INFLUENCE OF CALCIUM CHLORIDE ADDITION
ON THE PROPERTIES OF EMULSIONS FORMED
WITH MILK PROTEIN PRODUCTS

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TO MY PARENTS

ABSTRACT

The objective of this study was to investigate the effects of added $CaCl_2$ on (i) the adsorption behaviour of caseinate and whey protein concentrate (WPC) at the oil-water interfaces and (ii) the stability of emulsions formed with caseinate or WPC. The relationship between aggregation state of protein, due to Ca^{2+} binding, and emulsifying properties is discussed. The effects of addition of NaCl to the emulsions containing various concentrations of $CaCl_2$ were also explored.

Protein solutions and 30% soya oil, at pH 7.0, were mixed and homogenized at 207/34 bar and 55°C to form emulsions. $CaCl_2$ was added to protein solutions prior to emulsion formation or to the emulsions after they were made. The average particle size (d_{32} or d_{43}), the surface protein concentration, the composition of protein adsorbed layer at the interface and the creaming stability of emulsions were determined. The microstructure of emulsions was observed using the confocal laser microscopy.

The droplet sizes of emulsions made with sodium caseinate or WPC were similar and were independent of the protein concentration at concentration above 0.5%. The surface protein concentration of emulsions made with sodium caseinate, WPC or calcium caseinate generally increased with increase in the protein concentration, although the trends were different. The emulsions made with calcium caseinate had higher d_{32} and surface protein concentration than that of sodium caseinate or WPC.

In emulsions made with sodium caseinate at low protein concentrations, the adsorption of β-casein occurred in preference to α_s-casein, whereas α_s- (α_{s1}- + α_{s2}-)casein was found to adsorb in preference to β-casein at high protein concentrations. In calcium caseinate emulsions, the α_s-casein was adsorbed in preference to β-casein at all concentrations. In emulsions made with WPC, β-lactoglobulin adsorbed slightly in preference to α-lactalbumin. In emulsions made with mixtures of sodium caseinate and WPC (1:1), the adsorption of whey

proteins occurred in preference to caseins at low concentrations (< 3%), whereas caseins were adsorbed in preference to whey protein at high concentrations.

In emulsions made with calcium caseinate or WPC, the creaming stability of emulsions followed mainly the changes in particle size of emulsions. However, the creaming stability of emulsions made with sodium caseinate decreased markedly as the caseinate concentrations were increased above 2.0%. This was attributed to depletion flocculation occurring in these emulsions. Whey proteins did not retard this instability, due to depletion flocculation, in emulsions made with mixtures of caseinate and WPC

When $CaCl_2$ was added prior to or after emulsion formation, the d_{43} and surface protein concentration increased with increasing $CaCl_2$ concentration in emulsions made with 0.5 and 3.0% sodium caseinate. The adsorption of α_s-casein increased with increase in the concentration of $CaCl_2$, with a corresponding decrease in the adsorption of β-casein. The creaming stability of emulsions made with 0.5% caseinate decreased with increasing $CaCl_2$ concentration. However, the creaming stability increased with $CaCl_2$ concentration in 3.0% caseinate emulsion. The destabilising effects of $CaCl_2$ in emulsions made with sodium caseinate were reduced by the addition of 200 mM NaCl.

Addition of $CaCl_2$ to protein solutions prior to emulsion formation increased the d_{43} and surface protein concentration in emulsions made with 0.5 or 3.0% WPC. In this case, the adsorption of β-lactoglobulin occurred slightly in preference to α-lactalbumin. The creaming stability of emulsions decreased with increase in the concentration of $CaCl_2$. The addition of $CaCl_2$ to emulsions after emulsion formation also resulted in increases in d_{43} and surface protein concentration of emulsions made with 0.5% WPC and formation of gel-like network structure at high $CaCl_2$ concentrations. However, the stability of emulsion made with 3.0% WPC was not affected by the addition of $CaCl_2$.

Different aggregation mechanisms are involved depending upon whether Ca^{2+} is added to protein solution before emulsification or to the emulsion after its formation. Addition of Ca^{2+} to protein solution may lead to decrease in emulsifying capacity and subsequently result in protein bridging flocculation between emulsion droplets. Ca^{2+} bridging flocculation between emulsion droplets may be formed in emulsions that have Ca^{2+} added. The change in aggregation state of caseinate due to Ca^{2+} binding could retard the instability of emulsion due to depletion flocculation. The protein unfolding at the surface of emulsions made with low whey protein concentrations may promote the protein-Ca-protein bridges forming between protein-coated emulsion droplets, consequently forming gel-like network structure in emulsions.

ACKNOWLEDGMENT

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Chapter 1
INTRODUCTION

Milk proteins are well known surfactants and hence are used as ingredients in a wide range of formulated food systems (Mulvihill, 1992). They stabilize emulsions and foams because they are amphiphiles. They adsorb onto the oil-water interface and stabilize emulsions by a combination of charge repulsion and steric stabilization (Dickinson and Stainsby, 1982). The stability and rheological properties of emulsions stabilized by milk proteins are largely determined by the interactions between the droplets. The nature and strength of the interactions between droplets are dependent on the structure and composition of adsorbed layer at the oil-water interface (Dickinson, 1998).

In an aqueous environment, the proteins are held in their conformations by a number of different interactions, such as hydrophobic interactions, hydrogen bonding, ion-pair interactions (attractive and repulsive), coordination of metal ions, and van der Waals interactions (Dalgleish and Hunt, 1995). The major proteins in milk, the caseins (α_{s1}-, α_{s2}-, β- and κ-), are generally considered to have disordered and flexible structures. Sodium caseinate, a widely used food ingredient produced from milk casein, exists in aqueous solution at neutral pH as a mixture of casein monomers (Mulvihill, 1992) and small casein aggregates (so-called 'sub-micelles') (Pepper and Farrell, 1982). In contrast to the caseins, there are the whey proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins), which are characterized by well defined three-dimensional structures held together by disulfide bridges; these proteins are much more rigid than the caseins (Kinsella, 1984).

The milk proteins whether individual molecules or in the form of aggregates becomes rapidly adsorbed at the new oil/water interface during emulsification (Walstra and Smulders, 1997). However, the state of protein in bulk solution does influence its adsorption behaviour and composition of protein interfacial

layer, which subsequently influences the stability of emulsions (Dalgleish, 1995, Srinivasan *et al.*, 1996).

Milk proteins exist in bulk solution as aggregates in many situations, for example 'casein micelles', aggregated caseins in sodium caseinate after heating, in the presence of Ca^{2+}, high ionic strength and pH close to pI (Dalgleish and Hunt, 1995). However, they have received comparatively little research attention relative to other model emulsions. There are still many gaps in our understanding of the adsorption behaviour of aggregated proteins at the oil/water interface and their influence on stability of emulsions.

The binding of calcium ions to milk proteins have been studied under a variety of environmental conditions (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; Pappas and Rothwell, 1991). The binding of Ca^{2+} to protein reduces the electrostatic repulsion and interaction of the hydrophobic domains leads to formation of large aggregates (Swaisgood, 1992). This property will inevitably influence the adsorption behaviour of milk protein at the oil/water interface and the stability of emulsions formed by milk proteins.

Little information is available on the influence of Ca^{2+} on the adsorption behaviour of milk proteins at the oil/water interface and stability of milk protein–stabilized emulsions. No previous work has been reported on the effects of Ca^{2+} on the composition of protein interfacial layer and creaming stability of emulsions stabilized by milk proteins. Srinivasan *et al.* (1996) reported that addition of Ca^{2+} to sodium caseinate solution prior to emulsion formation caused the increases in the droplet size and the surface protein concentration of emulsions. Mulvihill and Murphy (1991) reported that highly-aggregated calcium caseinate had higher protein load at the interface of emulsion droplets. When Ca^{2+} is added to emulsions, the association of Ca^{2+} with adsorbed protein between the protein-coated oil droplets also results in larger particle size due to the aggregation of emulsion droplets (Dickinson *et al.*, 1992; Agboola and Dalgleish, 1995).

The main objective of this study was to investigate the effects of addition of $CaCl_2$ to the protein solution prior to making emulsions or to emulsions made in the absence of Ca^{2+} on the adsorption behaviour of proteins in oil-water emulsions. The effects on the stability of emulsions made with sodium caseinate (Chapter 5) or WPC (Chapter 6) were also studied. The relationship between aggregation state of protein, due to Ca^{2+} binding, and emulsifying properties is discussed. In addition, It is known that ionic strength influences the binding of Ca^{2+} to milk proteins (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; Baumy and Brule, 1988). Therefore, the effects of addition of NaCl to the emulsions containing various concentrations of Ca^{2+} were also explored. The emulsifying properties of sodium caseinate, WPC and calcium caseinate as well as of the binary protein mixtures are discussed in Chapter 4. The confocal microscopy was used to observe the microstructure of emulsions for examining the relationship between structure and creaming stability of emulsions.

Chapter 2
LITERATURE REVIEW

2.1 General characteristics of milk proteins

Normal bovine milk contains 30-35 g protein/L. Milk proteins are classified as either caseins or whey proteins. Table 2.1 shows some of the structural and chemical characteristics of these major milk proteins.

Table 2.1. Some structural and chemical characteristics of milk proteins

	Whey proteins			Caseins			
	β-lg	α-la	BSA	α_{s1}-	α_{s2}-	β-	κ-
Molecular weight (D)	18362	14194	65000	23612	25228	23980	19005
Total amino acids	162	123	581	199	207	209	169
Apolar amino acids, %	34.6	36	28	36	40	33	33
Isoionic point	5.2	4.3	5.3	4.96	5.27	5.2	5.54
Proline residues (mole/mole)	8	2	28	17	10	35	20
Lysine residues (mole/mole)	15	12	59	14	24	11	9
Phosphoryl groups (mole/mole)	0	0	0	8-9	10-13	5	1
Disulphide bonds (mole/mole)	2	4	17	0	1	0	1
Thiol groups (mole/mole)	1	0	1	0	0	0	0
Secondary structure (%)							
α-Helix	15	26	54	-	-	9	23
β-Sheet	50	14	18	-	-	25	31
β-Turns	18	-	20	-	-	-	24
Unordered	-	60	-	-	-	66	-
Native conformation	**Globular**			**Extended**			

Abbreviations used: β-lg = β-lactoglobulin; α-la = α-lactalbumin; BSA = bovine serum albumin; α_{s1}-casein; α_{s2}-casein; β-casein; κ-casein. (from Kinsella, 1984)

2.2 Physicochemical characteristics of milk proteins

2.2.1 Physicochemical characteristics of caseins

Casein comprises the largest fraction of bovine milk proteins (76-80% total protein). Bovine casein consists of four distinct proteins: α_{s1}-, α_{s2}-, β- and κ-caseins (12-15 g/L, 3-4 g/L, 9-11 g/L and 3-4 g/L in milk respectively).

α_{s1}-Casein possesses eight sites of post-translational phosphorylation; consequently, this protein exhibits interactions with calcium typical of the caseins. Another important characteristic is the clustering of polar and non-polar residues. These characteristics suggest a unique dipolar structure composed of a highly solvated, charged domain and a hydrophobic globular domain. Most likely the polar domain possesses a mixture of α-helix, β-structure, β-turns, and unordered structure. The flexible nature of the polar domain causes the molecular dimensions to be very sensitive to ionic strength and to binding of ions, particularly protons (H^+) and Ca^{2+}. In addition, intermolecular interactions between hydrophobic domains leads to self-association, or association with other caseins, these hydrophobic interactions become more important as the polar domain reduces the charges due to binding of Ca^{2+} to the orthophosphate groups, since this binding greatly reduces the dimensions of the polar domain. The intermolecular interactions then result in precipitation of isolated α_{s1}-casein or formation of micelles by interaction with κ-casein (Swaisgood, 1982, 1992).

The general characteristics of α_{s1}-casein are shared by the other calcium-sensitive caseins i.e. α_{s2}-casein and β-casein. Structures of both α_{s2}- and β-caseins are characterized by charged polar domains and hydrophobic domains. Like α_{s1}-casein, sequences in the polar domains, which may approach random coil secondary and tertiary structure, are such that clusters of seryl residues are phosphorylated. α_{s2}-Casein contains several phosphoseryl clusters and thus is the most hydrophilic, whereas β-casein contains only a single phosphoseryl cluster in the N-terminal sequence and the remaining large C-terminal sequence is very hydrophobic and flexible (Swaisgood, 1982, 1992).

The structure of κ-casein is clearly amphipathic, but without the anionic phosphate cluster in its polar domain. κ-Casein remains soluble in the presence of calcium at all temperatures. A physiological role played by this protein is the stabilization of calcium-sensitive caseins in the presence of calcium salts in milk. Also, the hydrophobic domain of κ-casein as compared to β-casein is less hydrophobic, has a lower frequency of prolyl residues and probably contains more secondary structure (Swaisgood, 1982, 1992).

2.2.1.1 Self-association of caseins

Caseins have strong tendency to association due to their high hydrophobicity and peculiar charge distribution. The major interactions involved in these processes are of electrostatic and hydrophobic nature. A delicate balance between these forces, depending on experimental conditions such as pH, temperature and ionic strength, determines the type and extent of association (Rollema, 1992). The associative properties of α_{s1}-casein and α_{s2}-casein are very dependent on the ionic strength, due to the anionic clusters in the polar domain. The molecular structure of β-casein is dominated by hydrophobic interactions of its surface and less sensitive to ionic strength than α_{s1}-casein and α_{s2}-casein. In the case of κ-casein, polymers of a fixed size are formed, irrespective of temperature and ionic strength (Swaisgood, 1992).

2.2.1.2 Interactions of caseins with calcium

Interaction of caseins with calcium is a very important characteristic of caseins. Many studies have shown that calcium binds primarily to the phosphoseryl residues (Swaisgood, 1992). This result is expected on the basis of their calcium sensitivities in relationship to composition and primary structures of the four caseins. The order of calcium concentration that initiates precipitation is: α_{s2}-casein < α_{s1}-casein < β-casein. κ-Casein is not precipitated by Ca^{2+}. Nevertheless, there is substantial evidence that calcium also binds to other sites, carboxylate residues in particular, besides the phosphoseryl residues. Calcium binding to caseins is affected by pH, temperature and ionic strength in a manner consistent with the effects on the Ca^{2+} concentration required to

initiate precipitation. Thus, equilibrium binding constants increase with increasing pH and temperature, but decrease with increasing ionic strength (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; Swaisgood, 1992). Binding of Ca^{2+} to high affinity phosphoseryl clusters in the polar domain alters its interaction with the hydrophobic domain bringing about a conformational change in that domain which allows some association to occur. Further binding to carboxyl residues throughout the structure reduces the electrostatic repulsion and interaction of the hydrophobic domains leads to formation of large aggregates (Swaisgood, 1992).

2.2.2 Physicochemical characteristics of whey proteins

The structure and chemical characteristics of the three main whey proteins are shown in Table 2.1. The primary structure of whey protein is typical of compact globular protein, with a rather uniform sequence distribution of non-polar, polar, and charged residues. Hence, these proteins fold intramolecularly, burying most of their hydrophobic residues so that extensive self-association or interaction with other proteins does not occur (Swaisgood, 1982).

α-Lactalbumin normally occurs as a monomer, the hydrodynamic radius of which indicates a nearly spherical, very compact globular protein (Brew and Grobler, 1992). α-Lactalbumin is a calcium-binding metalloprotein, which is also capable of binding zinc and probably other metals. The heat stability of α-lactalbumin is reduced by removal of Ca^{2+} (Fox, 1989).

β-Lactoglobulin, however, does undergo limited self-association; at the pH of milk a dimer is formed with a geometry resembling two impinging spheres. The structure of β-lactoglobulin is dependent on pH; thus, below pH 3.5 the dimer dissociates to a slightly expanded monomer, between pH 3.5 and 5.2 the dimer tetramerizes to give an octamer, and above pH 7.5 the dimer dissociates and undergoes a conformational change giving an expanded monomer (Swaigood, 1982). The functionality of β-lactoglobulin is greatly influenced by the presence of both a sulphydryl group and by conformational changes since this determines

the availability of the sulphydryl group for reactions. Thus, under appropriate conditions, β-lactoglobulin readily participates in sulphydryl-disulphide interchange reactions which affects many of its characteristics, such as solubility (Hambling *et al.,* 1992).

BSA contains numerous disulphides which impose conformational restrictions, but since the molecule contains no long-distance disulphide bond, it is relatively flexible. BSA binds several ligands at different sites. Binding of hydrophobic molecules, such as fatty acids, apparently occurs in hydrophobic pockets that can open and close to admit large insoluble hydrophobic molecules (Fox, 1989).

2.3 Caseinates and whey protein products

Sodium caseinate, usually prepared by solubilizing acid casein with NaOH, is the water-soluble casein most commonly used in foods. Another important caseinate is calcium caseinate that is produced by mixing casein slurry with $Ca(OH)_2$ (Mulvihill, 1992). Commercially available whey protein products contain from 35 to 85% protein that are produced from sweet or acid whey derived from cheese or casein production. The whey protein concentrates (WPCs) containing 35-55% protein are used largely in animal feed manufacture. WPC that contain > 70% protein are used extensively as functional and nutritional ingredients in medical, phamaceutical and human food products, such as infant formula, health food and drinks, high gel product applications and frozen foods (Morr and Ha, 1993). The composition of WPCs, although reflecting the source of the raw whey, can be influenced largely by the processing history (Morr and Ha, 1993).

2.4 Emulsifying properties of milk proteins

2.4.1 Introduction

Proteins lower the interfacial tension between oil and water, thereby facilitating the formation of dispersed droplets in aqueous media. Emulsions are comprised

of oil droplets (average range 0.5 to 5 μm diameter) enveloped by a continuous film of surfactant material that stabilizes the droplets, preventing flocculation and coalescence. Milk proteins impart long-term stability to emulsions by forming an adsorbed film at the interface which protects new droplets from aggregation and subsequent coalescence. The emulsion stability depends on a delicate balance between attractive intramolecular forces between molecules in the film and repulsive forces on the outer film surface that are determined by the nature of the interfacial film (Leman and Kinsella, 1989). A diversity of model systems and varying conditions have been used to determine the emulsifying properties of milk proteins (Kinsella, 1984). For determination of the relative emulsifying properties of proteins, especially milk proteins, and for studying the relations between protein structure and emulsifying properties, a standardized model system for optimum emulsion formation is needed.

A good emulsifying test should assess the relative emulsifying capacity of various proteins and facilitate determination of surface area, size distribution of droplet, and surface concentration of proteins. It should also provide control of all variables, including energy input during formation so that the true interfacial surface activity of the protein can be measured. Emulsion stability, the ability of the formed emulsion to remain unchanged over a period, is the criterion of most practical value (Kinsella, 1984; Halling, 1981). A variety of methods have been used to determine stability, and oil separation and creaming are among the indices of stability applied to model systems (Tornberg and Hermansson, 1977; Tornberg and Lundh, 1978; Leman and Kinsella, 1989).

2.4.2 Emulsion formation

Emulsion manufacture is a highly energetic and dynamic process. The formation of emulsion depends on energy input (Tornberg and Hermansson, 1977). A wide variety of apparatus have been used in making protein-stabilized emulsions. The mixers, valve homogenizers, ultrasonic equipment, and several types of blenders and homogenizers of varying sizes and shapes vary in their ability to form an emulsion. The particle size distribution of oil droplets varies

and frequently factors affecting emulsifying properties are overridden by the characteristics of the equipment used (Tornberg and Hermansson, 1977; Tornberg and Lundh, 1978; Leman *et al.* 1988). Tornberg and Hermansson (1977) and Tornberg and Lundh (1978) compared the results of several emulsifying procedures using different equipment for emulsions stabilized by milk proteins. The valve homogenization was more effective compared to the ultraturrax and omni-mixer in giving emulsions of high creaming stability. Therefore, the high pressure valve homogenizer has been widely used as the method for lab experiments and manufacturing food emulsions (Tornberg, 1978 a, 1978 b; Dickinson *et al.* 1984; Leman *et al.,* 1988; Mulvihill and Murphy, 1991; Euston *et al.* 1995; Srinivasan *et al.* 1996; Dickinson and Golding, 1997). The principle of the valve homogenizer was described by Tornberg and Lundh (1978). Droplets are disrupted by a combination of intense laminar and turbulent flow. The main factor affecting the emulsions droplet size distribution is pressure drop across the homogenizer valve, and increased turbulence on the low pressure side of the valve favours the formation of finer emulsions. The main experimental difficulty in using valve homogenizers is that small volumes cannot be homogenized. A high-pressure mini homogenizer and microfluidization have been developed which can produce emulsions of smaller particle size distribution (Dickinson and Stainsby, 1988).

2.4.3 Droplet size distribution and protein load

There are two important fundamental properties, droplet size distribution and protein load, which must be measured for studying the adsorption behaviour of proteins at interface and characterization of emulsions stabilized by proteins. The droplets in food emulsions are widely dispersed in size, and may range from 0.1 to 10 µm in any one system. The state of droplet size distribution reflects the emulsifying capacity of proteins, energy input during formation as well as the effects of various factors on surface activity of proteins such as pH, temperature, ionic strength and ratio of the two phases. In addition, the particle size distribution influences markedly the properties of food emulsions, such as stability, viscosity, texture and mouthfeel.

The droplet size of emulsions can be determined by several methods. These include microscopy, Coulter counter, spectroturbidimetry, electron microscopy (Liboff *et al.*,1988), electronic imaging system (Klemaszewski *et al.*, 1989) and light scattering. Studies using light microscopes are limited by the lack of magnification that hinders accurate sizing of the smallest emulsion droplets and are time consuming and tedious. Electron microscopy has also been applied to the study of emulsions, but it is difficult to be used as a routine method, because of difficulty in sample preparation, reproducibility and artifacts, although it may be useful for specific studies (Liboff *et al.* 1988; Agboola and Dalgleish, 1995; Sharma *et al.,* 1996). Klemaszewski *et al.* (1989) described an electronic imaging system where microscopic views are transferred electronically to a video monitor with magnification. However, problems of poor contrast and droplet flocculation can cause image distortion and artifacts. Leman and Kinsella (1989) reviewed other methods that determine droplet size of emulsions.

In recent years, most of the researchers have used the Malvern MasterSizer to measure droplet diameter (Hunt and Dalgleish, 1994, 1995; Agboola and Dalgleish, 1995; Euston *et al.,* 1995, 1996; Srinivasan *et al.,* 1996; Dickinson and Golding, 1997). The principle and measurement of droplet diameter using the Malvern MasterSizer are described in Chapter 3.

The proteins adsorbed at the interface form an interfacial film around oil droplets; the amount of protein at interface of droplets is a important factor that influences the properties of protein film (Dickinson and Stainsby, 1982). The amount of protein present at the interface per unit surface of dispersed phase is defined as protein load. It is usually expressed as milligrams of protein per unit area of the dispersed phase (mg/m^2). Protein load may determine the amount of protein required to make an emulsion with desired oil volume and droplet size. The protein load is related to creaming and coalescence stability, low protein load enhances the creaming and coalescence process (Dickinson and Stainsby, 1988).

The protein load of emulsion droplets depends on the concentration and type of protein as well as the condition for emulsion formation. The factors that affect the protein load include protein concentration, volume of oil, energy input, state of protein aggregation, pH, ionic strength, temperature and Ca^{2+} (Dickinson and Stainsby, 1988).

The amount of protein adsorbed on the interface of emulsion droplet suggests the state of protein adsorbed at the interface. If the protein load is <1 mg/m^2, it suggests the protein molecules are fully unfolded or there is adsorption of an extended polypeptide chain. If the protein load is 1 – 3 mg/m^2, there may be adsorption of a monolayer of globular proteins or unfolded molecules that are adsorbed in the conformation of trains, loops and tails. Protein load values over 5 mg/m^2 suggest adsorption of aggregates of proteins or multilayers of proteins. Some proteins of higher molecular weight may also give higher protein loads (Phillips, 1981; Hunt and Dalgleish, 1994; Dam *et al.*, 1995; Srinivasan *et al.*, 1996).

2.4.4 Adsorption behaviour of milk proteins at the oil/water interface

The extent to which a particular protein functions as a good emulsifier depends on its unique molecular properties and food within which it is present. Both in the laboratory and during food manufacture, it is possible to envisage several stages in the formation of a stabilizing protein film: 1) movement of protein molecules from the bulk phase to the interface, 2) adsorption at the interface, 3) rearrangement and unfolding at the interface and establishment of protein-protein interactions in the adsorbed layer (Dickinson and McClements, 1995).

In real processing situations, the dynamic adsorption processes taking place during the homogenization of emulsions, the dispersed system is subjected to large shear forces and turbulent flow. The movement of the protein emulsifier from the aqueous phase to the surface is usually very rapid (of the order of milliseconds); this is important for the formation of emulsions, where newly

formed interface must be covered quickly with a stabilizing layer in order to prevent immediate droplet coalescence (Dickinson *et al.,* 1989).

Proteins are amphipathic molecules containing both polar and non-polar parts (Swaisgood, 1992). By adsorbing at an oil-water interface, a protein can orientate itself so that a substantial proportion of the non-polar amino acids are in contact with the oil phase, and the polar groups are in contact with the aqueous phase (Dickinson, 1992). The major driving force for protein adsorption is the reduction in the contact area between non-polar groups and water, both at the oil-water interface and at the surface of the protein (Dickinson and Stainsby, 1982). By adsorbing at the interface, the protein reduces the free energy of the system and hence the interfacial tension. The effectiveness of any particular protein in lowering the tension depends on the number and type of contacts it makes with the interface (Dickinson *et al.,* 1988). A protein molecule that spreads out a lot, and thus has a substantial proportion of its non-polar residues in contact with the surface, is one which is also very effective in reducing the interfacial tension. The flexible proteins (caseins) with a higher proportion of non-polar groups are more effective at reducing the interfacial tension than rigid proteins with fewer non-polar groups (Dickinson and McClements, 1995).

The order of surface activity reported for the individual milk proteins is: β-casein > monodispersed casein micelles > serum albumin > α - lactalbumin > α_s-caseins = κ-casein > β-lactoglobulin > euglobulins (Mulvihill and Fox, 1989). Dickinson (1989) reported the interfacial tension and interfacial shear viscosity for adsorbed protein films at the n-hexadecane-water interface (shown in Table 2.2). The results revealed that the more hydrophobic β-casein is more interfacial active than α_{s1}-casein and that caseinates have surface properties, intermediate between these two.

Table 2.2. Interfacial tension (γ) and interfacial shear viscosity (η) for adsorbed protein films at the n-hexadecane – water interface (bulk protein concentration 10^{-3} wt %, ionic strength 0.005M, pH 7, 25°C, 30 hours) (from Dickinson, 1989)

Protein	*γ /mN m⁻¹*	*η /mN m⁻¹*
α_{s1}-casein	24.0	5
β-casein	22.4	0.5
κ-casein	23.0	200
Sodium caseinate	22.4	7.5
α - lactalbumin	28.5	300
Gelatin	35.2	120

In addition, the net charge of protein and charge distribution along the polypeptide chain also plays an important role in determining the rate and extent of protein adsorption. A protein molecule approaching an interface, which has protein molecules of the same type (and charge) already adsorbed there, will experience an electrostatic repulsion which will tend to oppose further adsorption. Therefore, the adsorption of protein will be affected as pH or ionic strength change (Tornberg, 1978 b). Little is known about the specific roles of hydrogen bond formation or van der Waals forces on the thermodynamics or kinetics of adsorption. An adsorbed protein will attempt to maximize the number of these interactions so as to reduce its overall free energy, but whether either type of interaction is more effective in the adsorbed state than in the unadsorbed state is not clear (Dickinson and McClements, 1995).

The kinetics of protein adsorption at an interface can be measured by monitoring surface concentration and surface pressure, i.e., depression of surface tension as a function of time. Proteins migrate to and adsorb at an interface to form a film very rapidly. The rate of surface pressure development reflects the diffusion of protein molecules to the interface (Dickinson *et al.,* 1988).

Because the thermodynamic environment of the interface and the bulk aqueous phase is different, the relative contributions of various forces which determine

protein configurational structure are altered. Once a protein adsorbed at an interface, protein molecules spread out, undergo unfolding and rearrangement to form a stabilizing film (Dalgleish, 1996). The rate and extent of unfolding depends on the flexibility of the protein molecule, i.e., on the strength of the forces maintaining the secondary and tertiary structure. A random coil protein such as β-casein has a flexible structure and it unfolds rapidly at the interface. It is reported that the casein adsorption layers at interface are rather extended layers, up to about 12 nm thick (Dalgleish, 1990), and whey proteins such as β-lactoglobulin, which give layers which are only about 2 nm thick. It can give some impression of how folded or unfolded the proteins are in their adsorbed states. Nevertheless, some workers have reported that α-lactalbumin and β-lactoglobulin change conformation and substantially unfold structure to some extent at the surface (Mackie, *et al.,* 1993; Dalgleish and Leaver, 1993; Dalgleish, 1995; Dickinson and McClements, 1995; Dalgleish, 1996; Fang and Dalgleish, 1998). The adsorbed whey protein structure lies somewhere intermediate between the native structure and the fully denatured state which may have a native-like secondary structure and an unfolded tertiary structure (Dickinson, 1998). Additionally, the partial unfolding of the globular protein structure following adsorption cause exposure of the reactive sulfhydryl group, leading to slow polymerization of the adsorbed protein in the aged layer *via* sulfhydryl-disulfide interchange (Dickinson and Matsumura, 1991; McClements *et al.,* 1993).

Further adsorption to an existing film depends on protein ability (mainly hydrophobicity and flexibility) to penetrate the film and compress already adsorbed proteins. Rapid coverage of newly formed droplets is responsible for the formation of finely dispersed emulsions. Experiments have shown that the protein concentration is no longer the limiting factor in determining the emulsion droplet size at concentrations > 1 wt% (Fang and Dalgleish, 1993 a; Hunt and Dalgleish, 1994). They infer that a protein concentration of 1 wt% is sufficient to provide monolayer coverage of the nascent interfacial area during homogenization. The addition of more protein to the system increases the

surface protein concentration only marginally. This slight increase is likely to be a result of closer packing of the adsorbed proteins in the monomolecular layer (Dalgleish *et al.*, 1995; Hunt and Dalgleish, 1994; Srinivasan *et al.*, 1996). Nylander and Wahlgren (1994) suggested that the adsorbed β-casein forms a rather tenuous, extended layer on oil-water interfaces, and this extended layer prevents further sequential adsorption of protein. However, the sharp increase in surface protein concentration upon the addition of more protein seems to suggest the formation of a secondary layer of adsorbed protein around the emulsion droplet. Similar result was reported by Srinivasan *et al.* (1996); they suggested that an increase in caseinate concentration may cause formation of casein aggregates in solution which may be subsequently adsorbed at the interface, resulting in high values of surface protein concentration.

Otherwise, Dickinson and McClements (1995) considered that an individual protein molecule arriving at the interface could unfold unimpeded by the presence of any surrounding molecules at low bulk protein concentration. In a concentrated system, on the other hand, an adsorbing molecule will probably have insufficient time to unfold before other protein molecules adsorb nearby. These neighbouring molecules in the adsorbed layer restrict subsequent unfolding through a combination of steric hindrance and electrostatic repulsion at the oil-water interface, and so the free energy gained by an isolated region is correspondingly reduced.

It is also demonstrable that the thickness of the adsorbed layer depends on the manner in which the emulsion is made, being highly dependent on the type of homogenizer used and on the ratio of protein to oil (Fang and Dalgleish, 1993 a). When the protein concentration is low relative to the amount of oil surface created during homogenization ($\Gamma = 1$ mg/m^2 or less), the casein must necessarily spread over a maximum area: this is reflected in the thickness of the adsorbed layer, which is about 5 nm. Conversely, in the presence of excess casein (where Γ is above 2 mg/m^2), there is sufficient protein to cover the oil surface without spreading, the adsorbed layer is found to be about 10 nm thick.

Such thicker layers can also be produced by adding caseinate to emulsions, which were formed in the presence of low concentrations of casein. Why are there those results? It was considered that it is result of casein molecules conformations change at the interface, depending on whether the molecules are spread or packed, because the caseins are rather flexible molecules (Dalgleish, 1996). Dalgleish (1998) considered that casein molecules appear to be stretched to their maximum extent, and the thickness of the adsorbed layer is less at an overall surface coverage of 1 mg/m^2. Conversely, the presence of excess casein increases the monolayer coverage to a maximum value of 3 mg/m^2, the parts of the molecules in contact with the interface adopt a more compact conformation, and the hydrophilic moieties protrude further from the interface.

The most susceptible bonds in β-casein are close to the N-terminus of the protein, showing that this part of the molecule must protrude into the solution. This is also the portion of the molecule that forms the hydrodynamically thick layer. The hydrophobic groups of the protein are adsorbed at oil interface. However, this reasoning cannot be applied to all proteins, even other caseins (Dalgleish, 1996), since they are likely to be less flexible than β-casein, and do not have such a pronounced inequality in the distribution of hydrophobic and hydrophilic residues in their primary structures. It has been suggested that α_{s1}-casein adsorbs to oil-water interface via peptides towards the middle of its sequence, rather than the end as in β-casein, and it may be this which causes the protein to form thinner absorbed layer than β-casein (shown in Figure 2.1) (Dalgleish, 1996).

2.4.5 Adsorption behaviour of aggregated milk protein particles

Milk proteins exist, in bulk solution, as aggregates in many situations, for example 'casein micelles', aggregated caseins in sodium caseinate after heating, in the presence of Ca^{2+}, high ionic strength and pH close to pl (Dalgleish and Hunt, 1995). Whatever the cause, the micelles, or fragments of them, are found at the oil-water interface, and they form a stabilizing layer

around the oil droplets. The thickness of this layer is less than the average dimensions of a casein micelle, especially if the homogenization has been vigorous, but the layer is equally clearly not simply a monolayer of casein (Dalgleish, 1995; Sharma *et al.,* 1996). Protein loads are much higher than in emulsions prepared using either simple caseinate or whey protein, and as a result the emulsifying power of micellar casein is less than that of an equivalent weight of caseinate (Dam *et al.,* 1995; Dalgleish, 1995; Sharma *et al.,* 1996). Mulvihill and Murphy (1991) found that the protein loads were dependent on the aggregation state of the emulsifying protein. The protein loads were highest for highly aggregated micellar casein-stabilized emulsions, lower for less aggregated ethanol precipitated casein and high calcium caseinate-stabilized emulsions, and lowest for the least aggregated low calcium, sodium and ammonium caseinate-stabilized emulsions (Mulvihill and Murphy, 1991). Singh *et al.* (1993) reported that both the κ-casein/whey protein-rich and κ-casein-depleted fractions separated from heated milk have higher protein load and lower emulsification capacities than those of the sodium caseinate or whey protein isolate. It would be suggested that the size of the protein aggregates in solution was more important for determining the emulsifying properties than the protein composition of the aggregates (Singh *et al.,* 1993).
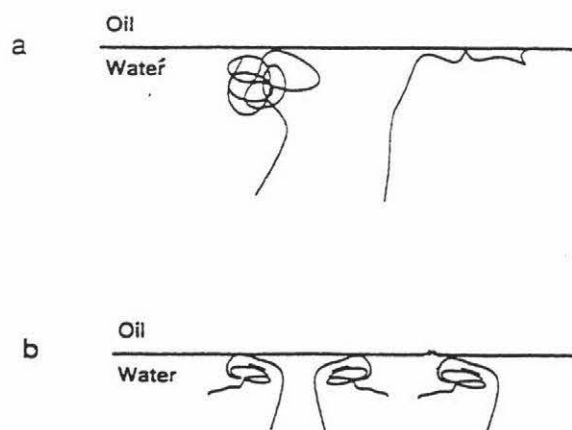
Figure 2.1 Indications of conformations at the interface for β- (a) and α_{s1}-caseins (b).For β-casein, the two suggestions are for conformations of the protein where most of the mass is near to the interface, but the N-terminal of the protein protrudes into solution. For the α_{s1}-caseins, the point of attachment is near the middle of the molecule and the tails of the protein protrude less. (from Dalgleish, 1996).

2.5 Interfacial composition and competitive adsorption

Whatever is the method used to make an emulsion, there is likely to be competition between the various surface-active components for adsorption at the newly created interface. There is competition, firstly, between the various proteins, which may differ in composition, in molecular size, or in state of aggregation, secondly, between these proteins and the various surfactants. Over the last few years, there have been important advances in the area of competitive adsorption (Robson and Dalgleish, 1987; Dickinson *et al.*, 1988 a; Dickinson, 1989; Dickinson *et al.*, 1991; Courthaudon *et al.*, 1991; Dickinson and Tanai, 1992; Euston *et al.*, 1995, 1996; Hunt and Dalgleish, 1994,1996; Fang and Dalgleish, 1996; Cornec *et al.*, 1998).

2.5.1 Competitive adsorption between milk proteins

A number of researchers reported that β-casein is adsorbed in preference to α_{s1}-casein and other proteins in emulsions stabilized by a mixture of milk proteins because it is most surface-active and hydrophobic (Dickinson *et al.*, 1988 a; Dickinson, 1989; Dickinson *et al.*, 1991; Euston *et al.*,1995). Dickinson *et al.* (1988 a) reported that β-casein displaced α_{s1}-casein rapidly from the emulsion droplet surface; α_{s1}-casein also displaced β-casein, but to a much lesser extent. None of the α_{s1}-casein at the droplet surface is irreversibly adsorbed; all of it can be displaced by β-casein. But Robson and Dalgleish (1987) considered that some of the α_{s1}-casein was irreversibly adsorbed. They demonstrated no preferential adsorption of β-casein in sodium caseinate-stabilized emulsions immediately after homogenization, but on aging, β-casein displaced some of the adsorbed α_{s1}-casein. Hunt and Dalgleish (1994) found no preference for β-casein or α_{s1}-casein in sodium caseinate-stabilized emulsions. This result can be explained in that the caseins which make up sodium caseinate are aggregated to some extent, which may affect their adsorption behaviour at interfaces.

Euston *et al.* (1995, 1996) and Srinivasan *et al.* (1996) reported that the preferential adsorption of β-casein in sodium caseinate was dependent on the concentration of protein used in making emulsions. At low protein concentration (< 2.0%), β-casein was adsorbed in preference to α_s-casein, whereas the larger amount of α_s-casein than β-casein was present at the interface at high protein concentrations. This change in adsorption behaviour of individual caseins may be related to the formation of casein aggregates in sodium caseinate solution or formation of β-casein micelles at higher protein concentrations which affected its adsorption behaviour (Euston *et al.*, 1995; Srinivasan *et al.*, 1996). Another possibility is that α_s-casein and β-casein are adsorbed at the surface as a complex containing almost equal proportions of α_s-casein and β-casein at high concentrations of protein (2.0 %) (Euston *et al.*, 1996).

