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Behaviour of Fat Globules and Membrane Proteins under Different Processing Environments as Related to Milk Powder Manufacture

Massey University

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN FOOD TECHNOLOGY

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AIQIAN YE

RIDDET CENTRE AND INSTITUTE OF FOOD, NUTRITION AND HUMAN HEALTH

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ABSTRACT

The objective of the first part in this study was to gain a better understanding of the protein components of the milk fat globule membrane (MFGM). In the second part, the influence of processing factors on the fat globules and the MFGM during the manufacture of whole milk powder were examined. Relationships between the state of the MFGM in whole milk powders and their reconstitutions properties were also explored.

The MFGM proteins, isolated from early-, mid- and late-season fresh whole milks, were characterized using one- and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. SDS-PAGE under reducing conditions showed the presence of about 40 protein bands, ranging in molecular weight from 15 to 200 kDa. The major MFGM proteins e.g., xanthan oxidase, butyrophilin, PAS 6 and PAS 7 constituted 60-70% of total MFGM proteins while 20-30% were minor proteins. Two-dimensional SDS-PAGE indicated that xanthine oxidase and butyrophilin might be complexed via intermolecular disulfide bonds in the natural MFGM. The examination of MFGM proteins heated at > 60 °C in the absence of skim milk proteins (caseins and whey proteins) showed that xanthine oxidase and butyrophilin interacted further to form very high molecular weight protein complexes, whereas PAS 6 and PAS 7 were relatively heat stable and did not form complexes.

Heat treatment of fresh whole milk in the temperature range 65-95 °C caused incorporation of β-lactoglobulin (β-lg) into the MFGM. Small amounts of α-lactalbumin (α-la) and κ-casein were also observed in the MFGM material of heated milk. The amounts of β-lg and α-la that associated with the MFGM increased with an increase in temperature up to 80 °C, and then remained almost constant. The maximum values for β-lg and α-la association with the MFGM were ~1.0 mg/g fat and ~0.2 mg/g fat, respectively. Association of β-lg and α-la with the MFGM was described by a first-order reaction (65-85 °C for β-lg and 70-80 °C for α-la) in the low temperature range and by a second-order reaction in the high temperature range (85-95 °C for β-lg and 80-95 °C for α-la). Arrhenius plots showed an abrupt change in temperature dependence of

the rate constants at 85 °C for β-lg and 80 °C for α-la. Of the major original MFGM proteins, xanthine oxidase and butyrophilin were not affected by the heat treatment of whole milk, whereas PAS 6 and PAS 7 decreased during heating. Interestingly, this behaviour is in contrast to that shown by these proteins in systems containing no skim milk proteins.

The changes in fat globule size and MFGM proteins during the manufacture of whole milk powder were determined using light scattering, SDS-PAGE, confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM).

Heat treatment of whole milk by direct stream injection (DSI) prior to evaporation caused a decrease in the fat globule size and an increase in the MFGM protein, through the association of caseins and whey proteins with the MFGM material.

Evaporation of milk by a multiple-effect falling film evaporator caused a gradual decrease in the fat globule size and an increase in the MFGM protein after each effect. Caseins dominated the total MFGM protein, indicating the adsorption of casein micelles to the newly formed surface of the fat globules during evaporation. When whole milk was preheated (95 °C for 20 s) before evaporation, the amounts of total MFGM protein were higher (~6 mg/m^2 compared to ~4 mg/m^2 for the non-preheated whole milk) because of association of whey proteins with the native MFGM proteins and casein micelles.

The average fat globule size decreased further upon homogenisation of the concentrated milk. The amount of MFGM protein (mg/m^2) of concentrated milk also increased after homogenisation, the extent of the increase being dependent upon the temperature and pressure of homogenisation. Furthermore, heat treatment of concentrated milk to 79 °C either before or after homogenisation also increased the amount of MFGM protein. However, at the same homogenisation temperature and pressure, the amounts of whey proteins in the MFGM of the concentrated milk that was heated after homogenisation were higher than the concentrated milk that was heated followed by homogenisation.

The amounts of the major native MFGM proteins did not change during homogenisation, indicating that the skim milk proteins did not displace the native MFGM proteins but adsorbed onto the newly formed surface.

The fat globule size of homogenized concentrated milk decreased after spray drying, while the amount of MFGM protein (mg/m^2) decreased slightly. Some "uncovered fat" was observed on the surface of powder particles. It is possible that the proteins do not adsorb to all newly formed fat surfaces during spray drying.

The reconstitution properties of whole milk powders produced using different processing treatments were determined. High homogenization pressure and temperature used before spray drying resulted in poor reconstitution properties of the powder, particularly when the heat treatment was carried out after homogenization. It is suggested that the proteins adsorbed at the fat globule surfaces during homogenisation of the concentrated milk and their subsequent aggregation during heat treatment play a key role in determining the reconstitution properties of whole milk powders.

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CHAPTER 1
INTRODUCTION

Whole milk powder (WMP) is a very important consumer product for the New Zealand dairy industry with a market value of about $500 million/year. It is a major thrust of the New Zealand dairy industry to increase the proportion of this product sold directly to the consumer. As WMP is generally used by the consumer as a fresh milk substitute and as a whitener in tea and coffee, its reconstitution properties in water over a wide range of temperatures are very important criteria for its acceptability. The functionality, particularly the reconstitution properties, of WMP can be highly variable and occasionally unpredictable, and solutions to functionality problems tend to be largely empirical in nature. In order to manufacture products that will function consistently and outperform those of the New Zealand dairy industry competitors, greater background knowledge of what is causing functionality problems is needed.

In the manufacture of WMP, milk is subjected to a range of processes, such as agitation, pumping, heating, concentration, homogenization and spray drying. These processing treatments cause a number of physical and chemical interactions of milk components. The interactions of the specific milk components, *i.e.* casein micelles, whey proteins, fat globules and minerals, during milk powder processing play a major role in determining functional properties when the powder is reconstituted in water.

Preheat treatment of milk, prior to evaporation, is the most important tool in the manufacture of specialized milk powders. The most obvious change during preheating is the denaturation of whey proteins and their association with the casein micelles. The kinetics of these reactions in skim milks has been studied previously (Dannenberg & Kessler, 1988; Anema & Mckenna, 1996; Oldfield et al., 1998 a). However, most of this work has focused on skim milk systems and there has been little work reported on the interactions of fat globules during the preheating step in relation to WMP manufacture.

The natural fat globules in milk are coated with a protective layer, generally known as the milk fat globule membrane (MFGM). The protein composition of the MFGM is very complex; over 40 different polypeptides, ranging in molecular weight from 15 to 240

kDa, have been observed (Mather, 2000). The state of major MFGM proteins and the structure of the MFGM are still unclear.

It is generally accepted that the MFGM is markedly affected by processing treatments, such as homogenization, heat treatment and spray drying during manufacture of powder. Protein-protein and protein-fat interactions involving the MFGM have been shown to influence the reconstitution properties of powder in water (Mol, 1975; Caric & Kalab, 1987; Ohba et la., 1989; de Ruyck, 1991; McKenna et al., 1999). Published work on interactions between fat globules and proteins in heated whole milk shows that whey proteins bind to fat globules during the heat treatment of whole milk, but there is some debate as to how these proteins are incorporated into the MFGM (McPherson et al., 1984; Dalgleish & Banks, 1991; Houlihan et al., 1992; Kim & Jimenez-Flores, 1995). The details of kinetics and mechanisms of binding of proteins with fat globules have not been fully elucidated (Corredig & Dalgleish, 1996; Sharma & Dalgleish, 1994). No systematic studies have been carried out on the behaviour of MFGM and fat globules during evaporation and spray drying.

Furthermore, in New Zealand, milk production is seasonal and there are changes in the composition of milk during the season. For example, the absolute concentration of protein is high at the beginning and end of the season and this may affect the functional properties of whole milk powder. Little is known about the seasonal changes in the MFGM composition and its interactions during processing treatments.

The overall aim of this thesis was to further our knowledge about the influence of processing factors on the fat globules and the MFGM during the manufacture of whole milk powders, and to understand the effects of changes in the fat globules and the MFGM proteins on the reconstitution properties of whole milk powders.

CHAPTER 2
LITERATURE REVIEW

2.1 General characteristics of milk components

Milk is a complex fluid containing many components in several states of dispersion. The main components of milk are fat, proteins, lactose, minerals and water. The fat, proteins, lactose and minerals make up the ~13% total solids content of milk. The general composition of whole milk is shown in Table 2.1. The fat component of milk is mainly triglycerides (98%), present as an emulsion of fat globules stabilised by a phospholipid and glycoprotein membrane. The protein content of milk can be divided into casein and whey proteins. In milk, caseins are present in colloidal suspension, where as whey proteins are present in solution. Lactose, a sugar found only in milk, is present in solution. Lactose is a disaccharide, containing the monosaccharides, glucose and galactose. The minerals of milk occur either in solution or are associated with the proteins, as either undissolved salts or bound ions.

Table 2.1. Typical raw milk composition

Component	Level in milk (% w/w)
Water	86.50
Fat	4.70
Protein	3.25
Casein	2.60
Whey protein	0.65
Lactose	4.80
Minerals	0.65

2.2 General characteristics of milk proteins

Milk proteins fall into two main categories: caseins and whey proteins. Caseins represent ~80% while whey proteins represent the rest (~20%) of the total milk proteins.

Several reviews covering the aspects of the chemistry of milk proteins have been published (Schnudt, 1980; Swaisgood, 1980, 1992; Whitney, 1988; Fox, 1988). Some of the properties of proteins in milk are presented in Table 2.2.

Table 2.2. General properties of milk proteins[1]

Attribute	Caseins				Whey proteins		
	α_{s1}-	α_{s2}-	β-	κ-	β-lg	α-la	BSA
Molecular wt. (Da)	23,614	25,230	23,983	19,023	18,363	14,176	66,267
Residues/molecule:							
Amino acids	199	207	209	169	162	123	582
Proline	17	10	35	20	8	2	34
Cysteine	0	2	0	2	5	8	35
Disulphide	0	1	0	1	2	4	17
Phosphoserine	8	11	5	1	0	0	0
Isoionic point	4.96	5.19	5.19	5.43	5.41	4.2-4.5	4.3
Hydrophobicity[2]	4.9	4.7	5.6	5.1	5.1	4.7	4.3

[1] adapted mainly from Walstra and Jenness, 1984

[2] kJ/residue

2.2.1 Caseins

Caseins comprise the largest fraction of bovine milk proteins (76-80% total protein). Caseins are defined as a group of phosphate containing milk-specific proteins that precipitate on acidification to pH 4.6. Bovine casein consists of four distinct proteins: α_{s1}-, α_{s2}-, β- and κ-caseins, together with some derivatives formed by the proteolysis of these caseins.

α_{s1}-Casein possesses eight sites of post-translational phosphorylation; consequently, this protein exhibits interactions with calcium which is 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 charge is reduced 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. The structures of both α_{s2}- and β-caseins are characterized by charged polar domains and hydrophobic domains. In 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 protein, 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).

2.2.2 Casein micelle

Caseins, unlike many globular proteins (whey proteins), are not present in milk as individual molecular structures, but rather as large protein complexes that also incorporate milk salts, particularly calcium and phosphate salts. Thus, the 'native'

structure is actually a protein complex, resulting from interaction of individual caseins, known as a casein micelle. They consist of ~92% protein (casein) and 8% inorganic salts, mainly calcium and phosphate. The approximate composition of bovine casein micelles is given in Table 2.3.

Table 2.3. Composition of casein micelles[1]

Component	Concentration (g/100 g)
Total casein	92
α_{s1}-Casein	33
α_{s2}-Casein	11
β-Casein	33
κ-Casein	11
Minor caseins	4
N-acetylneuraminic acid	0.2
Galactose	0.2
Galactosamine	0.2
Total inorganic constituents	8
Calcium	2.9
Magnesium	0.1
Inorganic phosphate	4.3
Sodium	0.1
Potassium	0.3
Citrate	0.4

[1]adapted from Schmidt, 1980 and McMahon & Brown, 1984

The micelles appear as more or less spherical particles with a relatively wide size distribution of 50-300nm as shown by electron microscopy (Schmidt et al., 1971). The average molecular weight of casein micelles, from sedimentation and diffusion experiments, is ~5.2×10^8 indicating that ~2.0×10^4 casein monomers are incorporated in the average micelle (Dawan et al., 1974; Bloomfield & Mead, 1975).

Casein micelles are highly hydrated and sponge-like colloidal particles containing about 3.7g water/g protein. The exact structure of the casein micelle has not been fully resolved with several models having been proposed in the past three decades (Schmidt, 1982; Walstra & Jennes, 1984; Holt, 1992; Horne, 1998). The submicellar structure of the casein micelle as proposed by Schmidt (1982) is shown in Fig. 2.1. According to this model, casein micelles are composed of discrete subunits linked through colloidal calcium phosphate. The protein composition of the subunits is variable; those containing higher amounts of κ-casein are orientated towards the outer surface while others containing little or no κ-casein are buried inside the micelle. This model was later modified by Walstra (Walstra & Jenness, 1984; Walstra, 1990), who incorporated the concept of steric stabilization of the micelle by κ-casein. In the proposed model (Fig. 2.2), most of the κ-casein is located at the outside, and the protruding chains of its C-terminal end give the micelle a hairy surface. The hairs are flexible and show perpetual Brownian motion. The effective thickness of the hairy layer is at least 5 nm. A small part of κ-casein is in the interior. The evidence for such a structure also comes from hydrodynamic studies (Walstra, 1979; Holt & Dalgleish, 1986; Horne & Davidson, 1986; Horne 1989) and from proton nuclear magnetic resonance (NMR) in D_2O (Griffin & Roberts, 1985; Rollema et al., 1988) which show that a part of κ-casein has considerable freedom of motion. κ-Casein hairs at the surfaces of casein micelles provide stabilization to the micelles towards flocculation (Walstra, 1990).

