Formation and stability of emulsions made with proteins and peptides

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Stellingen

1. Variaties in de vorming en stabiliteit van emulsies, bij gelijke condities gemaakt met eiwitten of peptiden, worden vooral bepaald door de effectieve molaire massa van deze stoffen.
   Dit proefschrift.

2. Een volledige opheldering van de relaties tussen de moleculaire structuur van eiwitten en peptiden en de vorming en stabiliteit van emulsies is niet mogelijk en ook niet zinvol.
   Dit proefschrift.

3. Versnelde tests leveren per definitie geen goede indicatie voor de lange-termijn stabiliteit van emulsies.

4. De opkomst van geautomatiseerde analysemethoden voor de bepaling van deeltjesgrootteverdelingen heeft bij veel onderzoekers ten onrechte de microscoop naar de achtergrond verdrongen.

5. Het voornaamste verschil tussen een schuim en een emulsie is de deeltjesgrootte.

6. Productontwikkeling is grensverleggend maar vaak ook grensoverschrijdend.

7. Functionele levensmiddelen moeten in de eerste plaats lekker zijn.

8. Alcohol heeft niet alleen op roomlikeur een destabiliserende werking.

Stellingen behorende bij het proefschrift:
Formation and stability of emulsions made with proteins and peptides,
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The formation and stabilization of oil-in-water emulsions using well-defined and well-characterized proteins and peptides was studied in order to elucidate the relation between their molecular and functional properties. The emulsions were formed with a high-pressure homogenizer. To study the effect of the homogenizer scale on the emulsion properties, emulsions were prepared with a laboratory and a small industrial homogenizer. The flow in the industrial homogenizer was shown to be turbulent. In the laboratory homogenizer, droplet break-up was found to occur in a bounded laminar type of flow, resulting in a poor operating efficiency. The effect of the flow type on the emulsion properties, however, appeared to be small, if the number of passes through the laboratory homogenizer was sufficiently high.

Proteins appeared to have good emulsion forming properties as long as protein aggregation was absent. In those cases, the recoalescence rate during homogenization was found to be similar and only small differences in the droplet size of emulsions could be determined. The surface excess of the emulsion droplets appeared to be governed by the conformational stability and the aggregated state of the proteins. Globular proteins with a high conformational stability yielded relatively low surface excesses, while a flexible random coil protein, like β-casein, yielded a relatively high surface excess. Protein aggregation may be due to physicochemical conditions and surface or heat denaturation. If protein aggregates were present, the emulsion droplets were also often aggregated. The droplet size, surface excess, and rate of recoalescence of these aggregated emulsions were usually found to be relatively high.

The emulsion forming properties of β-casein peptides appeared to be comparable or superior to those of intact proteins. Amphiphilic peptides without the hydrophobic C-terminal domain of β-casein yielded a relatively low surface excess, likely due to strong electrostatic interactions between the highly charged groups of the N-terminal end. The surface excess of emulsions made with hydrophobic peptides with a removed N-terminal domain was comparable to those of emulsions made with intact β-casein. The peptides were due to their relatively small molar mass more readily desorbed from the oil/water interface than intact proteins.

The coalescence stability of emulsions made with proteins was high even at low protein concentrations and appeared to be mainly determined by the surface excess of the droplets. The emulsion stabilizing properties of β-casein peptides were inferior to those of intact proteins probably due to their relatively low molar mass. Comparison of the stability of emulsions made with amphiphilic peptides with an intact or partially removed N-terminal domain showed that this domain was of great importance for providing stability against coalescence. The coalescence stability of emulsions made with hydrophobic peptides was relatively high, which was attributed to the high surface excess of the droplets. The electrostatic and steric interactions appeared to be of great importance for stabilizing emulsions made with peptides against coalescence as was indicated by the effect of changes in pH and ionic strength on the stability.

aan Jacco
aan mijn ouders

Contents

Chapter 1

Protein and peptide stabilized emulsions: introduction

1.1 General introduction

In many food products, proteins act as emulsifiers and emulsion stabilizers. Protein hydrolyzates or peptides are often used in emulsified clinical formulas to improve digestibility and to reduce allergenic reactions. The emulsion forming properties of proteins and peptides are usually good. Peptides are, however, generally less capable of stabilizing emulsions than proteins. Moreover, the suitability of proteins and peptides is strongly dependent on the type of food product in which they are used.

The functionality of proteins and peptides has been the subject of many studies. Most research has been done using model systems to be able to establish the behaviour of proteins and peptides in more complex, food products. The relation between the molecular and emulsion forming and stabilizing properties of proteins and peptides is, however, still little understood. A fundamental understanding is often hampered due to the use of protein and peptide mixtures with undefined properties. Also, often methods are used which poorly distinguish between the various aspects of emulsion formation and stabilization, or which yield results that hardly correlate with the properties of interest (Halling, 1981; Walstra, 1996).

For an effective and wide-spread use of proteins and peptides in emulsions, a good understanding of their functionality is essential. To obtain this understanding, the emulsion forming and stabilizing properties of purified proteins and peptides with well-defined and widely varying molecular properties have been studied.

1.2 Emulsion formation

Extensive reviews on emulsion formation are given by Walstra (1983, 1993) and Walstra and Smulders (1998). A brief summary is given below.

Emulsions are mixtures of at least two immiscible liquids of which one is dispersed as droplets into the other, which forms the continuous phase. To make an emulsion, droplets have to be generated from the interface between these liquids. This process is relatively easy. For a stable emulsion, however, the droplets have to be sufficiently small. The break-up of large droplets into smaller ones requires a large amount of energy. This energy is needed to overcome the Laplace pressure, which opposes the deformation and thus break-up of emulsion droplets. The Laplace pressure (p_L) is defined as the pressure difference between the inside and outside of a droplet and is given by

$$p_L = 4\frac{\gamma}{d} \tag{1.1}$$

where γ is the interfacial tension and d the droplet diameter.

Droplet deformation and break-up increases the interfacial area of emulsions. If present, surfactants adsorb at this newly created droplet interface. The adsorbed surfactants lower the interfacial tension, facilitating droplet break-up, and prevent immediate recoalescence of colliding droplets. The interfacial tension and stability of the newly formed emulsion droplets against recoalescence depend on the concentration and interfacial properties of the surfactant.

The processes of droplet break-up, surfactant adsorption, and droplet collision, possibly followed by recoalescence, occur simultaneously and repeatedly during emulsion formation. The droplet size of the emulsion formed is the result of a kind of steady state between droplet break-up and recoalescence and thus of the time-scales of the different processes. For example: the time elapsing between break-up and subsequent collision of droplets is the time available for surfactant adsorption, hence the time available for stabilizing the droplets against recoalescence. The time-scales of the various processes depend on the hydrodynamic conditions in the emulsifying equipment and the intensity of the emulsification process. For high-pressure homogenizers, the time-sales are generally in the order of microseconds.

1.3 Emulsion stability

The physical instability of emulsions may occur in various, often correlated forms. A brief overview of the various types is given below. Most of the data are taken from an extensive review on emulsion stability by Walstra (1996).

1.3.1 Creaming

A frequently occurring type of instability is emulsion creaming. The creaming rate of emulsion droplets is roughly given by the Stokes equation

$$v_s = \frac{a(\rho_d - \rho_c)d^2}{18\eta_c} \tag{1.2}$$

where v_s is the Stokes velocity, a the acceleration, ρ the density and η the viscosity of the disperse (subscript d) or continuous phase (subscript c). According to Stokes, the creaming velocity is governed by the equilibrium between the droplet buoyancy and the drag force acting on the droplets. The buoyancy force is given by

the density difference between the disperse and continuous phase, the droplet size, and the acceleration due to gravity or centrifugation. The drag force acting on moving droplets decreases the creaming rate and is dependent on droplet size and viscosity of the continuous phase.

Equation 1.2 may only be applied at a limited number of conditions. Factors affecting the creaming velocity are: the volume fraction of the disperse phase, the width of the droplet size distribution, the hydrodynamic and interfacial properties of the droplets, and the rheological properties of the continuous phase. Creaming is furthermore opposed by the Brownian or heat motion of droplets and by convection currents due to temperature gradients. As a result, food emulsions are generally stable with respect to creaming if the average droplet size is smaller than one micron.

The creaming stability of emulsions is often studied using accelerated creaming tests. However, many of the factors mentioned before are affected by the test conditions, thus changing the creaming stability of emulsions. The results of these tests should, therefore, be treated with caution.

1.3.2 Aggregation

When emulsions are aggregated, the droplets remain close for a prolonged period of time without rupture of the film separating them. Droplet aggregation results in an increased particle size, and hence in most cases in a decreased creaming stability. Some authors distinguish various types of aggregation according to the degree of reversibility using terms like aggregation, flocculation, and agglomeration. These types are, however, hard to distinguish and here only the general term aggregation will be used.

Droplet aggregation is governed by the interaction forces acting between droplets. According to the DLVO theory, the interaction free energy (V_{int}) between two droplets equals the sum of the van der Waals attraction (V_A) and the electrostatic repulsion (V_E). The magnitude of these forces depends on the distance between the droplets, the film thickness (h). An example is given in Figure 1.1.

Emulsion droplets aggregate if the attraction force exceeds the repulsion force, i.e. if the interaction free energy is negative. The interaction free energy as function of the distance between droplets often shows two minima. At a very short distance, the van der Waals attraction between droplets is very strong and a so-called primary minimum is found. The van der Waals attraction decreases rapidly with increasing film thickness, while the electrostatic repulsion can remain relatively high, resulting in a maximum in the interaction free energy. If the film thickness increases further, the electrostatic repulsion and to a lesser extent the van der

Waals attraction decrease, often resulting in a shallow secondary minimum. The presence of adsorbed surfactants often affects the relation between the interaction free energy and the film thickness, making the interactions much more complex (Walstra et al., 1999).

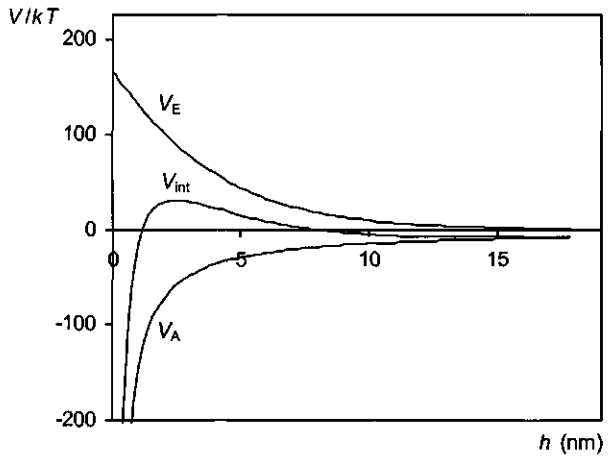

Figure 1.1 Example of the calculated interaction free energy (V_{int}), van der Waals attraction (V_A), and electrostatic repulsion (V_E) between two identical droplets as a function of interdroplet distance (h), according to DLVO theory.

Adsorbed polymers often induce a strong repulsion between droplets due to the formation of a thick layer of protruding polymer chains. At a droplet distance smaller than the effective thickness of this layer, a strong steric repulsion will occur due to a restricted mobility of the polymer chains. At a distance somewhat larger than the effective layer thickness, the repulsion can still be strong due to an increased osmotic pressure caused by overlapping polymer layers. The effective thickness of the adsorbed layer is determined by the polymer properties and the solvent quality. At a poor solvent quality, the adsorbed layer is relatively thin and attraction between polymer chains may occur due to a decreased osmotic pressure and an increased van der Waals attraction. The solvent quality is affected by temperature, ionic strength, and dielectric constant.

If the adsorbed polymers are polyelectrolytes, aggregation may occur below a certain degree of dissociation of the ionic groups and may thus depend on pH. Strong aggregation is also possible if polymers possess reactive groups which can form covalent cross-links with polymers adsorbed at other droplets. Non-covalent linkage of charged groups of adsorbed polymers by particularly divalent ions may also cause aggregation.

Other possible types of aggregation are bridging and depletion flocculation. Bridging flocculation may occur if the surface excess of polymers adsorbed at emulsion droplets is well below the plateau value or if surface-active particles are present in solution. In these cases, a single polymer or particle may link droplets by adsorption at two separate droplets. The resulting aggregates are also called homogenization clusters and are sometimes readily disrupted by slight agitation (Ogden et al., 1976).

Depletion flocculation may occur if relatively large polymers are present in solution. These polymers are, due to their size, depleted near the droplet interface. As a result, the polymer concentration is higher in the bulk phase than near the droplet interface, leading to an increased osmotic pressure of the bulk phase. Droplet aggregation decreases this osmotic pressure by reducing the size of the depleted region near the droplets. Depletion flocculation may occur with non-adsorbing polymers, like polysaccharides, or with small particles, like soap and protein micelles (Dickinson et al., 1997).

1.3.3 Coalescence

Coalescence occurs if the film between two emulsion droplets is ruptured and the droplets join to form a single, large droplet. Eventually, this process will result in a complete separation of an emulsion in an oil and water phase. For coalescence to occur, the film between droplets should be very thin for a prolonged period of time. Coalescence is, therefore, more likely to occur when emulsion droplets are creamed or aggregated.

The coalescence rate is governed by two processes: the rate at which a thin film between droplets is formed (either by aggregation or creaming), and the rate at which this film is ruptured. Usually, film rupture is the rate determining step, making coalescence a first-order rate process (van den Tempel, 1957). The film rupture rate increases with increasing film size, hence increasing droplet size. Therefore, the coalescence stability of emulsions often decreases in time.

Surface-active polyelectrolytes are generally effective stabilizers against coalescence. Polyelectrolytes adsorbed at emulsion droplets hamper the formation of a thin film due to the strong steric repulsion caused by the thick layer of adsorbed chains and due to the electrostatic repulsion by charged groups. The adsorbed amount should, however, be sufficiently high.

To study the coalescence stability of emulsions often accelerated tests, like centrifuge or high osmotic pressure tests, are used. The correlation of the results of these tests with the stability of emulsions stored under quiescent conditions tends, however, to be poor.

A special type of coalescence, partial coalescence or clumping, may occur if the disperse phase is partially crystallized. Crystals protruding into the continuous phase may penetrate other emulsion droplets, causing coalescence. Full coalescence is, however, prevented by the presence of a crystal network in the droplets and as a result the droplet shape is largely retained.

1.3.4 Ostwald ripening

Ostwald ripening or disproportionation is the type of instability that may occur if the disperse phase is (slightly) soluble in the continuous phase. The solubility of the disperse phase is proportional to the Laplace pressure and thus increases with decreasing droplet size. The concentration of disperse phase material will, therefore, be higher near small droplets than near large droplets. As a result, material diffuses from small to large droplets, resulting in a decreasing size of the small droplets and an increasing size of the large droplets.

Ostwald ripening is generally of little importance for the stability of oil-in-water emulsions as long as the molar mass of the oil molecules is sufficiently high in order to prevent solubilization into the aqueous phase (Davis and Smith, 1976; Taisne et al., 1996).

1.4 Interfacial properties of proteins and peptides

Proteins and peptides in solution generally have a high affinity for many types of surfaces, including oil/water interfaces, and readily adsorb at a much lower concentration than small-molecule surfactants (Walstra and de Roos, 1993). The interfacial tension of an oil/water interface with adsorbed proteins is, however, relatively high compared to those of an interface with adsorbed small-molecule surfactants. Small-molecule surfactants are able to form a closely packed layer at an interface, resulting in a relatively low interfacial tension (Walstra and Smulders, 1998). Proteins and peptides consist of amino acids with varying affinities for interfaces, hampering a very close packing.

Proteins and peptides are predominantly adsorbed at oil/water interfaces via their hydrophobic segments. Some studies suggest that the hydrophobic groups may even be dissolved in the oil phase (Graham and Phillips, 1979c; Walstra and de Roos, 1993). Adsorbed proteins often rearrange their molecular conformation to facilitate adsorption of hydrophobic groups at an interface, thus attaining an energetically most favourably conformation. Due to slow conformational changes after protein adsorption, the interfacial tension often changes in time even if no more protein adsorption occurs (Graham and Phillips, 1979a). These slow changes

often result in time-dependent emulsion properties (Dickinson et al., 1988a; McClements et al., 1993).

The extend of the rearrangements after adsorption is governed by the conformational flexibility of proteins and peptides and by the available interfacial area, hence surface excess (Walstra and de Roos, 1993). Flexible proteins, like caseins, adsorb in loops, trains and tails (Graham and Phillips, 1979c). Globular proteins mostly adsorb as relatively compact globules. The conformational changes are mostly larger if the surface excess is low (Norde and Favier, 1992).

Proteins and peptides often form a monolayer at oil/water interfaces, yielding a maximum surface excess. In some cases, multilayer adsorption occurs and no maximum surface excess is found. These multilayers are usually readily removed by lowering the protein concentration of the continuous phase (Graham and Phillips, 1979b). The adsorption of proteins in a monolayer is in general practically irreversible and, therefore, not an equilibrium process at the time-scale usually considered (Dalgleish, 1989). For desorption of a protein monolayer to occur, the protein concentration of the continuous phase has to be extremely low. Hence, only very few molecules have to desorb to obtain a new equilibrium (Walstra and de Roos, 1993). Even in this case, the rate of desorption is practically zero, since the difference in the chemical potential of proteins at the interface or in the bulk is very small, due to the extremely small protein concentrations in solution (Walstra et al., 1999). If desorption occurs, the desorption rate is found to increase with increasing surface pressure and to decrease with increasing molar weight (MacRitchie, 1985). Peptides are, therefore, likely to be more readily desorbed than intact proteins.

Adsorbed proteins may be desorbed by other proteins (Hunter et al., 1991) or by small-molecule surfactants (Oortwijn and Walstra, 1979; Feijter et al., 1987). The susceptibility for such a replacement is governed by the conformational flexibility and the propensity of proteins for intermolecular interactions (Dalgleish, 1989; Dickinson et al., 1989; Hunter et al., 1991).

If surfactants are adsorbed at an interface, the interface will have certain rheological properties. Shearing of the interface, i.e. deformation without affecting the interfacial area, is opposed by the surface shear viscosity. The surface shear viscosity of an interface with adsorbed proteins is governed by their intermolecular interactions (Graham and Phillips, 1976). The relation between surface shear viscosity and emulsion properties is, however, largely unclear (Walstra, 1996).

When an interface is deformed in dilatation, the interfacial area is enlarged without affecting its shape. If during dilatation the total amount of adsorbed protein remains constant, the change in interfacial tension is related to the change in interfacial area according

$$E_{sd} \equiv \frac{d\gamma}{d\ln a} \tag{1.3}$$

where E_{sd} is the surface dilational modulus and a the interfacial area. The surface dilational modulus is time and rate dependent (Walstra and de Roos, 1993).

In emulsions, the interface of droplets may be dilated by the flow of continuous phase along the droplet interface. The liquid flow can drag along surfactants adsorbed at the interface, resulting in an interfacial tension gradient. Due to this gradient, the resistance against further interfacial dilatation increases, slowing down and possibly even preventing further deformation. The opposite of this mechanism occurs when the interfacial tension gradient disappears. These two mechanisms are combined the so-called Gibbs-Marangoni effect.

The Gibbs-Marangoni effect is during emulsification of great importance for stabilizing colliding emulsion droplets against recoalescence (Walstra and de Roos, 1993; Walstra and Smulders, 1998). If two droplets approach, the film between the droplets drains, dragging along interfacial area. An interfacial tension gradient will be formed, if the film between the droplets becomes depleted from surface-active material. This gradient prevents further drainage of the film and upon its disappearance, liquid is dragged into the film, thus increasing the film thickness and preventing coalescence. The strength of this stabilizing mechanism is determined by the Gibbs elasticity of the film, which equals twice the surface dilational modulus.

1.5 Emulsion forming and stabilizing properties of proteins

Proteins are usually water soluble and only suitable for stabilizing oil-in-water emulsions (Walstra and de Roos, 1993). The properties of emulsions made with proteins are generally thought to be governed by protein properties like hydrophobicity, amphiphilicity, conformation and conformational stability, charge and molar mass (Kinsella, 1984). These molecular properties govern the ability of proteins to lower the interfacial tension during emulsification, hence the formation of small droplets, and to stabilize emulsions against recoalescence by affecting the rheological properties of the droplet interface. These properties determine furthermore the characteristics of the adsorbed layer, which stabilizes the droplets against coalescence and aggregation by steric and electrostatic repulsion. Some general trends found for the relation between the formation and stability of emulsions and the molecular properties of proteins will be discussed. Exceptions to these trends are, however, frequently reported.

Proteins mainly adsorb at oil/water interfaces via hydrophobic groups (Walstra and de Roos, 1993). The hydrophobicity, or more specific, the surface or effective hydrophobicity, and the distribution of hydrophobic groups or amphiphilicity are, therefore, often believed to be important factors governing the emulsion forming and stabilizing properties of proteins. Some positive relations between the surface hydrophobicity and the emulsion forming properties of proteins have been reported (Kato and Nakai, 1980). However, proteins with a high surface hydrophobicity tend to have poor emulsion forming properties due to their low solubility (Keshavarz and Nakai, 1979; Nakai, 1983). In other studies, no correlation could be found (Shimizu et al., 1986b; Saito and Taira, 1987). These discrepancies are probably due to the use of ambiguous experimental methods for determining the surface hydrophobicity of proteins (Shimizu et al., 1986b).

The conformation and conformational stability of proteins are dependent on the protein net charge, hence pH. At the isoelectric pH, the net charge of proteins equals zero and the intramolecular electrostatic repulsion is minimal. As a result, proteins have a compact conformation and a high conformational stability near this pH. The intermolecular repulsion is also small near the isoelectric pH, often resulting in a poor protein solubility, and hence poor emulsifying properties (Halling, 1981). A typical example is the emulsion forming properties of β-casein as function of pH (Touati et al., 1990). Whey proteins are aggregated near their isoelectric pH and as a result the emulsions made with these proteins are also aggregated and have a relatively high surface excess due to adsorption of protein aggregates (Shimizu et al., 1981). The droplets of emulsions made with β-lactoglobulin are relatively large and also aggregated near the isoelectric pH (Hunt and Dalgleish, 1995). Away from this pH, the pH hardly affects the size and surface excess of the emulsions made with this protein (Dickinson and Williams, 1994). Despite the decreased emulsion forming properties, the coalescence stability generally appears to be relatively high near the isoelectric pH of proteins (Halling, 1981).

The conformational stability of proteins governs the extend of unfolding after adsorption at an interface. This stability is usually high if intramolecular disulfide-bonds are present and is governed by physicochemical conditions (Dalgleish, 1989). The contribution of the conformational stability to the emulsion forming and stabilizing properties of proteins remains, however, largely obscure.

The ionic strength affects the functionality of proteins by shielding of the protein charge, hence by reducing the electrostatic repulsion. The effect of the ionic strength on the emulsion properties depends on the type of protein and the physicochemical conditions (Hunt and Dalgleish, 1996). At low ionic strengths, sometimes poor emulsion forming properties are found (Pearce and Kinsella, 1978), probably due to a salting-in effect. At a relatively high ionic strength,

emulsion droplets are often aggregated. Emulsion droplets tend to be less susceptible to this type of aggregation if the surface excess is relatively high (Dickinson et al., 1984).

Divalent ions like calcium ions may form intermolecular links between proteins. If formed between proteins adsorbed at droplet interfaces, these links often induce emulsion aggregation (Dickinson et al., 1992). The susceptibility for this type of aggregation depends on the calcium affinity of proteins and the presence of calcium before or after emulsification. For example, emulsions made with β-casein are sensitive for calcium induced aggregation if calcium is present before emulsification (Dickinson et al., 1992). If added after emulsification, calcium hardly affects the properties of emulsions with β-casein (Dickinson et al., 1998). Emulsions made with whey proteins are also susceptible to calcium induced aggregation (Rientjes and Walstra, 1993). For emulsions made with sodium caseinate, a relatively large droplet size and high surface excess is found in the presence of calcium (Dickinson et al., 1984; Srinivasan et al., 1996).

Droplet aggregation may also be induced by linkage of adsorbed proteins via covalent bonds. Some proteins possess reactive groups which may be exposed by denaturation, resulting in the formation of intermolecular bonds. An example is the time-dependent exchange of disulfide-bonds between adsorbed β-lactoglobulin molecules due to surface denaturation (Dickinson and Matsumura, 1991). Completion of these reactions requires a long period of time and is often the cause of time-dependent changes in the emulsion properties (Dickinson and Matsumura, 1991; McClements et al., 1993). Reactive groups of proteins may also be exposed by heat or pressure denaturation (Saito and Taira, 1987; Galazka et al., 1996).

The emulsion forming properties of heat denatured proteins are often found to be poor due to protein aggregation (Saito and Taira, 1987; Rientjes and Walstra, 1993). As a result, emulsions made with these proteins have a relatively large droplet size and high surface excess (Oortwijn and Walstra, 1979). The aggregates may be formed via covalent as well as non-covalent bonds (Sawyer, 1968; Iametti et al., 1995). The size of the aggregates, hence the effect on the emulsion forming properties depends on physicochemical conditions, like pH and ionic strength, and protein concentration during heating (Saito and Taira, 1987; Roefs and Kruif, 1994; Iametti et al., 1995). In some cases, heat denaturation improves the functionality of proteins. For example, the emulsion forming properties of soy proteins were superior after thermal treatment due to the irreversible dissociation of the proteins into sub-units (Nir et al., 1994). If the denaturation is reversible, usually no influence on emulsion properties can be determined.

Summarizing, it appears that protein solubility is one of the most important factors determining the functionality of proteins in emulsions (Halling, 1981). A

decrease in solubility on even a molecular scale, e.g. a slight increase in the effective molar mass of proteins due to aggregation, has apparently already a negative effect on the emulsion forming and stabilizing properties of proteins.

1.6 Emulsion forming and stabilizing properties of peptides

Instead of comparing the functional properties of proteins with varying molecular structures, the effect of modifying the molecular structure of a single protein on its functional properties is often used to study structure-function relations. Proteins are often modified by enzymatic hydrolysis, since it affects most properties which are thought to be of importance for protein functionality like molar mass, hydrophobicity, electrostatic charge, and molecular conformation (Kinsella, 1984).

The effect of enzymatic hydrolysis on the functional properties of proteins remains, despite of many studies, largely obscure due to the use of complex and poorly defined peptide mixtures. Few studies were done using pure peptides. For example Huang et al. (1996) found improved emulsion forming properties for a flexible, amphiphilic β-lactoglobulin peptide (f. 41-100)-S-S-(f. 149-162) compared to those of the intact protein. Shimizu et al. (1983) demonstrated the importance of the N-terminal domain of α_{s1}-casein for its emulsion forming properties. Removal of this hydrophobic domain resulted in a decreased affinity for oil/water interfaces and decreased emulsion forming properties. Removal of the even more hydrophobic C-terminal domain appeared to have less effect on the emulsion forming properties. Data available for relating the molecular properties of pure peptides to their emulsion forming and stabilizing properties are, however, limited and few general trends appear to be distinguishable.

An important factor determining the functionality of hydrolyzed proteins is the cleavage specificity of the enzyme used, since this determines the molecular properties of the peptides formed (e.g. Vojdani and Whitaker, 1994; Multilangi et al., 1996; Caessens et al., 1999c). The functionality of the hydrolyzates is also affected by the degree of hydrolysis. At a limited degree of hydrolysis often improved emulsifying properties are found (Chobert et al., 1988; Vojdani and Whitaker, 1994). This improvement is frequently due to an enhanced solubility of the hydrolyzed proteins, especially near the isoelectric pH (Shimizu et al., 1986a; Chobert et al., 1988; Multilangi et al., 1996). This effect is, however, not always obvious (e.g. Lee et al., 1987a; Chobert et al., 1988; Vojdani and Whitaker, 1994).

A further increase in the degree of hydrolysis generally results in decreasing emulsifying properties (Saito et al., 1993; Agboola and Dalgleish, 1996). Agboola and Dalgleish (1996) found a relatively low coalescence stability of emulsions

made with hydrolyzed sodium caseinate or β-lactoglobulin compared to those of emulsions made with the intact proteins. The stability of the emulsions decreased with an increasing degree of hydrolysis, hence decreasing molar mass. It is generally believed that peptides need to have a certain minimum molar mass to be able to stabilize an emulsion effectively (Lee et al., 1987a; Chobert et al., 1988).

In some cases, synergistic effects between peptides are observed, making the emulsifying behaviour of peptides even more complex. For example, the emulsion forming properties of a hydrophobic α_{s1}-casein peptide (f. 1-23) and a hydrophobic β-casein peptide (f. 193-209) were improved in the presence of the hydrophilic glycomacropeptide of κ-casein (Lee et al., 1987b; Shimizu et al., 1986a).

Finally it is worth mentioning the approach of Enser et al. (1990), Carey et al., (1994), and Saito et al. (1995) who studied the emulsifying properties of synthetic peptides. These peptides were designed to possess molecular properties which were thought to be of importance for their functionality. The size of these synthetic peptides is, however, limited and so far little evidence for relations between molecular and functional properties could be established.

1.7 Aim and outline of thesis

Although some general trends appear to be discernible, the relation between the molecular and functional properties of proteins and peptides is still largely obscure. To effectively use proteins and peptides in a wide range of emulsified products, a fundamental understanding of their functionality is required. To unravel this structure-function relation, a large, collaborative project was started, titled "Formation and stability of emulsions and foams with proteins and peptides in relation to their molecular properties".

This project was divided into three sub-projects. One project was concerned with the enzymatic hydrolysis of β-casein and β-lactoglobulin and a preliminary testing of the foam and emulsion forming and stabilizing properties of the obtained peptides. This work has been performed and reported by Caessens (1999). Some of the peptide fractions from this project were selected for a more thorough study in the other sub-projects. One of these projects was performed by van Kalsbeek (to be published), who studied the relation between the molecular and foam properties of these peptides and of several intact proteins. The work of the third sub-project is presented in this thesis and was focused on the emulsion forming and stabilizing properties of the same proteins and peptides.

The aim of this project is to elucidate the relation between the molecular and emulsion forming and stabilizing properties of proteins and peptides. To achieve

this, the functionality of several pure and well-defined proteins, β-casein, β-lactoglobulin, α-lactalbumin, lysozyme and ovalbumin, and of purified β-casein peptides was studied.

Most of the work described in this thesis is the result of small, laboratory-scale experiments. To ensure that the conclusions obtained with these laboratory-scale experiments are applicable to emulsions produced on an industrial scale, the properties of emulsions prepared on a laboratory and industrial scale high-pressure homogenizer were compared. The results of this comparison are presented in Chapter 3. Chapter 4 deals with the emulsion forming properties of pure proteins in relation to their molecular properties. The effect of physicochemical conditions, like pH and ionic strength, and heat denaturation on these properties is discussed. Similarly, the emulsion forming properties of β-casein peptides are described in Chapter 5. The emulsion forming properties of these peptides are compared to those of intact proteins. Chapter 6 is concerned with the coalescence stability of emulsions made with proteins and peptides. Differences in the properties of the studied proteins and peptides are highlighted. Finally in Chapter 7, an overview of the properties of proteins and peptides is given. A hypothesis of the relation between their molecular and emulsion forming and stabilizing properties is presented.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Proteins

Proteins were selected according to the following criteria: well-characterized molecular properties, widely varying molecular conformations and conformational stabilities, and availability in a high purity. Bovine β-casein (90% based on dry weight, 95% based on total nitrogen, w/w) was obtained from Eurial (France). Bovine β-lactoglobulin (more than 95% based on dry weight, w/w) was prepared by Caessens et al. (1997b). Bovine α-lactalbumin (90% based on dry weight, 95% based on total nitrogen, w/w) was kindly provided by NIZO (The Netherlands). Electrophoretically pure hen egg-white lysozyme was kindly supplied by NIVE (The Netherlands). Hen ovalbumin (more than 98%, w/w) was obtained from Sigma. Sodium caseinate (DMV-International, the Netherlands) and whey protein (Bipro, Le Sueur Isolates, USA) were used for large-scale experiments.

2.1.2 β-Casein peptides

Peptides of β-casein were prepared, fractionated, and characterized by Caessens and co-workers (1997a; 1999, 1999b). β-Casein was hydrolyzed by plasmin. During the hydrolysis, a precipitate was formed which was removed from the reaction mixture by centrifugation. This pellet (PEL1: f. 106/108/114-209) consisted of peptides originating from the hydrophobic N-terminal domain of β-casein. The supernatant of the reaction mixture was ultrafiltered using a membrane with a molecular weight cut-off of 5 kDa. The peptides in the retentate consisted mainly of amphiphilic peptides (RET1: f. 1/29-105/107) from the C-terminal domain of β-casein. These peptides were further fractionated by Ion Exchange Chromatography, yielding two fractions of amphiphilic peptides: IEC2 (f. 29-105/107), and IEC3 (f. 1-105/107).

2.1.3 Other materials

All solutions were made using distilled water. The emulsions were prepared with food grade soya oil (Reddy, the Netherlands) with a density (ρ) of 0.92 kg.m^{-3} and a refractive index (n_d) of 1.4733. For recoalescence experiments, triolein (Fluka, Germany; ρ = 0.91 kg.m^{-3} and n_d = 1.4692) and tricaprilin (Sigma; ρ = 0.95 kg.m^{-3} and n_d = 1.4466) were used. All other chemicals were of analytical grade and purchased from Merck.

2.2 Methods

2.2.1 Oil purification

Oil was made surfactant free using silica gel 60 (35-70 mesh). The silica gel was pre-dried overnight at 100 °C. The oil and 10 % (w/w) silicagel were stirred for two hours after which the oil was recovered by centrifugation and decantation. This procedure was repeated if necessary.

2.2.2 Interfacial tension

The interfacial tension of the oil/water interface was measured using the static Wilhelmy plate method to check the absence of surface-active components in the oil. The Wilhelmy plate was pre-wetted with the aqueous phase and then immersed in the oil phase. The force registered was corrected for buoyancy.

The interfacial tension of purified soya oil was about 29 $mN.m^{-1}$, which agrees well with literature data (Gaonkar, 1989). For purified tricaprilin and triolein an interfacial tension of 22 and 21 $mN.m^{-1}$, respectively, was found.

