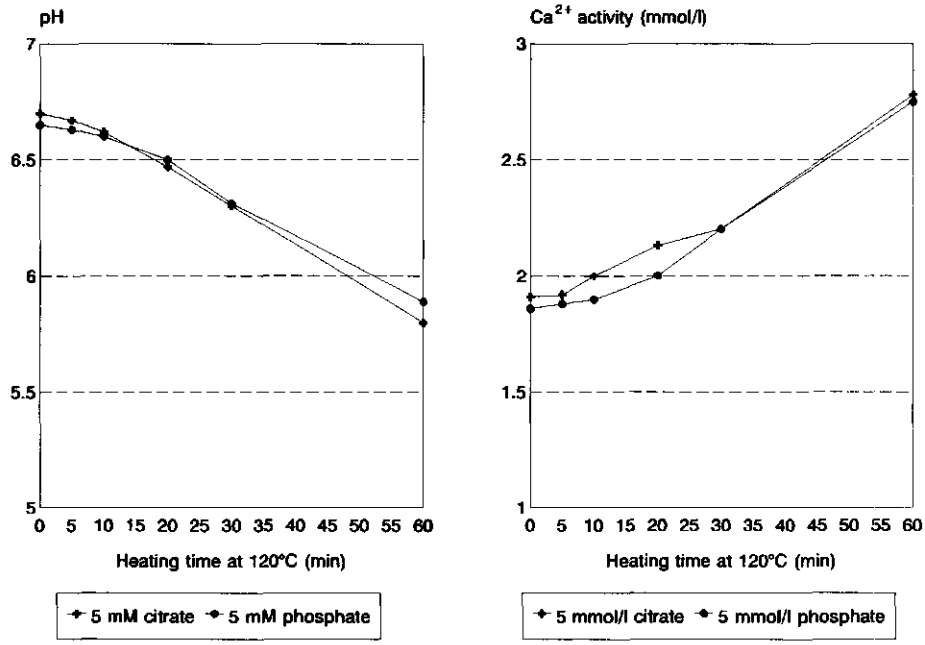

Figure 3.2 *(a) pH and (b) Ca^{2+}activity after heating at 120°C of oil-free caseinate systems with sucrose/lactose ratio (1.5) with added citrate (5 mmol/l) or phosphate (5 mmol/l) measured at 20°C.*

Acid formation

Acid formation and pH decrease during heating at 120°C, were determined in oil-free caseinate systems, containing citrate (5 mmol/l), pH 6.9, without sugar or with glucose or sucrose/lactose mixture (3:2) or malto-dextrin DE 19. Large differences were seen between products containing different type of sugars (Figure 3.3). The major contribution to acidity development in these caseinate emulsions came from thermal breakdown of sugars and concomitant formation of organic acids. The pH decline and acid formation in the sugar-free system was very low. Only 3.6 $mmol.l^{-1}$ were released during heating at 60 min 120°C. In comparison, acidity development was 50 $mmol.l^{-1}$ on addition of glucose, 18.6 $mmol.l^{-1}$ on addition of the sucrose/lactose mixture (3:2) and 13.5 $mmol.l^{-1}$ on addition of malto-dextrin DE 19.

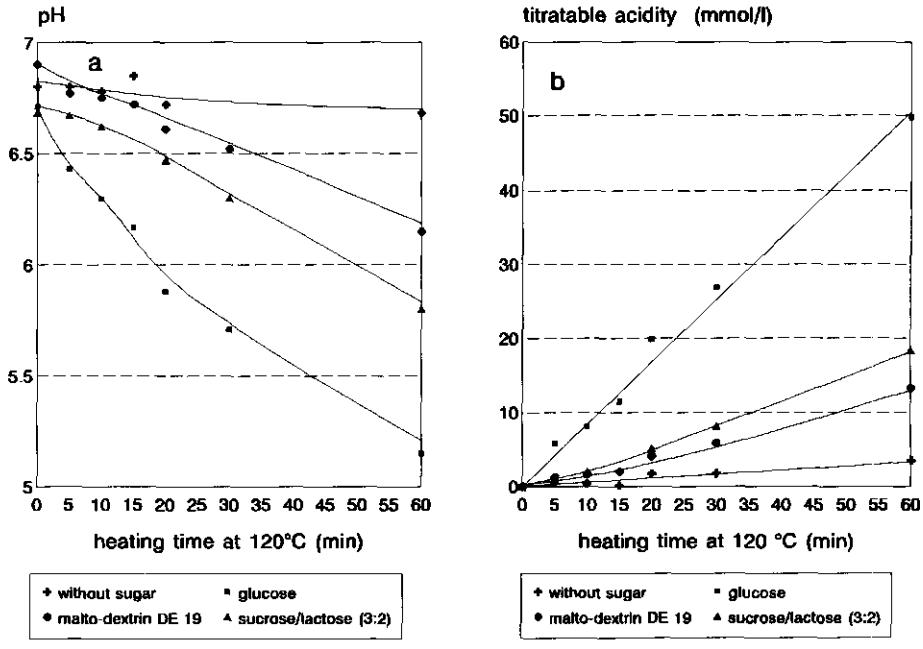

Figure 3.3 *a) pH (measured at 20°C) and b) titratable acidity (mmol/l) of oil-free caseinate systems containing different sugars as a function of heating time at 120°C.*

Acid formation during heating at 120°C seemed independent of initial pH in the range 6.2-7.2 in caseinate emulsions containing sucrose/lactose (3:2) and citrate (5 mmol/l) as stabilizing salt (the same will be valid for the oil-free systems). However, on addition of phosphate (5 mmol/l) as stabilizing salt to caseinate emulsion containing sucrose/lactose (3:2) at pH 6.1 and pH 7.4: titratable acidity was 3.6 and 17.4 mmol l^{-1} respectively at 60 min 120°C. The reaction involved was possibly the formation of a type of calcium phosphate with simultaneous release of H^+, which is known to proceed faster at high pH. The rate of pH-decrease was influenced by the buffering of the caseinate system (Figure 3.4). The change in buffering capacity at about pH 6.5 may be explained by the change in dissociation of phosphoserine (pK_a = 1.5 and 6.5) and histidine (pK_a = 6.4). However, salts, especially phosphates, will also affect buffering capacity (Walstra & Jenness, 1984; Lucey et al., 1993).

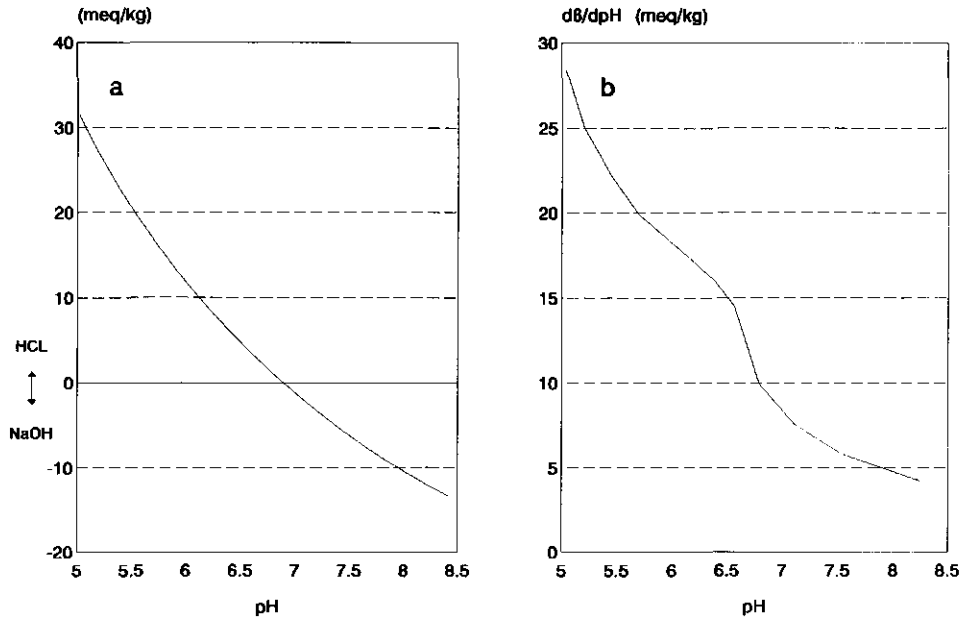

Figure 3.4 *a) Titration curve of caseinate emulsion (50 g oil/kg), sucrose/lactose (3:2), (5 mmol/l citrate) from initial pH 6.9 to 5.0 with HCl and from 6.9 to 8.4 with NaOH in mEq/kg and* ***b)*** *buffering capacity* dß/d*pH in mEq/kg.*

Association of salts with caseinate

Partitioning experiments were carried out to get a better understanding of changes in salt equilibria and colloidal or non-ultrafilterable salts. The effect of stabilizing salts (citrates and phosphates), pH and heating were studied. Experiments, with use of ceramic membrane (0.2 μm) were done at 20; 40; 60 and 80°C. We tried to determine salt partitioning at temperature of 120°C, but these experiments failed due to strong fouling of the membrane. Salt partition was also determined after prolonged heating during retort sterilization (20 and 60 min 120°C) followed by filtration at 20°C.

In a first experiment the effect of citrate was determined on the partitioning of salts in a caseinate emulsion containing various citrate concentrations (5, 10, 15 and 20 mmol/l) (Appendix 1). On increasing citrate, association of calcium and magnesium with caseinate was reduced and association of sodium and potassium was increased. Association of citrate, chloride and residual inorganic phosphate was negligible. About 20 to 70 percent of the calcium migrated through the microfiltration membrane (Figure 3.5).

The remainder was associated with the caseinate, probably as counterion. Residues of aspartic acid, glutamic acid and phosphoserine contribute to a negative charge of about -1.47 mEq. per g casein at pH 7.0. Lysine, arginine and histidine give a positive charge of about +0.81 mEq. per g casein, giving a total net charge of -0.66 mEq/ g casein (Walstra & Jenness, 1984). In this calculation any effect of mutual electrostatic interactions between amino acid residues on the caseinate, which will affect dissociation constants (Tanford, 1961), was not accounted for. The sum of the counterions remained almost constant and was: +0.64; +0.66; +0.62 and +0.60 (mEq/g protein) at citrate concentrations of 5, 10, 15 and 20 mmol/l, respectively. This amount of associated metal ions corresponds reasonably well with the net charge of caseinate at this pH (6.8-6.9). We found that when pH was changed in the region 6.5-7.5 filterable calcium was hardly affected. The amount of calcium in the filtrate was between 42 and 35 percent at 20°C in the caseinate emulsion containing 5 mol/l citrate.

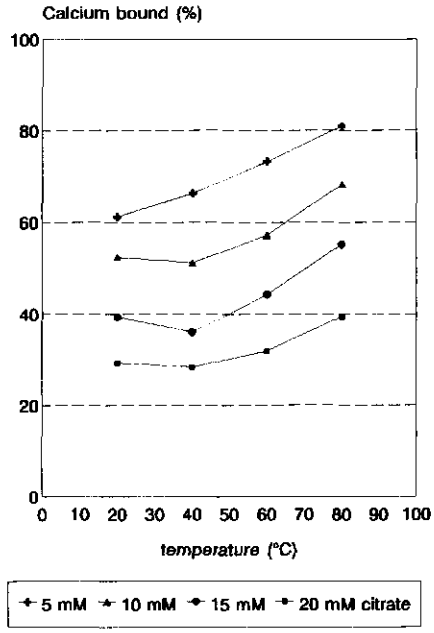

Figure 3.5 *Dependence on temperature and citrate concentration (5; 10; 15 and 20 mmol/l) of the partition of calcium in caseinate emulsions (50 g oil/kg). Calcium not passing the membrane expressed as % of total calcium (14 mmol/l).*

The effect of temperature on association of salts was less pronounced compared to effects of citrate addition. However, on heating up to 80°C, association of calcium and caseinate increased significantly, whereas association of magnesium, sodium and potassium was more or less constant. This increase is in agreement with the association constant of calcium and phosphate, which increases with increasing temperature (Holt et al., 1981). Heating of caseinate emulsions for 20 or 60 min at 120°C caused less significant changes in associated calcium, measured at 20°C. The diffusible calcium varied only from 61 to 60 and 57 percent of total calcium at 0, 20 and 60 min 120°C, respectively, in the emulsion containing 5 mmol/l citrate.

Association of calcium during heating seemed to be influenced both by dephosphorylation and by formation of small amounts of colloidal calcium phosphate. Dephosphorylation of the caseinate will cause a decrease in amount of counterions to caseinate. After cleavage of about 15 and 34 percent of the phosphate groups during 20 and 60 min heating, respectively, the net charge was calculated to be about -0.50 mEq/g protein at pH 6.6 and -0.33 mEq/g protein at pH 6.1, respectively. The counter-ions accounted for +0.59 mEq/g protein after 60 min heating at 120°C, consequently little colloidal calcium phosphate was probably formed. It was found that 75-80 percent of inorganic phosphate was diffusible.

Calculation of the ion equilibria from the composition of the filtrates, using a computer programme of Holt et al. (1981), allowed us to estimate Ca^{2+}, Mg^{2+}, HPO_4^{2-} and citrate^{3-}-activity and ionic strength (Appendix 2). Calcium ion-activity estimated from the ionic composition obtained by analysis of filtrate was in all products much lower than the activity measured with a calcium selective electrode. No good explanation could be found for this phenomenon. Perhaps it is due to the fact that the programme was developed for serum of milk. Although the amounts of salts in our filtrates were different from milk serum, types of salts were comparable. The inaccuracy in the determination of calcium, citrate and phosphate may have large effect, because of the high association constants. Furthermore, in our analysis carbonate was not accounted for, but this ion will only have a minor effect on the calculation of the Ca^{2+}-activity, because of the low association constant at neutral pH. From the figures, activity products of calcium and citrate or phosphate and also of magnesium and citrate or phosphate could be estimated. The activity product of calcium and citrate $(a_{Ca}2+)^3 * (a_{cit}3-)^2$ was between $3.5*10^{-19}$ and $1.4*10^{-18}$ mol^5 kg^{-5}, which is at most about 90 percent of saturation ($2.3*10^{-18}$ mol^5 kg^{-5}) of tricalcium citrate. This appears in agreement with the measurements, where citrate was found almost completely in the filtrate. The activity product of calcium and phosphate $(a_{Ca}2+)* (a_{HPO4}2-)$ was between $2.5*10^{-8}$ and $3.4*10^{-7}$ mol^2 kg^{-2}. The saturation product of dicalciumphosphate is $2.6*10^{-7}$ mol^2 kg^{-2}. Hence, a colloidal calcium phosphate may particularly be formed in conditions of 5 and 10 mmol/l citrate during intense heat treatment (60 min 120°C). This seems to fit with the results from the determination of filterable calcium and phosphate in these

conditions.

It appeared that salt partitioning in phosphate containing emulsions was completely different from citrate containing emulsions (Figure 3.6; Appendix 3). The most important difference was association of calcium and phosphate and caseinate on addition of inorganic phosphate. The fraction of calcium associated with caseinate was over 80 % in all conditions studied, as compared to 30-80 % in citrate containing emulsions. Association of phosphate, relative to the total amount was more or less constant, although the absolute amounts raised with increasing phosphate concentration. Association of magnesium and caseinate followed the same trend as calcium while association of sodium and potassium and caseinate seemed constant.

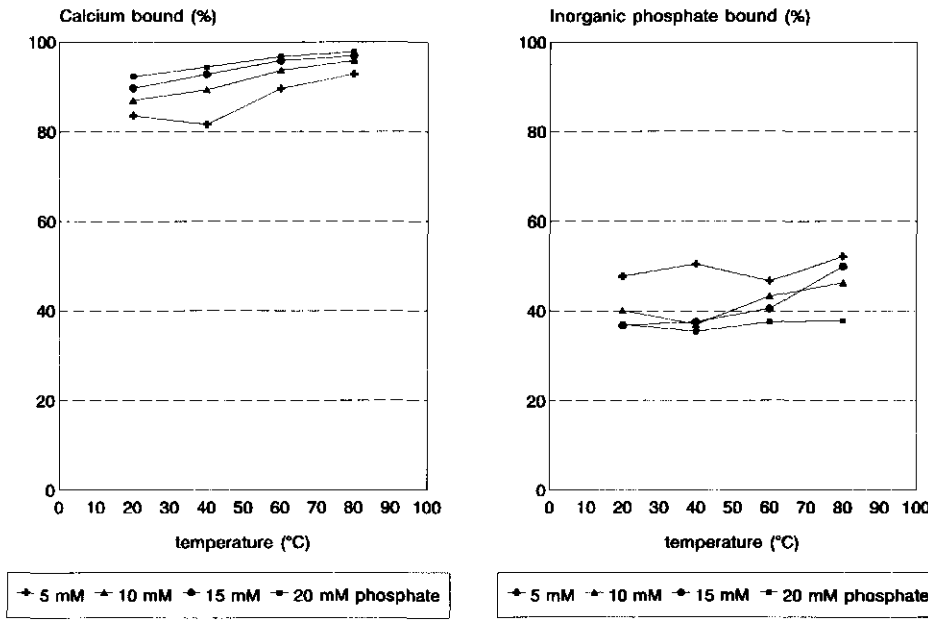

Figure 3.6 *Dependence on temperature and inorganic phosphate content (5; 10; 15 and 20 mmol/l added) of the partition of* **(a)** *calcium and* **(b)** *inorganic phosphate in caseinate emulsions (50 g oil/kg). Calcium and inorganic phosphate association expressed as % of total calcium (14 mmol/l) and inorganic phosphate (5; 10; 15 and 20 mmol/l) respectively.*

This resulted in a relatively high sum of associated metal ions of +0.75; 0.80; 0.79 and 0.85 (mEq/g protein) at phosphate concentrations of 5, 10, 15 and 20 mmol/l, respectively, all at 20°C and pH 6.7-6.9. These results indicate that the salts not only acted as counterions to caseinate, as in citrate containing samples, but some part became associated with caseinate. Probably a type of calcium or magnesium phosphate was formed at high phosphate concentrations. This was in agreement with calculation of the activity products (Appendix 4). The product of calcium and phosphate ($3.0*10^{-7}$ to $6.0*10^{-7}$ mol^2 kg^{-2}) was in excess of the solubility of dicalcium phosphate ($2.6*10^{-7}$ mol^2 kg^{-2}). Activity products of magnesium and phosphate were also calculated and these were lower than the solubility of magnesium and phosphate ($1.5*10^{-6}$ mol^2 kg^{-2})(Walstra & Jenness, 1984).

On raising the temperature from 20 to 80°C, filterable calcium and phosphate slightly decreased, indicating additional formation of calcium phosphate. This decrease was too small to make predictions on the stoichiometry of calcium phosphate formed. Elucidation of the type of complexes formed is even more complicated considering that phosphoserine residues could act both as binding site of Ca^{2+} and Mg^{2+} and are part of micellar calcium phosphate (Rollema, 1992).

During prolonged heating of phosphate containing caseinate emulsions, association of calcium and phosphate appeared to have altered somewhat. The fraction of calcium associated was hardly affected at all phosphate concentrations. About 84, 83 and 81 percent of calcium was associated after heating for 0, 20 and 60 min at 120°C in the emulsion containing 5 mmol/l phosphate. The fraction of inorganic phosphate, however, decreased from 48 to 42 and 36 percent, respectively during heating.

A number of changes were thought to affect partitioning in the inorganic phosphate containing emulsions. Heating for 60 min at 120°C solubilized about 30% of the organic phosphate groups. This may explain the decreased amounts of colloidal phosphate, because colloidal calcium-phosphate was probably in some way linked to the organic phosphate groups (Holt, 1995). This would not explain the unchanged binding of calcium. However Ca^{2+} may also act as counter-ion to caseinate in preference to monovalent cations. It was observed that associated sodium, potassium and magnesium were about 10 percent lowered during heat treatment (60 min at 120°C). The net charge after dephosphorylation of the caseinate was calculated to be -0.40 mEq/g protein at pH of about 6.3, which was about 35 percent lower than the initial net charge. Thus, both association of calcium phosphate and ratio of counterions to caseinate appeared to have changed during heating.

3.4 ^{1}H-NMR and ^{31}P-NMR measurements of caseinate systems

Using high resolution NMR spectroscopy, changes in structures of proteins on a molecular level can be studied at high protein concentrations and over a broad temperature range). Proton NMR spectroscopy was used for studying changes in micellar casein structure at temperature of 0-140°C (Rollema & Brinkhuis, 1989). From these results it was concluded that appreciable parts of the κ-casein but also the other caseins became flexible on heating, which would have implications for the stability of the casein micelle on heating (Walstra, 1990). Therefore, it seems interesting to study changes in segmental motion of the caseinate chains using both ^{1}H-NMR and ^{31}P-NMR. ^{31}P-NMR is used because serine phosphate groups possibly play a major role in the association of caseinate (Baumy et al., 1989; Rollema, 1992; Wahlgren et al, 1993).

At present caseinate was studied in oil-free systems containing malto-dextrin (166 g/kg), pH 6.8 in 10 % D_2O containing citrate in concentration of 5-15 mmol/l or phosphate 5-20 mmol/l. ^{1}H-NMR spectra with a relatively small linewidth were observed superposed on a broadened spectrum. This points to regions in the caseinate with substantial flexibility (Figure 3.7a). Resonances with small linewidth were seen both in the aliphatic part of the spectrum (5-0 ppm) and the aromatic part (9-6 ppm). In this region of the spectrum also resonances from amide protons are found. Dissociation of the caseinate aggregates by addition of urea-d_4 (6 mol/l) and EDTA (25 mmol/l) resulted in a much higher relative intensity of resonances at 0.9, 1.2 and 1.4 ppm and also in the aromatic region (Figure 3.7b). Presence of narrow peaks in the spectrum indicate that parts of the caseinate chains showed more rapid motions than those of caseinate aggregates as a whole. These results show that considerable parts of caseinate became more flexible on disruption of hydrophobic bonds by urea or by chelating bivalent ions using EDTA.

Proton-NMR spectra of caseinate malto-dextrin systems at different temperatures are shown in Figure 3.8. The intensity of the total spectrum increased with increasing temperature, indicating a high degree of flexibility of at least part of the caseinate at 90 and 120°C.