Holt (1992) suggested that the sub-structure of casein micelles may be depicted without requiring the existence of submicelles (Fig. 2.3). He claimed that the sub-structure consists of microgranules of calcium phosphate which are incorporated in a protein matrix which does not resemble a sub-micelle. The outer region of the micelle shows hairy layers which are considered to provide steric stability to the micelle (Holt, 1992; Holt & Horne, 1996).

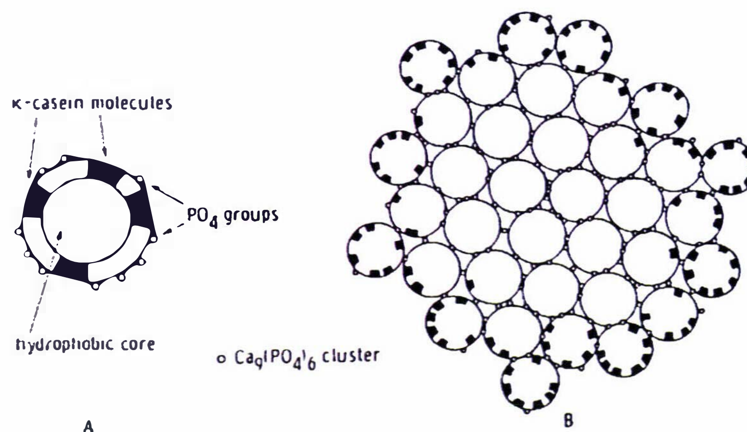

Fig. 2.1. Submicelle model of casein micelle as proposed by Schmidt (1982): A, submicelle, B, micelle.

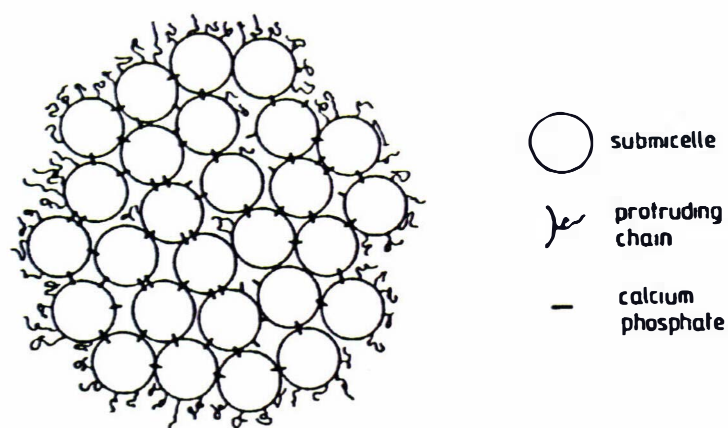

Fig. 2.2. Submicell model of casein micelle as proposed by Walstra (Walstra & Jenness, 1984; Walstra, 1990).

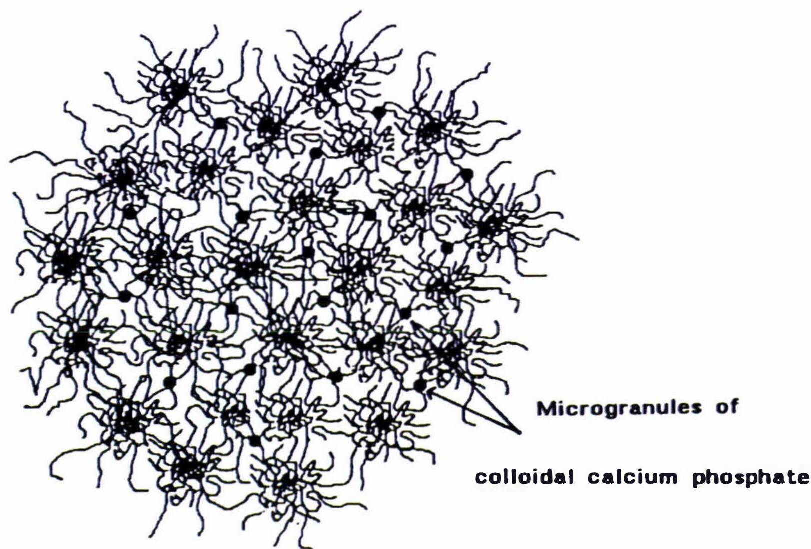

Fig. 2.3. Casein micelle model as proposed by Holt (1992).

Visser (1992) also claimed that the sub-micellar model proposed by Walstra and Jenness (1984) is an over simplification and cannot explain some phenomena that occur in the casein micelles. He proposed that the casein micelle is not based on the sub-micelles but is an aggregate of the individual caseins, in which κ-casein is located at the surface to stabilize the micelles by its charges and surface hydration layers. α_{s1}-, α_{s2}-, β-Caseins are randomly aggregated with a preference for α_{s1}-casein to form a skeleton of the overall structure probably in combination with the minor protein. β-Casein is aggregated and entrapped within the skeleton. He used this model to explain the phenomena that occurs in casein micelles, e.g. coagulation due to renneting.

Recently, Horne (1998) described a non-sub-micelle model as a dual-bonding model based on hydrophobic and electrostatic interactions. In this model (Fig. 2.4), bonding

occurs between the hydrophobic regions, depicted as rectangular bars, and by linkage of hydrophilic regions containing phosphoserine clusters to colloidal calcium phosphate clusters (CCP). Molecules of κ-casein limit further growth and are labelled with the letter 'K'.

Despite disagreement over the exact structure of the micelle, the concept of the casein micelle electrostatically and sterically stabilised by a 'hairy layer' coat of κ-casein appears to be universally accepted (Walstra, 1990; Swaisgood. 1992; Holt, 1998).

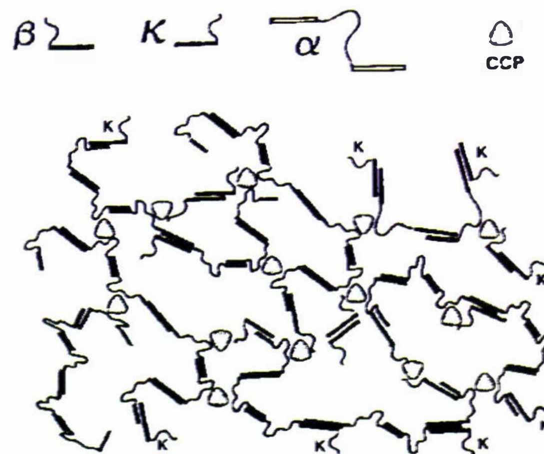

Fig. 2.4. Dual bonding model of casein micelle structure proposed by Horne (Horne, 1998).

2.2.3. Dissociation of casein micelles

Dissociation of casein micelles can be caused by one or other of the following treatments: cooling, heating, pH modification, chelation of calcium or treatment with chemicals, such as urea and sodium dodecyl sulphate (SDS).

Gradual removal of calcium (Ca^{2+}) from micelles by chelation with EDTA at a constant pH initially results in a release of caseins without a major effect on the size of casein micelles, but ultimately leads to complete micellar disaggregation (Lin et al., 1972; Munyua & Larsson-Raznikiewicz, 1980). The effect of depletion of Ca^{2+} on the composition and size distribution of casein micelles in milk was examined by Griffin et al. (1988). Partial removal of Ca^{2+} by EDTA and subsequent dialysis resulted in disaggregation of some caseins. However, the casein composition of the intact micelles and the number-frequency size distribution were essentially unchanged.

A more extensive dissociation of casein micelles can be induced by the addition of 6 M urea. In this way the micelles can be broken down into much smaller particles without rupturing the casein-calcium phosphate linkages. Aoki et al (1986) found that casein micelles disaggregated by urea could be fractionated by gel permeation chromatography into a calcium phosphate-containing protein fraction (α_{s1}-, α_{s2}-casein and β-caseins) and a fraction with very low calcium phosphate-containing proteins (β- and κ-caseins).

2.2.4. Whey proteins

Whey proteins are globular to ellipsoid in structure, relatively soluble and heat labile, with the exception of the proteose peptones (PP). The most important whey proteins are β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA) and immunoglobulins (Ig). The other proteins include lactoperoxidases, lysozyme, lactoferrin (Lf), lactollin and many others. These proteins have different properties as a result of differences in amino acid composition and spatial arrangement. The structure and properties of various whey proteins have been reviewed by Swaisgood (1982), Eigel et al. (1984), Mulvihill and Donovan (1987) and Whitney (1988) (Table 2.4).

Table 2.4. Characteristics of whey proteins[1]

Whey protein	Concentration in milk (g/kg)	MW (Da)	Isoelectric point (pH)	Disulphide bonds
β-Lactoglobulin	3.3	18,363	5.13	2
α-Lactalbumin	1.2	14,147	4.2-4.5	4
BSA	0.4	66,267	4.7-4.9	17
Immunoglobulin	0.7	$1.5\text{-}10\times10^5$	5.5-8.3	21

[1]adapted from Mulvihill & Donovan, 1987; Walstra & Jenness, 1984

β-Lactoglobulin

β-Lg, the major protein of whey, is the most extensively characterized and best described of all food proteins (McKenzie, 1971; Swaisgood, 1982; Hambling et al., 1992). Seven genetic variants (A, B, C, D, E, F and G) of β-lg have been identified with the B variant predominant in most breeds of Western cattle. The primary amino acid sequence of β-lg B consists of 162 amino acid residues with a calculated molecular weight of 18,277 Da (Brauniter et al., 1972). The β-lg monomer has one free thiol group (Cys^{121}) and two disulphide bridges (Cys^{106}-Cys^{119} and Cys^{66}-Cys^{160}) (Papiz et al., 1986). The A and B variants differ at positions 64 and 118, where Asp and Val in β-lg A are replaced by Gly and Ala in β-lg B. β-Lg, 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-lg is dependent on pH; 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-lg is greatly influenced by the presence of the sulphydryl group and by conformational changes since these determine the availability of the sulphydryl group for reactions. The free thiol group, Cys^{121}, and the disulphide bridge, Cys^{106}-Cys^{119}, are buried whereas the other disulphide bridge, Cys^{66}-Cys^{160}, is positioned on the outer surface in a mobile region of the molecule (Fig. 2.5). Thus, under appropriate conditions, β-lg readily

participates in sulphydryl-disulphide interchange reactions which affects many of its characteristics, such as solubility (Hambling *et al.,* 1992).

α-Lactalbumin

α-La accounts for approximately 25% of whey proteins. A total of three genetic variants (A, B and C) are known, of which the B variant predominates in Westen cattle. α-La B variant consists of 123 amino acid residues with a calculated molecular weight of 14,174 (Brew et al., 1970). It has four disulphide bonds linking residues 6 to 22, 28 to 111, 61 to 77, and 73 to 91. α-La binds calcium, which may stabilize the molecule against irreversible denaturation (Hiraoka & Sugai, 1984). Removal of calcium reduces the heat stability of the protein (Hiraoka & Sugai, 1984).

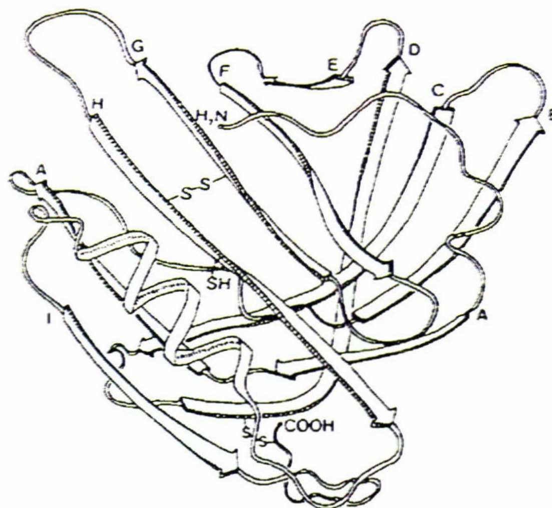

Fig. 2.5. Tertiary structure of β-lg. The arrows indicate strands of β-sheets, marked A-I (Papiz et al., 1986).

Bovine serum albumin and immunoglobulin

BSA is identical to the serum albumin found in the blood stream, and represents ~5% of the total whey proteins. BSA has the longest single polypeptide chain of all the whey proteins, consisting of 582 amino acid resides, and has a molecular weight of 66 kDa (Eigel et al., 1984). It has 17 disulfide bonds, which stabilize its tertiary structure, and one free thiol group at position 34.

Ig is a fraction of a complex heterogeneous mixture of large glycoproteins which possess antibody activity. These proteins represent ~10% of the whey proteins. Four distinct classes of Ig occur in bovine milk: IgM, IgA, IgE and IgG (Eigel et al., 1984). Two heavy (50-70 kDa) and two light (22.4 kDa) polypeptide chains, linked by disulfide bridges, form the basic subunit in each class. The molecular weight of Ig varies between ~150 and 900 kDa and the amino acid sequence is variable. These proteins are easily denatured by heat (Lyster, 1972).

2.3. General characteristics of milk fat globules

Bovine milk contains approximately 4-5% fat. More than 95% of the fat in cow's milk is secreted in the form of globules ranging in size from 0.1-10.0 μm in diameter. Each fat globule is surrounded by a membrane, the milk fat globule membrane, which maintains the integrity of the globules and renders them compatible with their aqueous environment (Mulder & Walstra, 1974; Keenan & Dylewski, 1995). The milk fat globules are important for the stability of milk and milk products, which concerns creaming, aggregation, coalescence and also some aspects of lipolysis. Milk and cream properties that are affected by the fat globules, include viscosity, colour and foaming. Furthermore, the properties of high-fat products may especially be dominated by the behaviour of the fat globules (Walstra, 1995). Major reviews on milk fat globules and their stability have been published by Mulder and Walstra (1974); McPherson and Kitchen, (1983); Walstra and Jenness (1984); Walstra (1995); Keenan and Dylewski, (1995).