It was found that preferential adsorption of caseinate over whey protein occurred in caseinate and whey protein mixed emulsions (Hunt and Dalgleish, 1994). The greater total protein surface concentration, observed in mixed emulsion made with 2.5 wt% protein, was a result of more adsorption of caseinate, not whey protein. It was considered that in the presence of even small amounts of adsorbed caseinate, whey protein can not form a secondary adsorbed layer. This may be due to steric hindrance afforded by the loops and tails from adsorbed caseinate, which are known to extend well into the aqueous phase.

Closs *et al.* (1993) found that β-lactoglobulin was adsorbed in preference to α-lactalbumin at the surface of oil droplets, in emulsions made with whey proteins, or with mixtures of β-lactoglobulin and α-lactalbumin. But, no preferential adsorption between β-lactoglobulin and α-lactalbumin was observed by other works (Dickinson *et al.*, 1989; Hunt and Dalgleish, 1994; Euston *et al.*, 1996). In addition, less β-lactoglobulin was displaced from surface of emulsion droplet than α-lactalbumin by β-casein (Dickinson *et al.*, 1989). Once the adsorbed β-lactoglobulin molecules have become partially unfolded and closely packed together at the interface, the slow linking together of subunits by covalent −S-S-

bonds will lead to the formation of a structured film which continues to strengthen irreversibly with time (Dickinson and Matsumaura, 1991).

2.5.2 Competitive adsorption between proteins and surfactants

Competitive adsorption between surfactants (small-molecule emulsifiers) and proteins in model oil-in-water emulsions has been reported widely in recent years. Because milk proteins and surfactants of smaller molecular weight are often present together in food emulsions, the interactions between proteins and surfactants have important influences on the properties of food emulsion. From studies on the interactions between milk protein and a variety of surfactants, it has been demonstrated that many surfactant molecules can displace protein from the oil-water interfaces. (Courthaudon *et al.,* 1991; Dickinson and Tanai, 1992; Fang and Dalgleish, 1996; Euston *et al.,*1995, 1996; Hunt and Dalgleish, 1996). The degree of displacement depends not only on the specific surfactant used, but also on when it is added (i.e., before or after emulsion formation). If the surfactant is introduced before emulsification, partial displacement is found, and if the surfactant is added after emulsification, complete displacement may occur (Courthaudon *et al.,* 1991 a).

The small molecule surfactants that are used to study competitive adsorption with milk proteins often are; polyoxyethylene sorbitan monostearate (PSM), diethyl glycol dodecyl ether (C12E8), Tween 20, Span 80, sorbitan monostearate (SM), dioleoylphosphatidylcholine (DOPC) and glycerol monostearate (GMS) as well as lecithin (Courthaudon *et al.,* 1991 a, b, c; Dickinson and Tanai, 1992; Chen et al., 1993; Dickinson and Iveson, 1993; Chen and Dickinson, 1993; Dickinson and Hong, 1995; Dickinson *et al.,* 1996; Cornec *et al.,* 1996; Fang and Dalgleish.,1996; Euston *et al.,*1995, 1996; Hunt and Dalgleish, 1996; Agboola *et al.,* 1998; Demetriades and McClements, 1998). This area has been reviewed by Dickinson (1997, 1998).

From these reports, we can obtain an impression that the small-molecule surfactants can displace proteins from an oil-water interface at different protein

and surfactant concentrations in caseinate-stabilized emulsion. The ability of displacement of water-soluble surfactants is greater than that of oil-soluble surfactants. The non-ionic surfactant has less ability of displacement. Some surfactants preferentially and selectively remove β-casein from the interface. Temperature affects the competitive adsorption. Increasing temperature reduce the displacement of proteins by surfactants from the interface. A distinct difference between these reports is that complete displacement of protein has been reported by some workers while only a partial protein displacement from the interface was found by others.

2.6 Emulsion stability

Emulsions are inherently unstable (Dickinson and Stainsby, 1982). Even in a system that appears to be perfectly stable, with a shelf-life of several years, the total number of droplets, their size distribution, and their arrangement in space, are all changing with time. Operationally, a stable emulsion is one that undergoes change rather slowly. In addition to their surface activity, proteins also have excellent emulsion stabilizing properties (Dickinson *et al.,* 1988). The capacity of proteins to stabilize emulsions is the most important criterion besides the emulsion formation in most food applications. The forces involved in stabilizing and destabilizing emulsion include van der Waals attractive forces, electrostatic interaction and steric factors related to the surface-active agents' osmotic effects, hydration forces and viscosity of the continuous medium (Leman and Kinsella, 1989). At pH values away from their isoelectric point, proteins are electrically charged, and so there is an electrostatic repulsion which prevents dispersed particles from closely approaching one another. With the possible exception of highly charged proteins, a predominant contribution to emulsion stabilization by protein comes from the steric stabilization mechanism. This is principally caused by a combination of excluded volume and osmotic pressure effects (Dickinson and McClements, 1995). The excluded volume effect occurs because two segments of protein can not occupy the same space and so their configurational entropy is reduced. The osmotic pressure effect

arises because the local concentration of protein increases when protein molecules overlap and so there is an osmotic driving force favouring separation of the particles. Protein molecules which form a dense viscoelastic interfacial layer, with extensive protein-protein interactions, tend to give adsorbed films that are more stable to rupture than proteins which form loose mobile structures (Dickinson and McClements, 1995).

The primary processes leading to instability are creaming, flocculation, coalescence and phase inversion. Creaming refers to the gravitational separation of emulsified droplets to form a densely packed phase without change in droplet size (Walstra, 1987). Flocculation denotes the aggregation of globules via interactions between adsorbed protein. Although this generally modifies the physical properties of the emulsion, the particle size distribution remains unchanged and the flocs can be readily dispersed because the interactions are weak (Walstra, 1987). There are two kind of flocculation, bridging flocculation and depletion flocculation, depending on the protein concentration on the interface of oil/water droplets (Walstra, 1987; Dickinson *et al.,* 1989; Dickinson and Golding, 1997). Coalescence, i.e., an increase in droplet size by accretion, gradually results in separation of the oil and aqueous phase. Unlike creaming and flocculation, it is always irreversible. Coalescence requires rupture of the stabilizing film at the oil/water interface, but this only occurs when the layer of continuous phase between the droplets has thinned to a certain critical thickness (Britten and Giroux; 1991; Das and Kinsella, 1993; Dickinson and Stainsby, 1988; Walstra, 1993).

An oil-in-water emulsion creams because the droplets are of lower density than the continuous phase. A contribution to the droplet density arises from the stabilizing adsorbed layer, which is usually more dense than the aqueous phase. As the film thickness is more-or-less independent of droplet size, this means that a typical food emulsion is polydisperse with respect to droplet density as well as with respect to droplet size (Dickinson and McClements, 1995). Therefore, in principle, creaming can be inhibited altogether by

decreasing particle size and raising the droplet density to match that of the aqueous phase. In addition, in commercial practice, creaming is often inhibited by increasing the viscosity of the continuous phase. In fairly dilute dispersion of spherical particles, creaming is usually described by Stokes' equation (Walstra, 1987).

$$V = 2g\Delta\rho\gamma^2/9\eta$$

Where V is the linear velocity, g is the acceleration due to gravity, $\Delta\rho$ is the density difference between particle and continuous phase, and η is the viscosity of the latter. One problem is that the continuous phase is often highly non-Newtonian; in fact, in many foods, it is not a single phase, but is physically inhomogeneous. Knowledge of the structure and rheology of the dispersion medium is often of greater importance than knowledge of the dispersed particles (Walstra, 1987). In addition, the creaming stability will be different from that predicted by Stokes' law in some flocculated emulsions, emulsions in which the net-work structure is formed (Syrbe *et al.*, 1998) or when the surfactant layer on the oil droplets significantly increases their effective density (Gouldby *et al.*, 1993).

Bridging flocculation will occur when single molecules become adsorbed simultaneously onto two surfaces or if emulsion droplet covered with adsorbed polymer are mixed with uncovered particles (Walstra, 1993). A prerequisite is that the concentration of the polymer in the solvent is very low, a condition that can be fulfilled because of the very high surface activity of many polymers. Consequently, the method of processing may determine, along with composition, whether bridging flocculation will actually occur (Walstra, 1993).

In addition to the effects of adsorbed macromolecules on stability with respect to flocculation, it is important to realise that unadsorbed macromolecules may be involved in what is called depletion flocculation, as discussed in detail by Dickinson and Stainsby (1988) and Walstra (1993). This type of flocculation

arises whenever colloidal particles are so close together that polymer molecules are excluded (deletion) from the region of continuous phase between them. The resulting osmotic pressure gradient causes the particles to flocculate spontaneously, since the mixing of solvent from between the particles with the bulk solution is associated with a decrease in free energy (Dickinson and Stainsby, 1988). In Asakura-Oosawa theory, the case of rigid spherical molecules in the gap between a pair of particle surfaces, depletion potential has the form:

$$U_d(h) = \begin{cases} -(3kTR\phi_s/d^3)(d-h)^2. & (h < d) \\ \\ 0 & (h \geq d) \end{cases}$$

where R is the particle radius, d is molecular diameter, h is gap and ϕ_s is solute volume fraction in bulk medium (Asakura and Oosawa, 1954). Figure 2.2 depicts the schematic explanation.
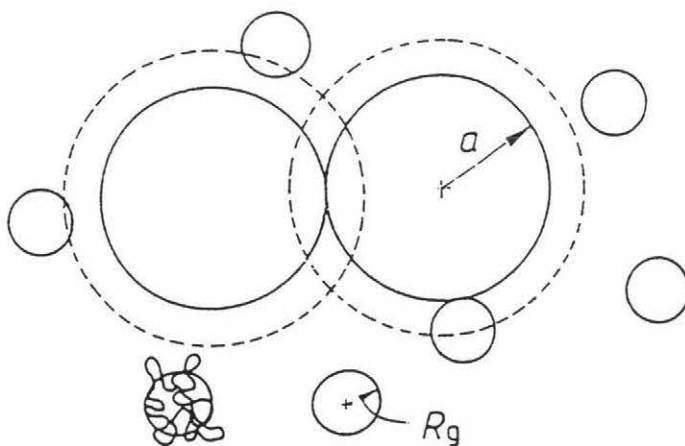

Figure 2.2: Schematic explanation of the depletion of macromolecules, with a radius of gyration (R_g), from the solution near spherical particles of radius, a, and of the resulting depletion flocculation (Walstra, 1993).

Several methods can be used to determine the rate of creaming (Leman and Kinsella, 1989). Most of methods are based on gravity-induced separation. Then the fat content of the lower emulsion phase is estimated by several methods, including gravitational estimation, measurement of viscosity, density, turbidity, particle size, conductivity or dielectric properties. But the rate of creaming in emulsion is slow, unless the droplets are large (greater than 5 µm). Therefore, accelerated tests are usually employed. Usually, a mild gravitational speed is employed to avoid breaking the interfacial film. Tornberg and Hermansson (1977) used 15 min of centrifugation at 180 g to determine the creaming stability. The details of this method are given in Chapter 3.

2.7 Factors affecting protein adsorption at oil-water interfaces and stability of protein-stabilized emulsions

2.7.1 Effect of energy input and homogenization pressure

The emulsifying properties of milk proteins are affected by energy input during emulsification (Tornberg and Hermansson, 1977; Tornberg, 1978 b, 1978 c; Leman *et al.* 1988; Murphy and Fox, 1991). Generally, greater energy input produces more stable emulsions up to a limit which is dependent on the type of protein and environmental factors, such as pH and ionic strength. Increase in homogenization pressure, which causes a decrease in particle size of emulsions and the formation of higher surface area per unit mass of protein, improves the stability of emulsions. However, prolonging the emulsification beyond an optimum time interval does little to improve the stability of most emulsions. If an emulsion is over processed, i.e., continual processing or excessive pressure, particle size will increase. This may reflect the fact that some proteins become progressively denatured and aggregate resulting in emulsion destabilization (Tornberg and Hermansson, 1977; Haque and Kinsella, 1987).

Srinivasan *et al.* (1996) reported that the d_{32} of emulsions made with soya oil (30%, w/w) and sodium caseinate (2.5% w/w, pH 7.0) decreased with increase

in homogenization pressure, whereas the surface protein concentration decreased sharply from 2.2 to1.5 mg/m^2. Mulvihill and Murphy (1991) reported similar results on various caseins or caseinates-stabilized emulsions. It was suggested that the decrease in surface concentration on increasing homogenization pressures (i.e., increasing oil surface area) might be attributed to increased spreading and rearrangement of adsorbed protein molecules at the interface. Relatively high surface concentration at low homogenization pressure, i.e., at smaller surface areas, might indicate that multilayers of proteins were formed at the interface, whereas at high homogenization pressure the layers of protein might be thinner and probably approaching a monolayer (Srinivasan *et al.*, 1996).

2.7.2 Effect of protein concentration

In general, the mean droplet size of protein-stabilized emulsions decreases and the protein load at oil/water interface increases with increasing the protein concentration at low concentration range. The emulsion droplet size does not change with protein concentration at high concentrations. The slight increase in protein load at high concentration range is likely to be a result of closer packing of the adsorbed proteins in the monomolecular layer (Phillips, 1981; Britten and Giroux, 1993; Fang and Dalgleish, 1993 a; Hunt and Dalgleish, 1994; Srinivasan *et al.*, 1996).

2.7.3 Effect of oil concentration

Increasing the concentration of oil in the emulsion leads to an increase in the mean droplet size and reduces the protein load; increasing oil concentration probably causes spreading of the adsorbed protein into thinner layers, resulting in a decrease in surface concentration (Tornberg, 1980; Srinivasan *et al.*, 1996).

2.7.4 Effect of pH

On lowering the pH from 7 to 6, there is enhanced flocculation of caseinate-stabilized emulsions because of reduced electrostatic repulsion between

proteins, which lead to a higher probability of bridging, appearing as more flocculated emulsions (Tornberg and Ediriweera, 1988).

The protein load of β-casein at the oil-water interface increases as the pH approaches the isoelectric point (*pI*). This behaviour is consistent with closer association of β-casein molecules in solution and at the interfaces (Dickinson and McClements, 1995). But, Tornberg (1978 a) reported that the protein load decreased with lowing of pH from 7 to 6 in caseinate-stabilized emulsions. One of the reasons for the contradictory results could be that the studies have not been standardized with regard to the protein: fat surface area ratio, which is a crucial factor in determining the properties of the protein adsorbed layer.

The stability of milk protein stabilized-emulsions changes with pH; caseinate and whey proteins can make stable emulsions under acidic conditions (pH 3) as well as at pH 7 (Hunt and Dalgleish, 1994 a; Agboola and Dalgleish, 1996 b; Demetriades *et al.*, 1997). The buffer had a large influence on the stability of emulsions made at pH 6: with citrate buffer, emulsions were unstable, but those made with imidazole were stable. The compositions of the adsorbed proteins were altered with changes of pH, α-lactalbumin was found to adsorb preferentially at lower pH. It is significant that this behavior depended on the concentration of protein (Hunt and Dalgleish, 1994 a).

2.7.5 Effect of ionic strength

Tornberg (1978 a, 1980) reported that protein load increased with salt added to the sodium caseinate–stabilized emulsions. It was considered that the protein adsorbed at the interface was composed of a monolayer, being more densely packed at the higher ionic strength.

Emulsions made with pure α_{s1}-casein have been found to be much more susceptible to flocculation by salts than β-casein emulsions (Casanova and Dickinson, 1998). NaCl addition produced extensive flocculation of α_{s1}-casein-coated oil droplets at ionic strength 0.1 M. With around half of the adsorbed

protein layer consisting of β-casein, emulsions remained stable at ionic strengths of 2 M NaCl. The poor salt stability of model α_{s1}-casein emulsions can be improved substantially by replacing with β-casein. It indicate that adsorbed β-casein may play a crucial role in sodium caseinate emulsions in protecting α_{s1}-casein coated droplets against flocculation by electrolytes (Casanova and Dickinson, 1998).

Dickinson *et al.* (1984) reported that the oil droplets were more easily flocculated at low sodium caseinate concentrations in the presence of NaCl. Hunt and Dalgleish (1995,1996) reported the effects of KCl on emulsions made with sodium caseinate and WPI. The presence of KCl at concentrations from 0 to 200 mmol/dm^3, the average droplet size (d_{43}) remained constant in emulsions made with sodium caseinate. But the particle size of emulsions stabilized by WPI increased with KCl concentration.

Increasing the concentration of KCl beyond 25 mmol/dm^3 also affected the surface composition of emulsion droplets made using caseinate at pH 7. The percentages of κ- and α_{s2}-caseins in the adsorbed protein remained constant as the concentration of KCl was increased up to 200 mmol/dm^3, but the adsorbed α_{s1}-casein increased at the expense of β-casein (Hunt and Dalgleish, 1995). This may be explained by the increased aggregation of β-casein as the ionic strength is increased, so that the increased micellization of the protein may cause it to be less surface active. The changes in the composition of the interfacial layers may therefore be related to the quaternary structures of the proteins. In addition, the concentration of KCl present at homogenization also affected the composition of the adsorbed layer of whey protein on the emulsion droplets. The α-lactalbumin was adsorbed preferentially with increasing of KCl concentration at pH 7 (Hunt and Dalgleish, 1995,1996). At both high and low ionic strengths, the displacement of α-lactalbumin was complete regardless of the age of the emulsion. The percentage of β-lactoglobulin which was not displaced by caseins increased with the age of the emulsion prior to the addition of caseinate (Hunt and Dalgleish, 1996). This suggests that disulphide bridges

were not formed directly between α-lactalbumin and β-lactoglobulin, leading to permanent adsorption of the former.

2.7.6 Effects of temperature and high pressure

Temperature-induced changes in the structure and interactions of adsorbed proteins at the oil-water interface or suspended in the aqueous phase will affect the stability and rheology of a protein-stabilized emulsion. Enhanced interfacial protein-protein interactions may lead to an increase in the thickness and mechanical strength of the adsorbed layer, thereby promoting stability against coalescence. On the other hand, increased protein-protein interactions will typically produce a reduction in stability against flocculation (Dickinson and McClements, 1995). Britten *et al.* (1994) reported that heat-denaturation of whey protein improved the emulsifying activity.

It was demonstrated that aggregate and emulsion gel may be formed on heating emulsions made with WPI (Jost *et al.,* 1986; Hunt and Dalgleish, 1995) at 90°C. The association of the droplets is triggered by the denaturation and subsequent aggregation of protein adsorbed at the surface of the droplets and in the continuous phase. A stable liquid-like emulsion may be converted into some sort of network structure (Dickinson, 1998). Similar results (formation of aggregate or emulsion gel) are also obtained in whey protein-stabilized emulsions by high-pressure treatment (Galazka *et al.,* 1995; Dumay *et al.,* 1996; Dickinson and James, 1998).

Increase in temperature reduces the displacement of adsorbed β-casein from the oil-water surface by surfactants (Dickinson and Tanai, 1992; Stevenson *et al.*, 1997). These results were obtained both at 40°C and 70°C, the 70°C may cause denaturation of proteins. Studies of heat denaturation of protein have shown that interactions among protein molecules involved in aggregate formation are initially hydrophobic in nature before being followed by the formation of covalent intermolecular disulphide bridges.

2.7.7 Effect of Ca^{2+}

There is much information available in the literature on the effects of Ca^{2+} on milk. But the association of Ca^{2+} with adsorbed proteins has been studied less than binding in solution. Addition of Ca^{2+} to emulsions can cause increase of the average particle sizes in oil-water emulsions made with both caseinate and whey protein and the formation of aggregates; increasing protein content caused the emulsions to become more stable after addition of Ca^{2+} (Dickinson *et al.,* 1992; Agboola and Dalgleish., 1995, 1996). The specific binding of Ca^{2+} to proteins is most likely responsible for the decrease in emulsion stability with increasing CaCl$_2$. Binding of Ca^{2+} to the caseins causes the thickness of the adsorbed layer to decrease, because of the reduction of the charge density and inter-chain repulsion in the extended protein molecules (Dalgleish, 1996).

Addition of Ca^{2+} to protein solution prior to emulsion formation caused destabilization of emulsion made with caseinate and increased the protein load (Srinivasan *et al.,* 1996). It may be due to formation of large casein particles/aggregates which subsequently may be adsorbed on to the oil surface (Srinivasan *et al.,* 1996).

When NaCl was added to emulsions destabilized by Ca^{2+}, the particle size of the emulsions decreased (Agboola and Dalgleish, 1995). The increased stability of the emulsions in the presence of NaCl could be attributed to competition by the cations for specific binding sites on phosphoserine groups of caseins, thereby reducing any destabilizing specific effects of Ca^{2+} (Agboola and Dalgleish, 1995).

2.7.8 Effect of ageing

Ageing causes an increase in the rigidity of the adsorbed protein layer at the interface. It was found that β-lactoglobulin displacement by β-casein or β-casein displacement by Tween 20 become more difficult as the oil-in-water emulsions mature (Hunt and Dalgleish, 1996; Stevenson *et al.,* 1997). This indicates that

the structure of the adsorbed protein continues to change after its initial interaction with the interface.

In the case of β-lactoglobulin, these changes could be ascribed to loss of the globular structure and, since the protein contains two disulphide bridges and a single free thiol group, to the formation of intermolecular disulphide bridges through disulphide exchange reactions. In contrast, β-casein molecules are relatively devoid of secondary structure and contain no cysteine residues. But the number of points of interaction between the adsorbed β-casein molecules and the interface, or between individual adsorbed protein molecules, increases during extended storage (Stevenson *et al.,* 1997).

Chapter 3
MATERIALS AND METHODS

3.1 Materials

Sodium caseinate (Alanate 180), WPC (Alanate 342) and calcium caseinate (Alanate 380) were obtained from the New Zealand Dairy Board, Wellington, New Zealand. Soya oil was purchased from Davis Trading Company, Palmerston North, New Zealand. All of the chemicals used were of analytical grade obtained from either BDH Chemicals (BDH Ltd, Poole, England) or Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

3.2 Emulsion preparation

Emulsions were prepared from chosen protein solutions and 30% soya oil. Protein solutions were prepared by adding the protein powder to the Milli-Q water (water purified by treatment with a Milli-Q apparatus, Millipore Corp. Bedford, MA), and then stirring for 60 min at room temperature (calcium caseinate at 50°C in a water bath) to ensure complete dispersion. In some cases, different concentrations of $CaCl_2$ were added to the protein solutions, and pH adjusted to 7.0 using 1 M NaOH or 1 M HCl. Appropriate quantities of soya oil were then mixed with the protein solution, to give 30% oil in the final emulsion. The mixture was heated to 55°C and homogenized in a two-stage valve homogenizer (Rannie a/s, Roholmsvej 8, DK 2620 Albertslund, Denmark) at 204 bar for the first stage and 34 bar for the second stage. In some cases, different amounts of $CaCl_2$ were added to the emulsions after they are made in the absence of $CaCl_2$. The emulsions were stored at 20°C. At least two separate emulsions were prepared for each treatment.

3.3 Determination of average particle size and specific surface area

A Malvern MasterSizer MSE (Malvern Instruments Ltd, Worcestershire, U.K.) was used to determine the volume-surface average diameter (d_{32}) and specific surface area (area per unit mass).

In this method, a low power laser beam is diffracted by the oil droplets in the solution and the diffracted light is collected over a range of scattering angles by series of semicircular photo-electric diodes. The sizes of oil droplets are divided into 22 classes across the sub-micron range 0.1-1.0 microns. The volume size distribution is calculated from the intensity of light diffracted at each angle using Lorenz-Mie theory. For the calculation of size distribution, the refractive index of the medium in which the particles are dispersed is required.

The parameters that were used to analyse the particle size distribution were defined by the presentation code 2NAD. Relative refractive index (N) was 1.095, i.e. the ratio of refractive index of emulsion particle (1.456) and that of the dispersion medium (1.33). The absorbance value of emulsion particle was 0.001. The following parameters were given by the MasterSizer.

d_{32}: the volume-surface mean diameter (also known as the Sauter mean diameter or d_{vs}) is given by

$$d_{32} = \frac{\Sigma \, d_i^3 \, N_i}{\Sigma \, d_i^2 \, N_i}$$

where N_i is the number of the particles with diameter d. d_1 is the minimum diameter in the distribution and d_2 is the maximum distribution diameter.

d_{43}: d_{43} is the weight-average diameter which is given by

$$d_{43} = \frac{\Sigma d_i \, W_i}{\Sigma W_i} = \frac{\Sigma d_i^4 \, N_i}{\Sigma d_i^3 \, N_i}$$

where W_i the weight of particles in the class size, is replaced by $d_i^3 N_i$ assuming that the density is constant for all sizes.

The specific surface area was also obtained from the MasterSizer results, and was used for the surface protein concentration calculations. The d_{32} and specific surface area values were accurate to within 3%.

3.4 Determination of surface protein concentration and composition

The surface protein concentration and composition were determined using the methods described by Srinivasan *et al.* (1996). Emulsions were centrifuged at 45,000 **g** for 40 min at 20°C in a temperature-controlled centrifuge (Sorvall RC5C, DuPont Co., Wilmington, DE). The subnatants were carefully removed using a syringe. The cream layer was dispersed in deionized water and recentrifuged at 45,000 **g** for 40 min. The subnatants were filtered sequentially through 0.45 and 0.22 μm filters (Millipore). The filtrates were analyzed separately for total protein using the Kjeldahl method (1026 Distilling Unit and 1007 Digestor Blorck, Tecator AB, Hoganas, Sweden). In the case of emulsions formed with calcium caseinate, a sediment was present after centrifugation. This sediment was taken out by a spoon for measuring total protein using the Kjeldahl method.

The surface protein concentration (mg/m^2) was calculated from the surface area of the oil droplets, determined by the MasterSizer, and the difference in the amount of protein used to prepare the emulsion and that measured in the subnatants and sediment (if present) after centrifugation.

Adsorbed protein (g) = total protein (g) taken for making an emulsion −
[protein (g) present in the subnatant + protein (g)
present in the sediment]

$$\text{Surface protein concentration (mg/m}^2) = \frac{\text{total (mg) protein adsorbed}}{\text{total fat surface area (m}^2)}$$

The composition of the protein adsorbed at the surface of the emulsion droplets was determined using SDS-PAGE. A certain amount of cream was spread on a filter paper, mixed with SDS buffer (0.5M Tris, 2% SDS, 0.05% mercaptoethanol, pH 6.8) and a portion (5 μL) was applied to the SDS gels previously prepared on Miniprotein II system (Bio-Rad Laboratories, Richmond, CA). The separation gels contained 15% acrylamide, made up in Tris/HCl buffer, pH 8.8, and stacking gels were composed of 4% acrylamide in Tris/HCl buffer, pH 6.8. After destaining, the gels were scanned on a laser densitometer (LKB Ultroscan XL, LKB Produkter AB, Bromma, Sweden). The percentage composition of each sample was determined by scanning the areas for α_s- (α_{s1} + α_{s2}), β- and κ-caseins and expressing the individual casein peaks as a fraction of the sum total.

3.5 Creaming stability

Immediately after preparation, the emulsions (30 g) were transferred into centrifuge tubes and maintained at 20°C for 24 hours. The samples were then centrifuged at 185 **g** for 15 min, a sample (5 g) from the lower phase was carefully removed using a syringe and analysed for fat content by the Mojonnier method. The stability rating was calculated as follows:

$$\text{Stability rating (\%)} = \frac{\text{fat in lower phase (\%)}}{\text{fat in original emulsion (\%)}} \times 100$$

3.6 Chemical analysis

Total protein was measured by determining the total nitrogen content by the macro-Kjeldahl method and multiplying it by the factor 6.38. The samples were

digested using a Kjeltec digester (Kjeltec 1007 Digester, Tecator, Sweden) and distilled using a Kjeltec system (Kjeltec 1026 Distilling Unit, Tecator, Sweden). The total fat in the emulsion was determined using the Mojonnier method.

3.7 Electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was described by Srinivasan *et al.* (1996).

Preparation of stock solutions
Acrylamide/Bis (30% TT, 2.67%C)
Acrylamide (30 g) and N, N-bis acrylamide (0.8 g) were dissolved in deionised water to give a final volume of 100 ml, and stored at 4 °C in a dark bottle.

1.5 M TRIS-HCl buffer, pH 8.8
TRIS (tris hydroxymethyl aminoethane, 18.15 g), obtained from USB (United States Biochemicals Corp. Cleveland, OH, USA), was dissolved in approximately 60 ml of deionised water, the pH adjusted to 8.8 with 1 M HCl and the volume made up to 100 ml with deionised water. The buffer was stored at 4°C.

0.5 M TRIS-HCl buffer, pH 6.8
TRIS (6 g) was dissolved in about 60 ml of deionised water. The pH of the solution was adjusted to 6.8 with 1 M HCl, and then the volume made up to 100ml with deionised water. The buffer was stored at 4°C.

10% SDS
SDS (10 g) was dissolved in deionised water with gentle stirring and the volume made up to 100 ml.

SDS-reducing buffer (sample buffer), 100 ml

To 50 ml of deionised water, 0.5 M Tris-HCl buffer (12.5 ml), glycerol (10 ml), 10% (w/v) SDS (20 ml), β-mercaptoethanol (5 ml) and 0.05% (w/v) bromophenol blue (2.5 ml) were added. The pH of the sample buffer was adjusted to 6.8.

5X Electrode buffer

Electrode buffer (5X) was prepared by dissolving TRIS (15 g), glycine (43.2 g) and SDS (3.0 g) in deionised water. After adjusting the pH to 8.3, the volume of the buffer was made up to 1 L and the buffer was stored at 4°C. For each elecrophoresis run, 60 ml of this electrode buffer was diluted with 240 ml deionised water.

Preparation of resolving gel (16%, w/v, 0.375 M TRIS, pH 8.8)

To prepare 10 ml of resolving gel buffer, the following solutions were mixed: deionised water (2.02 ml), 1.5 M Tris-HCl buffer (2.50 ml), 10% SDS (100 µl) solution and acrylamide/bis mixture (5.3 ml). The mixture was then degassed for 15 minutes. Immediately 5 µl of TEMED (tetramethylethylenediamine) and freshly prepared 10% (w/v) ammonium persulphate (50 µl) were added. After gentle mixing, 3.3 ml of the contents were poured between the electrophoresis casting plates (Bio-Red Mini Protein, Bio-Rad, richmond, CA, USA). A small quantity of deionized water was added to form an upper layer and the acrylamide solutions allowed to polymerize at 20°C for about 45 min. The water was then drained and dried with filter paper before pouring the stacking gel.

Preparation of stacking gel

To prepare 10 ml of stacking gel, the following solutions were added to 6.1 ml of deionised water, 0.5 M Tris-HCl buffer (2.5 ml), 10% SDS (100 µl) solution and 30% acrylamide/bis mixture (1.3 ml). The mixture was degassed for 15 min. Immediately TEMED (10 µl) and freshly prepared ammonium persulphate (50 µl) were added and 2.2 ml of this mixture was then poured on top of the resolving gel. The slot former (plastic comb) was immediately placed at the top of the stacking gel (between the plates) to form appropriate slots for the

samples. Polymerisation was carried out at 5°C overnight. Next day the plastic comb was removed with filter paper. Gel plates were then placed in the electrode chamber and samples were applied to the gel slots.

Sample preparation and running gels

Samples were dispersed in SDS sample buffer and the mixtures were heated in a water bath at 90°C for 5 min and then cooled to room temperature. The dilution of the sample (cream) with the sample buffer was dependent on the amount of protein present in the samples. The samples in sample buffer (5 µl) were applied to the slots of the SDS gel. The gels were run on a Mini-Protean system (Bio-Rad, Richmond, CA, USA) at 200 V using a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Richmond, CA, USA) until the tracking dye moved out of the gel. The approximate running time was about 60 min after which the gels were removed from the plates and transferred gently to staining solution.

Staining

The gels were put into plastic boxes and about 50 ml of comassie Blue R solution (1 g brilliant blue r was dissolved in 500 ml of isopropyl alcohol and 200 ml acetic acid and the contents made to 2 L with distilled water) was added. The gel boxes were put on a rocking table (made at the Food Technology Department workshop) so that the gels were uniformly stained with the staining solution.

Destaining

The staining solution was drained carefully after 1 hour and replaced with destaining solution which contained a mixture of 100 ml isopropyl alcohol and 100 ml acetic acid diluted to 1 L. The destaining solution was again changed after 1 hour with fresh destaining solution. Then the plastic box was left on the rocking table for 19 hours. After that the destaining solution was replaced with distilled water and the gel was scanned using the densitometer.

Densitometry

Quantitative determination of the component of the mixture separated by SDS-PAGE was performed by densitometry using a laser densitometer (LKB Ultroscan XL, LKB produkter, AB, Sweden). In the densitometer, the protein bands on the stained gel are scanned with a narrow beam of laser light and the absorbance at 522 nm is plotted as a function of track distance. The printout resulted in the form of a graph of individual peaks and table of individual peak areas. Standard protein solutions were run in conjunction with samples to aid identification of unknown protein bands in the sample.

3.8 Confocal laser microscopy

A Leica (Heidelburg, Germany) confocal scanning laser microscope with a 100 mm oil immersion objective lens and an Ar/Kr laser with an excitation line of 488 nm (in such a way that only the fluorescent wavelength band can reach the detector system) was used to determine the microstructure of emulsions. Emulsions were made as described above and about 3 ml of sample was taken in a test tube and Nile Blue (fluorescent dye) was mixed through and then placed on a microscope slide. The slide was then covered with a coverslip and observed under the microscope.

Analysis of 6 separate emulsions, made with 2.5% caseinate and 30% soya oil, showed that the variations were: $\pm$ 0.02 μm for d_{32} or d_{43}, ~ 4% for surface protein concentration, ~ 6% for stability rating, ~ 4% for α_s-casein, ~ 5% for β-casein and ~ 7% for κ-casein.

Chapter 4

FORMATION AND STABILITY OF OIL-IN-WATER EMULSIONS PREPARED USING COMMERCIAL SODIUM CASEINATE, WHEY PROTEIN CONCENTRATE AND CALCIUM CASEINATE

4.1 Introduction

Sodium caseinate, whey protein concentrate (WPC) and calcium caseinate are widely used in the food industry as functional ingredients, because of their excellent functional and nutritional properties (Mulvihill, 1992). One of the important properties is their ability to adsorb at oil-water interfaces and stabilize food emulsions (Dickinson and Stainsby, 1982). The adsorption behaviour of proteins and the stability of protein-stabilized emulsions depend largely on the molecular structure, interactions of the adsorbed protein and how these properties are influenced by environmental conditions (Dickinson and McClements, 1995).

Although some previous workers have studied the emulsifying properties of milk proteins and provided much valuable information (Tornberg and Lundh, 1978; Phillips, 1981; Robson and Dalgleish, 1987; Mulvihill and Muphy, 1991; Britten and Giroux, 1993; Fang and Dalgleish, 1993 a; Hunt and Dalgleish, 1994; Dickinson and Golding, 1997; Srinivasan *et al.,* 1996, 1999), some issues still remain unresolved. For example, β-casein, which is more surface active than other caseins, has been shown to adsorb in preference to α_{s1}-casein in emulsions stabilized by a model mixture of β-casein and α_{s1}-casein (Dickinson *et al.,* 1988). However, no preference for α_{s1}-casein or β-casein in sodium caseinate-stabilized emulsions was observed by Hunt and Dalgleish (1994). Euston *et al.,* (1995, 1996) and Srinivasan *et al.* (1996) reported that the preferential adsorption of β-casein in sodium caseinate was dependent on the concentration of protein used in making emulsions. At low protein concentration, β-casein was adsorbed in preference to α_s-casein, whereas the larger amount of α_s-casein than β-casein was present at the interface at high protein

concentrations. High amounts of κ-casein at surface were observed by Srinivasan *et al.* (1999), contrast to other reports (Hunt and Dalgleish, 1994). The structure of adsorbed caseins on the surface is not clear.

In emulsions formed with caseinate and whey protein, Hunt and Dalgleish (1994) reported that there was no preferential adsorption between caseinate and whey protein at low concentrations, but the amount of caseinate at surface was much more than whey protein at high concentrations. In addition, no preferential adsorption between β-lactoglobulin and α-lactalbumin was reported by some works (Dickinson *et al.*, 1989; Hunt and Dalgleish, 1994; Euston *et al.*, 1996). But Closs *et al.* (1995) found that β-lactoglobulin was adsorbed in preference to α-lactalbumin in emulsion formed with whey proteins, or with mixtures of β-lactoglobulin and α-lactalbumin. More information on the structure of individual protein in mixed films containing several different proteins is needed, especially when proteins are prone to self-assembly in concentrated solutions.

Very low creaming stability, observed in emulsions made with relatively high sodium caseinate concentration, has been attributed to depletion flocculation with respect to unbound or unadsorbed caseinate sub-micelles (Dam *et al.*, 1995; Dickinson and Golding, 1997; Srinivasan, 1999). However, the creaming stability of emulsions made with the mixtures of caseinate and whey protein is not known. It would be of interact to find out whether the flocculation is affected by the presence of non-aggregated proteins.

The emulsifying properties of calcium caseinate, in comparison to sodium caseinate are worth studying, in order to obtain knowledge about the behaviour of caseins at surface in the different state from different source. The caseins exists in a more aggregated state in calcium caseinate due to Ca^{2+} binding (Srinivasan *et al.*,1999).

The objective of this work is to study the adsorption behaviour of three commercial proteins: sodium caseinate, WPC and calcium caseinate, at oil-

water interface and to determine the stability of emulsions stabilized by these proteins, singly or in combination, at different concentrations. In particular, the adsorption behaviour and probable structures of adsorbed whey protein and caseins at low and high protein concentrations are discussed.

4.2 Results

4.2.1 Average particle size of emulsions

The average particle size (d_{32}) of the emulsions, made with WPC, sodium caseinate and calcium caseinate, as a function of protein concentration is shown in Figure 4.1. The average particle sizes (0.50 ± 0.03) were almost independent of the protein concentration in the range 1 - 5% in sodium caseinate and WPC-stabilized emulsions. The d_{32} values of emulsions stabilized by sodium caseinate and WPC were almost identical at a given protein concentration. At low protein concentrations (below 1.0%), the particle size were slightly larger. These results are consistent with the previous reports (Fang and Dalgleish, 1993 a; Mulvihill and Muphy, 1991; Britten and Giroux, 1993; Euston *et al.*, 1996; Srinivasan *et al.*, 1996). These values were significantly lower than that of calcium caseinate-stabilized emulsions. In emulsions made with calcium caseinate, the d_{32} decreased dramatically from 1.75 ± 0.02 to 0.75 ± 0.02 μm as the caseinate concentration was increased from 0.33 to 2%, then decreased slightly to 0.65 ± 0.02 μm with further increase in the concentration to 5%.