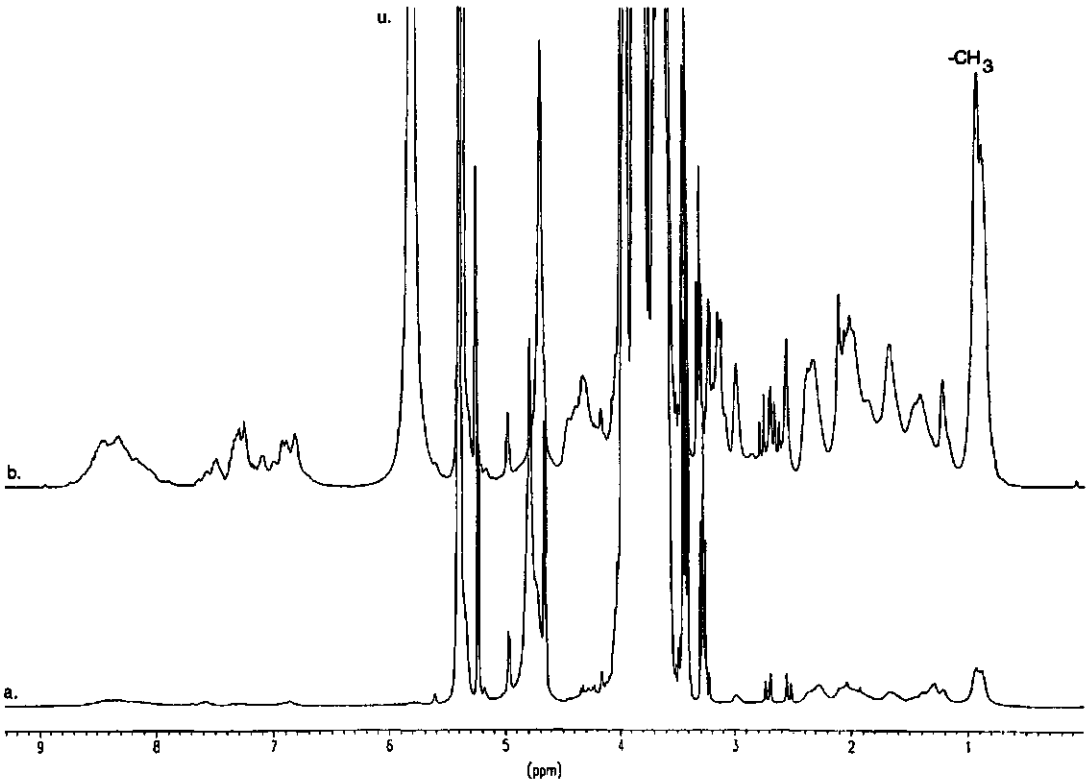

Figure 3.7 *(a) ^{1}H-NMR spectrum of an oil-free caseinate/malto-dextrin system in 10 % D_2O with citrate (5 mmol/l); pH 6.8; 25°C. (b) spectrum after addition of urea (6 mol/l) and EDTA 25 mmol/l). Protein concentration 55 g/kg. Chemical shift relative to external TSP (3-(trimethylsilyl)-propionic acid). Resonances at 3-6 ppm are from malto-dextrin; at 2.4 ppm and 2.6 ppm from citrate; resonance at 5.9 ppm (u denoted) originates from urea.*

Table 3.2 *Intensity of methyl resonance between 0.7 and 1.1 ppm in a proton-NMR spectrum of caseinate/malto-dextrin system (pH 6.8). Intensities are relative to that on addition of urea (6 mol/l) and EDTA (25 mmol/l).*

	Intensity -CH_3 resonance (%)		
	25°C	**90°C**	**120°C**
5 mmol/l citrate	12	36	68
10 mmol/l citrate	16	49	79
15 mmol/l citrate	18	58	88
25 mmol/l EDTA	18	61	95

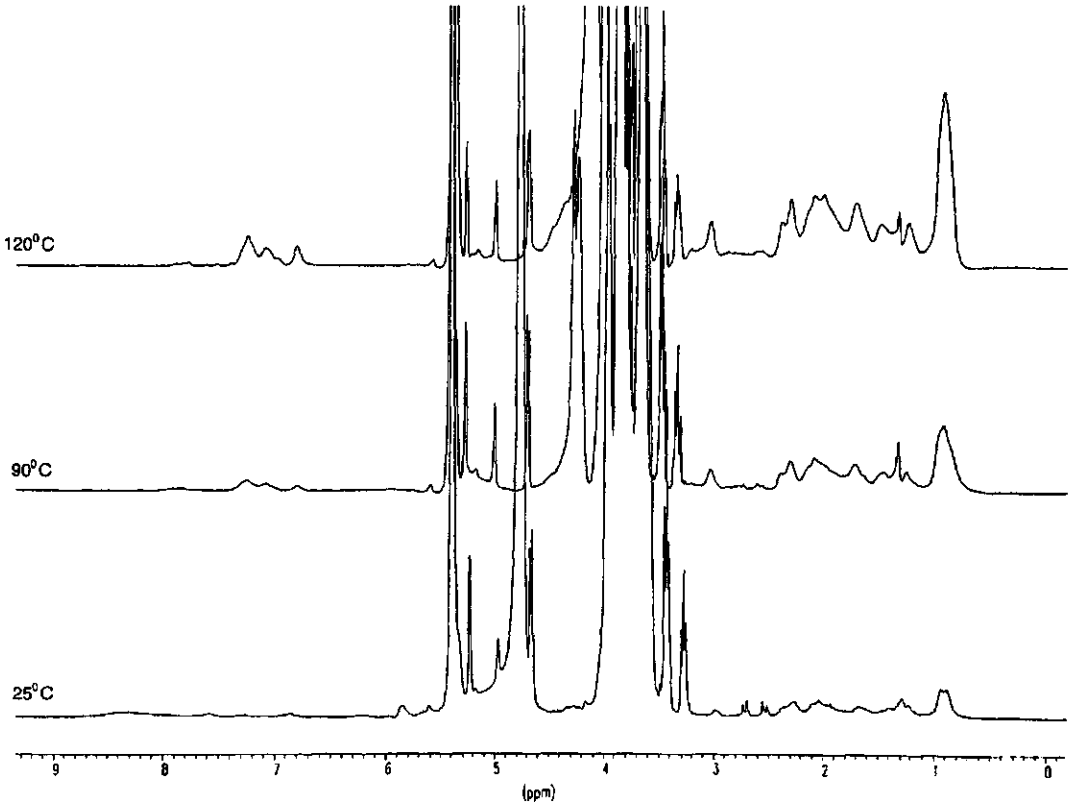

Figure 3.8 *^{1}H-NMR spectra of an oil-free caseinate/malto-dextrin system in 10% D_2O with citrate (5 mmol/l); pH 6.8 at 25, 90 and 120°C.*

In Table 3.2 intensity of resonance of -CH_3 protons from valine, leucine and iso-leucine residues between 0.7 and 1.1 ppm (Rollema et al, 1988) are given at 25, 90 and 120°C for samples with citrate (5, 10 and 15 mmol/l) as stabilizing salt. Intensity was calculated relative to intensity on addition of urea (6 mol/l) and EDTA (25 mmol/l) at 25°C. The changes on heating became pronounced at 90 and 120°C. At higher citrate concentration intensities were somewhat higher. After cooling to 25°C the spectra were the same as for the unheated samples, even after heating for 20-40 minutes at 120°C, showing reversibility with respect to mobility of the system. The relative intensity of peaks of CH_3 resonance of the sample with citrate (15 mmol/l) were comparable to the sample with EDTA (25 mmol/l), where all divalent ions were chelated.

The effect of inorganic phosphate addition on intensity of methyl resonance of the caseinate/malto-dextrin system was also measured at various temperatures.

Intensities of methyl peaks in phosphate samples at 25°C, 90°C and 120°C were comparable to peaks in citrate samples (Table 3.3). The results show clearly that adding of either citrate or phosphate caused no great difference with respect to mobility of the polypeptide chains on heating. After cooling from 120°C to 25°C relative intensity was about 0.12, independent of phosphate concentration and time after cooling (2-10 hours), indicating that changes on heating were completely reversible on heating.

Table 3.3 *Intensity of methyl resonance between 0.7 and 1.1 ppm in proton-NMR spectrum of caseinate/malto-dextrin system (pH 6.8). Intensities are relative to those on addition of urea (6 mol/l) and EDTA (25 mmol/l).*

	Intensity -CH_3 resonance (%)		
phosphate	**25°C**	**90°C**	**120°C**
5 mmol/l	10	24	74
10 mmol/l	11	30	84
15 mmol/l	12	34	92
20 mmol/l	13	43	100

By using ^{31}P NMR, the mobility of phosphorus in phosphoserine residues was also measured as a function of temperature for various concentrations of citrate and inorganic phosphate. In the ^{31}P-NMR spectra (Figure 3.9) a broad range of resonances of organic phosphorus and a narrow peak of anorganic phosphorus was seen. The Ser-P peaks have been assigned to the different phosphoserine clusters, and the chemical shift is modified by ionic conditions such as calcium (Baumy et al, 1989). Since we were not interested in changes of chemical shift on heating, all spectra are given relative to the resonance of inorganic phosphate. Only organic phosphorus could be measured quantitatively, because relaxation time (T_1) was by a factor 5 lower than inter-pulse delay. Intensity of the Ser-P peak was calculated relative to peak intensity after dissociation by urea (6 mol/l) and EDTA (25 mmol/l). The intensities of SerP-resonance at 25°C in samples with citrate (5 and 15 mmol/l) was about 90% of intensities after complete dissociation (Table 3.4). Raising the temperature to 90°C gave a decrease in intensity, hence less mobility, of the SerP group. After cooling the sample containing 5 mmol/l citrate from 90°C to 25°C, relative intensity was 0.80, indicating that the system responded more or less reversible. After heating for 40 min at 120°C and subsequent cooling to 25°C relative intensity was 0.51, because of release of phosphorus from caseinate.

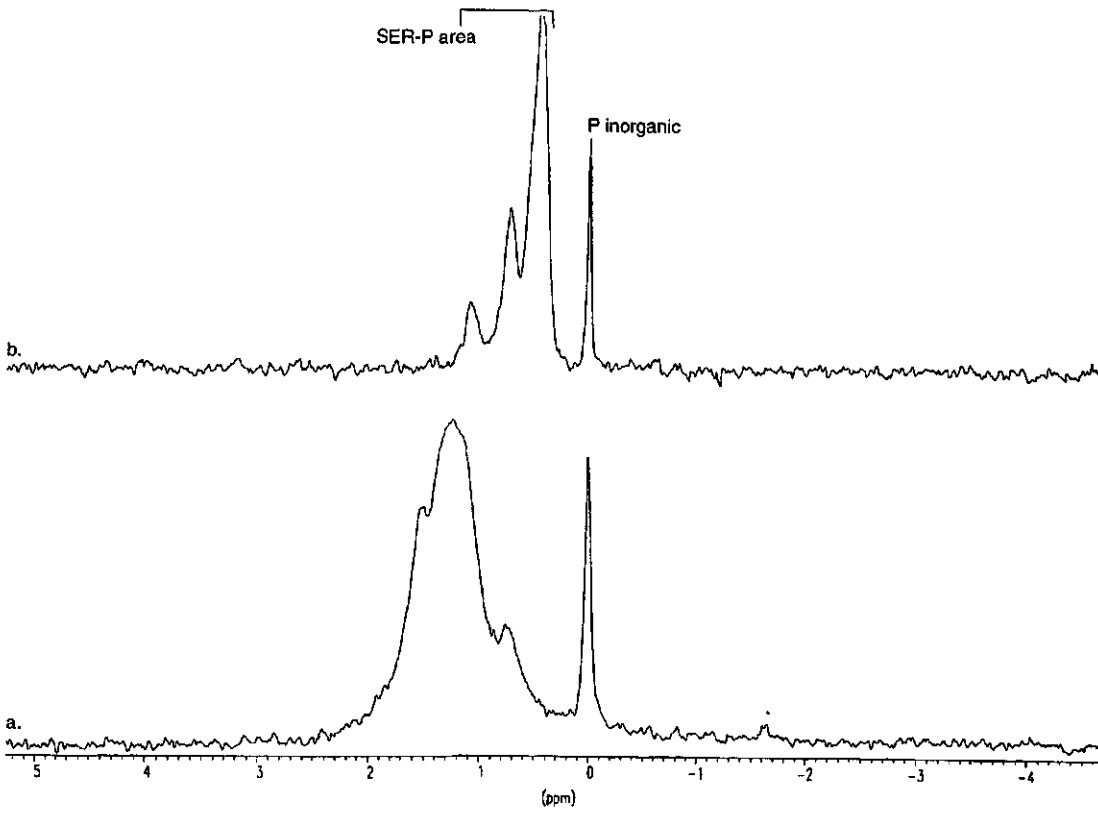

Figure 3.9 *(a) ^{31}P-NMR spectrum of an oil-free caseinate/malto-dextrin system in 10 % D_2O with citrate (5 mmol/l); pH 6.8. (b) spectrum after addition of urea (6 mol/l) and EDTA 25 mmol/l). Protein concentration 55 g/kg.*

Table 3.4 *Intensity of serine-P resonance in ^{31}P-NMR spectrum of caseinate malto-dextrin system. Intensities are relative to that on addition of urea (6 mol/l) and EDTA (25 mmol/l).*

	Intensity SerP resonance (%)	
citrate	**25°C**	**90°C**
5 mmol/l	86	63
15 mmol/l	93	75

On addition of phosphate, figures of relative intensity of Ser-P resonance in the spectra were also high, as in citrate stabilized caseinate systems at 25°C (Table 3.5). Heating to 90°C and 120°C gave a decrease in relative intensity of Ser-P resonance at 5 and 10 mmol/l phosphate but not significantly at 15 and 20 mmol/l phosphate. From this could be concluded that mobility of Ser-P group in phosphate samples (5 and 10 mmol/l) was lowered on heating, while they seemed not changed in 15 and 20 mmol/l phosphate samples. After cooling to 25°C and waiting for 10 hours, relative intensities were 0.75 and 0.78 for 5 and 20 mmol/l phosphate samples, respectively, indicating that phosphate-stabilized systems responded not as reversible on heating as citrate-stabilized caseinate systems.

Table 3.5 *Intensity of serine-P resonance in ^{31}P-NMR spectrum of an oil-free caseinate malto-dextrin system. Intensities are relative to that on addition of urea (6 mol/l) and EDTA (25 mmol/l).*

	Intensity Ser-P resonance (%)		
phosphate	**25°C**	**90°C**	**120°C**
5 mmol/l	88	52	55
10 mmol/l	84	67	67
15 mmol/l	85	84	83
20 mmol/l	85	88	87

The results of the NMR study show that the structure of the caseinate aggregates was strongly dependent on addition of salts and temperature. From the proton NMR spectra it may be concluded that in all caseinate solutions, with either citrate or phosphate, the mobility of a large fraction of the polypeptide chains increased with increasing temperature. The proton NMR spectra of caseinate solutions at high temperature (90 and 120°C) were comparable to the spectra of micellar casein systems (Rollema & Brinkhuis, 1989). The change in flexibility of casein molecules on heating seems therefore comparable in these two systems.
However, the presence of a Ser-P peak in the ^{31}P-NMR spectra of caseinate solutions showed clearly that the structure of caseinate was greatly different from casein micelles. In micellar systems no peak of organic phosphorus could be observed at all temperatures, from which was concluded that the SerP group was completely anchored, probably by colloidal calcium phosphate (Rollema, unpublished results). The mobility of the Ser-P group in caseinate appeared high at room temperature but decreased on heating to 90 and 120°C. These results indicate

that association of the caseinate via serine phosphate residues was low in the caseinate systems. The decrease on heating may be explained by anchoring of Ser-P groups to colloidal calcium phosphate, which seems possible, especially in conditions of added inorganic phosphate and relatively high Ca^{2+}-activity. Finally, the heat-induced changes as measured by proton and phosphorus NMR appear to be more or less reversible with temperature in caseinate systems with either citrate or phosphate.

3.5 Association of caseinate

Dissolved caseinate was defined as caseinate that does not sediment from oil-free caseinate systems, containing 5 mmol/l citrate during centrifugation at 200000 g for two hours. The non-sedimented caseinate from the systems with low (2 mmol/l), medium (14 mmol/l) and high (24 mmol/l) calcium concentration showed on analysis with HPLC peaks that could be identified with the major caseinate fractions. The supernatant casein composition from the oil-free systems with different calcium concentration, was similar to that for whole sodium caseinate (Table 3.6). Only at high calcium concentration (24 mmol/l) results were different. The amount of calcium sensitive α_{s1}-casein was decreased in the supernatant and therefore the amounts of α_{s2}- and κ-casein in the supernatant were raised and β-casein remained unaffected. However, it was expected from the ester phosphate content that incorporation of individual caseins into aggregates on addition of calcium would be in the order $\alpha_{s2} > \alpha_{s1} > \beta > \kappa$-casein (Aoki, 1989). Our slightly different results could also partly be explained by the relative high inaccuracy of the HPLC-method, especially for α_{s2}-casein (standard deviation was 34 %).

The fractions of caseinate in the sediment after centrifugation of oil-free caseinate systems were in agreement with findings of Mulvihill & Murphy (1991).

Table 3.6 *The mass proportions of various caseinate fractions (determined in duplicate by HPLC) in supernatant of oil-free caseinate systems and fraction sedimented during centrifugation.*

Sample	sediment (g/g)	κ	α_{s2}	α_{s1}	β
whole caseinate	< 0.05	12	8	32	48
caseinate system					
2 mmol/l calcium	< 0.05	11	7	32	50
14 mmol/l calcium	0.15	14	4	31	51
24 mmol/l calcium	0.30	22	15	17	46

They found also low levels of sediment (0.04) in caseinate systems at low calcium concentration and high level of sediment (0.37) at high calcium concentration, after centrifugation at somewhat different conditions (1h; 78000 g).

3.6 Conclusions

The above description of caseinate systems makes it clear that physical and chemical changes on heating can partly be explained, based on knowledge from studies of individual caseins in solution or micellar casein systems. However, some large differences were observed, probably due to the overall composition of the caseinate systems used in this study. Caseinate presumably is present in aggregates, of which structure and composition would depend on ionic conditions. The highest amount of caseinate sedimented (2 h centrifugation at 200000 g) from the dispersions with the highest calcium level, indicating that this caseinate was the most aggregated. The proportions of the individual caseinates in the supernatant did not seem to be very different from those in whole caseinate, if the calcium concentration was varied in the range of the caseinate systems we studied (maximum 14 mmol/l).

Heat treatment of caseinate systems causes a number of physical and chemical changes. The effects of temperature in the range 20-120°C and its heating time at 120°C were studied. When the temperature was raised from 20°C to 120°C, some changes in association of caseinate with salts and changes in motion of the caseinate segments, as measured with ^{1}H-NMR and ^{31}P-NMR were observed. Changes in caseinate on heating up to 120°C were greatly affected by the mineral composition. Chelation of Ca^{2+} and Mg^{2+}-ions through addition of citrate markedly reduced binding of these ions to caseinate and also ion activities were reduced. It was concluded that the metal ions, including calcium, only acted as counter ions to the caseinate. Because no inorganic phosphate appeared to be associated with caseinate, it is reasonable to assume that hardly any colloidal calcium phosphate was present in these citrate containing caseinate emulsions. These results were in agreement with ^{1}H-NMR and ^{31}P-NMR measurements. A relatively large fraction of the caseinate, including the serine-phosphate groups appeared flexible in the temperature region studied. Behaviour on addition of inorganic phosphates to the caseinate emulsions was somewhat different. Association of calcium and phosphate with caseinate increased with phosphate addition, demonstrating that calcium not only acted as counter ion of the caseinate, but that also a type of colloidal calcium phosphate was formed. This is in agreement with calculations of the activity products. It was found that the intrinsic solubility product of calcium phosphate was exceeded in all unheated and heated samples. Furthermore, mobility of the organic phosphate was lowered on heating, as observed in ^{31}P-NMR measurements. Nevertheless, mobility remained relatively high compared to micellar casein. These results confirm that the organic phosphate became somewhat immobilized on

heating. Such an immobilization of serine-phosphate groups and formation of colloidal calcium phosphate, may have implications for the structure and the stability during heating of caseinate in solution and caseinate adsorbed on emulsion droplets.

The changes in caseinate systems occurring between 20-120°C, such as pH, Ca^{2+}-activity and mobility of polypeptide chains, were reversible on cooling and seemed to take little time. Yamauchi et al. (1969) concluded the same, when they found reversible exchange of radiolabelled calcium in calcium caseinate dispersions. However, heating of caseinate systems to 120°C during longer time seemed to cause more permanent changes. During prolonged heating the negative charge of the caseinate was considerably reduced by dephosphorylation (e.g. 35% of the organic phosphorus was released at 120°C for 1h) which would probably be an important factor in the destabilization of the caseinate. Moreover pH changed in the direction of the isoelectric point of the caseinate because of acid formation from sugars. Taking into account both effects the net charge of caseinate would be changed from - 0.70 to - 0.31 mmol/g protein (after 1 h 120°C). Hence, one may expect that the stability of caseinate against aggregation decreases on heating because of reduced electrostatic repulsion between the caseinate residues. However, dephosphorylation during prolonged heating will cause calcium ions to be less effective in reducing the charge of the caseinate, hence in association and precipitation (Li-Chan & Nakai, 1989). Therefore, Ca^{2+}-activity and rate of pH decrease may in combination determine whether the caseinate will associate and eventually precipitate during heating.

It may be suggested that the strong association of calcium with phosphoserine groups on the caseinate play a major role in precipitation or coagulation of caseinate in solution or caseinate associated with emulsion droplets. Binding of calcium to caseinate effectively reduces the negative charge, which diminishes the electrostatic repulsion between the caseinate chains. Hydrophobic interactions may then lead to increased association and ultimately to precipitation of the caseinate (Rollema, 1992; Swaisgood, 1992). It was shown that, when the number of associated calcium ions was roughly equivalent to the number of phosphoserine residues in α_{s1}-casein and β-casein, precipitation of the individual caseins was initiated (Dalgleish et al., 1981). For κ-casein, calcium-induced association and precipitation was not observed because of the low phosphoserine content (Rollema, 1992).

Acknowledgements

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Chapter 4

Stability of Caseinate Emulsions during Heating

4.1 Introduction

Formulated foods for dietetic usage including caseinates, sugars, oil and salts are often heat sterilized. During processing and especially heat treatment of caseinate systems, they may coagulate, gel and phase separate (Cruijsen et al., 1994). A number of parameters may influence heat stability of caseinate stabilized emulsions. These include compositional factors such as type of sugars, pH and additives like stabilizing salts and lecithins. In this chapter, results of coagulation experiments during heating at 120°C are described and discussed. The aim of these experiments is to obtain qualitative information about possible mechanisms of coagulation.

4.2 Effect of pH

Heat stability of dairy based products is mostly studied as a function of initial pH. The heat coagulation time (HCT) pH profiles obtained from treatment at 120°C of caseinate systems with sucrose/lactose (3:2) and oil (0-250 g/kg) are presented here (Figure 4.1; results of 200 and 250 g oil/kg are not shown because of instability during heating up to 120°C). The HCT-pH profile of caseinate/sugar solution showed a progressive rise in HCT as pH increases over the range 6.3 to 7.3. At pH > 7.3 particles suddenly appeared in initial stage of heating. Emulsification of oil (50 - 250 g/kg), had a large effect on the HCT-pH plot. HCT decreased at all pH-values, when 100 g oil/kg or more was added; presumably, this is due to an increase of the effective volume fraction of caseinate particles. The effective volume fraction of caseinate systems was calculated from additional viscosity measurements and use of the Eilers equation, and was found to range from $\varphi_0 = 0.47$ in oil-free caseinate solutions to $\varphi_0 = 0.65$ when oil (250 g/kg) was emulsified (Appendix 5).

For preheated concentrated milk at initial pH of about pH 6.4, stability during heat treatment was strongly dependent on volume fraction in the range $\varphi_0 = 0.29$ to 0.46. The kinetics of coagulation of casein particles in those conditions where salt-induced coagulation is important was quantitatively explained by fractal aggregation theory (Nieuwenhuijse et al., 1992).

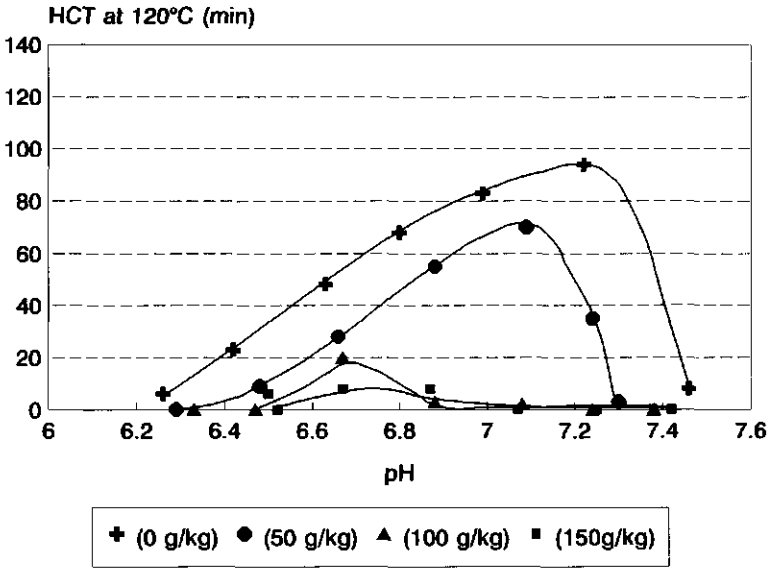

Figure 4.1 *HCT-pH plots of caseinate systems without and with emulsified oil (0-150 g/kg) (sucrose/lactose 3:2 and citrate 5 mmol/l).*

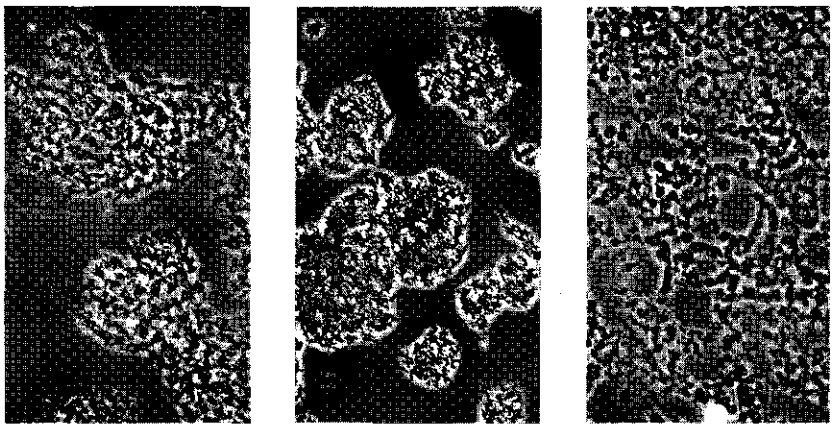

Figure 4.2 *Micrographs (40x) of heat-treated oil-free caseinate systems at point of coagulation, from initial pH 6.3 (left); 6.9 (middle) and 7.5 (right) (sucrose/lactose (3:2); citrate 5 mmol/l).*

On examination of light micrographs of coagulated material of caseinate solutions (Figure 4.2), various types of morphology were observed at different pH. At pH 6.3 to 6.9 (HCT 6 and 68 min respectively) a typically particle-like coagulation was seen, comparable to coagulation of not preheated concentrated milk at low pH (Nieuwenhuijse et al., 1991). At pH 7.5 (HCT = 8 min) droplet like structures were observed with a clear phase boundary ranging in size from about 1-5 μm. Larger droplets were often not spherical, indicating a low interfacial tension. They were thus thought to be phase droplets. The coagulated emulsions showed micrographs comparable to the oil-free solution, except that the emulsion droplets were enclosed in the coagulum and that the coagulum was more gel-like.