2.2.3 Emulsion preparation

Proteins and peptides were dissolved in 0.02 M HCl-imidazole or 0.03 M citrate buffer. The required ionic strength (typically 0.075 M) was obtained by adding sodium chloride to the buffer solution. A preservative (0.02 % sodium azide) was added if necessary.

A coarse pre-emulsion was prepared by mixing weighed amounts of oil and protein solution (oil volume fraction: φ = 0.2) using a magnetic stirrer. The pre-emulsion was homogenized with a laboratory scale high-pressure homogenizer (Delta Instruments, the Netherlands), operating at a pressure of 5 MPa, unless stated otherwise. Homogenization was usually repeated until a constant droplet size distribution was obtained (about 30 passes). During homogenization, the emulsions were cooled in ice-water to keep the temperature close to ambient temperature (typically 23-24 °C). Incorporation of air was avoided. Emulsions were prepared at least in duplicate. The oil fraction of the emulsions was occasionally determined using the Röse-Gottlieb method (IDF, 1987).

For large-scale experiments, emulsions were prepared using a small capacity, single-stage, industrial homogenizer (Rannie, Denmark; 100 $l.hr^{-1}$).

2.2.4 *Droplet size distribution*

2.2.4.1 *Characterization*

The droplet size distribution of emulsions can be characterized by an average size (e.g. diameter or volume) and a distribution width. Suppose that for emulsions with spherical droplets of diameter d, the number of droplets smaller than d is given by $F(d)$, then the number frequency distribution is given by $f(d) = \partial F(d)/\partial d$, and the volume frequency by $(1/6)\pi d^3 f(d)$. The n^{th} moment of the distribution is defined as

$$S_n = \int_0^\infty d^n f(d)\,\partial d \tag{2.1}$$

Any type of average diameter is then given by

$$d_{nm} = \left(\frac{S_n}{S_m}\right)^{1/(n-m)} \tag{2.2}$$

and the relative width or variation coefficient of the distribution by (Walstra, 1983)

$$c_n = \left(\frac{S_n S_{n+2}}{S_{n+1}^2} - 1\right)^{\frac{1}{2}} \tag{2.3}$$

An often used average, which is directly related to the interfacial area of droplets, is the volume-surface average or Sauter diameter (Walstra, 1965a):

$$d_{32} = \frac{S_3}{S_2} = \frac{\sum N_i d_i^3}{\sum N_i d_i^2} \tag{2.4}$$

with N_i and d_i the number and diameter of droplets in size class i, respectively. The relative width of the distribution is then given by (Walstra, 1968)

$$c_s = \sigma_2 = \left(\frac{S_2 S_4}{S_3^2} - 1\right)^{\frac{1}{2}} \tag{2.5}$$

and the specific interfacial area of emulsions by (Walstra, 1983)

$$A = \pi S_2 = \frac{6\varphi}{d_{32}} \tag{2.6}$$

Another useful average of the size distribution is the diameter d_{43}, which is related to the volume-surface average diameter and relative width as:

$$d_{43} = d_{32}\left(c_s^2 + 1\right) \tag{2.7}$$

2.2.4.2 Spectroturbidimetry

The spectroturbidimetric method has been described extensively by Walstra (1965a, 1968). Only a short description of the method will be given here.

A spherical droplet in an aqueous phase, illuminated by a beam of light of unit intensity, scatters an amount of light equal to $¼\pi d^2 Q$, where Q is the light scattering coefficient which is a function of

$$\rho = 2\pi \frac{d(n_d - n_c)}{\lambda} \tag{2.8}$$

where n_d and n_c are the refractive index of the disperse and continuous phase, respectively, and λ the wavelength of the light beam (in air).

The optical density (E) of a layer of thickness L containing N droplets per unit volume is according to the law of Lambert-Beer given by

$$E = \frac{1}{4}\pi d^2 NQL \log e \tag{2.9}$$

The optical density is thus proportional to d^2Q. Combining and rearranging Equation 2.8 and 2.9 yields

$$Z = \frac{Q^*}{\rho_{32}} = \frac{0.2443\,E\lambda}{L(n_d - n_c)\varphi} \tag{2.10}$$

where Z is the reduced turbidity, Q^* the average light scattering coefficient for a polydisperse emulsion corrected for forward scattering, and φ the oil volume fraction of the diluted sample, which is more readily determined than N. The volume-surface average of ρ_{32} is:

$$\rho_{32} = \frac{2\pi(n_d - n_c)d_{32}}{\lambda} \tag{2.11}$$

The quantity $\left(Q^{*}/\rho_{32}\right)$ as function of ρ_{32} has been calculated theoretically for assumed size frequency distributions. To determine a droplet size distribution, an experimentally obtained, reduced turbidity spectrum is compared to these calculated spectra. The best fitting spectrum yields the volume-surface average droplet size, the relative width and shape of the size distribution.

The reduced turbidity of emulsions can be determined if the refractive index of the continuous and disperse phase and the optical density as function of the wavelength are known. The refractive index was measured using an Abbe refractometer. The wavelength and temperature dependence of the refractive index was estimated using the relations found by Walstra (1965a, 1965b). The optical density of emulsions was measured with a Zeiss spectrophotometer (type M4 G II) with an attachment for turbidity measurements and an angle of acceptance of 1.5°. Before measurement, the emulsions were diluted with 0.3 % sodium dodecyl sulfate (SDS) solution (final optical density between 0.2 and 0.8) to avoid deviations due to multiple and dependent scattering. SDS was used to stabilize the droplets and to disperse any aggregates present. The absence of aggregates was checked by light microscopy. If protein micelles were present, emulsions were diluted with a solution of 0.375 % disodium ethylenediamine tetra-acetate (EDTA) and 0.125 % polyoxyethylene sorbitan monolaurate (Tween 20) of pH 10 to dissociate the micelles. The optical density was measured at wavelengths ranging from 380 to 1700 nm.

2.2.4.3 Coulter counter

The Coulter counter method is based on the registration of voltage pulses caused by particles suspended in an electrolyte solution, passing through a narrow orifice across which an electric field exists. The size of the voltage pulse is proportional to the particle volume. Hence, the size and number of pulses yields the droplet size distribution of an emulsion.

The size distribution of emulsions was determined using a Coulter Multisizer (type II) equipped with an orifice tube with an aperture of 30 or 100 μm. The emulsions were diluted with the electrolyte solution Isoton II (Coulter, England).

2.2.4.4 Laser light diffraction

The determination of a droplet size distribution by static laser light diffraction is based on the measurement of the forward diffraction pattern of a single-wavelength, laser light beam scattered by a diluted emulsion. This diffraction pattern is used for the calculation of the droplet size distribution.

The measurements were performed using a Coulter laser (LS 130). This system uses two methods. Laser light diffraction is used for determination of the size of droplets larger than 0.4 μm. For droplet sizes between 0.1 and 1.0 μm, PIDS-analysis is used, based on measurements of the extent of polarization of the scattered light.

2.2.4.5 Comparison of methods

Spectroturbidimetry is suitable for emulsions with a volume-surface average droplet size between about 0.2 and 15 μm and a monomodal size distribution (Walstra, 1968). The minimum droplet size, which can be measured using a Coulter counter equipped with an orifice with an aperture of 30 μm is 0.6 μm. This method is very sensitive for detecting irregularities in droplet size distributions. The Coulter laser appears to be mainly suited for emulsions with a volume-surface average droplet size larger than 1 μm. For emulsions with smaller droplets, the latter method is less suitable due to its poor discrimination power at small droplet sizes. The results of the diffraction method will, therefore, not be further discussed.

Generally, a good agreement is found between spectroturbidimetry and Coulter counter measurements, if the droplet size of the emulsions is sufficiently large (Walstra and Oortwijn, 1969; Boekel, 1980). For emulsions with relatively small droplets, the droplet size is overestimated when using a Coulter counter due to the presence of droplets which are not detectable (Table 2.1). The Coulter counter has, therefore, been used mainly as a check of the spectroturbidimetric method.

Table 2.1 Comparison of the volume-surface average diameter (d_{32}) as determined using spectroturbidimetry or Coulter counter: some typical results.

d_{32} (μm)	
spectroturbidimetry	Coulter counter
0.86	1.34
1.02	1.40
2.26	2.47
3.33	3.51
13.59	13.50

2.2.5 *Coalescence stability*

2.2.5.1 Methods

The coalescence stability of emulsions was studied using a turbidimetric method and a Coulter counter. The turbidity of an emulsion measured at a single wavelength may be used to estimate its volume-surface average droplet size. According to Equation 2.9 the turbidity, expressed as optical density, is given by (Walstra, 1969)

$$E = \text{constant} \sum N_i d_i^2 Q_i^* \tag{2.12}$$

The effective light scattering coefficient of a droplet in size class i, Q_i^*, is approximately constant if ρ_{32} is larger than 4. For a constant Q_i^* and an oil fraction proportional to $\Sigma N_i d_i^3$, Equation 2.12 converts to (Walstra, 1969)

$$\frac{E}{\varphi} = \text{constant} \frac{\sum N_i d_i^2}{\sum N_i d_i^3} = \frac{\text{constant}}{d_{32}} \tag{2.13}$$

The turbidity of diluted emulsions was measured at a wavelength of 380 and 1700 nm with the Zeiss spectrophotometer previously described. To meet the requirement of ρ_{32} larger than 4, the volume-surface average droplet size should be larger than about 1.7 μm and 7.1 μm for a wavelength of 380 and 1700 nm, respectively.

The Coulter counter was used for the determination of the droplet size by a direct measurement of the size distribution or by calculation using the number of droplets per unit volume of an emulsion. The Coulter counter and turbidity measurements were used to estimate the droplet size, and hence the coalescence stability of emulsions as function of time. The emulsions were stored at room temperature (i) under quiescent conditions and (ii) slowly rotating end-over-end with careful exclusion of air to establish the effect of creaming on the coalescence stability. Samples were taken at various times and diluted with 0.3 % SDS-solution to stabilize the droplets and to disperse any aggregates present. Samples of the emulsions stored under quiescent conditions were taken from undisturbed, single-use containers, which were gently mixed to disperse creamed droplets. Creaming appeared to have little effect on the coalescence stability of the emulsions at the experimental time-scales considered. Therefore, only the results of experiments performed under quiescent conditions will be discussed.

2.2.5.2 Comparison of methods

Some typical results for emulsions stored under quiescent conditions obtained with the methods described are shown in Table 2.2.

Table 2.2 Volume-surface average droplet size (d_{32}) of emulsions directly after formation and after a storage time (t) of 10 days under quiescent conditions. The droplet size was estimated using turbidimetric measurements (superscript ST) at a wavelength (λ) of 380 and 1700 nm, and Coulter counter measurements (superscript CC) of the droplet size and number of droplets per unit volume (N).

d_{32} (μm) at t = 0	d_{32} (μm) at t = 10 days			
	Turbidimetry		Coulter counter	
	λ = 380 nm	λ = 1700 nm	d_{32}	N
1.1^{ST}/ 1.4^{CC}	2.4	2.4	1.4	1.9
8.7	10.3	10.6	9.0	10.2

The droplet sizes estimated using turbidity measurements at a wavelength of 380 and 1700 nm were in good agreement. For the emulsion with the relatively small droplet size, the effective light scattering coefficient was not constant. Any variation in this coefficient appeared, however, to have little effect given the similarity of the results obtained at both wavelengths.

The droplet size distribution of emulsions stored under quiescent conditions as determined using a Coulter counter appeared to be hardly affected by droplet coalescence. The unstable droplets were most likely too large to be detected with the Coulter counter. These large droplets were in some cases visible as large oil droplets on top of the emulsion surface. The number of droplets per unit volume yielded results comparable to those of the turbidity measurements, if the emulsion droplet size was sufficiently large. For emulsions with small droplets, this method was less reliable due to presence of a large number of droplets with a size below the detection limit of the Coulter counter. Hence, the turbidity method appeared to yield the most reliable results. The coalescence stability of emulsions will, therefore, be discussed using the results of turbidity measurements at a wavelength of 380 nm. The other methods were used as an extra check.

2.2.6 Recoalescence rate

The rate of recoalescence during homogenization was studied by determining the change in the composition of emulsion droplets as function of the number of passes through a homogenizer as described by Taisne et al. (1996) and Smulders et al. (1999). The change in droplet composition was determined using turbidity measurements. According to the light scattering theory of Rayleigh-Gans,

the turbidity of an emulsion is related to the difference between the refractive index of the disperse and continuous phase according to (Walstra, 1965b)

$$F = n_c \sqrt{\frac{E}{\varphi}} \propto |n_d - n_c| \tag{2.14}$$

If an emulsion is diluted with solutions of varying refractive indices, the turbidity of a sample will be zero if the refractive index of the droplets equals the refractive index of the aqueous phase. An example is shown in Figure 2.1.

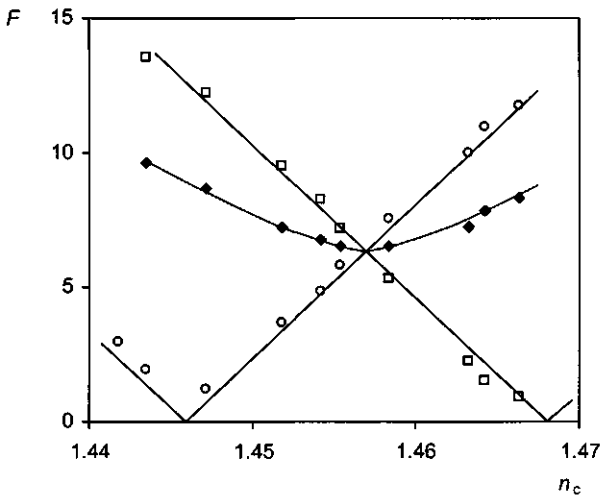

Figure 2.1 The turbidity indicated by parameter F of emulsions diluted in solutions with varying refractive indices (n_c). Emulsions with an oil phase of tricaprilin (O), or triolein (□), and an equivolume mixture of both emulsions without further homogenization (◆). Solid lines are calculated using Equation 2.14.

The recoalescence during homogenization is determined by measuring the turbidity of an emulsion prepared by mixing equal volumes of two emulsions made with different types of oil as function of the number of passes through a homogenizer. The factor F of the emulsion mixture is initially equal to the average of the factor F of the original emulsions. If recoalescence occurs, the oil in the emulsion droplets is mixed and as a result F decreases. The recoalescence rate can, therefore, be determined by studying the change in turbidity.

Emulsions made with tricaprilin and either triolein or soya oil (φ = 0.2), were prepared and homogenized separately, mixed in equal volumes, and subsequently

re-homogenized. During homogenization samples were taken and diluted in sucrose or fructose solutions with a refractive index equal to the refractive index of an equivolume mixture of the oil phases. The turbidity of these mixtures was measured with the previously described Zeiss spectrophotometer at a wavelength of 590 nm and used to determine the ratio F_p/F_0, where F_0, F_p are the parameter F after zero and p passes through the homogenizer, respectively. The decrease of ratio F_p/F_0 is a measure for the rate of recoalescence during emulsification. The ratio equals unity if the emulsions are stored under quiescent conditions, indicating the absence of Ostwald ripening, or if during emulsification recoalescence is absent. The ratio equals zero if the oil in the droplets is completely mixed, since in that case the refractive index of the disperse and continuous phase are equal.

2.2.7 Surface excess

2.2.7.1 Methods

The surface excess of emulsion droplets was measured using an indirect depletion method, and methods determining the amount of adsorbed protein directly. The measurement of the surface excess using the depletion method is based on the determination of the amount of unadsorbed protein and the interfacial area of the emulsion (Oortwijn and Walstra, 1979). The concentration of the protein solution before homogenization and the concentration of unadsorbed protein, combined with the specific interfacial area, yield the surface excess of emulsion droplets.

For determination of the concentration of unadsorbed protein, the emulsion droplets were separated from the aqueous phase by centrifugation at 13000 g for 20 minutes (Eppendorf Z231 M). The emulsions were generally stable against coalescence during this centrifugation procedure. For less stable emulsions, the major part of droplets was removed using a mild centrifugation step, followed by a more intensive centrifugation step to remove the smaller droplets. The protein concentration of the remaining aqueous phase and of the protein solution before homogenization were determined with BCA protein reagent (Pierce Ltd.), using a LKB-Biochrom 4060 spectrophotometer (Pharmacia). The reagent was calibrated for each specific protein or peptide. The calibration results were not affected by conformational changes of the proteins. The surface excess of each emulsion was determined at least in triplicate and was plotted as a function of bulk protein concentration over specific interfacial area, to facilitate comparison of emulsions with different interfacial areas (Walstra and de Roos, 1993).

To directly determine the amount of adsorbed proteins, the emulsions were washed repeatedly to remove unadsorbed proteins from the aqueous phase. To

wash emulsions, the emulsions were centrifuged to separate the droplets from the aqueous phase. The resulting cream layer was removed and redispersed in buffer solution, keeping the oil fraction equal to the oil fraction of the original emulsion. This procedure was repeated until approximately all unadsorbed protein was removed from the aqueous phase, which was usually after three washing procedures. The centrifugation speed was adjusted where necessary to facilitate easy removal and redispersion of the cream. The oil fraction and the droplet size distribution of the washed emulsions were determined using the Röse-Gottlieb method (IDF, 1987) and spectroturbidimetry, respectively.

The surface excess of the washed emulsion was determined using two methods. The emulsion was diluted with an equal volume of 15 % SDS-solution and heated to desorb the proteins from the droplet interface and to break the emulsion, as described by Hunt and Dalgleish (1994). The protein concentration of the resulting aqueous phase was measured and combined with the interfacial area of the emulsion used to calculate the surface excess. Determination of the surface excess using the second method involves direct measurement of the protein concentration by adding BCA-reagent to the emulsions. The emulsion droplets were removed by filtration (0.2 μm pore size) before photometric analysis.

2.2.7.2 Comparison of methods

The results obtained using the described methods were in good agreement as long as the surface excess was relatively high. For emulsions with a surface excess well below the maximum level, coalescence occurred during washing of the emulsions, making the direct methods less accurate. The depletion method was, therefore, selected as the most suitable method.

The main difficulty using the depletion method is the removal of all emulsion droplets from the aqueous phase by centrifugation. The surface excess of the remaining droplets has to be taken into account to avoid underestimation of the surface excess (Oortwijn and Walstra, 1979). The amount of oil in the aqueous phase after centrifugation was typically less than 0.2 % as determined with Röse-Gottlieb (IDF, 1987). This fraction appeared to have a negligible contribution to the surface excess as evidenced by the close agreement of the surface excess obtained using direct and indirect methods.

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Chapter 3

Formation of emulsions in a small laboratory and a large industrial scale high-pressure homogenizer

Abstract

The formation of oil-in-water emulsions with a laboratory or an industrial scale high-pressure homogenizer was compared, using proteins as emulsifier. In the industrial homogenizer, the emulsion droplets were broken up in a turbulent flow by inertial forces. In the laboratory homogenizer, the flow was found to be a bounded laminar flow-type and droplet break-up also appeared to occur via inertial forces. Compared to the industrial homogenizer, the operating efficiency of the laboratory homogenizer was small and many passes through this homogenizer were required to obtain a steady droplet size distribution. The homogenizer scale appeared to have little effect on the droplet size and recoalescence rate of emulsions made with proteins.

3.1 Introduction

In industry, emulsions are prepared using large-scale high-pressure homogenizers. The development of new emulsified products usually takes place on a much smaller scale. Fundamental studies are also performed on a smaller laboratory scale due to the use of often expensive and scarce materials. Examples of laboratory scale machines are: small-scale high-pressure homogenizers (e.g. Tornberg and Lundh, 1978; Burgaud et al., 1990), microfluidizers (e.g. Robin et al., 1993; Strawbridge et al., 1995), sonifiers and turbo-mixers (Walstra, 1974; Tornberg and Lundh, 1978).

From the literature it is known that in some cases the emulsion properties are affected by the type of emulsifying machine used. For example, milk homogenized with a microfluidizer has a bimodal droplet size distribution with a population of very small and of relatively large droplets, while milk homogenized with a high-pressure homogenizer has a monomodal size distribution (Strawbridge et al., 1995; Dalgleish et al., 1996). Bimodal droplet size distributions are also reported for emulsions prepared by sonification (Walstra, 1983). Emulsion formation is moreover affected by the dimensions of the emulsifying machine (Tornberg and Lundh, 1978; Walstra and Smulders, 1998). Walstra (1974) showed for example that the effect of the viscosity of the disperse and continuous phase depends on the type of high-pressure homogenizer.

Relations between the emulsifying conditions and the emulsion properties found on a laboratory scale might, therefore, not be valid for larger industrial scale processes. For the work reported here the influence of the dimensions of the used machine on the emulsion formation was studied for a small and a large scale high-pressure homogenizer. The aspects considered were: the mechanism of emulsion formation, and scale-up from laboratory to industrial scale processes. Some theoretical aspects of the emulsion formation will be discussed briefly. Emulsion formation was reviewed in greater detail by Walstra (1983, 1993) and Walstra and Smulders (1998).

3.2 Theory

A high-pressure homogenizer consists of a high-pressure pump and a valve with a narrow slit (Figure 3.1). A coarse pre-emulsion is forced through the narrow valve, resulting in a sharp increase in the flow velocity. The height of the valve lift and the flow velocity are determined by the homogenization pressure.

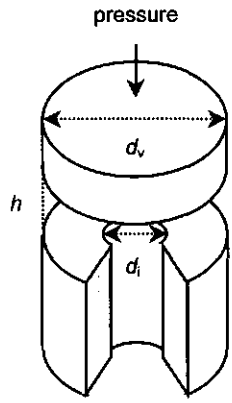

Figure 3.1 Model of a homogenizer valve. See text for explanation of symbols.

During the passage of emulsion droplets through the homogenizer valve, several processes occur simultaneously: deformation and possibly break-up of droplets, adsorption of surfactant at the newly formed interface, collision and possibly recoalescence of droplets (Walstra, 1993). Most of the droplet break-up occurs in the region just inside the valve, where the flow velocity is highest (Loo and Carleton, 1953; Phipps, 1974). About halfway across the homogenizer valve, the flow conditions are less severe and droplets are not broken up any further. Recoalescence of droplets is still possible as shown by the preliminary experiments of Loo and Carleton (1953) and Phipps (1974). The final droplet size of emulsions is determined by the balance between break-up and recoalescence of droplets, leading to a (quasi) steady state.

To break-up a droplet, the droplet must be deformed. The deformation of droplets is opposed by the Laplace pressure, which is given by

$$p_L = 4\frac{\gamma}{d} \tag{3.1}$$

where γ is the interfacial tension and d the droplet diameter. For break-up to occur, the stress acting on the droplets should counter act the Laplace pressure. This stress may be a shear or an inertial stress. Deformation and break-up of droplets by shear forces occurs in either a laminar flow regime or in a turbulent flow regime if the drop Reynolds number is smaller than unity (Walstra, 1983). The latter case is less relevant for this study and will not be further discussed.

The Reynolds number of the flow is defined as:

$$\mathrm{Re} = \frac{L\rho v}{\eta} \tag{3.2}$$

where L is the characteristic length, ρ the density, v the velocity, and η the viscosity. For homogenizer valves the characteristic lengh is given by $2*h$ (Kiefer, 1977), where h is the height or slit width of the homogenizer valve. The flow in the valve is laminar for Reynolds numbers smaller than about 1500 and turbulent for Reynolds numbers larger than about 3000 (Kiefer, 1977).

The velocity of emulsions in the homogenizer valve can be estimated using Bernoulli's law (Walstra, 1983), neglecting any energy losses due to friction. According to Bernoulli's law:

$$p_i + \frac{1}{2}\rho v_i^2 = p_v + \frac{1}{2}\rho v_v^2 \tag{3.3}$$

where p is the pressure and v the velocity in the valve inlet (i) or in the valve (v) of the homogenizer. Assuming that the pressure in the homogenizer valve is approximately zero, and that the velocity in the valve inlet can be neglected compared to the velocity inside the valve, Equation 3.3 reduces to

$$p_i = \frac{1}{2}\rho v_v^2 \tag{3.4}$$

The lift of the homogenizer valve can be obtained using Equation 3.4 and

$$Q = \pi d_i h v_v \tag{3.5}$$

where Q is the flow rate and d_i the diameter of the valve inlet. Knowing these properties, the flow regime in the valve can be determined.

In a laminar flow regime droplets are broken up if the Weber number exceeds a critical value (We_{cr}). The Weber number is defined as the ratio of the external stress over the Laplace pressure and is for a laminar flow given by

$$We = \frac{\eta_c G d}{2\gamma} \tag{3.6}$$

where η_c is the viscosity of the continuous phase and G the velocity gradient. The critical Weber number varies with the type of flow (e.g. simple shear or elongational flow) and with the viscosity ratio between the disperse and continuous phase (Grace, 1981; Bentley and Leal, 1986). The droplet size of emulsions formed by shear forces can be roughly estimated using (Walstra, 1993)

$$d \approx \frac{2\gamma We_{cr}}{\eta_c G} \tag{3.7}$$

In a turbulent flow, the flow is chaotic and eddies of various sizes are formed. The break-up of droplets depends on the scale and life-time of these eddies. For predicting the droplet size of emulsions formed in a turbulent flow, the Kolmogorov theory for isotropic turbulence can be applied, yielding for drop Reynolds numbers larger than unity (Walstra, 1993)

$$d \approx \frac{\gamma^{3/5}}{\varepsilon^{2/5}\rho^{1/5}} \tag{3.8}$$

where ε is the energy density.

The theories discussed so far are applicable to unbounded flow types as is the case in most large scale homogenizers. For very small, laboratory scale homogenizers, the dimensions of the valve are often of the same order of magnitude as the size of the emulsion droplets. In these homogenizers, the flow in the valve is a bounded laminar Poiseuille (or parabolic) type of flow and the relations described by Kiefer (1977) apply. The droplet size of emulsions formed in these types of homogenizers is approximately given by (Walstra, 1983)

$$d \approx \left(\frac{64 h^4 \gamma}{\rho_c v^2} \right)^{1/5} \tag{3.9}$$

where v is the average velocity in the valve and ρ_c the density of the continuous phase. Equation 3.9 is valid if

$$\frac{\rho_c v^2 h}{\gamma} > 64 \tag{3.10}$$

3.3 Materials and methods

Two types of high-pressure homogenizers have been used: a laboratory scale homogenizer (Delta Instruments, The Netherlands), and a small capacity, single-stage, industrial homogenizer (Rannie, Denmark). The properties of these homogenizers are given in Table 3.1. The actual valve diameter of the laboratory homogenizer is given by the circumference of the ball in contact with the valve (approximately 5 mm). The flow rate in this homogenizer decreases with increasing homogenization pressure.

Table 3.2 Properties of the laboratory and industrial high-pressure homogenizer

Properties	Laboratory homogenizer	Industrial homogenizer
valve type	ball	corrugated cone
inlet diameter (d_i)	5 mm	3 mm
valve diameter (d_v)	≈ 5 mm	13 mm
flow rate (Q)	5 - 8 $l.hr^{-1}$	100 $l.hr^{-1}$

With these homogenizers soya oil-in-water emulsions with an oil fraction of 0.2, a pH of 6.7, and an ionic strength of 0.075 M were prepared. The emulsifier was sodium caseinate (DMV-International, the Netherlands) or whey protein (Bipro, Le Sueur Isolates, USA) at various concentrations. The methods for preparing and characterizing the emulsions were described in Chapter 2.

3.4 Results and discussion

3.4.1 Properties of homogenizer valves

In Figure 3.2, the valve lifts calculated for the laboratory and the industrial high-pressure homogenizer are shown. The lifts were calculated neglecting energy losses due to friction. The friction, which depends on the type of flow in the valve, the Reynolds number, and the valve lift (Phipps, 1975; Kiefer, 1977), would result in a decrease in the linear flow rate and therefore an increase in the valve lift (Equation 3.5). The valve lifts shown in Figure 3.2 are thus underestimated, but comparison with data of Phipps (1971), who took into account friction losses, shows that the order of magnitude is correct. Phipps found for example for an industrial scale homogenizer comparable to the one used in this study, operating at a pressure of 30 MPa a valve lift of approximately 15 μm, which is slightly larger than the valve lift of 12 μm calculated here.

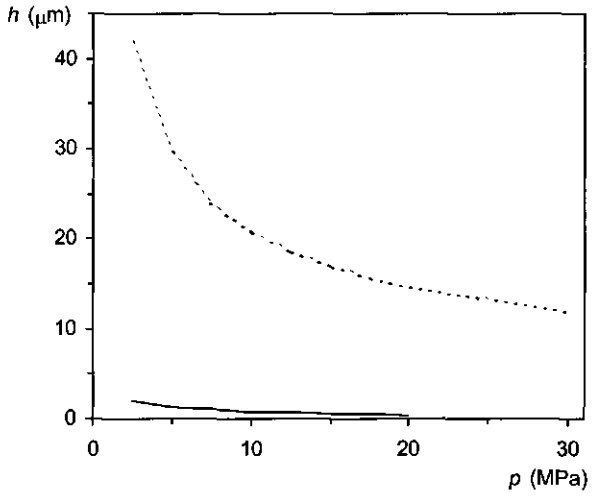

Figure 3.2 Calculated valve lift (h) of the laboratory scale (—) and the industrial scale (---) high-pressure homogenizer as a function of the homogenization pressure (p).

The valve lifts calculated for the laboratory homogenizer were rather small, though in good agreement with the values calculated by Tornberg and Lundh (1978). These small valve lifts are probably typical for most laboratory scale high-pressure homogenizers.

Using the calculated valve lifts, the Reynolds number of the flow in the valve of the industrial homogenizer was estimated to be approximately 5900, indicating a turbulent flow regime. The Reynolds number of the flow in the laboratory homogenizer ranged from approximately 280 for low pressures to 180 for high pressures. Hence, the flow regime in this homogenizer is laminar and for the most part elongational.

3.4.2 Mechanism of droplet break-up

The mechanism of the formation of emulsions in the homogenizers was studied using a surplus of sodium caseinate (c = 30 mg.ml^{-1}) as emulsifier. As shown in Figure 3.3, the relation between log d_{43} and log p of emulsions prepared in the industrial homogenizer was approximately linear with a slope of -0.61. This slope agrees well with the slope of -0.60 predicted by the Kolmogorov theory for droplet break-up by inertial forces in a turbulent flow (Walstra, 1993).

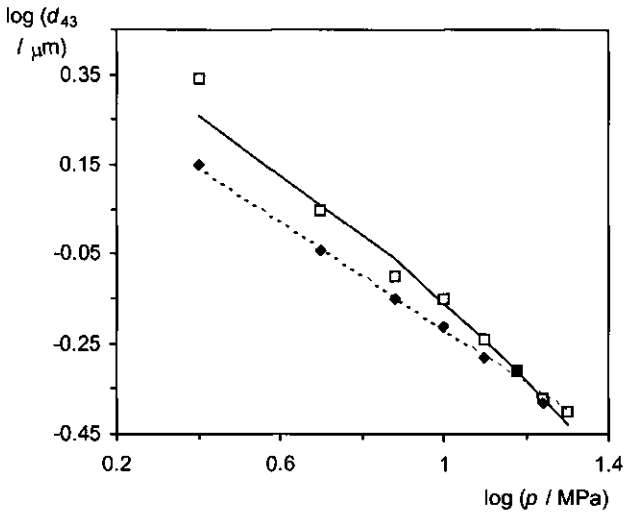

Figure 3.3 Average droplet size (d_{43}) of emulsions made with sodium caseinate (c = 30 mg.ml^{-1}) as a function of the homogenization pressure (p) prepared in the laboratory (□) or the industrial high-pressure homogenizer (◆). The best fit of the model of Kiefer (—) on the data of the laboratory homogenizer is indicated.

The relation between log d_{43} and log p of emulsions prepared in the laboratory homogenizer was not fully linear. For break-up of droplets in a laminar flow regime, a linear relation with a slope of -1 is expected (Walstra and Smulders, 1998). In this homogenizer the valve lift is, as shown before, of the same order of magnitude as the size of the emulsion droplets. Hence, break-up of droplets was likely to occur in a bounded type of laminar flow and the mechanism proposed by Kiefer (1977) possibly applies.

As shown in Figure 3.3, the model of Kiefer (Equation 3.9) appeared to be in good agreement with the measured droplet size of emulsions made with the laboratory homogenizer as a function of the homogenization pressure. The close fit indicates that break-up of droplets in this homogenizer was indeed likely to be due to inertial forces in a bounded Poisieulle type of flow. The best fit was obtained by taking an interfacial tension of 42 mN.m^{-1}, which is larger than the interfacial tension of a surfactant-free soya oil/water interface (γ = 29 mN.m^{-1}). This shows, that the model may be used for scaling of the droplet size as function of the homogenization pressure, the prediction of the absolute value of the droplet size is, however, uncertain.

Despite the differences in the mechanism of emulsion formation in the laboratory and industrial homogenizer, the emulsion droplet size appeared to be

only slightly affected by the homogenizer scale, especially at higher pressures. At relatively low pressures, the differences were found to be somewhat larger.

3.4.3 Operating efficiency of high-pressure homogenizers

In industry, emulsions are usually prepared with one or two passes through a high-pressure homogenizer. A high operating efficiency is therefore required. A measure for the operating efficiency is besides the droplet size, the relative width of the droplet size distribution as function of the number of passes through the homogenizer as shown in Figure 3.4.

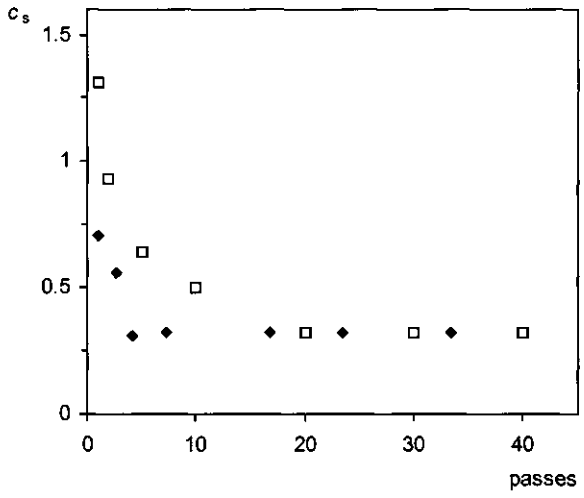

Figure 3.4 Relative width (c_s) of the droplet size distribution of emulsions made with sodium caseinate (c = 30 mg.ml^{-1}) as a function of the number of passes through the laboratory (□) or industrial (◆) high-pressure homogenizer at a pressure of 10 MPa.