2.3.1. Size distribution of milk fat globule

The size distributions found may depend greatly on the measurement method employed (Walstra et al., 1969). In fact, the shape of the size distribution curve is almost constant for individual milkings from same cow (Walstra, 1969; Hood, 1981). However, there are considerable differences in size distribution curves between cows of the same breed. A feed supplement that causes an increase in milk fat content usually causes an increase in fat globule size rather than in the number of globules (Hood, 1981). Average globule size decreases with advancing lactation, for instance from d_{vs} = 4.4 to d_{vs} = 2.9 μm (Mulder and Walstra, 1974).

Average globule size can be expressed in many ways (Table 2.4); it is more common to employ the volume-surface average diameter, d_{32} or $d_{vs} = = \sum dV/\sum dS = \sum d^3 dN/\sum d^2 dN$ and volume moment mean diameter, d_{43} or $d_{VM} = \sum dM/\sum dV = \sum d^4 dN/\sum d^3 dN$. The specific surface area of the fat globules is given by $A = 6\phi/d_{vs}$, where ϕ is the volume fraction of fat.

Table 2.4. Definitions of mean diameters[1]

Number, length mean diameter	$d_{NL} = \sum dL/\sum dN = \sum x dN/\sum dN$
Number surface mean diameter	$d_{NS} = \sqrt{(\sum dS/\sum dN)} = \sqrt{(\sum x^2 dN/\sum dN)}$
Number, volume mean diameter	$d_{NV} = \sqrt[3]{(\sum dV/\sum dN)} = \sqrt[3]{(\sum x^3 dN/\sum dN)}$
Length, surface mean diameter	$d_{LS} = dS/\sum dL = \sum x^2 dN/\sum x dN$
Length, volume mean diameter	$d_{LV} = \sqrt{(\sum dV/\sum dL)} = \sqrt{(\sum x^3 dN/\sum x dN)}$
Surface, volume mean diameter	$d_{VS} = \sum dV/\sum dS = \sum x^3 dN/\sum x^2 dN$
Volume, moment mean diameter	$d_{VM} = \sum dM/\sum dV = \sum x^4 dN/\sum x^3 dN$
Weight, moment mean diameter	$d_{WM} = \sum dM/\sum dW = \sum x dW/\sum dW = \sum x^4 dN/\sum x^3 dN$

[1]from Allen, 1990

2.3.2. Milk fat globule membrane

Each fat globule of milk is surrounded by a thin protective layer, usually called the milk fat globule membrane (MFGM). This membrane is primarily derived from the apical

cell membrane or the membrane of Golgi vacuoles and other materials of the lactating cell, although substantial reconformation probably occurs (Keenan et al., 1983a). The mass of the membrane is about 2% of that of the total fat globule. The membrane (approximately 10 nm thick) consists of a complex mixture of proteins, phospholipids, glycoproteins, triglycerides, cholesterol, enzymes and other minor components. The composition of the membrane is shown in Table 2.5.

Table 2.5. Average composition of milk fat globule membrane[1]

Components	mg per 100 g fat	mg per m^2	Percentage of membrane
Protein	900	4.5	48
Phospholipids	600	3.0	33
Cerebrosides	80	0.4	4
Gangliosides	20	0.1	1
Cholesterol	40	0.2	?
Neural glycerides	+	+	?
Hydrocarbons	20 ?	0.1	1
Ribonucleic acid	+	+	?
Carotenoids + Vaitamin A	0.04	2×10^{-4}	-
Fe	0.3	15×10^{-4}	-
Mo	0.05	2×10^{-4}	-
Cu	0.01	0.5×10^{-4}	-
Water	200	1.0	11
Total	>1860	>9.3	100

[1]from Walstra & Jenness (1984)

MFGM acts as a natural emulsifying agent enabling the fat to remain dispersed throughout the aqueous phase of milk (McPherson & Kitchen, 1983). All interactions between fat and plasma must occur across the membrane. The total area of the

membrane is considerable, and it contains reactive substances and enzymes. Consequently the membrane may determine such deteriorative reactions as lipolysis and autoxidation. The membrane also functions to minimise the flocculation and coalescence of fat globules, and it protects the fat against enzyme action. These observations apply to most milk products as well (Keenan et al., 1983b). Furthermore, variability of the stability of fat globules to flocculation, coalescence, fat oxidation and lipase action may be due to variability in membrane properties (Mulder & Walstra, 1974). The MFGM is markedly affected by processing treatments such as cooling, heating and homogenization of dairy products. Many properties of milk, such as its stability and acceptability are directly related to this unique membrane system (McPherson & Kitchen, 1983).

2.3.3. Proteins of milk fat globule membrane

The MFGM contains approximately 50% proteins (Table 2.5) and accounts for about 1% of the total milk proteins. However, the important function of this small amount of protein in helping to maintain the integrity of the membrane appears to outweigh its relatively low level in milk (McPherson & Kitchen, 1983). Most of the membrane proteins are highly specific. Their compositions, which are about as intricate as those of the plasma proteins, have been reviewed by McPherson and Kitchen, (1983); Keenan and Dylewski, (1995); Danthine et al. (2000) and Mather (2000).

Using a combination of electrofocussing and electrophoresis, Mather (1978) separated over 40 MFGM proteins, at least eight of which appeared to contain carbohydrate. Using silver staining, which is an order of magnitude more sensitive than Coomassie staining, over 60 different polypeptides from the MFGM can be detected after separation by SDS-PAGE. Most of these are minor, representing less than 0.5% of the total stainable proteins (Dylewski et al., 1984). The major proteins resolved, in order from the top of one-dimensional SDS gels, are shown in Table 2.6. Of the major proteins, six stain strongly with Coomassie blue (xanthine oxidase, PAS VI, butyrophilin, adipophilin (ADPH), Periodic acid schiff 6/7 (PAS 6/7) and fatty-acid binding protein (FABP)) are most often illustrated by investigators in publications. The characteristics, properties and amino acid sequences of these major MFGM proteins are reviewed and discussed in detail by Mather & Jack (1993) and Mather (2000).

The MFGM proteins may vary in amount with breed and stage of lactation. For example, butyrophilin and xanthine oxidase, accounted for significantly higher percentages of total membrane protein in milk from Holstein cows than in milk from Jersey cows. Both these proteins were high in membranes from animals in early lactation, they decreased in amount as lactation progressed to the midpoint, and then increased toward the end of lactation (Mondy & Keenan, 1993). However, butyrophilin and xanthine oxidase are present in constant molar proportions of about 4:1 in the MFGM regardless of variations due to breed and stage of lactation (Mondy & Keenan, 1993).

Table 2.6. Protein components in the MFGM[1]

Proteins	Molecular weight (kDa)	Percentage in MFGM (%)
MUC1	170-200	
Xanthine oxidase	155	20
PAS III	95-100	
PAS IV	76-78	
Butyrophilin	66-67	34-43
ADPH	52	
PAS 6 (Band 15)	50	
PAS 7 (Band 16)	48	
FABP	11-15	

[1]adapted from Keenan & Dylewski, (1995) and Mather (2000)

2.3.4. Isolation of MFGM material

Isolation of MFGM material involves four major steps: separation, removal of skim-milk components, destabilization and recovery of the membrane, which were reviewed by McPherson & Kitchen (1983) and Mather (2000). The separation of milk fat

globules is easily completed by centrifugation using a cream separator. After separation, skim milk components are removed from the cream, usually by washing at a given temperature with physiological buffers, in which either sucrose solution or milk salt buffer are used in order to minimise the losses of membrane components. It has been found that washing three times with buffers is sufficient to remove serum components and that further washing significantly reduce the yield of MFGM material (Anderson & Brooker, 1975; Mather et al., 1977).

There are a number of techniques which are available to release membrane material from the washed fat globules: (1) by physical agitation in a blender or homogenizer at reduced temperature (< 10 °C); (2) by freezing and thawing the washed globules (Keenan et al., 1970; Kobylka & Carraway, 1972); (3) by direct extraction with nonionic detergents (Patton, 1982), bile salts (Anderson et al., 1972; Patton et al., 1986) or polar aprotic solvents (Dapper et al., 1987). The released membrane is then collected by centrifugation (McPherson and Kitchen, 1983; Mather, 2000). Regardless of the extraction method employed, the cream is separated from fresh, warm milk as soon as possible after milking, because the MFGM components are prone to structural changes when fresh milk is cooled and held during long-term storage (Anderson et al., 1972; Mather, 2000)

2.3.5. Molecular organization and structure of the MFGM

Most workers who have attempted to purify individual MFGM proteins have observed a strong association between butyrophilin, xanthine oxidase and some minor MFGM proteins (e.g. several GTP-binding proteins) (Valivullah & Keenan, 1989; Ghosal et al., 1993). These complexes are dissociated only under strongly denaturing conditions. They probably occur naturally and are not artefacts induced by solubilization and isolation methods (Mather & Jack, 1993; Keenan & Dylewski, 1995).

Organization of material on the globule surface has been the subject of much experimentation and speculation over the past 75 years. Models of the surface material have progressed from an adsorbed layer or layers to the current view of a true biomembrane on the globule surface (King, 1955; Peereboom et al., 1969; McPherson & Kitchen, 1983; Keenan & Dylewski, 1995; Danthine et al., 2000). These models (Fig.

2.6) provide representation of the knowledge of the field at different periods. That there is a distinct asymmetric arrangement of constituents in MFGM has been documented by a number of studies. The carbohydrate moieties of glycoproteins (Monis et al., 1975; Horisberggcr et al., 1977; Sasaki & Keenan, 1979) and gangliosides (Tomich et al., 1976) are largely disposed on the globule surface. Certain enzymes intrinsic to the membrane have their active site accessible from one but not both faces of the membrane (Patton & Trams, 1971; Snow et al., 1980). Several of the membrane proteins are known to be exposed on the outer or inner membrane face (Mather & Keenan, 1974; Nielsen & Bjerrum, 1977; Patton & Hubert, 1983). Recent molecular cloning results have confirmed specific orientations of certain MFGM proteins. The primary structure of butyrophilin is consistent with a transmembrane orientation of this protein (Jack & Mather, 1990).

2.4. Effect of heat treatment on the fat globules and the MFGM protein

2.4.1. Size of fat globules

No change was observed in the globule size distribution when milk was heated with either batch heating or indirect heating (heat exchanger). However, when direct steam injection (DSI) followed by vacuum flash cooling was used, disruption of the milk fat globules occurred which resulted in a decrease in the average size of fat globules (Van Boekel and Folkerts, 1991).

2.4.2. Association of serum proteins with the MFGM

The effects of heat treatment on the serum proteins of milk have been studied extensively because these effects can influence the properties of the resulting products. However, relatively a few studies have focused on the heat-induced interactions between the proteins of MFGM and serum proteins, although these interactions are also known to influence the properties of dairy products.

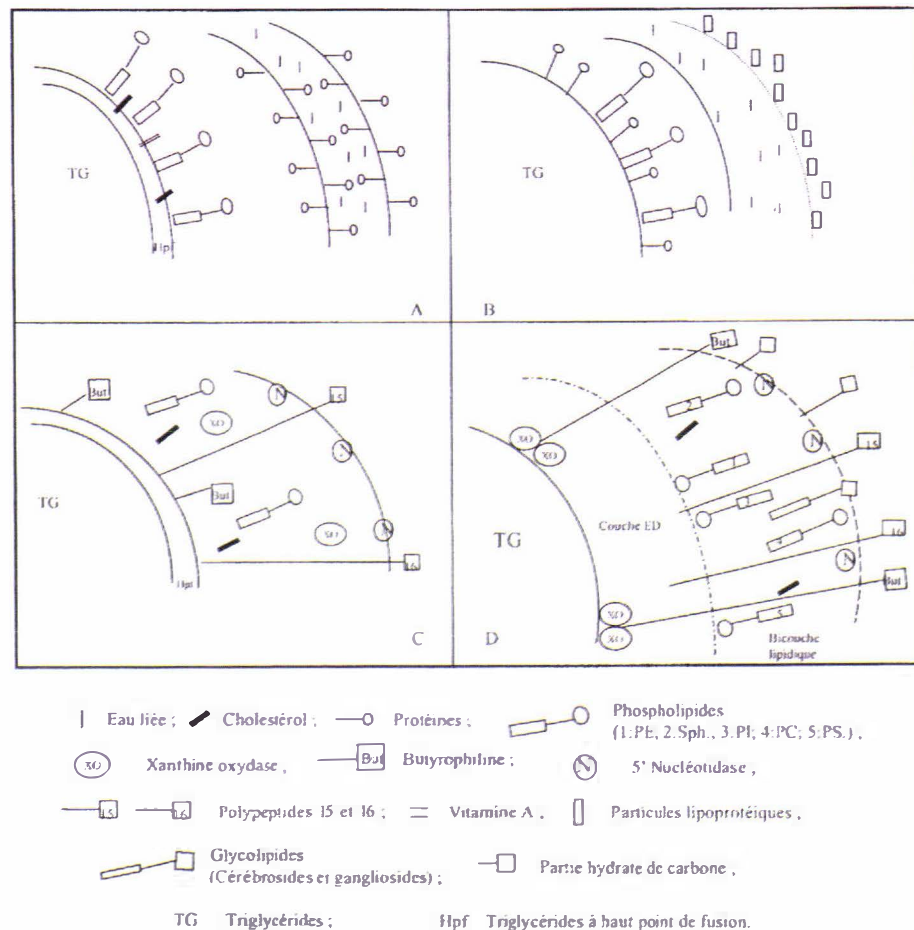

Fig. 2.6. Various stages in the evolution of the proposed model for milk fat globule membrane (adapted from Danthine et al., 2000). A: Model of King (1955); B: Model of Peereboom et al. (1969); C: Model of McPherson & Kitchen (1983); D: Model of Danthine et al. (2000).