The droplet size distributions, measured using Mastersizer, showed only a monomodal distribution with no evidence of aggregation for all emulsions containing sodium caseinate or WPC. However, the size distribution of emulsions formed with < 2% calcium caseinate showed bimobal distribution of particles, in agreement with the findings of Srinivasan *et al.* (1999).
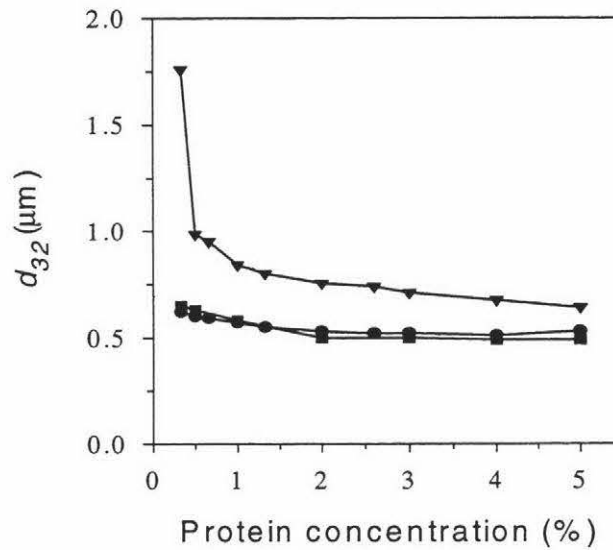
Figure 4.1: Changes in average particle size (d_{32}) of emulsions made with 30% soya oil, pH 7.0, and sodium caseinate (●), WPC (■) or calcium caseinate (▼) as a function of protein concentration. Each data point is the average of determinations on three separate emulsions.
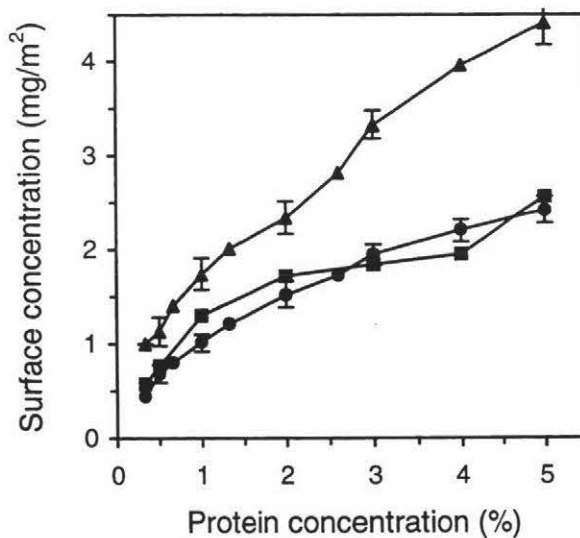

Figure 4.2: Changes in surface protein concentration (mg/m^2) of emulsions made with 30% soya oil, pH 7.0, and sodium caseinate (●), WPC (■) or calcium caseinate (▲) as a function of protein concentration. Each data point is the average of determinations on three separate emulsions.

4.2.2 Surface protein concentration

Figure 4.2 shows the changes in the surface protein concentration of emulsions formed with sodium caseinate, WPC or calcium caseinate with different concentrations of protein. At concentration below 1%, the surface protein concentration increased almost linearly with increase in protein concentration in all three emulsions. In emulsions formed with sodium caseinate, the surface protein concentration increased gradually with further increase in protein concentration (2.6 mg/m^2 at 5% protein). Emulsions containing WPC showed similar trend. Comparing with the emulsions formed with sodium caseinate, the surface protein concentrations were slightly higher when the protein concentrations were < 3.0%. At 3.0%, the surface concentrations were very similar (~ 2.0 mg/m^2) for both the caseinate and WPC stabilized emulsions. Beyond this point, the sodium caseinate stabilized emulsion had higher surface concentration than that of WPC emulsions. Similar trends have been reported (Fang and Dalgleish, 1993 a; Hunt and Dalgleish, 1994) although the values of surface concentrations were lower in this study.

The behaviour of calcium caseinate-stabilized emulsions was different; the surface protein concentration increased almost linearly from 1.2 to 4.4 mg/m^2 with increasing protein concentration from 0.33 to 5.0%. The values for surface concentration were higher than that of the emulsions formed with sodium caseinate or WPC at a given protein concentrations (Figure 4.2). This result is consistent with that reported by Srinivasan *et al.* (1999).

4.2.3 Creaming stability of emulsions

The stability rating of emulsions formed with sodium caseinate, WPC or calcium caseinate is shown in Figure 4.3. At low protein concentrations (< 1% in sodium caseinate or WPC emulsions, < 2% in calcium caseinate emulsion), the stability increased markedly with increasing protein concentration. It remained almost constant at higher concentrations in emulsions made with WPC or calcium caseinate. However, the stability of sodium caseinate emulsion decreased dramatically with increase in caseinate concentration from 1% to 5%; the decrease was very sharp between 1 and 3% protein.

The changes in the stability of emulsions made with sodium caseinate indicated that depletion flocculation of emulsion droplet occurred at higher solution protein concentration, in agreement with previous studies (Dickinson and Golding, 1997; Srinivasan, 1999). Figure 4.4 shows that the stability rating of emulsion started to decrease when the total concentration of protein used in making emulsion was 1%; at this point the caseinate concentration in aqueous phase (i.e. non-adsorbed caseinate) was 0.5%. At 2%, when the concentration in the aqueous phase was 1.5%, the stability was very low (25%).

The confocal micrographs of emulsion at 2% concentration showed clearly that the droplets were already joined together to form a network structure (Figure 4.5 C). More rigid network structure was formed with increasing caseinate concentration from 2 to 5% (Figure 4.5), which corresponded to increase in the concentrations in aqueous phase from 1.5 to 4.1% (Figure 4.4). Emulsion formed with < 1.0% caseinate showed no signs of flocculation, and the droplets were well separated (Figure 4.5 A). Dickinson and Golding (1997) reported that the extent of depletion flocculation is mainly dependent on the amount of unadsorbed protein, with the initial discernible onset of flocculation occurring at an aqueous caseinate concentration between 2 and 3% (w/w) in their case.

The stability rating of emulsions stabilized by WPC was higher than that of emulsions stabilized by calcium caseinate; the trends in both emulsions indicated that creaming stability was strongly dependent on the droplet size of emulsions (Figure 4.1). Figure 4.6 or Figure 4.7 show the confocal micrographs of emulsions made with WPC or calcium caseinate at various concentrations. At all concentrations, the emulsions made with WPC or calcium caseinate showed no flocculation and the droplets appeared to be separated and evenly distributed within the emulsions.
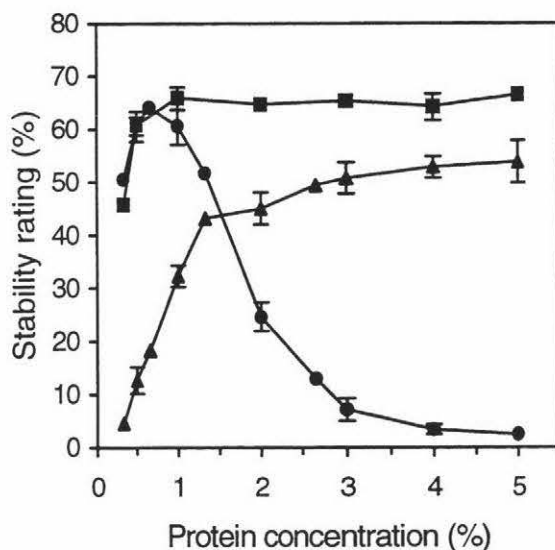
Figure 4.3: The effect of protein concentration on the stability rating (%) of emulsions made with 30% soya oil, pH 7.0, and sodium caseinate (●), WPC (■) or calcium caseinate (▲). Each data point is the average of determinations on three separate emulsions.
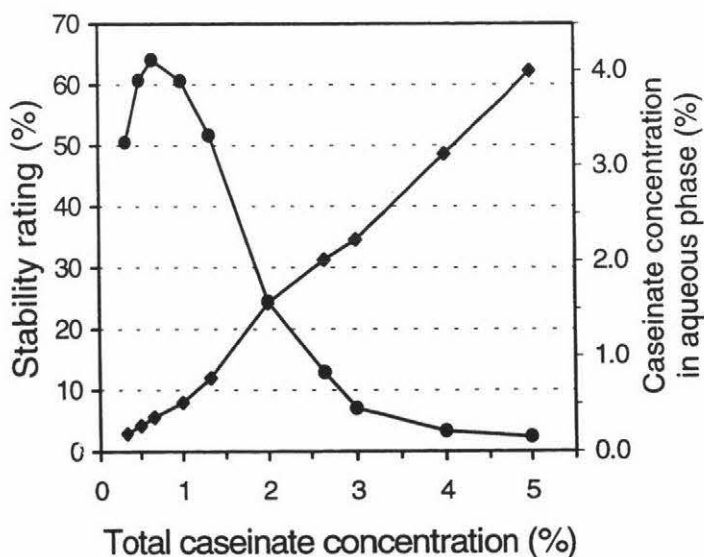

Figure 4.4: Changes in stability rating (●) and unadsorbed caseinate concentration in aqueous phase (■) of sodium caseinate emulsions (30% soya oil, pH 7.0) as a function of total caseinate concentration. Each data point is the average of determinations on three separate emulsions.
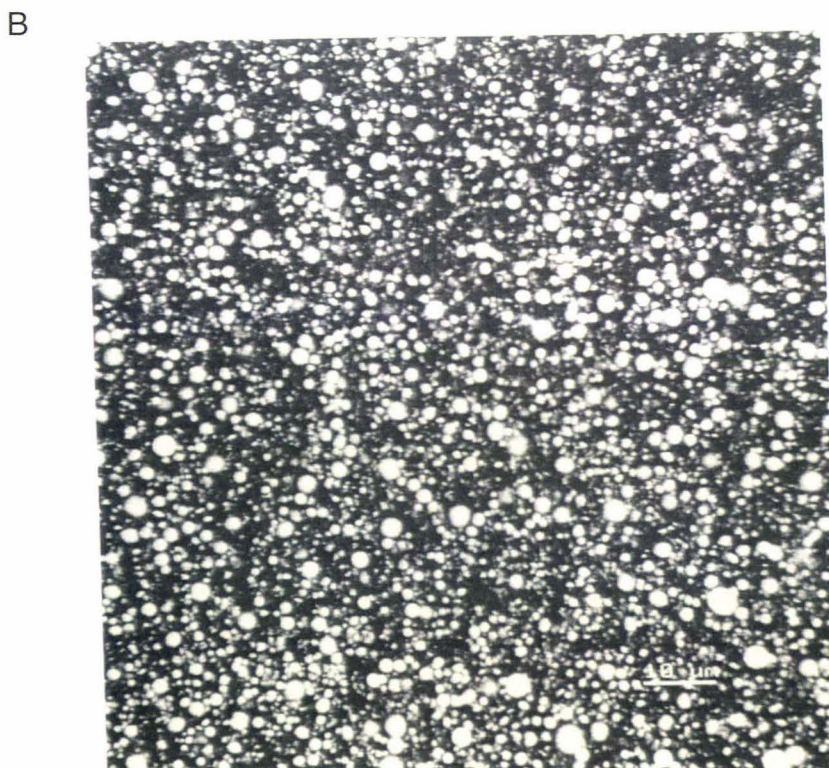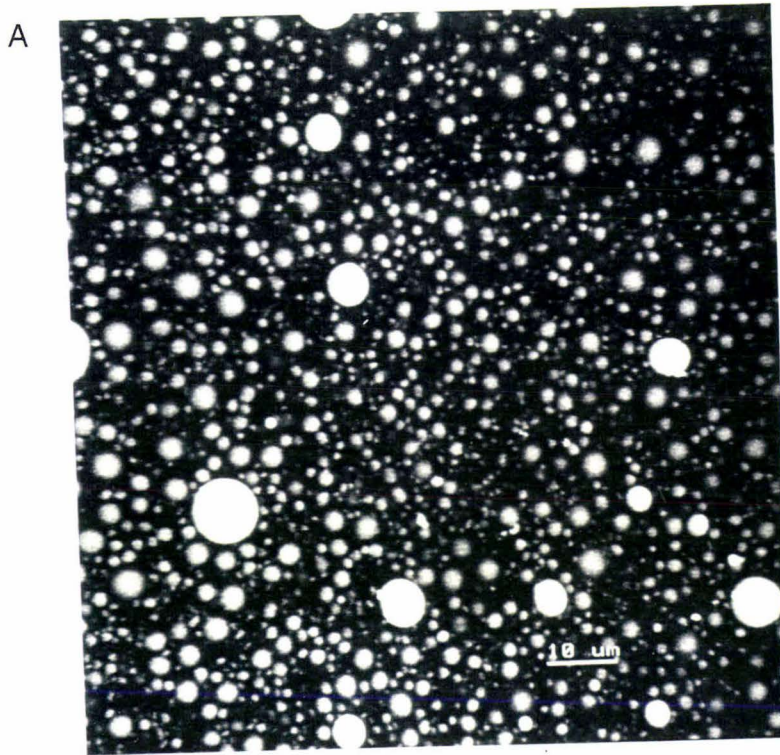
Figure 4.5: Confocal micrographs of oil-in-water emulsions (30% soya oil, pH 7.0) stabilized by 0.5% (A), 1% (B), 2% (C), 3% (D), 4% (E) and 5% (F) sodium caseinate.
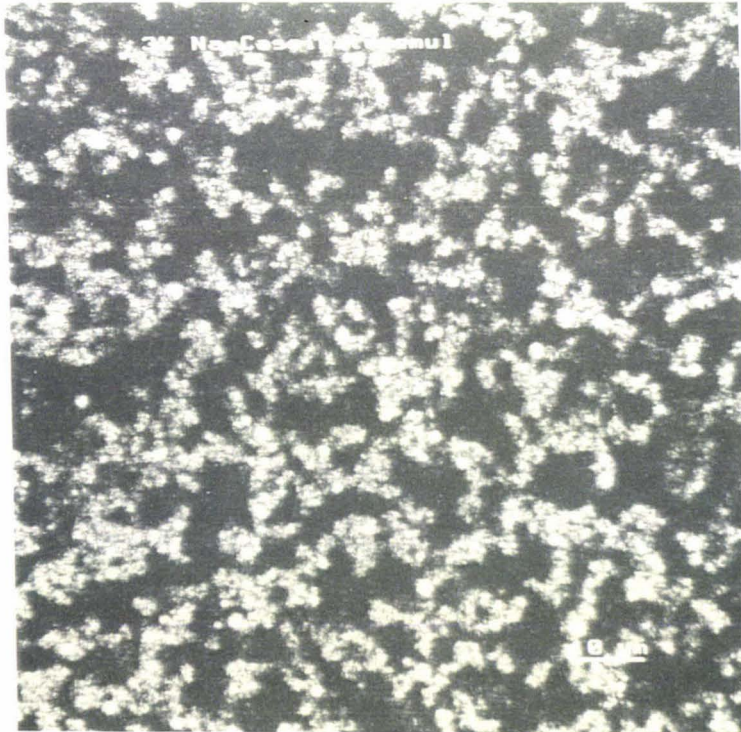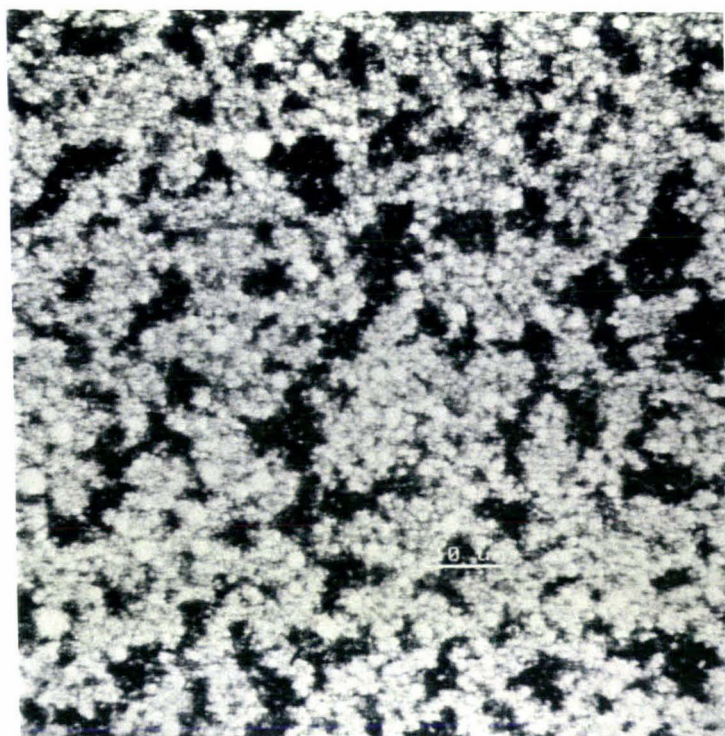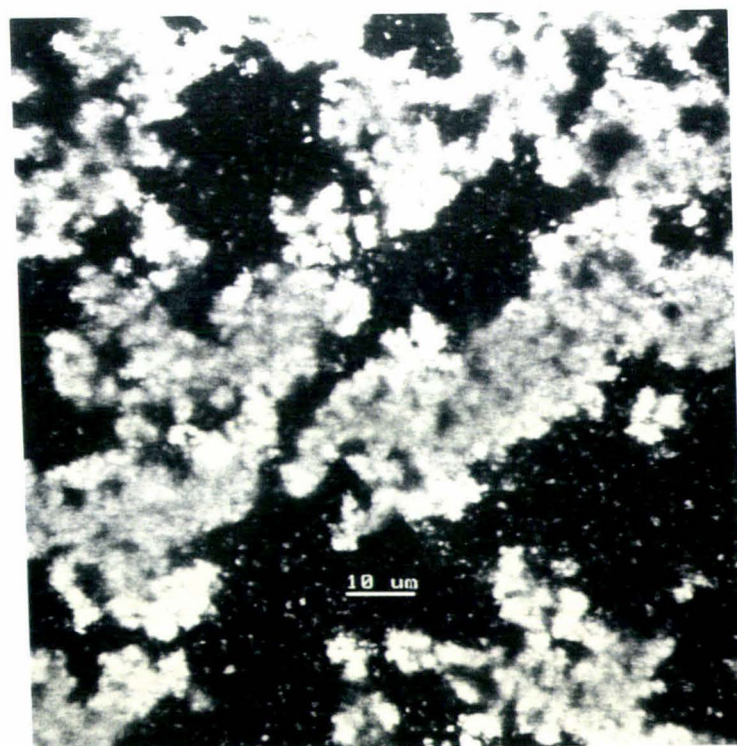
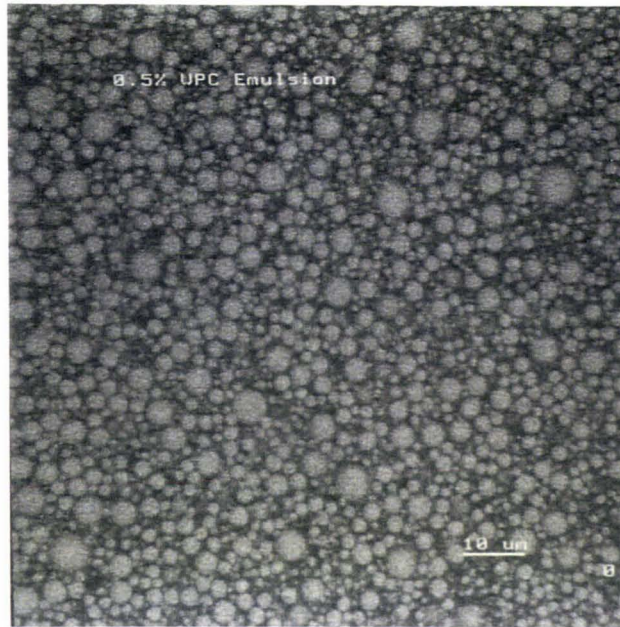C

D
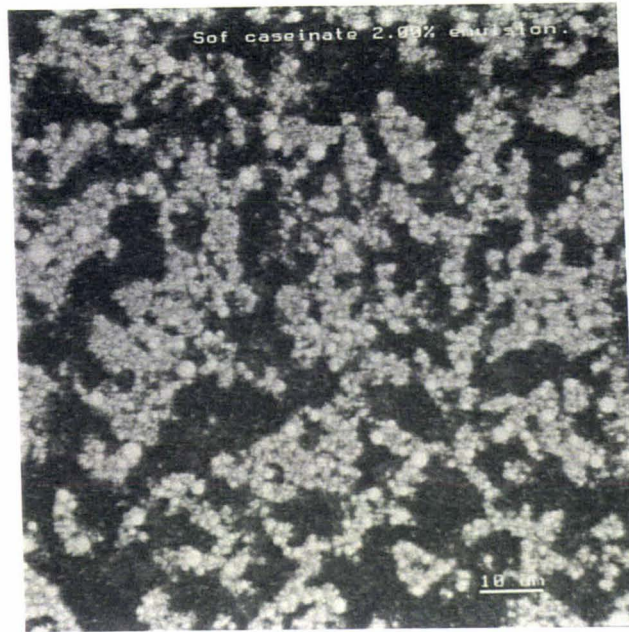
Figure 4.5: (Continued).
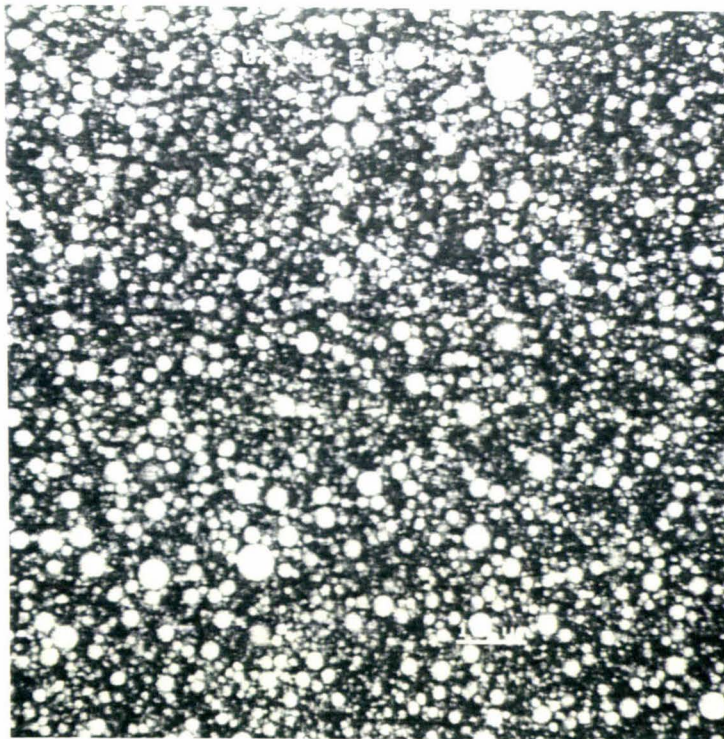
Figure 4.5: (Continued).

A

B

Figure 4.6: Confocal micrographs of oil-in-water emulsions (30% soya oil, pH 7.0) stabilized by 0.5% (A) and3% (B) WPC.
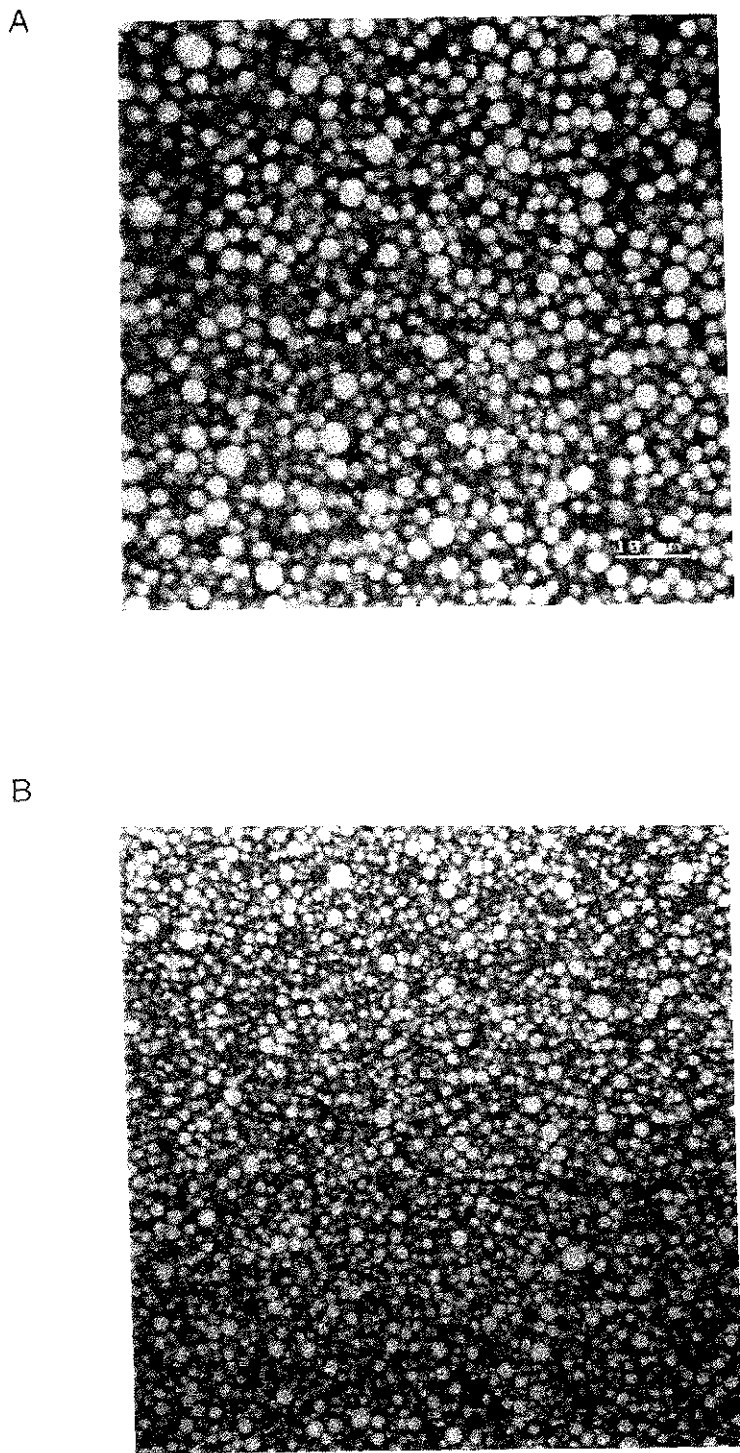
A

B

Figure 4.7: Confocal micrographs of oil-in-water emulsions (30% soya oil, pH 7.0) made with 0.5% (A), and 3% (B) calcium caseinate.

4.2.4 Composition of adsorbed protein layer at the oil/water interface

Emulsions were made using different concentrations of sodium caseinate or calcium caseinate (0.33 to 5.0%). The changes in the proportions of individual caseins in the cream phases of emulsions are shown in Figure 4.8. In the cream phase of emulsions made with sodium caseinate, the proportions of β-casein increased from $\sim$ 50 to $\sim$ 63%, and were higher than that in the original sodium caseinate, as the caseinate concentration was increased from 0.33 to 1.33%. The relative proportions of α_s-casein (α_{s1} + α_{s2}-casein) decreased from $\sim$ 32 to $\sim$ 19% and there were lower than that in the original caseinate. The proportions of κ-casein did not change and were close to that in original sodium caseinate at this range of concentrations. It indicated that the adsorption of β-casein occurred in preference to the adsorption of α_s-casein at low caseinate concentrations. However, when the caseinate concentrations were higher than 1.33%, the proportions of β-casein at interface decreased markedly (to $\sim$ 14% at 5% caseinate); the proportions of α_s-caseins increased to $\sim$ 52%, while the proportions of κ-casein also increased to $\sim$ 33%. The adsorption of α_s-caseins occurred in preference to β-casein at concentrations > 2.6% (Figure 4.8 A).

Figure 4.9 shows that the estimated surface concentrations of individual caseins changed with caseinate concentration. Maximum adsorption of β-casein occurred at caseinate concentrations of 1 to 2%. In contrast, the adsorption of α_s-caseins and κ-casein increased with increase in caseinate concentration.

The adsorption of α_s-casein at interface occurred largely in preference to adsorption of β-casein in emulsion made with calcium caseinate at all concentrations (Figure 4.8 B). The proportions of κ-casein ($\sim$ 8%) at interface was lower than the proportion in original calcium caseinate ($\sim$ 14%), which is consistent with the results of Srinivasan *et al* (1999). The proportions of α_s-casein, β-casein and κ-casein adsorbed at the interface remained unaffected by protein concentration.

The proportions of α-lactalbumin and β-lactoglobulin at the interface of emulsion droplets formed with WPC did not change with WPC concentration (Figure 4.10), However, the proportions of α-lactalbumin (~ 18%) at the interface were slight lower, and the relative proportions of β-lactoglobulin (~ 82%) were slight higher than that in the original WPC. The proportions of α-lactalbumin and β-lactoglobulin in original WPC used in this experiment were ~ 23% and ~ 77%, respectively. It indicated that there was a slight preference for adsorption of β-lactoglobulin. This result is agreement with that of Closs *et al.* (1993), who found that β-lactoglobulin was adsorbed in preference to α-lactalbumin on the oil droplets, in emulsions formed with whey proteins or mixtures of β-lactoglobulin and α-lactalbumin. However, no preferential adsorption between β-lactoglobulin and α-lactalbumin was observed in other works (Dickinson *et al.*, 1989; Hunt and Dalgleish, 1994; Euston *et al.*, 1996).
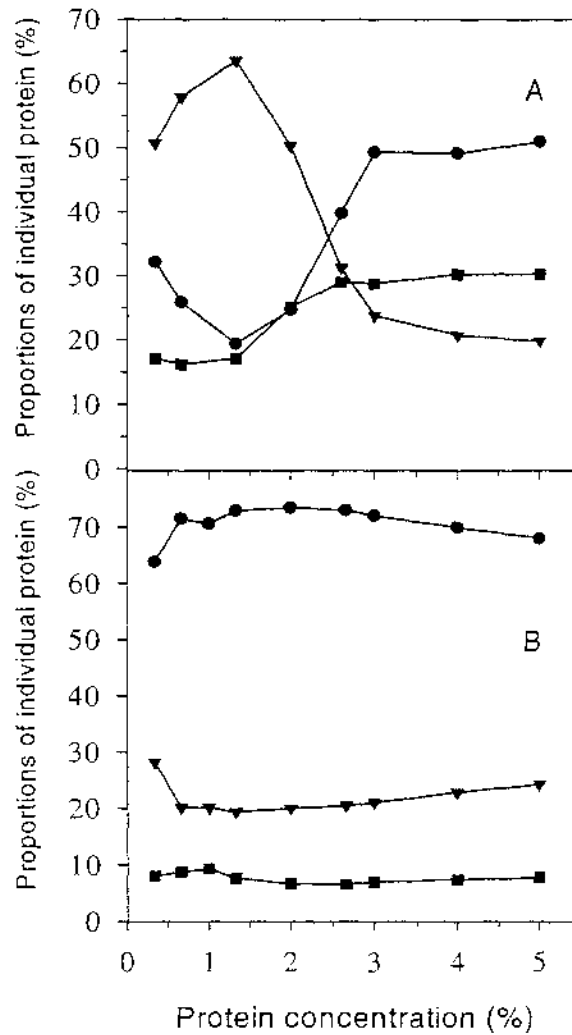
Figure 4.8: The effect of protein concentration on the relative proportions of α_s-casein (●), β-casein (▼) and κ-casein (■) at the droplet surface of emulsions made with 30% soya oil, pH 7.0, and sodium caseinate (A) or calcium caseinate (B). Each data point is the average of determinations on three separate emulsions.
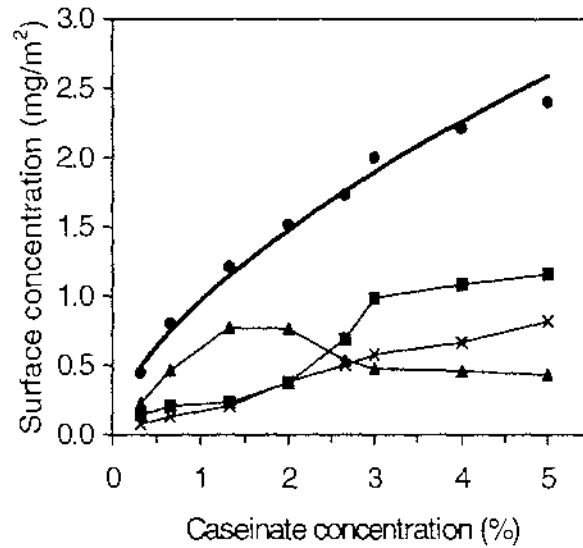
Figure 4.9: The estimated surface protein concentrations of α_s-casein (■), β-casein (▲) and κ-casein (×) in the cream phase of emulsions made with varying concentrations of sodium caseinate, and 30% soya oil, pH 7.0. Total surface protein concentrations (●).
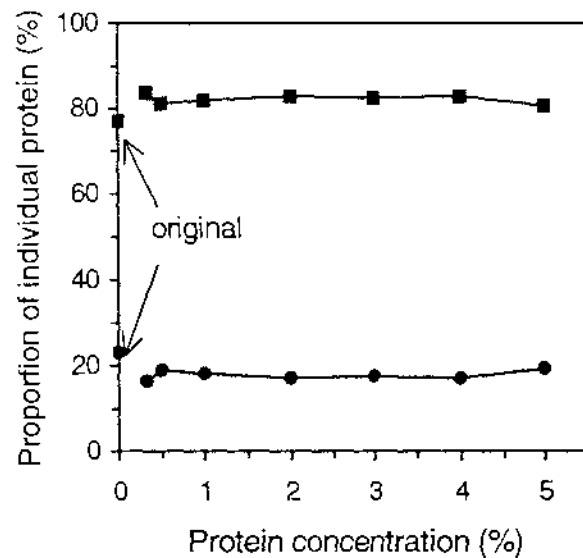

Figure 4.10: The effect of protein concentration on the relative proportions of α-lactalbumin (■) and β-lactoglobulin (●) at the droplet surface of emulsions made with 30% soya oil, pH 7.0, and WPC. Each data point is the average of determinations on two separate emulsions.

4.2.5 Mixtures of sodium caseinate and WPC

The proportions of sodium caseinate and WPC in the interfacial protein layers of emulsion droplets made with caseinate and WPC (1:1) at different concentrations of protein are shown in Figure 4.11. Whey protein adsorbed in preference the caseinate at protein concentrations below 3%. In contrast, the proportions of whey proteins on the interface were lower than that of caseinate at higher protein concentrations.

The estimated surface concentrations of caseinate and whey protein on the interface of emulsions made with binary protein mixture as a function of protein concentration are shown in Figure 4.12. It appears that the increase in whey proteins on the interface was mainly responsible for the increase in total surface protein concentration at protein concentration < 2%, whereas higher total surface coverage was because of increase in surface concentration of caseinate at > 2% protein. Similar trend of preferential adsorption of caseins in emulsions made with binary mixture of sodium caseinate and whey protein at high protein concentration has been observed by Hunt and Dalgleish (1994); however, they found that there was no difference in the adsorption of caseins and whey protein on the interface at low concentration of protein mixture.

Figure 4.13 shows the proportions of individual proteins on the surface of emulsion droplets made with mixture of caseinate and WPC. At low concentration of total protein ($\leq$ 1%), the proportions of adsorbed β-lactoglobulin and α-lactalbumin were higher than that in original protein mixture, while the proportions of all caseins were lower than that in the original protein, with β-casein being present in greater proportion. The proportion of β-lactoglobulin increased to a maximum at 2.0%, then decreased; α-lactalbumin and β-casein decreased and then remained constant; whereas α_s-casein and κ-casein increased with increase in concentration of protein mixture.

The changes in estimated surface concentration of these individual proteins in emulsions formed with binary protein mixture as a function of protein mixture

concentration is shown in Figure 4.14. The surface concentrations of α_s-casein and κ-casein increased with increase in protein concentration in the mixture; that of β-casein and α-lactalbumin almost remained constant and were very low. The surface concentration of β-lactoglobulin increased markedly with increasing protein concentration to 2%, remained constant at 2 to 4%, then increased again with further increase in protein concentration in the mixture.
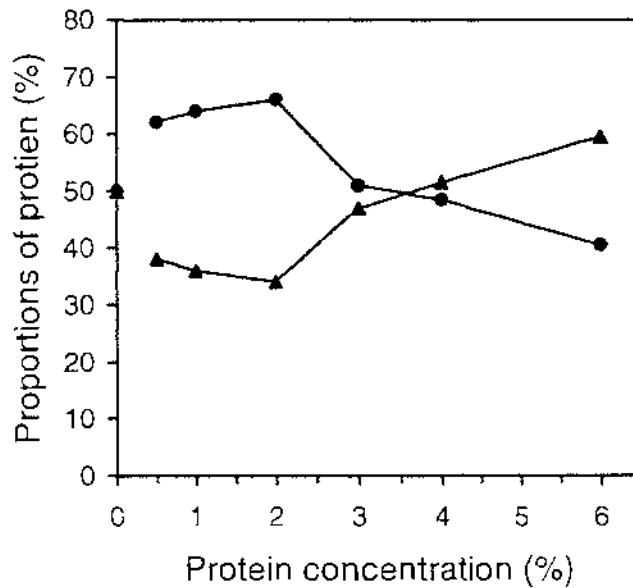

Figure 4.11: The proportions of caseinate (▲) and whey protein (●) at the droplet surface of emulsions made with binary mixture of sodium caseinate and WPC (1:1), 30% soya oil, pH 7.0, as a function of protein mixture concentration. Each data point is the average of determinations on two separate emulsions.

Figure 4.12: Changes in estimated surface concentrations of caseinate (▲) and whey protein (●) in the cream phase of emulsions made with protein mixtures of sodium caseinate and WPC (1:1), and 30% soya oil, pH 7.0, as a function of protein mixture concentration. Total surface protein concentration (◆).