The relative width of the droplet size distribution of emulsions prepared in the industrial homogenizer decreased faster than those of emulsions made in the laboratory homogenizer, indicating a higher operating efficiency of the large homogenizer. The relative width after a large number of passes was unaffected by the homogenizer scale. Similar results were found for the emulsion droplet size. For example, the droplet size of emulsions homogenized once at a pressure of 10 MPa was considerably smaller for emulsions prepared on an industrial scale (d_{43} = 1.0 μm) than for emulsions prepared on a laboratory scale (d_{43} = 2.9 μm). The difference in droplet size was much smaller after a large number of passes (Figure

3.3.). The operating efficiency of the homogenizers appeared to be unaffected by the type of protein or protein hydrolyzate used (results not shown).

The operating efficiency of homogenizers is determined by the flow regime in the valve and by the valve dimensions. In a turbulent flow droplet break-up may occur many times, while in a bounded laminar flow droplets are likely to be broken-up only once (Walstra and Smulders, 1998). The dimensions of the valve are also of importance, since these determine the size of the regions in the valve where the flow conditions are sufficiently severe for droplet break-up to occur. Both factors explain the relatively low operating efficiency of the small laboratory homogenizer.

3.4.4 Properties of emulsions made with proteins

The volume-surface average droplet size of emulsions made with sodium caseinate or whey protein prepared in the laboratory and the industrial homogenizer at a pressure of 5 MPa are shown in Figure 3.5. The emulsions were homogenized until a steady droplet size distribution was obtained.

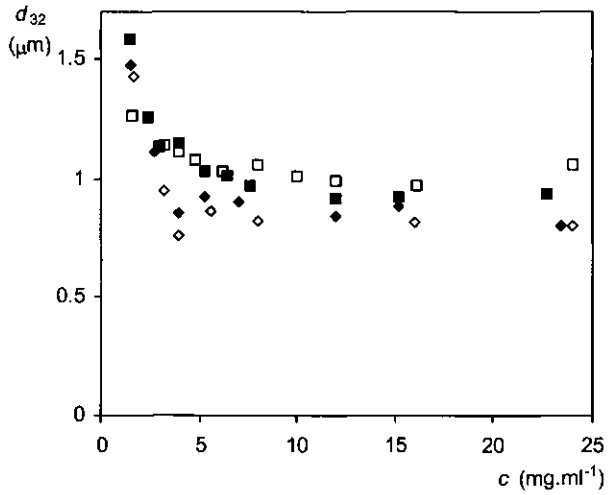

Figure 3.5 Volume-surface averaged droplet size (d_{32}) of emulsions made with varying concentrations (c) sodium caseinate (■) or whey protein (◆) prepared in a laboratory (open symbols) or industrial homogenizer (filled symbols), at a pressure of 5 MPa.

The droplet size of emulsions made with sodium caseinate was slightly larger than those of emulsions made with whey protein. The emulsion droplet size appeared to be hardly affected by the homogenizer scale. The difference in the

droplet size of emulsions made with sodium caseinate or whey protein appeared to be somewhat larger for emulsions made in the laboratory homogenizer than for those made in the industrial homogenizer. The differences were, however, very small, but might have been larger if the emulsions would have been prepared with only one or two passes through the homogenizer.

3.4.5 Recoalescence rate during homogenization

The rate of recoalescence during homogenization depends on the concentration and the interfacial properties of the surfactant. A potential third factor affecting the recoalescence rate is the type of homogenizer used. The recoalescence rate of emulsions made with whey protein during homogenization in the laboratory and the industrial homogenizer as function of the number of passes is shown in Figure 3.6. Similar results were obtained for emulsions made with sodium caseinate.

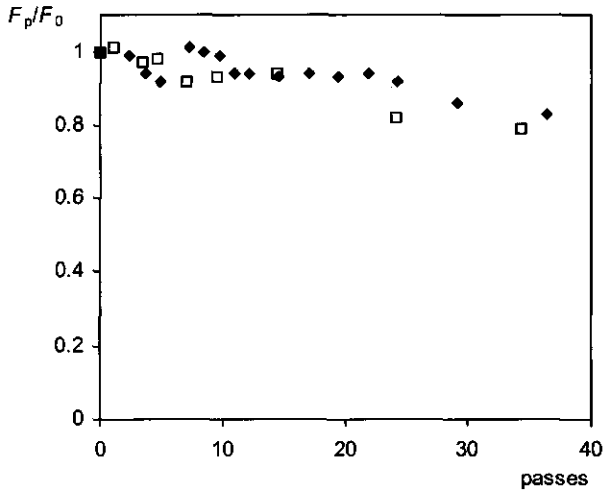

Figure 3.6 Ratio F_p/F_0 as a measure of recoalescence during homogenization of emulsions made whey protein (c = 4 mg.ml^{-1}) as function of the number of passes through the laboratory (□) or the industrial (◆) high-pressure homogenizer at a pressure of 5 MPa.

The recoalescence rate during homogenization of emulsions in the laboratory or industrial homogenizer was found to be very low under the conditions studied and appeared to be unaffected by the homogenizer scale. Since the conditions in a turbulent flow are more intense than in a laminar flow, the

recoalescence rate was expected to be higher in the industrial homogenizer than in the laboratory homogenizer. The recoalescence rate was, however, apparently not affected by the flow regime in the homogenizer valve.

3.5 Conclusions

The mechanism of emulsion formation and the operating efficiency of high-pressure homogenizers was shown to depend on the homogenizer scale. Nonetheless, the properties of the emulsions prepared in a laboratory or industrial scale homogenizer appeared to be rather similar, provided that the number of passes through the laboratory homogenizer was sufficiently large. Scale-up of laboratory experiments to industrial scale processes using high-pressure homogenizers, therefore, appeared to be allowed in the case studied here. Care should however be taken if emulsions are prepared with a few passes through the homogenizer or if using other types of emulsifying machines, like microfluidizers or sonifiers. The results may also be affected by the type of emulsifier, for example the use of small-molecule surfactants instead of proteins.

Chapter 4

Emulsion forming properties of proteins

Abstract

The emulsion forming properties of several milk and egg-white proteins with well-defined and widely varying molecular properties were studied to obtain a better understanding of the structure-function relation of proteins. The emulsion formation was studied by determining the droplet size distribution, surface excess and rate of recoalescence during emulsification as function of protein concentration and physicochemical conditions, like pH and ionic strength. The effect of heat denaturation on protein functionality was also studied.

Proteins generally possessed good emulsion forming properties as long as protein aggregation was absent. In those cases, only small differences in the droplet sizes could be determined and the recoalescence rates during emulsification were found to be similar. The surface excess of the droplets appeared to be governed by the conformational stability of the proteins. Globular proteins with a high conformational stability, like β-lactoglobulin, yielded a relatively low surface excess, while a flexible protein, like β-casein, yielded a relatively high surface excess.

The droplet size and recoalescence rate of aggregated emulsions were relatively large and appeared to be mainly governed by the aggregated state or effective molar mass of the proteins. The surface excess of the emulsions also seemed to increase with an increasing effective molar mass. Protein aggregation could be due to covalent and non-covalent intermolecular bonds, which may be induced by physicochemical conditions, heat and surface denaturation.

4.1 Introduction

Proteins generally have good emulsifying properties and are therefore often used in food emulsions. The emulsion forming properties are thought to depend on protein properties such as molar mass, hydrophobicity, conformation, conformational stability, and charge and on physicochemical conditions such as pH, and ionic strength (Kinsella, 1984). Despite numerous studies, the relation between the molecular properties of proteins and the processes occurring during emulsion formation remains still largely obscure. For an effective use of proteins in food emulsions, a basic understanding of this relation is required.

The processes occurring during emulsification are the deformation and break-up of droplets, the adsorption of proteins on the newly created oil/water interface and the collision and possibly recoalescence of droplets (Walstra, 1993). The final droplet size of emulsions is the result of a quasi steady state between droplet break-up and recoalescence.

To obtain a better understanding of the emulsion forming properties of proteins, several proteins with well characterized and widely varying molecular properties were selected. Some of these properties are shown in Table 4.1. A more detailed overview is given below. The relation between the molecular and emulsifying properties has been studied by comparing the droplet size, surface excess and recoalescence rate of emulsions made with these proteins under varying physicochemical conditions. The results of these experiments are discussed in this chapter. A detailed discussion of the fundamental principles governing the emulsion forming properties of proteins is given in Chapter 7.

Table 4.1 Molecular properties of the selected proteins (Swaisgood, 1982; Awade, 1996; Walstra et al., 1999).

Protein	Molar mass (Da)	Isoelectric pH
β-casein	24000	~ 5
β-lactoglobulin	18300	5.1
α-lactalbumin	14200	4.8
lysozyme	14500	10.7
ovalbumin	45000	4.5

4.2 Molecular properties of selected proteins

4.2.1 β-Casein

Bovine β-casein has little secondary structure (Creamer et al., 1981; Graham et al., 1984) and no intramolecular cross-links (Swaisgood, 1982), hence approximately random coil properties. The N-terminal domain (f. 1-43) of β-casein is highly hydrophilic and contains five phosphoseryl residues, while the C-terminal domain (f. 136-209) consists mainly of hydrophobic residues (Swaisgood, 1982). At the pH of normal bovine milk (pH 6.7), all the net charge of the protein is carried by the N-terminal domain, while the net charge of the C-terminal domain is nearly zero (Creamer, 1972). β-Casein is, due to the uneven distribution of charged and hydrophobic residues, highly amphiphilic (Swaisgood, 1982).

Like detergents, β-casein has a tendency to form micelles if the protein concentration is above the critical micelle concentration (Arima et al, 1979). The micellization occurs mainly via hydrophobic interactions between the C-terminal domains (Berry and Creamer, 1975) and is affected by physicochemical conditions like temperature, pH and ionic strength (Payens and Markwijk, 1963; Niki and Arima, 1969; Payens et al., 1969).

4.2.2 β-Lactoglobulin

β-Lactoglobulin is a globular whey protein with two disulfide-bonds and one thiol-group, which is buried in the interior of the molecule (Papiz et al., 1986). Under most conditions, β-lactoglobulin is in solution present in an associated state. This association is a rapid and reversible process and depends on physicochemical conditions (Townend et al., 1960b; McKenzie and Sawyer, 1967). At ambient temperature, β-lactoglobulin forms dimers between a pH of about 3.5 and 6.5, except at very low protein concentrations (Townend et al., 1960b; McKenzie and Sawyer, 1967). Cubic octamers are formed between pH 4.4 and 4.65 at low temperatures (Townend et al., 1960b; Townend and Timasheff, 1960; McKenzie and Sawyer, 1967). Below pH 3.5 and above pH 6.5, the dimers dissociate into monomers (Townend and Timasheff, 1960; Townend et al., 1960a). The equilibrium between monomers and dimers is governed by protein concentration, temperature and ionic strength (Aymard et al., 1996; Kella and Kinsella, 1988a).

β-Lactoglobulin is susceptible to denaturation at high pH and temperature. Above pH 7.5, conformational transitions occur, resulting in exposure of the free thiol-group and formation of intermolecular disulfide-bonds (Tanford et al., 1959; McKenzie and Sawyer, 1967; Kella and Kinsella, 1988a). A similar reaction occurs during heating. At high temperatures, β-lactoglobulin unfolds and protein

aggregates are formed via disulfide-bonds and non-covalent, presumably hydrophobic, interactions, making denaturation an irreversible process (Sawyer, 1968; Iametti et al., 1995). The formation of aggregates is dependent on protein concentration, pH, ionic strength, heating time and temperature (Sawyer et al., 1971; Roefs and Kruif, 1994; Boye et al., 1996).

4.2.3 α-Lactalbumin

α-Lactalbumin is a relatively small whey protein molecule, which contains four disulfide bridges (Brew et al., 1967). This protein has a high affinity for binding calcium ions (Hiraoka et al., 1980). The bound calcium ion stabilizes the native protein structure (Hiraoka et al., 1980) and increases the stability against thermal denaturation (Dolgikh et al., 1981; Kuwajima et al., 1986). Removal of the bound calcium by chelating agents induces conformational changes, yielding the so-called apo-α-lactalbumin. Apo-α-lactalbumin has a conformation which is intermediate between the native and denatured state, the so-called molten globule state (Dolgikh et al., 1981). The secondary structure of apo- and native α-lactalbumin is similar, while the apo-protein has a less compact conformation and a more flexible tertiary structure (Dolgikh et al., 1981; Segawa and Sugai, 1983). Below pH 2.5, bound calcium is released and a conformation similar to that of the apo-protein is adopted (Dolgikh et al., 1981; Permyakov et al., 1981).

4.2.4 Lysozyme

Hen egg-white lysozyme is an enzyme with antibacterial activity (McKenzie and White, 1991). The primary structure of lysozyme and α-lactalbumin are similar with a sequence homology of about 40 % (Brew et al., 1967). The three-dimensional structures of lysozyme and α-lactalbumin are also largely alike, mainly due to the similar locations of the four disulfide bridges (Brew et al., 1967). Some differences are, however, reported (Wilson et al., 1995; Urbanova et al., 1991). Lysozyme appears to have a more rigid conformation and a higher stability against heat denaturation due to stronger hydrophobic interactions (Barel et al., 1972; Acharya et al., 1989). Unlike α-lactalbumin, lysozyme has in solution at neutral pH a slight tendency to associate and form dimers (Deonier and Williams, 1970).

4.2.5 Ovalbumin

Hen egg-white ovalbumin is a large protein molecule, containing four buried thiol-groups (Nisbet et al., 1981). The conformation of ovalbumin is relatively unstable at neutral pH (Osuga and Feeney, 1977). Upon unfolding, the thiol-groups are exposed and polymers linked by disulfide bridges are formed (Kitabatake and Doi, 1987). At pH 2, ovalbumin is in a molten-globule state as indicated by the

increased conformational flexibility with retention of the native conformation (Koseki et al., 1988).

4.3 Droplet size of emulsions made with proteins

4.3.1 Emulsions made with β-casein

Emulsions with an oil fraction of 0.2 and varying protein concentrations (pH 6.7 and I = 0.075 M) were prepared using a laboratory high-pressure homogenizer operating at a pressure of 5 MPa. The volume-surface average droplet size of emulsions made with β-casein is shown in Figure 4.1.

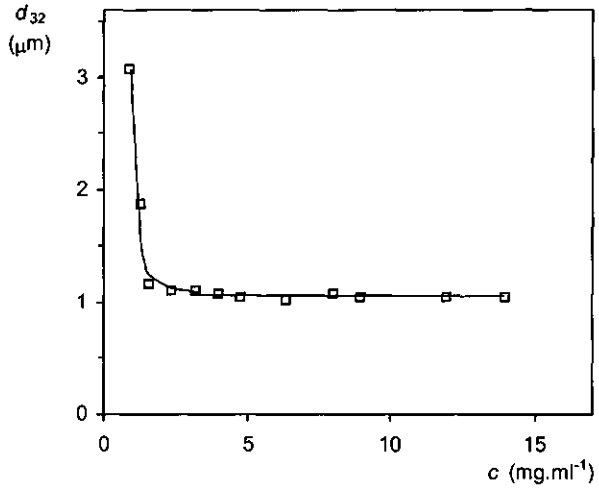

Figure 4.1 Volume-surface average droplet size (d_{32}) of emulsions prepared with varying concentrations (c) of β-casein (pH 6.7 and I = 0.075 M).

At low concentrations, the droplet size of emulsions made with β-casein decreased rapidly with increasing concentration. Above a concentration of about 4 mg.ml^{-1}, a surplus of protein was present and a constant droplet size was obtained. The constant droplet size and the minimum concentration required to obtain this droplet size were governed by the type of homogenizer (Chapter 3) and by the homogenization conditions (Figure 4.2).

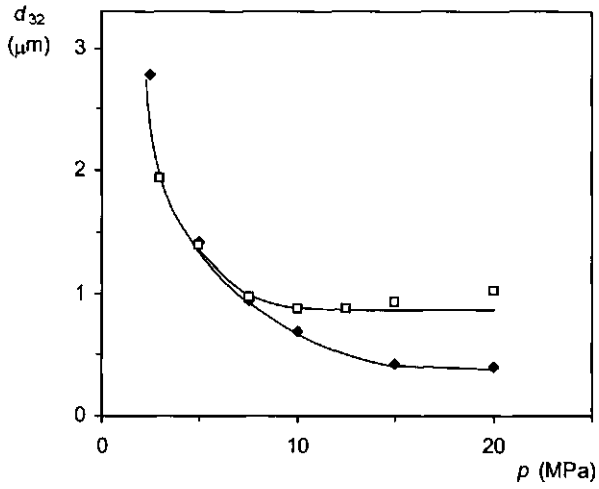

Figure 4.2 Volume-surface average droplet size (d_{32}) of emulsions made with β-casein as a function of homogenization pressure (p). Concentration: 4 mg.ml^{-1} (□), and 8 mg.ml^{-1} (◆).

In Figure 4.2, two regions can be distinguished. At low homogenization pressures, the emulsion droplet size was mainly determined by the homogenization conditions if a surplus of protein was present. If the protein concentration became limiting, the droplet size was determined by the concentration and independent of the pressure. At higher pressures, smaller droplets may be obtained, increasing the dependence on protein concentration.

In this study, the homogenization pressure used for preparing emulsions was relatively low to facilitate an accurate determination of the droplet size and surface excess. When comparing the emulsion forming properties of proteins, the absolute differences in their functionality will probably be larger if the emulsification conditions are more severe. The differences will, however, most likely remain relatively the same, facilitating a comparison of protein functionality.

4.3.2 Emulsions made with globular proteins

The droplet size of emulsions prepared with β-casein, β-lactoglobulin, or α-lactalbumin are shown in Figure 4.3a. The emulsion forming properties of these proteins improved in the order of β-casein, α-lactalbumin and β-lactoglobulin. The differences are, however, small and the proteins generally appeared to have good emulsion forming properties under the conditions studied.

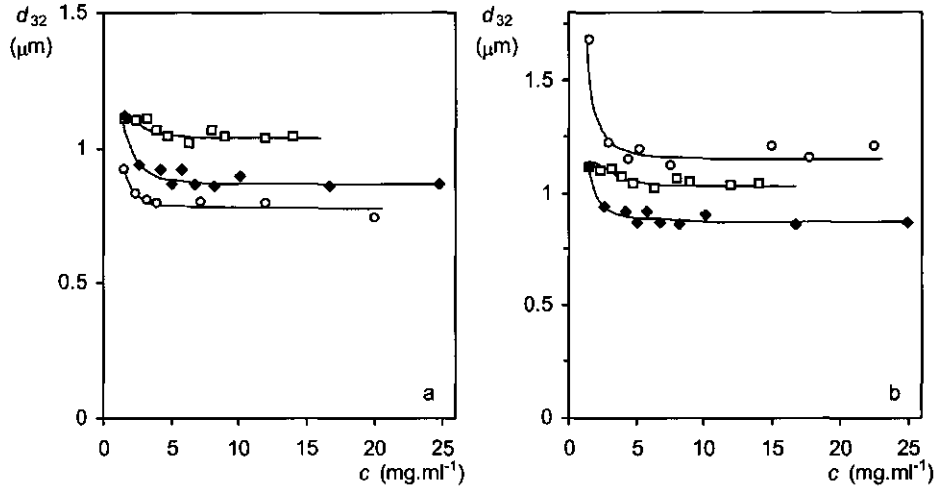

Figure 4.3 Volume-surface average droplet size (d_{32}) of emulsions as function of concentration (c) of: a, β-casein (□), β-lactoglobulin (○), and α-lactalbumin (◆); b, β-casein (□), α-lactalbumin (◆), and lysozyme (○); all at pH 6.7 and I = 0.075 M.

Comparison of our data with data from literature is hampered by the often contradicting results. For example, Chen and Dickinson (1993) found, in agreement with our results, superior emulsion forming properties for β-lactoglobulin compared to those of β-casein. In a similar study, the droplet sizes of emulsions made with the same proteins were found to be comparable (Chen et al., 1993). Kato et al. (1985), Dickinson et al. (1989) and Courthaudon et al. (1991) found, in contrast to our results, superior emulsion forming properties for α-lactalbumin compared to those of β-lactoglobulin. Dickinson et al. (1993b) found similar droplet sizes for emulsions made with these proteins. According to Shimizu et al. (1986b), the emulsion forming properties of β-casein were comparable to those of α-lactalbumin.

In general, the differences in the emulsion forming properties of β-casein, β-lactoglobulin and α-lactalbumin are found to be small. Deviations between literature date and our results may be due to variations in the properties of the studied proteins, like purity and degree of denaturation, and in the methods used. In literature, the emulsion forming properties of proteins are mostly studied at a single protein concentration, often using ambiguous methods. As shown in Figure 4.3, the dependence of the droplet size on protein concentration may vary among proteins. Determination of protein functionality at a single concentration may, therefore, result in erroneous conclusions.

The emulsions made with lysozyme were strongly aggregated and the size of the (deflocculated) emulsion droplets was found to be relatively large (Figure 4.3b). This relatively large droplet size is probably related to the state of aggregation as will be discussed later. The emulsion forming properties of lysozyme were, despite the similarities in their molecular structures (Brew et al., 1967), inferior to those of α-lactalbumin. In the literature, the emulsion forming properties of lysozyme are also generally found to be poor compared to those of other proteins, like caseins and whey proteins (Pearce and Kinsella, 1978; Kato et al., 1985). These inferior properties may be due to the high conformational stability of lysozyme (Norde and Favier, 1992).

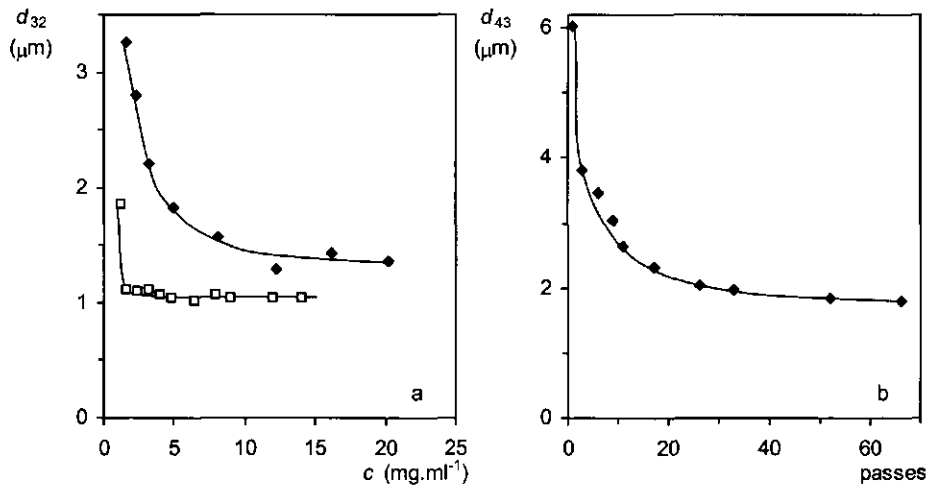

Figure 4.4 Average droplet size of emulsions made with β-casein (□) or ovalbumin (◆) as function of: a, concentration (*c*); b, number of passes through a high-pressure homogenizer at a concentration of 16 mg.ml^{-1}; all at pH 6.7 and I = 0.075 M.

As shown in Figure 4.4a, the droplet size of emulsions made with ovalbumin was relatively large and strongly dependent on protein concentration. Similar results are reported by Pearce and Kinsella (1978) and Kato et al. (1985). The emulsions made with ovalbumin were, like those made with lysozyme, strongly aggregated probably due to bridging flocculation via protein aggregates. Ovalbumin readily denatures at interfaces (Pearce and Kinsella, 1978; Kitabatake and Doi, 1987). Upon denaturation, reactive free thiol-groups are exposed, resulting in the formation of ovalbumin polymers via intermolecular disulfide-bonds (Kitabatake and Doi, 1987).

In case of progressing polymer formation, insoluble ovalbumin aggregates may be formed, decreasing the effective concentration and hence the emulsion forming properties of the protein. Pearce and Kinsella (1978) showed that the droplet size of emulsions made with ovalbumin increases if the emulsions are "homogenized" for a prolonged period of time using a type of ultra-turrax. In our study, emulsions made with ovalbumin appeared to be insensitive to such over-processing (Figure 4.4b), hence the processing time appeared to have little effect on the effective protein concentration. The negative effect of the processing time found by Pearce and Kinsella (1978) is likely to be due to the incorporation of air during the emulsification procedure.

4.4 Surface excess

4.4.1 Emulsions made with β-casein

In Figure 4.5, the surface excess of emulsion droplets made with β-casein is shown. The surface excess is given as function of protein concentration (c) over specific interfacial area (A) to facilitate comparison of the surface excess of emulsions stabilized with various proteins and differing interfacial areas.

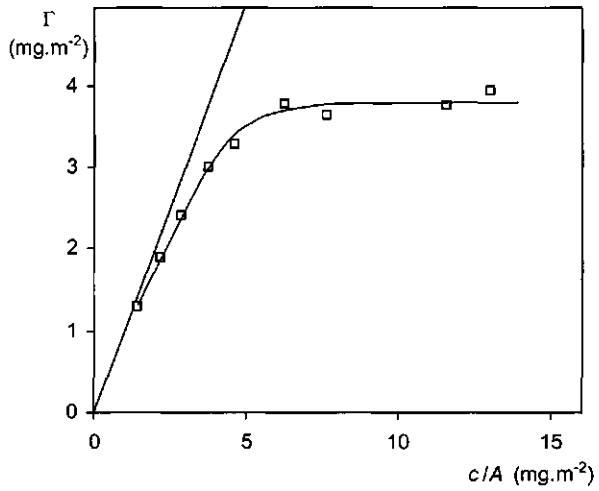

Figure 4.5 Surface excess (Γ) of emulsion droplets made with β-casein (pH 6.7 and I = 0.075 M) versus concentration over specific interfacial area (c/A). The straight line indicates the hypothetical surface excess obtained if all protein would be adsorbed onto the emulsion droplets.

At low protein concentrations, the surface excess of emulsion droplets made with β-casein increased rapidly with an increasing concentration and most of the β-casein available adsorbed at the droplet interface. At higher concentrations, the droplet interface became saturated with protein and a plateau surface excess was reached.

Proteins generally have a very high affinity for oil/water interfaces and readily adsorb at planar interfaces at much lower concentrations than small-molecule surfactants (Walstra and de Roos, 1993). In emulsions, the affinity for the oil/water interface appeared to be decreased and a relatively high concentration of unadsorbed protein (c_{eq}) was required to obtain a significant surface excess (Figure 4.6). Similar results were found for globular proteins.

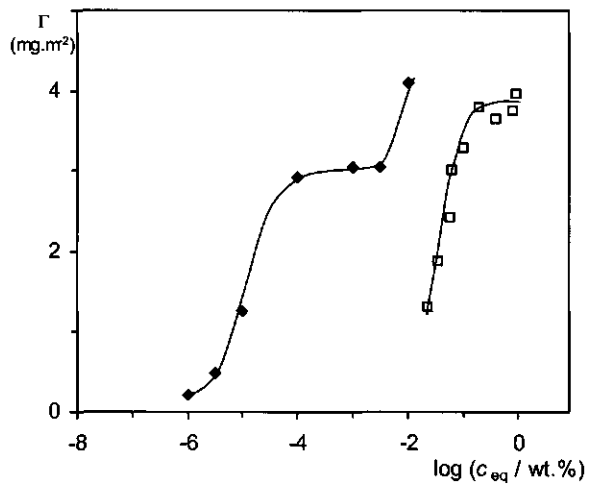

Figure 4.6 Surface excess (Γ) of β-casein for quiescent adsorption at a planar oil/water interface (◆; data taken from Graham and Phillips, 1979b) and at an emulsion droplet interface (□) as function of the equilibrium concentration of the aqueous phase (c_{eq}).

If β-casein was added to an emulsion with a low surface excess, the surface excess increased until the plateau surface excess was obtained. Apparently, in emulsions a barrier for protein adsorption is present and protein adsorption may only occur if the equilibrium concentration is sufficiently high. For example: the surface excess of an emulsion with an initial β-casein concentration over interfacial area of 2.2 $mg.m^{-2}$ increased from 2.0 $mg.m^{-2}$ to 3.8 $mg.m^{-2}$ if the β-casein concentration was increased to 12 $mg.m^{-2}$. Similar results were found by Dalgleish

(1990), who showed that the thickness of a layer of β-casein adsorbed at latex particles is not affected by the number of steps in which β-casein is added to the dispersion.

The cause of the relatively low "affinity" of β-casein for a droplet interface is unclear. An important difference between a planar and a droplet interface is that the interfacial area of emulsion droplets is, in contrast to a planar interface, correlated to the protein concentration and homogenization conditions. During emulsification, the droplet interface will be repeatedly expanded and compressed, which will likely enhance the unfolding of adsorbed proteins and hence affect the surface excess of the droplets. It appears to be unlikely that the curvature of the droplet interface affects the affinity of proteins for the interface.

The plateau surface excess of β-casein adsorbed at emulsion droplets appeared to be relatively high compared to those determined by Graham and Phillips (1979b) for β-casein adsorbed at a planar oil/water interface. At these interfaces, β-casein forms multilayers at high concentrations (Graham and Phillips, 1979b). Multilayer adsorption may be induced by the tendency of β-casein to form micelles in solution (Payens and Markwijk, 1963; Niki and Arima, 1969). To establish whether the high surface excess determined in this study was due to multilayer adsorption, the effect of washing of the emulsions on the surface excess was determined. To wash emulsions, the emulsion droplets were separated from the aqueous phase by centrifugation. The removed cream was diluted with buffer solution to regain the initial oil fraction. This procedure was repeated thrice to remove any unadsorbed protein from the aqueous phase. Due to the decreased protein concentration of the aqueous phase, any protein multilayer present will be desorbed (Walstra and de Roos, 1993).

As shown in Figure 4.7, the surface excess of emulsions made with β-casein was not affected by washing. Hence, the relatively high surface excess of the emulsion droplets could not be attributed to multilayer adsorption. Further evidence against multilayer formation or the adsorption of β-casein micelles was obtained by preparing and analyzing emulsions at 4 °C, where hydrophobic interactions are minimal and β-casein micelles are absent (Payens and Markwijk, 1963; Creamer, 1972). Again, similar surface excesses were found for emulsions prepared at ambient temperature and at 4 °C, suggesting monolayer adsorption at both temperatures. The presence of β-casein micelles appears to have little effect on the properties of the adsorbed protein layer (Dickinson, 1989).

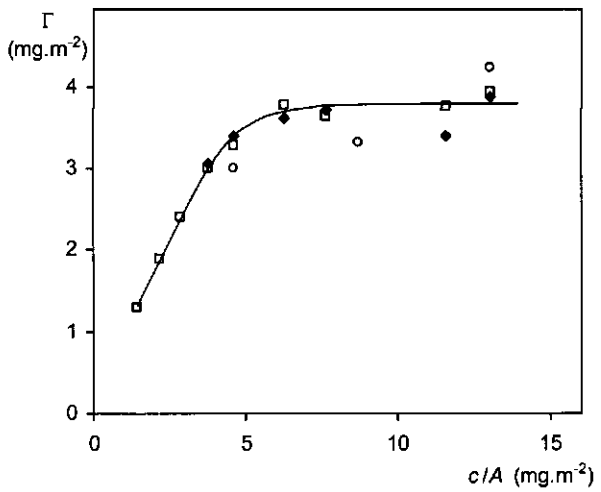

Figure 4.7 Surface excess (Γ) of emulsion droplets made with β-casein (pH 6.7 and I = 0.075 M) versus concentration over specific interfacial area (c/A): emulsions prepared at ambient temperature (□), washed emulsions (◆), and emulsions prepared and analyzed at 4 °C (○).

Few data are available on the surface excess of emulsion droplets as function of protein concentration. Interpretation of the surface excess obtained at a single protein concentration is complicated, since from most results it is not evident if sufficient protein was present to obtain a monolayer coverage. Also, the surface excess appears to depend on the type of disperse phase (Dickinson and Tanai, 1992; Dickinson et al., 1993b). In general, the surface excess of adsorbed protein monolayers varies between about 1 to 3 mg.m^{-2}. The plateau surface excess determined in our study for emulsions made with β-casein was thus relatively high, but appears to agree rather well with other studies. For example, Dickinson et al. (1988b) and Chen and Dickinson (1993) determined a surface excess for *n*-tetradecane emulsions (φ = 0.2) made with 4 mg.ml^{-1} β-casein with droplet sizes of 1.2 and 1.4 μm of about 2.9 mg.m^{-2} and 3.2 mg.m^{-2}, respectively.

The high surface excess of adsorbed β-casein is likely to be related to its low conformational stability. β-Casein is an approximately random coil protein with a highly flexible structure (Creamer et al., 1981; Swaisgood, 1982). Due to this high flexibility, the interfacial area occupied by β-casein molecules is strongly dependent on the surface excess (Feijter and Benjamins, 1982). At a high surface excess, a dense layer is formed which is much thicker than would be expected from the hydrodynamic radius of β-casein (4.6 nm) (Dalgleish, 1990). The flexible

conformation of β-casein allows the formation of a thick, "brush"-like layer with the hydrophobic C-terminal adsorbed at the interface and the charged N-terminal domain protruding far into the aqueous phase (Dalgleish and Leaver, 1991; Dickinson et al., 1993a; Beek et al., 1996). A schematic representation of the conformation of β-casein adsorbed at an oil/water interface is shown in Figure 4.8.