The MFGM proteins are known to be highly reactive. They contain large number of disulfide and sulfydryl groups (Cheng et al, 1988; Mather, 2000). For example, xanthine oxidase, one of the major MFGM proteins, contains 22 disulfide and 38 sulfydryl groups, four of which are detectable in the undenatured protein complex (Cheng et al., 1988). Early research has shown that membrane proteins will denature when milk is heated above 70 °C, and reactive groups may be exposed. Heating for a few minutes at 80 °C or a few seconds at 100 °C produces a noticeable quantity of H_2S (Mulder &

Walstra, 1974). Appell et al. (1982) reported that butyrophilin forms disulphide-stabilized complexes on heating at 58°C. The cysteine residues in membrane proteins seem to be very reactive, and disulfide interchange reactions may occur (van Boekel and Walstra, 1989). Furthermore, McPherson et al. (1981) found evidence which suggested that a lipoprotein complex was formed as a result of heat-induced interactions between β-lg, κ-casein and MFGM components.

Recent studies showed that heat treatment causes a number of changes in MFGM proteins, which include some denaturation and interactions with serum proteins via sulfydryl--disulfide interchange reactions (Dalgleish & Banks, 1991; Houlihan et al., 1992; Sharma & Dalgleish, 1993; Kim & Jimenez-Flores, 1995). Houlihan et al. (1992) analyzed the composition of MFGM after heating and found that the total protein of membrane increased, while lipid, phospholipid and triacylglycerol decreased when whole milk was heated at 80 °C for 2.5 to 20 min. The increase in the total MFGM protein is considered to be because of the association of serum proteins at the MFGM during heat treatment (Dalgleish & Banks, 1991; Houlihan et al., 1992; Sharma & Dalgleish, 1993; Kim & Jimenez-Flores, 1995).

Dalgleish & Banks (1991) reported that the bands of β-lg and α-la could be identified in the SDS-PAGE of MFGM material of heated whole milk, showing that both proteins associated with the MFGM. The amount of both β-lg and α-la in the membrane increased with heating time at ≥ 80 °C, but α-la was present at low levels (Dalgleish & Banks, 1991; Houlihan et al., 1992; Sharma & Dalgleish, 1993). The β-lg was also found in the MFGM when milk was heated at lower temperature (< 80 °C), but α-la was absent (Dalgleish & Banks, 1991). A low level of κ-casein was also observed in the MFGM of heated milk (Dalgleish & Banks, 1991; Houlihan et al., 1992).

The mechanism by which the serum proteins bind to the fat globule is not very clear. Dalgleish & Banks (1991) considered that there are two ways in which serum proteins may bind at the MFGM. They may bind to the proteins which are already present in the natural fat globule membrane system. Alternatively, the serum proteins may displace the original membrane material, either by directly competing, or because the heating

causes the original membrane to break down, leaving gaps through which the serum proteins (which may or may not be denatured at that stage) adsorb to the newly exposed fat surface (Dalgleish & Banks, 1991). Although their results cannot distinguish between these two mechanisms unambiguously, they do suggest that the former is more important.

Houlihan et al. (1992) considered from their studies that protein-protein interactions are the major forces in complex formation between MFGM and skim milk components in heated milk. It is possible that membrane lipids could also be involved. Spector and Fletcher (1970) have reported that β-lg can bind tightly to long-chain fatty acids, which are present in substantial amounts in MFGM phospholipids and triacylglycerols (Kitchen, 1977), and Brown (1984) found that β-lg interacted with both neutral and polar lipids. MFGM lipids could complex with β-lg, particularly if the hydrohobic regions of the lipids were accessible, which could occur as some membrane proteins were lost during heating.

Kim and Jimenez-Flores (1995) reported that heating of whole milk at 87 °C for 2.5 to 60 min resulted in a time-dependent increase in compounds of high molecular mass, which could not enter the gel, a sudden decrease of β-lg after just 2.5 min, and a gradual decrease of α-la under non-reducing conditions in the SDS-PAGE. However, in the reducing condition, the large aggregates seem to have been completely resolubilized, and most of β-lg and α-la were resolved again in their monomeric forms. After the proteins were reduced, monomer bands of β-lg and α-la in the heated sample (87 °C for 60 min) were resolved into the same fraction, but in lower concentrations than those in raw whole milk. These results suggest that these whey proteins were involved mainly, but not exclusively, in the formation of heat-induced disulfide linkages as dimer and trimer complexes of β-lg or as a complex of β-lg and κ-casein or α-la. The lower concentration of these serum proteins after heating of the milk suggests that other interactions exist between the serum and MFGM proteins.

Recently, Lee and Sherbon (2002) reported that heat treatment of milk resulted in a significant decrease in free SH groups in the MFGM, while the amount of SS groups

increased, indicating that free SH groups were involved in SS interactions. Their results suggested that proteins containing free SH groups and SS groups (β-lg and α-la) from the serum phase were incorporated gradually into the MFGM with heating.

With respect to the association of κ-casein with the MFGM, Houlihan et al. (1992) suggested that the MFGM is involved in heat-induced interactions with skim milk components, in particular β-lg and κ-casein, and that the levels of incorporation of these components into the membrane are dependent on the extent of the heat treatments. κ-Casein may interact directly with MFGM components, in addition to forming complexes with β-lg during heating. However, Dalgleish and Banks (1991) did not agreed that a κ-casein/β-lg complex could be formed on the globule surface, since the SDS-PAGE patterns showed little or no enhancement of the band attributable to κ-casein, in samples where conspicuous increases of β-lg had occurred. The amounts of casein associated with the fat appeared to be relatively constant, independent of the heat treatment. In fact caseins were present on the surfaces of the fat globules even in unheated milk. This may be a result of inadvertent partial homogenization during the handing of the milk. There was no evidence for enhanced binding of any caseins during heating (Dalgleish & Banks, 1991).

2.4.3. Change in the native MFGM proteins

Houlihan et al. (1992) reported that xanthine oxidase, PAS 6 (Band 15) and PAS 7 (Band 16 or 49 kDa) decreased in intensity in the MFGM isolated from milk that was heated at 80 °C. They also determined the resuspended washed cream that was heated at 80 °C for 20 min. The gross composition of the MFGM was not affected. The results indicate that the MFGM heated without serum proteins did not cause major compositional changes in the membrane. The authors suggested that PAS 6 and PAS 7 might be displaced from the membrane by whey proteins during heating. Kim and Jimenez-Flores (1995) also found that this band (PAS 7) decreased in intensity during the heating of milk. However, these authors interpreted this decrease as a breakdown in the structure of this protein and suggested that the 49 kDa protein may have been cleaved into smaller molecular weight proteins after heat treatment.

2.4.4. Quantities of β-lg and α-la associated with the MFGM

Corredig and Dalgleish (1996) reported that no significant difference was observed in the amount of whey protein deposited onto the fat globules as a function of time at 65, 75 and 85 °C in batch heating of milk in water baths. The interaction of whey protein with the MFGM was complete well before the time required to totally denature the serum proteins. The total amount of whey protein deposited onto the MFGM when whole milk was heated never reached values > 1 mg whey protein/g fat. These results demonstrated that although the strong binding of whey protein to MFGM was very rapid, the amounts of whey protein bound in this way were quite low, amounting to < 1% of the total serum protein in the milk (Corredig & Dalgleish, 1996). This strongly suggests that the interaction of whey proteins with intact milk fat globules is not simply displacement of the original membrane by adsorbing whey protein (Corredig & Dalgleish, 1996).

Corredig and Dalgleish, (1996) also reported that the quantity of α-la bound to the fat globules was lower after HTST treatment than for samples heated in the water bath, perhaps because of differences in the binding during the warm-up period. The quantity of α-la and β-lg bound to the fat globules did not increase with temperature > 85 °C. Therefore, they considered that the method of heating seemed to be important: it seems that it was not only the temperature of heating itself that played a main role in the interactions, but also the way it was attained (Corredig & Dalgleish, 1996).

In contrast to no change in globule size distribution when milk is heated with batch heating and indirect UHT heating, direct steam injection (DSI) heating caused the disruption of the milk fat globules (van Boekel and Folkerts, 1991). Large amounts of whey protein were also bound to the fat globules during heat treatment in DSI; these must have adsorbed to the fat-water interface, or bound to adsorbed casein, or bound to MFGM protein. Corredig and Dalgleish, (1996) reported that the amounts of both α-la (~0.65 mg/g fat) and β-lg (~2.3 mg/g fat) bound to the fat globules were about three times greater during heat treatment in DSI than those deposited (0.2 and 0.75 mg/g fat) in the indirectly heated milks. They also found that the amounts of both α-la (~0.2 mg/g

fat) and β-lg (~0.75 mg/g fat) bound to the fat globules in UHT of indirect heating were higher than that of HTST treatment (~0.075 and ~0.6 mg/g fat).

There has been very little published work on the kinetics of β-lg and α-la association with MFGM during heat treatment, although the aggregation kinetics of β-lg and α-la have been extensively studied (Lyster, 1970; Hillier & Lyster, 1979; Dannenberg & Kessler, 1988; Anema & McKenna, 1996; Oldfield et al., 1998 a, b). Reaction orders of pseudo first order (Dalgleish, 1990), 1.5 order (Dannengerg & Kessler, 1988; Anema & McKenna, 1996; Oldfield et al., 1998 a) and second order (Hillier & Lyster, 1979) have been obtained for the denaturation reactions of β-lg and α-la. Sharma and Dalgleish (1994) found that the kinetics of β-lg and α-la incorporation into the fat globule membrane fitted a pseudo-first order kinetic equation based on a site filling model. However, their kinetic parameters were limited to only 70 and 75 °C. In addition, the samples were homogenised prior to or after heating, and this would have caused the adsorption of some β-lg and α-la to the interface rather than heating itself.

2.5. Effect of evaporation on the fat globules and the MFGM protein

Falling film evaporators, which are predominant in dairy industry, are designed to remove as much water as possible from the milk at a low energy cost and with minimal heat-damage to the milk constituents. In the traditional design, the milk is boiled under vacuum in the first effect through the application of externally supplied heat to the first stage. Subsequent stages (or effects) are heated by the vapour generated from evaporation in the preceding effects. Treatment of milk in a falling-film evaporator may considerably disrupt fat globules (Mulder & Walstra, 1974). A thick protein layer, containing casein micelle was seen on the fat globule surface by the electron microscopy in concentrated milks (Schmind et al., 1971; de Felipe et al., 1991; Velez-Ruiz & Barbosa-Canovas, 2000). However, the concentrated milks used in these studies were homogenized before examination by electron microscopy or the commercial condensed milks were used, where processing conditions were unknown. There has

been no information available on the change in the MFGM during evaporation under the conditions of the powder manufacture.

2.6. Effect of homogenization on the milk fat globules and the MFGM protein

When milk is homogenized, the fat globules are broken up and reduced in size. The size reduction of fat globules involves the destruction of the original fat globule membrane. There are four readily recognizable prime variables of homogenisation which influence the results of homogenisation. These are the homogenisation pressure and emulsion flow rate, which are determined by the type of homogenizer, together with the temperature of the emulsion and its fat or oil volume fraction (Phipps, 1985). Homogenisation pressure is the main variable; as homogenisation pressure increases, globule size decreases. For milk or cream containing up to 12% fat, there is usually a linear inverse relationship between homogenisation pressure (P) and the fat globule size (d_{vs}), when these are plotted on logarithmic scales. As the amount of fat increases, the plots of log d_{vs}/log P become somewhat curvilinear and homogenisation becomes less effective (Goulden & Phipps, 1964). For normal whole milk, there is practically no change in globule size as the homogenisation temperature is increased. For higher fat content samples, increasing homogenisation temperature improves the homogenisation efficiency (Goulden & Phipps, 1964).

During homogenisation the surface area of fat globules is increased 4 to 7 times (Sommer, 1951; Fox et al., 1960; Mulder & Walstra, 1974), and therefore the quantity of the original membrane material is not sufficient to cover the newly created surface. It has been shown that on exiting the homogenizing valve, the newly formed fat globule surfaces are already covered with “new” surface layers. Obviously, the new surface layers are formed with the surface active material present in skim milk. Results from electron microscopy have shown that in addition to the natural fat globule membrane components, the surfaces of homogenized fat globules contain milk proteins i.e. casein micelles and sub-units of the casein micelles (Henstra & Schmidt, 1970; Darling & Butcher, 1978).

The composition of the newly formed or adsorbed surface layer depends on conditions during adsorption. In a review article, Walstra (1995) stated that the composition of the continuous phase, intensity of agitation, temperature, heat treatment and ratio of fat to protein are important variables. At approximation, proteins are adsorbed in proportion to their concentration. Oortwijn and Walstra (1979) reported that milk plasma gives a total protein load, $\Gamma = \sim 10$ mg/m^2, layer of casein and serum proteins, in which whey protein only gives 2.5 mg/m^2. However, the adsorption of caseins and serum proteins is affected by intensity of agitation. If agitation is very vigorous, as in a homogenization, the protein arrives by convection and larger particles are preferentially adsorbed (Walstra & Oortwijn, 1982). In recombined milk, Walstra and Oortwijn (1982) and Sharma et al. (1996 a) reported that caseins constituted more than 90% of the adsorbed proteins. More intense agitation (higher power density) thus gives thicker adsorbed layers containing relatively less serum protein, mainly because it leads to the formation of smaller globules. Small globules acquire much more protein per unit surface area due to the preponderance of large casein micelles at the surface of small fat globules (Walstra & Oortwijn, 1982).