Figure 4.13: Changes in the relative proportions of α_s-casein (◆), β-casein (●), κ-casein (▲),α-lactalbumin (□) and β-lactoglobulin (△) at the droplet surface of emulsions made with binary mixture of sodium caseinate and WPC (1:1), 30% soya oil, pH 7.0, as a function of protein mixture concentration. Each data point is the average of determinations on two separate emulsions.
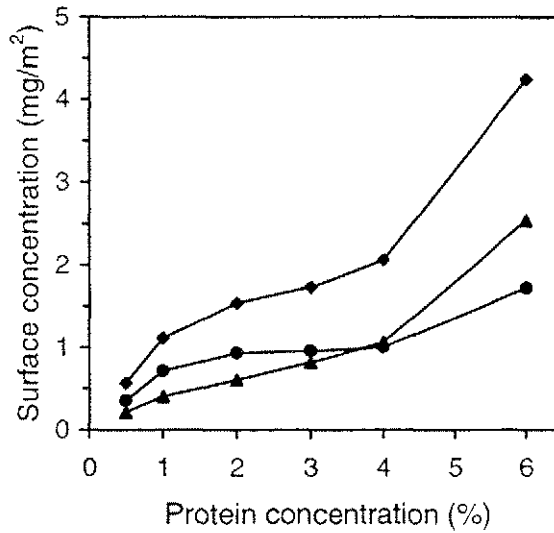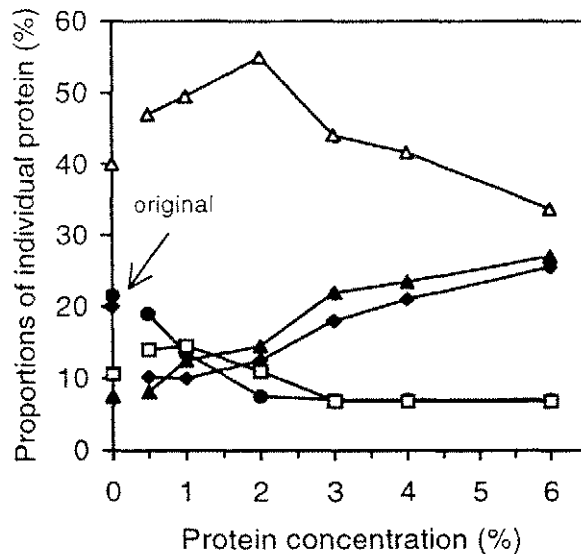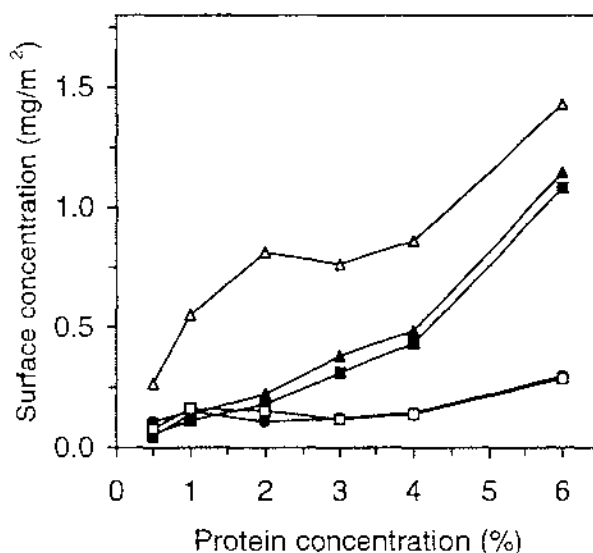
Figure 4.14: Changes in estimated surface concentrations of α_s-casein (■),β-casein (●),κ-casein (▲),α-lactalbumin (□) and β-lactoglobulin (△) in the cream phase of emulsions made with binary mixture of sodium caseinate and WPC (1:1), and 30% soya oil, pH 7.0, as a function of protein mixture concentration.

4.2.6 Creaming stability of emulsions made with binary protein mixture

The stability rating of emulsions formed with mixture of sodium caseinate and WPC (1:1) as a function of total protein concentration is shown in Figure 4.15. For comparison, this figure also shows the stability rating of emulsions made with caseinate or WPC alone. It was shown that the stability of emulsions formed with protein mixtures reached a maximum at 2%, and then decreased markedly with increasing the total protein concentration further. The trend in stability with concentration was similar to that of sodium caseinate emulsions, except that the plots shifted to higher concentration (about 2 fold). The stability rating values of 4% binary protein mixture emulsion was similar to that of 2% sodium caseinate emulsion. In addition, results obtained at 3 and 6% binary protein concentrations were comparable with 1.5 and 3% of sodium caseinate (Figure 4.15). It indicated that the stability of emulsions would be similar if

emulsions made with sodium caseinate alone or binary protein mixture (caseinate and WPC) contained identical concentration of sodium caseinate, and that the whey protein in the system did not affect the stability of emulsions. This suggests that depletion flocculation of emulsions caused by non-adsorbed caseinate in aqueous phase will occur as caseinate reaches critical concentration whether or not whey protein exists in system; whey protein, involved in formation of emulsions, did not affect the depletion flocculation phenomenon.

Figure 4.16 shows the confocal micrographs of emulsions made with binary mixture of WPC and sodium caseinate (1:1) at various concentrations. The aggregated network structure with respect to flocculation formed when concentrations of protein mixture were higher than 3%, corresponding with decrease in stability rating of emulsions (Figure 4.15).
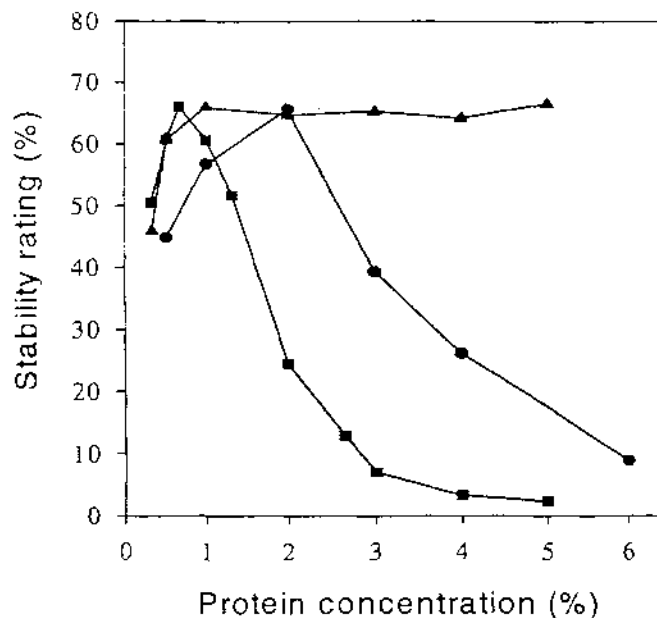

Figure 4.15: The stability rating (%) of emulsions made with binary mixture of sodium caseinate and WPC (1:1) (●), sodium caseinate (■) or WPC (▲), and 30% soya oil, pH 7.0, as a function of protein concentration. Each data point is the average of determinations on two separate emulsions.
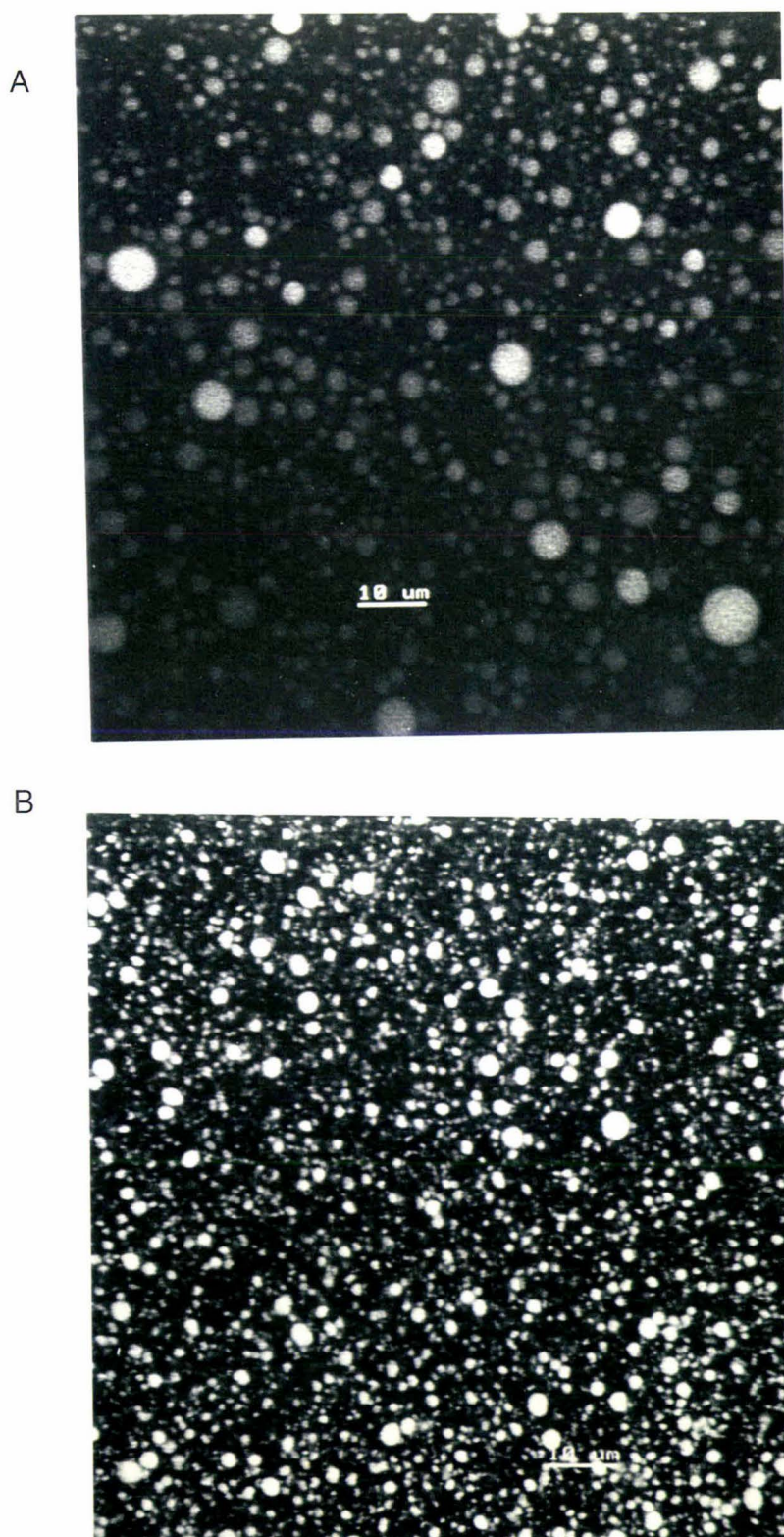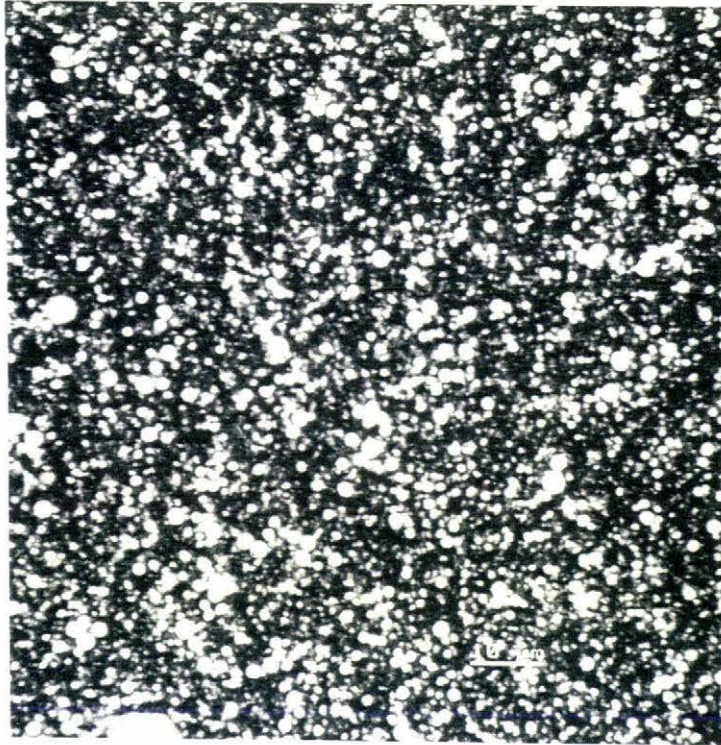
Figure 4.16: Confocal micrographs of oil-in-water emulsions (30% soya oil, pH 7.0) made with 0.5% (A), 1% (B), 2% (C), 3% (D), 4% (E) and 6% (F) binary mixtures of sodium caseinate and WPC (1:1).

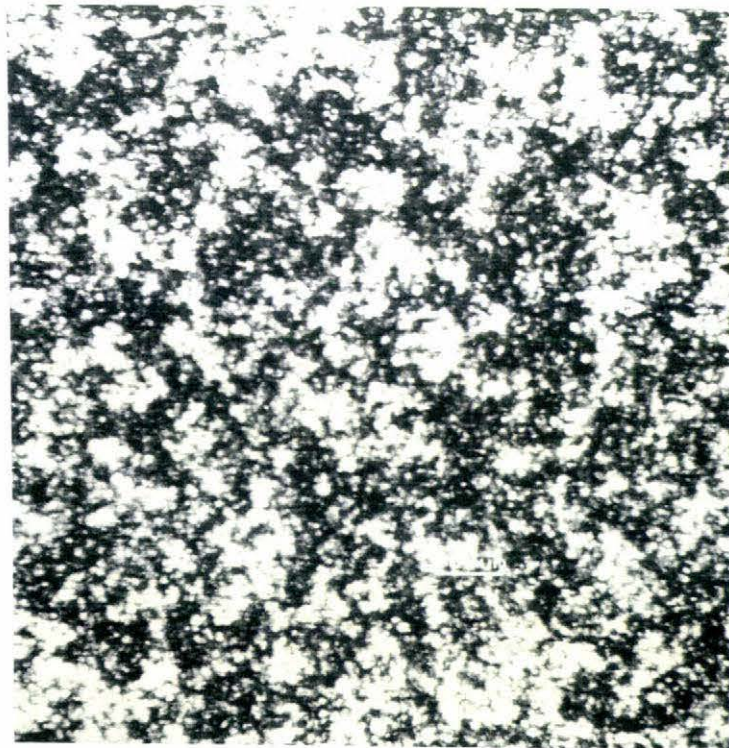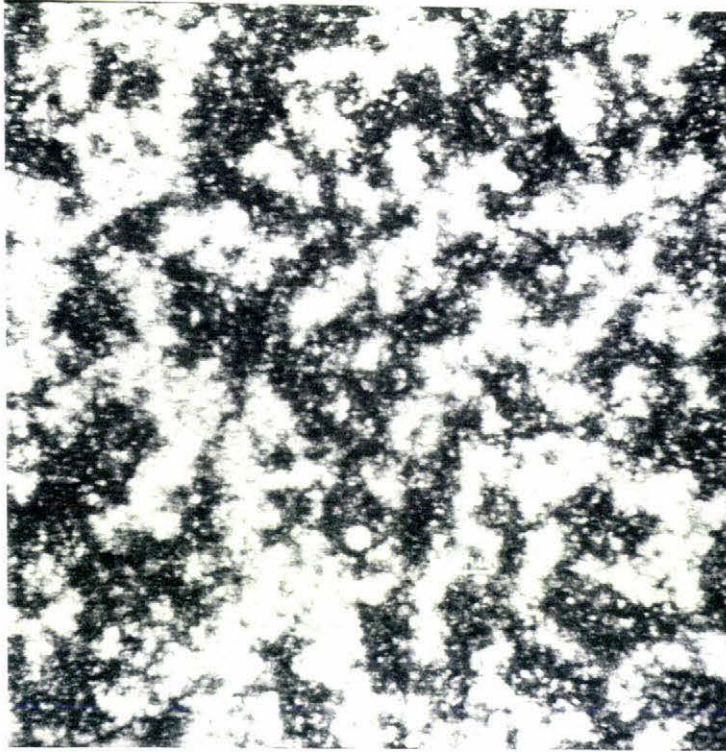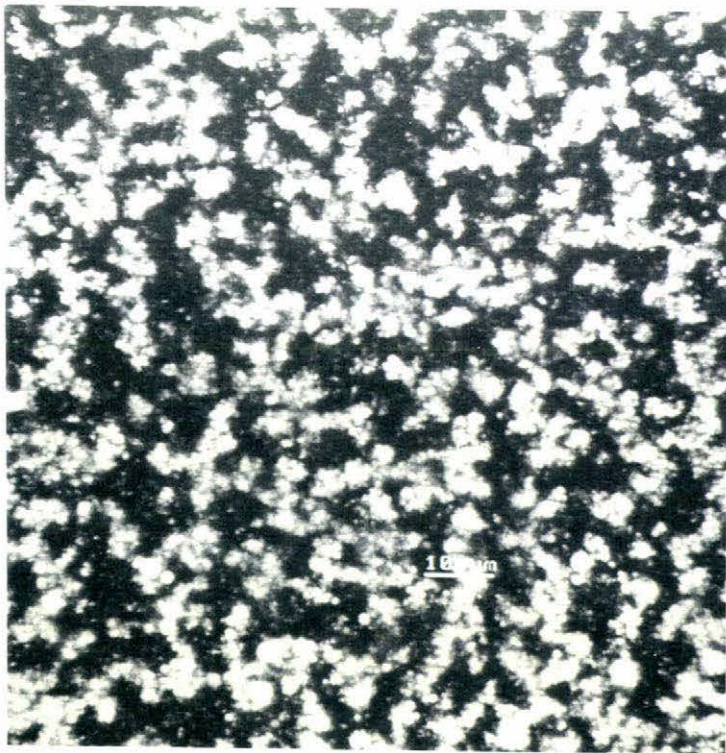**Figure. 4.16.** (Continued).

E

F

Figure. 4.16. (Continued).

4.3 Discussion

The droplet sizes of emulsions made with sodium caseinate and WPC were similar and were independent of the protein concentration 1.0% (Figure 4.1). This result is in agreement with previous reports (Fang and Dalgleish, 1993 a; Hunt and Dalgleish, 1994, Srinivasan *et al.,* 1996). Dalgleish (1995) described that whey proteins, α-lactalbumin or β-lactoglobulin, singly or in combination, are excellent emulsifying agents, and their emulsions are only a little less stable than those produced using caseins. For emulsions prepared under the same conditions (concentration of oil and protein, pH, homogenization pressure), the droplet sizes in the whey protein emulsions were found to be somewhat larger than those in the casein-stabilized emulsions when the concentration of protein is low (< 1%) (Figure 4.1). The droplet sizes mainly depends on the energy density of homogenization process (Tornberg and Lundh, 1978; Haque and Kinsella, 1989; Srinivasan *et al.,* 1996). The effects of protein concentration on droplet size of emulsions depend on the protein source and its emulsifying capacity. The emulsions stabilized by calcium caseinate had larger d_{32}, (Figure 4.1), which may be due to bridging flocculation between emulsion droplets, especially at low protein concentrations (Srinivasan *et al.,* 1999). It appears that the emulsifying capacity of this protein is lower because Ca^{2+} binding with caseins lead to formation of caseins aggregates and micelle-like particles in the calcium caseinate solution.

The increase in surface protein coverage with increase in protein concentration up to ~ 1% (Figure 4.2) may suggest that there was not enough protein to cover the new surface of oil droplets during homogenization. The present results are consistent with those of Hunt and Dalgleish (1994), although lower surface concentration were obtained in the present study at each given protein concentration. The surface concentration of sodium caseinate was lower than WPC at low protein concentrations, which may be attributed to greater flexibility of caseins than the globular whey proteins, i.e. the caseins can spread more extensively to cover more surface area than the whey protein molecules. It means that the adsorbed whey protein molecules on the surface are likely to be

closer to each other than casein molecules, although whey proteins also unfold to some extent (Dalgleish, 1996), once adsorbed to the interface of oil droplets. The surface concentration of whey proteins (Figure 4.2, Figure 4.12 and Figure 4.14), remained constant at ~ 1 to ~ 3% protein. This could be explained by assuming that saturated monomolecular layer was formed on the surface in this concentration range. The slight increase in surface concentration, if any, may be a result of closer packing of the adsorbed proteins in the monomolecular layer as protein concentration is increased. Significant further increase in surface coverage probably indicates the formation of protein mutilayers on the droplet surface at high concentrations.

The trend of surface coverage in sodium caseinate emulsions with protein concentration was quite different from that of WPC (Figure 4.2), in which there was no obvious period of saturated monomolecular layer. This may be because sodium caseinate exists in aqueous solution as small casein aggregates at neutral pH. The extent of casein aggregation increases with increasing the concentration of protein (Pepper and Farrell, 1982). It is possible that caseins are adsorbed to the droplet surface as aggregated structures (sub-micelles) (Euston *et al.,* 1995, 1996). Therefore, there does not appear to be the period of saturated adsorbed monomolecular layer like whey proteins and the surface concentration is greater that of whey proteins at high protein concentrations (Figure 4.2, Figure 4.12). This is supported by the results obtained for the composition of adsorbed protein layer in emulsions made with sodium caseinate (Figure 4.8, Figure 4.13). The preferential adsorption of β-casein, due to its high surface activity, seems to exist only at low concentrations (< 1%) where all caseins may exist as monomeric molecules. The adsorption of α_s-casein increased with increasing protein concentration. This may be explained by assuming that β-casein occupies more surface, as a molecule or smaller particle, due to its high surface activity, and α_s-casein and κ-casein may occupy less by forming larger particles; consequently, this will results in the amount of α_s-casein and κ-casein at the surface being larger than β-casein. The behaviour of the aggregated casein particles during homogenization may be an important

factor to result in above possibility due to different self-association mechanisms of individual caseins. There are two possibilities: one is that aggregated particles of β-casein are dissociated, while aggregated particles of α_s-casein and κ-caseins survive under the high shear, turbulent condition in the homogenizer. Another is that all casein aggregates are dissociated and subsequently α_s-casein and κ-casein associate again before adsorption at the surface, but β-casein does not. In addition, the high adsorption of α_s-casein and κ-casein may be related to fact that α_{s2}-casein and κ-casein may form inter- or intramolecular disulphide bonds at the surface. Previous workers considered that it was because the β-casein lose its surface activity and competitive ability due to aggregation in solution at high concentrations (Euston *et al.*, 1995, 1996; Srinivasan *et at.*, 1996, 1999).

In emulsions stabilized by binary mixture of sodium caseinate and WPC (Figure 4.12), trends of surface concentration of caseinate and whey protein were similar to the result for emulsions stabilized by each protein individually (Figure 4.2). But from Figure 4.11 and Figure 4.12, we can only know that the amount of whey protein at the surface was larger than caseinate at low protein concentrations whereas caseinate was present in greater amounts at high concentration ($\geq$ 4%). We are not able to judge the area occupied by each protein. The different amounts of adsorbed individual proteins at the surface (Figure 4.13 and Figure 4.14) may be mainly attributed to different state of protein molecular structure at the surface. Greater adsorption of whey proteins at low concentration may be due to less spreading of globular whey protein molecules on the surface, especially β-lactoglobulin may adsorb at the surface by a dimer structure (Mackie *et al.*, 1993). High adsorption of caseins (α_s- and κ-) at high protein concentration may be because they adsorb as aggregated structures on the surface, whereas β-casein and α-lactalbumin may adsorb in a monomolecular state. The situation may be similar to the observations of Sharma *et al.* (1996) on recombined milk, in which only ~ 10% whey protein in total adsorbed protein covered ~ 33% of the total surface area. In contrast, Hunt

and Dalgleish (1994) stated that the competitive adsorption between whey protein and caseinate was related to their different adsorption affinity at surface.

The linear increase in the surface coverage of emulsions made with calcium caseinate (Figure 4.2) with protein concentration may be because there was not enough protein available to cover the surface which was probably due to aggregation of caseins induced by Ca^{2+} binding. Alternatively, the greater aggregation due to increasing calcium caseinate concentration in solution may result in high surface concentration. The amount of larger aggregated particles in calcium caseinate solution increases with increase in protein concentration (Figure 4.17). The preferential adsorption of α_s-casein observed in calcium caseinate emulsions may be explained by that the aggregated protein particles adsorbed to the surface were rich in α_s-casein. The α_s-casein is more sensitive to Ca^{2+} (Dalgleish and Parker, 1980) and high proportion of α_s-casein existed in the large, sedimentable protein aggregates of calcium caseinate solution (Srinivasan *et al.*, 1999).

The creaming stability of emulsions formed with sodium caseinate, WPC or calcium caseinate increased with increasing protein concentration at low protein concentrations (Figure 4.3); this is mainly due to the decrease in the particle size of emulsions. However, the dramatic decrease in the creaming stability of sodium caseinate emulsions as concentration is increased may be attributed to depletion flocculation occurring in these emulsions. Dickinson and Golding (1997) reported that the extent of depletion flocculation is mainly dependent on the size and concentration of unbound or unadsorbed protein; the caseinate sub-micelles (15 - 20 nm) at 2 – 3% concentration in aqueous phase are responsible for the initial discernible flocculation in their case. In Asakura-Oosawa theory, the case of rigid spherical molecules in the gap between a pair of particle surfaces, depletion potential has the form:

$$U_d(h) = \begin{cases} -(3kTR\phi_s/d^3)(d-h)^2. & (h < d) \\ 0 & (h \geq d) \end{cases}$$

where R is the particle radius, d is molecular diameter, h is gap and ϕ_s is solute volume fraction in bulk medium (Asakura and Oosawa, 1954). For the size of emulsion droplets (R) of 0.5 μm, the caseinate sub-micelles (d) (15-20 nm) and 2 − 3% (ϕ_s = 0.02 - 0.03) concentration in aqueous (h = 0), the $U_d(h)$ ≈ 3KT. This value is sufficiently large enough to enhance flocculation and substantially change creaming stability (Dickinson, 1996). Any change in the molecular diameter (d) and solute volume fraction (ϕ_s) in aqueous phase will change the extent of flocculation.
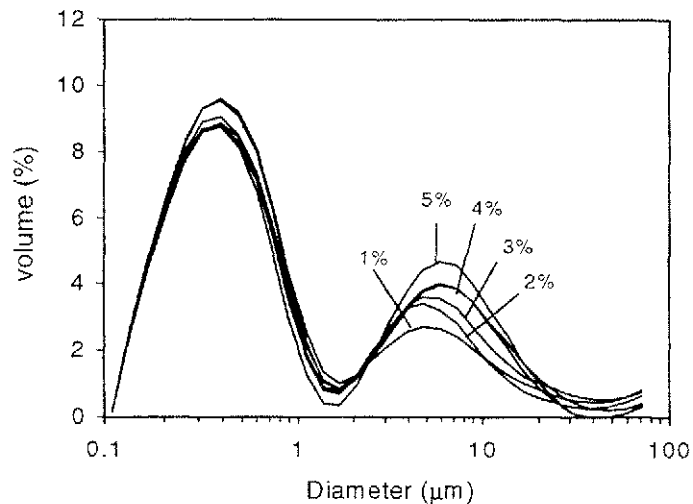

Figure 4.17: The particle size distribution of the calcium caseinate solution, pH 7.0 as a function of caseinate concentration, determined by MasterSizer.

This present results are consistent with Asakura-Oosawa theory. The depletion flocculation did not occur in emulsions made with WPC and calcium caseinate (Figure 4.3). This could be mainly because there were no suitable size of protein particles in the aqueous phase. Whey proteins exist in a molecular state in solution, the d may smaller than h, $U_d(h)$ = 0 in Asakura-Oosawa theory. In contrast, the large aggregated particle (d > 1μm) (Figure 4.17) are formed due to the binding of Ca^{2+} to caseins in calcium caseinate solution. The $U_d(h)$ ≈ 0.03KT as d is 1 μm, which is negligible and depletion flocculation does not occurred at this condition.

The creaming stability behaviour (Figure 4.15) of emulsions formed with binary protein mixtures demonstrates that the depletion flocculation is dependent on the amount of unadsorbed caseinate particles in the aqueous phase and that the whey protein molecules do not retard the instability of emulsions.

4.4 Conclusions

The droplet sizes of emulsions made with sodium caseinate or WPC were similar and were independent of the protein concentration at concentration above 0.5%. The surface protein concentration of emulsions made with sodium caseinate, WPC or calcium caseinate generally increased with increase in the protein concentration, although the trends were different. The emulsions made with calcium caseinate had higher d_{32} and surface protein concentration than that of sodium caseinate or WPC. In emulsions made with sodium caseinate at low protein concentrations, the adsorption of β-casein occurred in preference to α_s-casein, whereas α_s-(α_{s1}- + α_{s2}-)casein was found to adsorb in preference to β-casein at high protein concentrations. In calcium caseinate emulsions, the α_s-casein was adsorbed in preference to β-casein at all concentrations. In emulsions made with WPC, β-lactoglobulin adsorbed slightly in preference to α-lactalbumin. In emulsions made with mixtures of sodium caseinate and WPC (1:1), the adsorption of whey proteins occurred in preference to caseins at low concentrations (< 3%), whereas caseins were adsorbed in preference to whey protein at high concentrations. These results suggest that the individual proteins adsorbed at the interface of oil droplets as the aggregation state in protein solutions for making emulsions.

In emulsions made with calcium caseinate or WPC, the creaming stability of emulsions was mainly dependent upon the particle size of emulsions. However, the creaming stability of emulsions made with sodium caseinate decreased markedly as the caseinate concentrations were increased above 2.0%. This was attributed to depletion flocculation occurring in these emulsions. Whey proteins did not retard this instability, due to depletion flocculation, in emulsions made with mixtures of caseinate and WPC.

Chapter 5
INFLUENCE OF CaCl₂ ADDITION ON THE PROPERTIES OF EMULSIONS STABILIZED BY SODIUM CASEINATE

5.1 Introduction

The binding of calcium ion to caseins have been studied under a variety of environmental conditions (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981). The binding of Ca^{2+} to protein reduces the electrostatic repulsion and interaction of the hydrophobic domains, leading to formation of large aggregates (Swaisgood, 1992). This property will inevitably influence the adsorption behaviour of milk proteins at the oil/water interface and the stability of emulsions stabilized by milk proteins. Previous workers have shown that the adsorption behaviour of caseinate at the oil/water interface and the stability of emulsion stabilized by caseinate are significantly influenced by the state of aggregation of protein (Mulvihill and Murphy, 1991; Srinivasan *et al.*, 1996). Srinivasan *et al.* (1996) reported that addition of Ca^{2+} to caseinate solution prior to emulsion formation led to increase the droplet size and the surface protein concentration of emulsions. Mulvihill and Murphy (1991) reported that the high aggregated calcium caseinate had higher protein load at the interface of emulsion droplets. The association of Ca^{2+} protein-coated oil droplets also results in aggregation of emulsion droplets (Dickinson *et al.*, 1992; Agboola and Dalgleish, 1995).

Normally, the droplet size distribution, can reflect the stability of emulsion, but the changes in the creaming stability of emulsion stabilized by protein does not completely follow the droplets size (see Chapter 4). The state and concentration of both adsorbed and non-adsorbed protein, besides the droplet size of emulsions, are important factors that influence the stability of emulsions (Mulvihill and Murphy, 1991; Dickinson and Golding, 1997; Syrbe *et al.*, 1998). Results shown in Chapter 4 demonstrate that emulsions made with high caseinate concentrations (> 2%) were found to be more unstable than emulsions made with low caseinate concentrations. The destabilization was

attributed to so called 'depletion flocculation' which occurred due to the high concentration of non-adsorbed caseinate (Dickinson and Golding, 1997).

Little information is available on the details of influence of Ca^{2+} on the adsorption behaviour of caseinate at oil/water interface and stability of resulting emulsions (Agboola and Dalgleish, 1995). No previous studies have been reported on the effects of Ca^{2+} on the composition of protein interfacial layer and creaming stability of emulsions stabilized by caseinate.

The objective of this work was to study the effects of addition of $CaCl_2$ into the caseinate solutions prior to making the emulsions or to emulsions made in the absence of Ca^{2+} on the adsorption behaviour of caseinate and the stability of emulsions made with sodium caseinate. The relationship between aggregation state of caseins, due to Ca^{2+} binding, and their emulsifying properties is discussed. In addition, it is known that ionic strength influences the binding of Ca^{2+} to caseins (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981). Therefore, emulsions with added NaCl and containing various concentrations of Ca^{2+} were also studied. The confocal microscopy was used to observe the microstructure of emulsions for examining the relationship between structure and creaming stability of emulsions.

5.2 Results

5.2.1. Emulsion formation

Emulsions were made with 0.5 or 3% sodium caseinate, 30% soya oil at pH 7.0. Various concentrations of $CaCl_2$ were added into the caseinate solution prior to making the emulsion or to emulsions made in the absence of Ca^{2+}. 0 or 200 mM NaCl was added to emulsions after formation.

5.2.2.Average particle size and particle size distribution

The effects of the presence of $CaCl_2$ during emulsion formation or addition of $CaCl_2$ to the emulsions made in the absence of $CaCl_2$, on the average diameter (d_{43}) of emulsion droplets, made with 0.5 or 3.0% sodium caseinate at pH 7.0, are shown in Figure 5.1 and Figure 5.2. For emulsions made with 0.5% sodium caseinate (Figure 5.1), the d_{43} increased from 0.99 to 1.72 µm when the concentration of $CaCl_2$ (added to the caseinate solution) increased from 0 to 3 mM. The d_{43} of emulsions increased abruptly to 40.5 and 52.3 µm as the concentration of $CaCl_2$ increased to 4 and 5 mM. Addition of 200 mM NaCl to these emulsions had no significant effect on the d_{43} of emulsions containing up to 3 mM $CaCl_2$. However, the d_{43} of emulsions containing 4 and 5 mM $CaCl_2$ decreased markedly upon addition of NaCl.

When $CaCl_2$ was added to emulsions, made in the absence of $CaCl_2$, the d_{43} did not change with increase in $CaCl_2$ up to 2 mM, but increased significantly from 1.0 to 6.6 µm with further increase in $CaCl_2$ (from 3 to 5 mM). The d_{43} reduced to ~1.3 µm when 200 mM NaCl was added to emulsions (Figure 5.1 B).

In emulsions made with 3.0% sodium caseinate, the d_{43} remained unaffected with increase in $CaCl_2$ concentrations up to 15 mM, but increased markedly (from 0.81 to 30.8 µm) with further increase in $CaCl_2$ to 20 mM. Addition of 200 mM NaCl to emulsions did not significantly change the d_{43}, except in emulsions containing 18 or 20 mM $CaCl_2$ where a small decrease in d_{43} values was observed (Figure 5.2 A),

When CaCl$_2$ was added to emulsions made in absence of CaCl$_2$, the d_{43} increased gradually from 0.81 to 7.3 μm with increase in CaCl$_2$ from 0 to 20 mM. The d_{43} reduced to 0.81 μm when 200 mM NaCl was added to emulsions, except that emulsions containing 20 mM CaCl$_2$ still had higher d_{43} value (2.25 μm) compared with the control (no CaCl$_2$ or NaCl) (Figure 5.2 B).

The average particle size of caseinate emulsions where CaCl$_2$ was added before homogenization did not change with time by dilution and stirring in the MasterSizer, whereas it decreased with time for emulsions in which CaCl$_2$ was added after homogenization. The d_{43} decreased from 4.6 to 1.6 μm for emulsion containing 4 mM CaCl$_2$ or from 6.6 μm to 2.4 μm for emulsion with 5 mM CaCl$_2$ added, when the emulsions were stirred 5 min in the MasterSizer. Dickinson *et al* (1992) also reported that the aggregation of particles in emulsions due to calcium added after homogenization was reversible upon dilution.

Figure 5.3 shows the droplet size distributions of emulsions made with 0.5 or 3.0% caseinate, in which CaCl$_2$ was added prior to emulsion formation. The size distribution of droplets in emulsions made with both 0.5 and 3.0% caseinate was monomodal until a critical CaCl$_2$ concentration (4 mM in 0.5%, 20 mM in 3.0%). It indicated that there was no aggregation of droplets. Strong aggregation of droplets was induced by further increase in the concentration of CaCl$_2$ (Figure 5.3), in which the original particles were extensively aggregated.

In comparison with the above changes, the droplet distribution of emulsions in which CaCl$_2$ was added after emulsification showed a general reduction in the proportion of emulsion droplets in the original size range and an increase in proportion of large particles (Figure 5.4). This gave a bimodal size distribution as concentration of CaCl$_2$ was increased. However, the shape of the size distribution of small particles hardly changed although some very large particles were formed from aggregation of small ones. The difference in the emulsions made with 0.5 and 3.0% caseinate was merely the difference in critical

concentration of $CaCl_2$ required to change the particle size distribution (2 mM for 0.5%, 10 mM for 3.0%). This result is similar to that obtained by Agboola and Dalgleish (1995).