The coagula formed from the heated caseinate/sugar solution of initial pH 6.3-7.3 could not completely be dissolved in simulated filtrate (1:10), demineralized water (1:10), NaOH (1N) to pH 8.0, EDTA/Tween (0.04 mol/kg and 5 g/kg), dithiothreitol (DTT) (110 g/kg), urea (6 mol/kg), sodium-dodecylsulfate (SDS) (100 g/kg) or a mixture of DTT, SDS and urea. This indicates that covalent bonds are formed during heating. The coagulum formed at initial pH of 7.5 (HCT = 8 min), could immediately be dissolved in all dissolving agents, and in simulated filtrate the coagulum dissolved in a few (2-3) hours. After 12 hours, the coagulum had disappeared and the heated oil-free caseinate solution was clear, which points to a reversible type of coagulation or phase separation. The results of dissolution experiments of the emulsions gave some additional information. When only 50 g/kg oil was emulsified, results of dissolution experiments were comparable with results for the oil-free caseinate solution. When more oil (100-150 g/kg) was added (and the HCT was low), coagula formed at all initial pH-values could partly be dissolved in demineralized water and completely in EDTA/Tween (0.04 mol/l and 5 g/kg), but not in simulated filtrate. It appears that in the emulsions with low initial pH or at high effective volume fraction, where reactions become fast, a type of calcium or salt induced coagulum is formed on heating. At intermediate pH, the reaction leading to coagulation seems slow and eventually chemical cross-links are formed. At high pH 7.5, phase separation seems to occur.

4.3 Effect of stabilizing salts

4.3.1 Heat coagulation and solubility of coagula

Effect of phosphate

The ionic composition and especially the presence of Ca^{2+}-ions will probably influence heat stability. Therefore phosphate was added to the emulsions (50 g oil/kg). Phosphate (5; 10; 15 and 20 mmol/l) was added to caseinate emulsions in such a way that the ionic strength was hardly affected, and the pH was adjusted. When potassium phosphate was added, the amount of potassium chloride in the recipe was proportionally decreased, leaving the ionic strength almost constant. Phosphate addition to the caseinate emulsions caused a significant increase in HCT over the

entire pH range (Figure 4.3). There was no change in the shape of the HCT-pH profiles. The rather constant HCT in the pH range 6.7-7.5 may be explained by the pH changes during heating. The first pH-decline at initial pH 7.0 and 7.5 was relatively fast, probably because of the low buffering capacity above pH 6.5 (Figure 3.4), and afterwards pH decrease was slow. At initial pH 6.3 the coagulum was particle like, at all other pH values more gel-like. No phase separation phenomena were observed in the coagulated material. At 10 and 15 mmol/l phosphate and pH 6.3 coagula could be partly dissolved in simulated filtrate, demineralized water, EDTA/Tween solution and urea. At all other initial pH values and different phosphate levels coagula were insoluble in the various media, indicating covalent bond formation.

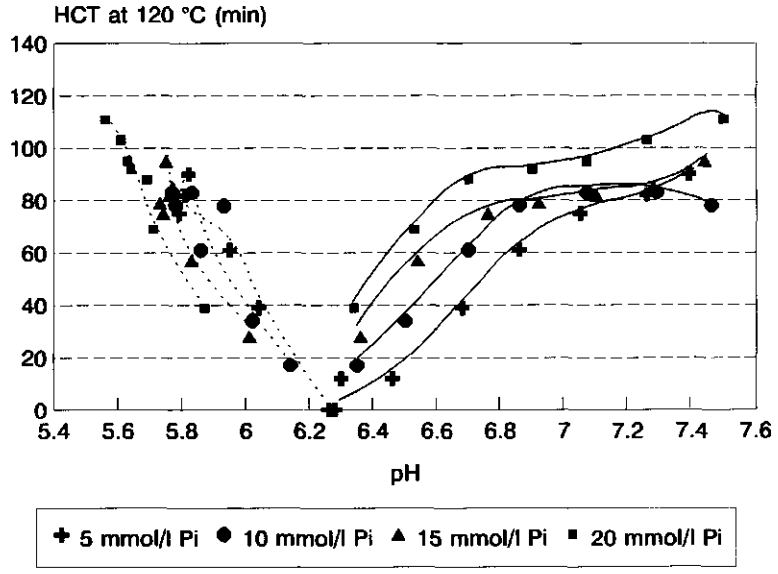

Figure 4.3 *HCT-pH profiles of caseinate emulsions (50 g oil/kg) with 5-20 mmol/l phosphate (sucrose/lactose ratio (3:2)). Initial pH (closed lines); pH at coagulation (dashed lines).*

The pH at coagulation was also determined and plotted in Figure 4.3: the relation between HCT and pH at coagulation was almost linear. Hence, coagulation was not merely a result of acid precipitation at a fixed pH, although it seems that acid formation during heating plays a major role. When the pH of a caseinate emulsion was readjusted to its original pH 6.9 after every 20 min heating, coagulation could be delayed from HCT = 58 min to HCT >200 min.

Effect of citrate

Adding citrate as stabilizing salt is another way to manipulate the Ca^{2+}-activity of caseinate emulsions. The final citrate concentrations in the emulsions were 5, 10, 15 and 20 mmol/l. When potassium citrate was added, the amount of potassium chloride in the emulsions was proportionally decreased, leaving pH and ionic strength almost constant. Addition of citrate had large effect on the heat stability of caseinate emulsions at a particular pH, the shape of HCT-pH profile and the pH at coagulation (Figure 4.4). Compare curve: 50 g/kg oil; 5 mmol/l citrate in Figure 4.1. Increasing the citrate concentration from 5 to 10 mmol/l caused considerable destabilisation at all pH values. The very low heat stability in the product with 10 mmol/l citrate over the pH range 6.3-7.5 was observed to be reproducible, and HCT-pH profiles of emulsions with intermediate citrate concentrations (6; 7 and 8 mmol/l), were in between these curves. When oil was omitted, the product was even more unstable during heating than the oil-containing product: in the pH range 6.9 to 7.5, these samples coagulated within 5-15 min at 120°C. Microscopically, the coagulated samples showed the same morphology as the previously shown phase separated sample (Figure 4.2). Raising the citrate concentration further to 15 and 20 mmol/l, enhanced heat stability and the shape of HCT-pH curve was also altered. The HCT-pH profile shape was changed in the direction of emulsions with added phosphate (Figure 4.3).

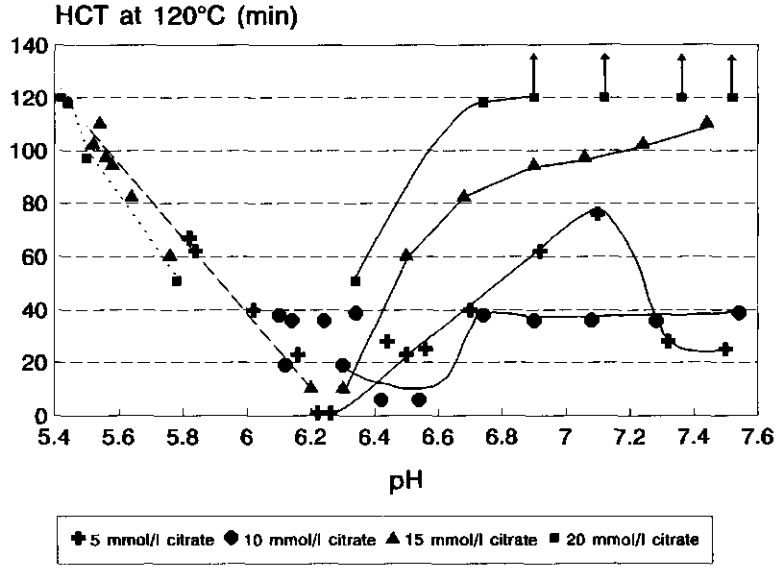

Figure 4.4 *HCT-pH profiles of caseinate emulsions (50 g oil/kg) with 5-20 mmol/l citrate (sucrose/lactose ratio; 3:2):Initial pH (closed lines); pH at coagulation (dashed lines). Arrows indicate HCT > 120 min.*

In Figure 4.4 complete different relations are shown between pH at coagulation and heat stability (HCT) indicating that different reactions cause coagulation. Products with 5, 15 and 20 mmol/l citrate show similar profiles, except for 5 mmol/l at initial pH >7.3. Product with 10 mmol/l citrate shows a dissimilar profile.

Dissolution experiments were performed on coagula from the above citrate (5, 10, 15 and 20 mmol/l) containing products. At low initial pH 6.3 or pH 6.5 and short heating time (< 15 min), coagula were (partly) soluble in all reagents indicating a type of salt-induced reaction. Other results of dissolution experiments of emulsions with 5 mmol/l citrate are given in section 4.2.

The dissolution behaviour of the heat induced coagula from the oil-free caseinate solution and emulsions (10 mmol/l) at initial pH 6.9- 7.5 was somewhat different but not contradictory. The coagula of the caseinate solution disappeared within 2-8 hours and could be solubilized in all reagents, indicating formation of a phase separated system. From the emulsions gel-like coagula were formed and these could only partly be solubilized in 6 M urea or 200 g/kg SDS solution and not in the other reagents (e.g., simulated filtrate, demineralized water and calcium chelating agent). Thus, it seems that during this somewhat longer heat treatment a limited number of covalent bonds were formed. The possibility of phase separation in the caseinate emulsions can, however, not be ruled out. Phase separation was not observed in products with 15 and 20 mmol/l citrate. At low initial pH (6.3) and short heating time (< 15 min), coagula were (partly) soluble in all reagents, pointing to salt-induced coagulation: at other pH values coagula were not soluble at all and were gel-like, pointing to chemical cross-linking.

4.3.2 Turbidity

The particles or aggregates formed in these caseinate systems appear to have a rather loose structure, which depend very much on the ionic conditions. It appeared that addition of citrates affected association or aggregation of caseinate enormously. To investigate whether the caseinate aggregates change their structure, turbidity was measured on heating (Table 4.1).

For the caseinate system at 5 mmol/l citrate, the initial turbidity was relatively high and increased further on heating indicating formation of structures of aggregated caseinate. On cooling and storage the caseinate appeared to disaggregate. Furthermore, the turbidity appeared to decrease with increasing pH (7.5) (results not shown). The systems with 10 or 15 mmol/l citrate were less turbid before heating and directly after heating, indicating a rather loose structure. It may be suggested that the stability of the caseinate aggregates in solution or at the oil interface depend on the size and composition of the aggregates. These aggregates may contain all caseins of which κ-casein appears to play a major role in colloidal stability of caseinate systems (Dalgleish, 1995).

Table 4.1 *Turbidity (E at 900 nm, measured at 20°C) of oil-free caseinate systems at various citrate concentration before and after heat treatment (20 min 120°C; sucrose/lactose (3:2); pH 6.8).*

	E_{900}		
citrate (mmol/l)	**before heating**	**directly after heating**	**24 h after heating**
5	0.524	2.422	0.110
10	0.060	0.572	0.056
15	0.057	0.076	0.054

4.3.3 Association of caseinate with the emulsion droplet surface

The stability of caseinate emulsion droplets may depend on the types and amounts of casein adsorbed. After homogenization, caseins on the surface layer possibly exchange with those in solution, so that the amount and composition of caseins on the surface layer could change upon altering environmental conditions. In this section we report on association of caseinate with oil globules using a depletion method and a desorption method with sodium dodecyl sulphate (SDS) followed by analysis with SDS-PAGE (Sharma & Dalgleish, 1993).

The amounts of caseinate adsorbed onto the emulsion droplets were analyzed in emulsions before and after heating for 20 min at 120°C. In emulsions with phosphate (5-20 mmol/l) as stabilizing salt, the amount of adsorbed caseinate was between 2 and 4 mg/m^2 and it decreased at high phosphate (20 mmol/l) and corresponding low Ca^{2+}-activity values (Table 4.2). A considerable increase in caseinate load on heating at 120°C was observed. This effect was thought to be due to polymerization or increased association of caseinate during heating. However, the results with phosphate (5 and 10 mmol/l) may perhaps be somewhat overestimated, because of sedimentation of a small part of material containing caseinate and oil, during centrifugation at 44000 g. In citrate containing caseinate emulsions a different effect was found on heating, especially at 15 and 20 mmol/l citrate. Before sterilization, the surface load was approximately 1-2.5 mg/m^2 and it increased far less on heating than the phosphate stabilized products. The results for unheated caseinate emulsion droplets were consistent with caseinate emulsions and model systems in which plateau values in surface load of 2.6-3.5 mg/m^2 were observed by others (Tornberg, 1978; Robson & Dalgleish, 1987; Mulvihill & Murphy, 1991; Fang & Dalgleish, 1993a; Singh et al., 1993). These authors also found increased protein loads in caseinate emulsions with increased Ca^{2+}-activity.

Table 4.2 *Surface load of droplets of caseinate emulsions (50 g oil/kg) with added sucrose/lactose (3:2), measured at 20°C before and after sterilization (20 min 120°C).*

	Γ protein (mg/m²)				
	before heating	**after heating**		**before heating**	**after heating**
phosphate (mmol/l)			**citrate (mmol/l)**		
5	3.7	33.5*	**5**	2.4	25.2*
10	3.7	33.4*	**10**	1.6	13.7
15	3.6	23.1	**15**	1.3	4.1
20	1.9	16.3	**20**	1.1	1.6

* small amounts of sediment were observed upon centrifugation.

Desorption method

The gel electrophoresis pattern of desorbed surface material of unheated caseinate emulsions with phosphate or citrate, showed all individual caseins to be present (Figure 4.5a and 4.5b). However, the ratio of individual caseins seemed to be different from that in whole caseinate. We found that especially the amount of α_{s2}-casein in the interfacial layer was raised and that of β-casein was decreased as compared to whole caseinate while the amounts of α_{s1}- and κ-casein seemed unaffected. In figure 4.5a an unknown band is seen above the caseins. Our results suggest that especially β-casein was depleted from the interfacial layer. This observation is rather surprising because β-casein is considered the most hydrophobic of the caseins. We checked with a β-caseinate stabilized emulsion whether β-casein could be desorbed by SDS from the interface, and this was indeed the case. A study of Robson & Dalgleish (1987) on changes of the caseinate emulsion interface suggested that the ratio of individual caseins was comparable to that in whole caseinate. However, no distinction could be made between α_{s1}- and α_{s2}-casein with the analytical FPLC-method used there. Furthermore their results were obtained in emulsions with rather high fat/protein ratios, butter fat (220 g/kg) and whole caseinate (15 g/kg). Therefore total caseinate load was very low, about 1 mg/m^2.

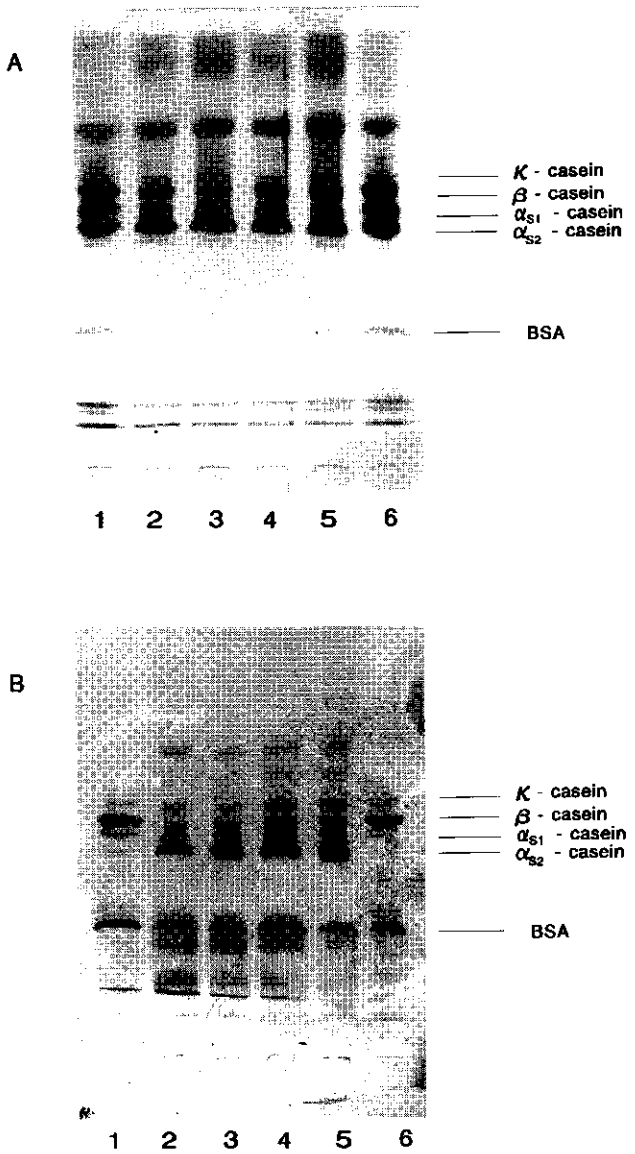

Figure 4.5 *(a) SDS-PAGE pattern of whole caseinate (lanes 1 and 6) and of surface caseinate obtained after desorption with SDS from caseinate emulsions (50 g oil/kg) with 5 mmol/l phosphate (lane 2); 10 mmol/l phosphate (lane 3); 15 mmol/l) phosphate (lane 4); 20 mmol/l phosphate (lane 5).*

(b) whole caseinate (lanes 1 and 6) and caseinate emulsions (50 g oil/kg) with 5 mmol/l) citrate (lane 2); 10 mmol/l citrate (lane 3); 15 mmol/l citrate (lane 4); 20 mmol/l citrate (lane 5). BSA was added as a standard.

Euston et al. (1995) showed that the ratio of the individual caseins on oil droplets depended on the ratio of protein concentration and surface area. In emulsions with whole caseinate (2.5 g/kg) and soya oil (200 g/kg) in deionized water; pH 7, the ratio of β- to α_s- casein at the surface was greater than in whole caseinate. However, at a protein concentration of 20 g/kg, there was no preference for β-casein. Dickinson et al. (1989) showed that the two major caseins α_{s1}- and β-casein displaced each other fairly rapidly. Hence the presence of individual caseins at the interface and exchange of them would depend on the protein concentration per unit oil surface are, ionic conditions and the way the emulsion droplet membrane was formed.
Our results seem to be in good agreement with those of Sharma & Dalgleish (1993), although they studied emulsion interfaces of heated and unheated homogenized milk. They also found that especially α_{s2}- and κ-casein were associated with the emulsion droplets of unheated homogenized milk after strong dissociation of micellar casein with a buffer containing EDTA (5 mmol/l) or both urea (6 mol/l) and EDTA (5 mmol/l).

When caseinate emulsions were heated at 120°C, bands of the individual caseins became less distinct on the electrophoresis gel and a large part of protein material was found in the slot of the gel, indicating that the caseins were polymerized. It was therefore difficult to measure changes at the emulsion droplet interface upon heating. If anything, there appeared to be no further changes in ratios of individual caseinates at the interface on heating.

Summarizing, the heat stability of these caseinate solutions and emulsions strongly depended on type of stabilizing salt used, initial pH and Ca^{2+}-activity. Salt-induced reactions can be responsible for coagulation at pH 6.3-6.5, independent of type of stabilizing salt used. At pH values higher than 6.7, the type and concentration of the stabilizing salt seemed the coagulation rate determining factor, but polymerization reactions seem to be the ultimate cause of coagulation. In caseinate systems at 10 mmol/l citrate and at 5 mmol/l citrate and pH 7.5, phase separation occurred, which appeared to depend on type and size of aggregates formed on heating.

4.4 Effect of lecithin

Heat stability of caseinate emulsions can be modified by addition of lecithins which is a permitted additive in most food and dietetic products. Since lecithin is a mixture of phospholipids, effects of crude soya lecithin and of various of its fractions on heat stability and association of caseinate with the emulsion droplets was studied.

4.4.1 Effect of crude lecithin on HCT-pH profile of caseinate emulsion.

Crude soya lecithin was added prior to homogenization in a ratio of 0.09 g phospholipids to 100 g of caseinate emulsion (citrate concentration 5 or 10 mmol/l). The HCT-pH profile of the emulsion with 5 mmol/l citrate hardly changed on addition of lecithin (Figure 4.6). The coagulation in the range pH 6.3-7.3, which appeared

mainly dependent on ionic conditions, was hardly affected. Moreover no significant changes in pH or Ca^{2+}-activity were measured on addition of lecithin. Addition of lecithin caused a positive effect at pH 7.5, probably by prevention of phase separation. This finding as such was not reported before. In studies of the effect of lecithin on heat stability of milk and concentrated milk it was suggested that interactions between phospholipids and caseins and also with whey proteins were enhancing stability (McCrae & Muir, 1992; Singh et al., 1992).

In caseinate emulsions containing lecithin and 10 mmol/l citrate it was found that heat stability improved over the pH range 6.5-7.5. Increase was highest at high pH values. In one of the previous sections it was concluded from dissolution experiments that phase separation is probably the cause of coagulation in these emulsions. This was confirmed in this experiment: on addition of lecithin phase separation was delayed and covalent bond formation seemed responsible for coagulation, because the coagulum could not be dissolved in any of the reagents used.

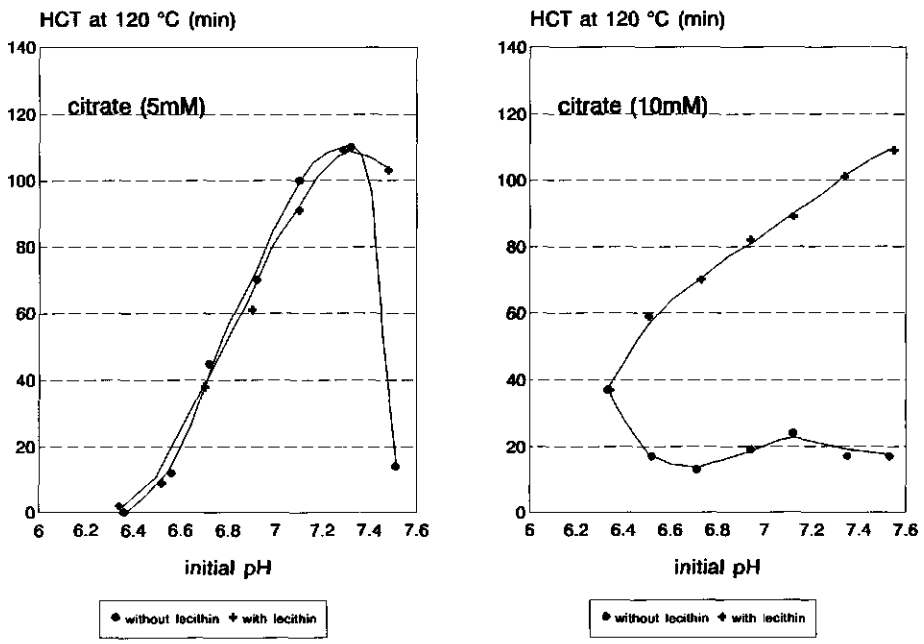

Figure 4.6 *Effect of crude soya lecithin (0.09 g phospholipids/100g) on HCT-pH profiles of caseinate emulsions (50 g oil/kg) with 5 and 10 mmol/l citrate; sucrose/lactose ratio 3:2.*

Crude soya lecithin was not capable to prevent phase separation of unhomogenized oil-free caseinate systems with 10 mmol/l citrate. At pH values 6.3-7.5, HCT at 120°C

of caseinate solutions with or without addition of crude soya lecithin was the same and low (< 15 min). Hence, it appears that crude lecithin only stabilizes the caseinate system in the case that the oil and lecithin were homogenized in the caseinate solution. This may seem surprising, but a similar effect was also found in the earlier mentioned study on heat stability of milks (McCrae & Muir, 1992). In our case, the beneficial effect of soya lecithin on heat stability was lost at a phospholipid concentration of 0.30 g/100g, where emulsion droplets appeared to be simultaneously coagulated and coalesced.