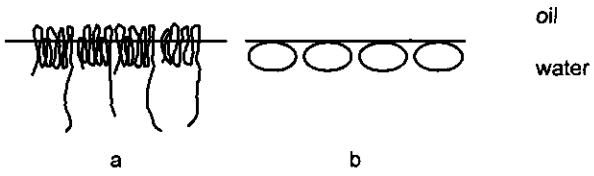

Figure 4.8 Schematic representation of the presumed molecular conformation at the oil/water interface of: a, β-casein; b, globular proteins like β-lactoglobulin and lysozyme.

4.4.2 Emulsions made with globular proteins

The adsorption isotherms of emulsions made with β-lactoglobulin or lysozyme were similar to those of emulsions made with β-casein, although the maximum surface excess of these emulsions was found to be relatively low (Figure 4.9). The surface excess of emulsions made with β-lactoglobulin found in this study was somewhat larger than the surface excess determined by Chen and Dickinson (1993) for *n*-tetradecane emulsions (φ = 0.2) made with β-lactoglobulin (c = 4 mg.ml^{-1}, d_{32} = 0.93 μm and Γ = 1.4 mg.m^{-2}). According to Graham and Phillips (1979b), the plateau surface excess of lysozyme at the air/water surface is about 2 mg.m^{-2}, which agrees well with our results. At a planar oil/water interface, however, no plateau surface excess could be determined by these authors.

The difference in surface excess of emulsions made with globular proteins, like β-lactoglobulin and lysozyme and a random coil protein, like β-casein is likely due to variations in their molecular flexibility. Globular proteins generally have a less flexible conformation and largely retain their conformation when adsorbed at interfaces. Various studies have shown that globular proteins form a relatively thin adsorbed layer, yielding a low surface excess as is shown in Figure 4.8. Compared to β-casein, β-lactoglobulin forms a much more compact and dense layer at oil/water interfaces (Dalgleish and Leaver, 1991; Atkinson et al., 1995). The layer of lysozyme adsorbed at a methylated silica surface is also relatively thin (Malmsten, 1994). The conformational stability, hence, appears to be an important factor determining the surface excess of emulsion droplets.

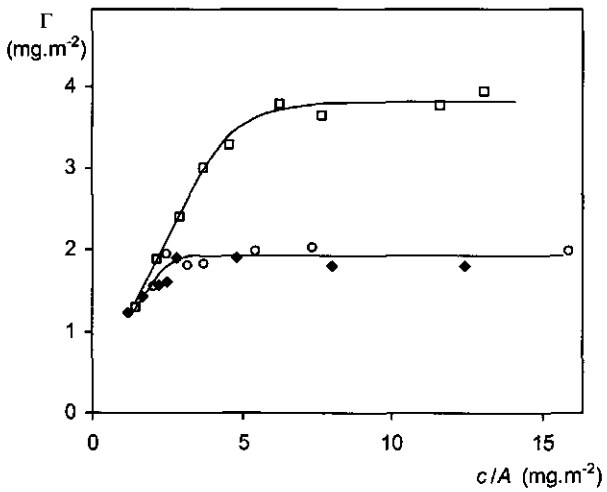

Figure 4.9 Surface excess (Γ) of emulsion droplets versus concentration over specific interfacial area (*c/A*) made with β-casein (□), β-lactoglobulin (◆) or lysozyme (○); all at pH 6.7 and *I* = 0.075 M.

Despite the high conformational stability of globular proteins, some unfolding will occur upon adsorption. Even a globular protein with a high conformational stability, like lysozyme, will adjust its conformation as is evident from the loss of enzymatic activity upon adsorption at emulsion droplets (de Roos and Walstra, 1996). The extend of the conformational changes depends on the type of interface and the surface excess (Graham and Phillips, 1979c; Norde and Favier, 1992; Vegt et al., 1996).

The surface excess of emulsions made with α-lactalbumin appeared to reach a plateau value at intermediate concentrations (Figure 4.10a). At higher concentrations, the surface excess increased with increasing protein concentration. After washing of the emulsions, the surface excess was similar to the surface excess of emulsions made with β-lactoglobulin. Hence, the high surface excess was obviously due to multilayer adsorption. α-Lactalbumin has a low conformational stability (Barel et al., 1972; Acharya et al., 1989) and tends to form aggregates in the unfolded state via hydrophobic interactions (Segawa and Sugai, 1983), which may explain multilayer adsorption. This presumed "interfacial aggregation" did, however, not induce droplet aggregation.

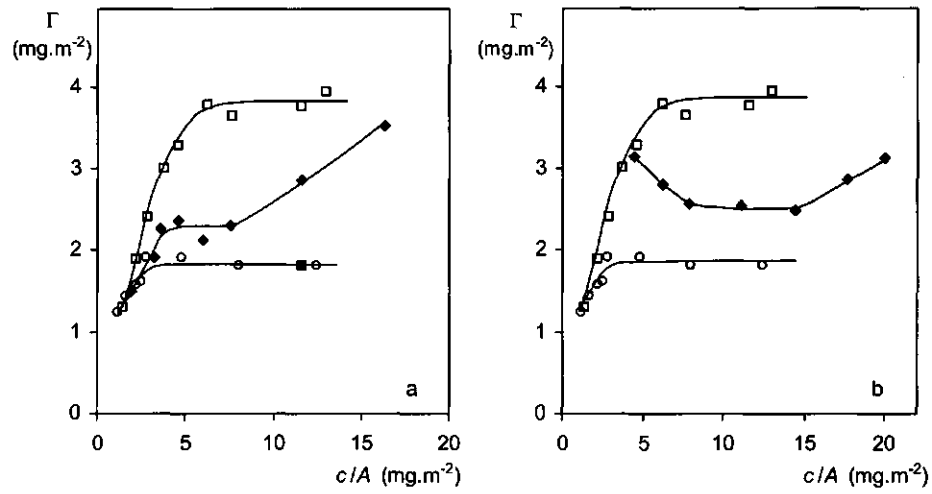

Figure 4.10 Surface excess (Γ) versus concentration over specific interfacial area (*c/A*) of: a, emulsions made with α-lactalbumin before (◆) and after washing (■); b, emulsions made with ovalbumin (◆); all at pH 6.7 and *I* = 0.075 M. The surface excess of emulsions with β-casein (□) and β-lactoglobulin (○) are shown as reference.

The surface excess of emulsions with α-lactalbumin after washing agrees well with literature data determined at relatively low concentrations. It is generally reported that the surface excess of emulsions made with α-lactalbumin is somewhat smaller or comparable to those of emulsions made with β-lactoglobulin (Dickinson et al., 1989; Couthaudon et al., 1991; Dickinson et al., 1993b; Matsumura et al., 1994). No data were available on the surface excess at high α-lactalbumin concentrations.

The surface excess of emulsion droplets with ovalbumin showed an unusual, but reproducible dependence on protein concentration and appeared to be relatively high for a monolayer of adsorbed globular proteins (Figure 4.10b). At air/water interfaces, the surface excess of an ovalbumin monolayer equals about 2 $mg.m^{-2}$ (Bull, 1972; Feijter and Benjamins, 1982). Mine et al. (1991) showed that the surface excess of ovalbumin at emulsion droplets is generally much higher due to multilayer adsorption. Ovalbumin readily denatures at interfaces, exposing free thiol-groups and initiating the formation of inter and intramolecular disulfide-bonds (Pearce and Kinsella, 1978; Kitabatake and Doi, 1987). The ensuing ovalbumin polymers result in a high surface excess. The size of the polymers is likely to depend on ovalbumin concentration, which might explain the unusual relation between surface excess and ovalbumin concentration.

To study the effect of the formation of intermolecular disulfide-bonds on the properties of emulsions made with ovalbumin, 5 mM of the thiol-blocking agent N-ethylmaleimide (NEM) was added to the ovalbumin solution prior to emulsification. Addition of NEM did not affect the droplet size distribution, the surface excess or the aggregation of the emulsions (results not shown). Kitabatake and Doi (1987) demonstrated that ovalbumin may form aggregates via covalent and non-covalent interactions upon denaturation. Apparently, the type of interaction causing polymer formation, has no significant effect on the emulsion properties. This agrees with results of Kitabataka and Doi (1987) who found little effect of the addition of NEM on the stability of foams made with ovalbumin.

4.5 Recoalescence during emulsification

4.5.1 Recoalescence stability of emulsions made with β-casein

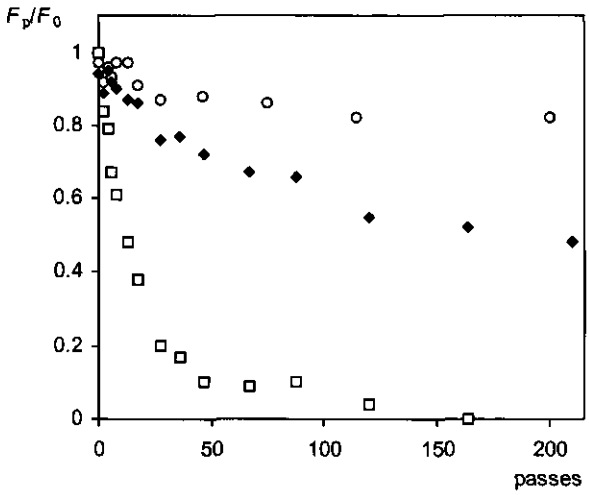

Figure 4.11 Ratio F_p/F_0 as a measure of recoalescence during homogenization of emulsions made with β-casein at pH 6.7 and I = 0.075 M. Concentrations: 1.6 mg.ml^{-1} (□); 4 mg.ml^{-1} (◆); and 12 mg.ml^{-1} (○).

The droplet size distribution of emulsions is governed by a kind of steady-state between droplet break-up and recoalescence during emulsification (Walstra, 1993). To establish whether variations in the droplet size of emulsions made with different proteins is due to differences in the ability of proteins to facilitate droplet break-up or to prevent immediate recoalescence, the rate of recoalescence during

emulsion formation was studied. In Figure 4.11, the ratio F_p/F_0 is shown as function of the number of passes through the homogenizer of an emulsion made with β-casein. The initial slope of the curve is a measure of the recoalescence rate during homogenization, with a steeper slope indicating a higher recoalescence rate.

The rate of recoalescence decreased with increasing protein concentration presumably due to an increasing surface excess of the emulsion droplets. However, even at a concentration where the droplets have a maximum surface excess, some recoalescence occurred. The recoalescence rate was also governed by the homogenization pressure and the oil fraction of the emulsions (results not shown).

Since the surface excess appeared to affect the recoalescence stability of emulsions, the recoalescence rate may depend on the decrease in droplet size during emulsification. The recoalescence of emulsions was studied using pre-emulsions, which were homogenized repeatedly until a steady droplet size was obtained. Subsequent re-homogenization during recoalescence experiments will only induce limited droplet break-up. The interfacial area of the emulsions, and hence the amount of adsorbed proteins will, therefore, remain approximately constant during these recoalescence experiments.

When making new emulsions, the initial droplet size is relatively large and little protein is adsorbed at the droplet interface. During emulsification of these coarse emulsions, the rate of droplet break-up will initially be high and a large amount of new interfacial area will be formed at which proteins will immediately absorb. The surface excess will nevertheless be relatively small and the adsorbed proteins will have little time to unfold to the most favourable conformation (Walstra and Smulders, 1998). The recoalescence rate may, therefore, be dependent on the extent of the increase in interfacial area. To study this effect, coarse pre-emulsions were prepared using a single pass through the homogenizer and the recoalescence rate was determined.

The recoalescence rate during re-homogenization of emulsions made with β-casein prepared using a single pass or multiple passes through the homogenizer is shown in Figure 4.12. The turbidity measurements were corrected for the decrease in emulsion droplet size during homogenization. The coarseness of the pre-emulsions appeared to have no significant effect on the recoalescence rate. Apparently, the time-scale required for stabilizing the droplets against immediate recoalescence is of the same order of magnitude as the time-scale of the emulsion forming process (say a few microseconds). Detailed calculations of the time-scales are hampered, since little is known about the time-scales of the processes

occurring during the formation of emulsions in a bounded type of laminar flow, which is prevalent in the laboratory homogenizer used.

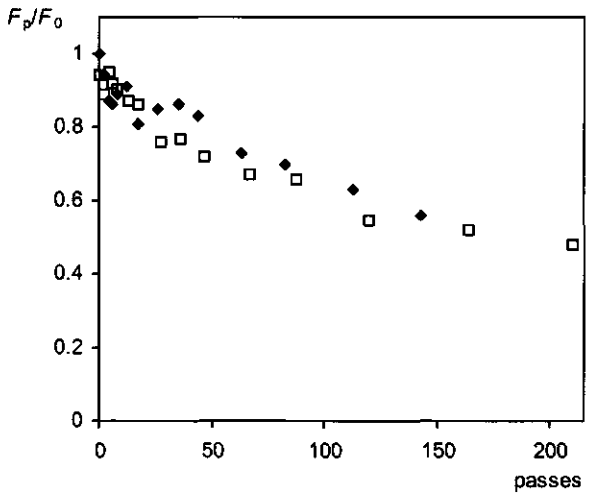

Figure 4.12 Ratio F_p/F_0 as a measure of recoalescence during homogenization of emulsions with 4 mg.ml^{-1} β-casein at pH 6.7 and I = 0.075 M. Homogenization procedure pre-emulsions: single pass (◆); multiple passes (□).

4.5.2 Recoalescence stability of emulsions made with globular proteins

In Figure 4.13, the recoalescence rates of emulsions made with β-casein, β-lactoglobulin or α-lactalbumin are compared at varying protein concentrations. The recoalescence rate of these emulsions appeared to be mainly determined by protein concentration. The protein properties seemed to have no significant effect on recoalescence stability under the conditions studied.

It was shown previously that the minimum droplet size of emulsions made with these proteins decreased in the order β-casein, α-lactalbumin and β-lactoglobulin. Since the recoalescence stabilities appeared to be similar, the differences in droplet sizes were likely to be due to variations in the abilities of these proteins to facilitate droplet break-up.

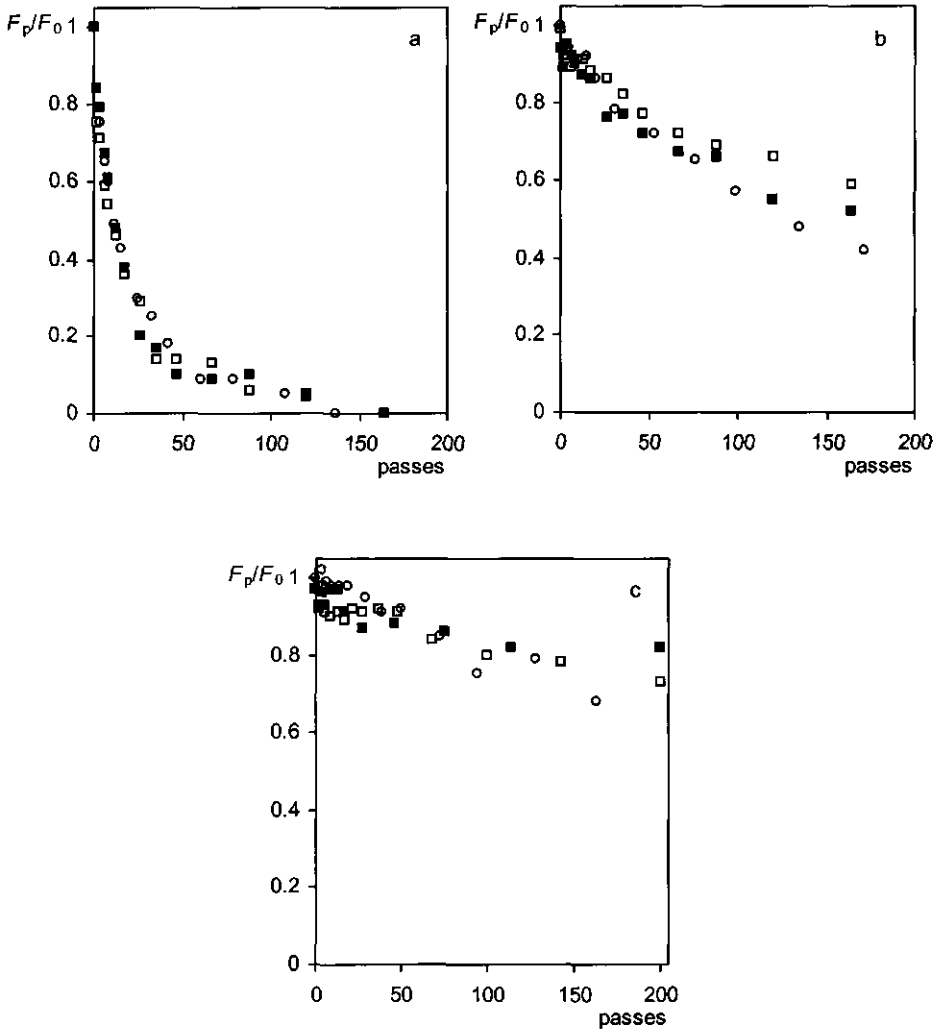

Figure 4.13 The recoalescence stability of emulsions made with β-casein (□), β-lactoglobulin (◆) or α-lactalbumin (○). Protein concentration: a, 1.6 $mg.ml^{-1}$, b, 4 $mg.ml^{-1}$, and c, 12 $mg.ml^{-1}$; all at pH 6.7 and I = 0.075 M.

The recoalescence rate during homogenization of emulsions made with lysozyme and ovalbumin appeared to be relatively high (Figure 4.14). The relatively inferior emulsion forming properties of these proteins is, therefore, likely to be at least partially due to their poor recoalescence stabilizing properties. As is also shown later, there appears to be a relation between droplet aggregation, droplet size, and recoalescence rate. Generally, aggregated emulsions were found to have relatively large droplets and poor recoalescence stabilities. It could be that aggregated droplets are less stable against recoalescence due to the relatively thin film separating the droplets. However, little is known about the state of aggregation during emulsification and hence the mechanism remains partly obscure.

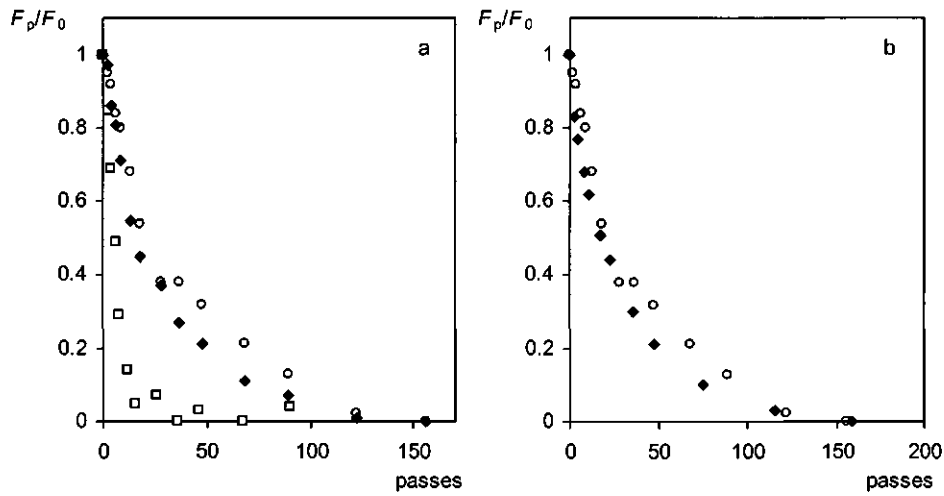

Figure 4.14 Ratio F_p/F_0 as a measure of recoalescence during homogenization of emulsions with: a, lysozyme with a concentration of 1.6 mg.ml^{-1} (□), 4 mg.ml^{-1} (◆), and 12 mg.ml^{-1} (○); b, lysozyme (○) and ovalbumin (◆) in a concentration of 12 mg.ml^{-1}; all at pH 6.7 and I = 0.075 M.

4.6 Effect of physicochemical conditions on emulsion formation

4.6.1 Emulsions made with β-casein

Many of the molecular properties of proteins are governed by physicochemical conditions. The pH affects amongst others the net charge, conformation and conformational stability of proteins. The effect of variations in pH on the emulsion forming properties of β-casein is shown in Figure 4.15.

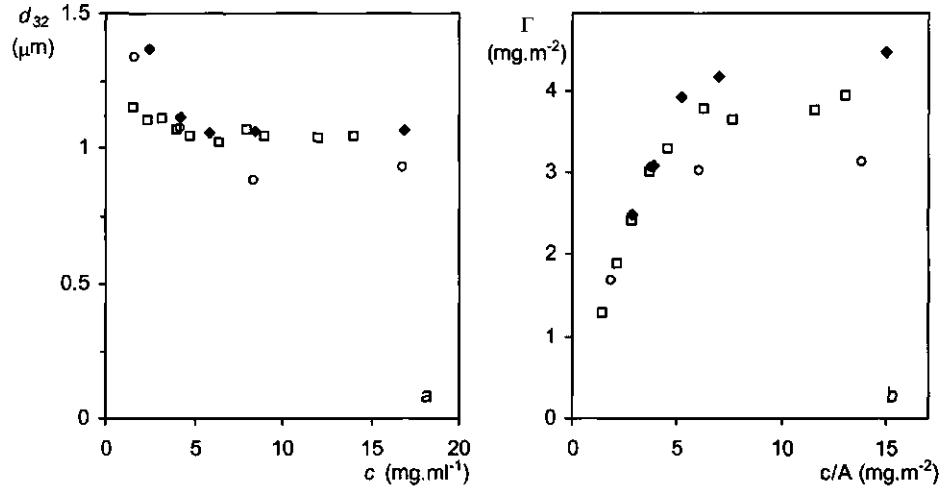

Figure 4.15 Emulsion forming properties of β-casein at pH 5.6 (◆), 6.7 (□), and 9.0 (○) as function of concentration (*c*): a, volume-surface average droplet size (d_{32}); b, surface excess (Γ); all at I = 0.075 M.

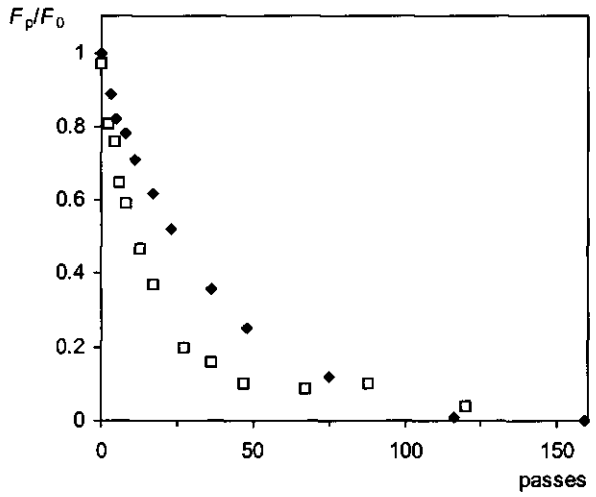

Figure 4.16 Ratio F_p/F_0 as a measure of recoalescence during homogenization of emulsions with 1.6 mg.ml^{-1} β-casein at pH 6.7 (□), and pH 9.0 (◆); I = 0.075 M.

The droplet sizes of emulsions made with β-casein at pH 5.6 and 9.0 were relatively large at a low protein concentration. At pH 5.6, the droplets were aggregated at this concentration which may have induced the relatively large droplet size. At higher concentrations, the droplet size of emulsions made with β-casein at pH 5.6 and 6.7 appeared to be similar, while at pH 9.0 smaller droplets were found. The recoalescence rate during homogenization was found to be somewhat lower at pH 9.0 than at pH 6.7 (Figure 4.16), which may partly explain the smaller droplet sizes at pH 9.0. The emulsion forming properties of β-casein at pH 6.7 were possibly affected by the presence of micelles. At pH 9.0, micellization of β-casein is impeded due to the high charge of the molecules (Niki and Arima, 1969). The absence of micelles may have improved the emulsion forming properties of β-casein at the high pH.

The surface excess of emulsion droplets with β-casein appeared to decrease with an increasing pH. This decrease is likely to be due to increasing electrostatic interactions between adsorbed β-casein molecules if the pH is further away from the isoelectric pH, hampering a close packing of β-casein at the droplet interface.

β-casein has a low solubility near its isoelectric pH (~ pH 5) due to the low charge of the proteins (Bingham, 1971). The emulsion forming properties of β-casein were very poor at this pH. For example: for an emulsion made with 8 $mg.ml^{-1}$ β-casein, a droplet size of 6.5 μm and surface excess of 43 $mg.m^{-2}$ was found. The emulsion was severely aggregated probably due to bridging flocculation by undissolved protein particles. The results of a range of experiments under various conditions (not shown) indicated that near the isoelectric pH the emulsion properties were mainly determined by the number and size of the undissolved particles and less by the protein properties.

The net charge of β-casein appeared to have a large effect on the emulsion forming properties of β-casein. This effect was studied further by varying the ionic strength of the emulsions. An increased ionic strength of 0.075 M to 0.150 M appeared to have no significant effect on the droplet size and surface excess of emulsions made with β-casein at pH 6.7. Dickinson et al (1998) showed likewise that the droplet size of emulsions made with β-casein was not affected up to an ionic strength of 0.2 M in the pH range of 5.5 to 7.0. At pH 9.0, the droplet size was neither found to be affected by an increase in ionic strength from 0.075 to 0.150 M, but the surface excess was found to be somewhat higher (Γ_{plat} = 3.1 $mg.m^{-2}$ and 3.5 $mg.m^{-2}$ at I = 0.075 M and 0.150 M, respectively). At a high ionic strength, the electrostatic interactions between proteins are relatively low, allowing a more dense packing of proteins at the droplet interface, hence a higher surface excess.

Especially at pH 9.0, where the charge of β-casein is high, the effect of increased shielding of the charge on the surface excess may be significant. However in general, the ionic strength appeared to have only a small effect on the emulsion forming properties of β-casein in the range studied.

4.6.2 Emulsions made with β-lactoglobulin

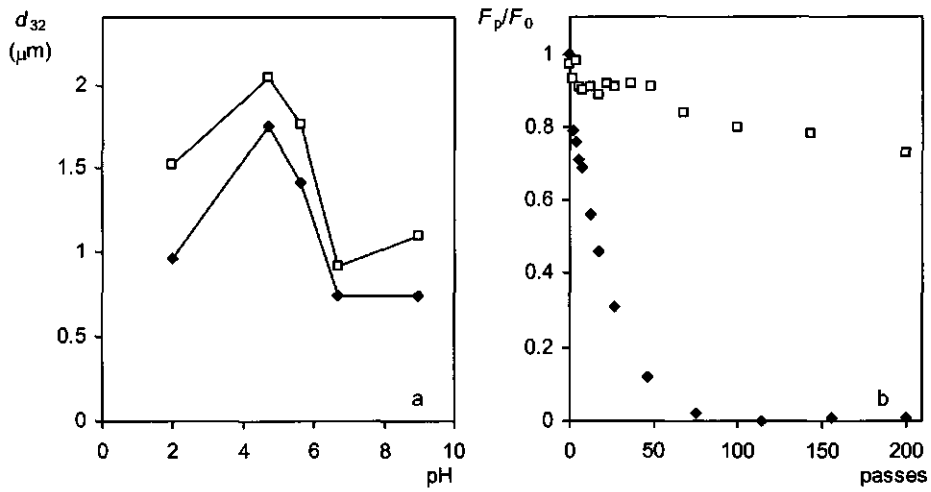

Figure 4.17 Emulsion forming properties of β-lactoglobulin as function of pH: a, volume-surface average diameter (d_{32}) at a concentration of 1.6 mg.ml^{-1} (□) and 16 mg.ml^{-1} (◆); b, ratio F_p/F_0 as a measure of recoalescence during homogenization of emulsions made at a concentration of 12 mg.ml^{-1} at pH 5.6 (◆) and pH 6.7 (□); all at I = 0.075 M.

The emulsion forming properties of β-lactoglobulin appeared to be superior under conditions prevalent in milk, i.e. pH 6.7 and an ionic strength of 0.075 M (Figure 4.17a). At pH 9.0 and a high β-lactoglobulin concentration, the droplet size of the emulsions was comparable to those of emulsions made at pH 6.7. At a low concentration, the droplets were at pH 9.0 somewhat larger and aggregated. The surface excess of the emulsions was similar at both pH values.

At pH 9.0, β-lactoglobulin is slowly and irreversibly denatured and aggregates linked by intermolecular disulfide-bonds are formed (Tanford et al., 1959; McKenzie and Sawyer, 1967; Kella and Kinsella, 1988). The resulting protein polymers may at low concentrations induce droplet aggregation by bridging

flocculation. At higher concentrations, the presence of polymers apparently did not have an effect on the emulsion properties.

The emulsion droplets made at pH 2.0 were relatively large and aggregated compared to those made at pH 6.7. At pH 2.0, β-lactoglobulin has a relatively high conformational stability (Kella and Kinsella, 1988b) and is in solution primarily present as monomers (Aymard et al., 1996), whereas at pH 6.7 β-lactoglobulin is mainly present as dimers (Townend et al., 1960b; McKenzie and Sawyer, 1967). Both the aggregated state as well as the conformational stability may have affected the emulsion forming properties of β-lactoglobulin.

The emulsion forming properties of β-lactoglobulin were poor near its isoelectric pH (pH 5.1). The emulsion droplets were relatively large and aggregated and the surface excess was found to be relatively high (Γ = 1.8 mg.m^{-2} at pH 6.7 and Γ = 2.1 mg.m^{-2} at pH 4.7 and pH 5.6). It was again observed that the aggregated emulsions were less stable against recoalescence than the emulsions without aggregates at pH 6.7 (Figure 4.17b), probably partly explaining their relatively large droplet size.

The results on the emulsion forming properties of β-lactoglobulin as a function of pH are in good agreement with the results reported in literature. The emulsion forming properties of β-lactoglobulin are generally found to be inferior near their isoelectric pH, mainly due to droplet aggregation (Klemaszewski et al., 1992; Chen and Dickinson, 1993; Dickinson and Williams, 1994). Chen and Dickinson (1993) reported a minimum droplet size at pH 7.0 (d_{32} = 0.93 μm), and a slightly larger droplet size at a lower pH (d_{32} = 1.23 μm and 1.29 μm at pH 5.0 and 3.0, respectively). The surfaces excess of the emulsions was found to be highest near the isoelectric pH of β-lactoglobulin (Γ = 1.6 mg.m^{-2} and 1.4 mg.m^{-2} at pH 5.0 and 7.0, respectively). Dickinson and Williams (1994) found no further effect of the pH on the droplet size in the pH-range 2.0 to 7.0.

The relatively poor emulsion forming properties near the isoelectric pH of β-lactoglobulin may have been due to the zero net charge of the molecules. At a zero net charge, proteins have a very compact conformation and electrostatic interactions between proteins are reduced. For β-lactoglobulin, this results in a decreased solubility near its isoelectric pH (de Wit and Kessel, 1996), hence an increased effective molar mass due to protein aggregation, which may in turn induce droplet aggregation. The relatively high surface excess found near the isoelectric pH may be due to decreased electrostatic and steric repulsions between adsorbed proteins.

The influence of the ionic strength on the emulsion forming properties of β-lactoglobulin was studied at varying pH values. At pH 6.7, the size of the

emulsion droplets increased with an increase in ionic strength from 0.075 M to 0.150 M (Figure 4.18). The emulsions were aggregated and the surface excess was relatively high (Γ = 1.8 mg.m^{-2} and 2.2 mg.m^{-2} at an ionic strength of 0.075 and 0.150, respectively). Both may be attributed to an increased shielding of the electrostatic charges of the proteins, resulting in a decreased electrostatic repulsion.

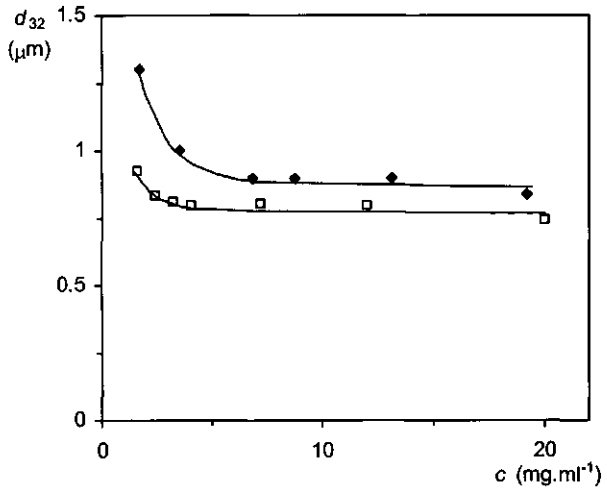

Figure 4.18 *Effect of ionic strength on the volume-surface average droplet size (d_{32}) of emulsions made with β-lactoglobulin at pH 6.7 and an ionic strength of 0.075 M (□), and 0.150 M (◆).*

In the range studied, the ionic strength did not affect the emulsion forming properties of β-lactoglobulin at pH values above and below 6.7. At a pH between 2.0 and 5.6, the ionic strength appear to have no significant effect on the aggregated state of the emulsions. At pH 9.0, the proteins are highly charged and an increase in ionic strength had apparently little impact on the electrostatic interactions between the proteins.

4.6.3 Emulsions made with α-lactalbumin

In Figure 4.19 the effect of pH on the emulsion forming properties of α-lactalbumin is shown. Again it was found that the emulsion forming properties were poor near the isoelectric pH of α-lactalbumin. The droplets were aggregated and the surface excess was relatively high, which was at high protein concentrations most likely due to multilayer adsorption. A similar effect of pH on the

emulsion forming properties of α-lactalbumin was found by Klemaszewski et al. (1992) for pH values ranging from 4.0 to 7.0.