Oortwijn and Walstra (1979) reported that high temperatures (up to 70 °C) during homogenization produced a thinner protein layer. For instance, the protein load (Γ) at 40 °C was ~10 mg/m^2, whereas at 60 °C Γ was ~6 mg/m^2. The explanation is that casein micelles presumably can spread faster over an O/W interface at a higher temperature (Walstra, 1995). However, a heat treatment of the milk before adsorption causes the adsorbed layer to be thicker (Oortwijn & Walstra, 1979; Sharma & Dalgleish, 1994). Part of the explanation is the association of serum proteins with the casein micelles caused by heating (Oortwijn & Walstra, 1979).

Cano-Ruiz and Richter (1997) reported that the protein load increased as homogenization pressure increased from 30 to 90 MPa but decreased with increase in the temperature from 65 to 85 °C. They also found that the composition of the proteins forming the milk fat globule membrane in homogenized milk was not affected by homogenization pressure or fat concentration, but significant differences in the composition of the milk fat globule membrane were caused by the heat treatment that

was applied before homogenization. The MFGM proteins in homogenized milk were composed of native membrane proteins, caseins, α-la and β-lg. Caseins represented about 70% of the proteins in the membrane after homogenization of milk. The ratio of adsorbed whey proteins in the MFGM was higher in milk heated at the higher temperature.

Keenan et al. (1983 a) and Cano-Ruiz and Richter (1997) reported that only about 10% of the surface of homogenized fat globules in milk was covered by their natural membrane proteins. In this connection, previous studies have demonstrated that part of the original membrane material is released into the plasma under conditions such as agitation and homogenization (Mulder and Walstra, 1974; Mangino and Brunner, 1975; Darling and Butcher, 1978). It was suggested that homogenization would disrupt the membrane and release the components into the serum phase because protein components with mol. wt greater than 50 kDa were found in serum phase after homogenization (Mangino and Brunner, 1975; Darling and Butcher, 1978). However, Keenan et al. (1983) reported that two of the major MFGM proteins, xanthine oxidase and butyrophilin, remained associated with lipid globules in homogenized milk.

Most of the previous work on the influence of homogenization conditions on the fat globules has been carried out on whole milk or cream. No information on the effect of homogenisation on highly concentrated milks (> 45%) is available in the literature. In the manufacture of whole milk powder, the homogenisation is normally carried out on the concentrated milk before or after heating prior to spray drying.

2.7. Properties of modified fat globules and the MFGM proteins

After homogenization of milk, the conspicuous change is that the size of the globules become smaller. Smaller fat globules are more stable with respect to creaming and coalescence. The MFGM or surface layer is different, causing the fat in final products to be far less prone to auto-oxidation but far more prone to lipolysis as compared to natural milk (Mulder & Walstra, 1974; Walstra & Jenness, 1984). The most marked difference in the MFGM between natural fat globules and fat globules after homogenization is that the latter are largely covered by micellar casein. These fat globules are considered more

or less like large casein micelles in their properties (Walstra, 1995). The fat globules covered by casein micelles will participate in any aggregation reaction of the casein micelles, such as those occur during renneting, acidification or at high temperatures. Furthermore, some research workers have reported that the micellar caseins adsorbed at the surface of fat globules show greater reactivity in aggregation reactions than the casein micelles in the plasma (Mol, 1975; Anderson et al., 1977; Walstra & Jenness, 1984; Sharma & Dalgeish, 1994; Singh et al., 1996; McKenna et al., 1999).

Mol (1975) reported that increasing the pressure during the homogenisation of concentrated milk increased the number of insoluble particles in the resulting whole milk powder. This suggests that the casein micelles adsorbed on to the fat globules may be less heat stable during following processes (e.g. drying) than the casein micelles normally present in milk plasma. He concluded that the combination of fat and protein is very likely to be much less heat-stable than that of protein alone. Anderson et al. (1977) also reported that the deterioration of stability in homogenized UHT creams coincides with a gradual increase in the amount of casein attached to the fat phase of the cream. The reason why the fat-casein complex is heat unstable remains unknown. Walstra and Jenness (1984) showed that homogenization of whole milk reduces the heat stability of the milk and this effect is enhanced with increasing homogenization pressure, increasing fat content, and milk concentration. Walstra (1995) explained that the shorter coagulation time after homogenisation is because the effective volume fraction of micellar casein is greatly increased due to the transfer of micellar casein to the fat globules, and the average size of the casein-like particles is increased due to homogenisation.

Sweetsur and Muir (1983) and McCrae and Muir, (1991) reported that the degree of instability to heating progressively increases as more protein is adsorbed at the fat-serum interface through the use of high homogenization pressures. The reduction in heat stability observed in milk, reconstituted from powder, in hot water and coffee, was probably directly related to the higher protein load on the fat globules. However, McCrae et al. (1994) suggested that increased fat surface area due to homogenization pressure rather than casein load affected homogenization-induced destabilization. It may

be inferred that the greater change in the structure of surface-adsorbed casein micelles (more spreading) probably lead to a greater decrease in the heat stability.

McKenna et al. (1999) reported that the increased number of casein micelles adsorbed onto the fat globules after homogenization had a detrimental effect on powder solubility. Increasing homogenization pressure during processing increased the formation of insoluble material on reconstitution in water and coffee. McKenna et al. (1999) considered that this effect is mainly due to the adsorption of casein micelles onto the fat globules and the subsequent clustering of fat globules possibly caused by the sharing of adsorbed casein micelles between two or more fat globules.

Singh et al. (1996) found that the maximum proportion of κ-casein that dissociated by heating (pH 7.1) recombined milk appeared to greater from the free casein micelles (70%) than from micelles attached to the surface of fat globules (25%). This was considered to be due to the difference in interaction forces between κ-casein and other casein components, at the fat globule surface compared with those in the free micelles. One possibility is that a proportion of κ-casein is adsorbed directly at the fat-water interface and thus is not readily dissociated during heating and pH changes. Sharma and Dalgeish (1993) and Sharma et al. (1996) also showed that, in unheated recombined milk when the adsorbed micelles were dissociated by EDTA, a significant proportion of κ-casein remained associated with the fat globule surface.

2.8. Fat globules and the MFGM in the milk powder

The fat in whole milk powder (WMP) can be observed by electron microscopy to be in globular (spherical) form within the particle and appears to be embedded in a matrix which consists mainly of a lactose glass interspersed with casein micelles (Buma, 1971; Buchheim, 1981). Recently, McKenna (1997) reported that the distribution and size of individual fat globules in the WMP particles can be clearly observed by confocal laser scanning microscopy (CLSM). In addition, the occasional presence of relatively large regions of fat on the surface and within some powder particles can be identified using CLSM (McKenna, 1997). Fat spread over part of the surface of particles of whole-milk

powder was also observed using electron microscopy by Buma (1971) and Buchheim (1981). Walstra (1995) suggested that the spreading of fat is likely to result from several fat globules coming into contact with the air-water surface of the drying droplet. This surface fat appeared to pool at the joining points of agglomerated powder particles (McKenna, 1997).

McKenna et al. (1999) compared some powders that contained different fat globule size distribution in the confocal micrograph. The fat globule distribution was fairly uniform throughout the particles is in some powders. A difference in the size of the globules from the outside to the inside of particles in which there were some larger, more coalesced fat globules near the surface of these particles that reduced in size towards the centre of the particle, were observed in the other powders (McKenna et al., 1999). Furthermore, McKenna et al., (1999) found that there were considerable fat globule clustering in powders after comparing the size distribution of powders in a dissociating buffer or in water. The extent of the fat globule clustering was different in the different powders, depending on the conditions of manufacture. The extent of the fat globule clustering appeared to be related to the solubility properties of powders (McKenna et al., 1999).

The details of proteins on the surface of fat globules in some powders were also observed by McKenna et al. (1999) using transmission electron microscopy (TEM). The most distinguishing feature in these micrographs was the significant quantity of casein micellar material associated with the fat globules and the interaction of these micelles with other fat globules to form protein-associated fat clusters. The amount of the protein adsorbed at the surface of fat globules and the number of protein-associated fat clusters were different between the powders manufactured under different conditions. In addition, the pronounced hair-like structures protruding from the casein micelles, which were probably aggregates of denatured whey protein and κ-casein, were also observed in the adsorbed casein micelles in some powders, which may be responsible for the increased aggregation within the powder (McKenna et al., 1999).

It is still uncentain whether the surface fat of powder particles 'free fat' or 'uncovered fat' or is covered by a protein layer (Buma, 1971, Walstra, 1995). Usually free fat is defined as that fraction of the fat which can be extracted with organic solvents under standardized conditions (Holm et al., 1925). Unfortunately, very different experimental conditions have been used by various workers to measure free fat and as no systematic evaluation of the methods has been carried out, the results may not be comparable. Several workers explained the occurrence of free fat in milk powder by assuming that such fat results from fat globules in which the protein membranes has been damaged in the processing step prior to spray drying (Mohr, 1961; Samhammer, 1966; King, 1955). However, Walstra (1995) believed that the term 'free fat' or 'uncovered fat' is a vague term, and that 'free fat' or 'uncovered fat' does not normally occur in liquid milk products. He considered that as soon as uncovered milk fat comes into contact with milk plasma, proteins will adsorb on to the fat surface and thus cover the fat in a very short time (~ 10 ms). Furthermore, Buma (1971) reported that more than the surface fat can be extracted by solvents; the extracted fat includes surface fat, fat in globules that are in contact with the particle surface, cracks in the particle and vacuoles and fat in globules. The amount extractable is thus higher for smaller powder particles, larger fat globules and lower water content of the powder.

In fact, the fat globules in milk powder particles have been subjected to a number of processing steps which have damaged all or the major part of the native membranes. These steps include the pasteurization, concentration, homogenization and spray drying. In particular, homogenization causes a significant change in the fat globules and the surface protein layer. Buma (1971) considered that it is rather unlikely that a dry fat globule membrane with a thickness of 5-10 nm, consisting of about 50% of lipids, would give more protection against fat solvents than a far thicker layer of non-fat milk solids in the powder particles. The proportion of free fat was observed not to correlate with most powder properties (e.g. rate of autoxidation, powder stickiness, rate of creaming after redispersion), except for a fairly weak correlation with powder dispersibility for proportions of free fat below 0.2 (Buma, 1971).

2.9. Role of fat globules and the MFGM proteins in influencing functional properties of the whole milk powder

The changes in fat globules and the MFGM proteins due to protein and fat interactions during processing have been shown to influence the reconstitution properties of the dried powder in water (Buma, 1971; Buma & Henstra, 1971; Mol, 1975; Caric & Kalab, 1987; de Ruyck, 1991; McKenna et al., 1999).

Free fat results seem to have some bearing on powder flow, cohesiveness and wetting. However, this influence is probably related only to the fat on the surface of the particle (Buma, 1971). Buma (1971) also observed that the solubility of spray dried WMP was better if its free fat content was higher, indicating that more highly homogenized milk would give lower free fat but may reduce powder solubility. An increase in homogenisation pressure during processing of powder decreases the content of free fat in the powder (de Vilder et al., 1979; Oldfield et al., 2000). However, the adsorption of casein micelles onto the fat globule surface during homogenization and the formation of clusters of fat globules predisposes the powder to the formation of insoluble material on reconstitution.

It has been shown by Mol (1975) that increasing the homogenisation pressure of concentrate prior to spray drying increased the number of insoluble particles in the resulting whole milk powder. Teehan et al. (1997) and McKenna et al. (1999) reported that poor stability in coffee was related to powders having a high degree of protein coverage on the fat globules. McKenna et al. (1999) also reported that the different extent of fat globule clustering in the powders appeared to be related to the solubility of the powders, in which greater fat globule clustering resulted in poor scores for all the solubility related functional tests, i.e., coffee sediment, hot sediment, cold sediment and slowly dissolving particles. Powder with relatively large numbers of very small fat globules, which had a greater amount of proteins associated with the fat globules, had lower values for solubility tests. Examination of the microstructure of sediment from coffee and hot water, using TEM, showed that the sediment was mainly comprised of extensively aggregated micelles and clusters of fat globules associated with proteins (McKenna et al., 1999). Most of the casein micelles were no longer discrete units but had spread and 'fused' with adjacent micelles to form an almost continuous protein network. The smaller fat globules within the structure were generally clustered together

and completely embedded in a continuous protein matrix. The adsorption of casein micelles onto the fat globules and subsequent clustering of fat globules, possibly caused by the sharing of adsorbed casein micelles between two or more fat globules, was a major contributing factor towards the poor functional properties of powder.

2.10. The aim and objectives of the thesis

The overall aim of this work is to further our knowledge about the influence of processing factors on component interactions during the manufacture of whole milk powders, and to understand the effects of component interactions on the reconstitution properties of whole milk powders. This knowledge will be advanced through developing an understanding of the behaviour of fat globules and their interactions during preheating, evaporation, homogenization of milk concentrate and spray drying. Relationships between the state of fat in whole milk powders and their reconstitution properties will then be explored. Specific objectives of this research are as follows:

- To characterize the polypeptides in the natural MFGM in whole milk.
- To identify the nature of interactions between milk serum proteins and MFGM components during the heating of whole milk obtained at different times during the New Zealand dairying season.
- To determine the kinetics (temperature and time dependence) of binding of whey proteins and caseins to fat globules in whole milk.
- To investigate the effects of evaporation (concentration) on fat globule-protein interactions.
- To examine the effects of homogenisation conditions (temperature, pressure and placement of homogenisation step, i.e. before or after heat treatment) and heat treatment of homogenized concentrate on fat globule-protein interactions.
- To determine the effects of spray drying on the interactions between fat globules and proteins.
- To explore the relationship between the state of fat (e.g. fat globule size, amount and type of proteins bound to fat globules) and reconstitution properties of whole milk powders.

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

Bulked fresh whole milk was collected from a single herd on a Massey University Dairy Farm, Palmerston North, New Zealand. Samples for laboratory experiments were collected in August, January to March and May, respectively. In New Zealand calving occurs in August and therefore these samples approximately correspond to early, mid and late lactation milks. The samples were not cooled and were used for experimental trials within 30 min of collection.