To investigate whether the changes in particle size distributions were due to coalescence or flocculation, the emulsions were diluted in 2% SDS solution before particle size measurement. The particle size distributions of unstable emulsions (i.e. increased particle sizes), due to addition of 5 mM $CaCl_2$ prior to emulsion formation, returned to monomodal distribution like that of emulsion containing 3 mM $CaCl_2$ (Figure 5.5 A). But they did not completely return to that of emulsion made in the absence of $CaCl_2$. It indicates that the formation of large particles was due to flocculation based on larger droplets formed by recoalescence during homogenization. However, in emulsions that had 5 mM $CaCl_2$ added after emulsion formation, the size distribution returned to that of original emulsion in the absence of Ca^{2+} (Figure 5.5 B), suggesting flocculation was responsible for increased particle size.
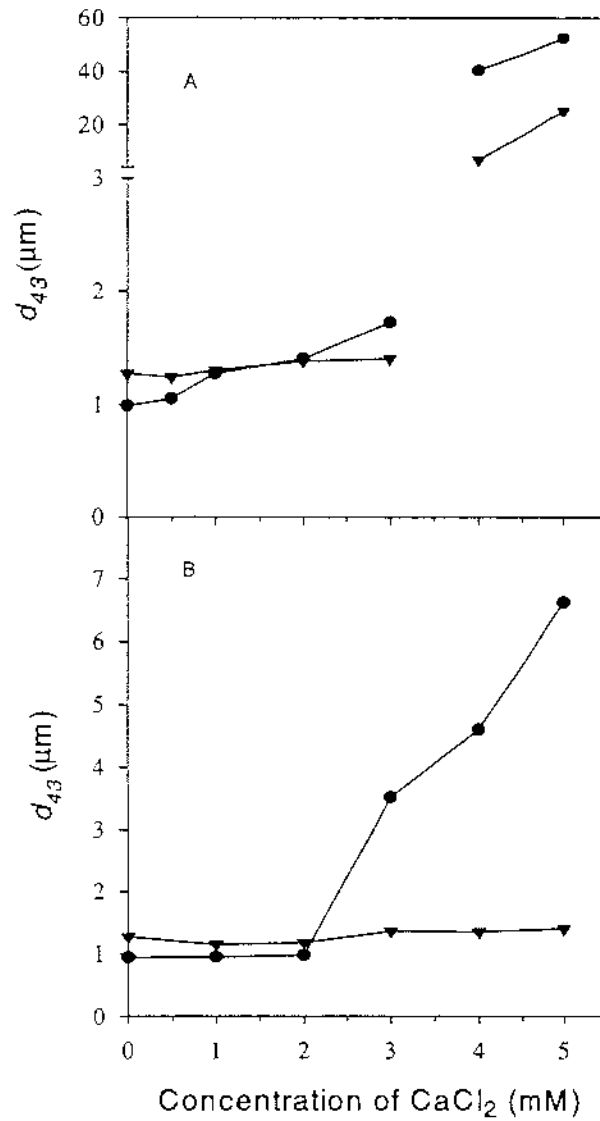
Figure 5.1: Changes in average particle diameter (d_{43}) (●) as a function of CaCl$_2$ concentration in emulsions made with 30% oil and 0.5% sodium caseinate. CaCl$_2$ was added before (A) or after (B) emulsion formation. 200 mM NaCl added to the emulsions at each CaCl$_2$ concentration (▼). Each data point is the average of determinations on two separate emulsions.
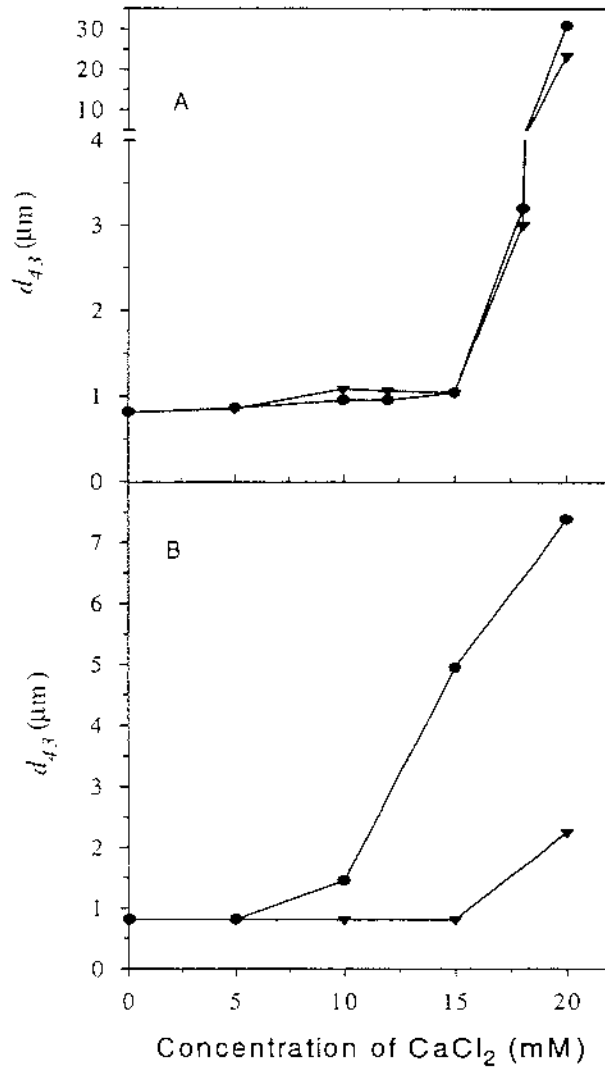
Figure 5.2: Changes in average particle diameter (d_{43}) (●) as a function of $CaCl_2$ concentration in emulsions made with 30% oil and 3.0% sodium caseinate. $CaCl_2$ was added before (A) or after (B) emulsion formation. 200 mM NaCl was added to the emulsions at each $CaCl_2$ concentration (▼). Each data point is the average of determinations on two separate emulsions.

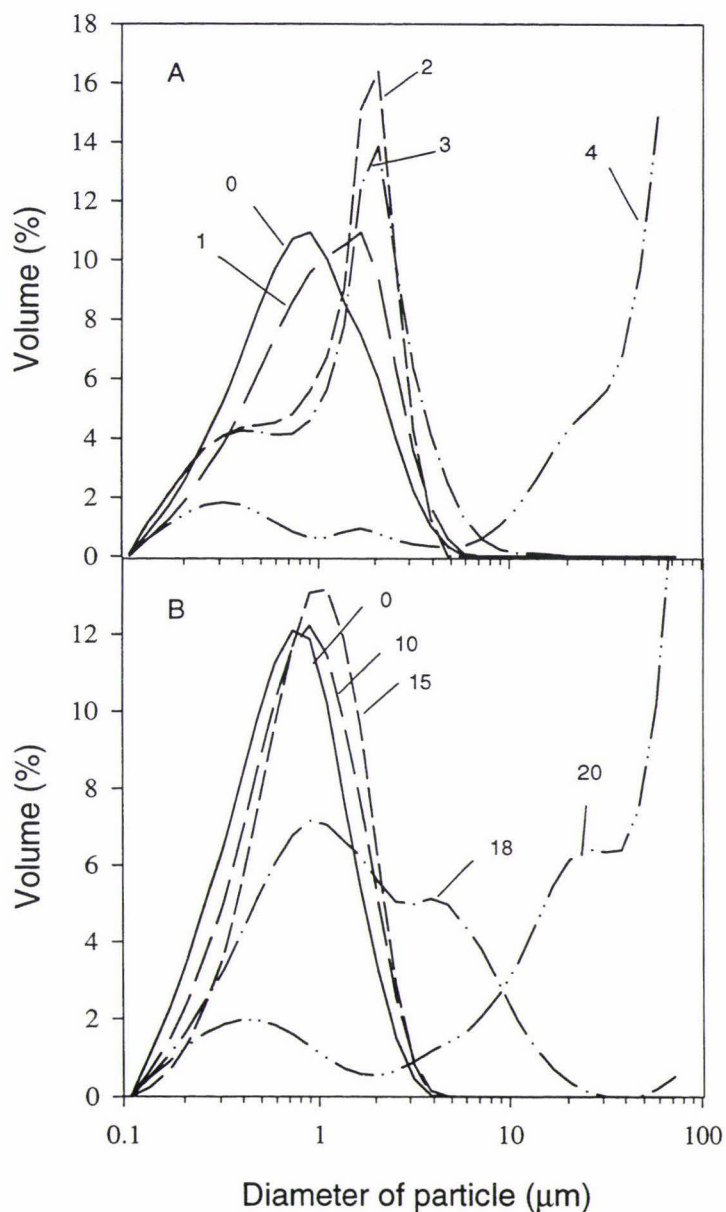**Figure 5.3:** Changes in the particle size distribution of emulsions (30% soya oil) made with 0.5% (A) or 3.0% (B) sodium caseinate. CaCl$_2$ was added before emulsion formation and different CaCl$_2$ concentrations (mM) are marked on the figures.
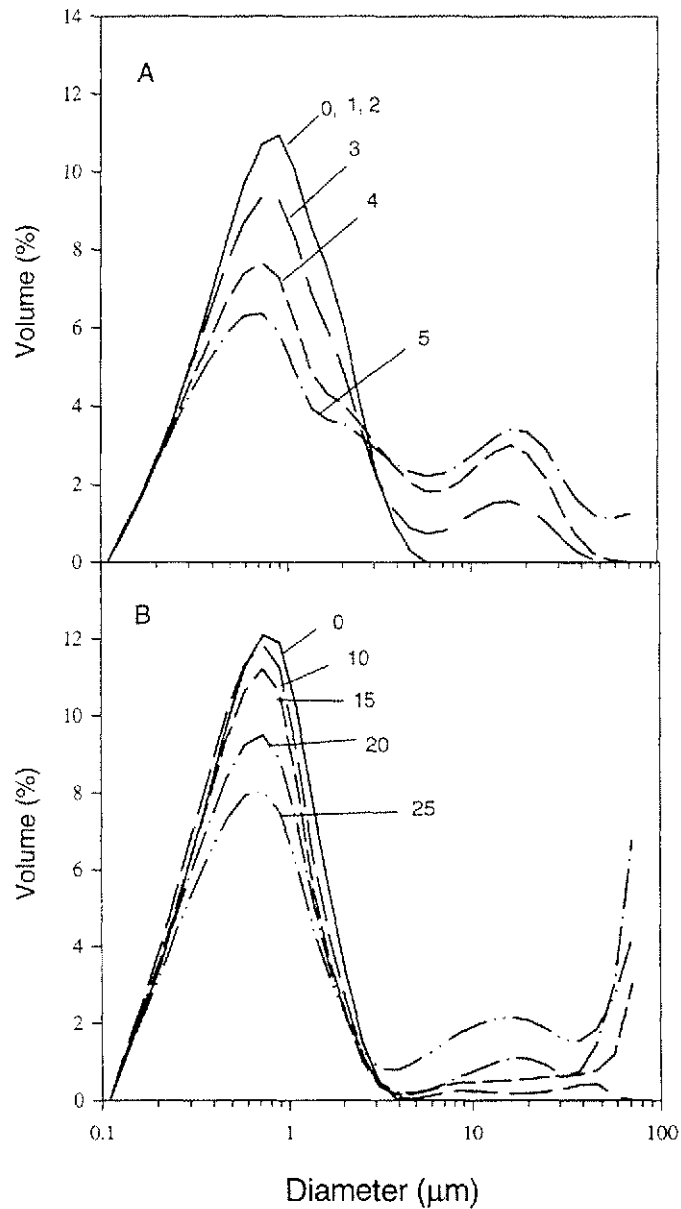
Figure 5.4: Changes in the particle size distribution of emulsions (30% soya oil) made with 0.5% (A) or 3.0% (B) sodium caseinate. CaCl₂ was added after emulsion formation and different CaCl₂ concentrations (mM) are marked on the figures.
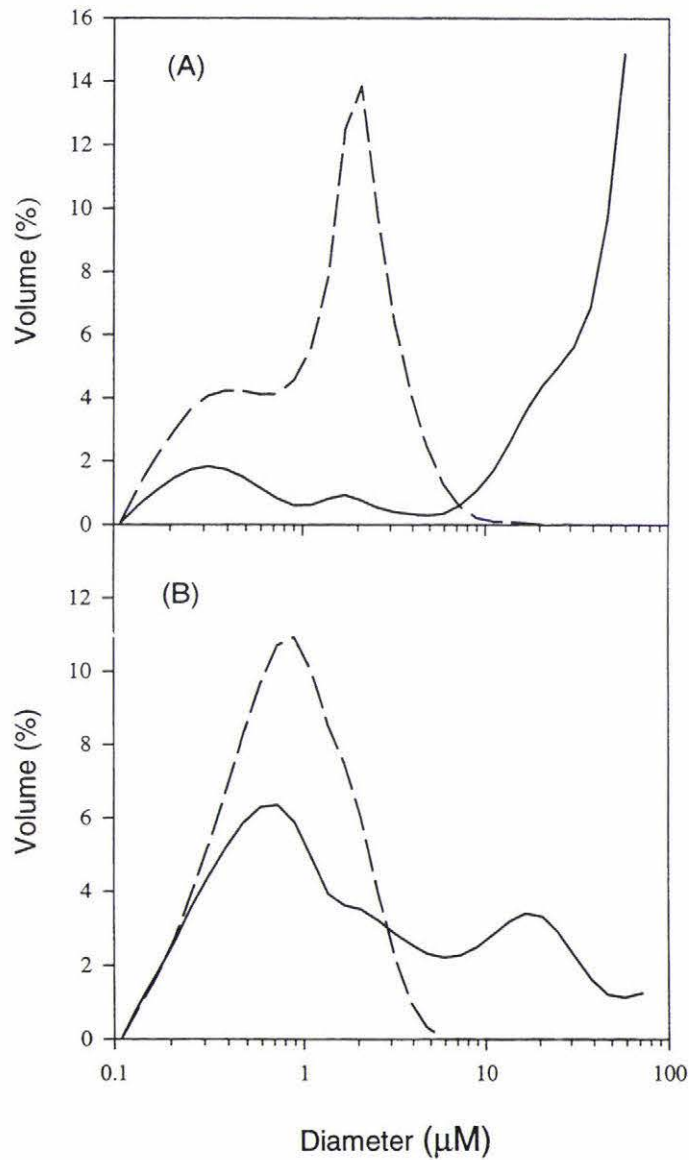
Figure 5.5: Particle size distribution of emulsions (30% soya oil) made with 0.5% sodium caseinate. 5 mM $CaCl_2$ was added before (A) or after (B) emulsion formation and the emulsions were diluted with 2% SDS solution (----) or with deionised water (——).

5.2.3. Surface protein concentration

The changes in the surface protein concentration of emulsions, made with 0.5% sodium caseinate, in the presence of $CaCl_2$ during emulsion formation and addition of $CaCl_2$ to the emulsion after emulsification are shown in Figure 5.6. As the concentration of $CaCl_2$ added to caseinate solution increased from 0 to 3 mM, the surface protein concentration increased gradually from 0.68 to 1.18 mg/m^2, but increased only slightly (to 1.25 mg/m^2) with further increase in $CaCl_2$ concentration to 5 mM. When 200 mM NaCl was added to the emulsions, the surface protein concentration increased markedly for emulsions containing below 2 mM $CaCl_2$. However, the surface protein concentration of emulsions containing > 2 mM $CaCl_2$ decreased upon addition of 200 mM NaCl (Figure 5.6 A). When $CaCl_2$ was added to the emulsions (made with 0.5% sodium caseinate), the changes in surface protein concentration were similar to those in emulsions made in the presence of $CaCl_2$ (Figure 5.6 B).

In emulsions made with 3.0% sodium caseinate, in the presence of $CaCl_2$, the surface protein concentration increased markedly from 2.0 to 7.0 mg/m^2 when the concentration of $CaCl_2$ increased from 0 to 20 mM (Figure 5.7 A). When 200 mM NaCl was added, the surface protein concentration increased slightly for emulsions containing below 10 mM $CaCl_2$, but it deceased for emulsions containing > 10 mM $CaCl_2$ (Figure 5.7 A). Generally, similar results were obtained when $CaCl_2$ was added to the emulsions after they were made (Figure 5.7 B).

Figure 5.6: Changes in surface protein concentration (mg/m²) as a function of CaCl₂ concentration in emulsions (30% soya oil) stabilized by 0.5% sodium caseinate. CaCl₂ was added before (A) or after (B) emulsion formation (●), and 200 mM NaCl was added to emulsions at each CaCl₂ concentration (▼). Each data point is the average of determinations on two separate emulsions.
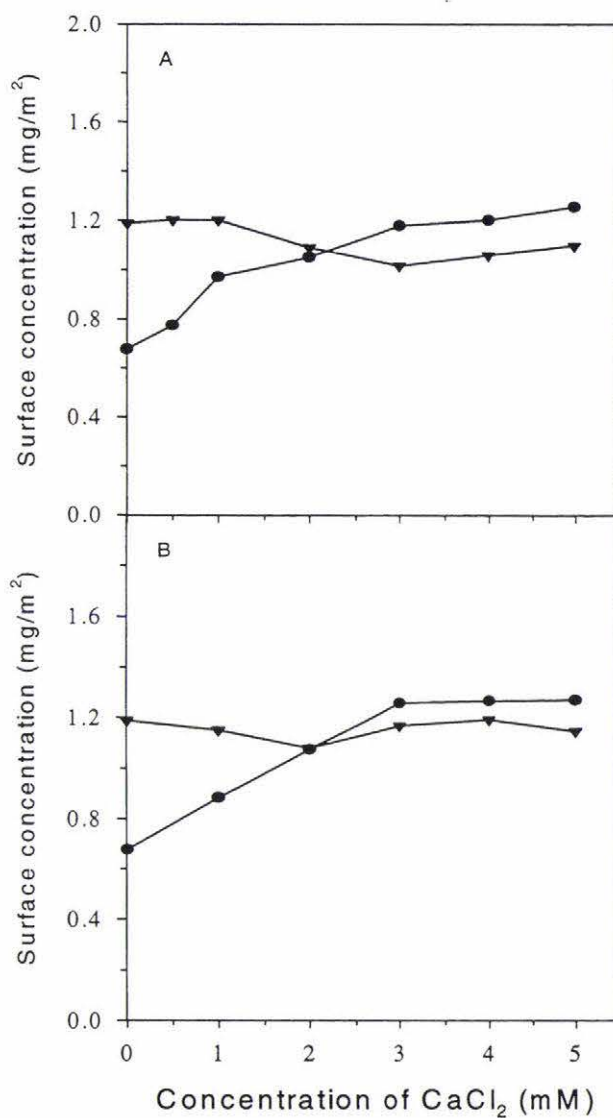
Figure 5.7: Changes in surface protein concentration (mg/m²) as a function of CaCl₂ concentration in emulsions (30% soya oil) stabilized by 3.0% sodium caseinate. CaCl₂ was added before (A) or after (B) emulsion formation (●), and 200 mM NaCl was added to emulsions at each CaCl₂ concentration (▼). Each data point is the average of determinations on two separate emulsions.
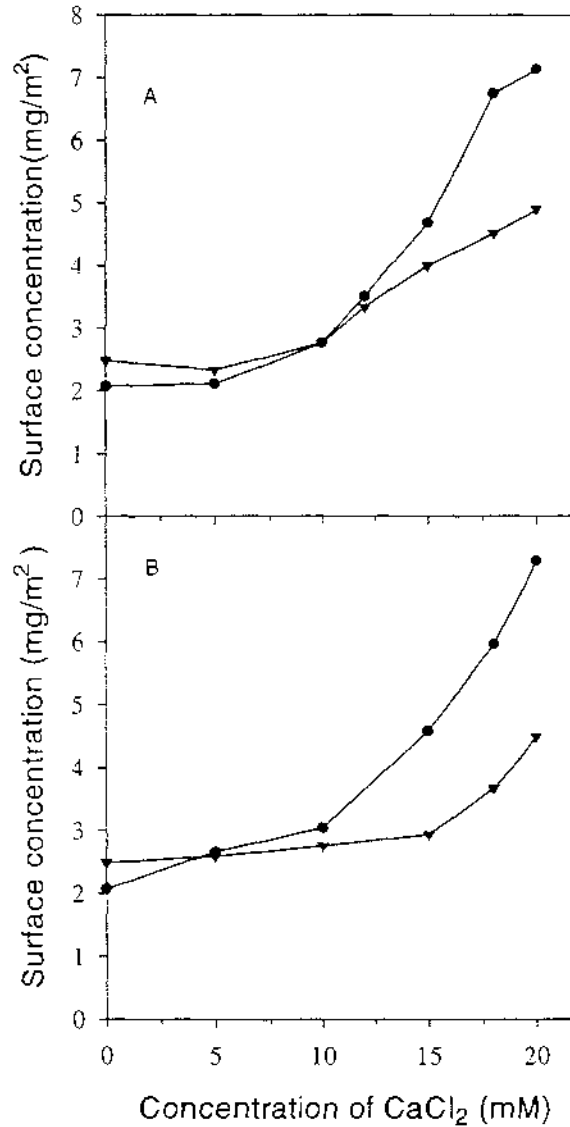
5.2.4. Composition of caseins at the oil/water interface (cream phase)

5.2.4.1. Emulsions made with 0.5% caseinate

The effect of addition of $CaCl_2$ prior to or after emulsion formation on the proportions of adsorbed caseins at the interface of emulsion droplets, made with 0.5% sodium caseinate, is shown in Figure 5.8. In emulsions made with 0.5% caseinate, in the absence of $CaCl_2$, the proportions of adsorbed caseins were: α_s-casein (α_{s1} + α_{s2}-caseins) ~ 35%, β-casein ~ 49% and κ-casein ~ 18% as compared with the proportions in the original caseinate solution (α_s-casein ~ 45%, β-casein ~ 41% and κ-casein ~ 14%). This suggests that β-casein was adsorbed in preference to α_s-casein under these conditions. As the concentration of $CaCl_2$ was increased from 0 to 3 mM, prior to emulsion formation, the relative proportion of α_s-casein increased from ~ 35 to ~ 55%. There were corresponding decreases in the proportions of adsorbed β-casein from ~ 50% to ~ 30%. The adsorbed κ-casein did not change with $CaCl_2$ concentration. When 200 mM NaCl was added to the emulsions after emulsion formation, the proportions of caseins at the interface did not change significantly, except in the case of emulsions containing with 0 or 5 mM $CaCl_2$.

In the case of emulsions in which $CaCl_2$ was added after they were made, the proportion of α_s-casein increased from ~ 35 to ~ 45%, while the proportion of β-casein decreased from ~ 50% to ~ 45% as $CaCl_2$ at 1 mM $CaCl_2$ addition (Figure 5.8 B). There were no further changes beyond 2 mM $CaCl_2$ addition. Addition of 200 mM NaCl to emulsions after emulsion formation did not significantly affect the interfacial composition of emulsion droplets made in the presence of $CaCl_2$ (Figure 5.8 B).

5.2.4.2. Emulsions made with 3.0% caseinate

The effect of addition of $CaCl_2$ prior to or after emulsion formation on the proportions of adsorbed caseins at interface of emulsion droplets, made with 3.0% sodium caseinate, is shown in Figure 5.9. The interfacial compositions (α_s-casein ~ 49%, β-casein ~ 23% and κ-casein ~ 27%) of emulsions made with

3.0% sodium caseinate in the absence of $CaCl_2$ was different from that of emulsions made with 0.5% sodium caseinate. It appeared that α_s-casein (α_{s1}- + α_{s2}-caseins) was adsorbed in preference to β-casein under these conditions (see Chapter 4). Low concentrations of $CaCl_2$ (< 10 mM) added prior to emulsion formation caused an increase in the proportion of α_s-casein and a decrease in the proportions of β- and κ-caseins. Further increase in $CaCl_2$ concentration resulted in a decrease in the proportion of α_s-casein with corresponding increases in β- and κ-caseins. Addition of 200 mM NaCl to emulsions did not significantly affect the interfacial composition of emulsion droplets made in the presence of $CaCl_2$ (Figure 5.9 A). Generally similar results of interfacial composition were found when $CaCl_2$ was added to emulsions after they were formed (Figure 5.9 B).

Figure 5.8: Effect of concentration of CaCl$_2$ on the relative proportions of α_s-casein (●), β-casein (▼) and κ-casein (■) at the droplet surface (cream phase) of emulsions formed with 30% soya oil and 0.5% sodium caseinate. CaCl$_2$ was added before (A) or after (B) emulsion formation. 200 mM NaCl was added to be emulsions at each CaCl$_2$ concentration, (——) without NaCl, (----) with NaCl. Each data point is the average of determinations on two separate emulsions.
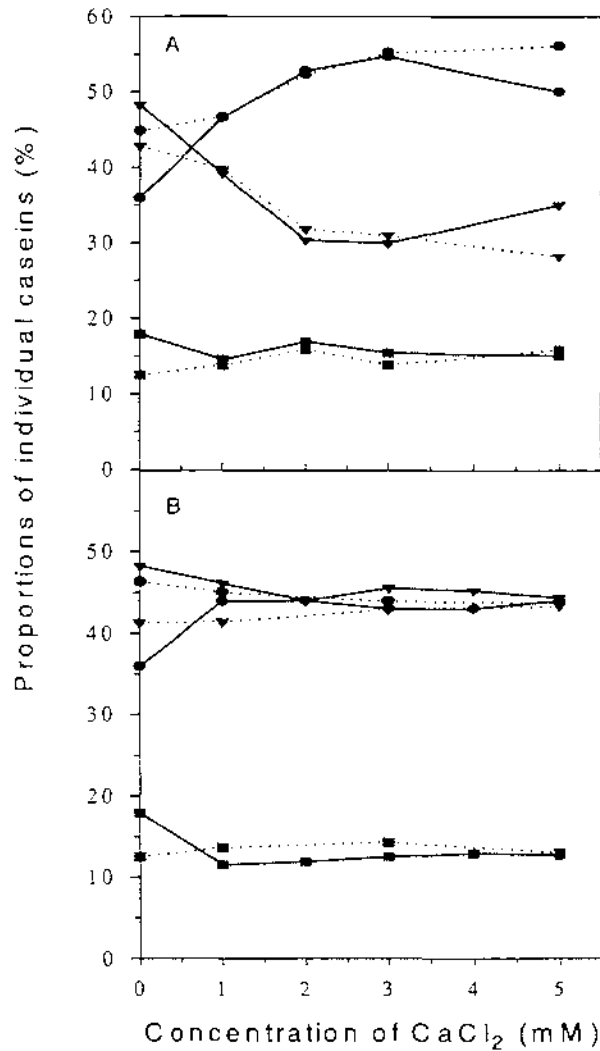
Figure 5.9: Effect of concentration of CaCl₂ on the relative proportions of αₛ-casein (●), β-casein (▼) and κ-casein (■) at the droplet surface (cream phase) of emulsions formed with 30% soya oil and 3.0% sodium caseinate. CaCl₂ was added before (A) or after (B) emulsion formation. 200 mM NaCl was added to the emulsions at each CaCl₂ concentration, (——) without NaCl, (----) with NaCl. Each data point is the average of determinations on two separate emulsions.
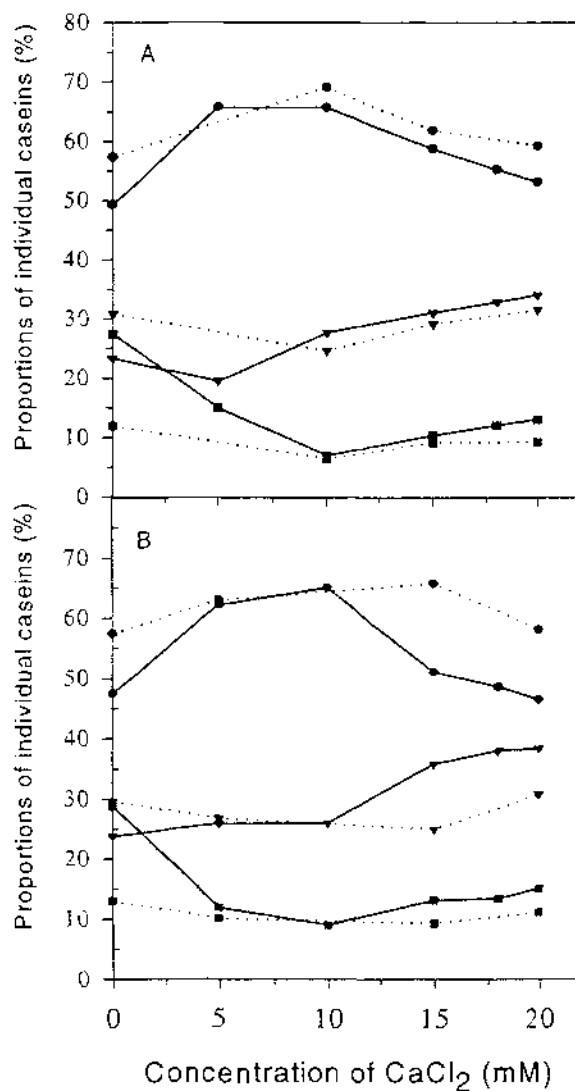
5.2.5. Creaming stability of emulsions

5.2.5.1. Emulsion made with 0.5% caseinate

The effect of addition of $CaCl_2$ prior to or after emulsion formation on creaming stability of emulsions made with 0.5% sodium caseinate is shown in Figure 5.10. In the case of $CaCl_2$ added prior to emulsion formation, increase in $CaCl_2$ from 0 to 5 mM caused a major decrease in the creaming stability of emulsions (from ~ 70 to ~ 2%), when $CaCl_2$ concentration was ≥ 4 mM. It indicates that the creaming stability strongly follows the particle size of emulsions under these conditions. The addition of $CaCl_2$ to emulsions after emulsion formation also resulted in a decrease in creaming stability, but this decrease (~ 70 to ~ 40%) was less marked than in the case of addition of $CaCl_2$ prior to emulsion formation (Figure 5.10 B). Addition of 200 mM NaCl to both emulsions had no considerable effect on creaming stability (Figure 5.10).

5.2.5.2. Emulsions made with 3.0% caseinate

The creaming stability of emulsions made with 3.0% caseinate, in the absence of both NaCl and $CaCl_2$, was considerably low due to the depletion flocculation (Dickinson and Golding, 1997; Srinivasan, 1999; Chapter 4). The addition of $CaCl_2$ both prior to and after emulsion formation dramatically influenced the stability rating of these emulsions (Figure 5.11). When $CaCl_2$ was added to the caseinate solution prior to emulsion formation, the stability rating increased markedly from ~ 7% to ~ 73% as the $CaCl_2$ concentration was increased from 0 to 12 mM. Between 12 to 18 mM $CaCl_2$, the stability rating remained close to 75%, but it decreased abruptly to ~ 10% as the $CaCl_2$ was further increased to 20 mM (Figure 5.11 A).

Compared to above results, the creaming stability of emulsions made with 3.0% caseinate was more sensitive to the addition of $CaCl_2$ to emulsions after they were made. The stability improved to ~ 70% at a $CaCl_2$ concentration lower than 10 mM (Figure 5.11 B), and did not decrease at high $CaCl_2$ concentrations, although the average particle size (d_{43}) markedly increased (Figure 5.2 B).

Figure 5.10: Changes in stability rating (%) as a function of CaCl$_2$ concentration in emulsions (30% soya oil) stabilized by 0.5% sodium caseinate. CaCl$_2$ was added before (A) or after (B) emulsion formation (●), and 200 mM NaCl was added to emulsions at each CaCl$_2$ concentration (▼). Each data point is the average of determinations on two separate emulsions.
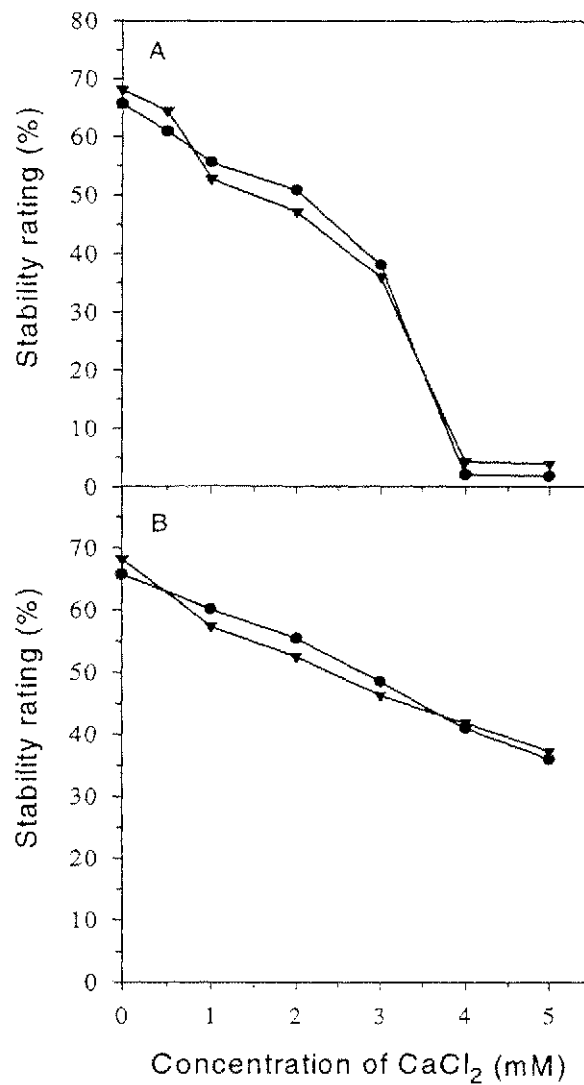
Figure 5.11: Changes in stability rating (%) as a function of CaCl₂ concentration of emulsions (30% soya oil) stabilized by 3.0% sodium caseinate. CaCl₂ was added before (A) or after (B) emulsion formation (●), and 200 mM NaCl was added to emulsions at each CaCl₂ concentration (▼). Each data point is the average of determinations on two separate emulsions.
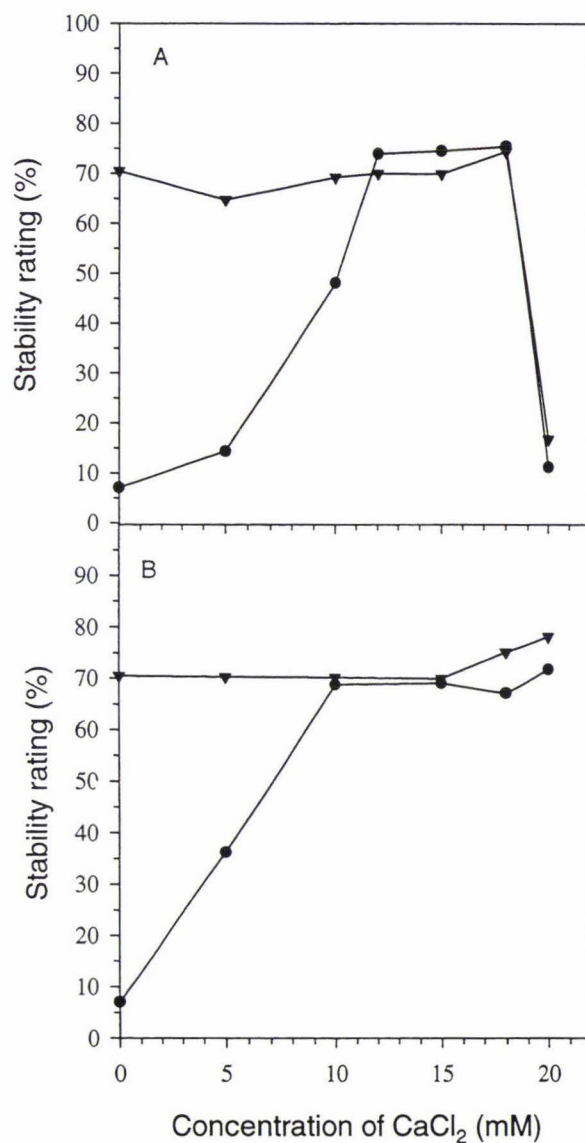
Addition of 200 mM NaCl to emulsions improved markedly the stability rating (to ~ 70%) of emulsions made with 3.0% caseinate in the absence of $CaCl_2$ and of those containing lower $CaCl_2$ concentrations (< 12 mM) regardless of the method of $CaCl_2$ addition

5.2.6. Microstructure of emulsions

The microstructure of these emulsions was examined using confocal microscopy to observe the state of droplet aggregation and providing a visual perspective of the aggregation. Emulsion droplets appeared to be homogeneous with no sign of flocculation when the concentration of $CaCl_2$ was lower than the critical concentration required to cause aggregation in emulsions in which $CaCl_2$ was added to 0.5% sodium caseinate solution before emulsification (Figure 5.12 B). Emulsions containing 5 mM $CaCl_2$ showed large number of small particles aggregated together and separated from aqueous phase (Figure 5.12 C). In microgragh of the emulsions in which the $CaCl_2$ was added after emulsion formation (Figure 5.13), we can see some aggregated droplet clusters mixed with separated small droplets in emulsions (0.5% caseinate) with 3 mM $CaCl_2$ added (Figure 5.13 B), in accordance with the particle size distribution of these emulsions (Figure 5.4). As the $CaCl_2$ concentration was further increased, more clusters (about 10 – 15 μm) were formed from droplet aggregation (Figure 5.13 D). It was different from that in emulsions in which $CaCl_2$ was added before homogenization, in which much larger aggregates involving large numbers of droplets were formed (Figure 5.12 C).

Addition of 12 mM $CaCl_2$ resulted in fine and homogeneous state of emulsion droplets as compared with significant flocculated structure in the emulsion made with 3.0% caseinate in the absence of $CaCl_2$ (Figure 5.14 A, B, C). Presence of such change in flocculation was responsible for the enhanced creaming stability of emulsions (Figure 5.11). However, strong flocculation was found again in emulsions made with 3.0% caseinate in presence of 20 mM $CaCl_2$ (Figure 5.13 D).

Figure 5.15 demonstrates that the changes in microstructures of emulsions made with 3.0% caseinate when CaCl$_2$ was added after homogenization. Addition of 10 mM CaCl$_2$ prevented emulsion droplets from flocculation (Figure 5.15 A); addition of 5 mM CaCl$_2$ showed some improvement (Figure 5.15 B). The addition of 25 mM CaCl$_2$ resulted in the loss of the homogeneous state, formed large aggregates (Figure 5.15 D).

Figure 5.12: Confocal micrographs of 0.5% sodium caseinate-stabilized oil-in-water emulsions; 0 mM (A), 3 mM (B) or 5 mM (C) CaCl₂ were added to caseinate solution prior to emulsion formation.

Figure 5.12: (Continued).

Figure 5.13: Confocal micrographs of 0.5% sodium caseinate-stabilized oil-in-water emulsions; 0 mM (A), 3 mM (B), or 10 mM (C) CaCl$_2$ were added to emulsion.

B

C

Figure 5.13: (Continued).

A

B

Figure 5.14: Confocal micrographs of 3.0% sodium caseinate-stabilized oil-in-water emulsions; 0 mM (A), 5 mM (B), 12 mM (C) or 20 mM (D) CaCl₂ were added to caseinate solution prior to emulsion formation.