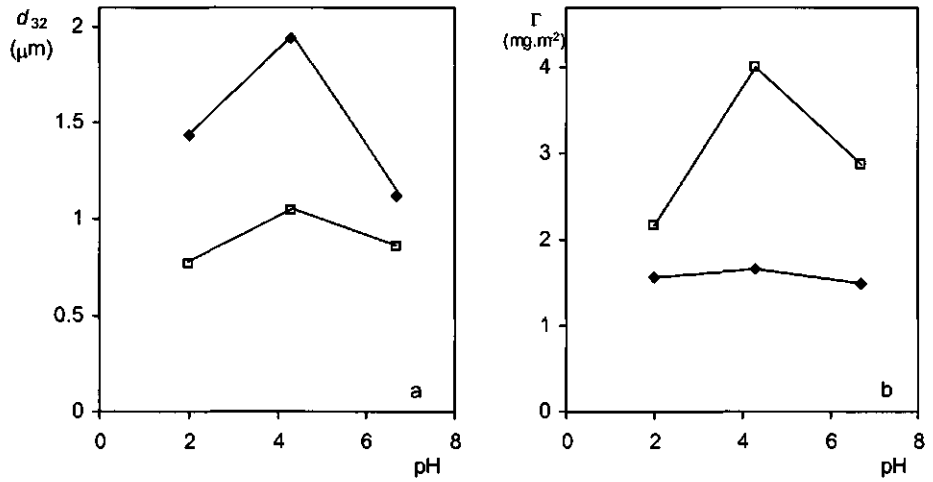

Figure 4.19 The emulsion forming properties of α-lactalbumin as function of pH at a concentration of 1.6 mg.ml^{-1} (◆) and 16 mg.ml^{-1} (□): a, volume-surface average droplet size (d_{32}); b, surface excess (Γ); I = 0.075 M.

At pH 2.0 and a low α-lactalbumin concentration, the emulsion droplets were relatively large and aggregated. At a high concentration, the emulsion forming properties of α-lactalbumin appeared to be slightly superior to those at pH 6.7. The surface excess was relatively low at this pH, which agrees with the results of Matsumura et al. (1994). At pH 2.0, α-lactalbumin is in a molten globule state with a relatively high conformational flexibility. The increased flexibility may have a positive effect on the emulsion forming properties of α-lactalbumin, other factors can, however, not be excluded.

The ionic strength appeared to have little effect on the emulsion forming properties of α-lactalbumin at pH 6.7. An increase in ionic strength from 0.075 M to 0.150 M did not affect the droplet size or surface excess.

4.6.4 Emulsions made with lysozyme

The influence of pH and ionic strength on the emulsion forming properties of lysozyme is shown in Table 4.2 for a concentration of 16 mg.ml^{-1}. Similar results were found for lower lysozyme concentrations and the emulsions were aggregated under all conditions studied. Electrostatic interactions appeared to have a large effect on the emulsion forming properties of lysozyme. Decreasing these

interactions by increasing the pH or the ionic strength, resulted in larger droplet sizes and higher surface excesses. Similar results were found by Klemaszewski et al. (1992). Apparently, the tendency of lysozyme to form aggregates has a negative effect on its emulsion forming properties.

Table 4.2 The emulsion forming properties of lysozyme as function of pH and ionic strength at a concentration of 16 mg.ml^{-1}.

	I = 0.075 M		I = 0.150 M	
pH	d_{32} (μm)	Γ (mg.m^{-2})	d_{32} (μm)	Γ (mg.m^{-2})
6.7	1.18	1.93	1.46	3.50
9.0	1.43	3.44	1.66	4.04

4.6.5 Emulsions made with ovalbumin

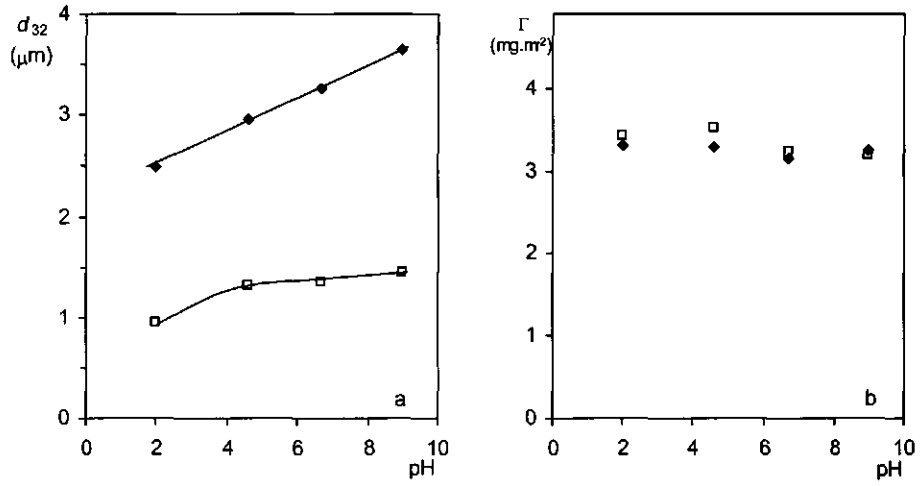

Figure 4.20 The emulsion forming properties of ovalbumin as function of pH at a concentration of 1.6 mg.ml^{-1} (◆) and 16 mg.ml^{-1} (□): a, volume-surface average droplet size (d_{32}); b, surface excess (Γ). I = 0.075 M.

The emulsion forming properties of ovalbumin as function of pH are shown in Figure 4.20. All emulsions were aggregated in the pH-range studied. Especially at pH 9.0, the aggregation was severe and time-dependent as indicated by an increasing emulsion viscosity and the formation of skin-like structures during storage. The droplet size of the emulsions was found to increase with increasing pH, while the surface excess was hardly affected by pH. Mine et al. (1991) found for emulsions made with ovalbumin a similar pH dependence of the droplet size,

but a decreasing surface excess with increasing pH. An increase in the ionic strength from 0.075 to 0.150 M appeared to have little effect on the emulsion properties at pH 6.7.

The emulsion forming properties of ovalbumin were not significantly deteriorated near its isoelectric pH. At pH 2.0, where the protein has a flexible, molten globule conformation (Mine et al., 1991), the emulsion forming properties of ovalbumin were found to be superior. In contrast, α-lactalbumin appeared to have slightly inferior emulsion forming properties in the molten-globule state. These contradicting results indicate that there appears to be no universal relation between molecular properties as charge, conformation, and conformational stability and the emulsion forming properties. Some general trends are discernible, but if a basic understanding of the structure-function relation of proteins is desired, a comprehensive knowledge of protein properties and the effect of physicochemical conditions would be required.

4.7 Effect of heat denaturation on emulsion properties

4.7.1 Emulsions made with β-lactoglobulin

To study the effect of conformational changes on the emulsion forming properties of proteins, β-lactoglobulin was heat denatured. β-Lactoglobulin solutions were heated at 85 °C for 20 minutes prior to emulsification. The pH of the solutions was kept at pH 7.5 to limit the aggregation of β-lactoglobulin. Above pH 7.0, the aggregation of β-lactoglobulin due to heat denaturation is limited, resulting in a decreased number of intermolecular disulfide exchange reactions and hence limiting the formation of covalently linked polymers (de Wit et al., 1988).

The droplet size of emulsions made with heat-denatured β-lactoglobulin was larger than those of emulsions made with the native protein (Figure 4.21) and a relative high protein concentration was required to obtain a constant droplet size. The emulsions with heat-denatured β-lactoglobulin were aggregated. Droplet aggregation was strongest at low concentrations, probably due to bridging flocculation. At higher concentrations, the extent of aggregation appeared to be relatively low. The surface excess of emulsions with denatured β-lactoglobulin was relatively high, which was likely due to the adsorption of protein aggregates. The viscosity of the emulsions increased during storage, probably due to continuing intermolecular reactions.

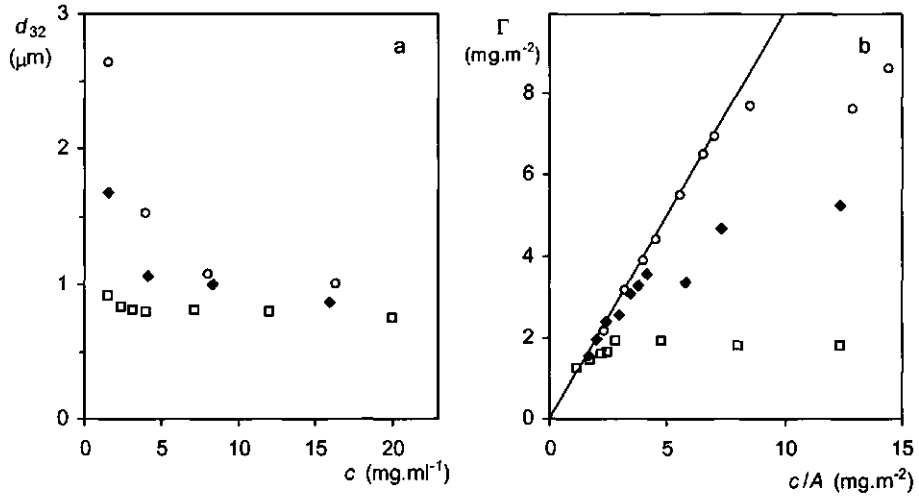

Figure 4.21 Properties of emulsions made with β-lactoglobulin in the native state (□), heat-denatured (○), and heat-denatured in presence of 2 mM NEM (◆): a, volume-surface average droplet size (d_{32}), b, surface excess (Γ); all at pH 6.7 and I = 0.075 M. Solid line indicates surface excess of emulsion droplets that would result if the proteins would be completely adsorbed.

It is generally found that the droplet size and surface excess of emulsions made with heat-denatured proteins are relatively large as a result of protein aggregation (Oortwijn and Walstra, 1979; Saito and Taira, 1987; Rientjes and Walstra, 1993). During heat denaturation, β-lactoglobulin unfolds, exposing a free thiol-group. This thiol-group may react with other proteins and as a result aggregates linked via disulfide-bonds are formed (Sawyer, 1968; Iametti et al., 1995). The size of the aggregates is dependent on protein concentration and on physicochemical conditions (Sawyer et al., 1971; Saito and Taira, 1987; Boye et al., 1996).

To study the effect of aggregate size on emulsion properties, β-lactoglobulin solutions with varying concentrations were heated and subsequently used for making emulsions with similar protein concentrations (Figure 4.22). The droplet size and the aggregated state of the emulsions generally appeared to increase with an increasing concentration at which the proteins were denatured, presumably due to an increasing size of the protein aggregates. Hence, the emulsion forming properties of β-lactoglobulin appear to be directly linked to the size of the aggregates or the effective molar mass of the proteins. The surface excess was

not significantly affected by the concentration at which the proteins were denatured and similar to those shown in Figure 4.21b.

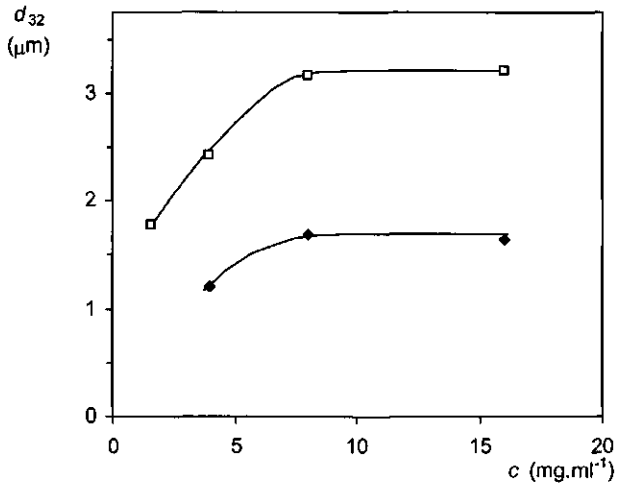

Figure 4.22 Volume-surface average droplet size (d_{32}) of emulsions prepared with 1.6 mg.ml^{-1} (□) or 4 mg.ml^{-1} (◆) heat-denatured β-lactoglobulin. The concentration (*c*) at which the protein solutions were heated is shown at the x-axis.

In this study, we were mainly interested in the relation between the conformation and emulsion forming properties of β-lactoglobulin. It was, therefore, attempted to prevent aggregation by adding NEM to the protein solutions prior to heating. The emulsion forming properties of β-lactoglobulin denatured in presence of NEM were slightly superior to those of β-lactoglobulin denatured without NEM (Figure 4.21). The droplets were, however, still relatively large and aggregated and the surface excess was high, most likely due to adsorption of aggregates. As McClements et al. (1993) showed, NEM is capable of preventing the formation of disulfide-bridges, but aggregation of β-lactoglobulin via non-covalent bonds will still occur. Consequently, heat denaturation of β-lactoglobulin is not a suitable tool for studying the effect of conformational changes on the emulsion formation.

4.7.2 Emulsions made with α-lactalbumin

α-Lactalbumin was heat denatured (85 °C, 20 min) to study the effect of conformational changes on its emulsion forming properties. At higher protein concentrations, the droplet size of emulsions made with heat-denatured α-lactalbumin was comparable to those of emulsions made with native proteins

(Figure 4.23a). At a concentration of 1.6 mg.ml^{-1} heat-denatured α-lactalbumin, the emulsion droplets were slightly aggregated and relatively large, while at higher concentrations no significant effect was found. The surface excess of emulsions made with heat-denatured α-lactalbumin appeared to be comparable or slightly larger than those of emulsions made with native α-lactalbumin (Figure 4.23b). The limited effect of heat denaturation on the emulsion forming properties of α-lactalbumin was presumably due to the ability of α-lactalbumin to renature after heating (Rüegg et al., 1977; de Wit and Klarenbeek, 1984). Any change in the state of aggregation and the surface excess may have been due to the presence of some slight impurities (e.g. β-lactoglobulin).

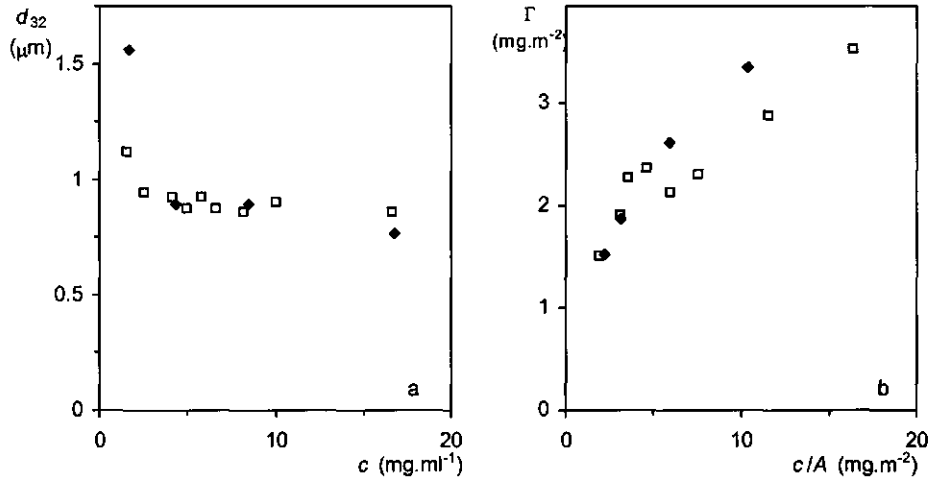

Figure 4.23 Emulsion forming properties of native (□), and heat-denatured (◆) α-lactalbumin: a, volume-surface average droplet size (d_{32}); b, surface excess (Γ). All at pH 6.7 and I = 0.075M.

4.8 Conclusions

Some general relations between the molecular and emulsion forming properties of proteins could be established. Proteins appear to have good emulsion forming properties as long as the protein is well soluble and aggregates are absent. Under these circumstances, only small differences in emulsion forming properties could be determined. These difference are likely to be even smaller if the proteins are less pure.

In the presence of aggregates, e.g. due to previous heat denaturation, usually relatively large droplet sizes and high recoalescence rates were found,

which were probably due to the increased effective molar mass of the proteins. Generally it was found that the droplet size and surface excess increased with increasing aggregate size. The tendency for protein aggregation appeared mainly to be determined by molecular properties, like electrostatic charge, conformational stability, hydrophobicity, and the presence of thiol-groups. Physicochemical conditions, like pH and ionic strength, mainly affected the emulsion forming properties via their effect on intermolecular protein interactions, hence protein aggregation.

The surface excess of emulsion droplets appeared to be mainly determined by the conformational stability of proteins and, again, the presence of aggregates. Proteins with a high conformational stability yielded emulsion droplets with a relatively low surface excess. A flexible protein conformation, on the other hand, allows the formation of a dense, and closely packed layer, hence a high surface excess. The surface excess of emulsion droplets was high in the presence of aggregates, which may be due to physicochemical conditions, surface or heat denaturation. A high surface excess may also be caused by multilayer adsorption.

Chapter 5

Emulsion forming properties of β-casein peptides

Abstract

Oil-in-water emulsions were prepared at various physicochemical conditions using purified and well-characterized β-casein peptides. An attempt was made to relate the molecular properties of these peptides to their emulsion forming properties.

The emulsion forming properties of amphiphilic β-casein peptides (f. 1/29-105/107) were superior to those of intact β-casein. At pH 6.7, emulsions made with these peptides had a smaller average droplet size and a lower surface excess than those made with the intact protein. The emulsion forming properties of hydrophobic β-casein peptides (f. 106/108-209) were at pH 9.0 comparable to those of intact β-casein at this pH.

Comparison of the properties of emulsions made with either β-casein peptides of the intact protein appeared to indicate that the surface excess of the droplets was largely determined by the hydrophobic C-terminal domain of β-casein. Due to their relatively small molar mass, the peptides were more readily desorbed from the oil/water interface than intact β-casein.

5.1 Introduction

The functionality of proteins is thought to be governed by molecular properties as size, amphiphilicity, number and distribution of hydrophobic groups, electrostatic charge, conformation and conformational stability (Kinsella, 1984). In the previous chapter, the relation between the molecular and emulsion forming properties of proteins was studied by comparing the functionality of proteins with widely varying molecular properties. In this chapter, the effect of controlled modification of the molecular structure of a single protein by enzymatic hydrolysis on the emulsion formation is studied.

Most work on enzymatically modified proteins describes the functionality of hydrolyzate mixtures. To elucidate structure-function relations, well-defined peptide fractions are required. Data on the functionality of pure peptides are, however, limited. For example, Huang et al. (1996) compared the emulsion forming properties of intact β-lactoglobulin and a β-lactoglobulin peptide (f. 41-100)-S-S-(f. 149-162). The authors suggested that the superior emulsion forming properties of this peptide were due to its increased solubility, amphiphilicity and conformational flexibility. Enser et al. (1990), Carey et al. (1994), and Saito et al. (1995) studied the emulsifying properties of synthetic peptides designed to form amphipathic secondary structures, which were assumed to yield an optimal functionality. However, no clear relation between the secondary structure and the emulsion formation could be established. The functionality of these peptides appeared to be mainly determined by their solubility (Saito et al., 1995) and molar mass (Enser et al., 1990; Carey et al., 1994). A similar relation between molar mass and emulsion formation was found for amphiphilic α_{s1}-casein peptides (f. 1-23) (Shimizu et al., 1986a), and hydrophilic (f. 1-25) and hydrophobic (f. 193-209) β-casein peptides (Lee et al., 1987a). The inferior emulsion forming properties of these peptides appeared to be mainly due to their relatively small molar mass.

In this study, the emulsion forming properties of purified β-casein peptides were investigated. β-Casein has some distinct and well-known features and thereby offers excellent opportunities for studying structure-function relations. A noteworthy feature of β-casein is the uneven distribution of hydrophobic and electrostatically charged residues along the polypeptide chain (Swaisgood, 1982), making the protein strongly amphiphilic (Figure 5.1). Of interest is also its flexible and approximately random coil structure (Creamer et al., 1981; Graham et al., 1984).

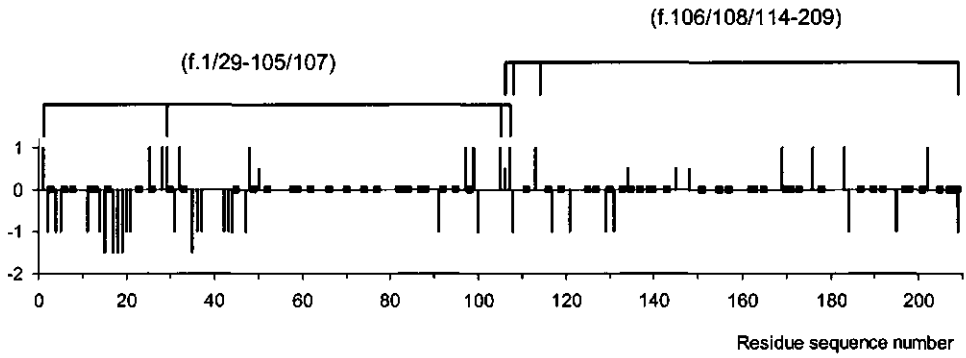

Figure 5.1 Schematic representation of the peptide chain of β-casein at pH 6.7. The positive and negative charges are indicated by vertical bars, with the long negative bars and the small positive bars denoting the phosphoseryl and histidine residues, respectively. The black squares represent the hydrophobic amino acids. (After Walstra et al., 1999). The peptide fractions are indicated.

The β-casein peptides used in this study were kindly provided by Caessens and co-workers. β-Casein was hydrolyzed by plasmin and the resulting hydrolyzates were fractionated and characterized (Caessens et al., 1997a; 1999b). Of these fractions, a group of amphiphilic and a group of hydrophobic peptides were selected for further research. The amphiphilic peptides (RET1: f. 1/29-105/107) originate from the highly charged N-terminal domain of β-casein. This group of peptides was further fractionated, yielding two fractions of amphiphilic peptides IEC2 (f. 29-105/107) and IEC3 (f. 1-105/107). Both fractions consist of peptides with a highly charged N-terminal and a hydrophobic C-terminal domain, resembling miniature β-casein molecules. The main difference between the fractions IEC2 and IEC3 is the size of the charged N-terminal head and the number of bulky and highly charged phosphoseryl residues present (1 and 5 residues, respectively). The second group of peptides originates from the C-terminal domain of β-casein (PEL1: f. 106/108/114-209). These peptides contain mainly hydrophobic amino acids and at neutral pH only a few charged groups. The molar masses of the peptide fractions are summarized in Table 5.1.

The wide variation in the molecular properties of the β-casein peptides was expected to result in significantly different emulsion forming properties, enabling a detailed study of the relation between functionality and molecular structure. To elucidate this relation, the emulsion forming properties of the peptides were studied at various physicochemical conditions and compared with those of intact β-casein.

Table 5.1 Molar masses of β-casein and selected β-casein peptides (Caessens et al., 1997a; 1999b).

Fraction		Molar mass (Da)
β-casein		24040
amphiphilic peptides RET1		
IEC2:	f. 29-105	8740
	f. 29-107	9000
IEC3:	f. 1-105	12200
	f. 1-107	12460
hydrophobic peptides PEL1		
	f. 106-209	11820
	f. 108-209	11560
	f. 114-209	10830

5.2 Emulsion forming properties of amphiphilic β-casein peptides

5.2.1 Droplet size

Figure 5.2 shows the volume-surface average droplet size of oil-in-water emulsions made with β-casein and amphiphilic β-casein peptides as a function of concentration at pH 6.7 and an ionic strength of 0.075 M. At a low peptide concentration, the droplet size of emulsions made with amphiphilic peptides RET1 (f. 1/29-105/107) was slightly smaller than those of emulsions made with β-casein. Increasing the concentration resulted in a decreasing average droplet size of emulsions with amphiphilic peptides, whereas the droplet size of emulsions with intact β-casein remained approximately constant. At a surplus concentration, the average droplet size of the peptide stabilized emulsions (d_{32} = 0.69 μm) was smaller than those of emulsions made with any of the proteins studied (e.g. d_{32} = 1.08 μm and 0.79 μm for emulsions with β-casein and β-lactoglobulin, respectively). Further fractionation of the amphiphilic peptides in IEC2 (f. 29-105/107) and IEC3 (f. 1-105/107) with a small and a large electrostatically charged head, respectively, had no significant effect on the emulsion droplet size.

Results similar to those described here, were reported by Caessens and co-workers (1997a; 1999b) who made a preliminary study of the emulsion forming properties of the same peptide fractions. Caessens et al. (1999a) studied the secondary structure of β-casein and the amphiphilic peptides IEC2 and IEC3 in solution and adsorbed at hydrophobic Teflon particles using far-ultraviolet circular dichroism (pH 6.7 and I = 0.020 M). Approximately 70-80 % of the secondary structure of β-casein and β-casein peptides in solution consisted of a random coil structure. The secondary structures of the adsorbed protein and peptides were more ordered and contained less random coil structure (approximately 65 %, 55%,

and 50% for β-casein and the peptides IEC2 and IEC3, respectively). The change in the secondary structure after adsorption was smaller at an ionic strength of 0.075 M.

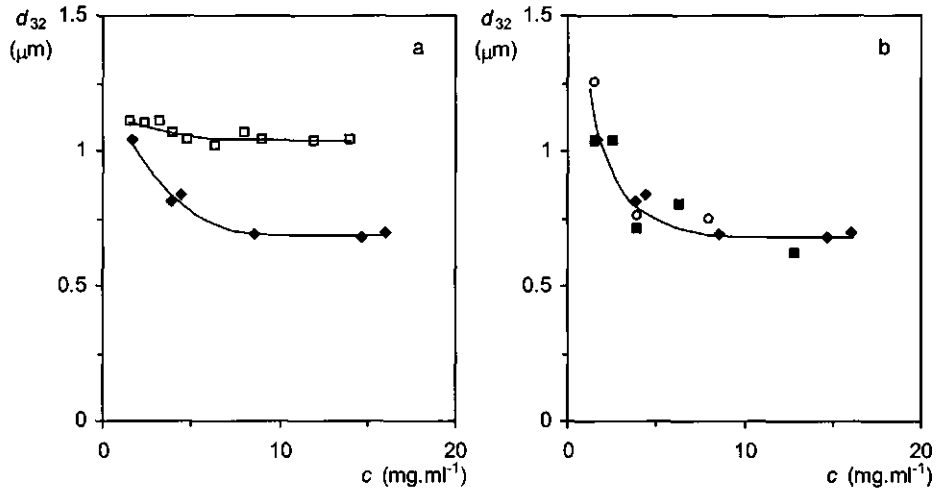

Figure 5.2 *Volume-surface average droplet size (d_{32}) of emulsions as function of concentration (c) of: a, amphiphilic peptides RET1 (◆) and β-casein (□); b, amphiphilic peptides RET1 (◆), IEC2 (○), and IEC3 (■); all at pH 6.7 and I = 0.075 M.*

Despite the similar secondary structures of β-casein and amphiphilic peptides, the droplet size of the emulsions was found to be distinctly different. The secondary structure, therefore, appeared to have a negligible effect on the emulsion forming properties. The secondary structures were, however, rather similar and the available data are limited.

5.2.2 Recoalescence rate during homogenization

The droplet size distribution of freshly made emulsions is often the result of a balance between droplet break-up and recoalescence during emulsification (Walstra, 1993). To better understand the superior emulsion forming properties of the amphiphilic β-casein peptides, the rate of recoalescence during the homogenization process was determined. In Figure 5.3, the recoalescence of emulsion droplets is shown as function of the number of passes through the homogenizer. The initial slope of the curve is a measure of the rate of recoalescence during homogenization, with a steeper slope indicating a higher rate.

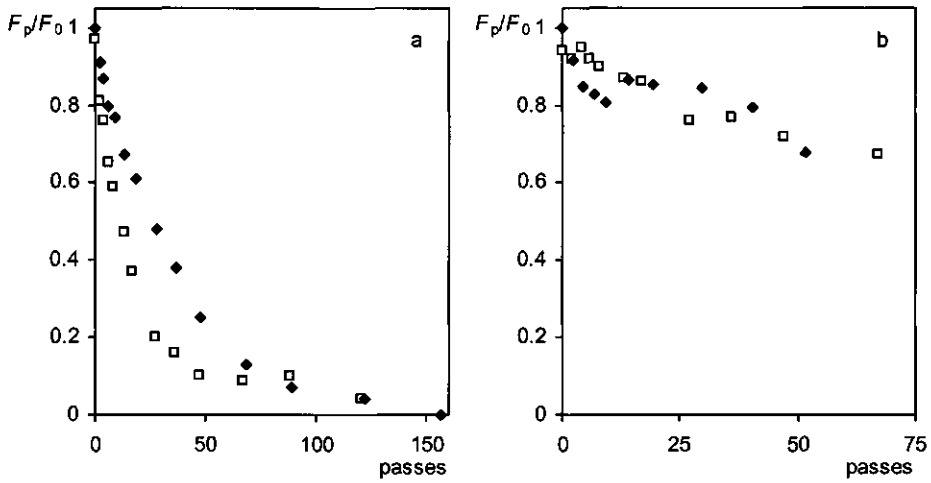

Figure 5.3 Ratio F_p/F_0 as a measure of recoalescence during homogenization of emulsions with β-casein (□) and amphiphilic peptides RET1 (◆) at pH 6.7 and I = 0.075 M and a concentration of: a, 1.6 mg.ml^{-1} and b, 4 mg.ml^{-1}.

The recoalescence rate of emulsions at a concentration of 1.6 mg.ml^{-1} was smaller for emulsions made with amphiphilic peptides RET1 than for emulsions made with β-casein. At a concentration of 4 mg.ml^{-1}, the recoalescence rate was relatively low and similar for both emulsions. The recoalescence rate is expected to decrease to approximately zero, if the concentration would be increased further as was demonstrated in Chapter 4.

The superior stability against recoalescence of emulsions with amphiphilic peptides at a low concentration may explain the relatively small droplet size of these emulsions at this concentration. At a higher concentration, the difference in the droplet size of the peptide and protein stabilized emulsions seemed to be due to the superior droplet break-up properties of the peptides, since at these concentrations the recoalescence rates appeared to be similar. The exact cause of the superior emulsion forming properties of the amphiphilic peptides remains, however, obscure due to differences in the droplet size and surface excess of emulsions made with amphiphilic peptides or intact proteins.

5.2.3 Surface excess

In Figure 5.4, the surface excess of emulsions made with β-casein or amphiphilic β-casein peptides is shown as a function of the concentration per unit specific interfacial area. The surface excess of emulsion droplets made with the amphiphilic peptides RET1 was for all concentrations lower than those of

emulsions made with intact β-casein. The maximum surface excess was reached at approximately the same concentration per unit interfacial area. At concentrations below this maximum, the surface excess of emulsions made with amphiphilic peptides appeared to increase relatively less steeply with concentration. The relative increase in surface excess was, however, comparable. The surface excess of emulsion droplets with the amphiphilic peptides RET1 and IEC3 seemed similar, while the surface excess of emulsions with the peptide fraction IEC2 appeared to be slightly lower at high concentrations (Figure 5.4b). The data available are, however, limited.

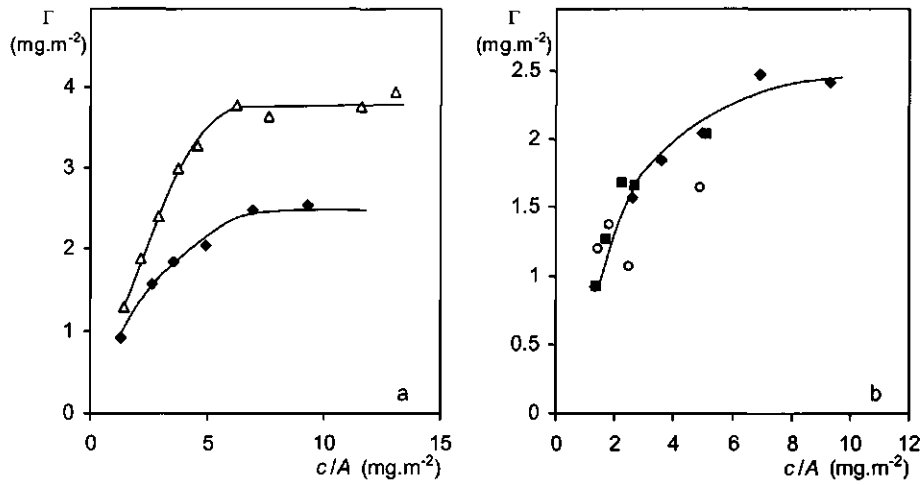

Figure 5.4 Surface excess (Γ) of emulsion droplets versus concentration over specific interfacial area (*c/A*), at pH 6.7 and *I* = 0.075 M with: a, β-casein (□) and amphiphilic peptides RET1 (◆); b, amphiphilic peptides RET1 (◆), IEC2 (○), and IEC3 (■).

Caessens et al. (1999a) determined the surface excess of β-casein and the amphiphilic β-casein peptides IEC2 and IEC3 adsorbed at hydrophobic Teflon particles at pH 6.7 and an ionic strength of 0.020 M. The maximum surface excess of β-casein adsorbed at these particles was about 3.0 $mg.m^{-2}$. For both peptide fractions a surface excesses of 2.0 $mg.m^{-2}$ was found. These surface excesses are lower than those found in this research (Γ = 3.8 $mg.m^{-2}$ and 2.5 $mg.m^{-2}$ for emulsions with β-casein or amphiphilic peptides RET1, respectively), but qualitatively comparable. The surface excesses of β-casein and a β-casein peptide (f. 1-93) adsorbed at hydrophobized silica found by Nylander and Wahlgren (1994) also agreed qualitatively well with our results. Any absolute differences are

probably due to differences in the physicochemical conditions, and in the nature of the surfaces and interfaces. Especially the ability of the hydrophobic groups of proteins and peptides to protrude into the oil phase (Walstra and de Roos, 1993) may affect the surface excess of emulsion droplets.

The molecular conformation of intact β-casein adsorbed at oil/water interfaces has been the focus of many studies. It is generally believed that at an oil/water interface the hydrophobic C-terminal domain of β-casein forms a dense layer close to the interface, whereas the hydrophilic N-terminal domain extends far into the surrounding aqueous phase forming a brush-like layer (Dalgleish and Leaver, 1991; Dickinson, 1994; Beek et al., 1996). Partial removal of the N-terminal or C-terminal domain of β-casein will affect the conformation of the resulting peptides at the oil/water interface. Nylander and Wahlgren (1994) studied the adsorption of β-casein and an amphiphilic β-casein peptide (f. 1-93) on hydrophobized silica particles. They postulated that the hydrophobic C-terminal domain is essential for orienting the hydrophilic N-terminal domain of β-casein. Removal of a part of this hydrophobic domain would result in a flat and less protruding conformation of the β-casein peptides at the interface and thereby a lower surface excess.