Bulked whole milk was also collected from Fonterra Co-operative Group in November for the manufacture of whole milk powder on the pilot plant at the Fonterra Research Centre (FRC), Palmerston North.

All the chemicals used were of analytical grade obtained from either BDH Chemicals (BDH Ltd., Poole, England) or Sigma Chemical Co. (St. Louis, MO, USA) unless specified otherwise. The water used in all experiments was purified by reverse osmosis followed by treatment with a Milli-Q apparatus (Millipose Corp., Bedford, MA 01730, USA).

3.2. Heat treatment of whole milk samples

Samples of fresh whole milk for laboratory experiments were heated in a pilot-scale, indirect-heated UHT plant (Alfa-laval, Australia) to a desired temperature in the range 65 to 95 °C and held for 0 to 60 min in a temperature-controlled water bath. It took < 2 s to heat the milk to the desired temperature in this UHT plant. After holding the samples for set times they were rapidly cooled to about 20 °C in an ice bath.

3.3. Manufacture of whole milk powder

The different processing conditions for the experimental trial are shown in Fig. 3.1. There were 16 experimental runs. Bulked whole milk was standardized to 3.4% fat, 3.2% protein, 4.8% lactose, 12.0% total solids and 8.5% SNF in the pilot plant of the Fonterra Research Center, Palmerston North. Milk was pasteurized at 72 °C for 15 s before further processing. Sample of bulked whole milk and standardized milk were collected for analysis.

3.3.1. Preheat treatment of milk

Preheating was carried out by a two-stage, direct steam injection (DSI) process to bring the milk to the desired temperature (95 °C). The milk was then passed through holding tubes (residence time 20 s) to the vacuum vessel where it was flash-cooled to ~70 °C prior to entry into the first stage evaporator. For the non-preheating process run, the milk was heated to ~70 °C by DSI prior to entry to the evaporator.

3.3.2. Evaporation

For each process run, about 1750 L of standardised milk was passed through a three-effect, pilot scale, falling film evaporator (Wiegand, Karlsrudhe, Germany) with a nominal evaporative capacity of 1600 l/h (Fig. 3.2). The temperature of milk in the first, second and third effects was ~72 °C, ~63 °C and ~51 °C, respectively. The milk was evaporated to ~49% total solids. The final concentrate was either held at 50 °C or heated to either 65 or 79 °C prior to homogenization. Samples were collected after each effect of the evaporation treatment and immediately cooled to about 30 °C in a water bath prior to further analysis.

3.3.3. Homogenization of milk

The concentrated milk samples from the evaporator were homogenized in a single stage homogenizer with pressures of either 40 bar or 70 bar and temperatures of either 50, 65 or 79 °C. The samples that were homogenized at 50 °C were heated to either 65 °C or 79 °C in a heat exchanger after homogenization. Samples were collected after each treatment and immediately cooled to about 30 °C in a water bath before further analysis.

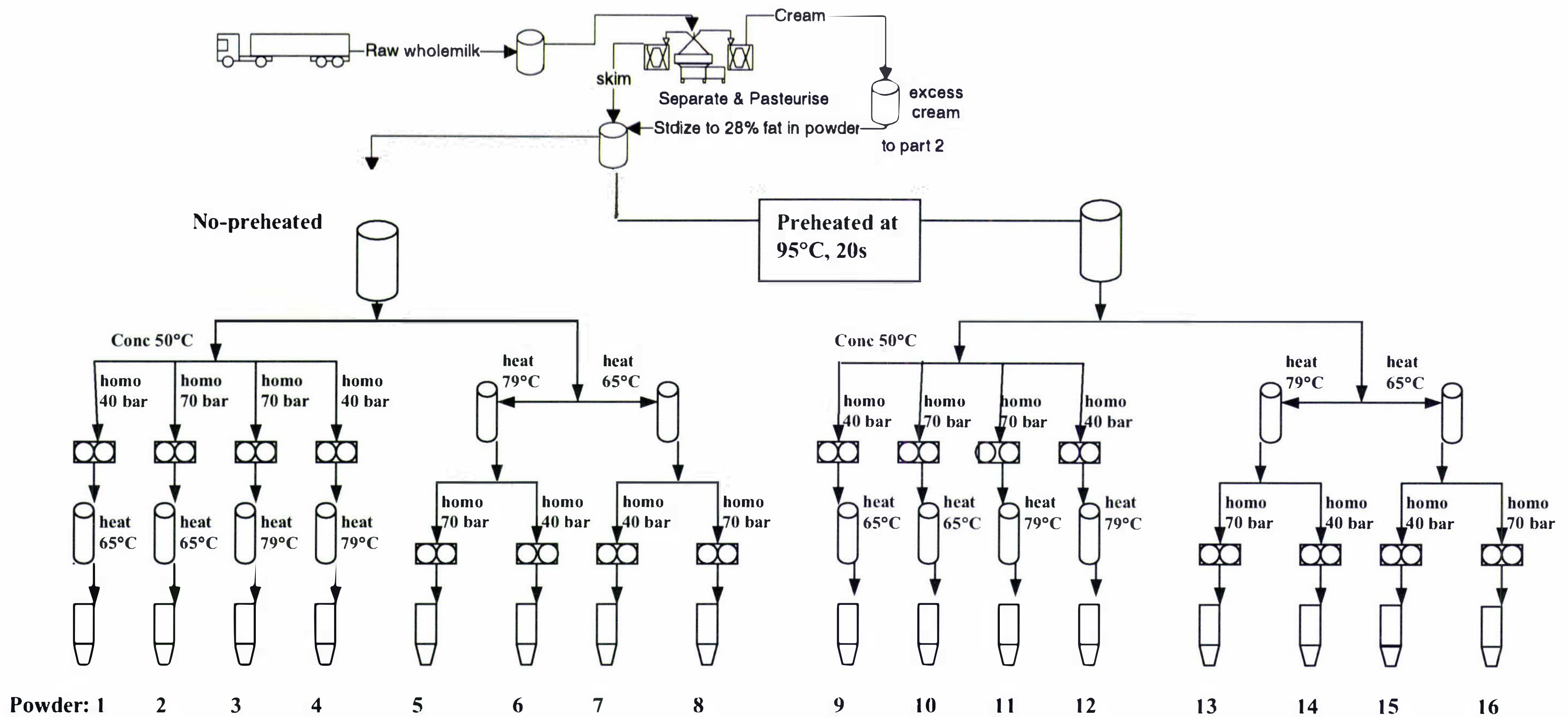

Fig. 3.1. Schematic diagram of the experimental runs for the milk powder manufacturing trial.

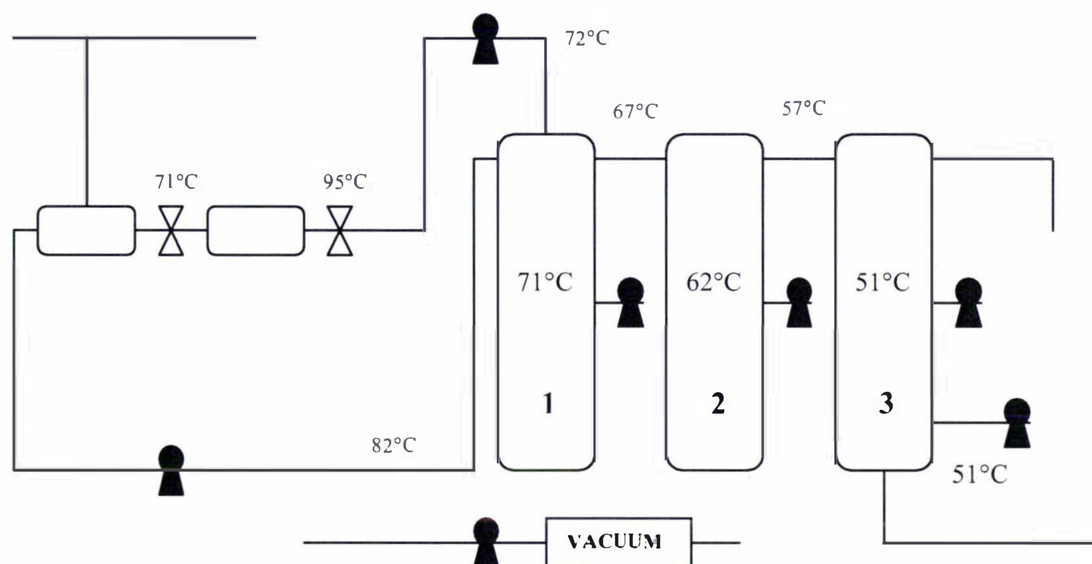

Fig. 3.2. Schematic diagram of the three-effect, pilot scale, falling film evaporator in Fonterra Research Centre pilot plant.

3.3.4. Spray drying of concentrated milk

The concentrated milk samples (~49% total solids) at temperatures of either 65 °C or 79 °C after homogenization were dried in a disc atomizer Integral Fluid Bed Drier. The experimental conditions for the dryer are shown in Table 3.1. The milk powder samples were collected and packed in plastic bags for storage at 20 °C prior to further analysis.

Table 3.1. The experimental conditions for the Integral Fluid Bed Drier

Feed rate	130 l/h
Feed press	100 bar
Disc speed	16000 rpm
Drier inlet temp	180 °C
Drier inlet air flow	2700 kg/h
Chamber temp	80 °C
Chamber pressure	-0.1 H_2O
Fluid bed inlet temp	75 °C
Fluid bed inlet airflow	1200 M^3/h
Fines return	Bed

3.4. Isolation of milk fat globule membrane material

Prior to centrifugation, milk concentrate samples and milk powder samples were diluted to ~12% solids with Milli-Q water. All unhomogenised milk samples (both unheated and heated) were centrifuged at 15,000 *g* for 20 min at 20 °C in a temperature-controlled centrifuge (Sorvall RC5C, DuPont Co., Wilmington, DE), while homogenized milk samples and diluted milk powder samples were centrifuged at 45,000 *g* for 20 min. Three fractions were obtained, i.e. top (cream) layer, middle layer and sediment. The top layer (cream) was removed from the centrifuge tube using a spatula. The middle layer was then decanted off leaving behind the sediment. For one set of experiments, the three layers were weighed and analysed for total protein and fat content.

The cream was washed with simulated milk ultrafiltrate (SMUF) (Jenness & Koops, 1962) to remove protein material that is not bound to the fat globule membrane. The cream was suspended in 10 volumes of SMUF or in 10 volumes of SMUF containing 6M urea and 50 mM EDTA. The mixture was left at room temperature for 1 h,

recentrifuged at 15,000 *g* for 20 min at 20 °C and then the top layer was collected. This washing step was repeated twice.

To study the effects of heat treatment on MFGM, the cream washed in SMUF solution was resuspended in SMUF to yield a suspension with a fat content similar to that of the original milk. Sub-samples (200 ml) were heated at temperatures in the range 50 to 80 °C in a thermostatically controlled water bath and immediately cooled to about 20 °C in an ice bath. The heated samples were then centrifuged at 15,000 *g* for 20 min at 20 °C; the cream layer was removed and stored at 5 °C.

3.5. Characterization of MFGM protein components

The composition of the individual proteins in the washed cream were determined by polyacrylamide gel electrophoresis (PAGE). The washed cream was dispersed (1: 2 wt/wt) in 0.5 M Tris-HCl buffer, containing 10% glycerol, 2% (w/v) SDS and 0.05% Bromophenol blue. SDS displaced the MFGM proteins from the oil-water interface (de Feijter, Benjamins, & Tamboer, 1987) and stabilised the protein molecules in extended conformations. For PAGE under non-reducing conditions, the samples were heated at 45 °C for 5 min in a water bath. For reducing conditions, 5% β-mercaptoethanol was added to the samples followed by heating at 95 °C for 5min in a boiling water bath.

Under non-reducing conditions, non-covalently linked protein aggregates were dispersed and migrated into the resolving gel, but disulphide-linked aggregates stayed at the top of the stacking and resolving gels. On the other hand, under reducing conditions both non-covalently linked and disulphide-linked aggregates were dispersed and migrated into the resolving gel. Comparison of SDS-PAGE patterns under non-reducing and reducing conditions enables disulfide-linked complexes formed by heat treatment to be detected.

After heating in the SDS buffer, a further centrifugation at 2500 *g* for 30 min was performed before PAGE analysis to remove the fat from the sample. 10 μl of subnatant was then loaded onto the SDS-gel and the gel was run in 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). The SDS-PAGE systems have been described by Singh and Creamer (1991). The protein bands were fixed and stained using a solution of Coomassie blue R-250. After the gels had been stained and destained, the proteins of the MFGM were identified by comparing with molecular weight standard proteins obtained from Bio-Rad Laboratories (Hercules, CA 94547, USA), and with the results reported previously (Keenan & Dylewski, 1995; Mather, 2000). In addition, the gels were scanned using an Ultrascan XL laser densitometer and the results were analysed using an LKB 2400 GelScanXL software program (LKB Produkter AB, Bromma, Sweden) to obtain quantitative results.

The quantification of the β-lg and α-la was carried out using their respective standard curves. Standard samples of purified β-lg and α-la (Sigma Chemical Co., St. Louis, MO) were run on the electrophoresis gels in various amounts in the range 0.25-10 μg. A satisfactory linear plot (R^2 = 0.99), obtained between the integrated peak areas and the sample concentration, was then used to quantify the serum proteins in the samples derived from the fat globule surface.