C

D
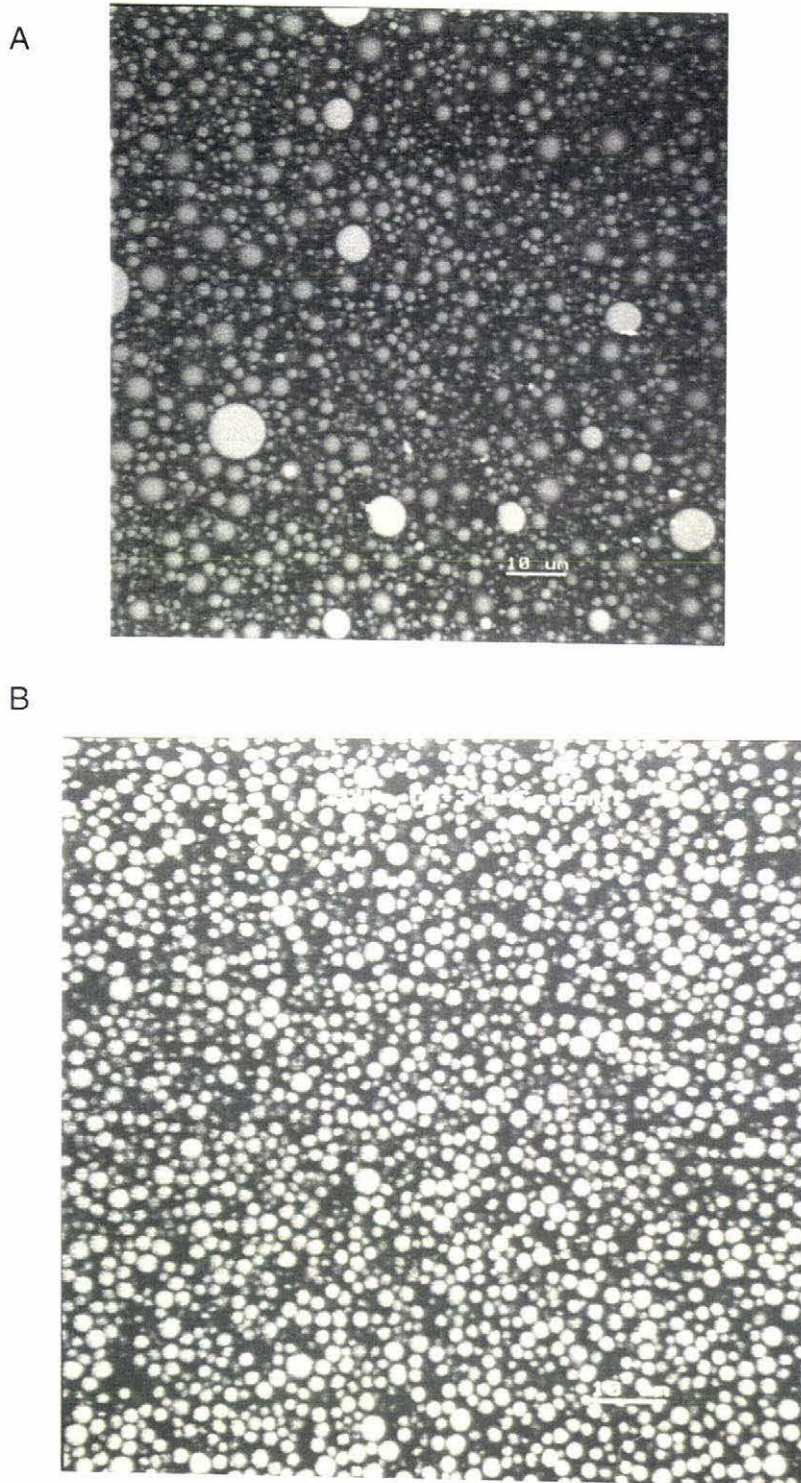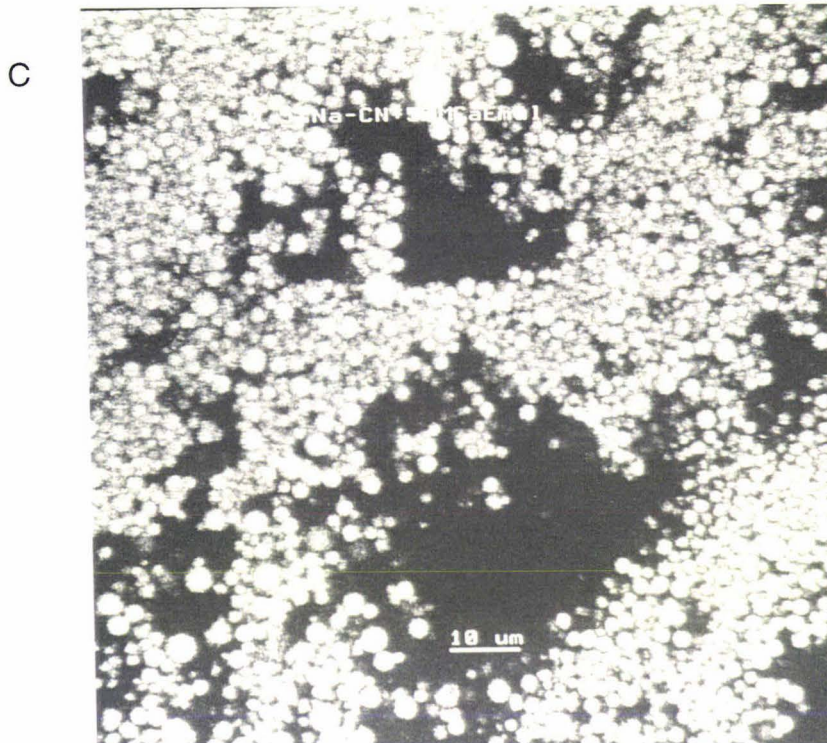

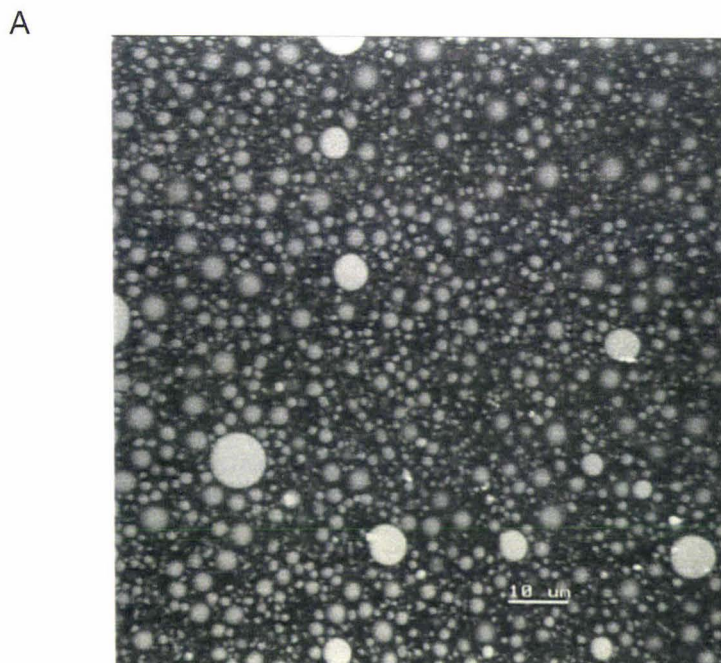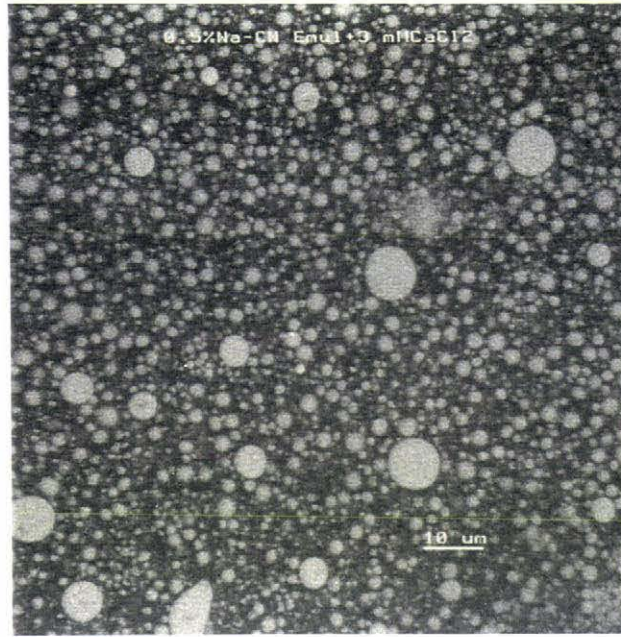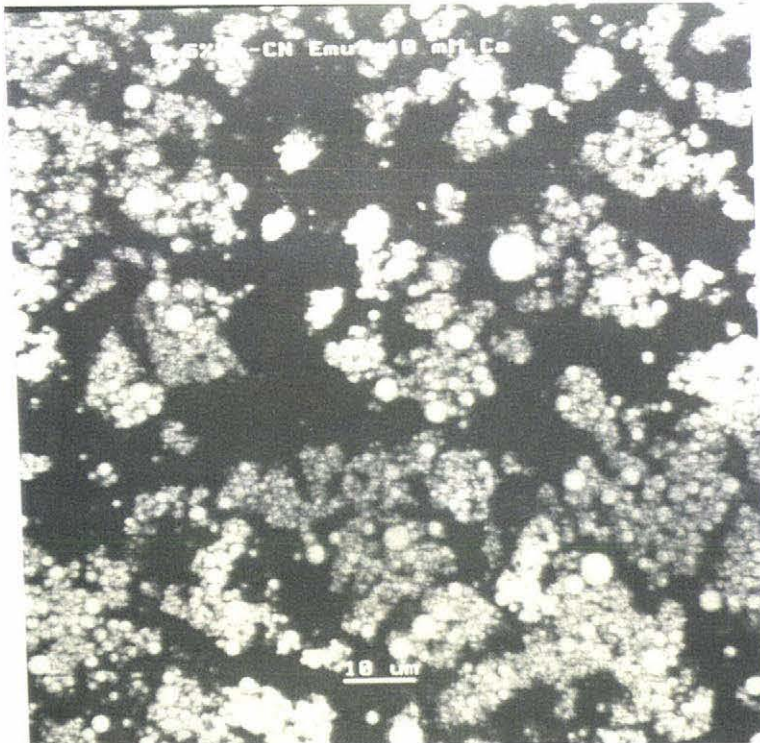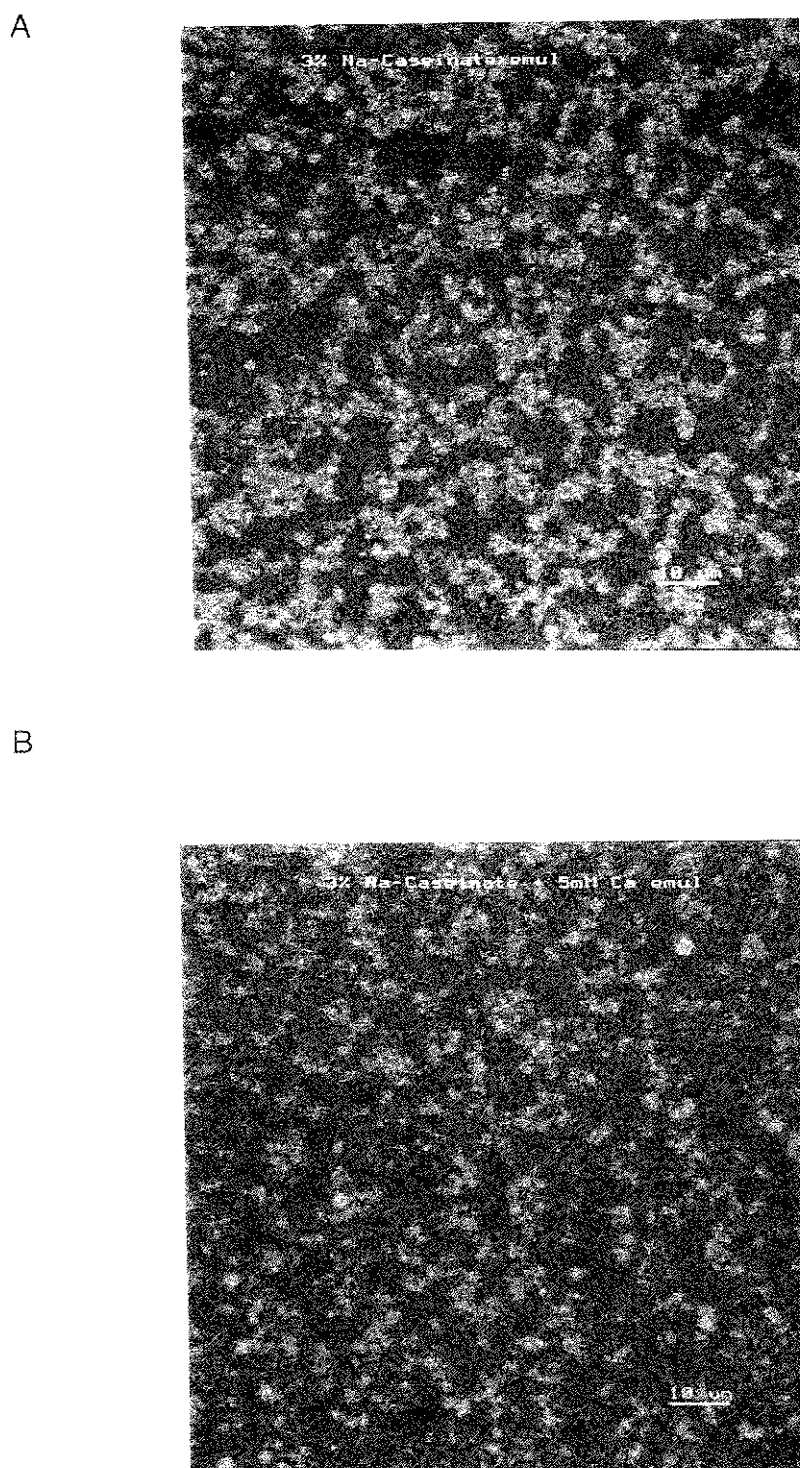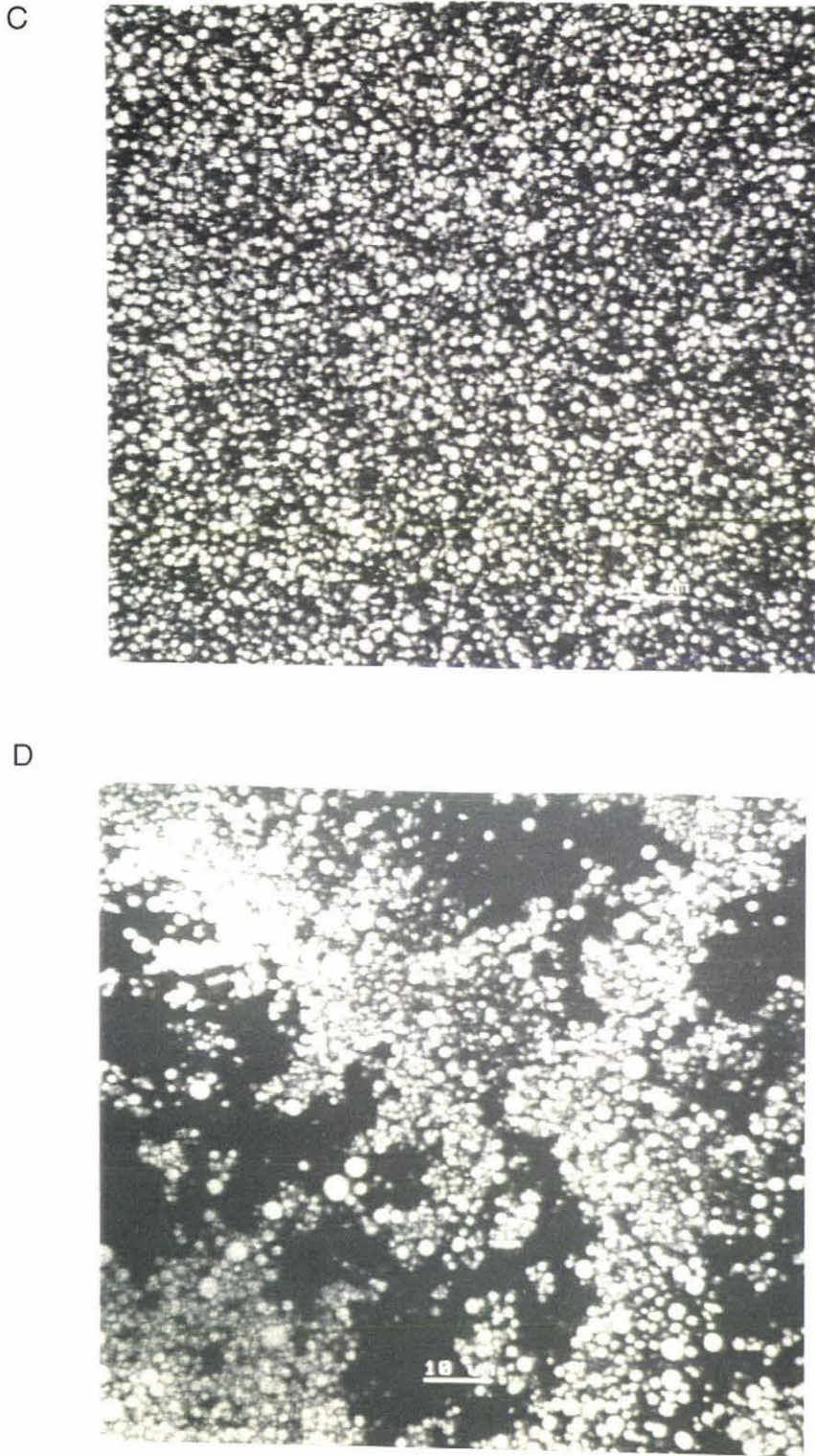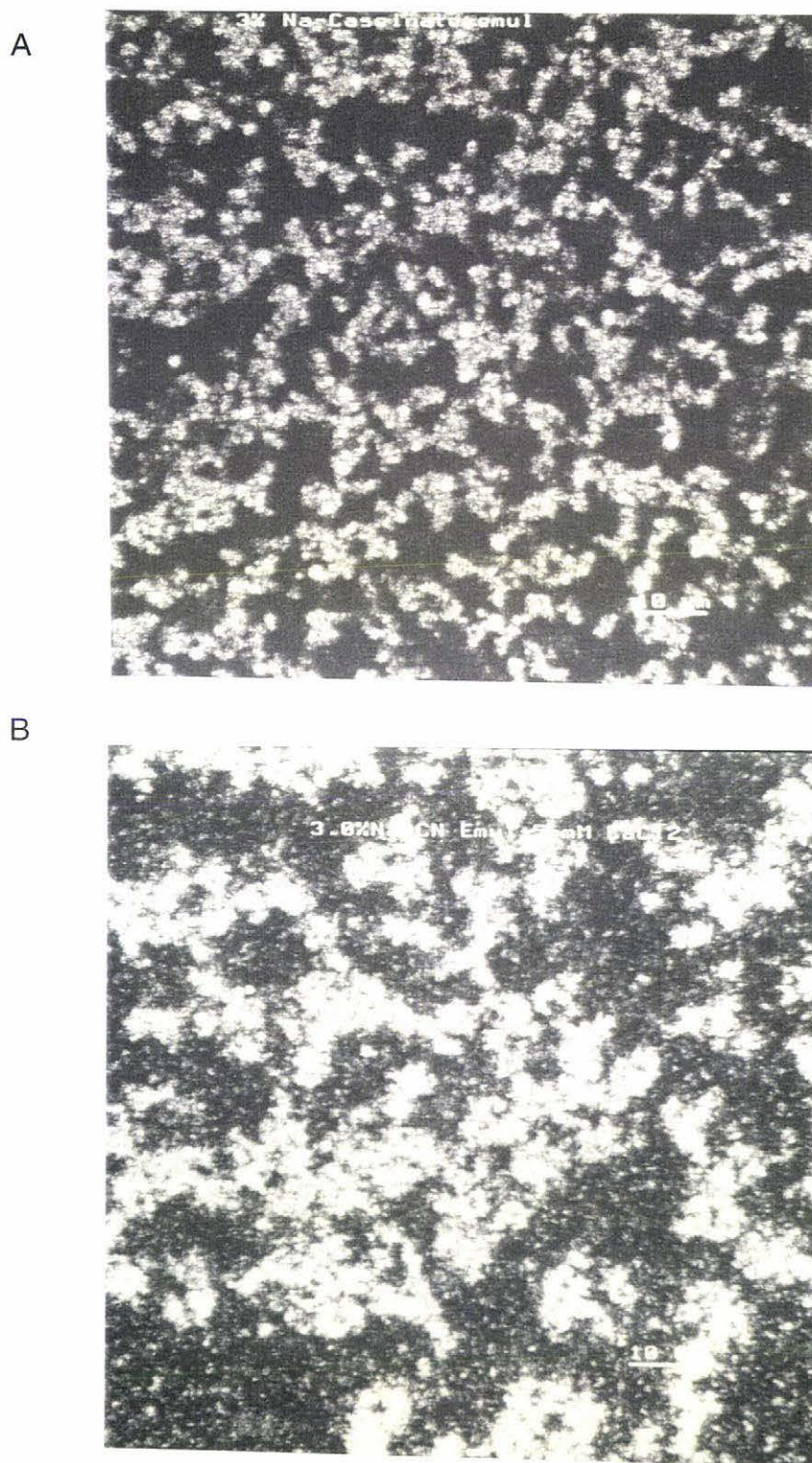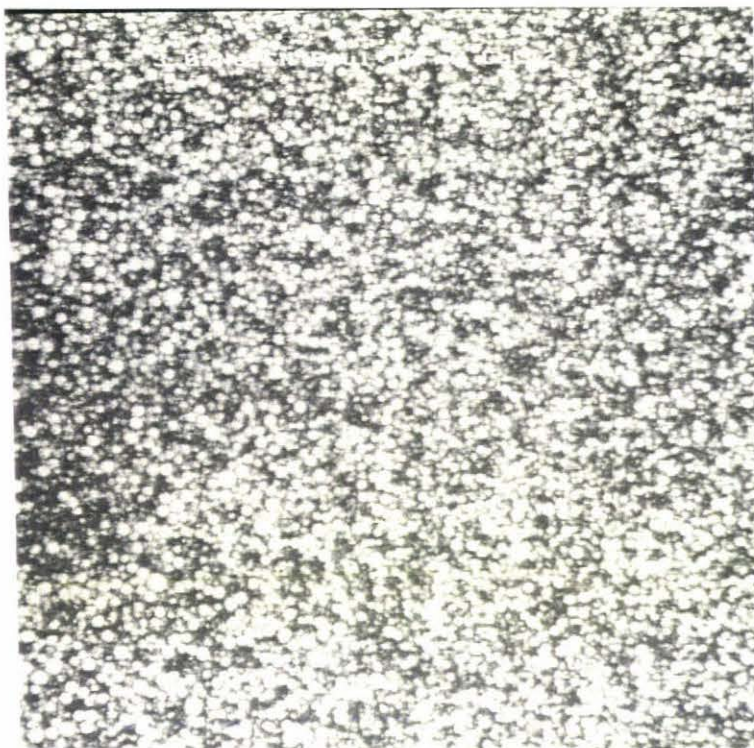**Figure 5.14:** (Continued).

A

B

Figure 5.15: Confocal micrographs of 3.0% sodium caseinate-stabilized oil-in-water emulsions; 0 mM (A), 5 mM (B), 10 mM (C) or 25 mM (D) CaCl$_2$ were added to emulsion.
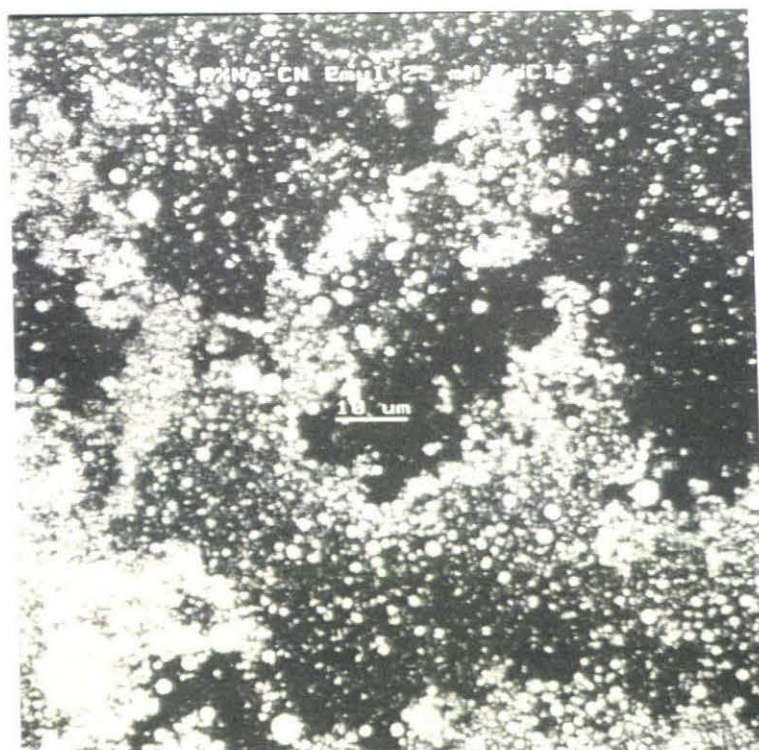
C

D

Figure 5.15: (Continued).

5.3 Discussion

From the present results, we got a strong impression about the sensitive effect of Ca^{2+} on the adsorption behaviour of caseinate at interface of emulsion droplets and the stability of the caseinate-stabilized emulsions. It is well known that, when Ca^{2+} is added to caseinate solution, Ca^{2+} binding with phosphoserine of caseins reduces electrostatic repulsion between the molecules (Horne and Dalgleish, 1980). This reduction in electrostatic repulsion results in the formation of casein aggregates in solution (Swaisgood, 1992). The increase in droplet size of emulsions (Figure 5.1 A and Figure 5.2 A) upon addition of low concentrations of $CaCl_2$ to caseinate solution prior to emulsification may be attributed to coalescence of the smaller droplets during homogenization. Formation of aggregated caseins, due to Ca^{2+} binding, leads to a decrease in emulsifying capacity of caseins. This aggregation may slow down the transport of protein to the interface and inhibit the protein molecules undergoing spreading and rearrangement effectively. As a result, caseinate may not able to cover immediately all new surface area of oil droplet created during homogenization and achieve a state of lowest free energy. Some smaller droplets that have higher surface free energy would coalesce into larger droplets. These reasons may be responsible for the increase in the average droplet size during homogenization in emulsions formed with caseinate solution containing $CaCl_2$. Dickinson *et al.* (1992) suggested that the addition of Ca^{2+} to β-casein prior to homogenization decreased its emulsifying capacity and led to coalescence during homogenization, rather than bridging flocculation.

A large increase in the aggregation of emulsion droplets as the concentration of Ca^{2+} was further increased (Figure 5.1 A and Figure 5.2 A) is mainly because of protein bridging flocculation between droplets. It was confirmed by the action of SDS on these droplets (Figure 5.5) and microstructure observations (Figure 5.12 and Figure 5.14). In addition, this flocculation can not be reversed by dilution with stirring in the MasterSizer. In theory, the bridging flocculation is restricted to low concentrations of a protein with multiple anchor sites and a spatial extension beyond the range of the repulsive barrier between the

spatial extension beyond the range of the repulsive barrier between the emulsion particles. Under these conditions the surfaces of the emulsion particles are only partially covered and approaching particles easily become linked by protein bridges, at distances that prevent any impact of the repulsive barrier (Dickinson and Stainsby, 1982, 1988; Walstra, 1993). The decrease in the extent of flexibility of protein, caused by further aggregation of caseins, lead to insufficient amount of protein being available to cover the total surface of oil droplets. The aggregated particles of caseins adsorbed at the oil droplet surface occupy an extent of space, extended away from the interface. The outside part of protein particle extending away from the interface might have some protein molecules that are still able to adsorb. Further coalescence is prevented by steric repulsive (or distance barrier) of larger protein particles that have already been adsorbed. Therefore, choosing bridging flocculation to minimize the interfacial free energy is the most possible way. However, some Ca^{2+} bridging flocculation between droplets could not be avoided.

In literature, there is no satisfying theory to quantify the conditions favouring coalescence or bridging flocculation. However, it might be inferred that adsorption of individual molecules at interface is involved in coalescence, whereas the adsorption of large aggregated particle is usually involved in bridging flocculation under the conditions when the protein concentration is insufficient to cover the surface during homogenization.

Here, a question arises as to how an aggregated particle of protein adsorb to interface of oil droplets? The increase in surface protein concentration of emulsions with an increase in concentration of Ca^{2+} (Figure 5.6 A and Figure 5.7 A) suggests that the aggregated particles could adsorb to the interface. The adsorption behaviour of calcium caseinate (see Chapter 4) and previous reports support this conclusion (Mulvihill and Murphy, 1991; Srinivasan *et al.*, 1996, 1999). From the preferential adsorption of α_s-casein at the interface of emulsion droplets made with caseinate solution containing Ca^{2+} (Figure 5.8 A and Figure 5.9 A), it could be inferred that the caseinate was directly adsorbed at the

the high shear, turbulent conditions in the homogenizer, and then adsorb a monomolecular protein to the interface and continuously form protein multilayers (the state of aggregation) promoted by Ca^{2+}? The preferential adsorption of α_s-casein at the interface is also observed in emulsions that had Ca^{2+} added after emulsion formation (Figure 5.8 B and Figure 5.9 B). The formation of flocculation in emulsions suggests that caseinate adsorbed as aggregated protein particle, and did not adsorb as individual molecules at the interface. It has been observed that casein micelles adsorb on the surface of oil droplets (Oortwijn and Walstra, 1979; Dam *et al.*, 1995; Dalgleish, 1995; Sharma *et al.*, 1996). The micelle structure rearranges before the micelle interacts with fat surface (Dalgleish, 1995; Sharma *et al.*, 1996). Therefore, we could assume that the aggregated protein particles probably dissociate during homogenization, but they re-associate before adsorbing to the surface of oil droplet. It means that the time required for protein associating by a number of different interactions is shorter than the adsorption of protein to surface, although it is likely some association could still occur when they are adsorbed. The re-association in a new condition and environment lead to different composition and structure of protein at the surface.

The flocculation by calcium bridges between protein molecules adsorbed on different emulsion droplets may be responsible for aggregation in emulsions in which calcium was added after homogenization (Figure 5.1 B and Figure 5.2 B). Dickinson *et al.* (1992) reported that the particle size distribution of β-casein-stabilized emulsion, in which calcium was added after homogenization, was less sensitive than that of emulsions made in the presence of calcium. They inferred that emulsions stabilized by proteins with a high affinity for calcium are more prone to flocculation by calcium bridges between protein molecules adsorbed on different emulsion droplets when calcium is added after homogenization, and the flocculation is reversible upon dilution. A marked increase in surface protein coverage at low calcium concentration (Figure 5.6 and Figure 5.7), indicates that some Ca^{2+} might be involved in binding with caseins in the aqueous phase and forming calcium bridges between caseins unadsorbed in aqueous phase and adsorbed on interface of emulsion droplets.

The different mechanism of aggregation between the addition of Ca^{2+} before and after emulsification can be confirmed by whether this aggregation is reversible when the aggregated emulsions are diluted with water in the MasterSizer. The flocculation by protein bridging due to insufficient protein would be irreversible on dilution with water, whereas the flocculation caused by calcium bridging would be reversible upon dilution. The extent of decrease in the average particle size upon addition of NaCl in aggregated emulsions can also distinguish between different aggregation mechanisms. Most of the aggregated particles in emulsions were dissociated by NaCl addition in the case when $CaCl_2$ was added after homogenisation, whereas dissociation occurred to a limited extent in emulsions in which $CaCl_2$ was added before homogenization (Figure 5.1 and 5.2). It may be because Na^+ competed directly with specific sites for Ca^{2+} binding between the droplets in the former case, but only provided little more protein to the interface from aqueous phase, due to dissociation of aggregated caseins, in the latter case. The strength of Ca^{2+} binding to both α_{s1}-casein and β-casein is inversely related to the ionic strength as dictated by the molarity of NaCl (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981).

The presence of Ca^{2+} increased the surface protein coverage of emulsion droplets, not only prior to homogenization, but also when $CaCl_2$ was added to preformed emulsions (Figure 5.6, Figure 5.7). This is attributed to aggregation of caseins due to binding of Ca^{2+} to caseins. The aggregation of caseins in caseinate solution containing Ca^{2+} is due to the binding of Ca^{2+} to caseins in the polar domains (Swaigood, 1992). Mulvihill and Murphy (1991) found that surface protein concentrations were related to the state of aggregation in the caseins/caseinates used in emulsion preparation, with higher protein loads for high-calcium caseinate-stabilized emulsion than less aggregated sodium caseinate. Srinivasan *et al.* (1996) also reported that protein load of caseinate-stabilized emulsions increased with increase in Ca^{2+} concentration in the caseinate solution. No previous work has been reported on the surface coverage as affected by the addition of Ca^{2+} after emulsification. It may suggest that the binding of Ca^{2+} to caseins promotes interactions between the caseins in

the aqueous phase and that adsorbed at the interface of droplets. In addition, the binding of Ca^{2+} to caseins adsorbed at the interface may cause more compact packing of adsorbed caseins (Horne and Leaver, 1995; Dickinson, 1998), which may allow more protein to be adsorbed at interface of droplets.

The addition of NaCl to the emulsions made in the absence of Ca^{2+} or containing low Ca^{2+} levels leads to an increase in the surface protein coverage (Figure 5.6). Increasing the ionic strength may reduce the electrostatic repulsions between the adsorbed film and arriving molecules, thereby increasing the rate of adsorption and consequently increasing the proportions of protein particles irreversibly adsorbed at the interface. More compact packing of molecules at the interface is also facilitated at higher ionic strength (Tornberg, 1978 a). This would also cause an increase in surface protein concentration with increase in NaCl concentration. The surface protein concentration showed a slight decrease in emulsions containing higher concentration of Ca^{2+} as NaCl was added (Figure 5.6 and 5.7). This could be attributed to competition by the cation for specific binding sites on phosphoserine groups of caseins, thereby reducing the amounts of casein aggregate adsorbed at the interface.

The present results clearly showed that the addition of Ca^{2+} to emulsions made with caseinate, both before and after homogenization, enhanced the adsorption of α_s-casein (α_{s1}- + α_{s2}-caseins) at the interface of droplets (Figure 5.8 and Figure 5.9). This may be because the binding of Ca^{2+} to α_s-casein is more favorable than to β-casein. The binding capacity of α_{s1}-casein to Ca^{2+} is higher than β-casein (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; Pappas and Rothwell, 1991). Srinivasan *et al.* (1999) reported that the large aggregates in calcium caseinate consist mainly of α_s-caseins. The increase in protein concentration on the surface of droplets was mainly because of the increase in adsorbed α_s-casein when Ca^{2+} was added. The environmental conditions were favorable for further adsorption of α_s-casein. When the concentration of Ca^{2+} was high enough, other caseins, besides α_s-casein, were also adsorbed. The

proportion of κ-casein at interface decreased with the addition of Ca^{2+} (Figure 5.8 and Figure 5.9); this may be because Ca^{2+} does not bind to κ-casein.

The addition of NaCl (raising the ionic strength of environment) to emulsions increased the adsorption of α_s-casein (α_{s1}- + α_{s2}-caseins) (Figure 5.8 and Figure 5.9). Previous studies have shown that the degree of α_{s1}-casein self-association is higher than that of β-casein at high ionic strength (Swaisgood, 1992); self-aggregated α_{s1}-casein molecules may subsequently become adsorbed, resulting in a high concentration of α_{s1}-casein at the interface.

The present results demonstrate that the creaming stability of emulsions made with 0.5% sodium caseinate decreased with increase in the concentration of Ca^{2+} (Figure 5.10). Creaming stability is mainly dependent on the changes in the average particle size of emulsions, as d_{43} increased with increasing the concentration of Ca^{2+}.

In emulsions formed with relatively high concentrations of sodium caseinate, the creaming stability improved markedly by $CaCl_2$ addition both before and after emulsification (Figure 5.11). The instability of the emulsions made with sodium caseinate in absence of Ca^{2+} and NaCl was attributed to depletion flocculation, which has been discussed in Chapter 4. Dickinson and Golding (1997) reported that the extent of deletion flocculation is mainly dependent on the size and concentration of unbound or unadsorbed protein; the caseinate sub-micelles (15 - 20 nm) and 2 – 3% concentration in aqueous phase were responsible for the initial discernible flocculation in their case.

In present results, emulsions containing 12 mM (added before emulsification) or 10 mM Ca^{2+} (added after emulsification), which had about 1.7% caseinate concentration in the aqueous phase of emulsions, showed no flocculation (Figure 5.14 C, Figure 5.15 D). However, this concentration (~ 1.7%) in aqueous phase had already caused a strong flocculation in emulsions made with sodium caseinate (~ 2.5%) in the absence of Ca^{2+} (see Chapter 4, Figure

4.4). This suggests that the depletion flocculation was probably prevented largely by the particle size of protein in the aqueous phase, not the changes in the concentration of unadsorbed protein in this condition. It is consistent with Asakura-Oosawa theory (Asakura and Oosawa, 1954). In Asakura-Oosawa theory (see Chapter 4), if unadsorbed protein in aqueous phase (solute volume fraction ϕ_s) is 0.017 (~ 1.7%), while the caseinate sub-micelles remain at 15-20 nm (*d*), then $U_d(h) \approx 2.5KT$. This value is large enough (Dickinson, 1996), which depletion flocculation may occur.

It was also found that the flocculation behaviour of the caseinate-stabilized emulsions was sensitive to ionic strength (Figure 5.11). The change in the affinity between the unadsorbed protein in aqueous phase and the protein adsorbed at the interface of droplets is likely a factor that improved the stability from the depletion flocculation, besides the decrease in protein concentration and size of the unadsorbed species as discussed by Srinivasan (1999). This idea is based on fact that adding salt to an aqueous continuous phase diminishes the electrostatic repulsion and decreases the steric repulsion of casein (Walstra, 1987). The caseins can not be excluded from the region between the emulsion droplets, especially the emulsion droplets in which α_{s1}-casein is adsorbed predominately at the interface. The α_{s1}-casein possesses the more charge and is more self-associated at high ionic strengths (Swaisgood, 1992; Dickinson *et al*, 1998). In principle, the addition of salt is advantage to bridging flocculation not to depletion flocculation in protein-stabilized emulsion. However, Dickinson (1998) considered that the depletion flocculation of caseinate-stabilized emulsions is rather insensitive to ionic strength (up to 0.5 M NaCl).

5.4 Conclusions

Addition of Ca^{2+} has a considerable influence on the adsorption behaviour of sodium caseinate in the oil/water interface and on the creaming stability of resulting emulsions. When CaCl$_2$ was added prior to or after emulsion

formation, the particle size and surface protein concentration increased with increasing $CaCl_2$ concentration in emulsions made with 0.5 and 3.0% sodium caseinate. The adsorption of α_s-casein increased with increase in the concentration of $CaCl_2$, with a corresponding decrease in the adsorption of β-casein. The creaming stability of emulsions made with 0.5% caseinate decreased with increasing $CaCl_2$ concentration. However, the creaming stability increased with $CaCl_2$ concentration in 3.0% caseinate emulsion.

Different aggregation mechanisms are involved depending upon whether Ca^{2+} is added to protein solution before emulsification or to the emulsion after its formation. Addition of Ca^{2+} to caseinate solution may lead to a decrease in emulsifying capacity and subsequently resulting in protein bridging flocculation between emulsion droplets. Ca^{2+} bridging flocculation may be formed in emulsions that have Ca^{2+} added. The presence of Ca^{2+} retards the depletion flocculation in emulsions made with high concentration of sodium caseinate, not only during homogenization but also when Ca^{2+} is added to preformed emulsions. Addition of Ca^{2+} increases mainly the adsorption of α_s-casein at the interface of oil/water emulsion droplet, implying that the aggregated protein particles are adsorbed on the droplet surface. The effects of Ca^{2+} in emulsions made with sodium caseinate could be reduced by the addition of NaCl. The confocal microscopic observations are an effective examination method for supporting the results determined by other methods in the study of the stability change of emulsions induced by Ca^{2+}.