5.3 Emulsion forming properties of hydrophobic peptides

5.3.1 Effect of solubility on emulsion formation

The solubility of the hydrophobic peptides PEL1 (f. 106/108/114-209) at pH 6.7 and an ionic strength of 0.075 M was relatively small. At a peptide concentration of 1.6 $mg.ml^{-1}$, insoluble particles remained visible in the solution even after storage for an extended period of time at 4 °C. The removal of the N-terminal domain of β-casein markedly decreased the net electrostatic charge and somewhat increased the hydrophobicity of these peptides (Table 5.2). This most likely resulted in increased intermolecular interactions, hence decreased solubility.

Table 5.2 The estimated net charge and hydrophobicity of β-casein and hydrophobic β-casein peptides at pH 6.7.

Fraction	Charged residues (mol%)	Net charge	Hydrophobic residues (mol%)
β-casein	24	-13.0	30
hydrophobic peptides			
PEL1			
f. 106-209	19	0	34
f. 108-209	18	-1.5	34
f. 114-209	16	-1.5	34

The emulsions prepared with the hydrophobic peptide solutions containing undissolved particles were strongly aggregated and immediately separated into a curd-like cream and a clear aqueous solution. This aggregation was probably due to bridging flocculation by undissolved peptide particles. The size of the single emulsion droplets was relatively large and almost all of the peptides present were adsorbed at the droplet interface, yielding a high surface excess. For example, for a peptide concentration of 10 $mg.ml^{-1}$ a droplet size of 2.5 μm and a surface excess of 16 $mg.m^{-2}$ was found. The emulsions were stable against coalescence during a one week period, probably due to the high surface excess of the droplets.

The formation of emulsions with the poorly soluble hydrophobic peptides was highly irreproducible and appeared to be rather governed by the properties of the undissolved particles, like size and number, than by the molecular properties of the peptides. The discussion of the emulsifying properties of the hydrophobic peptides will therefore be limited to the emulsifying properties at conditions were these peptides were fully soluble, i.e. at pH 9.0.

5.3.2 Droplet size

The average droplet sizes of emulsions made with hydrophobic peptides or intact β-casein at pH 9.0 were found to be similar and at high concentrations smaller than those of emulsions made with intact β-casein at pH 6.7 (Figure 5.5). As discussed in Chapter 4, the improved emulsion forming properties of β-casein at pH 9.0 might be due to the absence of β-casein micelles at this pH (Niki and Arima, 1969). The minimum droplet size of emulsions made with hydrophobic peptides was 0.89 μm, which is larger than those of emulsions made with amphiphilic peptides (d_{32} = 0.69 μm) or with β-lactoglobulin (d_{32} = 0.82 μm) at pH 6.7.

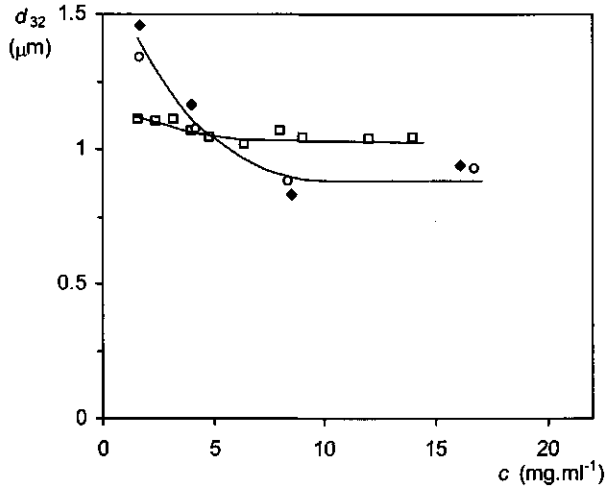

Figure 5.5 Volume-surface average droplet size (d_{32}) of emulsions as function of concentration (c) with hydrophobic peptides PEL1 (◆) at pH 9.0, and intact β-casein at pH 6.7 (□) and pH 9.0 (○); I = 0.075 M.

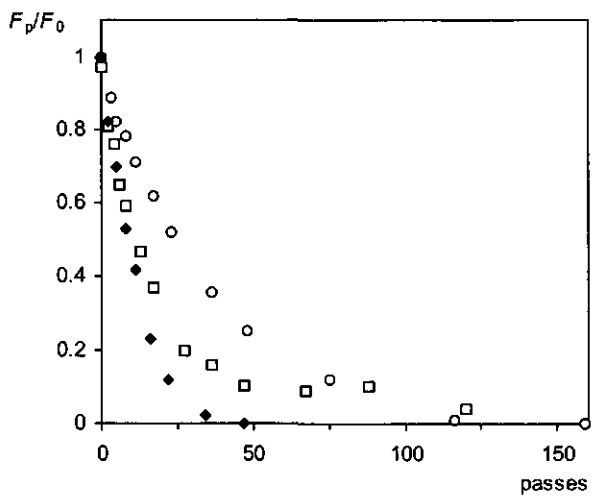

Figure 5.6 Ratio F_p/F_0 as a measure of recoalescence during homogenization of emulsions with 1.6 mg.ml^{-1} hydrophobic peptides PEL1 (◆) at pH 9.0, and intact β-casein at pH 6.7 (□) and pH 9.0 (○); I = 0.075 M.

5.3.3 *Recoalescence rate during homogenization*

The rate of recoalescence during homogenization of emulsions made with hydrophobic peptides PEL1 or intact β-casein is shown in Figure 5.6. The initial slope of the ratio F_p/F_0 as function of the number of passes through the homogenizer was at pH 9.0 steeper for emulsions with hydrophobic peptides than for emulsions with intact β-casein. The relatively poor stability against recoalescence of emulsions made with the hydrophobic peptides may explain their relatively large droplet size at a low peptide concentration.

The recoalescence rate of emulsions made with hydrophobic peptides was comparable to those of emulsions made with β-casein at pH 6.7. Considering the relatively small droplet size of the emulsions with β-casein at the latter pH, these results suggest that the droplet break-up properties of hydrophobic peptides were inferior compared to those of intact β-casein at pH 6.7. At high concentrations, the droplet size of emulsions with hydrophobic peptides was found to be smaller than those of emulsions with β-casein at pH 6.7, suggesting the opposite. The recoalescence rate was, however, not determined at high peptide concentrations due to the limited amount of peptides available.

5.3.4 *Surface excess*

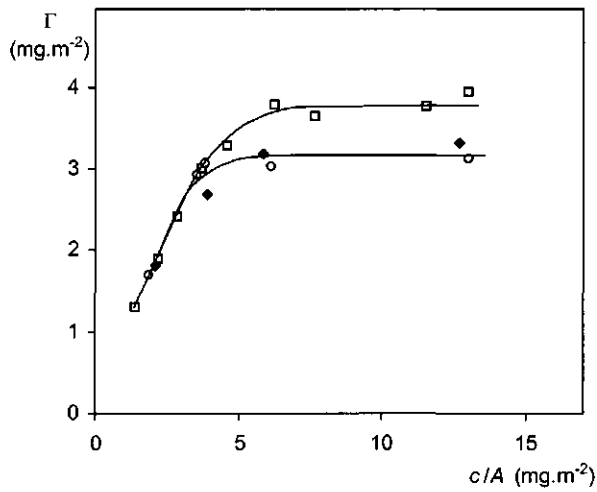

Figure 5.7 Surface excess (Γ) of emulsion droplets versus concentration over specific interfacial area (*c*/*A*) with: hydrophobic peptides PEL1 (◆) at pH 9.0, and intact β-casein at pH 6.7 (□) and pH 9.0 (○); *I* = 0.075 M.

As shown in Figure 5.7, the surface excess of emulsion droplets made with the hydrophobic peptides PEL1 or with intact β-casein was at pH 9.0 found to be similar. The surface excess of emulsion droplets with β-casein was higher at pH 6.7 than at pH 9.0. Caessens et al. (1999a) found similar results for the surface excess of β-casein and hydrophobic β-casein peptides adsorbed at hydrophobic Teflon particles. At pH 9.0, the surface excesses of the particles with the hydrophobic peptides PEL1 and β-casein appeared to be 2.7 $mg.m^{-2}$, which was also lower than the surface excess of β-casein adsorbed at these particles at pH 6.7. The relatively low surface excess at pH 9.0 was likely to be due to the high electrostatic charge of β-casein at this pH, resulting in a relatively strong inter and intramolecular repulsion between the adsorbed molecules.

The surface excess of emulsions with the hydrophobic peptides PEL1 was relatively high compared to those of emulsions with the amphiphilic peptides RET1. To establish whether the high surface excess was due to multilayer formation, the effect of washing of the emulsions on the surface excess was determined. To wash the emulsions, the emulsion droplets were separated from the continuous phase by centrifugation. The resulting cream was removed and redispersed in buffer solution and the same procedure was repeated. The surface excess was determined after each washing step and is shown in Table 5.3. The surface excess of the emulsions made with the peptides PEL1 was found to be lower after each washing procedure.

Table 5.3 Surface excess (Γ) of emulsion droplets made with 8 $mg.ml^{-1}$ hydrophobic (PEL1) or amphiphilic (RET1) peptides after washing with buffer solution (pH 9.0 and 6.7, respectively; I = 0.075 M).

	Γ ($mg.m^{-2}$)	
Number of washing steps	Hydrophobic peptides (PEL1, pH 9.0)	Amphiphilic peptides (RET1, pH 6.7)
0	3.2	2.0
1	2.8	1.7
2	2.5	oil separation

The adsorption of proteins at interfaces in a monolayer is often considered to be virtually irreversible. Due to the high affinity of proteins for interfaces, desorption only occurs if the protein concentration in the bulk is extremely low. When in that case desorption occurs, the desorption rate is usually very low and only very few molecules need to desorb to establish a new equilibrium (Walstra, 1996). Proteins adsorbed at the interface in multilayers are usually much more readily desorbed. Decreasing the bulk concentration leads in most cases to removal of the multilayers (Walstra and de Roos, 1993). It can, therefore, not be

ruled out that the decreasing surface excess of emulsions with hydrophobic peptides upon washing is due to desorption of peptide multilayers.

The surface excess of emulsions made with the amphiphilic peptides RET1 is relatively low and multilayer adsorption appears to be unlikely. The surface excess of these emulsions also decreased after washing (Table 5.2). After washing the emulsions twice, the surface excess was even too low for stabilizing the droplets against coalescence, resulting in oil separation. The decrease in surface excess was in this case most likely due to peptide desorption.

The formation of multilayers at the oil/water interface of emulsions made with hydrophobic peptides was further studied by preparing and analyzing these emulsions at 4 °C. At this temperature, the hydrophobic interactions between the peptides are minimal, making multilayer adsorption less likely. The results are shown in Table 5.4.

Table 5.4 Average size (d_{32}) and surface excess (Γ) of emulsion droplets made with hydrophobic peptides PEL1 (c = 8 mg.ml^{-1}) prepared at different temperatures (T) and at pH 9.0 and I = 0.075 M.

T (°C)	d_{32} (μm)	Γ (mg.m^{-2})
4	1.20	2.9
22	0.89	3.2

The droplet size and surface excess were both affected by preparing the emulsions at a low temperature. The relatively large droplet size was probably due to the relatively high viscosity of the oil phase at 4 °C. The relatively low surface excess of the emulsion prepared at 4 °C might be an indication of some multilayer formation. The difference is, however, small and extensive multilayer adsorption appears to be unlikely.

MacRitchie (1981; 1985) showed that for air/water interfaces the desorption rate of proteins is governed by molar mass and physicochemical conditions. The desorption rate of proteins increased with decreasing molar mass and is minimal at a pH near the isoelectric pH of proteins due to a minimal electrostatic repulsion. In Chapter 4 was shown, that the adsorption of β-casein at emulsion droplets was practically irreversible. The more reversible adsorption of β-casein peptides might be due to their relatively small molar mass. The relatively high electrostatic charge of the amphiphilic peptides may have increased their susceptibility for desorption from the oil/water interface even further.

Generally it can be concluded that despite the more reversible adsorption, the emulsion forming properties of the hydrophobic peptides and intact β-casein were very similar. The similar surface excesses indicate that the hydrophobic

peptides form, like β-casein, a densely packed layer at the oil/water interface, suggesting that the surface excess is primarily determined by the flexible, hydrophobic region of the β-casein molecules. The contribution of the N-terminal domain to the surface excess of emulsions made with intact β-casein, therefore, appears to be small. However, it should be kept in mind that for emulsions with hydrophobic peptides, the molar concentration of peptides adsorbed at the oil/water interface is approximately twice the molar concentration of β-casein at the interface. The conformation of the peptides adsorbed at the oil/water interface might therefore be very different from those of the intact protein.

5.4 Effect of physicochemical conditions on emulsion forming properties of β-casein peptides

5.4.1 Emulsions made with amphiphilic peptides

The functional properties of proteins and peptides are affected by inter and intramolecular interactions, which in turn are influenced by physicochemical conditions, like pH and ionic strength. The effect of the physicochemical conditions on the emulsion forming properties of β-casein peptides was studied for a limited pH and ionic strength range.

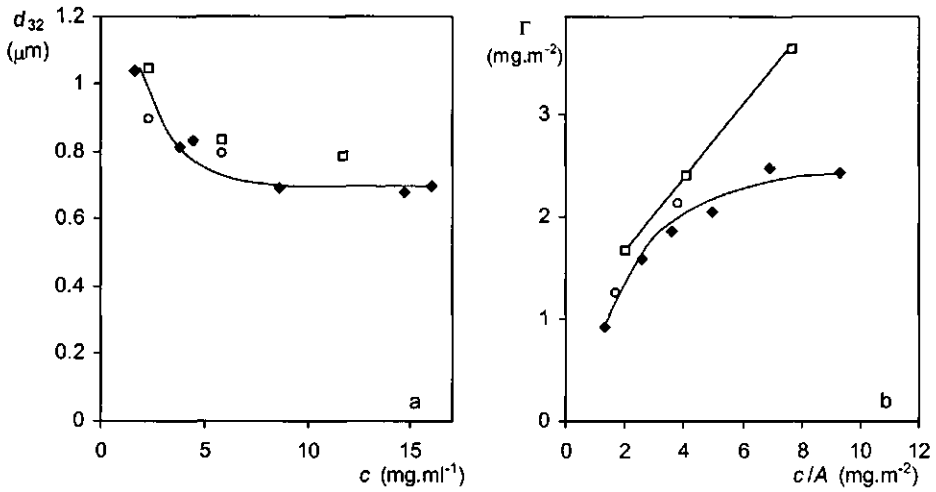

Figure 5.8 Properties of emulsions with amphiphilic peptides RET1 at pH 6.7 and I = 0.075 M (◆) or I = 0.150 M (□), and at pH 9.0 and I = 0.075 M (○): a, average droplet size (d_{32}); b, surface excess (Γ).

The properties of emulsions made with the amphiphilic peptides RET1 at varying pH and ionic strength are shown in Figure 5.8. At a pH between 4.0 and 5.5, the amphiphilic and also the hydrophobic peptides did not fully dissolve and emulsions with properties similar to those made with hydrophobic peptides at pH 6.7 were formed. The properties of these emulsions will, therefore, not be discussed further.

The droplet size of emulsions made with amphiphilic peptides increased slightly with increasing ionic strength and the emulsions were slightly aggregated. It was shown in Chapter 4, that generally the droplet size of aggregated emulsions was found to be relatively large. These large droplets may at least partly be due to a high recoalescence rate during homogenization of these aggregated emulsions. Droplet aggregation appeared to be caused by a decrease in the solubility of proteins and peptides, even if this decrease is on a molecular level.

The surface excess of emulsions made with amphiphilic peptides at an ionic strength of 0.150 M was high and no maximum was found in the concentration range studied. The apparent absence of a maximum and the high surface excess indicate the formation of multilayers at the oil/water interface. The aggregation of these emulsions and the multilayer adsorption of these peptides are likely to be due to changes in the molecular interactions. At a high ionic strength, the electrostatic charges of proteins and peptides are more shielded, leading to decreased inter and intramolecular repulsions and thus to increased hydrophobic interactions and probably decreased solubility.

The droplet size of emulsions made with amphiphilic peptides at pH 9.0 appeared to be larger than those made at pH 6.7, while the surface excess appeared to be unaffected by pH. The emulsions were not aggregated. Increasing the charge of the already highly charged amphiphilic peptides seemed to have, therefore, only a slight effect on the formation of emulsions.

5.4.2 Emulsions made with hydrophobic β-casein peptides

The average droplet size of emulsions made with the hydrophobic peptides PEL1 at pH 9.0 was not affected by changing the ionic strength from 0.075 M to 0.150 M (Figure 5.9). It is noteworthy that, regardless of the unaffected droplet size, the emulsion droplets were aggregated and had a relatively high surface excess at the high ionic strength. A similar, but smaller increase in surface excess was found for emulsions made with intact β-casein at the same physicochemical conditions (Chapter 4). At the high ionic strength, the charge of the peptides was most likely more shielded allowing either a denser peptide packing or the formation of peptide multilayers at the oil/water interface, resulting in a higher surface excess.

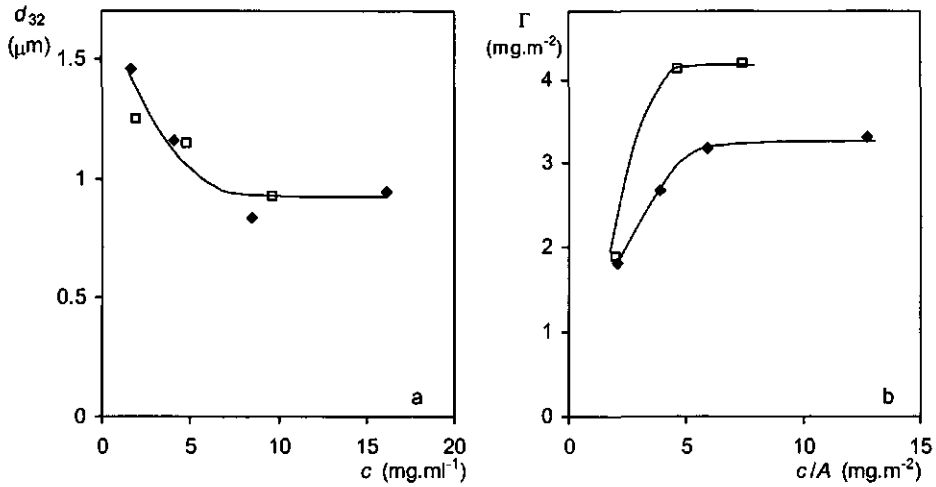

Figure 5.9 Properties of emulsions with hydrophobic peptides PEL1 at pH 9.0 and I = 0.075 M (◆) or I = 0.150 M (□): a, average droplet size (d_{32}); b, surface excess (Γ).

5.5 Conclusions

The emulsion forming properties of the studied peptides of β-casein were significantly affected by their molecular properties. Some of the properties found to affect the peptide functionality were: charge, amphiphilicity, solubility, hydrophobicity, and molar mass.

The amphiphilic peptides formed emulsions with a smaller droplet size than intact β-casein. The surface excess of emulsion droplets with these peptides was relatively low due to their high electrostatic charge. Increasing the ionic strength decreased the electrostatic repulsion, resulting in droplet aggregation and an increased surface excess of the emulsion droplets.

The emulsion forming properties of the hydrophobic peptides were strongly related to their solubility. Under physicochemical conditions where their solubility was low, the emulsion forming properties of the hydrophobic peptides were poor. When the peptides were completely soluble, their emulsion forming properties were comparable to those of intact β-casein at similar conditions. The relatively high surface excess of emulsions with hydrophobic peptides appears to be mainly due to hydrophobic C-terminal domain of β-casein.

In general, the emulsion forming properties of the β-casein peptides studied appeared to be somewhat better or comparable to those of intact proteins if their solubility is high. The peptides were, however, much more readily desorbed form

the droplet interface than intact proteins, which was probably due to their relatively low molar mass.

Chapter 6

Coalescence stability of emulsions made with proteins and peptides

Abstract

The emulsion stabilizing properties of proteins and peptides with respect to coalescence were studied under quiescent conditions. The coalescence stability of emulsions made with proteins was high even at low protein concentrations. At the conditions studied (pH 6.7 and an ionic strength of 0.075 M), the coalescence stability appeared to be mainly determined by the surface excess of the emulsion droplets.

Emulsions made with β-casein peptides were less stable against coalescence than emulsions made with intact proteins, probably due to the relatively low molar mass of the peptides. The coalescence stability of emulsions made with peptides appeared to be mainly governed by electrostatic and steric interactions. The N-terminal domain of the amphiphilic β-casein peptides was found to be of great importance for stabilizing emulsions against coalescence. The relatively high coalescence stability of emulsions made with hydrophobic peptides appeared mainly to be due to the high surface excess of the droplets. The contribution of the electrostatic and steric interactions was further studied by determining the effect of changes in pH and ionic strength on the coalescence stability.

6.1 Introduction

Food emulsions often need to be stable for a long period of time, say more than one year. During this time only minor stability defects are acceptable. The most important types of instability that may occur in food emulsions with a liquid oil phase are: creaming, aggregation, and coalescence (Walstra and de Roos, 1993). Creaming or sedimentation of emulsions occurs due to a density difference between the disperse and continuous phase. The creaming stability of emulsions is determined by the droplet size distribution, and hence the emulsion formation. Larger droplets cream more rapidly, while creaming of very small droplets is hampered by Brownian motion and weak convection currents. Generally, it is found that food emulsions with droplets smaller than one micron are stable with respect to creaming (Walstra, 1996).

The aggregation of emulsions is mostly determined by the properties of the proteins and peptides, stabilizing the droplets. Emulsion aggregation usually occurs if these proteins and peptides also have a tendency to form aggregates in solution (Walstra and de Roos, 1993).

Coalescence occurs when the thin film between two droplets ruptures and the droplets join to form one single, large droplet. Coalescence is an irreversible process and leads eventually to the complete break-down of an emulsion, making it an important type of instability. Usually, coalescence follows first order kinetics, indicating that the coalescence rate determining step is the rate at which the film between droplets is ruptured (van den Tempel, 1957). The likeliness of film rupture increases with increasing size and lifetime of the film, thus when emulsion droplets are relatively large or when droplets are aggregated or creamed (Walstra, 1996).

Literature data on the coalescence stability of emulsions made with proteins and peptides is very limited. It is generally found that the rate of coalescence of protein stabilized emulsions is very low as long as the droplet size is sufficiently small and the surface excess is sufficiently high, and when the droplets are not aggregated or creamed (Walstra and de Roos, 1993). To accelerate coalescence, centrifuge test are often used or some form of shearing is applied. These tests often induce coalescence, which would not occur under quiescent storage conditions. The results of these studies are, therefore, difficult to correlate to the coalescence stability of emulsions stored under practical conditions (Dickinson, 1992; Walstra, 1996).

To obtain a better understanding of the emulsion stabilizing properties of proteins and peptides, the coalescence stability of emulsions was studied in relation to the molecular properties of the proteins and peptides used. An other type of emulsion instability, aggregation, has been discussed in previous chapters.

The coalescence stability was determined by measurement of the change in droplet size during storage of emulsions at ambient temperature and under quiescent conditions. The studied proteins and peptides were chosen for their widely varying molecular properties. Their molecular and emulsion forming properties have been described in Chapters 4 and 5.

6.2 Coalescence stability of emulsions made with proteins

The emulsion stabilizing properties with respect to coalescence were studied for the proteins: β-casein, β-lactoglobulin, α-lactalbumin, lysozyme, and ovalbumin. Some typical results are shown in Figure 6.1.

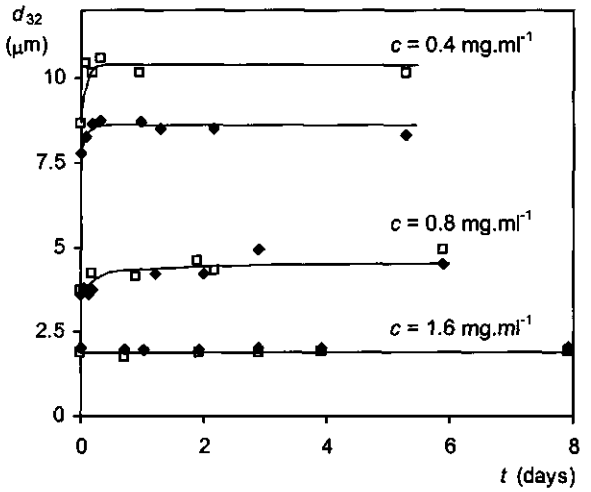

Figure 6.1 Volume-surface average droplet size (d_{32}) of emulsions made with β-casein (□) or β-lactoglobulin (◆) as function of storage time (t); all at pH 6.7 and I = 0.075 M.

The coalescence stability of emulsions made with the studied proteins appeared to be similar. At a concentration of 1.6 mg.ml^{-1} and higher, the emulsions were stable against coalescence. At lower concentrations, some coalescence was found to occur shortly after emulsion formation. After storage for one or two days, the coalescence stability appeared to increase and hardly any change in droplet size could be determined.

These results appear to disagree with the film rupture theory, which predicts an increasing coalescence rate with increasing film size, hence increasing droplet size (Dickinson, 1992; Walstra, 1996). The increased coalescence stability during

storage might be due to time-dependent intermolecular interactions between proteins adsorbed at emulsion droplets, increasing the strength of the film between droplets (Dickinson and Williams, 1994). Dickinson et al. (1988a) showed for example that the coalescence stability of individual oil droplets in contact with a planar oil/water interface increased if the interface was aged. The improved coalescence stability of emulsions made with globular proteins, especially those made with β-lactoglobulin or ovalbumin, may be due to the formation of a protein network. For emulsions made with β-casein, the formation of such a network is, however, unlikely.

The stability of emulsions may also increase due to an increase in the droplet surface excess as a result of coalescence (Oortwijn and Walstra, 1979). Dickinson et al. (1988a) observed an increased coalescence stability of oil droplets with a low surface excess at a planar interface with increasing protein concentration. A higher surface excess would increase the steric and electrostatic repulsion between droplets, hence increase the film thickness and film stability.

At a concentration of 1.6 $mg.ml^{-1}$, the surface excess of protein stabilized emulsions was found to be approximately 1.3 $mg.m^{-2}$ for most of the proteins studied (Chapter 4). Apparently, this surface excess is sufficiently high for stabilizing the droplets against coalescence. At lower protein concentrations, the surface excess was not determined due to the inaccuracy of the method. However, suppose that the emulsion droplet size during emulsification equals the minimum achievable droplet size for a certain protein, i.e. the droplet size is fully determined by the homogenizing conditions. For an emulsion made with β-lactoglobulin, the minimum droplet size is approximately 0.8 μm. If in an emulsion made with 0.8 $mg.ml^{-1}$, all protein would be adsorbed at the droplets, the surface excess would be 0.5 $mg.m^{-2}$. This surface excess is, apparently, too low for stabilizing the droplets against (re)coalescence, resulting in a rapid increase in droplet size and surface excess. It is however, remarkable that the droplet size of the emulsions after a few days of storage ($d_{32} \approx 5$ μm) is much larger than the droplet size required for obtaining a plateau surface excess ($\Gamma = 1.8$ $mg.m^{-2}$ and $d_{32} \approx 2$ μm). The cause of this discrepancy is unclear.

6.3 Coalescence stability of emulsions made with amphiphilic β-casein peptides

6.3.1 Peptide fraction RET1

The fraction RET1 (f. 1/29-105/107) consists of amphiphilic peptides originating from the N-terminal domain of β-casein (Caessens et al., 1997a). These peptides have a charged, hydrophilic head and a hydrophobic tail, and compared to intact β-casein good emulsion forming properties. The coalescence stability of emulsions made with these peptides as indicated by the change in emulsion droplet size during storage is shown in Figure 6.2.

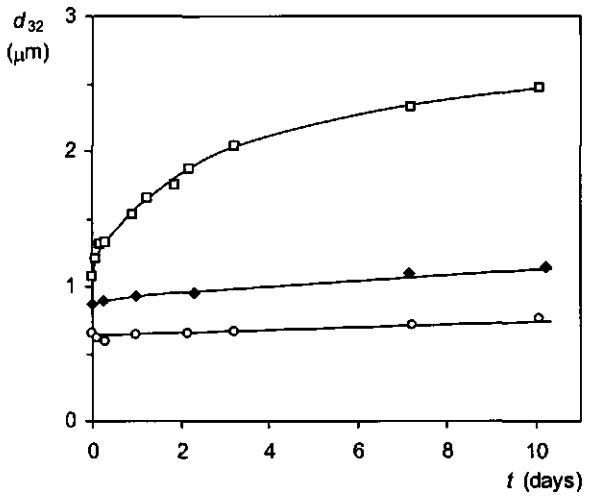

Figure 6.2 Volume-surface average droplet size (d_{32}) of emulsions prepared with amphiphilic β-casein peptides RET1 (f. 1/29-105/107) as function of time (t). Concentration (c): 1.6 mg.ml^{-1} (□), 4 mg.ml^{-1} (◆), and 8 mg.ml^{-1} (○); all at pH 6.7 and I = 0.075 M.

The coalescence stability of emulsions made with amphiphilic β-casein peptides increased with increasing peptide concentration, but even at a concentration of 8 mg.ml^{-1} some coalescence occurred. The emulsions made with these peptides were obviously less stable against coalescence than those made with intact proteins. This agrees well with literature data, where often poor emulsion stabilizing properties of peptides compared to those of intact proteins are reported (Agboola and Dalgleish, 1996; Caessens, 1997a; Agboola et al., 1998).

Similar to protein stabilized emulsions, the coalescence stability appeared to be time-dependent, especially at low peptide concentrations. The droplet size of emulsions with a peptide concentration of 1.6 mg.ml^{-1} increased rapidly during the first few days after emulsification. After this period, the coalescence rate decreased and became roughly comparable to the coalescence rate of emulsions with higher peptide concentrations. A similar time-dependence was found by Agboola et al. (1998) for emulsions stabilized with hydrolyzed whey proteins.

As discussed before, the decrease in coalescence rate was likely to be caused by an increase in surface excess due to a decreasing interfacial area of the emulsions. In Table 6.1, the estimated surface excess of emulsion droplets during storage is shown. The surface excess as function of time was calculated assuming negligible peptide desorption due to coalescence and might, therefore, be somewhat overestimated.

Table 6.1 Estimated surface excess of emulsion droplets made with the amphiphilic peptides RET1 during storage under quiescent conditions.

	Surface excess (mg.m^{-2})		
Concentration	1.6 mg.ml^{-1}	4 mg.ml^{-1}	8 mg.ml^{-1}
Storage time (days)			
0	0.9	1.6	2.0
2	1.5		
10	2.1	2.1	2.4

The surface excess measured directly after emulsion formation was higher for a higher peptide concentration. After 2 days of storage, the estimated surface excess of an emulsion with a peptide concentration of 1.6 mg.ml^{-1} was comparable to the initial surface excess of the emulsion made with a concentration of 4 mg.ml^{-1}, while the coalescence stability of the latter emulsion appeared to be higher. Similar results were obtained at longer storage times and at higher concentrations, suggesting that the coalescence stability was not directly related to the surface excess. The droplets of the emulsions with the low peptide concentration were, however, larger, which possibly explains the lower coalescence stability.

The emulsions made with 1.6 mg.ml^{-1} amphiphilic peptides were visibly creamed after two days of storage. The emulsions with higher peptide concentrations showed no discernible creaming within the experimental time-scale. The creaming of the emulsions appeared to have little effect on the coalescence stability, since the coalescence rate decreased rather than increased in time. This conclusion is also reached when comparing the Laplace pressure and the pressure

exerted by the buoyancy force on the creamed droplets. The buoyancy pressure acting on a droplet on the top of a creamed layer is given by (Walstra, 1996)

$$p_B = \frac{F_B}{a} = \frac{g\pi d^2 h(\rho_d - \rho_c)}{\pi d^2} \qquad (6.1)$$

where p_B and F_B are the buoyancy pressure and force, respectively, and h the height of the creamed layer. For an emulsion with a droplet size of 2 μm, an interfacial tension of 15 $mN.m^{-1}$ and a creamed layer of 5 mm, the Laplace pressure (4 γ/d) and buoyancy pressure equal 30 kPa and 4 Pa, respectively, showing that the pressure exerted on the creamed droplets is negligible compared to the Laplace pressure of the droplets.

When the emulsion properties of intact β-casein and the amphiphilic peptides are compared, the importance of distinguishing between the emulsion forming and stabilizing properties of proteins and peptides is obvious. The emulsion forming properties of the amphiphilic peptides were superior to those of intact β-casein, while the emulsion stabilizing properties of the peptides with respect to coalescence were clearly inferior. The major difference in the molecular properties of intact β-casein and the amphiphilic peptides RET1 is the molar mass and the size of the hydrophobic C-terminal tail. The effect of these properties on the emulsion stability will be discussed later.

6.3.2 Peptide fractions IEC2 and IEC3

The amphiphilic β-casein peptides RET1 were further fractionated, yielding the fractions IEC2 and IEC3. The fractions IEC2 (f. 29-105/107) and IEC3 (f. 1-105/107) consist of amphiphilic peptides with a small and a large electrostatically charged, hydrophilic head, respectively. The coalescence stability of emulsions made with these fractions is shown in Figure 6.3.

The emulsion forming properties of the peptide fractions were comparable as is indicated by the similar droplet sizes directly after emulsification. The emulsion stabilizing properties with respect to coalescence appeared to increase in the order of IEC2, RET1, and IEC3. The coalescence stability of emulsions made with the peptides IEC2 was relatively poor at low peptide concentrations and during the first day after emulsion formation. The emulsions made with the peptides IEC3 were relatively stable with respect to coalescence, especially at a high concentration where no change in droplet size could be detected. These results are generally in agreement with the preliminary results of Caessens et al. (1999b) on the coalescence stability of emulsions with similar peptide fractions.