Two dimensional PAGE (first SDS-PAGE non-reducing and then SDS-PAGE reducing) was used to identify the components in the protein complexes in MFGM samples. After the first-dimension separation (described above) the sample wells were dried using strips of filter paper and filled with SDS stacking gel solution, and this was allowed to set (about 30 min). The gel was removed from between the glass plates and carefully cut so that each strip contained all the protein bands from each sample, including any material that did not migrate into the stacking gel. Two of the strips were stained to confirm that the separation in the "first dimension" was satisfactory. One of the unstained strips was placed in hot (94 °C) SDS sample buffer containing 1% of β-mercaptoethanol for 30 s. The strip was then removed from the hot sample buffer and washed with a little water to remove excess β-mercaptoethanol solution, and the surplus water was blotted from the strip surface with filter paper strips. The gel strip was placed on one of the glass plates and perpendicular to the spaces (0.75 mm), the second plate was placed over it and the two plates were assembled into the gel-setting equipment.

SDS resolving gel solution (3.3 ml) was then carefully poured between the plates, leaving a space of about 12 mm between the top of the gel solution and the bottom of the gel strip. The gel solution was then overlaid with water in the usual fashion and the gel strip was completely immersed in this water. This provided further washing off of excess β-mercaptoethanol from the surface of the strip (the β-mercaptoethanol would have inhibited the acrylamide polymerisation). After the resolving gel had set, the water was removed, the equipment was flushed with a little stacking gel solution and the space was filled with stacking gel solution, with the equipment tilted at about 30°, so that no bubbles were trapped beneath the gel strip. A standard well-forming comb, which had most of the teeth removed, was then inserted so that the wells formed were level with the centre of the gel strip and at least 4 mm distant from the gel strip. After loading the appropriate chamber buffer and the control sample, the proteins were electrophoresed in the "second dimension". The resultant gel was then stained, destained and photographed as described above.

3.6. Protein and fat content

The total protein content of cream, that had been washed with SMUF, was determined using the Kjeldahl method (AOAC, 1974) by determining total nitrogen and multiplying it by a factor of 6.38. The samples were digested using a Kjeltec 1007 digester (Tecator, Sweden) and distilled using a Kjeltec 1026 Distilling Unit (Tecator, Sweden). The total fat content of the whole milk and the washed cream was determined using the Mojonnier method for milk (International Dairy Federation, IDF 1C: 1987) and cream (IDF 16C: 1987), respectively.

3.7. Average fat globule size

A Malvern MasterSizer MSE (Malvern Instruments Ltd, Worcestershire, UK) was used to determine the fat globule size distribution using the presentation code 2NAD. The relative refractive index (N) i.e. the ratio of refractive index of fat globule (1.456) and that of the dispersion medium (1.33) was 1.095. The milk sample was mixed with 2% SDS and 50 mM ETDA solution to dissociate the casein micelles, prior to fat globule size measurements.

3.8. Confocal laser microscopy

A Leica (Heidelburg, Germany) confocal scanning laser microscope (CSLM) with a 100 mm oil immersion objective lens and an Ar/Kr laser with an excitation line of 488 nm (set up in such a way that only the fluorescent wavelength band can reach the detector system) was used to determine the microstructure of milk samples. A liquid sample (3 ml) was taken in a test tube and Nile Blue (fluorescent dye) was mixed through and then placed on a microscope slide. Concentrated milk samples and powder samples were prepared for microscopy by dispersing the samples into a glycerol-based solution containing 10 mg/L of Nile Blue and placing this solution on a microscope slide. The slide was then covered with a coverslip and observed under the microscope. Each sample was prepared in duplicate on two different occasions. Multiple fields were viewed and typical fields were acquired and stored as TIFF (format) files.

3.9. Transmission electron microscopy

The sample was mixed with warm melted (35–40 °C) 3% low-temperature gelling agarose in a 1:1 ratio. This solution was poured on to a microscope slide, allowed to set, and chopped into 1 mm^3 cubes. The cubes were put into a bijoux bottle containing 3% glutaraldehyde in 0.2M sodium cacodylate buffer. This was kept at 5 °C for 24 hours. The sample with glutaraldehyde were then rinsed twice with 0.2 M sodium cacodylate buffer rinses over 2 hours. The agarose embedded samples were placed in 1% osmium tetroxide (1 ml) overnight at room temperature. The samples were rinsed twice with distilled water and placed in 1% uranyl acetate (1 ml) for 30 minutes and then rinsed twice with distilled water. The dehydration process was carried out at 5 °C in 25% acetone (15 min) then in 50%, 70% and 90% acetone for (30 min each) followed by 100% acetone (3 changes over 90 min). The acetone was replaced with Procure 812 embedding resin, and put on rollers for 24 h. A cube of the sample was place into an embedding capsule and this was cured at 60 °C for 48 h. The embedded samples were then sectioned to a thickness of 90 nm using the Reichert Ultracut microtome. These sections were mounted on 3 mm copper grids and stained with lead citrate before

examination in a Philips transmission electron microscope (TEM) (Philips, NL-5600 MD Eindhoven, The Netherlands) at an acceleration voltage of 60 kV.

3.10. Determination of functional properties of whole milk powder

3.10.1. Solubility index

The solubility index (SI) of the powders was measured according to the American Dry Milk Institute (1965) method.

3.10.2. Cold (25 °C) sediment

A method as described by McKenna et al. (1999) was used. Milk powder was mixed into water at 25 °C. After a 2 min hydration time the milk was poured through a stainless steel mesh. The residue (sludge) deposited on the mesh was weighed. The reconstituted milk was then poured into a glass test tube, inverted and drained. After 2 min, the residue, slowly dissolving particles (SDPs) deposited on the inner surface of the test tube were compared with a standard chart and graded from A (few SDPs) to E. The remaining milk was poured into centrifuge tubes. The sediment was measured as milliliters of sediment remaining in the calibrated sample tube after centrifugation at 160 *g* for 5 min.

3.10.3. Hot (85 °C) sediment

Milk powder was mixed into water at 85 °C. After 15 min hydration time the milk was poured through a stainless steel mesh. The reconsitituted milk was poured into centrifuge tubes. The sediment was measured as milliliters of sediment remaining in the calibrated sample tube after centrifugation at 160 *g* for 5 min.

3.10.4. Hot coffee sediment

A method similar to that described by Teehan et al. (1997) was used to measure the stability of milk powder in hot coffee. Milk powder was mixed into a standard coffee solution (80 °C). After a 10 min standing time, the sample was re-mixed, poured into a centrifuge tube and centrifuged at 160 *g* for 5min. The coffee sediment was measured in milliliters.

3.10.5. Dispersibility

A method as described by McKenna et al. (1999) was used to measure dispersibility. Powder was mixed into water at 45 °C. The reconstituted milk was immediately poured through a mesh under vacuum. The residue remaining on the mesh was compared with a standard chart and assigned a grade from 1 to 7.

3.11. Statistical analysis

Results were analysed statistically using the Minitab 12 for Window package. Differences were considered significant at $P \leq 0.05$.

CHAPTER 4
CHARACTERIZATION OF PROTEIN COMPONENTS OF NATURAL AND HEAT-TREATED MILK FAT GLOBULE MEMBRANES

4.1. Introduction

The protein composition of the MFGM is very complex; over 40 different polypeptides, ranging in molecular weight from 15 to 240 kDa, have been observed, among which at least 8 are glycoproteins (McPherson, Dash, & Kitchen, 1984). The major classes of polypeptides have molecular weights of 155, 67, 50 and 49 kDa which were identified as xanthine oxidase, butyrophilin, PAS 6 (band 15) and PAS 7 (band 16), respectively (Mather, 2000). Furthermore, Mondy and Keenan (1993) reported that the amounts of protein components in the MFGM vary with the stage of lactation. There were greater amounts of butyrophilin and xanthine oxidase in early and late lactation milks compared with mid lactation milks.

The molecular state of the various polypeptides in the MFGM is not very clear (Keenan & Dylewski, 1995). Valivullah and Keenan (1989) reported that butyrophilin and xanthine oxidase are cross-linked with each other and have a nearest neighbour relationship. Xanthine oxidase which exists in dimeric form under physiological conditions, may be self-associated via hydrophobic interactions (Cheng et al., 1988). Since the butyrophilin and xanthine oxidase content of membranes was not related to milk fat globule diameter, it is likely that these proteins alone are not involved in anchoring the membrane to the fat globule surface (Mondy & Keenan, 1993). Mather and Jack (1993) proposed that butyrophilin associates in a supramolecular complex with other proteins of MFGM via hydrophobic or hydrophilic interactions and, possibly, intermolecular disulfide bonds. Proteins that may interact with butyrophilin include the phospholipid-binding protein MFG-E8, xanthine oxidase and an unidentified protein of 52 kDa.

The cysteine and cystine residues in the MFGM have been reported to be highly reactive in the membrane environment (Van Boekel & Walstra, 1989). Appell, Keenan

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and Low (1982) reported that an integral butyrophilin forms disulphide-stabilized complexes on heating at 58 °C. Cheng et al. (1988) reported that xanthine oxidase contained 22 disulfide and 38 sulfhydryl groups, four of which were detectable in the undenatured protein complex.

Previous studies on the MFGM fraction in dairy systems indicate that heat-induced interactions occur between MFGM components and skim milk proteins. MFGM-β-lactoglobulin or MFGM-α-lactalbumin complexes, possibly involving sulphydryl-disulphide interchange reaction, form in heated whole milk systems (Dalgleish & Banks, 1991; Houlihan et al., 1992; Kim & Jimenz-Flores, 1995). However, no information is available on the heat-induced changes in MFGM protein in the absence of serum proteins.

The objectives of the experimental work reported in this chapter were to further characterize the polypeptides in MFGM, to explore the relationship among these polypeptides, and to determine interactions among the polypeptides of MFGM during heating in the absence of serum proteins. Furthermore, seasonal variations in MFGM polypeptides were determined under New Zealand farming conditions.

4.2. Results and discussion

4.2.1. Characterization of the MFGM proteins isolated from whole milk

Sample of fresh whole milk were collected from a Massey University Dairy Farm as described in Section 3.1. MFGM material was isolated from these milk samples as described in Section 3.5. The total protein content of the MFGM and the surface protein coverage were significantly lower in mid season milk (6.4 mg/g fat and 1.28 mg/m^2 respectively) than in either early season milk (8.3 mg/g fat and 1.85 mg/m^2) or late season milk (7.8 mg/g fat and 1.59 mg/m^2) (Table 4.1). The values for total protein content compare reasonably well with those of Patton and Huston (1986) (10 mg/g globule mass) and Mulder and Walstra (1974) (9 mg/g fat globules). However, McPherson et al. (1984) reported that the yield of membrane protein was only 2.7 mg/g fat. This low value may be due to the different extraction procedure used, in which the sample containing the membrane material was placed on top at a sucrose solution and

centrifuged. The MFGM material was then removed from the sample-sucrose interface. Keenan & Dylewski (1995) have noted that in order to obtain valid analytical data, milk fat globules must be washed sufficiently to remove occluded serum constituents, yet extensive washing may also remove loosely associated but nonetheless true membrane constituents.

Fig. 4.1A illustrates the polypeptide patterns of MFGM, isolated from early, mid and late season milks, as determined by SDS-PAGE (15% acrylamide) under reducing conditions. About 37 protein bands were observed in the gel, in which there were 10 major bands, ranging in molecular weight from 47 to 200 kDa. No bands corresponding to casein and whey proteins were observed (Fig. 4.1A, lane 1 to 6), indicating that these proteins were entirely removed by the washing procedure used. SDS-PAGE with lower acrylamide content (8%) was run for greater separation of the higher molecular weight polypeptides (Fig. 4.1B).

Table 4. 1: Characteristics of milk fat globules in early, mid and late season milks[1]

	Early season milk (n = 4)	Mid season milk (n = 8)	Late season milk (n = 4)
Total protein in MFGM (mg/g fat)	$8.3 \pm 0.36^{a,2,3}$	6.4 ± 0.39^{b}	7.8 ± 0.23^{a}
Surface protein coverage of milk fat globule (mg/m^2)	1.85 ± 0.10^{a}	1.28 ± 0.10^{b}	1.59 ± 0.08^{c}
d_{32} (μm)	1.30 ± 0.2^{a}	1.20 ± 0.2^{b}	1.21 ± 0.2^{b}
Specific fat surface area (m^2/mg)	4.5 ± 0.2^{a}	5.0 ± 0.2^{b}	4.9 ± 0.2^{b}

[1] Early, mid and late season milks were collected in August, January to March and May, respectively.
[2] Data are means ± sample standard deviations.
[3] Different superscripts within a row indicate significant differences ($P < 0.05$).