4.4.2 Association of caseinate with the emulsion droplet surface.

The amount of protein associated with oil-globules containing crude lecithin (0.09 g/100g emulsion) added before or after homogenization was measured using the depletion method (Table 4.3). The average droplet size in the sample with added lecithin before homogenization was small compared to the control (without lecithin), indicating that the phospholipids became associated with the interface. No coalescence was observed during heat treatment. Surface protein loads of all three unheated samples were comparable and also comparable to results of Fang & Dalgleish (1993b). In the sample with lecithin added before homogenization, caseinate at the surface was slightly reduced.
After heating, the surface load was significantly less when lecithin was added prior to homogenization, as compared to the other samples, indicating that the interfacial layer was affected by lecithin addition. The surface load seemed not to be affected by the lecithin compared to the control when lecithin was added afterwards.

Table 4.3 *Effect of crude soya lecithin on HCT, average droplet size, surface protein load of caseinate emulsion droplets, measured at 20°C before and after heat treatment (20 min 120°C) (duplicate experiments, standard deviation was 0.5 mg/m²).*

	d_{vs} (μm)	Γ (mg/m²) before heating	after heating	HCT (min at 120°C)
control	0.56	2.1	4.4	15
control + lecithin added before homogenization	0.23	1.5	1.6	65
control + lecithin added after homogenization	0.58	2.1	3.6	24

With the depletion method used, partitioning of the phospholipids from crude lecithin could not be measured precisely. No phospholipid could be detected in the subnatant of samples with lecithin added either before or after homogenization when measured as phosphorus in oil after extraction according to the Röse-Gottlieb method. Judging from these results, the conclusion would be that all added phospholipids, being 0.8 mg/m^2 were associated with the oil. However, these experiments can not discriminate between phospholipids located at the interface itself or being associated with the protein at the interface.

The composition of individual caseins was determined using the desorption method with SDS. All caseins were present at the surface but the proportion was different from whole caseinate. They remained unchanged on addition of lecithin either before or after homogenization (Figure 4.7). SDS gel electrophoresis of adsorbed caseinate of heated emulsions showed polymerization to such an extent that individual caseinates could not be distinguished after treatment with SDS and dithiothreitol.

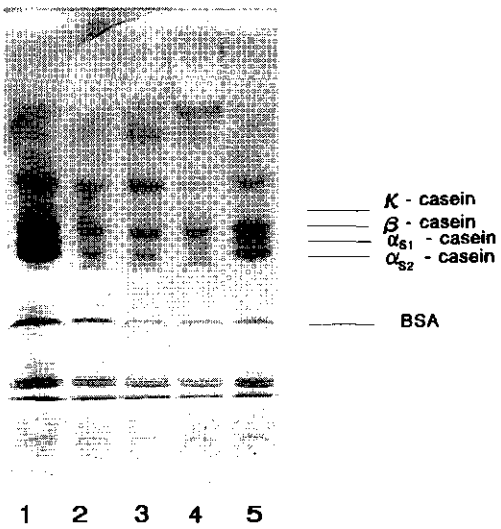

Figure 4.7 *SDS-PAGE pattern of whole caseinate (lanes 1 and 5) and surface caseinate obtained after desorption with SDS from caseinate emulsion (no lecithin added) (lane 2), lecithin added after homogenization (lane 3) and lecithin added prior to homogenization (lane 4). BSA was added as a standard.*

These results on types and amounts of caseinate at the interface and particle size show that phospholipids were probably associated with the emulsion droplets but did not affect caseinate composition at the interface when crude soya lecithin was added prior to homogenization. However, the amount of caseinate increased on heating.

When lecithin was added after homogenization, the phospholipids were probably not associated with the oil, because particle size and amount and type of caseinate were comparable to the control without lecithin in unheated and heated samples.
When lecithin was added afterwards, heat stability, protein load and individual caseins at the interface seemed unaffected. Therefore, the stabilizing effect of lecithin in caseinate emulsions must be attributed to alteration of the composition of the emulsion droplet membrane during homogenization. It appeared that the emulsion droplets were protected against phase separation and coagulation by addition of lecithins prior to homogenization, through lowering of the surface load of caseinate. McCrae & Muir (1991) found the same relationship between heat stability of recombined milk and initial surface protein concentration.

4.4.3 Effect of various fractions of soya lecithin on the heat stability of caseinate emulsion

In order to obtain additional information on the mechanism of stabilization by lecithin, various fractions of phospholipids were tested. Fractions of soya lecithin were added prior to homogenization at a concentration of phospholipids (0.09 g/100g) to caseinate emulsion containing 10 mmol/l citrate. Concentration and composition of crude soya lecithin (Topcithin), a lecithin rich in phosphatidylcholine (Epikuron 200), a lecithin rich in polar phospholipids (Epikuron 110) and a lecithin with lysolecithin (150 g/kg) (Emulfluid E) were shown in Table 2.2. The non-phospholipid fraction of the lysolecithin-rich lecithin consisted mainly of water and triglycerides, though it contained also some glycolipids and carbohydrates (Singh et al., 1992). On addition of crude lecithin (Topcithin) or lecithin rich in polar phospholipids (Epikuron 110) or a hydrolysed lecithin (Emulfluid E) heat stability compared with control was enhanced over pH range 6.3-7.5 (Figure 4.8). Stability of caseinate emulsion with Emulfluid E was even higher than with crude lecithin. On addition of Epikuron 200 with the apolar pure phosphatidylcholine (PC), heat coagulation time was even decreased compared to the control. When coagula of PC containing emulsions were studied microscopically, emulsion droplets were enlarged and coagulated compared with droplets before heating, indicating coalescence of the emulsion droplets during heat treatment. These results seemed consistent with those of McCrae & Muir (1992) for recombined milk. They found also a stabilizing effect of the more polar fractions of lecithin on heat stability and a destabilizing effect of the apolar phosphatidylcholine. Concerning the destabilizing effect of phosphatidylcholine no results on possible changes of emulsion droplet size distribution were given there. The fact that different fractions of lecithin or the enzymic modified lecithin resulted in different HCT-profiles suggests that adsorption of phospholipids on caseinate droplets as such was not responsible for improvement of heat stability. However, phospholipid composition of the lecithin fraction may determine its stabilizing effect through interaction of caseinates possibly via polar residues coupled to the polar groups of the phospholipids

(salt bridges).

Summarizing: the stabilizing mechanism of lecithin in these caseinate emulsions in preventing coagulation or phase separation is not fully clear yet. Effects of crude lecithin and fractions of lecithin suggest that interactions occur between certain fractions of lecithin and caseinates, probably the more polar fractions. Moreover, addition of lecithin prior to homogenization gave alteration of the surface load of caseinate and decreased sensitivity towards phase separation or coagulation.

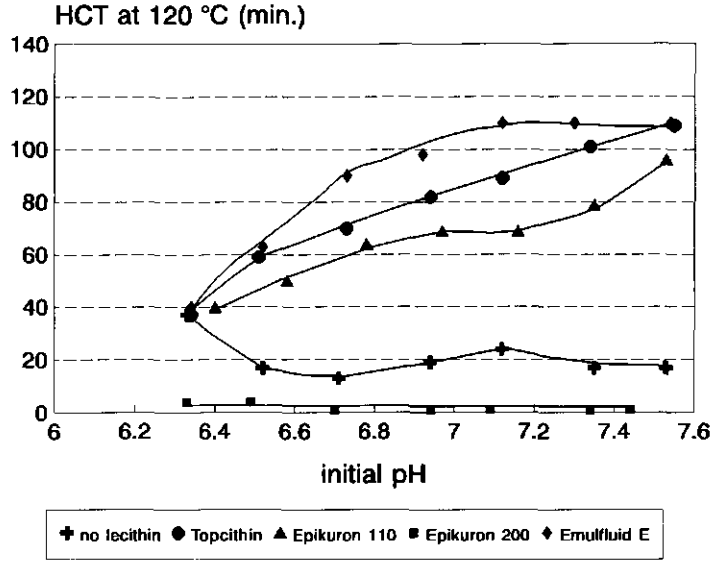

Figure 4.8 *HCT-pH profiles of caseinate emulsions (50 g oil/kg) with fractions of crude soya lecithin (0.09 g/100g) added to the oil, (5 mmol/l citrate; sucrose/lactose ratio 3:2).*

4.5 Effect of sugars

Sugars are considered to be a factor affecting heat stability of caseinate emulsions. De Koning (unpublished results, 1982) found that heat stability of sodium caseinate emulsions (20 g caseinate/kg) with paraffin oil (120 g/kg) in NaCl (14 mmol/l) (pH 7.0) was high at 130°C (HCT> 100 min). After addition of lactose (40 g/kg) heat coagulation time (HCT) was reduced to 35 minutes. Thus, reducing sugars like lactose may be considered as a destabilizing factor. Other sugars normally included in dietetic formulations are sucrose, glucose syrups and malto-dextrins. In chapter 5,

effects of malto-dextrins on heat stability and phase separation will be given. In this section effects of different reducing and non-reducing sugars on heat stability and chemical cross-linking will be demonstrated.

4.5.1 Heat stability

In Figure 4.9, HCT-pH profiles of caseinate emulsions with 5 mmol/l phosphate are given on addition of sucrose, glucose and sucrose/lactose mixture (3:2). Stability of emulsions with (non-reducing) sucrose was comparable to emulsions without sugar at all pH values. When sucrose was partly replaced by lactose, a reducing sugar, stability decreased at all pH values. Complete replacement of sucrose by glucose caused a further decrease in stability at pH 7.2-7.5. In all these samples chemical cross-linking seemed the ultimate cause of coagulation, because coagula could not be dissolved in the reagents, except for coagulated samples at low initial pH (6.3) and low HCT (< 20 min). These coagulates could partly be dissolved in demineralized water and urea.

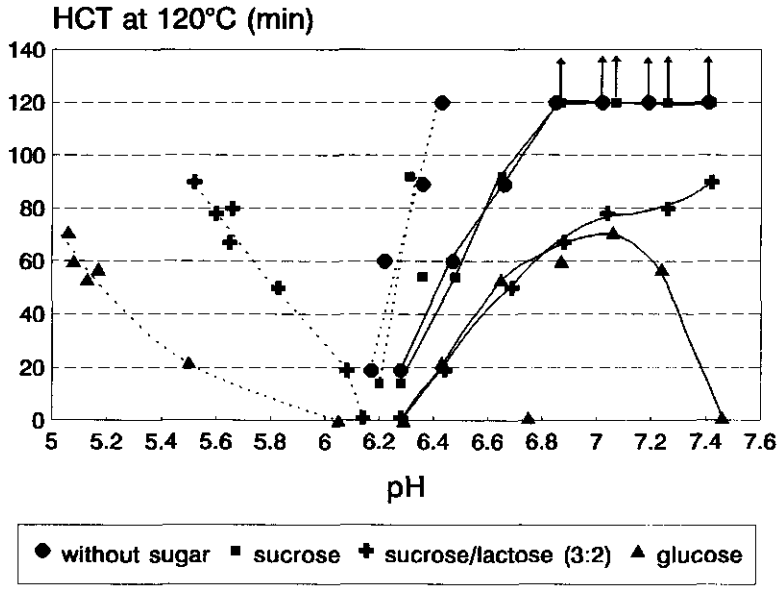

Figure 4.9 *HCT-pH profiles of caseinate emulsions (50 g oil/kg) with different sugars (phosphate, 5 mmol/l). Initial pH (closed lines); pH at coagulation (dashed lines); Arrows indicate HCT > 120 min.*

In section 4.2 and 4.3, it was concluded that coagulation is not only a result of acid precipitation at a certain pH-value, but it seemed clear that acid formation during heating is somehow responsible for rate of coagulation and HCT of these caseinate emulsions. These present results show indeed that coagulation depended both on the presence of sugar, type of sugar used and initial pH. There is, however, not an unequivocal relationship between HCT and pH at coagulation. In emulsions without sugar or with sucrose, coagulated within 120 min, pH at coagulation was high, between 6.2 and 6.4. On the other hand, when glucose had been added, pH at coagulation of most samples was between 5.0 and 5.5.

4.5.2 Chemical cross-linking of caseinate

Covalent cross-linking occurred during heating of caseinate emulsions. When caseinate systems were heated with reducing sugars such as the sucrose/lactose (3:2) mixture, a typical brown pigment was formed after a relatively short heating time at 120°C. In a caseinate system with only the non-reducing sucrose, a pink pigment was formed after more than, say, 60 min. These changes in colours may be indicative of polymerization reactions. Caseinate polymerization during heating can be estimated by quantifying bands on SDS-polyacrylamide gels after solubilization in a solution of sodium dodecyl sulfate (SDS) and dithiothreitol (DTT). This method does not distinguish between Maillard reactions and cross-linking of amino acids. The formation of the cross-linking amino acid lysinoalanine (LAL) during heating was studied in somewhat more detail.

4.5.2.1 SDS-gel electrophoresis

Crosslinking can qualitatively be estimated from the decrease in monomeric caseinate on SDS-polyacrylamide gels. Excess of sodium dodecyl sulfate (SDS) (200 g/kg) and dithiothreitol DTT (110 g/kg) were added to the heated oil-free caseinate system with citrate (10 mmol/l) to rupture all but covalent bonds. Urea was omitted in the dissolution medium. After heating for 20 min at 120°C of a caseinate system containing sucrose/lactose (3:2), a compressed band was formed in the slot of the gel (Figure 4.10b). This could be attributed to protein polymers or to advanced Maillard products (Guo et al., 1989; Law et al., 1994; Prabhakaram & Ortwerth, 1994). After 30-60 min heating a marked smearing of all bands was observed, which may be attributed to a wider range of charges on the caseinate after dephosphorylation (Li-Chan & Nakai, 1989; Law et al., 1994). When samples with the non-reducing sugar sucrose were subjected to SDS-gel electrophoresis, accumulation of proteinaceous material in the slot was also observed for the same heating conditions (Figure 4.10a).

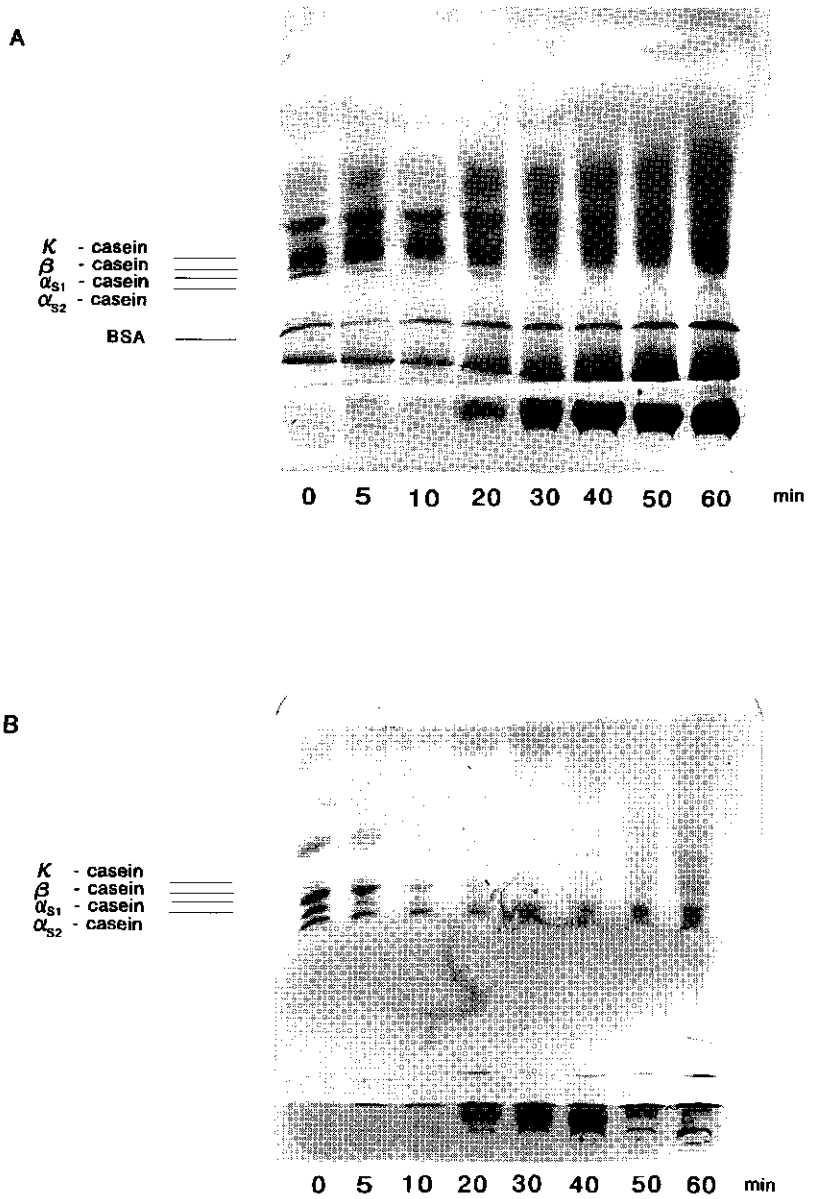

Figure 4.10 *SDS-PAGE pattern of oil-free caseinate systems heated during 0-60 min at 120°C treated with sodium-dodecyl-sulphate (SDS) and dithiothreitol (DTT).* ***(a)*** *With added sucrose;* ***(b)*** *with added sucrose/lactose (3:2).*

4.5.2.2 Formation of lysinoalanine

The extent of formation of the cross-linking amino acid LAL and the effects of pH and of adding reducing or non-reducing sugars was measured, following heating of oil-free caseinate systems at 120°C. LAL can be indicative for chemical crosslinking reactions between caseinate residues, although in the acid hydrolysates of heated caseinate systems unknown other compounds, like the o-phthaladehyde-positive compound designated X, were detected (Figure 4.11). In the unheated caseinate systems fairly large amounts of LAL (620 mg/kg protein= 2.7 mmol/kg protein) were found, indicating that the caseinate had been subjected to a relatively intense heat treatment during its manufacture. This was also reported by other workers (Henle et al., 1993). The compound X was not found in the original caseinate, but was formed after heating for about 30 min in the caseinate system. "X" might be histidino-alanine (HAL), also found by Henle et al. (1993) in heat-treated milk protein containing products. Unfortunately, no HAL-standard or other technique was available to check the identity of the compound.

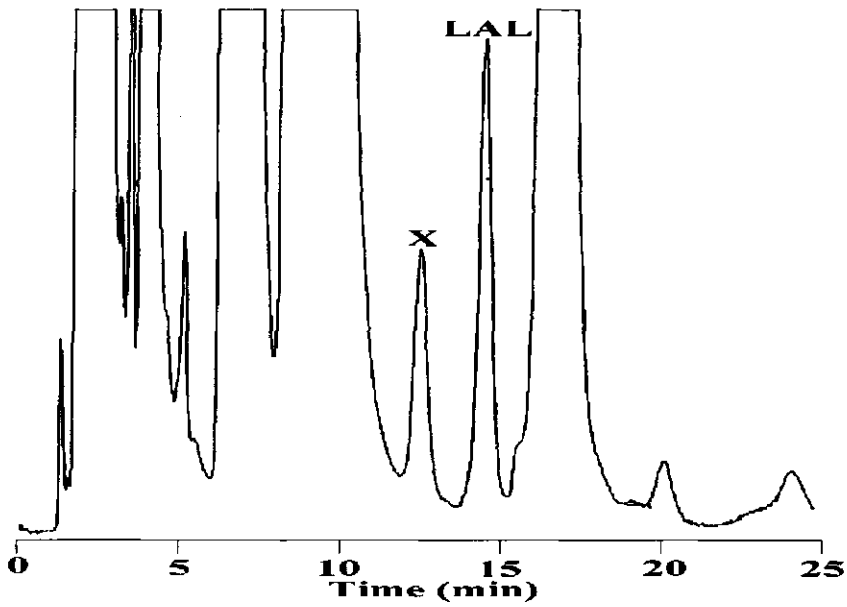

Figure 4.11 *HPLC chromatogram of acid hydrolysate of heated fat free caseinate system (60 min 120°C). Lysinoalanine (LAL) and the unknown component "X" are indicated.*

In the caseinate system with sucrose/lactose (3:2) and phosphate (5 mmol/l) as stabilizing salt, the amounts of LAL increased with heating time up to 30 min at 120°C (Figure 4.12). At longer heating times up to 120 min, no additional LAL was formed. This seemed to correspond remarkably well with pH decrease during heating. LAL-formation was limited when pH was pH 6.5 or lower. The peak area of compound X increased further on heating at longer heating times (120 min).

The pH dependent LAL formation was also found in other milk systems (Watanabe & Klostermeyer, 1977; Kleyn & Klostermeyer, 1980; Fritsch et al., 1983). However, in one of these studies with micellar casein no plateau values were found. LAL formation increased steadily with heating time, probably due to the relatively high buffering capacity of milk-systems. When milk was heated for 30 min at 120°C, the pH decreased by only 0.1 pH unit (van Boekel et al., 1989), while in our caseinate systems pH decreased by 0.3 to 1.0 pH units.

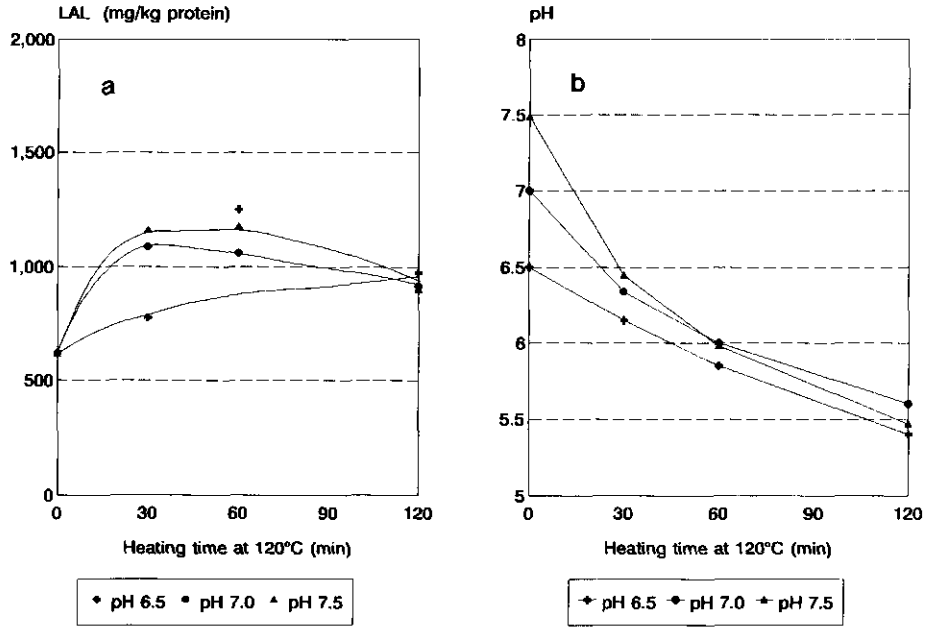

Figure 4.12 *(a) Formation of Lysinoalanine (LAL) (mg/kg protein) and (b) decrease in pH in oil-free caseinate systems, phosphate (5 mmol/l) at initial pH 6.5, 7.0 and 7.5, containing sucrose/lactose (3:2), heat treated at 120°C.*

When the non-reducing sugar sucrose was added instead of lactose to the caseinate system, LAL concentration increased enormously, from 640 to 1900 mg/kg protein. The pH decrease in these conditions was rather slow, as expected: from initially 6.7 to 6.3 after 60 min at 120°C.

Our results thus indicate that formation of lysinoalanine is very pH dependent. If acid production during heating is high, the pH will reach a value below which rate of LAL-formation is very slow. The Maillard reaction may also compete with the formation of LAL through the formation of a Schiff base in the early stage of the reaction sequence of the Maillard reaction. Also in caseinate systems it is quite possible that a large part of lysine residues is involved in Maillard reaction and can not form any further LAL (Fritsch et al., 1983).