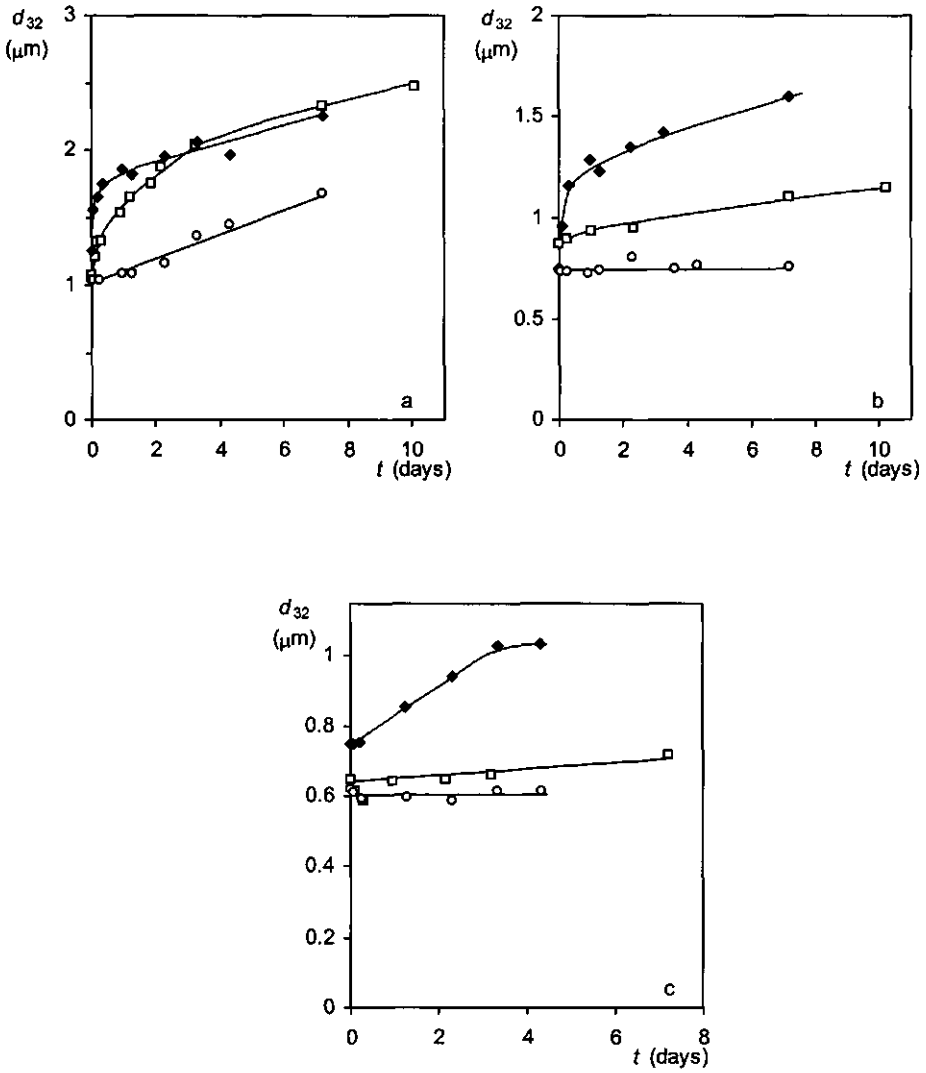

Figure 6.3 Volume-surface average droplet size (d_{32}) of emulsions made with the amphiphilic β-casein peptides RET1 (f. 1/29-105/107) (□), IEC2 (f. 29-105/107) (◆), and IEC3 (f. 1-105/107) (○) as function of time (t). Concentration: a, 1.6 mg.ml^{-1}; b, 4 mg.ml^{-1}; c, 8 mg.ml^{-1}; all at pH 6.7 and I = 0.075 M.

As shown in Chapter 5, the surface excesses of emulsions with RET1 and IEC3 were similar, while the surface excess of emulsions with IEC2 appeared to be somewhat lower. The relatively low surface excess of the latter emulsions might explain their inferior coalescence stability. The difference in coalescence stability of emulsions made with RET1 and IEC3 can, however, not be explained by differences in the surface excess. Apparently, some other peptide properties are also of importance for the coalescence stability of the emulsions.

The main differences between the molecular properties of the peptides IEC2 and IEC3 are the electrostatic charge, the number of phosphoseryl-groups present (1 and 5, respectively), and the size of the hydrophilic head (Table 6.2). Several studies have shown that the hydrophilic N-terminal domain of β-casein adsorbed at oil/water interfaces forms a tail, that protrudes far into the aqueous phase (Dalgleish and Leaver, 1991; Dickinson, 1994; Beek et al., 1996). It appears to be likely, that especially the peptides IEC3 will adopt a similar conformation at the oil/water interface to minimize the electrostatic and steric repulsion exerted by the voluminous and charged groups of the N-terminal, particularly when the surface excess is relatively high.

Table 6.2 Percentage of charged and hydrophobic residues and estimated net charge of β-casein and β-casein peptides at pH 6.7.

Fraction		Charged residues (mol%)	Net charge	Hydrophobic residues (mol%)
β-casein		24	-13.0	30
amphiphilic peptides RET1				
IEC2:	f. 29-105	25	-4.0	26
	f. 29-107	27	-2.5	25
IEC3:	f. 1-105	32	-13.0	27
	f. 1-107	34	-11.5	26
hydrophobic peptides PEL1				
	f. 106-209	19	0	34
	f. 108-209	18	-1.5	34
	f. 114-209	16	-1.5	34

The thickness of the adsorbed β-casein layer decreases when the phosphoseryl-groups of β-casein are removed (Dalgleish, 1990; Leaver and Horne, 1993; Husband et al., 1997). Emulsions made with dephosphorylated β-casein are, moreover, less stable against coalescence than emulsions made with intact β-casein, suggesting that the N-terminal tail is of importance for stabilizing droplets (Husband et al., 1997). Nylander and Wahlgren (1994) showed that peptides of

β-casein adopt a less extended conformation adsorbed at silica particles if the C-terminal end is removed. From the peptides IEC2, the C-terminal as well as a part of the N-terminal end were removed. It appears to be likely that these peptides will, therefore, have a relatively flat conformation at the oil/water interface due to decreased intra and intermolecular electrostatic and steric repulsions. This seems to be confirmed by the relatively low surface excess of emulsions made with these peptides. The low electrostatic and steric repulsion allows the formation of a relatively thin film between approaching droplets, increasing the possibility of coalescence. Apparently, the charged N-terminal domain is an important molecular property for stabilizing emulsions droplets against coalescence. The relatively poor emulsion stabilizing properties of the peptides IEC2 may be due to the partial removal of this domain.

The stabilizing properties of peptides are, besides the N-terminal domain, likely to be determined by other molecular properties as well. The molecular properties of the peptides IEC3 are with the exception of the molar mass very similar to those of intact β-casein. It therefore appears likely that the decreased stability of emulsions made with β-casein peptides was mainly due to the decreased molar mass.

In general, the molecular properties of peptides seemed to be much more important for the stability of emulsions than those of intact proteins as indicated by the difference in the emulsion stabilizing properties of the peptides IEC2 and IEC3. Apparently, the molecular properties become much more critical when the molar mass is relatively low.

Finally, it should be noted that the amphiphilic peptides RET1, IEC2, and IEC3 contained small traces of hydrophobic impurities. Removal of these impurities seemed to improve the coalescence stability of emulsions made with these peptides. The differences in the coalescence rate, however, appeared to be relatively the same (Caessens et al., 1999b). It is therefore expected that, although the coalescence rate might have been somewhat overestimated, the general trends found in this study are valid.

6.4 Coalescence stability of emulsions made with hydrophobic β-casein peptides

The coalescence stability of emulsions made with the hydrophobic β-casein peptides PEL1 (f. 106/108/114-209) at pH 9.0 appeared to be relatively high compared to those of emulsions made with amphiphilic peptides, but somewhat lower than those of emulsions made with intact β-casein (Figure 6.4). At a peptide concentration of 1.6 $mg.ml^{-1}$, where the surface excess of the droplets is similar to

those of emulsions made with intact β-casein, some coalescence occurred. At higher concentrations, the emulsions were stable against coalescence over the experimental time-scale, even when the surface excess of the droplets was below the maximum level.

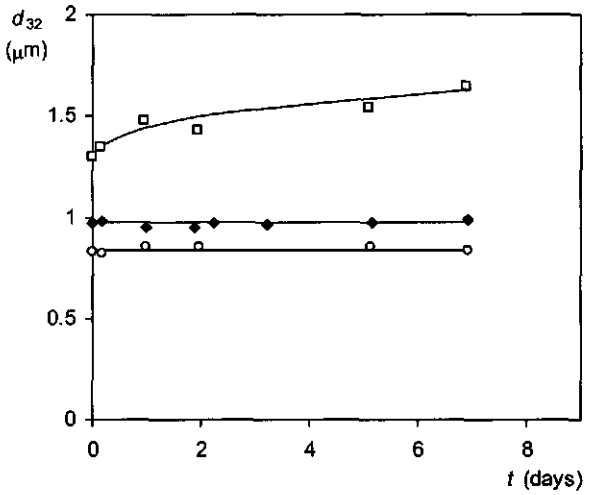

Figure 6.4 *Volume-surface average droplet size (d_{32}) of emulsions prepared with hydrophobic β-casein peptides PEL1 (f. 106/108/114-209) as function of time (t). Concentration: 1.6 mg.ml^{-1} (□), 4 mg.ml^{-1} (◆), and 8 mg.ml^{-1} (○); all at pH 9.0 and I = 0.075 M.*

The relatively poor coalescence stability at a low peptide concentration was likely to be due to the relatively low molar mass of the hydrophobic peptides. The relatively high coalescence stability of emulsions made with a higher concentration of these peptides appeared to be caused by steric interactions due to the relatively high surface excess of the droplets. It is however likely that electrostatic interactions also have contributed to the coalescence stability of the emulsions, since the peptides will have a significant charge at a high pH. To study the importance of the electrostatic and steric interactions in more detail, the effect of changes in pH and ionic strength on the coalescence stability were determined.

6.5 Effect of physicochemical conditions on coalescence stability of emulsions made with β-casein peptides

6.5.1 Emulsions made with amphiphilic peptides

The emulsion stabilizing properties of the amphiphilic peptides RET1 appeared to be markedly affected by changes in the electrostatic interactions between the emulsion droplets (Figure 6.5). Increasing the ionic strength from 0.075 M to 0.150 M decreased the coalescence stability of the emulsions, especially at a low peptide concentration. The coalescence stability of emulsions with amphiphilic peptides improved if the pH had been increased from pH 6.7 to 9.0, whereby the net charge of the peptides was increased.

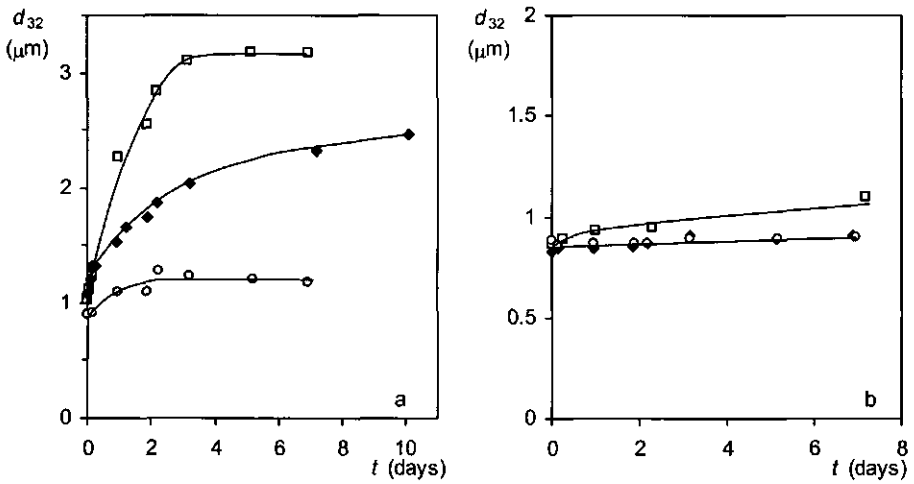

Figure 6.5 Volume-surface average droplet size (d_{32}) of emulsions as function of time (t) made with: a, 1.6 mg.ml^{-1}; b, 4 mg.ml^{-1} amphiphilic peptides RET1 (f. 1/29-105/107). Physicochemical conditions: pH 6.7 and I = 0.075 M (◆), pH 6.7 and I = 0.150 M (□), and pH 9.0 and I = 0.075 M (○).

At an ionic strength of 0.150 M, the emulsions were found to be aggregated. Increasing the ionic strength results in decreased electrostatic repulsions due to an increased shielding of peptide charges, hence in decreased inter and intramolecular electrostatic repulsions. As a result, emulsions are often aggregated at a high ionic strength, especially when the surface excess is low (Dalgleish, 1990; Brooksbank et al., 1993).

If the electrostatic repulsion is relatively low, the conformation of the peptides adsorbed at the emulsion droplets is likely to be less extended and more

flat. Measurements of the thickness of a layer of β-casein adsorbed at polystyrene latex particles showed that the layer thickness decreased with an increasing ionic strength (Dalgleish, 1990; Brooksbank et al., 1993). The repulsion distance between approaching droplets will, therefore, be smaller, resulting in a lower coalescence stability. A pH increase from 6.7 to 9.0 has an opposite effect. The net charge of the peptides increases with the increasing pH, resulting in increased inter and intramolecular repulsions, hence an increased coalescence stability.

6.5.2 *Emulsions made with hydrophobic peptides*

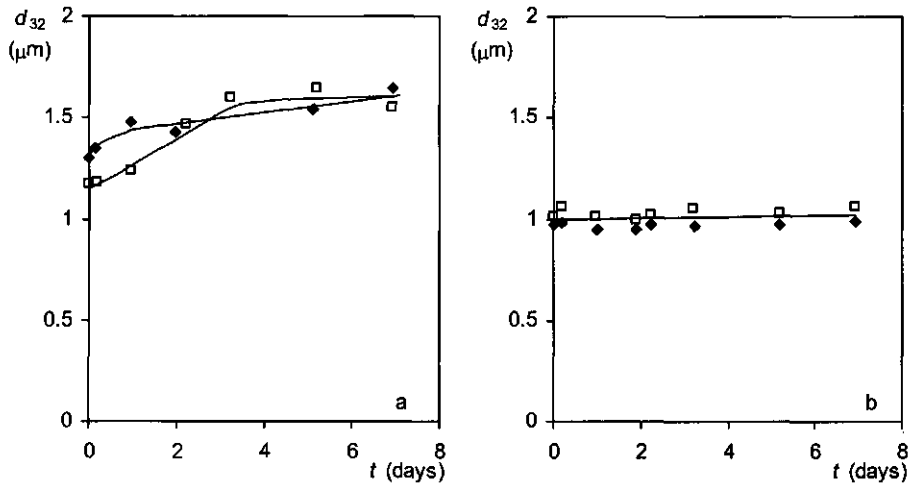

Figure 6.6 Volume-surface average droplet size (d_{32}) of emulsions as function of time (t) made with: a, 1.6 mg.ml^{-1}; b, 4 mg.ml^{-1} hydrophobic peptides PEL1 (f. 106/108/114-209). Physicochemical conditions: I = 0.075 M (◆), and I = 0.150 M (□); all at pH 9.0.

The effect of the ionic strength on the coalescence stability of emulsions made with hydrophobic peptides PEL1 is shown in Figure 6.6. The ionic strength did not significantly effect the coalescence stability of emulsions made with hydrophobic peptides during the experimental time-scale. Increasing the ionic strength resulted in aggregated emulsion droplets and an increased surface excess (Chapter 5). The thick layer of adsorbed peptides provided apparently enough steric repulsion to stabilize the droplets against coalescence despite of their aggregated state.

6.6 Conclusions

The coalescence stability of emulsions made with proteins was high even at low protein concentrations. Under the conditions studied, the molecular properties of the proteins appeared to have no significant effect on the emulsion stability. The main parameter affecting the coalescence stability seemed to be the surface excess of the emulsion droplets.

The emulsion stabilizing properties of the peptides of β-casein were inferior to those of the intact proteins, which was probably due to their low molar mass. Their stabilizing properties appeared to be strongly dependent on the molecular properties of the peptides. The coalescence stability appeared to be mainly determined by the strength of the electrostatic and steric repulsion exerted between the emulsion droplets, hence the peptide charge, the thickness of the peptide layer adsorbed at the droplet interface and the physicochemical conditions.

Chapter 7

General discussion and conclusions

7.1 Introduction

In the previous chapters, the emulsion forming and stabilizing properties of selected proteins and peptides have been described. These proteins and peptides were selected for their well-known and varying molecular properties. β-Casein is a flexible, random coil protein with a highly charged N-terminal head and a hydrophobic C-terminal tail. β-Lactoglobulin is a globular protein with a relatively stable conformation and a tendency for intermolecular association. α-Lactalbumin and lysozyme are globular proteins with predominantly similar molecular structures, but with varying electrostatic charges and conformational stabilities. Ovalbumin, finally, is a relatively large, globular molecule with a low conformational stability and is prone to aggregation upon unfolding.

The studied peptides were fractionated and purified hydrolyzates of β-casein. The peptide fraction RET1 (f. 1/29-105/107) consisted of amphiphilic peptides originating from the N-terminal domain of β-casein. This fraction was further purified to obtain the fractions IEC2 (f. 29-105/107) and IEC3 (f. 1-105/107) with a small and large charged head, respectively. Also, the emulsion forming and stabilizing properties of the fraction PEL1 (f. 106/108/114-209) consisting of predominantly hydrophobic peptides originating from the C-terminal domain of β-casein were studied.

In literature, many studies concerned with the emulsion forming and stabilizing properties of proteins and peptides have been reported. The relation between the molecular and emulsion properties of proteins and peptides remains, however, largely obscure, which is at least partly due to many of the results being contradictory. Reasons for these contradictions are the use of poorly defined protein and peptide mixtures and of inadequate experimental methods. Interpretation of the structure-function relation is also hampered by the often poor distinction of the different processes occurring during emulsion formation and stabilization.

The aim of this thesis was to elucidate the relation between the formation and stability of emulsions and the molecular properties of proteins and peptides. To achieve this objective, the emulsion forming and stabilizing properties of selected proteins and peptides were studied under varying physicochemical conditions. In this chapter, the main results are summarized and the relation between the molecular and emulsion properties of proteins and peptides is discussed.

7.2 High-pressure homogenizers

The emulsion forming properties of proteins and peptides were studied using a small laboratory high-pressure homogenizer. Large-scale experiments were hampered due to the use of expensive and scarce proteins and peptides. To ascertain that the structure-function relations found with small-scale experiments were applicable to the practically more relevant large-scale industrial processes, the mechanism of emulsion formation in a laboratory and a small industrial high-pressure homogenizer was studied.

During emulsion formation, droplets are deformed and broken up, surfactants adsorb at the droplet interface, and droplets collide and possibly recoalesce (Walstra, 1993). The mechanism and time-scales of these processes are governed by the hydrodynamic conditions prevalent inside the homogenizer valve. The flow regime in the industrial homogenizer was determined to be turbulent (Chapter 3). In the laboratory homogenizer, the flow was found to be laminar due to the limited dimensions of the valve and droplet break-up appeared to occur via the mechanism proposed by Kiefer (1977) for break-up in a bounded laminar flow.

Comparison of the droplet size, surface excess and recoalescence rate of emulsions made with sodium caseinate or whey proteins in either the laboratory or industrial homogenizer showed that the different flow conditions appeared to have little effect on the emulsion properties if the number of passes through the homogenizer was sufficiently high. The operating efficiency of the laboratory homogenizer was found to be inferior to that of the industrial homogenizer. To obtain a steady droplet size distribution, only a few passes through the industrial homogenizer were required. Emulsions prepared with the laboratory homogenizer using only one or two passes had a very wide droplet size distribution and were insufficiently stable against creaming.

To compensate the poor operating efficiency of the laboratory homogenizer, the emulsions were, in contrast to common industrial processes, prepared using a large number of passes through the homogenizer (typically about 30). As shown in Figure 7.1, the number of passes has, however, little influence on the relative difference in the emulsion forming properties of proteins and peptides. The recoalescence rate also appeared to be unaffected by the number of passes used for the preparation of the pre-emulsions (Chapter 4). It is therefore expected that the conclusions obtained in this study using laboratory scale experiments are also applicable to larger scale, industrial processes.

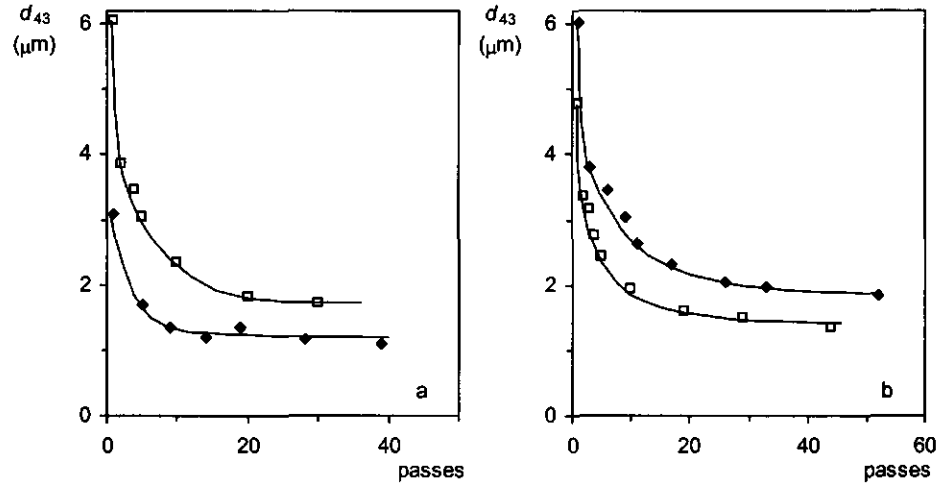

Figure 7.1 Average droplet size (d_{43}) as function of the number of passes through a laboratory homogenizer for emulsions made with: a, 4 mg.ml^{-1} β-casein (□) or amphiphilic β-casein peptides RET1 (◆); b, 16 mg.ml^{-1} β-casein (□) or ovalbumin (◆); all at pH 6.7 and I = 0.075 M.

7.3 Emulsion forming properties of proteins and peptides

7.3.1 Droplet size and recoalescence rate of aggregated emulsions

An overview of the emulsion forming properties of proteins and peptides at pH 6.7 and an ionic strength of 0.075 M is given in Table 7.1. The recoalescence rate is calculated from the initial slope of the ratio F_p/F_0 versus the number of passes through the homogenizer, -d (F_p/F_0) / d passes. With the method used, the actual rate of recoalescence (v_{reco}) can only be determined during the first few passes through the homogenizer. After these first passes, the oil in the emulsion droplets will be partly mixed and the change in the composition of the oil of the recoalescing droplets will be smaller, thus yielding an apparently lower recoalescence rate.

To obtain a better understanding of the functionality of proteins and peptides, a distinction has to be made between aggregated and non-aggregated emulsions. It was generally found that the droplet size of and recoalescence rate in emulsions that showed aggregation were relatively large compared to those of non-aggregating emulsions. The droplet size of aggregated emulsions was determined after deflocculation with SDS. The absence of aggregates was confirmed by microscopic analysis. Examples of aggregated emulsions are those made with lysozyme and ovalbumin.

Table 7.1 Overview of the emulsion forming properties of proteins and peptides at pH 6.7 and I = 0.075 M. Indicated are the plateau droplet size ($d_{32,\ plat}$) and surface excess (Γ_{plat}), the recoalescence rate (v_{reco}) at a concentration of c = 1.6 mg.ml^{-1}, and the presence of aggregates.

Protein or peptide	$d_{32,\ plat}$ (μm)	v_{reco} (passes^{-1})	Γ_{plat} (mg.m^{-2})	aggregates
β-casein	1.05 / 0.91[a]	0.06 / 0.03[a]	3.77 / 3.07[a]	no
β-lactoglobulin	0.79	0.06	1.86	no
α-lactalbumin	0.88	0.06	multilayers	no
lysozyme	1.18	0.09	1.93	yes
ovalbumin	1.37	0.04[b]	multilayers	yes
RET1 (f. 1/29-105/107)	0.69	0.03	2.51	no
PEL1 (f. 106/108/114-209)[a]	0.89	0.06	3.25	no

[a] Measured at pH 9.0.

[b] Measured at c = 12 mg.ml^{-1}.

The relatively high recoalescence rate of aggregating emulsions may at least partly explain the relatively large droplet size of these emulsions. It remains uncertain why aggregating emulsions are less stable against recoalescence. One cause may be the relatively small distance separating aggregated emulsion droplets. However, droplet aggregates would most likely readily be disrupted by hydrodynamic forces while passing the homogenizer valve. Another factor affecting the recoalescence rate may be protein aggregation. Protein aggregation decreases the effective protein concentration and may thus result in a relatively high recoalescence rate. Generally, emulsions are found to be aggregated if the proteins in solution also tend to aggregate (Walstra and de Roos, 1993).

Apart from a low recoalescence stability, the relatively large droplet size of aggregated emulsions would likely also be due to a limited ability of proteins to facilitate droplet break-up. The inferior break-up properties may, like the inferior recoalescence stability, be due to a relatively small effective protein concentration. Consider for example emulsions made with 12 mg.ml^{-1} β-lactoglobulin at pH 5.6 and pH 6.7 with droplet sizes of 1.4 μm and 0.8 μm, respectively (Chapter 4). If the rate of droplet break-up would have been similar for both emulsions, the emulsion made at pH 5.6 should have been completely recoalesced after a single pass through the homogenizer (v_{reco} of order 1 passes^{-1}) to agree with an increase in droplet size from 0.8 to 1.4 μm. The recoalescence rate at pH 5.6 was, however, found to be 0.06 passes^{-1}.

The aggregation of proteins, and subsequently of emulsion droplets may be caused by covalent and non-covalent intermolecular interactions. Proteins may be linked by covalent bonds if free thiol-groups are present. β-Lactoglobulin and ovalbumin contain one buried thiol-group (Papiz et al., 1986) and four buried thiol-

groups (Nisbet et al., 1981), respectively. For a reaction to occur, the thiol-group needs to be exposed. The formation of covalently linked aggregates is therefore governed by the conformational stability of proteins. Ovalbumin has a low conformational stability and readily denatures when adsorbed at interfaces, making the protein prone to aggregation (Osuga and Feeney, 1977; Pearce and Kinsella, 1978; Kitabatake and Doi, 1987). The susceptibility of ovalbumin to aggregation probably explains its relatively poor emulsion forming properties. β-Lactoglobulin has a relatively high conformational stability. Adsorbed at an oil/water interface, β-lactoglobulin slowly unfolds and polymers linked by disulfide-bridges are gradually formed (time-scale typically seconds till days) (Dickinson and Matsumura, 1991). Polymerization will most likely be negligible during emulsion formation, explaining the good emulsion forming properties of β-lactoglobulin.

The aggregation of emulsion droplets due to protein aggregation via non-covalent interactions is less readily predicted from the molecular properties of proteins than those due to protein aggregation via covalent interactions. Some proteins are known to associate in solution. For example, both lysozyme and β-lactoglobulin have a tendency to form dimers at neutral pH (Townend et al., 1960a; McKenzie and Sawyer, 1967; Deonier and Williams, 1970). Emulsions made with β-lactoglobulin were, however, aggregate free, while emulsions made with lysozyme were found to be aggregated. β-Casein forms micelles in solution at neutral pH and room temperature (Arima et al, 1979). The presence of these micelles appears to have little effect on the emulsion forming properties of β-casein (Dickinson, 1989). However, the slightly improved emulsion forming properties of β-casein at pH 9.0 (Chapter 4) may have been due to the absence of micelles at this pH (Niki and Arima, 1969). The emulsion forming properties of α-lactalbumin are also noteworthy. At high concentrations, α-lactalbumin formed multilayers at the emulsion droplets (Chapter 4). The multilayer adsorption was probably due to the tendency of α-lactalbumin to form aggregates upon surface denaturation via hydrophobic interactions (Segawa and Sugai, 1983). The association of α-lactalbumin did, however, not induce aggregation of emulsion droplets.

The aggregation of emulsions was generally stronger if the electrostatic interactions between proteins or peptides were smaller. The electrostatic interactions are governed by pH and ionic strength. Near their isoelectric pH, proteins have little charge and are generally prone to aggregation, resulting in relatively poor emulsion properties. See for example, the poor emulsion forming properties of β-casein, β-lactoglobulin and α-lactalbumin near their isoelectric pH (Chapter 4). The charge of proteins and peptides increases at a pH away from the isoelectric pH, often resulting in improved emulsion forming properties. For

example, the hydrophobic β-casein peptides PEL1 (f. 106/108/114-209) possessed good emulsion forming properties if the pH, hence the peptide charge, was sufficiently high (Chapter 5).

An increase in ionic strength results in decreased electrostatic interactions between proteins, possibly resulting in protein and droplet aggregation. The susceptibility of proteins for changes in ionic strength depends on the type of protein. For example emulsions made with β-casein appeared to be less sensitive to an increase in ionic strength from 0.075 M to 0.150 M than emulsions made with β-lactoglobulin.

The aggregation of emulsions may depend on protein concentration. For example, the droplet size of emulsions made with β-lactoglobulin was similar at pH 6.7 and pH 9.0 if the concentration was sufficiently high (Chapter 4). At a low protein concentration, the droplets of emulsions made at pH 9.0 were aggregated and relatively large compared to those of the non-aggregated emulsions at pH 6.7. Emulsion aggregation at low protein concentrations may be due to bridging during homogenization (Ogden et al., 1976).

Emulsion aggregation is also affected by the size of protein aggregates. It was shown that the emulsion forming properties of β-lactoglobulin were poor after heat denaturation (Chapter 4). The emulsion forming properties appeared to decrease with increasing concentration at which the proteins were denatured. During heat denaturation of β-lactoglobulin, aggregates are formed. The size of these aggregates depends on protein concentration and physicochemical conditions during heating (Sawyer et al., 1971; Saito and Taira, 1987; Boye et al., 1996). At a higher concentration, larger aggregates are formed resulting in a lower effective protein concentration and most likely in more bridging flocculation. Heat denaturation of proteins appeared to have little effect on the emulsion properties, if protein aggregation was absent. See for example, the effect of heat denaturation on the emulsion forming properties of α-lactalbumin (Chapter 4).

7.3.2 Droplet size and recoalescence rate of non-aggregated emulsions

Proteins and peptides appeared to have good emulsion forming properties as long as intermolecular associations were limited. In these cases, the recoalescence stability of emulsions made with the selected proteins appeared to be rather similar and only small differences in the droplet sizes could be determined (Table 7.1). These variations in the droplet sizes were, hence, likely to be due to differences in the abilities of proteins to facilitate droplet break-up.

The recoalescence rates of emulsions made with the β-casein peptides RET1 and PEL1 were found to be lower and higher, respectively, than those of emulsions made with intact β-casein. The superior recoalescence stability of

emulsions made with amphiphilic peptides RET1 may explain the relatively small droplet size of these emulsions. Considering the difference in droplet size of emulsions made with the peptides RET1 and intact β-casein, it appears likely that the peptides also possessed superior droplet break-up properties. The break-up properties of the hydrophobic peptides PEL1 seemed to be superior to those of β-casein as well: the droplet size of emulsions made with these peptides was found to be comparable to those of emulsions made with intact β-casein, despite of the inferior recoalescence stabilizing properties of these peptides. The improved droplet forming properties of β-casein peptides may have been due to their relatively small molar mass.

To elucidate the cause of the differences in emulsion forming properties of proteins and peptides, a basic understanding of the processes governing emulsion formation is required. During emulsification, droplets are deformed and possibly broken up. This process is opposed by the Laplace pressure of the droplets (Chapter 1). Proteins and peptides adsorbed at a droplet interface lower the interfacial tension, hence the Laplace pressure, making droplet break-up more easy. The equilibrium interfacial tension of a fully covered oil/water interface is approximately similar for most proteins and peptides due to steric interactions between adsorbed polypeptide segments (Walstra and Smulders, 1998). Droplet break-up is, however, a dynamic process of a very short time-scale and is governed by the dynamic interfacial tension of the oil/water interface. The dynamic or effective interfacial tension during droplet deformation is likely to be more dependent on the molecular properties of proteins and peptides, explaining the differences in their droplet break-up properties.

The effective interfacial tension is governed by the surface excess of the emulsion droplets during deformation. During deformation, the droplet interface is expanded, resulting in a decreased surface excess. Proteins and peptides will adsorb at the newly created interface. Nevertheless the surface excess will most likely be lower, and hence the interfacial tension higher during deformation than under equilibrium conditions.

Another factor governing the effective interfacial tension is the conformation of the adsorbed proteins and peptides. The time-scale of the droplet deformation process is too short (typically order of μs) to allow substantial conformational changes of the adsorbed proteins (order of minutes). As a result, the interactions between proteins and peptides and the droplet interface are not optimal, resulting in a relatively high interfacial tension.

The extent of droplet deformation and hence the increase in interfacial tension depends on the rheological properties of the interface. During droplet deformation, liquid will flow along the droplet interface dragging along adsorbed

proteins and peptides, resulting in an interfacial tension gradient. This gradient enhances the resistance against further interfacial deformation. Once the flow of liquid along the droplet interface has stopped, the interfacial tension gradient will disappear, dragging along liquid bordering the interface. This is the so-called Marangoni effect. The magnitude of the interfacial gradient and thus the Marangoni effect is governed by the surface dilational modulus. The surface dilational modulus relates the change in interfacial tension to the change in interfacial area during droplet deformation and is given by (Lucassen-Reynders and Kuijpers, 1992).

$$E_{sd} = \frac{-\mathrm{d}\gamma/\mathrm{d}\ln\Gamma}{\left(1+2\zeta+2\zeta^2\right)^{1/2}} \tag{7.1}$$

$$\zeta = \frac{\mathrm{d}m}{\mathrm{d}\Gamma}\left(D/2\omega\right)^{1/2} \tag{7.2}$$

$$\omega = \frac{\mathrm{d}\ln a}{\mathrm{d}t} \tag{7.3}$$

where γ is the interfacial tension, Γ the surface excess, m the molar concentration, D the diffusion coefficient of the proteins, ω a characteristic time-scale, a the interfacial area, and t the time. At the conditions prevalent during emulsification, the surface dilational modulus is mainly determined by the numerator in Equation 7.1, yielding (Walstra and Smulders, 1998)

$$E_{sd} \approx -\frac{\mathrm{d}\gamma}{\mathrm{d}\ln\Gamma} \tag{7.4}$$

According to Janssen et al. (1994), a dilatational resistance to droplet deformation can be expressed in an effective interfacial tension prevalent during deformation, approximately given by

$$\gamma_{eff} = \gamma + bE_{sd} \tag{7.5}$$

where b is a constant. According to theory, b should equal unity, but for near equilibrium conditions and simple shear flow, a value of about 0.23 was observed (Janssen et al., 1994).