Chapter 6

INFLUENCE OF CaCl₂ ADDITION ON THE PROPERTIES OF EMULSIONS STABILIZED BY WHEY PROTEIN CONCENTRATE

6.1 Introduction

Commercial WPC that contain >70% protein are used extensively as functional and nutritional ingredients in medical, pharmaceutical, and food products (Morr and Ha, 1993). Whey proteins are often used in food emulsion systems because of their ability to stabilize oil-in-water emulsions (Dickinson, 1992; Dalgleish, 1996 b). They adsorb to an oil-water interface and stabilize emulsions by a combination of charge repulsion and steric stabilization (Dickinson and Stainsby, 1982). The appearance, rheology and stability of protein-stabilized emulsions depend largely on the molecular structure and interactions of the adsorbed protein and how these interactions are influenced by environmental conditions (Dickinson and McClements, 1995). Whey proteins (β-lactoglobulin, α-lactalbumin, BSA and Ig), are characterized by well defined three-dimensional structures held together by disulfide bridges; these proteins are much more rigid than the caseins (Kinsella, 1984). It is known the both of the major whey proteins, β-lactoglobulin and α-lactalbumin, adsorb to oil-water interfaces and are capable of giving stable emulsions (Dalgleish, 1996 b). Indeed, singly or in combination, these proteins are excellent emulsifying agents, and their emulsions are only a little less stable than those produced using caseins under same conditions (Hunt and Dalgleish, 1994).

The binding of calcium ion to whey proteins have been studied under a variety of environmental conditions (Baumy and Brule, 1988; Pappas and Rothwell, 1991). The binding of Ca²⁺ to whey protein reduces the electrostatic repulsion and interaction of the hydrophobic domains (Baumy and Brule, 1988). This property may inevitably influences the adsorption behaviour of protein at the oil-water interface and the stability of emulsion formed with whey proteins. The association of Ca²⁺ with adsorbed protein between the protein-coated oil

droplets results in aggregation of emulsion droplets (Agboola and Dalgleish, 1995).

It is known that whey proteins can form gels by heating, high Ca^{2+} concentration or high ionic strength in solution (Mulivihill and Kinsella, 1988; Barbut and Foegeding, 1993). Particle gels can be formed on heat treatment of whey proteins-stabilized emulsions (Jost *et al.*, 1986; McClements *et al.*, 1993 a; Dickinson and Hong, 1995). The formation of a gel-like network structure in aggregated protein-stabilized emulsions may retard the creaming (Dickinson, 1998; Syrbe *et al.*, 1998).

Less information is available on the details of influence of Ca^{2+} on the adsorption behaviour of whey proteins at the oil-water interfaces and stability of emulsions formed with whey proteins, especially as the concentrations of Ca^{2+} are changed in emulsions made with different concentrations of protein. No previous studies are reported on the effect of Ca^{2+} on the composition of protein interfacial layers and creaming stability of emulsions stabilized by whey proteins.

The objective of this present work was to study the effects of changing the concentration of $CaCl_2$ added into protein solution prior to making emulsions or to emulsions made in the absence of $CaCl_2$ on the adsorption behaviour of whey proteins and the stability of emulsions made with WPC. The relationship between aggregation state of protein due to Ca^{2+} binding and their emulsifying properties is discussed. In addition, it is known that ionic strength influences the binding of Ca^{2+} to whey protein (Baumy and Brule, 1988). Therefore, experiments were also carried out in which NaCl was added to the emulsions made with WPC. The confocal microscopy was used to observe the microstructure of emulsions for examining the relationship between structure and creaming stability of emulsions.

6.2 Results

6.2.1 Emulsion formation

Emulsions were made with 0.5 or 3.0% WPC, 30% soya oil, at pH 7.0 with homogenization at 207/34 bar. Various amounts of $CaCl_2$ were added into the protein solution prior to making emulsions or to emulsions made in the absence of calcium. In some case, NaCl (200 mM) was added to emulsions after formation.

6.2.2 Average particle size and particle size distribution

In emulsions, made with 0.5% WPC, the d_{43} increased slightly when the concentration of $CaCl_2$, added to the protein solution prior to emulsification, increased from 0 to 3 mM, but a marked increase was observed as the concentration of $CaCl_2$ was further increased to 5 mM (Figure 6.1 A). The d_{43} values did not change when 200 mM NaCl was added to the emulsions. However, addition of SDS solution to emulsions containing $CaCl_2$ caused a marked decrease in d_{43} (to about 1.4 μm in emulsions containing 4, 5 or even 10 mM $CaCl_2$) but did not change the particle size of emulsions containing ≤ 3 mM $CaCl_2$. The d_{43} values did not fully return to that of emulsion without $CaCl_2$ after SDS treatment. It indicated that the measured large particles were not only formed by the aggregation of droplets, but also some larger droplets were formed by coalescence from small droplets during homogenization.

When $CaCl_2$ was added to emulsions after they were made, the d_{43} increased gradually from 1.0 to 2.2 μm with increase in the concentrations of $CaCl_2$ from 0 to 5 mM. The d_{43} reduced back to ~1.3 μm when 200 mM NaCl was added to emulsions (Figure 6.1 B). It indicated that the aggregates present in emulsions made with WPC, induced by $CaCl_2$, could be dissociated by NaCl. The d_{43} values fully returned to that of emulsion without added $CaCl_2$ after SDS treatment.

The d_{43} of emulsion droplets, made with 3.0% WPC, increased slightly from 0.7 to 1.0 μm with increase in the concentration of $CaCl_2$ from 0 to 10 mM, but

increased dramatically (from 5.9 to 34.3 µm) as the $CaCl_2$ concentration was further increased to 20 mM (Figure 6.2 A). When 200 mM NaCl was added to emulsions, the d_{43} decreased, to some extent, at 18 and 20 mM $CaCl_2$. Addition of $CaCl_2$ to emulsions, made with 3% WPC, had no effect on d_{43} (Figure 6.2 B).

Figure 6.3 shows that the d_{43} values of emulsions made with 0.5% WPC did not increase linearly with $CaCl_2$ added after emulsification. The particle size of emulsions reached a plateau at above 20 mM $CaCl_2$. However, in emulsions formed with 3.0% WPC, the droplet size of emulsions remained constant as $CaCl_2$ concentration was increased even to 100 mM. These results are similar to those of Agboola and Dalgleish (1995), who reported that addition of Ca^{2+} to emulsions after emulsification, made with β-lactoglobulin, had much smaller effect at high protein concentrations (1.5 and 2%) than at 1% protein concentration.

To emphasize different mechanisms of destabilization between the addition of Ca^{2+} prior to and after emulsification, the particle size distributions in both emulsions, made with 0.5% WPC, at various concentrations of $CaCl_2$ are shown in Figure 6.4 and Figure 6.5 respectively. As $CaCl_2$ was added to protein solutions prior to emulsion formation, the size distribution of droplets in emulsions showed a monomodal distribution; at 3 mM, particle size distribution shifted slightly to larger sizes in correspondence with slight increase in d_{43}. It indicated that there was no aggregation of droplets of emulsions before this $CaCl_2$ concentration; the coalescence of oil droplets during homogenization may have caused the increase in particle size at 3 mM $CaCl_2$. Aggregation was induced by further increase in the concentration of $CaCl_2$, resulting in bimodal and broad size distribution (Figure 6.4). When these emulsions were diluted with SDS, the bimodal and broad size distribution of emulsions (10 mM $CaCl_2$) altered back to monomodal distributions that were very similar to that of emulsion containing 3 mM Ca^{2+}.

Compared with above changes, the particle size distribution of emulsions that had various amounts of $CaCl_2$ added after emulsion formation showed a general reduction in the proportion of emulsion droplets in original size range and an increase in proportion of large particles correspondingly, which were formed due to aggregation of small ones. This gave a bimodal size distribution as concentration of $CaCl_2$ increased above 10 mM (Figure 6.5). There were no further changes in the particle size distribution beyond the 20 mM added $CaCl_2$; most particles were in the range 11 - 20 μm. Very large particles observed in Figure 6.4 were not observed in these emulsions.

Figure 6.1: Changes in average particle diameter (d_{43}) as a function of CaCl$_2$ concentration in emulsions made with 30% soya oil and 0.5% WPC (●). CaCl$_2$ was added before (A) or after (B) emulsion formation. NaCl (200 mM) was added to emulsions at each CaCl$_2$ concentration (▼). Emulsions were diluted by SDS solution (■). Each data point is the average of determinations on two separate emulsions.
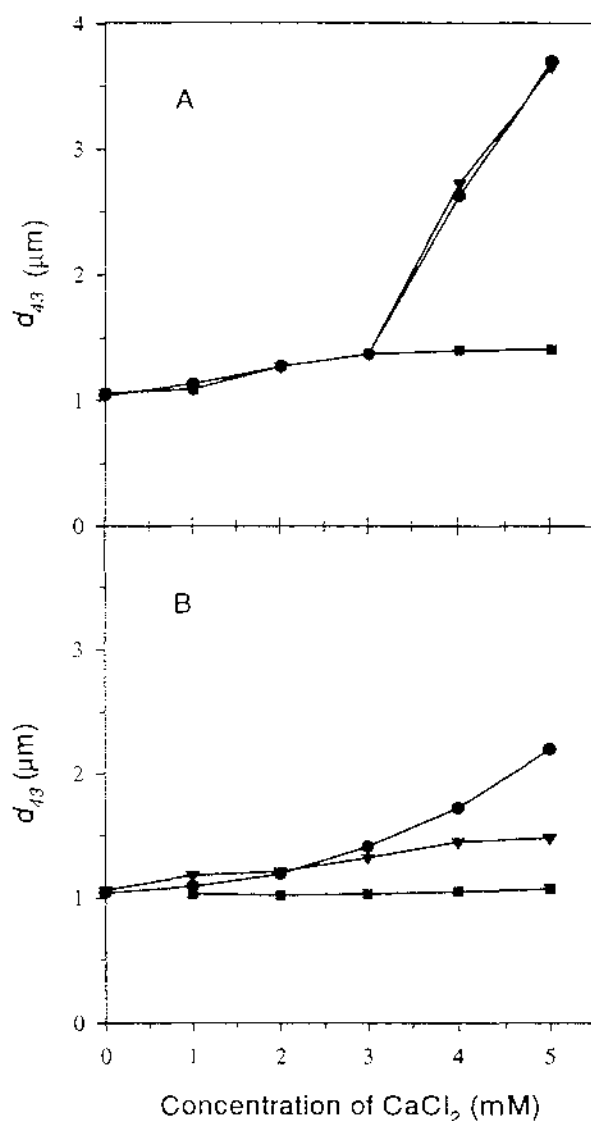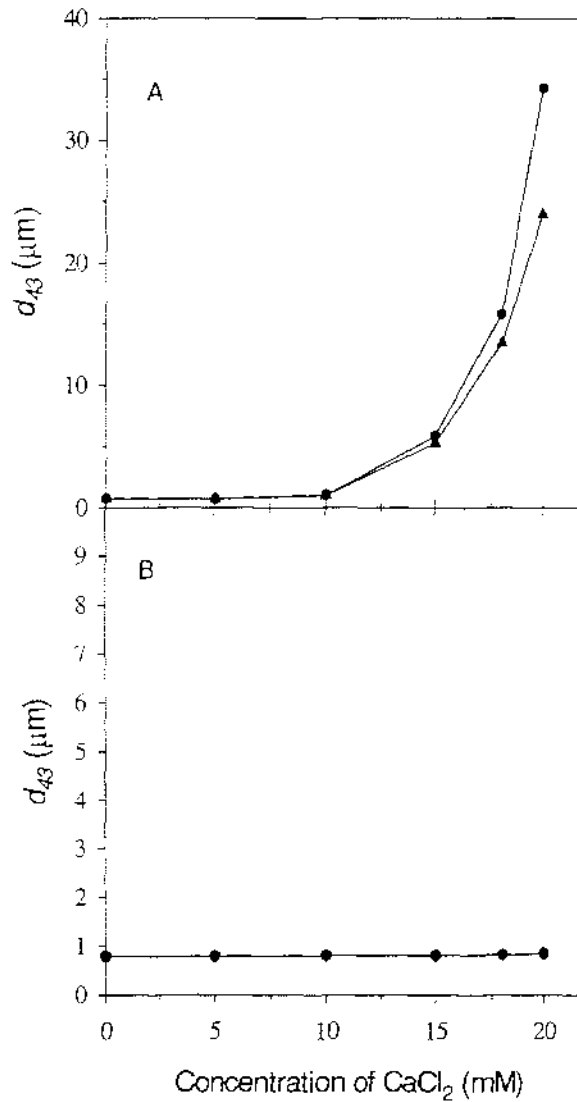
Figure 6.2: Changes in average particle diameter (d_{43}) as a function of $CaCl_2$ concentration in emulsions made with 30% soya oil and 3.0% WPC (●). $CaCl_2$ was added before (A) or after (B) emulsion formation. NaCl (200 mM) was added to emulsions at each $CaCl_2$ concentration (▲). Each data point is the average of determinations on two separate emulsions.
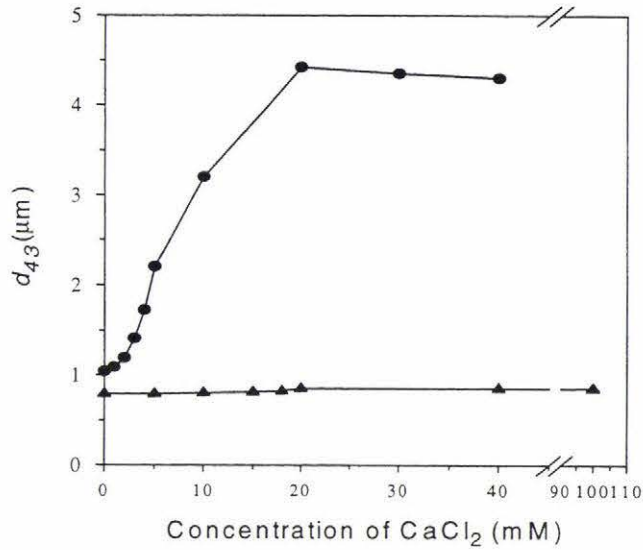
Figure 6.3: Changes in average particle diameter (d_{43}) as a function of $CaCl_2$ concentration in emulsions (30% soya oil) made with 0.5% (●) and 3.0% (▲) WPC. $CaCl_2$ was added to emulsions after emulsion formation. Each data point is the average of determinations on two separate emulsions.

Figure 6.4: Particle size distributions of emulsions (30% soya oil) made with 0.5% WPC. $CaCl_2$ was added before emulsion formation and different concentrations of $CaCl_2$ (mM) are marked on the figure (10 D: emulsion containing 10 mM $CaCl_2$ dispersed in SDS).
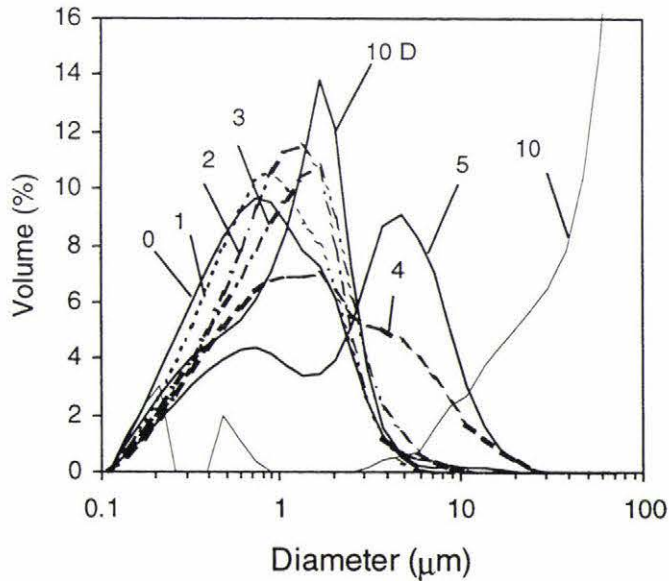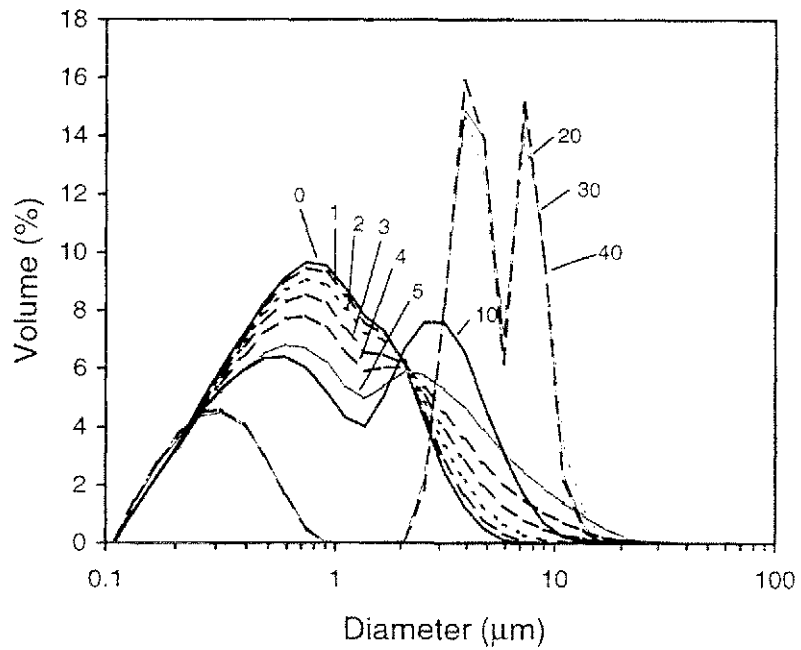
Figure 6.5: Particle size distribution of emulsions (30% soya oil) made with 0.5% WPC. CaCl₂ was added after emulsion formation and different concentrations of CaCl₂ (mM) are marked on the figure.

6.2.3 Surface protein concentration

In emulsions, made with 0.5% WPC in the presence of CaCl$_2$, the surface protein concentration increased from 0.76 to 0.95 mg/m^2 as CaCl$_2$ concentration was increased from 0 to 3 mM, but did not increase with further increase in CaCl$_2$ up to 5 mM (Figure 6.6 A). When 200 mM NaCl was added to emulsions, there was no change in the surface concentration, except a slight increase was observed for emulsions made in the absence of Ca^{2+} (Figure 6.6 A).

When CaCl$_2$ was added after emulsion formation, the surface protein concentration increased from 0.76 to 1.00 mg/m^2 with increase in CaCl$_2$ from 0 to 5 mM. Addition of 200 mM NaCl caused a slight increase in surface concentration of emulsions at low Ca^{2+} concentrations, and a slight decrease at 5 mM CaCl$_2$ (Figure 6.6 B).

In emulsions, made with 3.0% WPC in the presence of CaCl$_2$, the surface protein concentration increased markedly from 1.84 to 7.2 mg/m^2 when the concentration of CaCl$_2$ added to WPC solution increased from 0 to 20 mM (Figure 6.7 A). Addition of 200 mM NaCl to emulsions had no significant effect on the surface concentration. Addition of CaCl$_2$ to emulsions had no significant effect on the surface protein concentration of 3% WPC-stabilized emulsions (Figure 6.7 B). Addition of 200 mM NaCl did not change the surface protein concentrations of emulsion (Figure 6.7 B).

Figure 6.6: Changes in surface protein concentration (mg/m^2) as a function of $CaCl_2$ concentration in emulsions (30% soya oil) stabilized by 0.5% WPC (●). $CaCl_2$ was added before (A) or after (B) emulsion formation. NaCl (200 mM) was added to emulsions at each $CaCl_2$ concentration (▼). Each data point is the average of determinations on two separate emulsions.
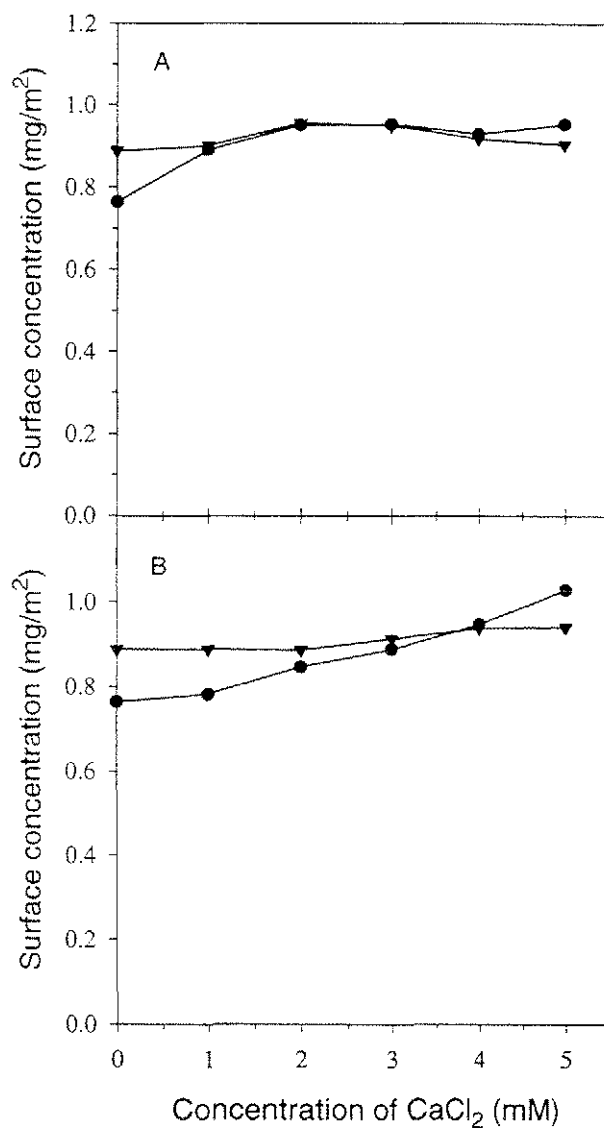
Figure 6.7: Changes in surface protein concentration (mg/m^2) as a function of CaCl$_2$ concentration in emulsions (30% soya oil) stabilized by 3.0% WPC (●). CaCl$_2$ was added before (A) or after (B) emulsion formation. NaCl (200 mM) was added to emulsions at each CaCl$_2$ concentration (▼). Each data point is the average of determinations on two separate emulsions.
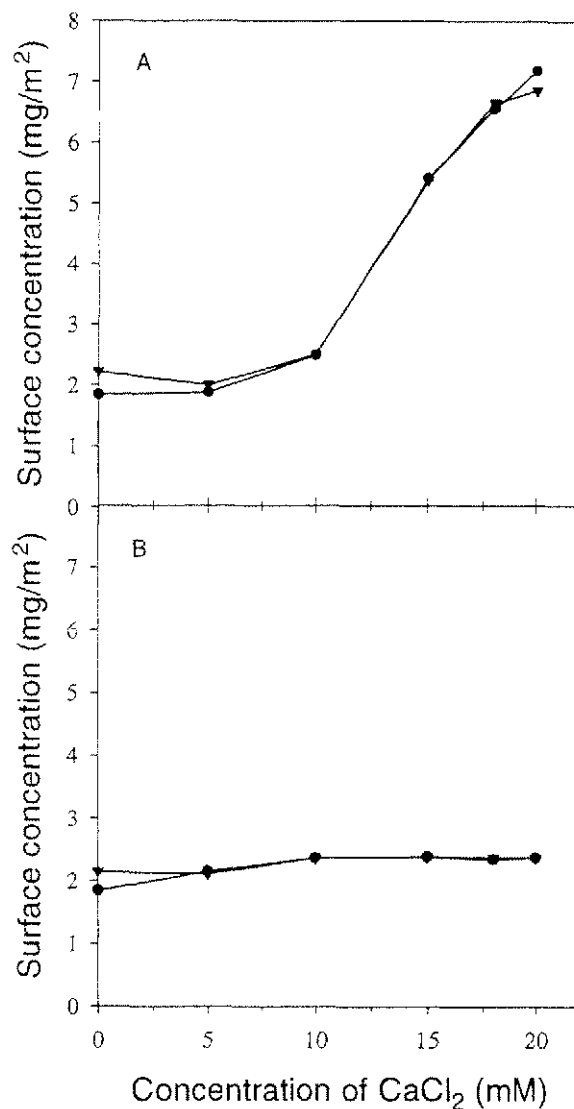
6.2.4 Composition of protein layer at the oil/water interface (cream phase)

In emulsions made with 0.5% or 3% WPC in the absence of $CaCl_2$, the proportions of adsorbed α-lactalbumin and β-lactoglobulin were: α-lactalbumin ~ 18%, β-lactoglobulin ~ 82% as compared with the proportions in the original WPC solution (α-lactalbumin ~ 25%, β-lactoglobulin ~ 75%). This suggests that β-lactoglobulin was adsorbed slightly in preference to α-lactalbumin under these conditions. As $CaCl_2$ was added to protein solution prior to emulsion formation, the proportion of α-lactalbumin decreased to ~ 10% with increase in $CaCl_2$ concentration from 0 to 5 mM in emulsions made with 0.5% WPC, or from 0 to 20 mM in the case of 3% WPC (Figure 6.8 A and Figure 6.9 A). There were corresponding increases in the proportions of adsorbed β-lactoglobulin from ~ 82% to ~ 90%. Addition of $CaCl_2$ to emulsions had no significant effect on the proportions of α-lactalbumin and β-lactoglobulin (Figure 6.8 B and Figure 6.9 B). When NaCl (200 mM) was added to emulsions containing $CaCl_2$ (added before or after emulsion formation), the proportions of α-lactalbumin and β-lactoglobulin did not change significantly (Figure 6.8, Figure 6.9).
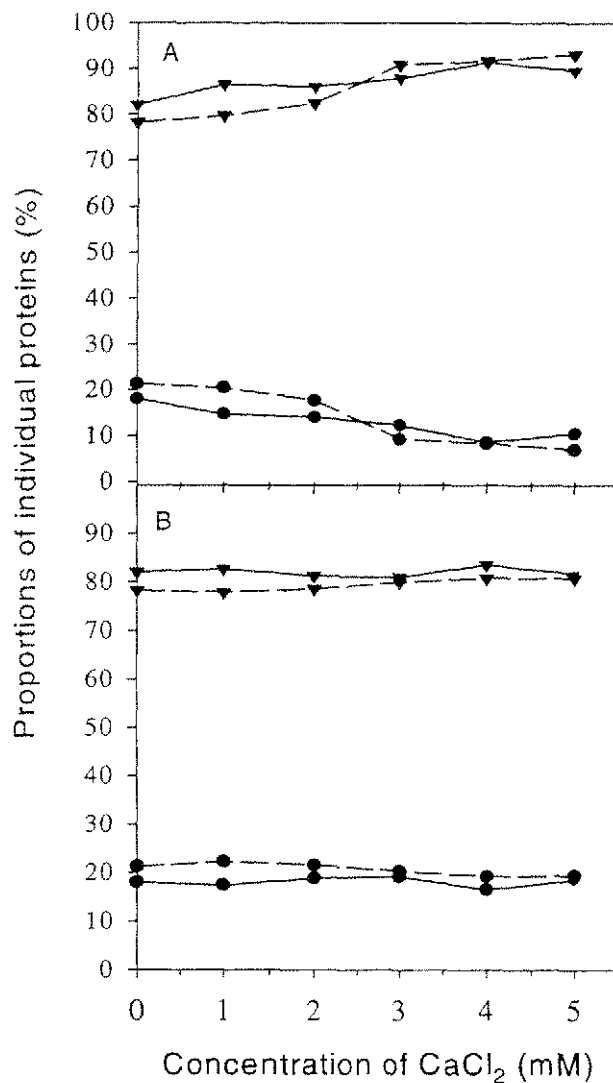
Figure 6.8: Changes in the proportions of α-lactalbumin (●) and β-lactoglobulin (▼) at the droplet surface (cream phase) of emulsions formed with 30% soya oil and 0.5% WPC as a function of $CaCl_2$ concentration. $CaCl_2$ was added before (A) or after (B) emulsion formation. NaCl (200 mM) was added to emulsions at each $CaCl_2$ concentration. (____) without NaCl, (----) with NaCl. Each data point is the average of determinations on two separate emulsions.
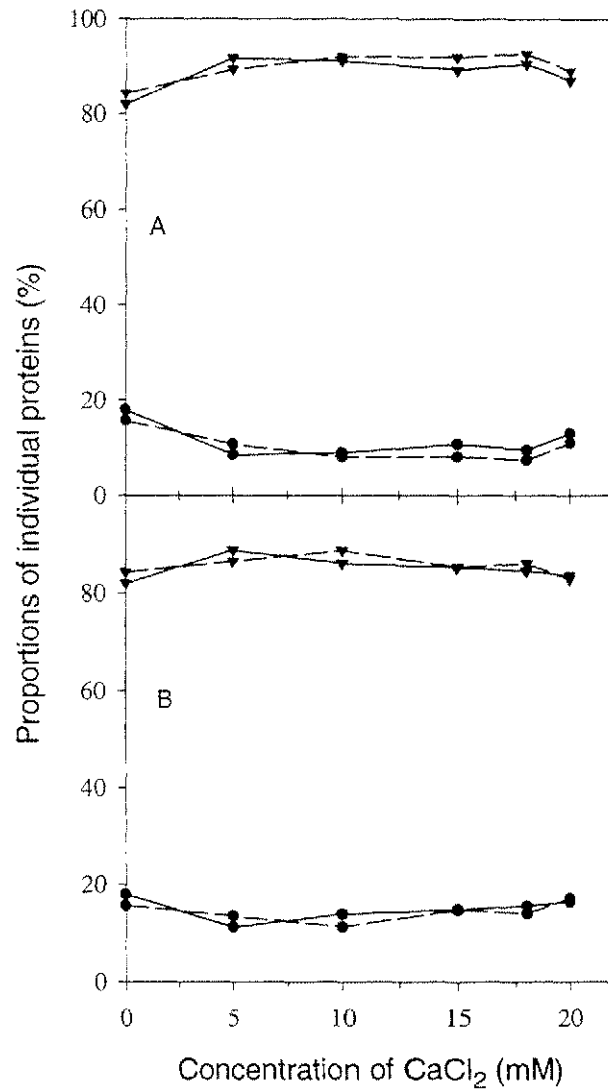
Figure 6.9: Changes in the proportions of α-lactalbumin (●) and β-lactoglobulin (▼) at the droplet surface (cream phase) of emulsions formed with 30% soya oil and 3.0% WPC as a function of CaCl₂ concentration. CaCl₂ was added before (A) or after (B) emulsion formation. NaCl (200 mM) was added to emulsions at each CaCl₂ concentration. (——) without NaCl, (----) with NaCl. Each data point is the average of determinations on two separate emulsions.

6.2.5 Creaming stability of emulsions

The effect of addition of $CaCl_2$ prior to or after emulsion formation on creaming stability of emulsions made with 0.5% and 3% WPC is shown in Figure 6.10 and Figure 6.11. In emulsions where $CaCl_2$ was added prior to emulsion formation, the creaming stability of emulsions made with 0.5% WPC decreased from ~ 60 to ~ 30% with $CaCl_2$ addition up to 5 mM. In emulsions made with 3% WPC, the stability decreased slightly at low $CaCl_2$ concentrations (< 15 mM), but decreased dramatically from ~ 70% to ~ 7% beyond 15 mM (Figure 6.11 A). It indicates that the changes in creaming stability of WPC-stabilized emulsions with $CaCl_2$ addition were dependent on their particle sizes under these conditions.

Addition of $CaCl_2$ to emulsions also resulted in a decrease in the creaming stability of emulsions made with 0.5% WPC (Figure 6.10 B). But the decrease (~ 60% to ~ 40%) was less marked than that of addition of $CaCl_2$ prior to emulsion formation, especially at higher concentrations of $CaCl_2$ (> 3 mM), where the creaming stability remained almost constant, despite the particle size still increased. It indicated that the creaming stability did not completely follow the particle size of aggregated emulsions in this condition. It implies that the different aggregation mechanisms exist between $CaCl_2$ added to protein solution prior to emulsion formation or added to emulsions after they were made. In emulsions made with 3% WPC, addition of $CaCl_2$ to emulsions did not significantly affect the stability rating of emulsions (Figure 6.11 B).

Addition of 200 mM NaCl to emulsions, made with 0.5% or 3% WPC and containing $CaCl_2$, had no significant effect on creaming stability (Figure 6.10 and Figure 6.11).

Figure 6.10: Changes in stability rating (%) of emulsions (30% soya oil) stabilized by 0.5% WPC as a function of $CaCl_2$ concentration (●). $CaCl_2$ was added before (A) or after (B) emulsion formation. 200 mM NaCl was added to emulsions at each $CaCl_2$ concentration (▼). Each data point is the average of determinations on two separate emulsions.
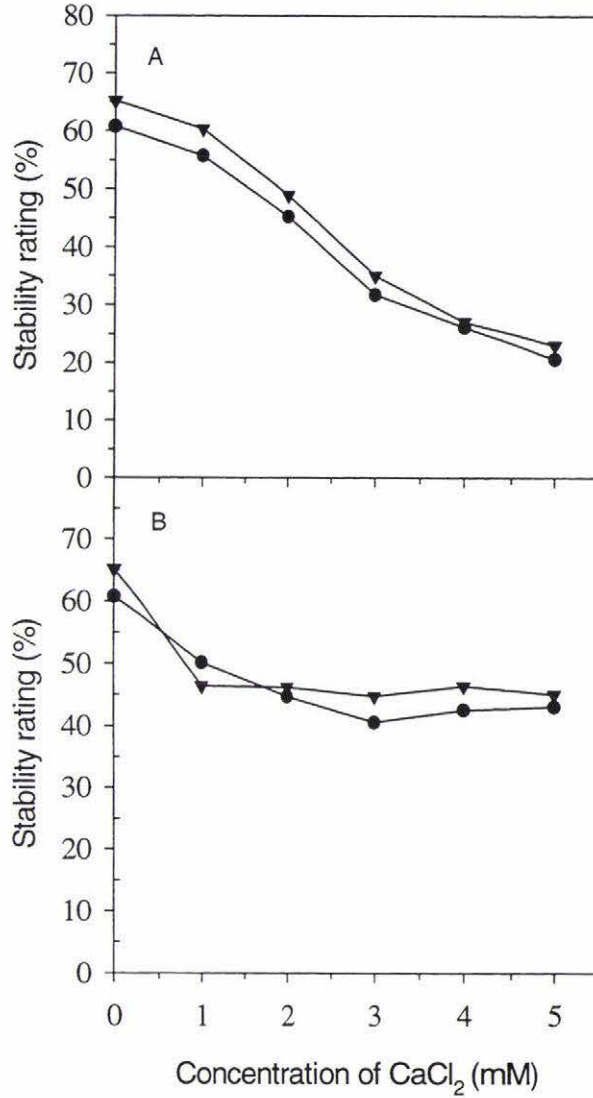
Figure 6.11: Changes in stability rating (%) of emulsions (30% soya oil) stabilized by 3.0% WPC as a function of $CaCl_2$ concentration (●). $CaCl_2$ was added before (A) or after (B) emulsion formation, 200 mM NaCl was added to emulsions at each $CaCl_2$ concentration (▼). Each data point is the average of determinations on two separate emulsions.
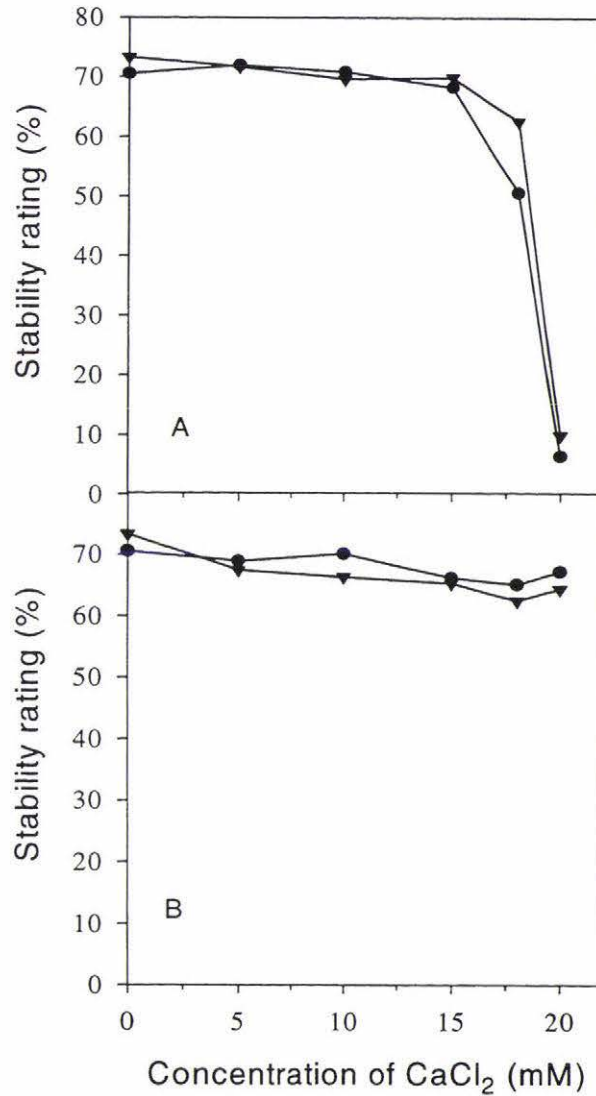
6.2.6 Microstructure of emulsions

The microstructure of these emulsions was examined using confocal microscopy for investigating the state of droplet aggregation and providing a visual perspective of the aggregation. In the micrographs, it is clear that there were different changes in the particles of emulsions, depending upon the mode of $CaCl_2$ addition. Figure 6.12 B shows that the emulsion droplets remained homogeneous with no sign of flocculation when 3 mM $CaCl_2$ was added to 0.5% WPC solution before emulsification. At 5 mM $CaCl_2$, in addition to some droplets around 10 μm, small particles that were aggregated together were observed (Figure 6.12 C). Higher $CaCl_2$ concentration (10 mM) caused greater aggregation of small particles, rather than the formation of larger droplets (Figure 6.12 D). This observation was in agreement with the results observed for particle size distribution of emulsions (Figure 6.5). Similar changes in emulsion particles with increase in $CaCl_2$ concentration were found in the emulsions made with 3.0% WPC when $CaCl_2$ was added to WPC solution (Figure 6.13 A B C D).