4.6 Effect of formaldehyde

Treatment of caseinates by the cross-linking agent formaldehyde (25 mmol/l), enhanced stability of caseinate emulsion with citrate concentrations of 5 and 10 mmol/l during heating in the entire pH range 6.3-7.5 (results not shown). This may be explained by increase of net charge of caseinate residues, reduction of available lysine or, maybe, formation of intramolecular cross-links of caseinate particles (Aoki & Kako, 1984; Singh & Fox, 1985; Nieuwenhuijse et al., 1991). It can be calculated that if all added formaldehyde reacts with positively charged ϵ-NH_2 groups on lysine residues (40 mmol/l), the net negative charge of caseinate will be about 0.84, 0.99 and 1.02 mEq. per g protein at pH 6.5, 7.0 and 7.5, respectively. Hence, the net negative charge will decrease by a factor of about 1.5 and the electrostatic repulsion in first approximation by 1.5^2 (Walstra & Jenness, 1984). The reduction of available lysine may affect formation of Maillard reaction products or cross-linked amino acids that are of importance for heat-induced coagulation in conditions were HCT is high, such as in the case that phase separation or salt induced coagulation is slow or absent. Small amounts of lysinoalanine formed on addition of formaldehyde in caseinate emulsions (Table 4.4). However, these changes might be caused by the changes in pH. Hence, the ability of formaldehyde to increase the heat stability of caseinate emulsions and solutions in conditions where salt-induced coagulation, covalent binding and phase separation were the cause of coagulation cannot provide any additional evidence for the mechanisms involved.

Table 4.4 *Lysinoalanine-formation during heating 20 min at 120°C of oil-free caseinate system with malto dextrin DE 19 (120 g/kg), calcium (2 mmol/l) and initial pH 6.8.*

formaldehyde (mmol/l)	lysinoalanine (LAL) (mg/kg protein)	initial pH	end pH
unheated	400	6.8	-
0	1340	6.8	6.3
5	1230	6.8	6.1
10	1120	6.8	6.1
50	880	6.6	6.2
100	630	6.4	6.0

4.6 Possible mechanisms of coagulation (Discussion)

Caseinate dispersions without sugar were very stable to heat at pH 6.7-7.0, but once oil and sugars were added or pH was changed either to lower or higher values, the caseinate system became very sensitive to heating. During heat treatment the caseinate in solution or caseinate coated emulsion droplets were destabilized and aggregated to form a coagulum. Both charge-charge repulsion and steric repulsion of caseinate residues were thought to be important in stability of these emulsions (Dalgleish, 1995).

The caseinate emulsions were very susceptible to heating when the volume fraction oil was rather high. When oil (100-250 g/kg) was added, caseinate emulsions coagulated within 20 min at 120°C or even during heating-up. From dissolution of coagula of these emulsions by EDTA it was concluded that a salt-induced reaction was the cause of coagulation. The rather high activity of Ca^{2+}-ions, and probably also Mg^{2+}-ions, would be relevant for stability of caseinate emulsions during heating. Binding of calcium to caseinates reduces their negative charge, which diminishes electrostatic repulsion between the molecules. Through calcium binding, association of caseinate will be increased, probably through interaction of the hydrophobic domains. Further binding of calcium may eventually lead to aggregation or precipitation of caseinate (Rollema, 1992; Swaisgood, 1992). In experiments with individual caseins it was found that the Ca^{2+}-concentration required to initiate precipitation of caseins was roughly equivalent to the number of phosphoserine residues (Parker & Dalgleish, 1981; Dalgleish & Parker, 1980 ; Holt, 1985). In our caseinate systems a mixture of different salts was added making comparison with data from literature difficult. Some results on partitioning were obtained for caseinate emulsions, in conditions where salt induced reaction leads to fast coagulation. In these

partitioning experiments it was shown that maximum calcium association was about 0.15 mmol/g caseinate and maximum magnesium association was 0.07 mmol/g caseinate. The sum of these values comes close to the number of phosphoserine residues for unheated whole caseinate (0.26 mmol/g protein) (Swaisgood, 1992).

In conditions where salt-induced reactions seemed not responsible for fast coagulation, aggregation was determined by changes during heating in properties of the continuous phase and of the emulsion droplet surface layer. Aggregation of caseinate stabilized emulsions may be induced in several ways: (i) by dephosphorylation of the caseinate which lowers the net surface charge, (ii) formation of acids from sugars changing the pH towards the isoelectric point of the caseinate, (iii) by changes of Ca^{2+}-activity as induced by calcium chelating salts, citrates and phosphates (iv) by (chemical) polymerization.

The extent of destabilization of caseinate solutions and emulsions is definitely affected by dephosphorylation. Cleavage of phosphate groups will affect charge distribution on caseinate molecules and sensitivity of caseinate against precipitation by acid or calcium ions (Hekken & Strange, 1993). In our study, a significant correlation was found between time of coagulation and dephosphorylation of caseinate during prolonged heating. It was found that approximately 30 percent of the caseinate phosphates were released after heating for 60 min at 120°C. From this, net charge was calculated to be about 35 percent smaller as compared to the initial net charge.

It is reasonable to assume that with continued heating the protective effect of the negative charge on the caseinate gradually diminishes, probably as a result of decreased pH, increased Ca^{2+}-activity and, of course, dephosphorylation. During heating rather large amounts of acid were formed because of the high content of reducing sugars in most emulsions (e.g. the pH changed from 6.7 to 5.9 during 60 min at 120°C in caseinate emulsions with sucrose/lactose 3:2). At the same time Ca^{2+}-activity increased from about 1.8 mmol/l to 2.7 mmol/l. The pH at coagulation seemed (except where type of sugar was changed) especially to depend on initial Ca^{2+}-activity and less on initial pH. The decrease in pH was not found to be the ultimate cause of coagulation, but the pH at coagulation of most of these emulsions (with sucrose/lactose 3:2) was between pH 5.4-6.0, stressing the importance of the pH decline. Furthermore we demonstrated that if the pH of the caseinate emulsion was readjusted periodically (every 20 min) to its original value, coagulation was delayed more or less infinitely.

Addition of stabilizing salts, citrates and phosphates, had great influence on Ca^{2+}-activity and salt partitioning and thus on electrostatic interactions. On addition of citrate a large part of calcium was chelated by citrate, giving a low Ca^{2+}-activity from maximum 2 mmol/l at 5 mmol/l citrate to 0.3 mmol/l at 20 mmol/l citrate and decreased association of calcium and magnesium to caseinate, while all salts seemed dissolved. Apparently, phosphate addition gave somewhat different changes. Effect of phosphate on Ca^{2+} -activity was comparable to citrate addition, also during heating. However association of calcium and magnesium and anorganic phosphate with

caseinate was increased, probably by formation of a type of calcium and magnesium phosphate. These results suggests that ionic species that decrease the net negative charge, such as H^+ions, but also Ca^{2+} and Mg^{2+}-ions, may be the most important parameters determining instability of caseinate emulsions. When during prolonged heating electrostatic and steric repulsion of caseinate residues in solution or at emulsions droplets is reduced, caseinate chains can approach each other more closely or make more frequent contact and possibly form covalent bonds (Walstra, 1990).

Lowering of the concentration of citrates and phosphates was accompanied by a parallel increase in initial total surface protein concentration. During heating the surface protein load further increased, especially at high Ca^{2+}-activity. Therefore surface protein load seemed very important for heat stability. This relationship was also found in recombined milk (McCrae & Muir, 1991). We tried to measure protein load during heating at 120°C by separation of the emulsion droplets by micro-filtration using a ceramic membrane. Unfortunately, the experiments failed because in these conditions caseinate itself was not completely filterable, so that the inaccuracy of the results was large. However, it is unlikely that such measurements would give a total different view. The composition of caseinate complexes on emulsion droplet interface was found to be slightly different from that of a caseinate solution. From desorption experiments with SDS at 20°C we found that the amounts of α_{s2}-casein in the interfacial layer was higher and that of β-casein was lower compared to whole caseinate, and the composition appeared to be quite similar after heating (20 min 120°C). The increased α_{s2}- casein amount in the interfacial layer could be of importance, since α_{s2} contains more phosphoserine residues than the other caseins. The phosphoserine content of the individual caseins decreases in the order $\alpha_{s2} > \alpha_{s1} > \beta > \kappa$ and corresponds with aggregation or precipitation of caseins on calcium addition (Rollema, 1992).

Involvement of chemical cross-linking reactions in our caseinate systems was shown in dissolution experiments, but the formation of cross-links seemed not to be rate determining in coagulation. From SDS-gel electrophoresis it was shown that polymerization of caseinate could already be observed after a heat treatment of 15 min at 120°C in emulsions containing a lactose/sucrose mixture or sucrose. The observation that on addition of non-reducing sugar heat stability could be improved, whereas the time at which polymerization started was comparable to the samples with reducing sugars, indicate that polymerization was not the rate determining factor in coagulation of these caseinate emulsions. It was found that formation of the cross-linked amino acid lysinoalanine (LAL) stopped when pH decreased below 6.5. Such a blocking of LAL formation at reduced pH value (<6.5) is at variance with the low pH at coagulation of caseinate emulsions (5.4-6.0), where covalent bonds were found to be always present at point of coagulation. Moreover, the highest LAL values (2000 mg/kg protein) were measured in the most stable caseinate emulsions with added sucrose (because of limited pH decrease). Only 10-20 percent of caseinate molecules could be involved in inter- or intramolecular cross-linking at this high LAL level. It

was found that the peak area of the unknown compound X in the chromatogram, which may be the cross-linked amino acid histinoalanine (HAL) increased further on heating, also when pH was below 6.5. This was also found by Henle et al. (1993). Thus, it appears that the reactions described here, represent only a small part of possible chemical cross-linking reactions of proteins.

A phase separated system was obtained on heating caseinate-sugar solutions or emulsions up to 120°C under some ionic conditions. The system was manifested by the appearance of coagulated spherical inhomogeneities of 1-5 μm, as microscopically observed directly after heating. The spherical inhomogeneities tended to disappear on cooling or on dilution. We hesitated in considering this phenomenon as a true phase separation, because only caseinates were present. But the clear phase boundary and the observed low interfacial tension of the phase droplets prompted us to consider this phenomenon as phase separation or incompatibility of the caseinates in solution or caseinate particles. A number of changes were observed upon heating and cooling for the caseinate systems that might partly explain the actual cause of phase separation. We found marked differences in phase separation phenomena during heating on addition of phosphate as compared to citrate. These differences could, at first sight, not be attributed to differences in Ca^{2+}-activity or the composition of the interfacial layer of the caseinate droplets. In emulsions with increasing phosphate, the amount of calcium phosphate associated with caseinate particles at ambient temperature was increased. On raising temperature, in the range 20-80°C, the amount of calcium phosphate associated with caseinate was further increased and may probably be even higher during heat treatment at 120°C. From proton NMR spectra it was concluded that mobility of polypeptide chains on addition of phosphate or citrate was high, when heated to 90 or 120°C. From P-NMR spectra was observed that at relatively low citrate and phosphate concentrations the mobility of Ser-P groups at 90°C or 120°C was somewhat lowered. From turbidity measurements it appeared that in most conditions caseinate had a loose, disaggregated structure, but aggregates were formed on heating. The properties (size and casein and calcium phosphate composition) of the formed caseinate aggregates may contribute to changed sensitivity of the caseinate system towards phase separation.

All in all, at least three types of reactions appear to be involved in heat causing coagulation of sugar containing caseinate emulsions:

(i) aggregation of caseinate coated emulsion droplets induced by calcium ions;

(ii) lowering of negative charge on caseinate residues by dephosphorylation, acid formation and association of calcium-ions with caseinate, followed by covalent bond formation.

(iii) phase separation, depending on environmental conditions like pH and type of stabilizing salts used.

In contrast to these caseinate emulsions, the reactions causing heat coagulation in normal milk and concentrated milk (in pH region of minimum stability) are formation of a κ-casein/β-lactoglobulin complex and dissociation of the complex from the casein micelles and subsequent precipitation of κ-casein depleted micelles by calcium ions (van Boekel et al., 1989; Singh & Creamer, 1992). Based on the above reactions in caseinate emulsions, the dependence of heat coagulation on addition of stabilizing salts like phosphate and citrate can be explained, as well as effects on addition of lecithin.

Addition of stabilizing salts

During heating, dephosphorylation of caseinate, decrease in pH and increase in Ca^{2+}-activity are probably the rate determining factors in heat coagulation of caseinate emulsions. The initial Ca^{2+}-activity may determine the pH needed for coagulation, while the rate of pH decline determines the time before that pH is reached. At that point caseinate chains can make more frequent contact, thus promoting covalent bonding eventually leading to coagulation. The shape of the HCT-pH profiles of phosphate containing emulsions was characterized by a progressive rise in HCT as pH increased. Increasing phosphate levels in caseinate emulsions resulted in enhanced heat stability. At phosphate concentration 5 and 10 mmol/l, heat coagulation time depended more on initial pH and on initial Ca^{2+}-activity, whereas at 15 and 20 mmol/l phosphate, coagulation depended more on final pH and initial Ca^{2+}-activity. Obviously, the largest part of the stabilizing effect of phosphate can be explained by the decrease in the Ca^{2+}-activity, thus increasing net negative charges on caseinate and, maybe, lowering the caseinate surface load.

The mechanism of heat coagulation of caseinate emulsions with citrate can be explained in the same manner as emulsions with phosphate. Citrate also lowers Ca^{2+}-activity, thus increasing electrostatic repulsions between caseinate chains and it also decreases the caseinate surface load on the emulsion droplets and thereby sensitivity towards heat treatment. However, at 10 mmol/l citrate, phase separation was induced on heating. This could only be explained by changes in the state of aggregation of the caseinate in those conditions. Clearly, the caseinates particles were changed on heating: mobility of the polypeptide chains increased, mobility of the serine phosphate groups was somewhat decreased and turbidity increased. Furthermore, calcium phosphate was absent in these caseinate particles, which must have large effect on stability during heating. The calcium phosphate content probably determines the state of aggregation and structure of the caseinate particles. The variation in calcium phosphate of the caseinate particles may therefore explain differences in stability of citrate and phosphate containing emulsions.

Addition of lecithin

The stability of caseinate emulsions could be enhanced by addition of crude soya lecithin. Analysis of the changes that occurred during heating indicated that the amount of caseinate in the emulsion droplet membrane was decreased on addition of lecithin and remained low during heat treatment. If desorption or reduction of caseinate from the surface by phospholipids were the main factor determining the stabilizing effect of lecithins, no great difference between fractions of lecithins would be expected. Since we found remarkable differences on addition of these lecithin fractions, an explanation could be in the nature of the interactions between phospholipids and caseinate. McCrae & Muir (1992) found similar effects of fractions of lecithin on heat stability in recombined milk. They suggested that hydrophobic interactions between caseins and phospholipids may occur, in particular with the alkyl chains of the lysolecithin and probably not with the choline head group (McCrae & Muir, 1992; Singh et al., 1992; Barrat et al., 1974). Although caseins have a high affinity for hydrophobic surfaces, the caseins exist mainly in solution in its native form in milk and not on the milk fat globules. But, when casein was homogenized together with phosphatidylcholine, the mixed casein/phosphatidylcholine membrane formed a thick layer compared with membrane of the caseins or phosphatidylcholine alone (Fang & Dalgleish, 1995). This effect of homogenization may also explain the difference in stabilizing action of lecithin in the emulsions and the unhomogenized oil-free caseinate systems. However, evidence for importance of hydrophobic interactions was obtained at ambient temperatures, though it is known that interactions of hydrophobic nature increase with increasing temperature (Lyklema, 1991).

The possibility of interaction of caseinate with the negatively charged region of phospholipids cannot be excluded. Addition of phospholipids may therefore increase the overall negative charge of the caseinate of the membrane resulting in an increased stability during heating probably through preventing phase separation. Such interactions of an electrostatic nature would therefore depend on pH and ionic strength. Indeed, we found some effects of pH and Ca^{2+}-activity on the stabilizing effect of phospholipids in caseinate emulsions. Phase separation by phospholipids was prevented in conditions when Ca^{2+}-activity was about 1 mmol/l and pH 6.5-7.5 at 10 mmol/l citrate and when Ca^{2+}-activity was about 1.5 mmol/l and pH 7.5 at 5 mmol/l citrate. On the other hand, no stabilization was found on addition of lecithin around pH 6.3 - 6.5. If interactions between phospholipids and caseinate are likely to occur, phospholipids may affect association and dissociation of caseinate aggregates at ambient temperature and during heat treatment. The phospholipids can also interfere in polymerization reactions. However, when surface material of heated caseinate emulsion with added lecithin was subjected to SDS gel electrophoresis, some polymerized caseinate was found in the slot of the gel. The extent of polymerization seemed somewhat lower, but unfortunately quantitative analysis was not possible.

In conclusion, a clear stabilizing effect of lecithins during heat treatment was found only when added prior to homogenization. Only polar lecithin fractions seemed effective in stabilization which can be explained qualitatively, by suggesting interactions between certain phospholipids and caseinate. The nature of the interactions involved needs further research. Irrespective of the mechanism involved, usage of lecithin offers a possibility to stabilize caseinate emulsions during heating when added prior to homogenization.

Acknowledgements

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Chapter 5

Effect of malto-dextrins on stability of caseinate emulsions during heating

5.1 Introduction

The preceding chapter described the behaviour of caseinate systems during heat treatment in the presence of mono- and disaccharides. It was found that under some ionic conditions of Ca^{2+}-activity and pH a phase separated system was obtained during heating which disappeared on cooling. The present chapter explores the behaviour of caseinate emulsions and oil-free caseinate systems in the presence of malto-dextrins. Malto-dextrins are partially hydrolysed starch preparations giving mixtures of species ranging from glucose to long polymeric chains, which may have a distinct effect on heat stability. Here we give results on the effect of commercial malto-dextrins and fractions thereof on sensitivity of caseinate stabilized emulsions towards phase separation and eventual coagulation, during heating at 120°C. From these results we would like to gain some understanding of the mechanisms involved, so that phase separation can be controlled. However, we are aware that our experimental system is far too intricate to use theoretical models applicable to simple systems (a solution of two monodisperse uncharged homopolymers).

5.2 Effect of malto-dextrin molar mass on heat stability

Profiles of heat-coagulation time against pH show that, in a given mass concentration of malto-dextrin, sensitivity towards coagulation increased with decreasing reducing power of the malto-dextrins (MD) (Figure 5.1). The commercially characterization according to their 'dextrose equivalent' (DE) is used, being DE 6, DE 12, DE 19 and DE 32 for these malto-dextrins.

Heat stability of the caseinate emulsion (caseinate 54 g/kg; carbohydrate 166 g/kg; oil 50 g/kg; citrate 5 mmol/l) with malto-dextrin DE 19 was comparable to the emulsion with the sucrose/lactose (3:2) mixture (compare Figure 4.1). The malto-dextrin DE 12 reduced the stability strongly and the emulsion with malto-dextrin DE 6 became already demixed during preparation of the emulsion.

In the coagulated samples droplet like structures were observed with a clear phase boundary, similar to the phase droplets described in chapter 4 (see Figure 4.2). The larger droplets were often not spherical, indicating a low interfacial tension. Sometimes separate phase droplets were seen, sometimes an interconnected droplet pattern.

relatively high concentration in the phase droplets must have a large effect on heat stability. The mere effect of protein concentration was clear from the following observation. We found an enormous decrease in coagulation time, from 100-120 min at 120°C when the protein concentration was 55-75 g/kg, to 0 min at 120°C when the protein concentration was 100-125 g/kg, in malto-dextrin free emulsions (50 g oil/kg) at pH 6.9.

The extent of destabilization due to heating of caseinate emulsions containing sucrose/lactose (3:2) appeared to increase with increasing surface protein concentration. The malto-dextrin may possibly contribute to the decrease in stability of caseinate emulsions during heating because of increased deposition of caseinate at the emulsion droplets under conditions of incompatibility, which is known as prewetting (Tolstoguzov, 1991; Tsapkina et al., 1992). It is observed that the presence of a surface can induce multi-layer adsorption, when the composition of the polymer system is already close to the two-phase state of the system. This results in an increase in the degree of protein saturation of the adsorbed layer and simultaneously, formation of multi-layers (Tsapkina et al., 1992).

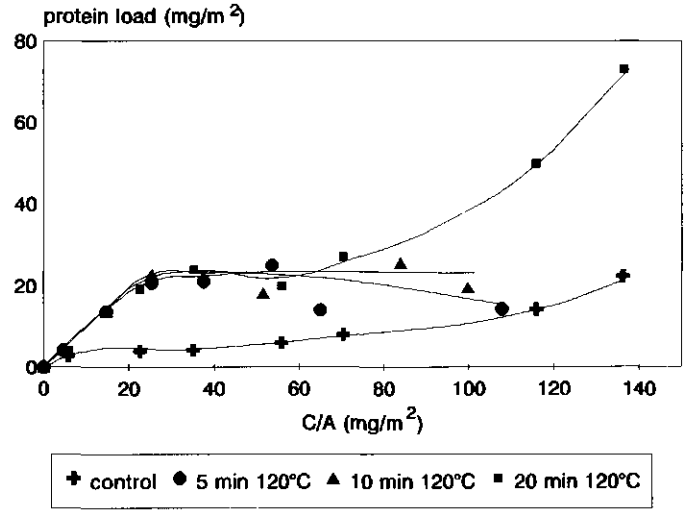

Figure 5.2 *Effect of heating on the surface protein concentration in caseinate emulsions (50 g oil/kg) as a function of the available protein concentration expressed per m^2 oil-droplet-surface, with malto-dextrin DE 19.*

The thickness of the layer formed on the emulsion droplets was expressed as the surface excess and was given as function of the C/A, where C is the protein concentration in the emulsion and A is the specific interfacial area which depends on C. The plot of the protein surface excess as a function of C/A after various heating times at 120°C of emulsions containing malto-dextrin DE 19 is shown in Figure 5.2. It may be noted that there are three parts in the isotherm in the unheated emulsions (control). In the first region (C/A < 20 mg/m^2) the protein load increased towards the plateau value of $\Gamma_{protein}$= 4-6 mg/m^2 in the second region. In the third region the protein load increased further, indicating protein adsorption in multi-layers. After heat treatment at 120°C the protein load in the second region increased towards 20 mg/m^2 at C/A = 20-80 mg/m^2. This value of the protein load seemed not very dependent on the time of heat treatment (up to 20 min). In the third region (C/A > 80 mg/m^2), the protein load was increased up to $\Gamma_{protein}$= 70 mg/m^2 when heated during 20 min at 120°C. Unfortunately, no results were obtained at high C/A for the other heat treatments.

The formation of multi-layers of the caseinate on the oil-droplets on heating in such a caseinate malto-dextrin system at the oil-water surface appears to be prewetting. The results may be due to the presence of malto-dextrins and the caseinate covered oil droplets. The malto-dextrins may induce a segregative type of phase separation or increase the sensitivity towards association or aggregation of the caseinate. From the adsorption isotherm at low C/A can be deduced that in the presence of the oil droplets, the caseinate becomes close to saturation of the adsorbing layer on heating. Moreover, the prewetting may lead to increased sensitivity towards coagulation. However, the heat coagulation times of oil-free caseinate systems containing malto-dextrin (DE 6; 12; 19) were comparable to those of the emulsions (50 g oil/kg), indicating that the presence of the rather low fraction of oil was of minor importance in determining the reaction rate leading to coagulation. However, at high oil fraction, the coagulation rate of these caseinate emulsions will probably be affected by this prewetting phenomenon.

5.3 Effect of fractionation of malto-dextrin

We replaced the malto-dextrin DE 12 by a permeate of this malto-dextrin after ultra-filtration (UF) with a membrane having a cut-off value of 2000 Da. On ultra-filtration, the molecular-weight distribution of the malto-dextrin was changed (Table 5.2). Weight average was lowered from about 20 kDa in the original malto-dextrin towards 3 kDa in the filtrate. Polydispersity M_w/M_n changed from 12.51 to 3.28.

The heat coagulation time (HCT) of the emulsion made from the fractionated malto-dextrin increased at all pH-values measured (Figure 5.3). The results suggest that the molar mass distribution and especially the longer malto-dextrin chains (e.g. > 100 kDa) determine the sensitivity of the caseinate system towards phase separation and enhanced coagulation during heat treatment.