Another process occurring during emulsification, is the close encounter of emulsion droplets, possibly followed by "recoalescence". The mechanism stabilizing droplets against recoalescence is still little understood. However, colloidal interactions appear to have little effect on the recoalescence stability. The colloidal repulsion between droplets with adsorbed proteins and peptides is most likely a few orders of magnitude smaller than the force acting on colliding droplets (Walstra and Smulders, 1998). This hypothesis was confirmed by Taisne et al. (1996). They showed that the coalescence stability of emulsions made with SDS decreased if the electrostatic repulsion was decreased by an increase in ionic strength. The initial emulsion droplet size, hence recoalescence rate was, however, unaffected by the increase in ionic strength.

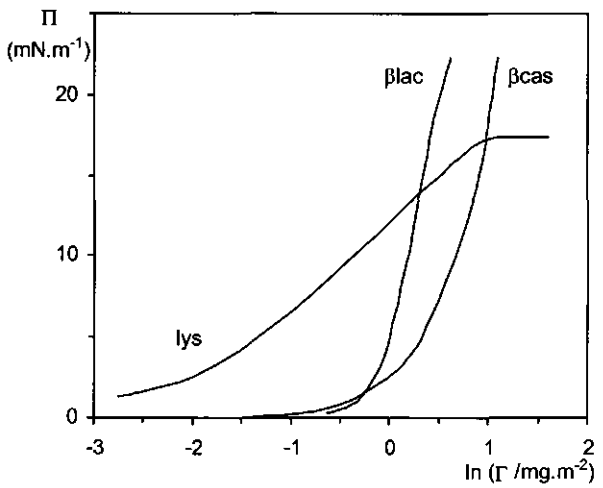

Figure 7.2 Surface pressure (Π) as function of surface excess (Γ) of β-lactoglobulin adsorbed at an air/water surface and β-casein and lysozyme at an oil/water interface. Data taken from Mitchell et al. (1970) and Graham and Phillips (1979b).

The recoalescence stability of emulsion droplets is likely to be governed by the dilational rheological properties of the oil/water interface (Walstra and Smulders, 1998). If droplets collide, liquid is forced out of the film between droplets, resulting in an interfacial tension gradient. This gradient will slow down and possibly stop the flow of liquid leaving the film, hence slow down the thinning of the film between approaching droplets. Once the thinning of the film has stopped, the interfacial tension gradient will tend to disappear. The gradient will partly disappear due to adsorption of proteins and peptides at the interface. These surfactants will, however, become soon depleted from the thin film between the droplets. The

disappearance of the remaining gradient induces a flow of liquid into the film and as a result the droplets are driven apart. This effect is the so-called Gibbs-Marangoni effect. The magnitude of this effect and the decrease of the flim thinning rate are determined by the surface dilational modulus (Walstra and Smulders, 1998).

The surface dilational modulus thus appears to be an important property governing emulsion formation. Under equilibrium conditions, this modulus may be calculated from the slope of the surface pressure ($\Pi = \gamma_0 - \gamma$) versus surface excess curve. Examples of some of these curves are given in Figure 7.2. The surface dilational moduli shown in Table 7.2 were calculated from the slope of these curves. Since the surface excess during emulsification is unknown, the surface excess of the droplets under equilibrium conditions was used for the calculations.

Table 7.2 Droplet size (d_{32}), surface excess (Γ), recoalescence rate (v_{reco}), and calculated surface dilational modulus (E_{sd}) of emulsions made with different proteins; all at pH 6.7 and I = 0.075 M.

Protein	c (mg.ml^{-1})	d_{32} (μm)	Γ (mg.m^{-2})	v_{reco} (passes^{-1})	E_{sd} (mN.m^{-1})
β-casein	1.6	1.11	1.30	0.06	6
	4.0	1.05	3.00	0.005	37
β-lactoglobulin	1.6	0.92	1.24	0.06	29
	4.0	0.79	1.86	0.005	25
lysozyme	1.6	1.68	1.75	0.09	6
	4.0	1.18	1.93	0.04	4

According to the results in Table 7.2, there appears to be no clear relation between the surface dilational modulus, droplet size and recoalescence rate of emulsions made with proteins. The surface dilational modulus was, however, calculated from data obtained under equilibrium conditions and, in the case of β-lactoglobulin, at an air/water interface. The conformation of adsorbed proteins and peptides is, moreover, dependent on the rate and time-scale of the interfacial deformation, making the surface dilational modulus time-dependent (Walstra and de Roos, 1993).

Unfortunately, little is known about the surface dilational modulus during emulsification. It may well be that under dynamic conditions, the relation between surface pressure and surface excess is completely different from the curves shown in Figure 7.2. For a similar surface excess, the surface pressure is likely to be substantially smaller during droplet deformation than at static conditions and is probably related to the conformational stability of proteins and peptides. Elucidating the relation between the emulsion forming and molecular properties of proteins and

peptides will, however, be complicated as long as the interfacial properties at the very short time-scales prevalent during emulsification are unknown.

7.3.3 Surface excess of emulsion droplets

The plateau surface excess of emulsion droplets made with globular proteins is typically about 2 $mg.m^{-2}$, while the surface excess of emulsions made with random coil proteins and peptides tends to be somewhat higher (Table 7.1). The plateau surface excess was usually reached at a higher concentration than is required for obtaining a constant droplet size (Figure 7.3). This difference was most likely due to differences in the time-scales of the various processes during emulsification which affects the dependence on protein concentration. Calculations of these time-scales are, however, hampered due to the lack of theories describing the time-scales prevalent in a bounded type of laminar flow.

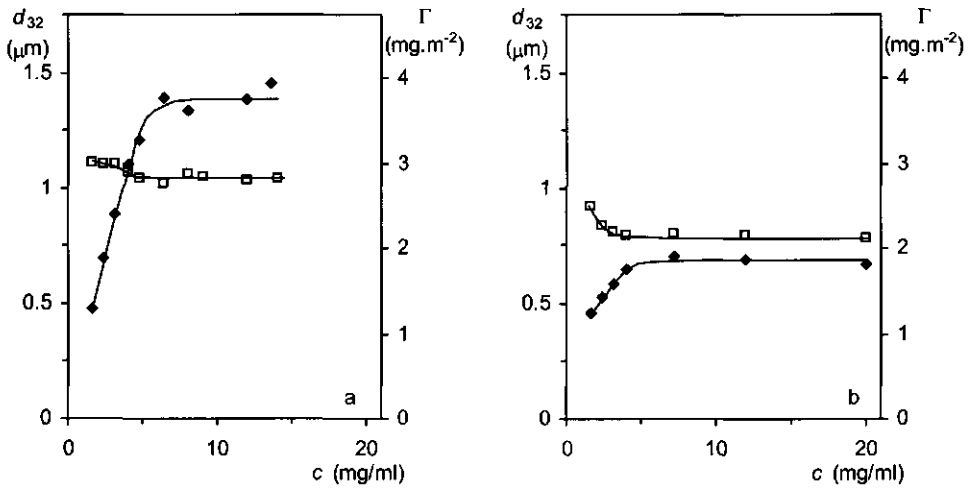

Figure 7.3 Volume-surface average droplet size (d_{32}, □) and surface excess (Γ,◆) of emulsions made with: a, β-casein; b, β-lactoglobulin; all at pH 6.7 and 0.075 M.

The affinity of proteins for the interface of emulsion droplets appeared to be relatively low. It was shown (Chapter 4), that compared to a planar interface, a relatively high protein concentration was required to obtain a significant surface excess at emulsion droplets. In general, the surface excess of emulsions appeared to be affected by the molar mass of individual proteins and peptides. For example, the relatively low surface excess of emulsions made with β-casein peptides compared to those of emulsions made with intact β-casein, was probably due to the relatively small molar mass of the peptides. In the presence of protein

aggregates, the surface excess was generally found to be high. See for example the high surface excess of emulsions made heat-denatured β-lactoglobulin (Chapter 4). These high surface excesses may have been due to multilayer adsorption. In these cases, the plateau surface excess was usually less well-defined. Proteins adsorbed in a multilayer may be linked non-covalently or covalently, as observed for α-lactalbumin and heat-denatured β-lactoglobulin, respectively.

The conformation and conformational stability of proteins and peptides appear to be an important factor determining the surface excess of emulsion droplets. Globular proteins, like β-lactoglobulin form a relatively thin and dense adsorbed layer (Dalgleish and Leaver, 1991; Atkinson et al., 1995), resulting in a relatively low surface excess. A flexible protein, like β-casein may form a thick, brush-like adsorbed layer with protein parts extending far into the aqueous phase (Dalgleish and Leaver, 1991; Dickinson et al., 1993; Beek et al., 1996), yielding a relatively high surface excess. The surface excess of emulsions made with the flexible, random coil peptides of β-casein was also found to be relatively high.

The surface excess of emulsion droplets was also found to be affected by protein and peptide charge. A high electrostatic charge may hamper a close packing at the droplet interface, resulting in a relatively low surface excess. The surface excess of emulsions made with β-casein was, for example, found to be lower if the pH was further away from the isoelectric pH. The surface excess of emulsions made with the amphiphilic peptides RET1 was, probably due to their high charge, found to be lower than those of emulsions made with the hydrophobic peptides PEL1. If the electrostatic interactions were reduced, for example at a pH near the isoelectric pH of proteins, or more shielded due to a high ionic strength, usually a relatively high monolayer surface excess or multilayer adsorption was found (Chapter 4).

7.4 Coalescence stability of emulsions made with proteins and peptides

Emulsion destabilization may occur in several forms. One of the most important types of instability is droplet coalescence, since coalescence will eventually result in a complete separation of an emulsion into an oil and a water phase. Droplet coalescence occurs when the film separating droplets ruptures and is governed by the thickness and life span of the film. Emulsion droplets are mainly stabilized against coalescence by the steric and electrostatic repulsion acting between adsorbed surfactants (Walstra, 1996).

Emulsions made with proteins appeared to have a high coalescence stability if the surface excess of the droplets was sufficiently high (Chapter 6). The

stabilizing properties of β-casein peptides with respect to coalescence appeared to be inferior to those of intact proteins. The surface excess of emulsions stabilized with peptides was found to be higher than those of emulsions made with globular proteins. This would suggest that the layer of peptides adsorbed at the droplet interface should be sufficient to stabilize droplets against coalescence. It was however shown in Chapter 5 that β-casein peptides were readily desorbed from the droplet interface upon washing of the emulsions. This decreased affinity of peptides for the oil/water interface is likely due to their relatively low molar mass (Walstra and de Roos, 1993) and may explain the inferior coalescence stability of emulsions made with peptides. The inferior stability of emulsions made with β-casein peptides may also be due to their highly flexible conformation, which may make desorption from an interface more easy.

The molar mass of some of the β-casein peptides is only slightly smaller than those of intact α-lactalbumin and lysozyme. This appears to indicate that the molar mass is only critical below a certain value. It is noteworthy that the surface excess of emulsions made with α-lactalbumin was also found to decrease upon emulsion washing (Chapter 4). This decrease was attributed to the desorption of α-lactalbumin multilayers and appeared not to be related to its relatively small molar mass. Multilayer adsorption of α-lactalbumin appeared likely, since no real plateau surface excess could be determined.

The coalescence stabilizing properties of the hydrophobic peptides PEL1 were superior to those of the amphiphilic peptides RET1. The surface excess of emulsions made with hydrophobic peptides was high compared to those of emulsions made with amphiphilic peptides, which may explain the superior emulsion stabilizing properties of the hydrophobic peptides. Apparently, the relatively high hydrophobicity and relatively low charge of the hydrophobic peptides, allowed the formation of a thick adsorbed layer which provided sufficient steric repulsion to make the droplets relatively stable against coalescence.

The coalescence stability of emulsions made with the amphiphilic peptides RET1 appeared to be greatly affected by electrostatic interactions. The importance of the electrostatic repulsion is demonstrated by the difference in coalescence stability of emulsions made with the amphiphilic peptides IEC2 (f. 29-105/107) and IEC3 (f. 1-105/107) with a small and large charged, hydrophilic head, respectively. The emulsions made with the peptides IEC3 were more stable against coalescence than those made with the peptide fraction RET1, which consists of a mixture of the fractions IEC2 and IEC3. The emulsions made with the peptides IEC2 were least stable against coalescence. Further evidence showing the effect of electrostatic interactions on the coalescence stability was obtained by varying pH and ionic strength. The coalescence stability of emulsions made with amphiphilic peptides

RET1 was found to decrease with increasing ionic strength and to increase with increasing peptide charge as a result of an increased pH.

It is noteworthy, that despite their good emulsion forming properties, the peptides possessed relatively poor emulsion stabilizing properties. These results clearly demonstrate the fundamental difference between the recoalescence and coalescence stability of emulsions.

7.5 Conclusions

Some general relations between the molecular and emulsion forming and stabilizing properties of proteins and peptides could be established. The emulsion forming properties of proteins and peptides appeared to be mainly governed by their molecular solubility or effective molar mass. The emulsion formation was generally relatively poor if proteins and peptides had a poor solubility or a tendency to associate (or aggregate). The solubility or tendency to associate appeared to depend on hydrophobicity, charge, presence of thiol groups and conformational stability of proteins and peptides. Predicting the associated state of proteins and peptides is, however, very difficult.

The differences in the emulsion forming properties of proteins and peptides in the absence of aggregates are most likely due to differences in the rheological properties of the oil/water interface during droplet deformation. A thorough understanding of the emulsion forming properties is hampered due to the lack of data concerning the properties of proteins and peptides under the circumstances (very short time scales) prevalent during emulsification.

The surface excess of emulsion droplets was found to be mainly governed by the conformational stability of and electrostatic interactions between proteins and peptides. Multilayer adsorption often occurred if the proteins and peptides had a tendency to associate.

The coalescence stability of emulsions appeared to be predominantly determined by the molar mass of proteins and peptides and by electrostatic and steric interactions. It appeared that if the molar mass was relatively small, the importance of steric and electrostatic repulsions for stabilizing droplets against coalescence is overriding.

Generally it can be concluded that some of the structure-function relations governing the emulsion forming and stabilizing properties of proteins and peptides have been elucidated in this study. The relation between emulsion and molecular properties is, however, still not completely understood. If a full understanding of the functionality of proteins and peptides would be required, their molecular properties

should be known under all circumstances, including those prevalent during emulsification.

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Symbols and abbreviations

Roman symbols

Symbol	*Description*	*Unit*
a	acceleration	$m.s^{-2}$
a	interfacial area	m^2
A	specific interfacial area	m^2
b	constant	-
c_{eq}	equilibrium concentration in aqueous phase	$mg.ml^{-1}$
c_n	relative width of droplet size distribution weighed with the n^{th} moment of the distribution	-
c_s	relative width of surface-weighed size distribution	-
d	droplet diameter	m
d	diameter of homogenizer inlet or valve	m
D	diffusion coefficient	$m^2.s^{-1}$
d_{32}	volume-surface average droplet diameter	m
d_{43}	volume-weighed diameter	m
d_i	diameter of homogenizer valve inlet	m
d_{nm}	average diameter derived from n^{th} over m^{th} moment	m
d_v	diameter of homogenizer valve	m
E	optical density	-
E_{sd}	surface dilational modulus	$N.m^{-1}$
F	turbidity according to Rayleigh-Gans	-
F_B	buoyancy force	N
F_0	initial turbidity	-
F_p	turbidity after p passes through a homogenizer	-
g	gravitational acceleration	$m.s^{-2}$
G	velocity gradient	s^{-1}
h	distance between particles	m
h	height or slit width of homogenizer valve	m
h	layer height	m
I	ionic strength	M
L	characteristic length	m
L	layer thickness	m
m	molar concentration	$mol.m^{-3}$
n	refractive index	-
N	number of droplets per unit volume	l^{-1}
p	homogenization pressure	Pa
p_B	buoyancy pressure	Pa
p_L	Laplace pressure	Pa
Q	flow rate	$m^3.s^{-1}$
Q	light scattering coefficient	-
Q^*	effective light scattering coefficient	-
$\overline{Q^*}$	average light scattering coefficient corrected for polydisperse droplet sizes and forward scattering	-
Re	Reynolds number	-
S_n	n^{th} moment of particle size distribution	m^{n-3}
t	time	s

Symbol	*Description*	*Unit*
t	storage time	days
T	temperature	°C
v	velocity	$m.s^{-1}$
$\bar{v}$	average velocity in homogenizer valve	$m.s^{-1}$
v_{reco}	*recoalescence rate*	$passes^{-1}$
v_s	Stokes velocity	$m.s^{-1}$
V_A	van der Waals attraction	J
V_E	electrostatic repulsion	J
V_{int}	interaction free energy	J
We_{cr}	critical Weber number	-
Z	reduced turbidity	-

Greek symbols

Symbol	*Description*	*Unit*
ε	energy density	$W.m^3$
γ	interfacial tension	$mN.m^{-1}$
γ_0	interfacial tension of a clean oil/water interface	$mN.m^{-1}$
γ_{eff}	effective interfacial tension	$mN.m^{-1}$
Γ	surface excess	$mg.m^{-2}$
η	viscosity	$N.s.m^{-2}$
φ	oil volume fraction	-
λ	wavelength	m
Π	surface pressure	$mN.m^{-1}$
ρ	density	$kg.m^{-3}$
ρ_{32}	spectroturbidimetric size parameter	-
ω	time scale	s^{-1}

Abbreviations

Abbreviation	*Description*
EDTA	disodium ethylenediamine tetra-acetate
IEC	ion exchange chromatography
NEM	N-ethylmaleimide
PEL	*pellet*
RET	retentate
SDS	sodium dodecyl sulphate
Tween 20	polyoxyethylene sorbitan monolaurate

Subscripts

Subscript	*Description*
0	zero passes through a homogenizer
c	continuous phase
CC	Coulter counter
d	disperse phase
i	in homogenizer valve inlet
i	size class
p	number of passes through a homogenizer
plat	plateau
ST	spectroturbidimetric measurement
v	in homogenizer valve

Summary / samenvatting

Summary

The aim of the study described in this thesis was to establish the relation between the molecular properties of proteins and peptides and the formation and stability of emulsions. A better understanding of this relation may result in a more effective and wide-spread use of proteins and peptides in emulsions. To achieve this, the emulsion forming and stabilizing properties of proteins and peptides with well-defined and varying molecular properties were studied at various physicochemical conditions. Selected were a random coil protein, β-casein, and four globular proteins with varying conformational stabilities and number of reactive thiol-groups: β-lactoglobulin, α-lactalbumin, lysozyme, and ovalbumin. The studied peptides were hydrolyzate fractions of β-casein with either amphiphilic or hydrophobic properties.

To establish the emulsion forming properties of these proteins and peptides, the droplet size, rate of recoalescence during homogenization, and surface excess were determined as a function of protein concentration, homogenization intensity and some other variables. Most emulsions were prepared using a small, laboratory scale high-pressure homogenizer. To elucidate the effect of the experimental scale on the emulsion properties, the mechanism of the formation of emulsions in a laboratory and industrial high-pressure homogenizer was studied. The flow in the valve of the small industrial homogenizer was shown to be turbulent. The flow in the valve of the laboratory homogenizer appeared to be a bounded laminar flow, due to the small dimensions of the homogenizer valve.

The operating efficiency of the laboratory homogenizer was poor compared to that of the industrial homogenizer. The droplet size and the width of the size distribution were relatively large for emulsions prepared with one or two passes through the small homogenizer. If the number of passes was high, the differences in the droplet size distribution and the recoalescence rate of emulsions made with either homogenizer were found to be small. Comparison of the emulsion forming properties of sodium caseinate and whey protein showed that the relative differences in these properties were similar for emulsion prepared in either homogenizer. Hence, the scale of the homogenizer appeared to have no significant effect on the emulsion forming properties of proteins of peptides under the experimental conditions of this study, allowing scale-up of the results obtained with laboratory experiments to larger scale, industrial processes.

The emulsion forming properties of proteins were generally good as long as protein aggregation was absent, as was observed for emulsions made with β-casein, β-lactoglobulin and α-lactalbumin at pH 6.7 and an ionic strength of 0.075 M. Under these circumstances, only small differences in the droplet size of

emulsions could be determined and similar recoalescence rates during homogenization were found. The surface excess of the emulsions appeared to be governed by the conformational stability of the proteins. A globular protein like β-lactoglobulin yielded a relatively low surface excess, while the highly flexible structure of β-casein allowed the formation of a thick protein layer at the droplet interface.

When the emulsions were aggregated, generally relatively large droplet sizes and recoalescence rates were found. The droplet size and recoalescence rate appeared to be mainly governed by the aggregated state or effective molar mass of the proteins. The surface excess of aggregated emulsions was often found to be high, presumably due to adsorption of protein aggregates or multilayers. The tendency for protein aggregation appeared to be determined by such molecular properties as electrostatic charge, hydrophobicity, conformational stability, and the presence of thiol-groups. Protein aggregation may be induced by some physicochemical conditions, and by heat and surface denaturation. The effect of pH and ionic strength on the emulsion forming properties varied amongst proteins. However, generally, proteins appeared to have inferior emulsion forming properties near their isoelectric pH.

The emulsion forming properties of the β-casein peptides studied were found to be comparable or superior to those of intact proteins if their solubility was high. The droplet size of emulsions made with amphiphilic β-casein peptides (f. 1/29-105/107) was smaller than those of emulsions made with intact proteins. The surface excess of the emulsion droplets made with peptides was found to be relatively low, which appeared to be due to their high electrostatic charge preventing a dense packing of the peptides at the interface. Shielding of these electrostatic charges by increasing the ionic strength resulted in an increased surface excess and in aggregation of the emulsion droplets.

The emulsion forming properties of the hydrophobic β-casein peptides (f. 106/108-209) were studied at pH 9.0, where the peptides were well soluble. At physicochemical conditions where the peptide solubility was poor, the emulsion forming properties were found to be poor. At pH 9.0, the properties of emulsions made with hydrophobic peptides were comparable to those of emulsions made with intact β-casein. The similar surface excesses of these emulsions appear to indicate that the C-terminal domain of β-casein is the main factor determining the surface excess of the droplets. The hydrophobic, as well as the amphiphilic peptides, were more readily desorbed from the oil/water interface than intact proteins, probably due to their relatively low molar mass.

Proteins were shown to have good emulsion stabilizing properties with respect to coalescence as the surface excess was sufficiently high. Emulsions

made with β-casein peptides were, however, less stable against coalescence, presumably due to their relatively low molar mass. The coalescence stability of emulsions made with amphiphilic peptides appeared to be mainly governed by electrostatic and steric interactions. It was shown that the N-terminal domain of these peptides was of great importance for the coalescence stability. The coalescence stability of emulsions made with the hydrophobic β-casein peptides was relatively high, most likely due to the relatively high surface excess of the droplets.

Generally, it appeared that an important molecular property governing the emulsion forming and stabilizing properties of proteins and peptides was their effective molar mass. The emulsion formation was generally relatively poor if proteins and peptides had a relatively large effective molar mass and the coalescence stability of emulsions made with peptides was relatively low due to their low molar mass. The effective molar mass may in some cases be predicted from the molecular properties of proteins and peptides. However in many cases, the relation between the molecular properties and the aggregated state of proteins and peptides is obscure.

The differences in the properties of non-aggregating emulsions were most likely due to differences in the surface dilatational properties of the oil/water interface during droplet deformation. A thorough understanding of the formation of emulsions is, however, hampered due to the lack of data on the properties of proteins and peptides at the extremely short time-scales of the emulsification processes.

Generally it may be concluded that some of the structure-function relations governing the emulsion forming and stabilizing properties of proteins and peptides have been elucidated in this study. However, if a thorough understanding of their functionality is required, the molecular properties of proteins and peptides should be known under all relevant circumstances, including those prevalent during homogenization.

Vorming en stabiliteit van emulsies gemaakt met eiwitten en peptiden

Samenvatting

Het doel van het onderzoek dat in dit proefschrift wordt beschreven, is het verkrijgen van inzicht in de relatie tussen de moleculaire eigenschappen van eiwitten en peptiden en de vorming en stabilisering van emulsies. Een beter begrip van deze relatie leidt mogelijk tot een breder en effectiever gebruik van eiwitten en peptiden in emulsies. Om meer inzicht te verkrijgen in deze structuur-functie relatie, is de vorming en stabiliteit van emulsies gemaakt met eiwitten en peptiden met goed gedefinieerde en uiteenlopende moleculaire eigenschappen onderzocht bij diverse fysisch-chemische omstandigheden. Geselecteerd waren β-caseïne, een flexibel eiwit met nauwelijks enige secondaire structuur en een viertal globulaire eiwitten met uiteenlopende conformatiestabiliteit en verschillende aantallen reactieve thiolgroepen, te weten β-lactoglobuline, α-lactalbumine, lysozym en ovalbumine. De onderzochte peptiden waren hydrolysaten van β-caseïne met sterk amfifiele of hydrofobe eigenschappen.

De emulsievormende eigenschappen van deze eiwitten en peptiden werden onderzocht door de druppelgrootte, de recoalescentiesnelheid tijdens het homogeniseren en de eiwitbelading van de emulsies als functie van de eiwit concentratie, homogenisatie intensiteit en enkele andere variabelen te bepalen. De meeste emulsies zijn gemaakt met een kleinschalige, laboratorium hoge-druk homogenisator. Om inzicht te verkrijgen in de invloed van de experimentele schaalgrootte op de emulsie-eigenschappen, is het mechanisme van de vorming van emulsies met een laboratorium- en een kleine industriële homogenisator onderzocht. In de industriële homogenisator bleek de stroming in de homogenisator klep turbulent te zijn. De stroming in de klep van de laboratorium-homogenisator bleek door de geringe afmetingen, een begrensde laminaire stroming te zijn.

De laboratorium-homogenisator was aanzienlijk minder effectief in het vormen van emulsies dan de industriële homogenisator. De druppelgrootte en de breedte van de druppelgrootteverdeling van emulsies gemaakt met één of twee passages door de laboratorium-homogenisator waren groot in vergelijking tot die van emulsies gemaakt met een gelijk aantal passages door de industriële homogenisator. De verschillen in de emulsie-eigenschappen waren echter klein wanneer het aantal passages door de laboratorium-homogenisator groot was. Vergelijking van de emulsievormende eigenschappen van natriumcaseïnaat en wei-eiwit laat zien dat de relatieve verschillen in de emulsie-eigenschappen onafhankelijk lijken te zijn van de grootte van de homogenisator. De

homogenisator-grootte heeft dus blijkbaar geen significant effect op de emulsievormende eigenschappen van eiwitten en peptiden onder de hier onderzochte experimentele omstandigheden, wat het opschalen van laboratorium-experimenten naar grootschalige, industriële processen mogelijk maakt.

De emulsievormende eigenschappen van eiwitten waren in het algemeen goed zo lang de eiwitten niet waren geaggregeerd, wat het geval was voor emulsies gemaakt met β-caseïne, β-lactoglobuline of α-lactalbumine bij pH 6,7 en een ionsterkte van 0,075 M. Onder deze omstandigheden werden slechts kleine verschillen in druppelgrootte gevonden en bleek de recoalescentiesnelheid tijdens het homogeniseren nagenoeg gelijk te zijn. De eiwitbelading van deze niet-geaggregeerde emulsies leek vooral te worden bepaald door de conformatiestabiliteit van de eiwitten. Een globulair eiwit als β-lactoglobuline bleek een relatief lage eiwitbelading te geven, terwijl een flexibel eiwit als β-caseïne een dikke geadsorbeerde laag op het grensvlak vormt.

Voor geaggregeerde emulsies werden in het algemeen relatief grote druppels en hoge recoalescentiesnelheden gevonden. De druppelgrootte en de recoalescentiesnelheid bleken vooral te worden bepaald door de mate van aggregatie of wel door de effectieve molaire massa van de eiwitten. De eiwitbelading van geaggregeerde emulsies was in de meeste gevallen hoog, waarschijnlijk door de adsorptie van eiwitaggregaten of -multilagen. De neiging tot eiwitaggregatie werd vooral bepaald door moleculaire eigenschappen als elektrostatische lading, hydrofobiciteit, conformatiestabiliteit en de aanwezigheid van reactieve thiolgroepen. Mogelijke oorzaken van eiwitaggregatie waren de fysisch-chemische omstandigheden en hitte- en oppervlaktedenaturatie. Het effect van de pH en ionsterkte op de emulsievormende eigenschappen varieerde per eiwit. De emulsievormende eigenschappen van eiwitten waren in het algemeen relatief slecht nabij de isoelektrische pH van de eiwitten.

De emulsievormende eigenschappen van de onderzochte β-caseïne-peptiden waren in het algemeen vergelijkbaar met of superieur aan die van intacte eiwitten zolang de oplosbaar van de peptiden goed was. De druppelgrootte van emulsies gemaakt met amfifiele β-caseïne-peptiden (f. 1/29-105/107) was kleiner dan die van emulsies gemaakt met intacte eiwitten. De belading van deze emulsies was relatief laag, waarschijnlijk door de hoge elektrostatische lading van de peptiden die een dichte pakking op het grensvlak verhinderen. Het afschermen van de elektrostatische lading door het verhogen van de ionsterkte leidde tot een toename in de grensvlakbelading en tot aggregatie van de emulsiedruppels.

De emulsievormende eigenschappen van de hydrofobe peptiden van β-caseïne (f. 106/108-209) zijn onderzocht bij pH 9,0, waarbij deze peptiden volledig oplosbaar zijn. De emulsievormende eigenschappen van deze peptiden

bleken matig te zijn onder fysisch-chemische omstandigheden waarbij de oplosbaarheid gering was. De eigenschappen van de emulsies gemaakt met hydrofobe peptiden waren bij pH 9,0 vergelijkbaar met die van emulsies gemaakt met intacte β-caseïne. Het C-terminale domein van β-caseïne leek sterk bepalend te zijn voor de eiwitbelading, gezien de grote overeenkomsten in de belading van emulsies gemaakt met hydrofobe peptiden of met intacte β-caseïne. Zowel de hydrofobe als de amififiele peptiden bleken gemakkelijker te desorberen van het olie/water grensvlak dan intacte eiwitten. Dit werd waarschijnlijk veroorzaakt door de relatief lage molaire massa van de peptiden.

De coalescentiestabiliteit van emulsies gemaakt met eiwitten blijkt uitstekend te zijn zo lang de eiwitbelading voldoende hoog is. De emulsies gemaakt met β-caseïne-peptiden waren echter duidelijk minder stabiel tegen coalescentie, waarschijnlijk door de geringe molaire massa van deze peptiden. De coalescentiestabiliteit van emulsies gemaakt met amfifiele peptiden bleek voornamelijk te worden bepaald door elektrostatische en sterische interacties. Het N-terminale domein van deze peptiden leek een belangrijke bijdrage te leveren aan de stabiliteit van de emulsies tegen coalescentie. De emulsies gemaakt met de hydrofobe β-caseïne-peptiden hadden een relatief hoge coalescentiestabiliteit, die waarschijnlijk was veroorzaakt door de relatief hoge belading van de druppels.

In het algemeen, leken de emulsievormende en -stabiliserende eigenschappen van eiwitten en peptiden vooral te worden bepaald door de effectieve molaire massa. De emulsievorming was in het algemeen matig als de effectieve molaire massa van de eiwitten en peptiden relatief groot was. De coalescentiestabiliteit van emulsies gemaakt met peptiden was relatief slecht, waarschijnlijk door hun lage molaire massa. De effectieve molaire massa kan in sommige gevallen worden voorspeld aan de hand van de moleculaire eigenschappen van eiwitten en peptiden. In de meeste gevallen is echter de relatie tussen de moleculaire eigenschappen en de mate van aggregatie van eiwitten en peptiden onduidelijk.

De verschillen in de eigenschappen van niet-geaggregeerde emulsies was waarschijnlijk vooral veroorzaakt door verschillen in de reologische dilatatie-eigenschappen van het olie/water grensvlak tijdens het vervormen van de druppels. Een fundamenteel begrip van de vorming van emulsies wordt echter gehinderd door een gebrek aan gegevens die gelden bij de zeer korte tijdschalen van de processen die optreden tijdens het emulgeren.

In het algemeen kan worden geconcludeerd dat in dit onderzoek enkele van de structuur-functie relaties die bepalend zijn voor de vorming en stabilisering van emulsies met eiwitten en peptiden zijn opgehelderd. Echter, indien een diepgaand begrip van de structuur-functie relatie gewenst is, dan dienen de moleculaire

eigenschappen van eiwitten en peptiden bekend te zijn onder alle relevante omstandigheden, en met name onder de omstandigheden die optreden tijdens het homogeniseren.

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Ine

Curriculum vitae

Ine Smulders werd op 1 maart 1969 geboren te Tilburg. In 1987 behaalde zij het VWO-diploma aan het St. Paulus Lyceum te Tilburg. In hetzelfde jaar begon zij met de studie Levensmiddelentechnologie aan de toenmalige Landbouwuniversiteit (thans Wageningen Universiteit) te Wageningen. Een onderdeel van deze studie waren een stageperiode aan de Canterbury University te Christchurch, Nieuw Zeeland en afstudeervakken Proceskunde en Levensmiddelennatuurkunde. Het doctoraalexamen werd behaald op 23 augustus 1993.

Van januari 1994 tot januari 1998 werkt ze als assistent in opleiding (AIO) bij de toenmalige sectie Zuivel en Levensmiddelennatuurkunde, vakgroep Levensmiddelentechnologie van de Landbouwuniversiteit te Wageningen. Het in deze periode uitgevoerde onderzoek staat beschreven in dit proefschrift.

Vanaf april 1998 is zij werkzaam als productontwikkelaar bij Creamy Creation, onderdeel van DMV, Campina Melkunie te Rijkevoort.