The micrograghs of emulsions, made with 0.5% WPC, with various amounts of $CaCl_2$ added are shown in Figure 6.14. The homogeneous and stable oil droplets still appeared at 5 mM $CaCl_2$ added emulsion (Figure 6.14 B). However, at 10 mM $CaCl_2$ added, a network structure of flocculated droplets was formed (Figure 6.14 C). This network structure was enhanced at higher $CaCl_2$ concentration (30 mM) (Figure 6.14 D). This flocculation structure was obviously different from the appearance of flocculation in emulsion made with WPC with $CaCl_2$ added before emulsification (Figure 6.12). It was also different from that of flocculation in emulsions made with sodium caseinate with $CaCl_2$ added to emulsion (see Chapter 5), in which the amorphous aggregated clusters (about 10 – 15 μm) were formed from some droplets (see Chapter 5, Figure 5.13). Microscopy on emulsions made with 3% WPC indicate that $CaCl_2$ added to emulsion had no effect on the appearance of emulsion droplets even

at 20 or 100 mM $CaCl_2$ (Figure 6.15 A or B). The emulsions appeared to be homogeneous with no sign of flocculation.

Agboola and Dalgleish (1995) observed the structure of flocculated emulsions induced by Ca^{2+}, using electron microscopy; they also found that emulsions made with caseinate formed amorphous aggregated particles, whereas those containing β-lactoglobulin appeared to produce fine strands of aggregates.

A

B

Figure 6.12: Confocal micrographs of 0.5% WPC-stabilized oil-in-water emulsions; 0 mM (A), 3 mM (B), 5 mM (C) or 10 mM (D) CaCl$_2$ was added to protein solution prior to emulsion formation.

Figure 6.12: (Continued).

Figure 6.13: Confocal micrographs of 3.0% WPC-stabilized oil-in-water emulsions; 0 mM (A), 5 mM (B), 15 mM (C) or 20 mM (D) CaCl₂ was added to protein solution prior to emulsion formation.

Figure 6.13: (Continued).

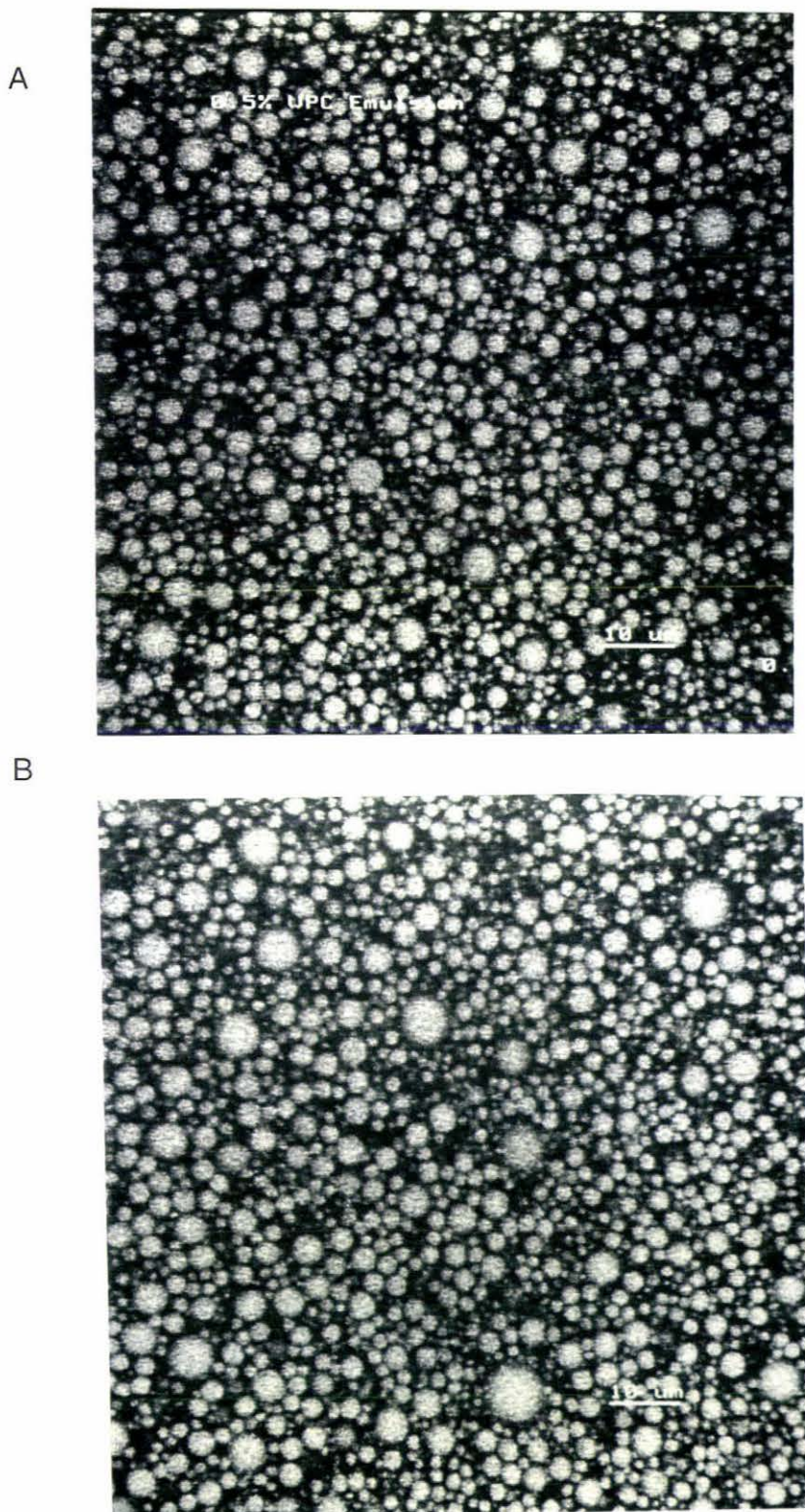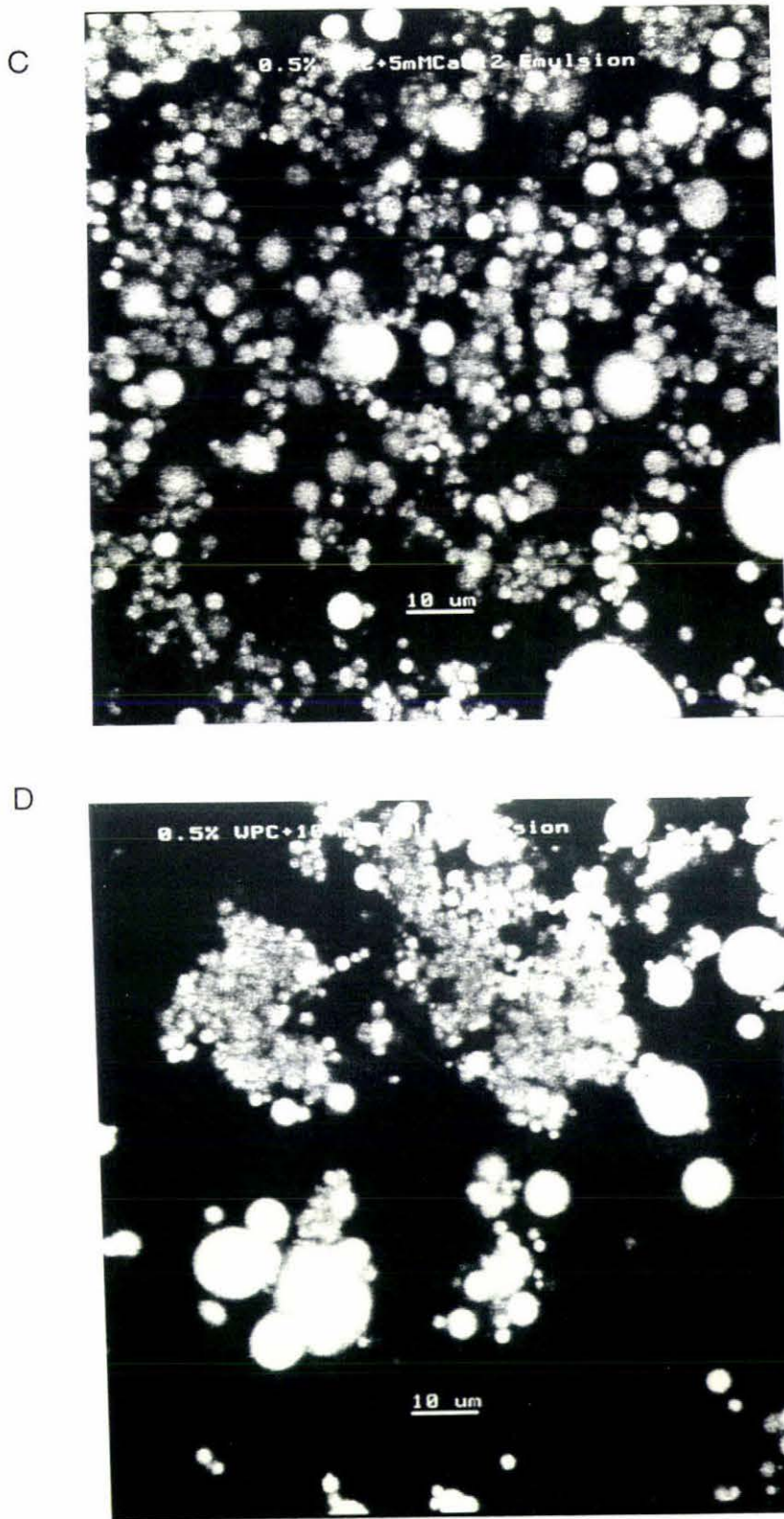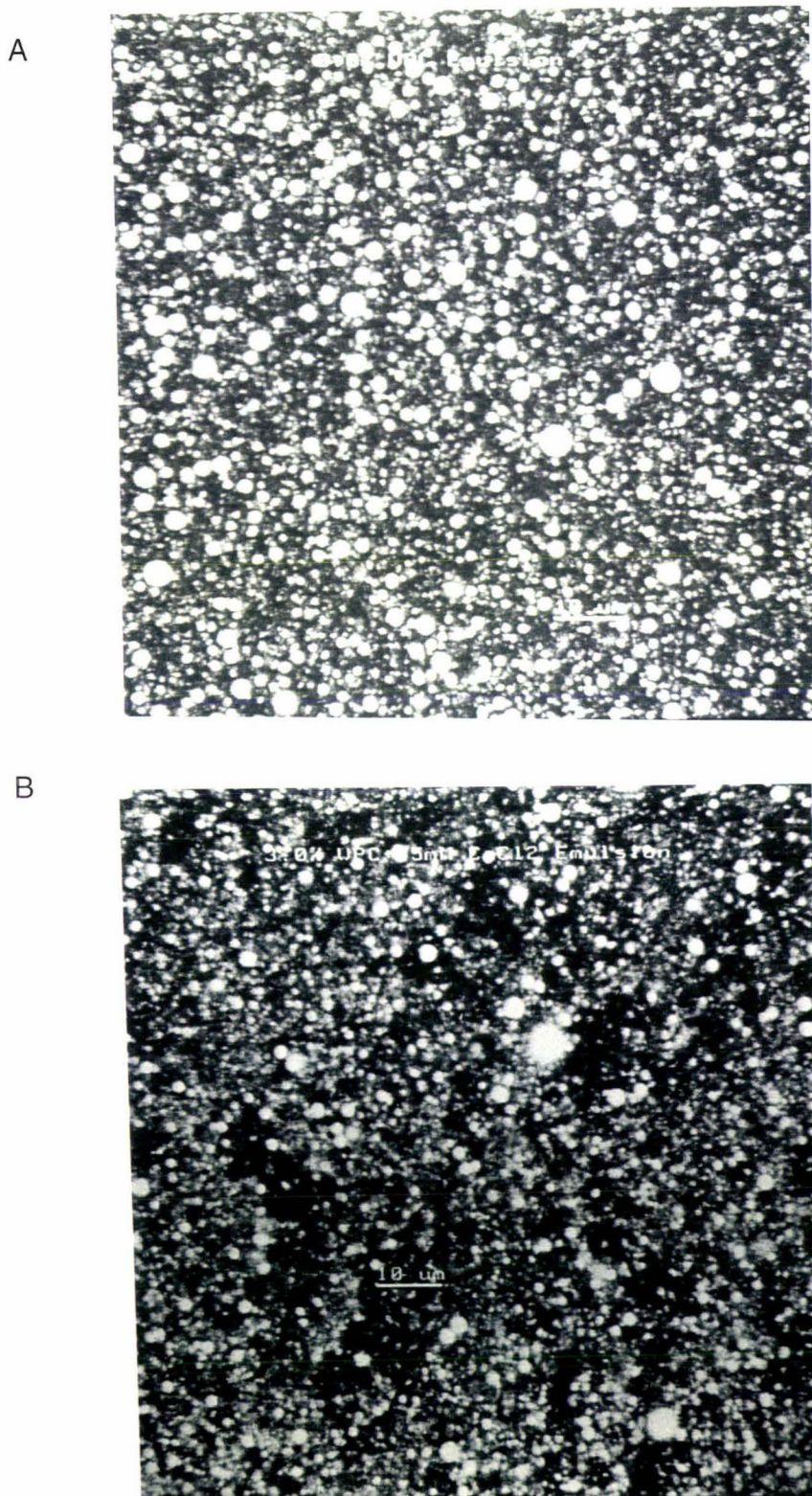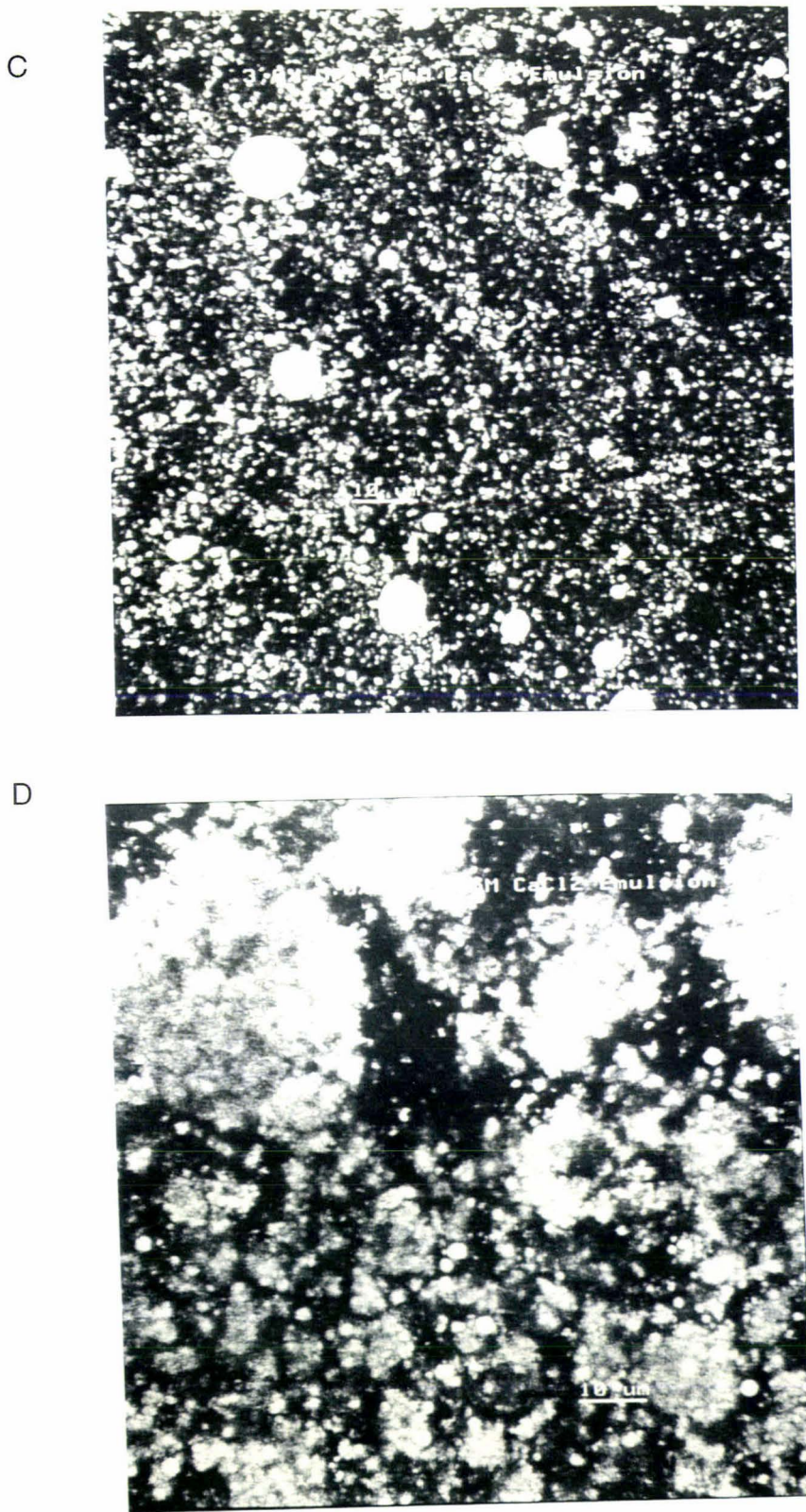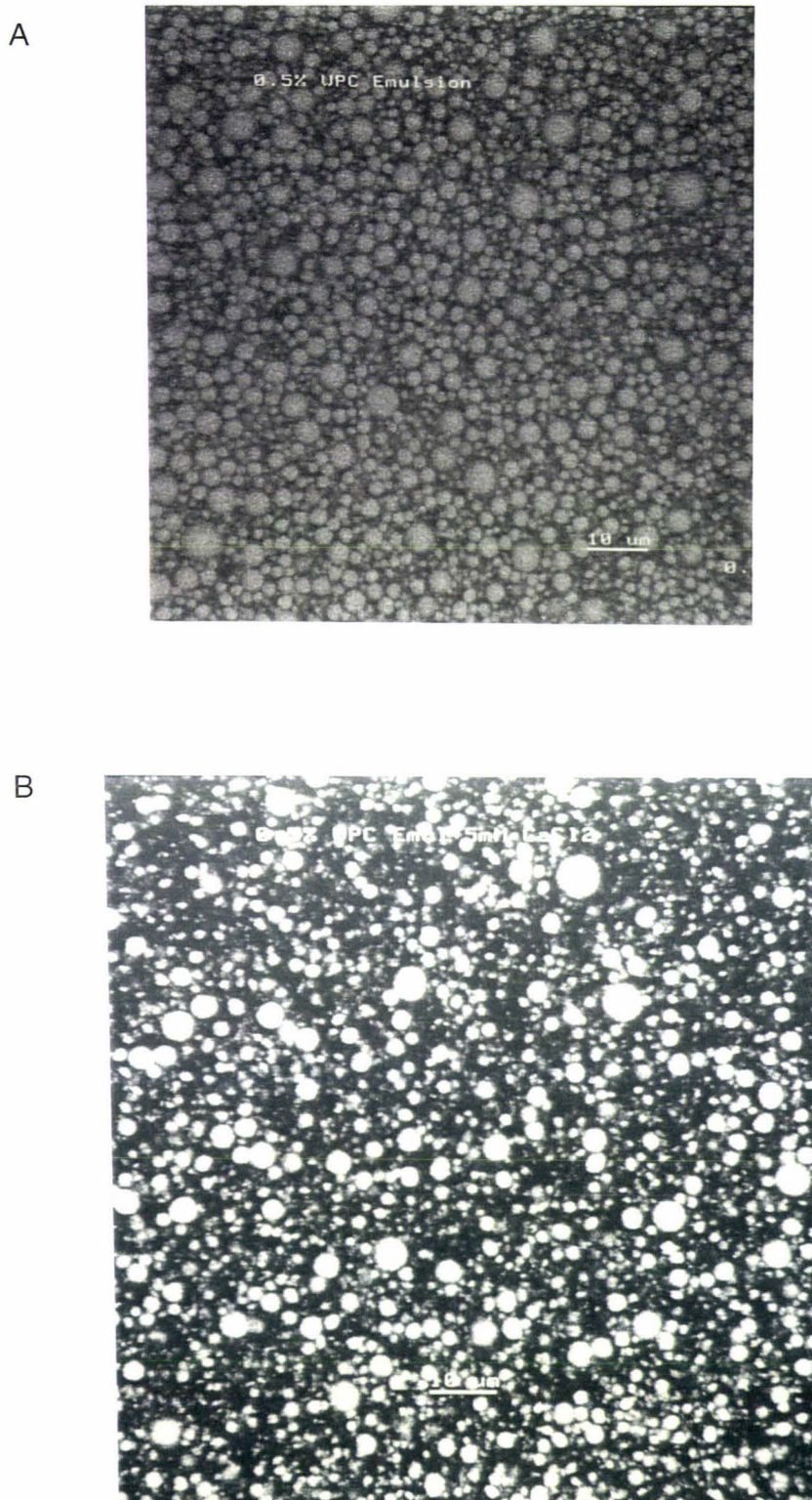**Figure 6.14:** Confocal micrographs of 0.5% WPC-stabilized oil-in-water emulsions; 0 mM (A), 5 mM (B), 10 mM (C) or 30 mM (D) CaCl$_2$ was added to emulsions after they were formed.
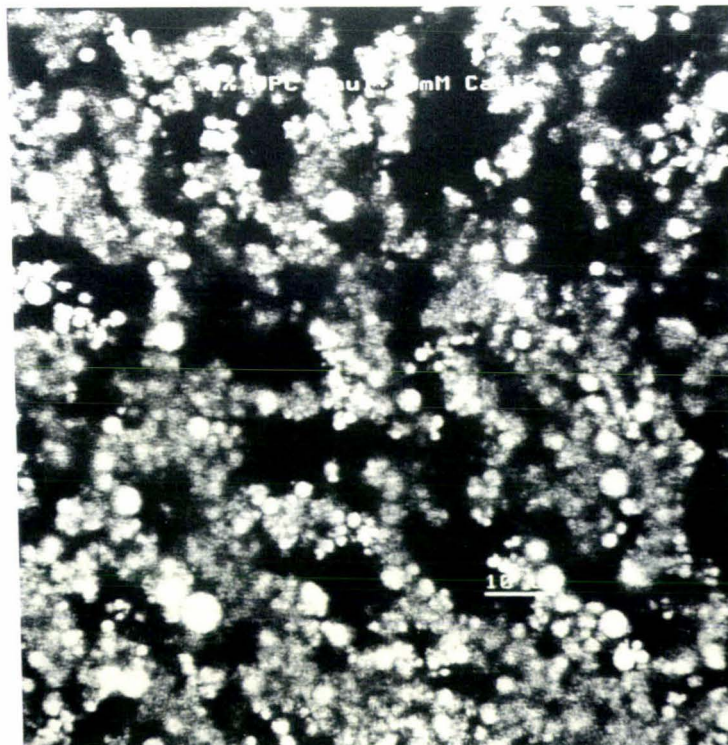
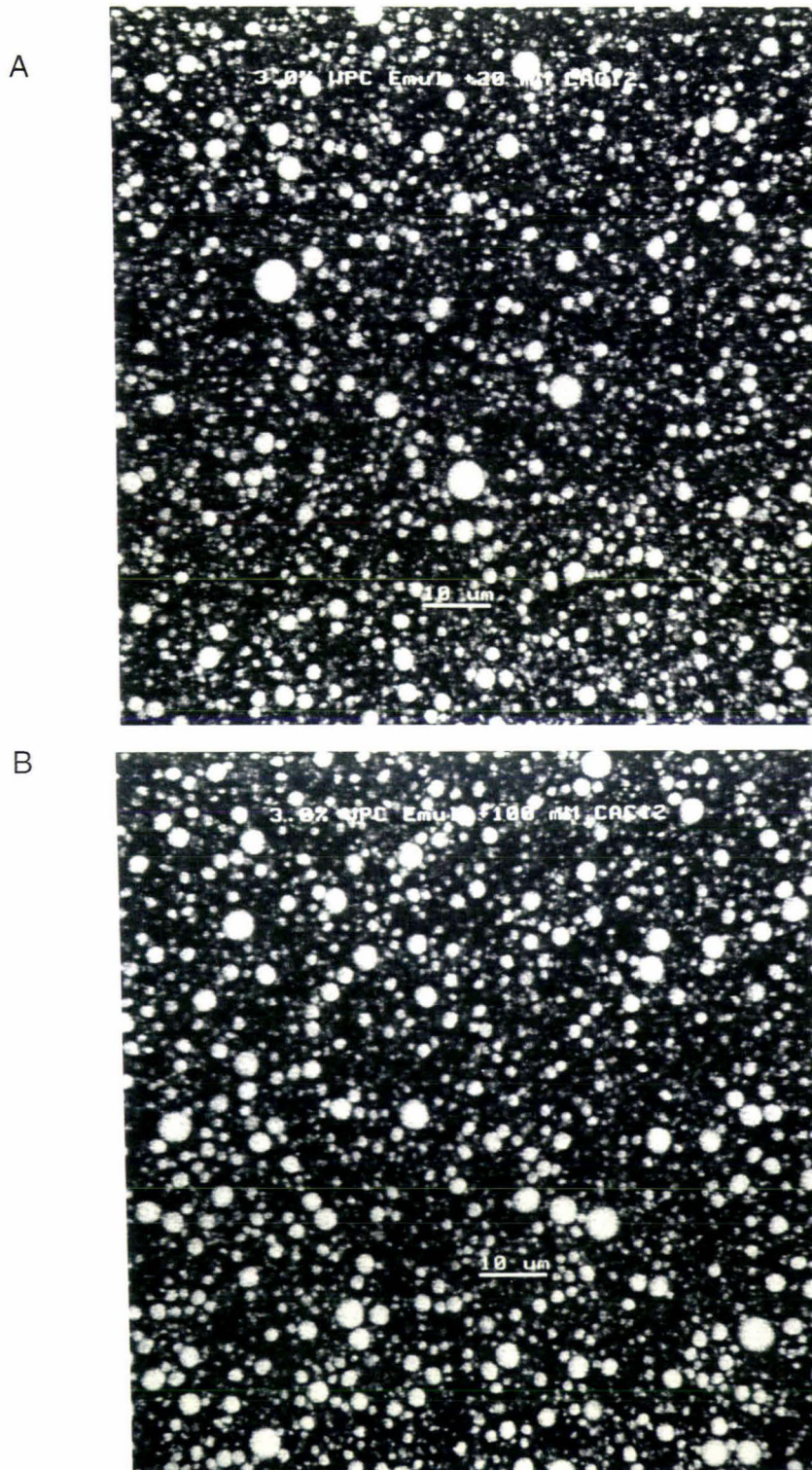**Figure 6.14**: (Continued).

Figure 6.15: Confocal micrographs of 3.0% WPC-stabilized oil-in-water emulsions; 20 mM (A), 100 mM (B) CaCl₂ was added to emulsions after they were formed.

6.3 Discussion

The present results (Figure 6.1 A and Figure 6.2 A) show that the addition of $CaCl_2$ to WPC solution prior to emulsification influences the particle size of emulsions, especially at high $CaCl_2$ concentrations. This may be attributed to binding of Ca^{2+} with whey proteins in solution, subsequently leading to a decrease in their emulsifying capacity. When $CaCl_2$ is added to WPC solution, Ca^{2+} can interact with β-lactoglobulin and α-lactalbumin (Pappas and Bothwell, 1991) via electrostatic interactions, involving ionic amino acid groups of the polypeptide chains, i.e., the free carboxylic groups of aspartic and glutamic acids (Baumy and Brule, 1988; Pappas and Bothwell, 1991). Ca^{2+} binding to protein reduces electrostatic repulsion between the molecules (Horne and Dalgleish, 1980) and increase the potential for inter-molecular associations (Kuhn and Foegeding, 1991). Xiong (1992) reported that the addition of calcium increased the aggregation of whey proteins. Therefore, the conformations and quaternary structures of β-lactoglobulin and α-lactalbumin may have already changed from that of original proteins to more associated structures due to addition of Ca^{2+} before they adsorb on the droplet surface. These changes do not appear to decrease their adsorption at the emulsion droplets, but an increase in surface concentration of protein is observed (Figure 6.6 A and Figure 6.7 A). The transport of protein to the interface may be affected; the protein molecules may not undergo spreading and rearrangement effectively. Consequently, the protein might not able to cover immediately all new surface area of oil droplets created during homogenization, and achieve a state of lowest free energy. Some smaller droplets that have higher surface free energy would coalesce to larger droplets. These reasons might be responsible for the decrease in the emulsifying capacity of whey protein and coalescence during homogenization in emulsions made with WPC solution containing low levels of $CaCl_2$.

It is considered that strong droplet aggregation in emulsions made with WPC containing high concentrations of $CaCl_2$ (Figure 6.1 A and Figure 6.2 A) may

mainly be because of the protein bridging flocculation between emulsion droplets since it could not been reversed by dilution in the stirrer of the MasterSizer, but could be dissociated by SDS solution (Figure 6.1). The mechanism of protein bridging flocculation has been discussed in Chapter 5.

Binding of Ca^{2+} to β-lactoglobulin and α-lactalbumin is weak compared to that to the caseins (Patocka and Jelen, 1991; Pappas and Rothwell, 1991). In fact, treatment of β-lactoglobulin at 25°C with calcium does not bring about any aggregation (Pappas and Rothwell, 1991). This is in agreement with our results that the extent of aggregation in emulsions made with WPC containing Ca^{2+} was lower than emulsions made with caseinate in presence of Ca^{2+} (see Chapter 5). However, it was found that strong aggregation occurred and surface protein concentration increased in emulsions made with 3.0% WPC containing high concentrations of $CaCl_2$ (Figure 6.2 A and Figure 6.7 A). It suggests that large protein aggregates, due to Ca^{2+} binding, form at this concentration of protein, which may be attributed to a decrease in whey protein aggregation temperature due to presence of Ca^{2+}. Barbut and Foegeding (1993) reported that the initial temperature required for whey protein aggregate formation decreased from 72°C to 45°C in protein solution (4%) containing 10 mM $CaCl_2$; this must be the result of $CaCl_2$ mediated protein-protein interactions. Since 55°C was used during homogenization for making emulsion in present experiments, aggregation due to Ca^{2+} binding might occur and be enhanced before adsorption to oil droplets.

The addition of $CaCl_2$ to emulsions formed with 0.5% WPC after emulsification also affected the particle size of emulsions (Figure 6.1 B). Agboola and Dalgleish, (1995, 1996a) reported that the change in the stability of β-lactoglobulin (chief whey protein) stabilized emulsion containing $CaCl_2$ was because of the binding of Ca^{2+} to β-lactoglobulin. However, they did not explain the low sensitivity to Ca^{2+} in emulsions made with higher concentrations of β-lactoglobulin (Agboola and Dalgleish, 1995). In our observation, the Ca^{2+} addition (ever 100 mM) did not change the droplet size of emulsion formed with

3.0% WPC (Figure 6.3). It has been mentioned that the Ca^{2+} binding to β-lactoglobulin and α-lactalbumin is weak at room temperature. But strong Ca^{2+} binding and aggregation in β-lactoglobulin after heating indicate that calcium-binding groups, i.e. carboxyl groups, may become exposed during the heat-induced unfolding of the molecule of β-lactoglobulin (Mulvihill and Kinsella, 1988; Pappas and Rothwell, 1991). An extent of unfolding induced by heating or pressure is a pre-condition for Ca^{2+} induced aggregation and gelation of whey proteins (Pappas and Rothwell, 1991; Barbut and Foegeding, 1993).

It is known that adsorption onto interfaces tends to cause protein to denature (Dickinson, 1989; Dalgleish, 1995). When β-lactoglobulin and α-lactalbumin are adsorbed to an oil-water interface, the unfolded structure increases (Dalgleish, 1996; Fang and Dalgleish, 1998). The unfolded structures on the surface of an emulsion droplet offer more chance to form protein-Ca-protein cross-links between emulsion droplets. This may be the reason why a considerable aggregation occurred in emulsions made with 0.5% WPC as Ca^{2+} was added to emulsions after emulsification without heating (Figure 6.3). However, protein molecules adsorbing from a concentrated solution may not unfold much even if they reach the fresh interface first (Dickinson *et al.*, 1988). The less sensitivity of Ca^{2+} in emulsions made with higher concentration of whey protein may be attributed to the less spreading and unfolding at high surface protein concentrations. Our results may offer an evidence that the adsorbed whey protein unfolds or denatures on surfaces at low concentrations, but there is less unfolding at high concentrations.

The decrease in the average particle size of Ca^{2+}-induced aggregated emulsions made with WPC when NaCl was added (Figure 6.1 B) may be attributed to Na^+ competing with Ca^{2+} at specific binding sites. Baumy and Brule (1988) reported that Na^+ might bind to whey proteins in solution at place of other metal cations. Patocka and Jelen (1991) also reported the possibility of Na^+ competing with Ca^{2+} for negative groups as well as for any specific metal binding sites of polypeptide chains in solutions of β-lactoglobulin.

The presence of $CaCl_2$ increased the surface protein coverage of emulsion droplets made with WPC, not only during homogenization, but also when the $CaCl_2$ is added to emulsions after emulsion formation (Figure 6.6 and Figure 6.7). The increase in surface protein concentration may be attributed to more compact packing of the adsorbed protein molecules, resulting from decreased repulsive interactions between adsorbed proteins or between adsorbed and non-adsorbed protein molecules with an increase in Ca^{2+} concentration. In addition, aggregated whey protein in solution induced by Ca^{2+} adsorbed to the interface may lead to high surface coverage in emulsions made with WPC in the presence of $CaCl_2$ prior to emulsification, especially in emulsions made with high concentration of WPC (Figure 6.7 A).

With respect to the effect of Ca^{2+} on the composition of protein layer at the oil-water interface of emulsions made with WPC in presence of $CaCl_2$, it is shown that β-lactoglobulin was adsorbed slightly in preference to α-lactalbumin (Figure 6.8 A and Figure 6.9 A). This may be attributed to the slightly higher calcium-binding capacity of β-lactoglobulin than α-lactalbumin. Pappas and Rothwell (1991) reported that the moles of calcium bound to 10^5 g of β-lactoglobulin were 24.6 and 35.3 for calcium concentration of 10 and 20 mmoles/litre, respectively, whereas the moles of calcium bound to 10^5 g of α-lactalbumin were found to be 23.3 and 31.0 for calcium concentrations of 10 and 20 mmoles/litre, respectively.

The microstructures of aggregated emulsions, induced by added Ca^{2+}, demonstrate a sort of gel-like network structure (Figure 6.14). Previous studies have observed that an emulsion gel was formed in a moderately concentrated emulsion stabilized by an adsorbed layer of a globular protein like β-lactoglobulin by heading, high-pressure treatment or addition of second polymer (*e.g.* gelatin) (Jost *et al.*, 1986; McClements *et al.*, 1993 a; Dickinson and Hong, 1995; Dumay *et al.*, 1996, Dickinson, 1998). The emulsion gel is a particle gel composed of aggregated protein-coated oil droplets. The association of the

droplets is triggered by the denaturation and subsequent aggregation of protein adsorbed at the surface of the droplets and in the continuous phase between droplets. A stable liquid-like emulsion may be converted into an emulsion gel by causing the protein-coated droplets to become joined together into some sort of network structure (Dickinson, 1998).

The formation of a gel needs some conditions – two important factors are: protein concentration and protein-protein interaction. The local protein concentration is effectively increased many fold by the adsorption of protein to surface. It is calculated that the protein concentration in the interfacial layer is more than 30% by weight, even though the bulk concentration may be as low as 0.5%, calculated from surface protein concentration (3 mg/m^2) and protein layer - thickness (2-10 nm) (Dalgleish and Hunt, 1995). Such high concentrations make interprotein interactions very likely. The formation of protein-Ca-protein cross-links between protein-coated emulsion droplets, induced by added CaCl$_2$, because of unfolding or denaturation of whey protein at surfaces, has been discussed above.

From the above description, it is entirely probable that a weak emulsion gel is formed in the aggregated WPC–stabilized emulsions, induced by added Ca^{2+}. In relation to gelation in whey protein solutions with respect to added Ca^{2+}, Mulvihill and Kinsella (1988) observed that WPI (10% protein, pH 7.0, 80°C for 30 min) did not form thermally-induced gels unless the protein suspension had added salts and CaCl$_2$ under certain conditions. Barbut and Foegeding (1993) reported that Ca^{2+} could induce the gelation of pre-heated WPI suspension at 25°C; the gel had a fine-stranded protein matrix. It was because there is still sufficient protein unfolding and/or aggregation to allow Ca^{2+}-induced gelation (Pappas and Rothwell, 1991; Barbut and Foegeding, 1993).

The trends (Figure 10 A and Figure 11 A) in creaming stability of emulsions made with WPC in the presence of CaCl$_2$ suggested that the stability was mainly dependent on the particle size of emulsions. Increase in the particle size

due to coalescence and flocculation induced by Ca^{2+} lead to decrease in creaming stability. However, in the case of the addition of $CaCl_2$ to emulsions after emulsion formation, the creaming stability reached a plateau at high concentration of Ca^{2+} (Figure 6.10 B), although the d_{43} still increased. It may be attributed to the formation of network structure in emulsions (Figure 6.14). Strong destabilization can turn into pseudostability state in systems with high volume ratios of colloid particles (Syrbe *et al.*, 1998). Destabilization makes the particles sticky, so that they aggregate into a particle network. If the attractive potential between particles is strong enough, the time-scale of rearrangement into a close packing can reach the order of months (Ploehn and Russel, 1990), thereby retarding the decrease in creaming stability.

6.4 Conclusions

Addition of $CaCl_2$ to protein solution prior to emulsion formation increased the particle size and surface protein concentration in emulsions made with 0.5 or 3.0% WPC. The adsorption of β-lactoglobulin occurred slightly in preference to α-lactalbumin. The creaming stability of emulsions decreased with increase in the concentration of $CaCl_2$. Relatively high concentration of Ca^{2+} may induce aggregation of whey proteins in solution, leading to a decrease in emulsifying capacity and consequentially resulting in formation of protein bridging flocculation between emulsion droplets during emulsion formation.

The protein unfolding at the surface of emulsion droplets made with low WPC concentration may promote protein-Ca-protein forming between protein-coated emulsion droplets. Addition of Ca^{2+} to emulsions after emulsion formation leads to aggregation of emulsion droplets and formation of gel-like network structure in emulsions made with low concentration of WPC. The network structure prevents further decrease in creaming stability of emulsions. The emulsions made with high WPC concentrations are stable to addition of Ca^{2+}. The presence of Ca^{2+} increased the surface coverage of emulsion made with WPC. The confocal microscopic observations are an effective examination method for supporting the results determined by other methods in the study of the stability change of emulsions induced by Ca^{2+}.

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