Table 5.2 *Molar mass distribution M by weight of fractions of malto-dextrin (MD) with DE 12 through filtration (membrane cut-off value of 2000 Da).*

M (kDa)	MD (DE 12)	concentrate	filtrate
>1000	-	-	-
500-1000	-	-	-
250-500	0.5	0.5	-
100-250	5	5.5	-
50-100	5	6	-
25-50	10	12	1
10-25	15.5	18	6
5-10	11	11.5	9
<5	53	46.5	84
M_n	1590	1790	967
M_w	19900	22200	3200
M_w/M_n	12.5	12.4	3.28
% > 100 kDa	5.5	6.0	0.01

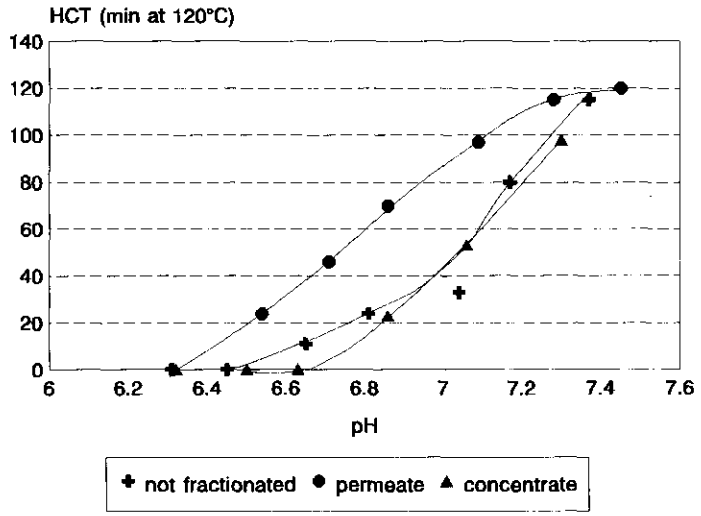

Figure 5.3 *HCT-pH plots of caseinate emulsions (50 g oil/kg) after fractionation of malto-dextrin DE 12 with a membrane of cut-off value 2000; 5 mmol/l citrate.*

The influence of using the permeate of malto-dextrin DE 19 on the surface excess after heating is shown in Figure 5.4. The surface excess in all regions appeared dependent on fractionation. In the second region the protein load increased from 4-6 mg/m^2 in the control and 10 mg/m^2 in the emulsions containing the low molar mass fraction of malto-dextrin, towards 20 mg/m^2 using the whole malto-dextrin. In the third region the surface excess in the fractionated malto-dextrin remained rather low: $\Gamma_{protein} < 20$ mg/m^2.

Besides the chain-length distribution of the malto-dextrin, its concentration seemed to affect the surface excess. At low malto-dextrin DE 19 concentration (50-100 g/kg) the protein surface concentration increased in the second region from $\Gamma_{protein} = 5$ mg/m^2 before heat treatment, to $\Gamma_{protein} = 10$ mg/m^2 after heat treatment. When malto-dextrin concentration was further raised from 166 g/kg to 250 g/kg the protein load in this intermediate region increased from initially $\Gamma_{protein} = 15$ mg/m^2 in the unheated sample to $\Gamma_{protein} = 50$ mg/m^2 after heating. Thus, the composition of the caseinate malto-dextrin mixture solution determines the partition between the serum phase and the droplet interface during heat treatment. These changes induced by the malto-dextrins and the emulsified oil may contribute to the increased susceptibility of caseinate emulsions to instability during heating.

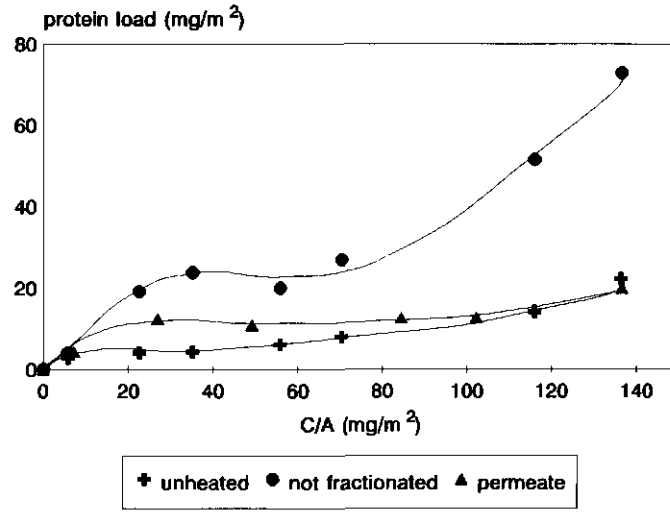

Figure 5.4 *Effect of fractionation of malto-dextrin DE 19 and heating (20 min 120°C) on the surface protein concentration in caseinate emulsions (50 g oil/kg) as a function of the available protein concentration expressed per m^2 oil-droplet-surface.*

5.4 Effect of salts

The tendency towards phase separation of aqueous mixtures of proteins and uncharged polysaccharides is generally very small or absent, due to the entropy of mixing of the counterions. The counterions effect largely disappears, however, on adding salt, thereby increasing the tendency for phase separation. These polyelectrolyte effects are normally important in the region I= 100 mmol/l or higher. Addition of extra salt to such mixtures will generally result in an increase in segregation (Snoeren et al., 1975; Picullel, 1994; Picullel et al., in press; Tolstoguzov, 1991; Tolstoguzov, 1994).

It would be interesting to investigate the effect of ionic strength, since we observed large effects of pH and Ca^{2+}-activity on phase separation and stability during heating in caseinate systems containing either lactose/sucrose mixtures or malto-dextrins. The ionic strength was changed by omitting KCl from the emulsions or by adding more KCl. The ionic strength was calculated from the composition of the filtrate, using a computer program by Holt et al. (1981).

The HCT-pH profiles are given in Figure 5.5 for caseinate emulsions of various ionic strength, containing malto-dextrin DE 19. Lowering the ionic strength increased the stability at pH > 7.3 compared to the reference. When the ionic strength was raised, the stability decreased remarkably over the whole pH range.

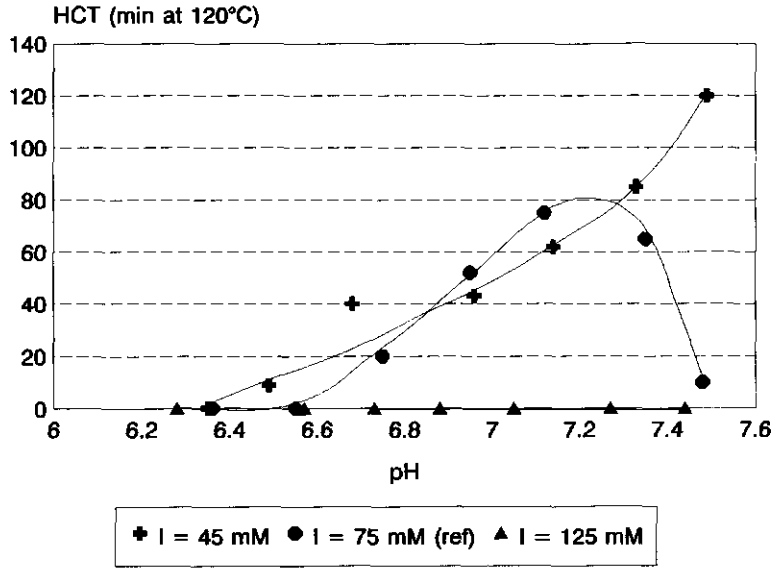

Figure 5.5 *HCT-pH plots of caseinate emulsions (50 g oil/kg) with ionic strength 45, 75 (reference) and 125 mmol/l; malto-dextrin DE 19; 5 mmol/l citrate.*

From measurement of the Ca^{2+}-activity and composition of the filtrate of the emulsion, it could be deduced that the ionic equilibria were altered significantly when the potassium chloride concentration was changed (Table 5.3). The change in Ca^{2+}-activity may therefore be as important as the change in ionic strength itself. The changes in ionic strength, calcium ions and temperature will determine the extent of association of the caseins via electrostatic and hydrophobic forces (Rollema, 1992). The specific binding of calcium to the phosphoserine residues in the polar domain would alter its interaction with the hydrophobic domain, thus allowing some association to occur. The calcium concentration that initiates association and precipitation would decrease with increasing temperature but increase with increasing ionic strength. The caseins would start to precipitate when the number of bound calcium ions is roughly equivalent to the number of phosphoserine residues (Swaisgood, 1992; Rollema, 1992).

Table 5.3 *Calculated and measured Ca^{2+}-activities of caseinate emulsions (50 g oil/kg); malto-dextrin DE 19; 5 mmol/l citrate; pH 6.7.*

Ionic strength (mmol/l)	**Ca^{2+}-activity (mmol/l)**	
	measured	**calculated**
45	1.29	0.63
75	1.71	0.82
125	1.86	1.08

Similar experiments at ambient temperature with oil-free caseinate systems with extra NaCl added (0.15 mol/l) showed the same tendency towards aggregation or demixing of the caseinate on addition of the various malto-dextrins. However, experimental phase diagrams constructed on observations like cloudiness of the solutions could not be produced, since most of the calcium containing caseinate systems were not transparent. Moreover, to improve understanding of the phase behaviour of caseinate malto-dextrin systems during heating, one would need observation of the changes in morphology at 120°C. This seems impossible with current techniques.

5.5 Conclusions

The results obtained give the conditions at which caseinate malto-dextrin mixtures and emulsions thereof are sensitive towards phase separation and coagulation during heating. The phase separation was merely characterised in practical terms of heat coagulation times (HCT), composition of the phases and by microscopy. In these systems it is difficult to decide whether phase separation originates from the polymers

(e.g. caseinates and malto-dextrins) or from the caseinate particles. This may be an intermediate situation. Although the thermo dynamics of such systems is of central importance, the result of phase separation appeared to depend mainly on the kinetics of the phase separation process. However, we were not able to characterize changes in the sizes of the phase droplets of these complicated systems at the temperature of heating. The results illustrate the importance of phase behaviour in caseinate malto-dextrin mixtures. Phase separation results in a system with locally high protein concentration, which appears to have a large effect on heat stability. Furthermore, it appears that the caseinate covered emulsion droplets induce adsorption in multi-layers during heating.

Acknowledgements

I would like to thank Robert Memelink and Bertus Dunnewind for their work on the deposition of protein on the oil droplets in the malto-dextrin containing caseinate emulsions, Hannemieke Luyten, Pauline Kip and Lydia van Mourik for studying phase behaviour in the mixtures and Arthur Kuif for analysis of the molar mass distribution of the malto-dextrins.

Chapter 6

Effect of Lecithin on Coalescence Stability of Caseinate Emulsions

6.1. Introduction

The occurrence of coalescence in any emulsion is mostly undesirable. Droplets merge to form large droplets, and eventually separated oil is seen at the top of the product. Caseinate emulsions are expected to be stable with respect to coalescence in conditions where enough protein is used to cover the emulsions droplets. The adsorbed caseinate layer provides electrostatic and steric repulsion, thus keeping the droplets apart. The surface composition and the protein concentration of the surface layer will therefore be of importance in relation to coalescence (Walstra, 1988; Dalgleish, 1989). Coalescence occurs when the film between emulsion droplets ruptures. This may happen if droplets stay close for a long time, which means that the same factors involved in aggregation or coagulation of the caseinate emulsions are also of importance for coalescence phenomena. However coalescence may also increase when the oil droplets are large and the interfacial tension is low (Walstra, 1988).
On addition of lecithin, stability with respect to coagulation of caseinate emulsions can be improved (see chapter 4). However, when the crude lecithin concentration exceeded about 1.5 g/kg, the emulsion droplets coalesced and simultaneously stability against coagulation decreased dramatically. In this chapter, the effects of lecithins and fractions of lecithins on coalescence of caseinate stabilized emulsions during heating, and possible mechanisms involved will be described.

6.2 Effect of various lecithin fractions.

In this section the results are described of the effect of various soya lecithin fractions on coalescence of caseinate emulsions heated at 120°C. The caseinate emulsions (model B: 40 g/kg soya oil, 40 g/kg caseinate, 120 g/kg carbohydrate, pH 6.7, Ca^{2+}-activity 0.1 mmol/l) were prepared as described in section 2.2. The overall composition is listed in table 2.1. Lecithin was added to the oil at 60-70°C and combined with the caseinate-malto-dextrin solution prior to homogenization. Homogenization was performed before pasteurization at a pressure of 10 MPa.

Coalescence of the phospholipid containing caseinate emulsions was only found when homogenization took place before pasteurization. A similar effect was observed in UHT processing of cream. When homogenization was performed before (UHT) heat treatment, coagulation occurred during sterilization, and not when the emulsion was homogenized afterwards. This effect was attributed to adsorption of caseins on the oil droplets during homogenization and subsequent coagulation of the casein

covered globules during heat treatment (Melsen & Walstra, 1989; van Boekel & Walstra, 1995). However, in our samples only coalescence and no coagulation was observed. It is clear that the homogenization step is essential in stability of the phospholipid containing emulsions and that the stability may be determined by the droplet surface layer.

The results presented in Fig. 6.1 show that in the absence of lecithin the caseinate emulsions were stable towards coalescence during heating. On addition of crude soya lecithin prior to homogenization, coalescence markedly increased. In the unheated emulsions all droplets were smaller than 5 μm, as observed by microscope. After heat treatment of lecithin containing emulsions droplets up to 20 μm were observed. Surprisingly, the average droplet particle size reached a plateau value during heating. In most systems described in literature, the average globule size gradually increased with time, roughly following first order kinetics. Above a certain "critical size" coalescence may be accelerated and the emulsion breaks (Boyd, Parkinson & Sherman, 1972; van Boekel & Walstra, 1981; Dickinson, Murray & Stainsby, 1988; Das & Kinsella, 1990). In this study, however, rate of coalescence seemed independent of initial droplet size. On varying homogenization pressure (5-10 MPa), resulting in different droplet sizes, no difference in rate of coalescence was found. The coalescence seemed hardly accelerated by heat-induced chemical changes, such as dephosphorylation of the caseinate and decrease of pH. Rate of coalescence appeared, if anything, to correlate negatively with rate of dephosphorylation or pH-decrease. Moreover, no coagulation was observed during prolonged heat treatments in all conditions studied.

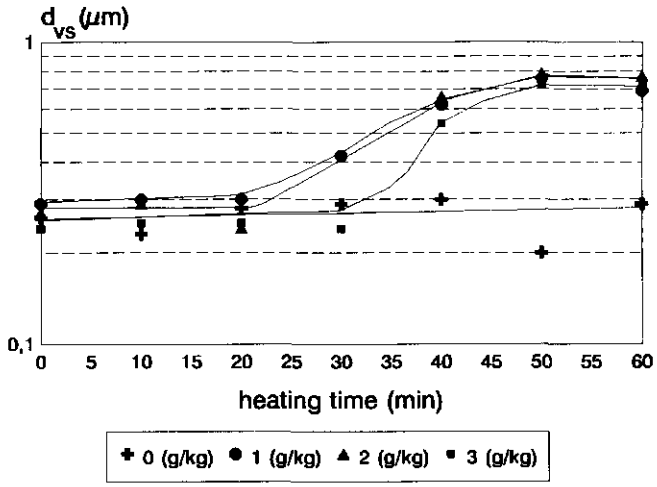

Figure 6.1 *Effect of crude soya lecithin on coalescence of caseinate emulsions during heating at 120°C, (0-3 g phospholipids/kg). d_{vs} was determined by spectroturbidimetry (Walstra, 1968).*

It is conceivable that during emulsification, adsorption of lecithin and caseinate results in two different types of interfacial layers: (i) the lecithin is adsorbed at the interface and the caseinate on top, or (ii) caseinate and lecithin are both in contact with the oil. The minimum surface coverage for protein stabilized emulsions is about 1 mg/m^2. Below this value droplets mostly aggregate and coalesce. The maximum adsorption of whole caseinate in different conditions of pH and ionic strength is about 3-4 mg/m^2 (Tornberg, 1978; Walstra,1988; Dickinson, Murray & Stainsby, 1988; Fang & Dalgleish, 1993a; Courthaudon et al., 1991; Mertens, 1989). The protein coverage found in the lecithin-free product was $\Gamma_{protein}$= 1.8 mg/m^2. This surface protein concentration $\Gamma_{protein}$(in mg/m^2) is generally primarily determined by C/A (in mg/m^2), where C is the protein concentration in the emulsion and A is the specific surface area ($A = 6 * \varphi / d_{vs}$, where φ is volume fraction of oil). Here, C = 40 mg/ml; $A = 6 * 0.04 / 0.48 = 0.5$ m^2/ml and C/A = 80 mg/m^2. Despite the relatively high concentration of caseinate per unit oil surface area C/A the surface protein concentration was lower than the plateau values found in the studies cited, where C/A < 10 mg/m^2. This difference may be attributed to the homogenization conditions or to uncertainties in the methods of particle size analysis.

The phospholipids may adsorb preferentially at the surface, because they can yield lower interfacial tension than the caseinates. The interfacial tension between most protein solutions and triglyceride oil is about > 10 mN m^{-1}. Phospholipids can reduce interfacial tension more, if present in sufficient quantities. When different phospholipids (0.02-0.7 g/kg) were added, interfacial tension was reduced to less than 5 mN m^{-1} at a macroscopic o/w interface (van der Meeren et al., 1995). The phospholipids are therefore supposedly located at the interface. Indeed, on addition of lecithin, the droplet size upon homogenization was decreased (e.g. from d_{vs}= 0.48 μm to d_{vs}= 0.29 μm), indicating that the effective interfacial tension during emulsification was lower.

It was thought that on addition of lecithin, the properties of the emulsion and the surface layer in particular were affected. On addition of crude lecithin, the surface protein concentration $\Gamma_{protein}$ was 0.7 mg/m^2. This seemed hardly to depend on lecithin concentration (1-3 g/kg). After a heat treatment of 60 min at 120°C, $\Gamma_{protein}$ was 0.4-0.9 mg/m^2. Without lecithin, the surface protein concentrations before and after heating were 1.8 and 4.2 mg/m^2, respectively. Changes in composition of individual caseinates in the surface layer were not determined in the samples with crude lecithin, but only in the samples with a phosphatidylcholine-rich fraction; this is reported in the next section. We also tried to measure the amounts of phospholipids at the interface. After centrifugation (44 000 g, 1 h) all phospholipids were found in the cream layer, indicating that the phospholipids were associated with the emulsion droplets and not with the serum phase. From this, the phospholipid loads at 1, 2 and 3 g of phospholipids/kg were assumed to be $\Gamma_{phospholipids}$ = 0.8, 1.6 and 2.4 mg/m^2, respectively.

According to Fang & Dalgleish (1993b) the surface coverage for a close-packed phospholipid monolayer will be about 1.75 mg/m^2. If it is assumed that all the phospholipids are adsorbed at the oil surface in a monolayer, it can be calculated from the phospholipids load that the maximum oil surface covered by phospholipids for 1, 2 and 3 g of lecithin/kg would have been 45%, 90% and >100%, respectively. This would indicate that a mixed layer of phospholipids and caseinates was formed when less than 2 g of lecithin/kg had been added. From 3 g of lecithin/kg onwards, either a mixed layer, or a phospholipids layer with caseinate on top may be formed.

It is postulated that when both phospholipids and caseinates were present in the interfacial layer (in about equal amounts), the low interfacial tension and rather low protein surface coverage, which would diminish inter-droplet repulsion may cause coalescence. However, interfacial tensions at the oil-water interface are not known for various phospholipids, especially not at emulsion droplets. Moreover interfacial tensions are not known in combination with caseinates and also not at higher temperature.

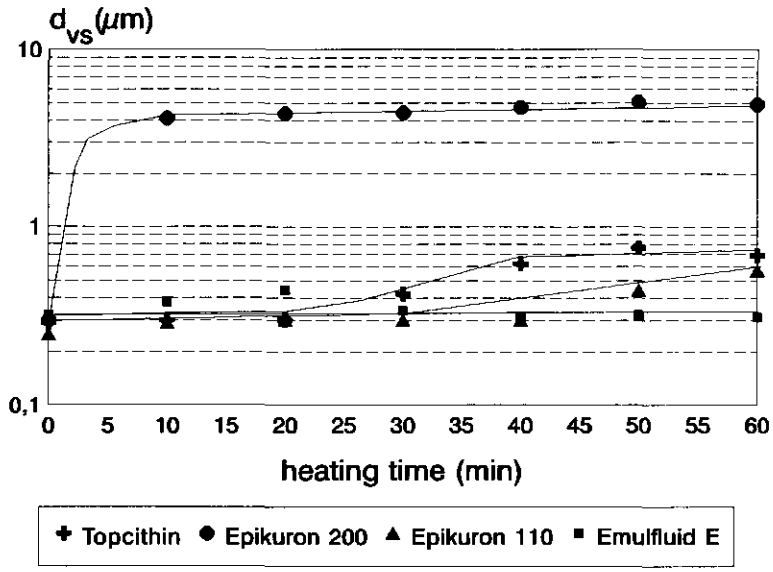

Figure 6.2 *Effect of different soya lecithin fractions (1.3 g phospholipids/kg) on coalescence of caseinate emulsions during heating at 120°C. d_{vs} was determined by spectroturbidimetry (Walstra, 1968).*

Addition of various lecithin fractions gave different rates of coalescence (Fig 6.2). Addition of lecithin fractions was corrected for phospholipid content to obtain the same concentration in the emulsions (1.3 g phospholipids/kg). Incorporation of the hydrolysed lecithin (Emulfluid E) and a polar fraction (Epikuron 110), resulted in a somewhat lower rate of coalescence as compared to the crude soya lecithin (Topcithin), whereas the phosphatidylcholine-rich fraction (Epikuron 200) caused increased coalescence. The initial droplet size (d_{vs} = 0.25-0.32 μm), the initial caseinate surface load ($\Gamma_{protein}$ 0.7-1.4 mg/m^2) and caseinate loads after heat treatment of 60 min at 120°C ($\Gamma_{protein}$ 0.8-1.6 mg/m^2) seemed hardly to depend on the type of lecithin used. Van der Meeren et al., (in press) found in emulsions stabilized by soya lecithins only, or fractions derived from it, a slight positive correlation between anionic phospholipid content and their ability to stabilize the emulsion with respect to coalescence at ambient temperature. It was also suggested that the presence of some minor components, such as glycolipids could affect coalescence rate.

Our results indicate that coalescence stability of caseinate emulsions depends on the type of lecithin fraction added. Probably, the mechanism involved in stabilization of the caseinate emulsions varies somewhat among phospholipids fractions, maybe because the residues coupled to the phosphate group differ. Moreover, environmental conditions may affect repulsive forces caused by the phospholipids and the sensitivity towards coalescence. It has been found that the effect of salts (sodium chloride and calcium chloride) and pH on behaviour of emulsions containing only various lecithins (phosphatidylcholine, phosphatidic acid) could almost quantitatively be explained by reduction of the negative charge; addition of salts and lowering of pH resulted in aggregation, followed by coalescence of the droplets (Washington, 1990; Bergenståhl, 1988; van der Meeren et al, in press). If we accept the mixed caseinate-phospholipids structure described before, we may get an idea of the contribution of different phospholipids on repulsive forces. The polar phospholipids would cause stronger repulsion than the more apolar phospholipids, in conditions of rather low ionic strength. Since phospholipid covered surfaces would create strong electrostatic repulsive forces only at short range (3 nm), addition of phospholipids would generally decrease repulsion between caseinate-stabilized droplets, and give relatively unstable emulsions.

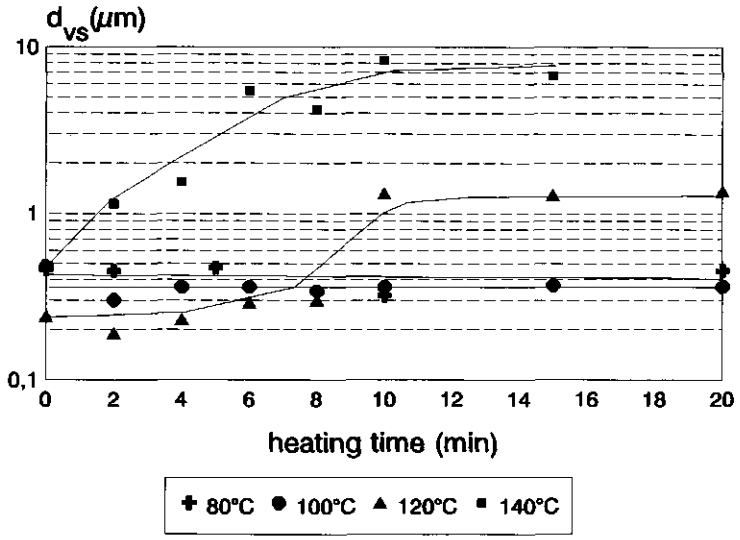

Figure 6.3 *Effect of heating on coalescence of phosphatidylcholine containing caseinate emulsions during heating at 80, 100, 120 and 140°C. d_{vs} was determined by spectroturbidimetry (Walstra, 1968).*

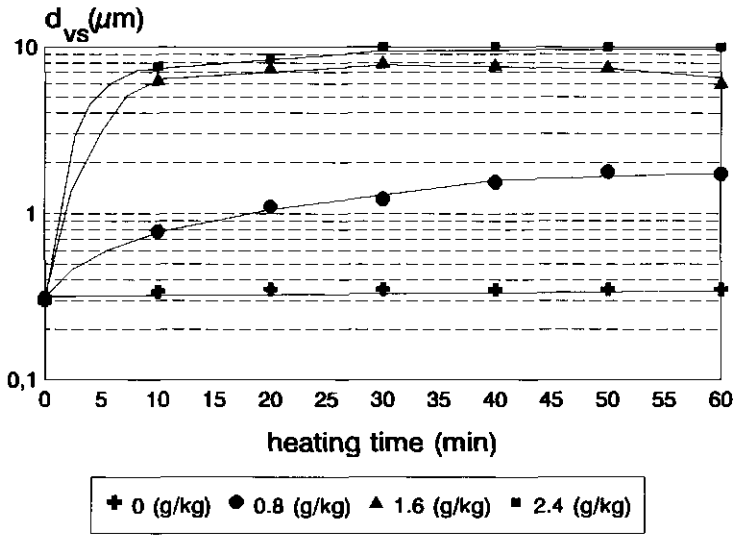

Figure 6.4 *Effect of phosphatidylcholine on coalescence of caseinate emulsions during heating at 120°C.d_{vs} was determined by spectroturbidimetry (Walstra, 1968).*

6.3 Effect of phosphatidylcholine

Phosphatidylcholine (PC) is present in high proportion (about 50%) in crude soya lecithin. In other sources its content can be even higher. In egg lecithin a PC concentration of about 80 percent was reported (McCrae & Muir, 1992). It was found that the phosphatidylcholine rich fraction Epikuron 200 (>99% PC) induced destabilisation enormously (see Fig 6.2). The effect of heating temperature on coalescence of a PC containing (0.8 g/kg) caseinate emulsion is illustrated in Fig 6.3. Coalescence was only observed in the emulsions heated at 120°C and 140°C. Coalescence of the emulsions droplets did not show first order kinetics, but proceeded in a manner depending on temperature. Boyd, Parkinson & Sherman (1972) and Alander & Warnheim (1989) suggested that the rate of coalescence in low molecular weight surfactant stabilized emulsions at a certain temperature is related to the so called phase inversion temperature (PIT). The phenomenon arises from the temperature dependent hydrophilicity of the surfactant, which determines its solubility in the oil and water phases. At the phase inversion temperature the solubility of the surfactant in oil and water is about equal, which goes along with very low interfacial tensions and an oil-in-water emulsion will be transferred in a water-in-oil type of emulsion.

Phosphatidylcholine (0.8-2.4 g/kg) induced more pronounced coalescence than crude soya lecithin (compare Figures 6.1 and 6.4). The effect of PC concentration on emulsion stability was also judged from creaming in an accelerated creaming test, described in section 2.5.3 (Table 6.1). We see that if the mean droplet size d_{vs} had increased during heat treatment from initially 0.3 μm to 1.7 μm or higher, the oil content in the lower layer decreased enormously. Hence, the addition of PC caused considerable change in the homogeneity of the emulsion.

Table 6.1 *Effect of phosphatidylcholine concentration on particle size and creaming of caseinate emulsion. The accelerated creaming test (40°C; 30 min. 200 g) was performed before and after heat treatment (60 min 120°C) and creaming was normalized on initial creaming, where ϕ is the proportion of oil which was not creamed. Initial droplet size d_{vs}= 0.3 μm.*

phosphatidyl-choline (g/kg)	$d_{vs,60}$ (μm)	ϕ_0 (-)	ϕ_{60} (-)	ϕ_{60}/ϕ_0 (-)
0.0	0.35	0.70	0.71	1.01
0.8	1.72	0.76	0.11	0.14
1.6	6.13	0.75	0.03	0.04
2.4	10.0	0.74	0.04	0.05

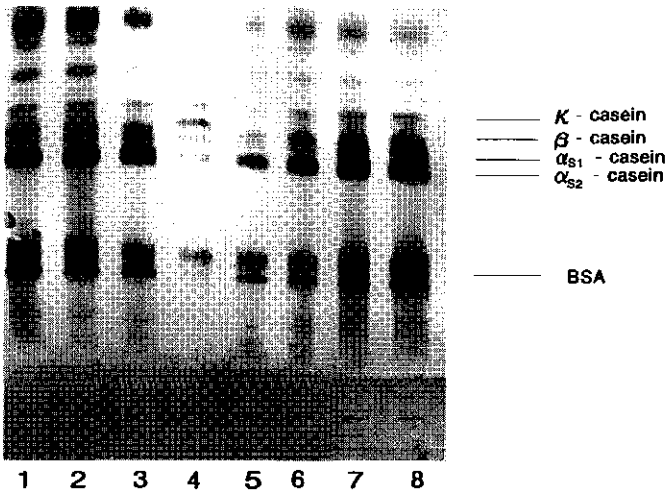

Figure 6.5 *SDS-PAGE pattern of whole caseinate (lane 4) and surface caseinate obtained after desorption with SDS from caseinate emulsions with 0 g/kg PC (lanes 1 and 2); 0.8 g/kg PC (lane 3); 1.6 g/kg PC (lanes 5 and 6); 2.4 g/kg PC (lanes 7 and 8);. BSA was added as standard.*

Table 6.2 *Effect of calcium concentration on coalescence of caseinate emulsions with phosphatidylcholine (0.8 g/kg) during heating (20 min 120°C).*

calcium (mmol/l)	**$d_{vs,20/dvs,0}$ (-)**	**ϕ_{20}/ϕ_0 (-)**	**$\Gamma_{protein,0}$ (mg/m^2)**
2	4.7	0.09	1.0
5	1.3	0.79	1.4
10	1.5	0.67	2.9

It was investigated whether changes in interfacial composition occurred during heating. The caseinate at the surface was initially $\Gamma_{protein}$= 1.0 mg/m^2 at 0.8 g phosphatidylcholine/kg and seemed hardly affected by heating, which was also found for crude lecithin. Again it seemed that the sensitivity towards coalescence was related to low interfacial protein coverage.
The composition of the individual caseins in the surface layer seemed hardly affected by phosphatidylcholine addition (Fig 6.5). After desorption of the surface layers with SDS and analysis with SDS-gel electrophoresis with added DTT, we found in all emulsions that the proportion of α_{s2}-casein was high compared to whole caseinate while that of β-casein was relatively low and that of α_{s1}- and κ-casein remained unaffected.

Phospholipid containing caseinate emulsions seemed more susceptible to coalescence when the calcium content was low and Ca^{2+}-activity may therefore (indirectly) be a factor determining coalescence. The response of the PC containing caseinate emulsion to calcium was high (Table 6.2). Coalescence of the emulsion was already prevented when calcium chloride was added at concentrations of 5 and 10 mmol/l calcium. These calcium concentrations (2, 5 and 10 mmol/l) correspond to Ca^{2+}-activities of 0.10, 0.36 and 1.10 mmol/l respectively. The calcium addition seemed also positively correlated with the initial surface protein load and probably therefore with repulsion from the caseinate residues at the interface.

It appears that the effect of Ca^{2+}-activity on coalescence is through increasing surface protein load, which would increase steric and electrostatic repulsion. This seems more important than screening of the negative groups at the caseinate or the phospholipids, which would reduce electrostatic repulsion. Mertens (1989) also found in phospholipid-free caseinate emulsions (250 g oil/kg; 2 g protein/kg; pH 5-9) that coalescence stability at ambient temperature was better correlated with surface protein concentration than with (possibly increased) electrostatic repulsion at high pH , although small differences in protein load $\Gamma_{protein}$ (3.2-3.5 mg/m^2) were measured. At low protein coverage $\Gamma_{protein}$ (1-2 mg/m^2), electrostatic interactions seemed more important. In the same set of experiments Mertens found that at low surface protein concentration, coalescence rate increased on increasing salt concentration (from 0-200 mmol/l). It was suggested that in these conditions the decreased electrostatic repulsion at high ionic strength was responsible for this increased sensitivity to coalescence.
We found a significant decrease in coalescence rate (estimated by particle sizing and accelerated creaming test) when pH was raised from pH 6.5 to 7.5 both in phosphatidylcholine and crude lecithin containing caseinate emulsions ($\Gamma_{protein}$= 1 mg/m^2). From these observations it appears that coalescence of caseinate emulsions may be susceptible to changes in ionic strength or pH in conditions where caseinate surface load is low or absent. This was further demonstrated in systems where the interfacial layer was almost depleted from caseinate, which could be achieved with emulsions having a low caseinate content or when the caseinate seemed displaced by

adding excess phospholipids (Courthaudon et al., 1991; Dickinson, Murray & Stainsby, 1988; Mertens, 1989). Courthaudon et al. (1991) found that the casein in β-casein emulsions (soya oil, 200 g/kg; protein, 4 g/kg) was displaced above a molar ratio of lecithin to protein of 16 (A = 1.5 m^2/ml). In our caseinate emulsions this ratio was only between 0.5 and 2 (A = 0.5 m^2/ml), indicating that compositions were far different.

In other laboratories it was found that when the interfacial layer consisted only of phospholipids, stability was decreased by raising the ionic strength (van der Meeren et al, in press). At the same time the interfacial tension (between iso-octane and water) was reduced from about 5 mNm^{-1} in pure water to less than 1 mNm^{-1} when ionic strength was raised to 100 mM . It was suggested that the lowering of the interfacial tension on addition of salt was the major cause of coalescence (Hofman & Stein, 1991; Gaonkar & Borwankar, 1991; van der Meeren et al, 1995). Coalescence of these lecithin stabilized emulsions seemed also very pH dependent. When initial pH was below pH 8 the phosphatidylcholine containing emulsion droplets coalesced during heat treatment (20 min 121°C) (Chaturvedi, Patel & Lodhi, 1992). During this heat treatment the initial pH 8 dropped to pH 5.5, probably leading to less repulsion of the emulsion droplets and coalescence.

Returning to our caseinate emulsions, we studied their stability upon addition of chemical reagents such as formaldehyde and dithiothreitol. Although these additives are not legally permitted, they can give more insight in the mechanism of coalescence of caseinate emulsions. Addition of formaldehyde (25 mmol/l) to crude lecithin and phosphatidylcholine containing caseinate emulsions gave a better stability during heat treatment ($d_{vs,20/dvs,0}$ about 1.5). The protein surface load was not affected by addition of formaldehyde. Therefore, it seemed that the explanation for the stabilizing action of formaldehyde was not due to formation of (intra)molecular cross-links or increased association. The explanation of the action of formaldehyde may be found in an increase of charge on caseinate residues (Aoki & Kako, 1984; Singh & Fox, 1985). A destabilizing effect during heat treatment was obtained on addition of dithiothreitol (0.5 mmol/l). Dithiothreitol was added to disrupt all possible S-S bridges involving α_{s2}- and κ-casein. As a consequence, part of the caseinate possibly dissociated from the interfacial layer so that the droplets became unstable and coalesced. However, we have no experimental evidence, e.g. from determination of surface protein load, to support this view. Our view that the stability of the phospholipid containing caseinate emulsions is mainly governed by repulsion (electrostatic and steric) of the caseinate is supported by the observations that agents which may increase both charge and association of the caseinate improved coalescence stability whereas an agent that may dissociate caseinate decreased stability.

6.4. Conclusions

From the above results it may be deduced that phospholipids give less stable caseinate emulsions during heating. Phospholipids may cause less adsorption of caseinate or possibly adsorption of caseinate in another way, which may largely affect repulsion between the emulsion droplets. The interfacial tension when using such caseinate phospholipid mixtures is an intricate subject, and it may certainly be important in affecting coalescence during heating. Clearly, further work would be required to investigate the effect of modification of the surface layer by phospholipids. It should be noted that all factors mentioned above may depend on type of phospholipids and the way they are added to the caseinate emulsions.

Acknowledgements

Cecile Peeters and Marjolein v.d. Krogt are thanked for assistance in the determination of coalescence of lecithin containing caseinate emulsions during heat treatment.

Appendix 1

Partitioning of calcium, magnesium, sodium, potassium, inorganic phosphate, citrate and chloride in caseinate emulsions (50 g oil/kg), containing 5-20 mmol/l citrate, pH 6.8-6.9 at various heating conditions. Values were recalculated to mmol/g protein. Total amounts of individual salt were calculated in mmol/l water.

	Ca	Mg	Na	K	P_i	Cit	Cl
	(mmol/g protein)						
total							
(mmol/l water)	**17**	**10**	**44**	**40**	**3**		
(mmol/g protein)	**0.23**	**0.14**	**0.59**	**0.55**	**0.04**		
citrate (6 mmol/l water)/chloride (61 mmol/l water)							
20°C	0.15	0.08	0.08	0.10	0.01	-0.02	-0.02
40°C	0.16	0.08	0.07	0.11	0.01	-0.01	0.01
60°C	0.18	0.07	0.06	0.06	0.02	-0.02	-0.04
80°C	0.18	0.08	0.10	0.11	0.02	0.00	0.04
heating at 120°C[1]							
20 minutes	0.15	0.08	0.08	0.02	0.01	-0.02	-0.03
60 minutes	0.13	0.07	0.11	0.14	0.03	0.01	0.09
citrate (12 mmol/l water)/chloride (43 mmol/l water)							
20°C	0.12	0.07	0.13	0.15	0.01	0.01	0.01
40°C	0.12	0.06	0.10	0.11	0.01	-0.01	-0.03
60°C	0.13	0.05	0.07	0.08	0.01	-0.02	-0.06
80°C	0.15	0.05	0.11	0.14	0.01	0.01	-0.01
heating at 120°C[1]							
20 minutes	0.10	0.05	0.09	0.04	0.00	0.00	0.01
60 minutes	0.10	0.05	0.12	0.16	0.03	0.02	0.05
citrate (18 mmol/l water)/chloride (30 mmol/l water)							
20°C	0.09	0.05	0.17	0.17	0.00	-0.02	-0.01
40°C	0.09	0.04	0.14	0.15	0.00	-0.03	-0.02
60°C	0.11	0.04	0.14	0.14	0.01	-0.03	-0.02
80°C	0.12	0.04	0.15	0.17	0.01	0.01	0.00
heating at 120°C[1]							
20 minutes	0.09	0.05	0.16	0.12	0.00	-0.01	0.00
60 minutes	0.08	0.05	0.13	0.16	0.03	0.02	0.05
citrate (24 mmol/l water)/chloride (24 mmol/l water)							
20°C	0.06	0.04	0.16	0.24	0.00	0.04	0.01
40°C	0.06	0.03	0.15	0.22	0.00	0.01	0.00
60°C	0.07	0.03	0.16	0.24	0.00	0.02	0.01
80°C	0.09	0.03	0.17	0.25	0.00	0.04	0.01
heating at 120°C[1]							
20 minutes	0.06	0.04	0.15	0.23	0.01	0.05	0.01
60 minutes	0.06	0.04	0.14	0.24	0.03	0.08	0.04

[1] Filtered at 20°C

[2] Coefficient of variation for the respective analyses: Ca 5%; Na 5%; K 5%; Mg 5%; $P_{inorganic}$ 1%; citrate 5%; Cl 1%.

Appendix 2

Calculated activities of calcium, magnesium, citrate and anorganic phosphate from composition of filtrate (20°C) of unheated and heated caseinate emulsions (50 g oil/kg) containing 5-20 mmol/l citrate, with a computer program based on the model of Holt et al., (1981) and activity products of $Ca_3Citrate_2$, $Mg_3Citrate_2$, $CaHPO_4$, $MgHPO_4$.

Sample	pH	$a(Ca^{2+})^1$ (mmol/kg)	$a(Ca^{2+})$ (mmol/kg)	$a(Mg^{2+})$ (mmol/kg)	$a(Cit^{3-})$ (mmol/kg)	$a(HPO_4^{2-})$ (mmol/kg)	$a(Ca^{2+})^3a(Cit^{3-})^2$ (mol^5/kg^5)	$a(Mg^{2+})^3a(Cit^{3-})^2$ (mol^5/kg^5)	$a(Ca^{2+})a(HPO_4^{2-})$ (mol^2/kg^2)	$a(Mg^{2+})a(HPO_4^{2-})$ (mol^2/kg^2)	Ionic strength (mmol/l)
unheated											
Citrate											
(6 mmol/l)	6.8	1.5	0.89	0.74	0.02	0.20	$3.5\ 10^{-19}$	$2.0\ 10^{-19}$	$1.8\ 10^{-7}$	$1.5\ 10^{-7}$	81
(12 mmol/l)	6.9	0.9	0.47	0.39	0.07	0.19	$4.7\ 10^{-19}$	$2.7\ 10^{-19}$	$0.9\ 10^{-7}$	$0.7\ 10^{-7}$	68
(18 mmol/l)	6.8	0.6	0.17	0.14	0.30	0.27	$4.3\ 10^{-19}$	$2.4\ 10^{-19}$	$0.5\ 10^{-7}$	$0.4\ 10^{-7}$	71
(26 mmol/l)	6.9	0.3	0.09	0.07	0.70	0.27	$3.6\ 10^{-19}$	$1.7\ 10^{-19}$	$0.2\ 10^{-7}$	$0.2\ 10^{-7}$	84
20 min 120°C											
Citrate											
(6 mmol/l)	n.d.	n.d.	0.92	0.77	0.03	0.19	$5.0\ 10^{-19}$	$2.9\ 10^{-19}$	$1.8\ 10^{-7}$	$1.5\ 10^{-7}$	81
(12 mmol/l)	n.d.	n.d.	0.71	0.58	0.06	0.25	$11.0\ 10^{-19}$	$6.1\ 10^{-19}$	$0.9\ 10^{-7}$	$0.7\ 10^{-7}$	68
(18 mmol/l)	n.d.	n.d.	0.26	0.22	0.21	0.25	$7.8\ 10^{-19}$	$4.7\ 10^{-19}$	$0.5\ 10^{-7}$	$0.4\ 10^{-7}$	71
(26 mmol/l)	6.6	0.4	0.15	0.12	0.44	0.43	$6.9\ 10^{-19}$	$3.7\ 10^{-19}$	$0.2\ 10^{-7}$	$0.2\ 10^{-7}$	84
60 min 120°C											
Citrate											
(6 mmol/l)	6.1	1.8	1.23	0.95	0.02	0.27	$6.2\ 10^{-19}$	$2.9\ 10^{-19}$	$3.4\ 10^{-7}$	$2.6\ 10^{-7}$	81
(12 mmol/l)	6.1	1.2	0.73	0.59	0.06	0.31	$13.0\ 10^{-19}$	$7.2\ 10^{-19}$	$2.3\ 10^{-7}$	$1.8\ 10^{-7}$	73
(18 mmol/l)	6.1	0.9	0.39	0.32	0.16	0.34	$14.0\ 10^{-19}$	$7.8\ 10^{-19}$	$1.3\ 10^{-7}$	$1.1\ 10^{-7}$	72
(26 mmol/l)	6.1	0.5	0.20	0.16	0.39	0.37	$13.0\ 10^{-19}$	$6.9\ 10^{-19}$	$0.7\ 10^{-7}$	$0.6\ 10^{-7}$	81

[1] Ca^{2+}-activity and pH measured with ion-selective electrode in the emulsion. The other activities were calculated. Carbonate was not accounted for in the calculation. At ionic strength values calculated here, the activity coefficient γ, calculated according to the Debye Hűckel theory, was 0.80; 0.42 and 0.14 for mono-, bi- and trivalent ions, respectively (Chang, 1981).

Appendix 3

Partitioning of calcium, magnesium, sodium, potassium, inorganic phosphate and chloride in caseinate emulsions (50 g oil/kg) containing 5-20 mmol/l inorganic phosphate at various heating conditions. Values were recalculated to mmol/g protein. Total amounts of individual salts were calculated to mmol/l water.

	Ca	Mg	Na	K	$P_{inorganic}$	Cl
	(mmol/g protein)					
total						
(mmol/l water)	**17**	**10**	**47**	**41**		
(mmol/g protein)	**0.22**	**0.13**	**0.63**	**0.54**		
$P_{inorganic}$ (9 mmol/l water)/chloride (76 mmol/l water)						
20°C	0.18	0.10	0.10	0.09	0.05	0.01
40°C	0.18	0.10	0.13	0.13	0.05	0.09
60°C	0.19	0.11	0.12	0.10	0.05	0.08
80°C	0.20	0.11	0.04	0.04	0.06	-0.04
heating at 120°C[1]						
20 minutes	0.18	0.09	0.08	0.08	0.05	0.03
60 minutes	0.18	0.09	0.11	0.12	0.05	0.13
$P_{inorganic}$ (16 mmol/l water)/chloride (62 mmol/l water)						
20°C	0.19	0.10	0.11	0.11	0.08	(0.13)
40°C	0.19	0.10	0.08	0.08	0.08	-0.02
60°C	0.20	0.11	0.10	0.09	0.09	-0.03
80°C	0.21	0.12	0.05	0.06	0.10	-0.05
heating at 120°C[1]						
20 minutes	0.18	0.08	0.08	0.10	0.07	0.03
60 minutes	0.17	0.08	0.09	0.10	0.06	0.06
$P_{inorganic}$ (22 mmol/l water)/chloride (48 mmol/l water)						
20°C	0.20	0.10	0.10	0.09	0.11	-0.02
40°C	0.20	0.11	0.08	0.08	0.11	-0.04
60°C	0.21	0.12	0.10	0.08	0.12	-0.03
80°C	0.21	0.12	0.15	0.13	0.15	0.04
heating at 120°C[1]						
20 minutes	0.18	0.09	0.09	0.08	0.11	-0.04
60 minutes	0.17	0.08	0.07	0.07	0.13	-0.03
$P_{inorganic}$ (28 mmol/l water)[4]chloride (35 mmol/l water)						
20°C	0.21	0.11	0.08[2]	0.13[3]	0.14	-0.01
40°C	0.21	0.11	0.08[2]	0.12[3]	0.13	-0.01
60°C	0.22	0.12	0.08[2]	0.12[3]	0.14	-0.01
80°C	0.22	0.12	0.07[2]	0.11[3]	0.14	-0.02
heating at 120°C[1]						
20 minutes	0.19	0.10	0.10[2]	0.14[3]	0.12	0.01
60 minutes	0.18	0.09	0.09[2]	0.13[3]	0.09	0.02

[1] Filtered at 20°C

[2] Total sodium content (39 mmol/l water)

[3] Total potassium content (47 mmol/l water)

[4] Coefficient of variation for the respective analyses: Ca 5%; Na 5%; K 5%; Mg 5%; $P_{inorganic}$ 1%; citrate 5%; Cl 1%.

Appendix 4

Calculated activities of calcium, magnesium and anorganic phosphate from composition of filtrate (20°C) of unheated and heated caseinate emulsions (50 g oil/kg) containing 5-20 mmol/l inorganic phosphate, with a computer program based on the model of Holt et al., (1981) and activity products of $CaHPO_4$, $MgHPO_4$.

Sample	pH	$a(Ca^{2+})$[1] (mmol/kg)	$a(Ca^{2+})$ (mmol/kg)	$a(Mg^{2+})$ (mmol/kg)	$a(HPO_4^{2-})$ (mmol/kg)	$a(Ca^{2+})a(HPO_4^{2-})$ (mol^2/kg^2)	$a(Mg^{2+})a(HPO_4^{2-})$ (mol^2/kg^2)	Ionic strength (mmol/l)
unheated **Phosphate**								
(9 mmol/l)	6.7	1.4	0.76	0.84	0.39	$3.0\ 10^{-7}$	$3.3\ 10^{-7}$	85
(16 mmol/l)	6.8	1.0	0.54	0.69	0.84	$4.5\ 10^{-7}$	$5.8\ 10^{-7}$	81
(22 mmol/l)	6.9	0.7	0.40	0.60	1.44	$5.8\ 10^{-7}$	$8.7\ 10^{-7}$	81
(28 mmol/l)	6.9	0.5	0.28	0.47	2.15	$6.0\ 10^{-7}$	$10.1\ 10^{-7}$	78
20 min 120°C **Phosphate**								
(9 mmol/l)	6.5	1.5	0.87	0.89	0.60	$5.2\ 10^{-7}$	$5.3\ 10^{-7}$	85
(16 mmol/l)	6.6	1.1	0.55	0.70	1.05	$5.8\ 10^{-7}$	$7.4\ 10^{-7}$	79
(22 mmol/l)	6.6	0.7	0.39	0.55	1.47	$5.8\ 10^{-7}$	$8.1\ 10^{-7}$	71
(28 mmol/l)	6.7	0.6	0.30	0.45	2.05	$6.1\ 10^{-7}$	$9.3\ 10^{-7}$	70
60 min 120°C **Phosphate**								
(9 mmol/l)	6.2	1.7	0.98	1.04	0.36	$3.5\ 10^{-7}$	$3.8\ 10^{-7}$	81
(16 mmol/l)	6.3	1.2	0.79	0.93	0.55	$4.3\ 10^{-7}$	$5.1\ 10^{-7}$	82
(22 mmol/l)	6.3	0.9	0.62	0.81	0.77	$4.8\ 10^{-7}$	$6.3\ 10^{-7}$	76
(28 mmol/l)	6.3	0.8	0.56	0.74	1.04	$5.8\ 10^{-7}$	$7.7\ 10^{-7}$	74

[1] Ca^{2+}-activity and pH measured with ion-selective electrode in the emulsion. The other activities were calculated. Carbonate was not accounted for in the calculation. At ionic strength values calculated here, the activity coefficient γ, calculated according to the Debye Hűckel theory, was 0.80; 0.42 and 0.14 for mono-, bi- and trivalent ions, respectively (Chang, 1981).

Appendix 5

The effective volume fraction of particles φ can be calculated from the Eilers equation and measurement of viscosity η. (Walstra & Jenness, 1984). For η_0 figures on concentrated milk were taken, φ_m was assumed to be 0.79 (Nieuwenhuijse et al., 1992)

$$\eta = \eta_0 \left(1 + \frac{1.25 \cdot \varphi}{1 - \varphi/\varphi_m} \right)^2$$

Volume fraction of caseinate particles in caseinates emulsions: oil (0-100g/kg), calculated from viscosity η at 20°C with controlled stress rheometer (Carrimed CSl 100) at shear rate of 10-100 s^{-1} (measurements not shown).

oil (g/kg)	η (mPa.s)	φ_0
0	8.8	0.47
50	12.5	0.50
100	19.3	0.56
150	26.3	0.59
200	37.5	0.62
250	53.0	0.65

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List of symbols

a	activity	mol/l
a_d	radius of emulsion droplet	m
A	Area	m^2
A_h	composite Hamaker constant	J
C	concentration	g/kg or mol/l
d_{vs}	volume-surface average diameter	m
E	optical density	-
g	acceleration due to gravity	m/s^2
G_a	London van der Waals interaction energy	J
G_r	electrostatic interaction energy	J
h	droplet separation distance	m
I	ionic strength	mol/l
k	Boltzmann constant	J/K
M_n	number average molecular weight	Da
M_w	weight average molecular weight	Da
T	temperature	°C or °K
α	fraction of encounters leading to coalescence	-
Γ	surface load	mg/m^2
γ	activity coefficient	-
η	viscosity	Pa.s
κ	inverse of the electric double layer thickness	m^{-1}
ϕ	proportion of oil which was not creamed	-
ψ_0	surface potential	V
φ	effective volume fraction	-

Abbreviations

BSA	bovine serum albumin
DE	dextrose equivalent
DHA	dehydroalanine
DTT	dithiothreitol
EDTA	ethylene-diamine-tetra-acetate
FPLC	fast protein liquid chromatography
GPC	gel permeation chromatography
HAL	histidinoalanine
HCT	heat coagulation time
HMF	5-hydroxymethyl-2-furaldehyde
HPLC	high performance liquid chromatography
LAL	lysinoalanine
NMR	nuclear magnetic resonance
MD	malto-dextrin
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PIT	phase inversion temperature
PVA	polyvinyl alcohol
SDS	sodium dodecyl sulfate
SER-P	serine phosphate
TCA	tri-chloroacetic acid
TFA	tri-fluoroacetic acid
UF	ultrafiltration
UHT	ultra high temperature

Summary

Caseinate stabilized emulsions for dietetic use must be able to withstand relatively high temperatures during industrial manufacture to ensure sterility and a long shelf-life. The process during manufacturing includes homogenization, in which the oil is distributed in small droplets through a continuous phase (an aqueous solution of caseinates, sugars and salts), and a sterilization step at about 120°C. A number of physical and chemical changes may occur during sterilization: **(i)** coagulation of the caseinate covered emulsion droplets, which may result in a curd-like material; **(ii)** coalescence, which involves the fusion of the oil droplets and **(iii)** phase separation, where one of the phases formed may be a gel-like material. It is the composition of the emulsions and the heat-induced changes in caseinate, which appear to determine the stability of the emulsions. It is quite well possible to produce physically stable products in industrial practise, but development is often done by trial and error, selecting recipies and processing methods. The aim of this study was therefore to obtain a better understanding of the heat-induced changes of the caseinate stabilized emulsions and the role of conditions like pH and types of stabilizing salts added, in order to improve control of emulsion stability during its manufacture and storage.

In chapter 1 the formation of protein stabilized emulsions and the properties of the interfacial layers are reviewed and discussed. It also describes types of interactions and changes in the emulsions which may lead to either aggregation, coalescence or phase separation of biopolymer systems. Emulsions of caseinates or individual caseins were reviewed and discussed. This also applies when heat-induced changes were considered.

In chapter 3 a number of physical and chemical heat-induced changes in caseinate systems are described. At room temperature, caseinate is present as aggregates, of which structure and composition appeared to depend on ionic conditions such as calcium-ion activity. In the caseinate emulsions a relatively large part of the calcium was associated with caseinate. It may be suggested that the strong association between calcium and the serine phosphate groups of the caseinate, reduces the charge of the caseinate very effectively, thereby promoting hydrophobic interaction, which may, in turn lead to increased association and ultimately to precipitation of the caseinate. These reactions will be more important at high temperature. Caseinate bound calcium and Ca^{2+}-activity were influenced by adding stabilizing salts like citrates and phosphates. When the citrate concentration was raised, the proportion of calcium bound to the caseinate decreased sharply and the calcium appeared only present as counter-ion to the caseinate. On addition of phosphates, the proportion of caseinate-linked calcium was high at all

concentrations applied. It was concluded that calcium is present both as counter-ion to the caseinate and as a type of calcium phosphate linked to caseinate. The results of proton and phosphorus NMR studies on oil-free caseinate systems showed that the structure of the caseinate aggregates was strongly dependent on temperature. The short-time mobility of a large part of the polypeptide chains increased with increasing temperature. The mobility of the serine-phosphate group was relatively high at room temperature, but decreased somewhat on heating. It appeared that the organic phosphate group becomes somewhat more anchored on heating. This may be due to the increased association of calcium and (organic) phosphate on heating. Furthermore it appeared that the changes in the caseinate were more or less reversible with temperature as measured by these NMR techniques. Prolonged heating of caseinate systems causes a number of changes that may contribute to the destabilization of the caseinate. It was shown that the pH decreased during heat treatment because of formation of acids from sugars. Furthermore, caseinate was partly dephosphorylated at 120°C probably resulting in less electrostatic repulsion between the caseinate chains. Thus, with continued heating, the protective effect of the negative charge on the caseinate seemed gradually diminished. However, because of dephosphorylation of the caseinate, association of calcium and caseinate was lowered during prolonged heating, possibly increasing stability. The knowledge about these changes was used to interpret the mechanisms involved in destabilization of the emulsions during heating.

Heat stability was determined by an objective heat stability test, using a falling-ball viscometer, called Klaro-graph. Heat coagulation time (HCT)-pH profiles were determined for caseinate emulsions and oil-free caseinate systems with different content of oil, type of sugars and type of stabilizing salts. The coagula formed on heating were subjected to dissolution tests with various reagents. This is described in chapter 4. From the results, a number of heat-induced coagulation reactions could be distinguished in the caseinate emulsions. Caseinate emulsions with high volume fraction of oil and relatively high Ca^{2+}-activity were very susceptible to heating. The destabilization could be attributed to a salt-induced reaction. The high Ca^{2+}-activity was implicated as the major cause of destabilization. The binding of calcium to caseinates in these conditions would diminish electrostatic repulsion between the molecules, which may eventually lead to aggregation or precipitation of the caseinate.

When the volume fraction of oil in the caseinate was low, HCT was largely determined by changes of pH and initial Ca^{2+}-activity. The HCT of the caseinate emulsions increased progressively with increasing pH. Furthermore, the susceptibility to heating decreased at all pH values by addition of permitted stabilizing salts like citrates and inorganic phosphates. The shape of these HCT-pH profiles and the effect of either citrates or phosphates could be attributed to lowering of the surface charge during heating, because of decrease of pH, changes in Ca^{2+}-activity and dephosphorylation of the caseinate. The coagula formed in these caseinate emulsions were insoluble in dissociating agents, suggesting that

covalent polymerization occurs in the coagulation process. Involvement of chemical cross-linking reactions was also shown in experiments with SDS gel electrophoresis and determination of the cross-linked amino acid lysinoalanine (LAL), but the formation of crosslinks seemed not to be rate determining in coagulation. During homogenization part of the caseinates was transferred to the newly formed interface. Caseinate could be associated with the oil-droplets in the form of caseinate aggregates or fragments thereof. Composition and properties of these new interfacial layers may to some extent determine the destabilization of the caseinate emulsions during heating. However, it was found that behaviour of caseinate emulsions containing 50 g oil/kg and oil-free caseinate systems was comparable. Furthermore, the proportions of the individual caseins associated with the emulsion droplets, seemed not greatly different from whole caseinate. Only the relative amount of α_{s2}-casein at the oil-interface appeared to be raised as compared to whole caseinate, and that of β-casein was lowered. The relative proportions of the individual caseinates also appeared to be hardly affected by addition of either citrates or phosphates. On the other hand, total amounts of caseinates at the interface were affected by addition of the stabilizing salts; they decreased when phosphate and citrate concentrations were raised. The susceptibility towards heating was correlated with the interfacial caseinate concentration. However, it remained unclear whether the relatively low caseinate load or a specific effect caused by added salts was responsible for the stabilization.

Addition of sugars to caseinate emulsions can reduce their stability during heating and result in phase separation. The latter was manifested by the appearance of spherical inhomogeneities, considered to be phase droplets, which tended to disappear on cooling or on dilution. The phase droplets were formed in caseinate systems containing either lactose/sucrose mixtures or malto-dextrins. In chapter 5 special attention was paid to this phenomenon. The appearance of these phase droplets was accompanied by multi-layer formation of caseinate on the oil-droplets and a decreased stability to heating. This was explained by enhanced protein-protein interactions in the phase droplets at high temperature. The susceptibility towards phase separation and heat was correlated to the chain-length distribution of malto-dextrins. The heat stability of the malto-dextrin containing caseinate emulsions could be enhanced by using malto-dextrin with a higher degree of hydrolysis or by removing the high molar mass malto-dextrins by ultrafiltration.

Crude soya lecithin enhanced the stability of caseinate emulsions during heating probably through preventing phase separation. This effect was only found when lecithin was added prior to homogenization. Although lecithin modified the protein surface concentration, adsorption of the phospholipids at the interface as such, seemed not responsible for the stabilizing effect. Since it was deduced that the polar fractions of the soya lecithin seem responsible for the stabilizing effect, the interactions of caseinate with the negatively charged region of phospholipids cannot be excluded.

When the soya lecithin concentration exceeds a maximum value, the stability decreases and the emulsion droplets are found to coalesce as described in chapter 6. From the various lecithin fractions, phosphatidylcholine, destabilizes the caseinate emulsions most. Coalescence is directly followed by creaming of the enlarged emulsion droplets and is probably partly due to lowering of the interfacial tension on addition of phosphatidylcholine. Destabilization of the phosphatidylcholine containing caseinate emulsions was especially found when protein surface load of the emulsion-droplets was low at low Ca^{2+}-activity. The phosphatidylcholine is not only found to change the surface load but seemed also to change the structure of the caseinate layer.

Samenvatting

Emulsies gestabiliseerd met caseïnaat worden gebruikt bij het samenstellen van dieetvoedingen. Deze caseïnaatemulsies dienen bij de industriële bereiding relatief hoge temperaturen te kunnen weerstaan om verzekerd te zijn van steriliteit en een lange houdbaarheid. Het bereidingsproces omvat een homogenisatiestap, waarbij de olie fijn verdeeld wordt in de continue fase, bestaande uit een waterige oplossing van caseïnaten, suikers en zouten, en een sterilisatiestap bij 120°C. Tijdens het sterilisatieproces kunnen een aantal belangrijke fysisch-chemische veranderingen optreden: **(i)** coagulatie van de met caseïnaat bedekte emulsiedruppels, welke kan resulteren in een wrongel-achtig materiaal; **(ii)** coalescentie oftewel samenvloeiing van de emulsiedruppels en **(iii)** fasescheiding, die tot afscheiding van een gelachtig materiaal leidt. De stabiliteit van de emulsies lijkt bepaald te worden door de hittegeïnduceerde veranderingen in het caseïnaat en de samenstelling van de emulsies. De industriële bereiding van de caseïnaatemulsies biedt vele mogelijkheden om fysisch stabiele produkten te maken, maar dit gebeurt veelal door te proberen welke combinatie van receptuur en werkwijze tot een goed resultaat leidt. Doel van dit onderzoek was daarom een beter inzicht te krijgen van hitte-geïnduceerde veranderingen van het caseïnaat en de rol van omstandigheden als pH en toegevoegde stabilisatiezouten daarin.

In het eerste hoofdstuk wordt een overzicht gegeven van de vorming van door eiwit gestabiliseerde emulsies en van de eigenschappen van de gevormde oppervlaktelaagjes van de emulsiedruppels. Het beschrijft ook de veranderingen in het soort van interacties die kunnen leiden tot aggregatie of coalescentie van emulsies of tot fasescheiding van biopolymeer-mengsels. Voor zover mogelijk, wordt een overzicht gegeven van emulsies met caseïnaten of individuele caseïnes en veranderingen daarin tijdens verhitting.

In hoofdstuk 3 worden een aantal fysische en chemische hittegeïnduceerde veranderingen in caseïnaat systemen beschreven. Bij kamertemperatuur is het caseïnaat aanwezig in de vorm van aggregaten waarvan structuur en samenstelling afhankelijk bleken van ionaire samenstelling zoals de calciumionactiviteit. In de onderzochte caseïnaatemulsies bleek een belangrijk deel van het calcium geassocieerd met het caseïnaat. De sterke associatie van calcium met de serine-fosfaat groepen van caseïnaat werd verondersteld de lading van het caseïnaat sterk te reduceren, waardoor hydrofobe bindingen gevormd worden, die kunnen leiden tot een versterkte associatie van de eiwitketens en uiteindelijk tot precipitatie van het caseïnaat. Deze reacties zullen belangrijker worden bij verhoging van de temperatuur. Door toevoeging van stabilisatiezouten zoals citraten en fosfaten, werd met name het aan caseïnaat gebonden calcium en daarmee de calciumionactiviteit beïnvloed. Verhoging van de citraatconcentratie had een verlaging van het aan

caseïnaat gebonden calcium en van de calciumionactiviteit tot gevolg. Calcium bleek alleen aanwezig als tegenion van het caseïnaat. Door toevoeging van fosfaten bleek het aan caseïnaat gebonden calcium in alle gevallen relatief hoog en onveranderd. Het calcium bleek zowel aanwezig als tegenion van het caseïnaat en als een vorm van caseïnaatgebonden calciumfosfaat. Uit de resultaten van proton- en fosfor-NMR-experimenten van olievrije caseïnaatsystemen bleek dat de structuur van de caseïnaataggregaten afhankelijk was van de temperatuur. De beweeglijkheid (op zeer korte tijdschaal) van een groot deel van de polypeptideketens nam duidelijk toe met toenemende temperatuur. De beweeglijkheid van de serine-fosfaat groep bleek relatief hoog bij kamertemperatuur maar nam iets af door verhitting. Dit werd toegeschreven aan de verdere verankering van de organische fosfaatgroep als gevolg van verhitting, wat mogelijk veroorzaakt werd door de verhoogde associatie van calcium en (organisch) fosfaat bij hogere temperatuur. Uit de experimenten bleek dat veranderingen in het caseïnaat, zoals gemeten met NMR, als gevolg van temperatuurwijzigingen reversibel zijn. Langdurige verhitting van caseïnaat-systemen veroorzaakte veranderingen die bijdragen aan de destabilisatie van het caseïnaat. De pH nam tijdens verhitting sterk af als gevolg van zuurvorming uit suikers. Verder werd bij 120°C het caseïnaat gedeeltelijk gedefosforyleerd, hetgeen resulteert in minder electrostatische repulsie tussen de caseïnaatstaarten. Hierdoor werd het beschermende effect van de negatieve lading van het caseïnaat geleidelijk verminderd. Het bleek echter dat door defosforylatie de associatie van calcium en caseïnaat afnam door verhitting, wat op zich stabiliserend kan werken. De kennis van deze veranderingen werd gebruikt voor de interpretatie van de mechanismen die betrokken zijn bij de destabilisatie van de emulsies tijdens verhitting.

De hittestabiliteit werd bepaald met een objectieve hittestabiliteit meting door gebruik te maken van de zogenaamde Klaro-graph. Dit is een vallende kogel-viscositeitsmeter waarmee de hittecoagulatietijd (HCT) als functie van de pH gemeten werd voor caseïnaatemulsies en olievrije systemen. De HCT werd bepaald voor produkten met verschillend oliegehalte, type suiker en type stabilisatiezout. De aard van de coagula werd onderzocht door middel van oplosproeven die uitgevoerd werden met verschillende reagentia. Dit is beschreven in hoofdstuk 4. Een aantal hittegeïnduceerde coagulatiereacties kon onderscheiden worden in de hittestabiliteitmetingen. Caseïnaatemulsies met een hoge volumefractie aan olie en een relatief hoge calciumionactiviteit bleken zeer gevoelig voor verhitting. De destabilisatie kon toegeschreven worden aan een zoutgeïnduceerde reactie, waarbij de hoge calciumionactiviteit gezien werd als belangrijkste oorzaak. De associatie van calcium en caseïnaat zal onder deze omstandigheden de electrostatische lading verminderen wat kan leiden tot aggregatie of zelfs precipitatie van het caseïnaat. In de caseïnaatemulsies met lage volumefractie aan olie bleek de HCT vooral afhankelijk van veranderingen in pH en de calciumionactiviteit. De hittecoagulatie-tijd neemt vrij sterk toe met toenemende pH. Verder kan door toevoeging van toegestane stabilisatiezouten als citraten en fosfaten de gevoeligheid voor verhitting

sterk verlaagd worden. Het verloop van de HCT-pH curves en het effect van citraat en anorganisch fosfaat kon toegeschreven worden aan de afname van de oppervlaktelading tijdens verhitting door de pH-verlaging, veranderingen in calcium-ion-activiteit en defosforylering van caseïnaat. Onder deze verhittingsomstandigheden worden chemische bindingen gevormd tussen de caseïnemoleculen. Polymerisatie van caseïne werd aangetoond door toepassing van verschillende dissociatiemedia, SDS-gelelectroforese en bepaling van het aminozuur lysinoalanine (LAL). De vorming van chemische bindingen bleek echter niet de hittecoagulatietijd bij de verschillende pH-waarden te bepalen.

Verwacht werd dat homogenisatie van olie in de caseïnaatdispersies een belangrijk effect zou hebben op de gevoeligheid voor verhitting. Tijdens homogenisatie adsorbeert namelijk een deel van het caseïnaat op het nieuw gevormde grensvlak waarbij het geassocieerd kan zijn in de vorm van aggregaten of fragmenten daarvan. De samenstelling en eigenschappen van de grenslaag zou in zekere mate de destabilisatie van de caseïnaat emulsies kunnen bepalen. Uit de waarnemingen bleek echter dat het gedrag van olievrije caseïnaatdispersies en van emulsies met 50 g olie/kg vergelijkbaar was. Verder waren de verhoudingen van de individuele caseïnes in de grenslaag niet erg verschillend van caseïnaat. Alleen de relatieve hoeveelheid α_{s2}-caseïne op het olie-water grensvlak bleek wat verhoogd ten opzichte van volledig caseïnaat en dat van β-caseïne was iets verlaagd. Bovendien bleken de verhoudingen tussen de caseïnes nauwelijks afhankelijk te zijn van toevoeging van citraten of fosfaten in verschillende concentraties. Deze stabilisatiezouten bleken wel invloed te hebben op de hoeveelheid caseïnaat in het grensvlak. Deze hoeveelheid bleek af te nemen door verhoging van de concentratie van fosfaat of citraat. De caseïnaatconcentratie in de grenslaag bleek gecorreleerd met de gevoeligheid bij verhitting. Uit de experimenten werd niet duidelijk of de relatief lage caseïnaatconcentratie in de olie-water grenslaag bepalend was voor de stabiliteit van de emulsie of de invloed van de stabilisatiezouten op het opgeloste caseïnaat.

Afhankelijk van de aard van de suiker kan toevoeging ervan aan caseïnaat-emulsies de stabiliteit tijdens verhitting reduceren en aanleiding geven tot fasescheiding. Dit komt tot uiting in afgeronde "deeltjes" of fasedruppels, die neigen tot oplossen na afkoelen of na verdunnen. Deze fasedruppels werden zowel gevormd in caseïnaatsystemen met lactose/sacharose mengsels als met malto-dextrinen. In hoofdstuk 5 werd aandacht besteed aan dit verschijnsel. Het vóórkomen van deze fase-druppels bleek samen te gaan met een verhoogde belading van het caseïnaat op de olie-druppels en een verlaagde stabiliteit tijdens verhitting. Dit laatste werd verklaard door de plaatselijk verhoogde eiwitconcentratie, en daardoor sneller verlopende eiwit-eiwit interacties, in de fase-druppels bij hoge temperatuur. De gevoeligheid voor fasescheiding en verhitting bleek af te hangen van de ketenlengteverdeling van de malto-dextrinen. De stabiliteit tijdens verhitten kon verhoogd worden door gebruik te maken van malto-

dextrinen met verhoogde hydrolysegraad of door gebruik van het permeaat na ultrafiltratie van de malto-dextrinen.

De stabiliteit van caseïnaatemulsies tijdens verhitting kon verhoogd worden door toevoeging van ruwe soja-lecithine. De soja-lecithine verhindert waarschijnlijk het optreden van fase-scheiding. Dit effect werd alleen waargenomen indien de lecithine voor de homogenisatie aan de olie toegevoegd werd. Hoewel toevoeging van lecithine effect had op de concentratie aan eiwit in de grenslaag leek adsorptie van lecithine aan het grenvlak als zodanig niet verantwoordelijk voor het stabiliserende effect. Aangezien vooral polaire fracties van soja-lecithine de hittestabiliteit verhogen, wordt een verklaring gezocht in interacties tussen het negatief geladen gebied van fosfolipiden en het caseïnaat.

Boven een bepaalde soja-lecithine concentratie blijkt de stabiliteit van caseïnaatemulsies tijdens verhitten af te nemen en de emulsiedruppels samen te vloeien, zoals beschreven in hoofdstuk 6. Van de verschillende lecithine fracties blijkt de fosfatidylcholine de emulsies het meest te destabiliseren. Coalescentie werd direct gevolgd door oproming van de vergrootte emulsiedruppels en werd waarschijnlijk gedeeltelijk veroorzaakt door de verlaging van de grensvlakspanning door toevoeging van fosfatidylcholine. Destabilisatie van de caseïnaatemulsies met fosfatidylcholine werd met name waargenomen indien de eiwitbelading van de emulsiedruppels laag was bij een verlaagde calciumionactiviteit. De fosfatidylcholine blijkt niet alleen in staat te zijn om de belading van de emulsiedruppels te verlagen, maar lijkt ook de structuur van het geadsorbeerde caseïnaat te wijzigen.

Curriculum vitae

Hans Cruijsen werd geboren op 14 september 1962 in Oeffelt. In 1979 behaalde hij het diploma Hoger Algemeen Voortgezet Onderwijs aan het Elzendaalcollege te Boxmeer. In datzelfde jaar begon hij met de laboratoriumopleiding HBO-B te Venlo. Deze werd voltooid in 1982. Daarna begon hij de studie levensmiddelentechnologie aan de toenmalige Landbouwhogeschool te Wageningen. Hij koos voor de afstudeervakken levensmiddelenmicrobiologie, toxicologie en zuivelkunde. In september 1987 slaagde hij voor het doctoraalexamen. In dezelfde maand trad hij als research medewerker in dienst bij Nutricia te Zoetermeer. In dit dienstverband is het in dit proefschrift beschreven onderzoek uitgevoerd.

Nawoord

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