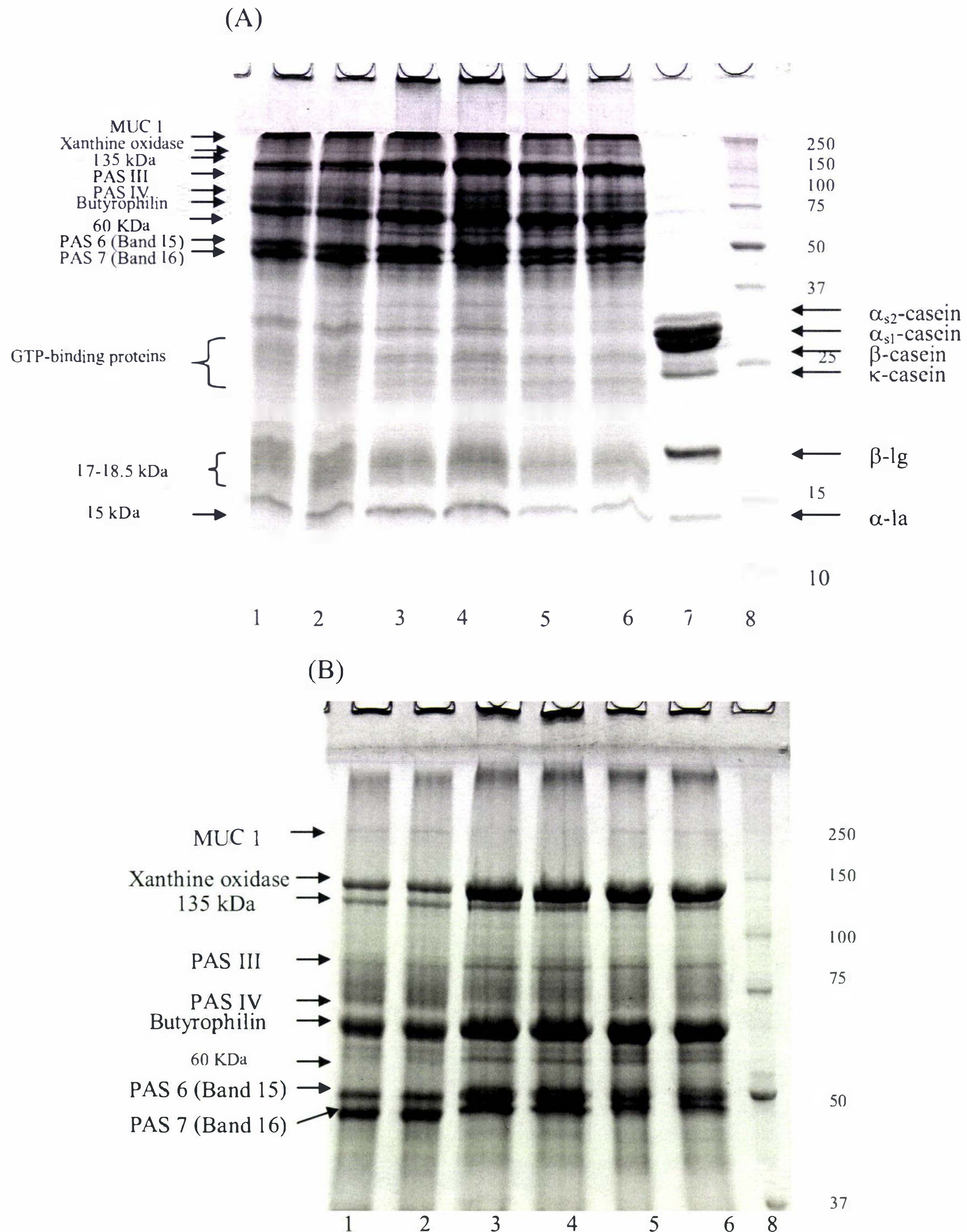


Fig. 4.1. SDS-PAGE patterns (15% (a) and 8% (b) acrylamide gel), obtained under reducing conditions, of MFGM material from fresh whole milk. Lane 1, 2, MFGM from mid season milk; lane 3, 4, MFGM from early season milk; lane 5, 6, MFGM from late season milk; lane 7, whole milk protein; lane 8, standard proteins. Membrane polypeptides are named according to Mather (2000).

The apparent molecular mass of major bands on the SDS-PAGE was estimated from the mobility of proteins in the gel when compared with the mobilidy of molecular weight standards. The approximate quantities of these polypeptides in the MFGM were determined by scanning the stained bands (Fig. 4.1). The nomenclature recommended by Mather (2000) is used generally to identify the polypeptides. Of the major polypeptides resolved, the bands corresponding to mucin 1 (Mr, 194 kDa), xanthine oxidise (Mr, 145 kDa), PAS III (Mr, 94 kDa), PAS IV (Mr, 78 kDa), butyrophilin (Mr, 67 kDa), PAS 6 (band15) (Mr, 50 kDa) and PAS 7 (band16) (Mr, 47 kDa) were identified. These polypeptide patterns are in good agreement with those described by Keenan and Dylewski (1995) and Mather (2000). A sharp band with Mr 135 kDa, with slightly greater mobility than xanthine oxidase, was observed (Fig. 4.1). This band has not been identified previously. A faint band with Mr ~ 60 kDa was also observed, although it was not as obvious in the mid season sample as in the early and late season samples.

There were at least 10-12 bands, between 44 and 20 kDa, some of them may be the GTP binding proteins. The other bands have not been reported previously and their identify is unknown. Ghosal, Ankrapp and Keenan (1993) detected 6 polypeptides of Mr, 28, 27.5, 26, 25, 23.5 and 21 kDa, when blots were probed with guanosine-5'-(γ-^{35}S-thio)triphosphate (GTPrS). They proposed that these polypeptides were GTP binding proteins.

A number of diffuse and faint bands with Mr about 17 - 18 kDa with slightly greater mobility than β-lg were also observed (Fig. 4.1); these polypeptides have also not been reported previously (Keenan & Dylewski, 1995; Mather, 2000). In addition, Fig. 4.1 shows the presence of a polypeptide with Mr 15 kDa and slightly lower mobility than α-lactalbumin. This is likely to be the same protein described by Patton and Huston (1986); in their study, this polypeptide had a Mr ~14 kDa. This constituent may be the mammary-derived growth inhibitor (FABP, MDGI) (Bohmer et al., 1987; Brandt et al., 1988, Mather, 2000). Brandt et al. (1988) purified and identified a ~13 kDa polypeptide from MFGM, which may play a role in the mechanism of growth inhibition exerted by mammary-derived growth inhibitor. A considerable proportion of protein material

(approximately 10%) remained on top of the stacking and resolving gels (Fig. 4.1A, B); however, the nature of this material is unknown.

No major difference in protein patterns on the SDS gel was observed among MFGM of mid, early and late season milks (Fig. 4.1). However, there was a significant difference ($P < 0.05$) in the percentage of some of the MFGM components between the milks (Table 4.2). It must be pointed out that the percentages reported in Table 4.2 are based on staining intensity, which may vary between protein species. Hence, it may not represent actual protein content, but will highlight differences between samples and changes during processing. Table 4.2 showed that ~10% of the MFGM material remained on top of the resolving gel (high molecular weight protein aggregates), 60-70% represented major proteins while 20-30% were minor proteins. These values were similar for all milks. Based solely on the staining intensity, xanthine oxidase and butyrophilin accounted for 11-12% and ~15-16% respectively of the MFGM polypeptides in early and late season milks, compared with only ~7% and ~10% of the MFGM polypeptides in mid season milk. Using molecular weights of 145 kDa for xanthine oxidase and 66.7 kDa for butyrophilin, the xanthine oxidase to butyrophilin molar ratio (about 1:3) was, however, similar for early, mid and late season milks. The percentage of PAS 7 was lower in the early and late season MFGM than that in mid season MFGM. These results are in agreement with the report of Mondy and Keenan (1993), who reported that both xanthine oxidase and butyrophilin were high in membrane samples from early lactation, both decreased in amount as lactation progressed to the midpoint, and that xanthine oxidase and butyrophilin were present in constant molar proportions of about 1:4. The constant molar ratio of xanthine oxidase to butyrophilin in all season milks may suggest that the two proteins were cross-linked together in some form.

Table 4.2. Protein components in the MFGM obtained from early, mid and late season milks[1].

Proteins	Molecular weight (kDa)	Percentage of total protein in MFGM (%)[2]		
		Early season milk (n = 4)	Mid season milk (n = 8)	Late season milk (n = 4)
High molecular weight material		10 ± 2.0[a,3,4]	10 ± 2.0[a]	9 ± 2.0[a]
Xanthine oxidase	145	12 ± 1.5[a]	7 ± 1.2[b]	11.2 ± 1.5[a]
Unknown	135	4 ± 0.55[a]	3 ± 0.56[a]	4 ± 0.5[a]
PAS III	93.7	4.2 ± 0.31[a]	4.5 ± 0.25[a]	4.5 ± 0.3[a]
PAS IV	78.4	4 ± 0.29[a]	5 ± 0.28[a]	4.2 ± 0.3[a]
Butyrophilin	66.7	16 ± 1.5[a]	10 ± 2.0[b]	15 ± 1.5[a]
Unknown	60.0	4 ± 0.3[a]	2 ± 0.25[a]	3 ± 0.3[a]
PAS 6 (Band 15)	50.9	9 ± 1.5[a]	8 ± 1.5[a]	9 ± 1.3[a]
PAS 7 (Band 16)	47.2	10 ± 2.5[a]	14 ± 2.8[b]	8 ± 1.3[a]
Unknown	17-18	3 ± 0.25[a]	5 ± 0.3[a]	2 ± 0.25[a]
FABP	15	2 ± 0.26[a]	3 ± 0.30[a]	2 ± 0.25[a]
Other minor proteins		22 ± 5.0[a]	28 ± 5.0[a]	28 ± 5.0[a]

[1] Early, mid and late season milks were collected in August, January to March and May, respectively.
[2] Data is based solely on staining intensity.
[3] Data are means ± sample standard deviations.
[4] Different superscripts within a row indicate significant differences ($P < 0.05$).

Mulder and Walstra (1974) reported that the milk fat globule diameter is maximal very early in lactation, but decreased progressively throughout lactation. The results of our study show a slightly different trend; namely that the fat globule diameter in early season milk was larger than both the mid and late season milk (Table 4.1). This suggests that higher levels of xanthine oxidase, butyrophilin and total MFGM proteins observed in early season milk as compared with mid season milk were not related to the milk fat globule size.

Fig. 4.2 shows the polypeptide patterns of MFGM, isolated from mid, early and late season milk, as determined by SDS-PAGE (15% acrylamide) under non-reducing conditions. Under non-reducing conditions, non-covalently linked protein complexes were dispersed and migrated into the resolving gel, but covalently-linked complexes remained at the top of the stacking and resolving gels or only partially entered the gels. Large quantities of these covalently-linked complexes can be seen in all milks. Four bands (labelled A, B, C and D), ranging from 60 to 150 kDa, were observed on the non-reducing SDS-PAGE (Fig. 4.2). These bands had very low intensity compared to the protein bands in this MW range on the SDS-PAGE under reducing conditions (Fig. 4.1). The intensity of PAS 6 and 7 remained somewhat similar to that observed in the reducing SDS-PAGE (compare Fig. 4.2 with Fig. 4.1A).

The polypeptide complexes observed in SDS-PAGE under non-reducing conditions were further characterized using 2D SDS-PAGE. An excised gel lane of polypeptide patterns of MFGM under non-reducing conditions was immersed in warm (95°C) solution of β-mercaptoethanol in SDS sample buffer, and used as the sample for the second SDS dimension (Fig. 4.3). High molecular weight aggregates that remained at the top of the stacking gel were resolved into faint spots in the second dimension, corresponding to xanthine oxidase, PAS III and butyrophilin. Material caught at the top of resolving gels resulted in intense spots corresponding to xanthine oxidase and butyrophilin and a faint spot with the same mobility as PAS 6 (50 kDa). It was observed that bands A, B, C and D in the first dimension (non-reducing condition) resolved to give the spots corresponding to xanthine oxidase, PAS III, PAS IV and butyrophilin in the second dimension (reducing condition), respectively. Most of PAS 6 entered the

second dimension (reducing condition) from the original location in the first dimension (non-reducing). In addition, a faint spot corresponding to the 15 kDa polypeptide (arrow) resolved from around 25 kDa (location of GPT binding proteins) was observed on second dimension (reducing). Similar patterns on 2D SDS-PAGE were observed with the early and late season milk samples.

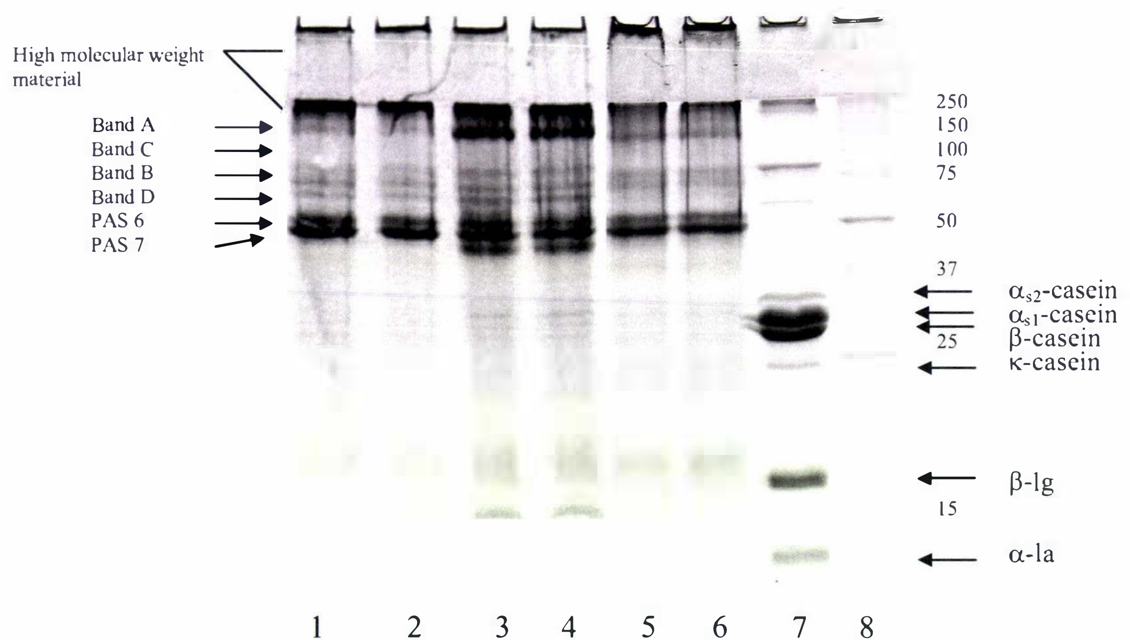


Fig. 4.2. SDS-PAGE patterns (15% acrylamide gel), obtained under non-reducing conditions, of MFGM material from fresh whole milk. Lane 1, 2, MFGM from mid season milk; lane 3, 4, MFGM from early season milk; lane 5, 6, MFGM from late season milk; lane 7, whole milk protein; lane 8, standard proteins.

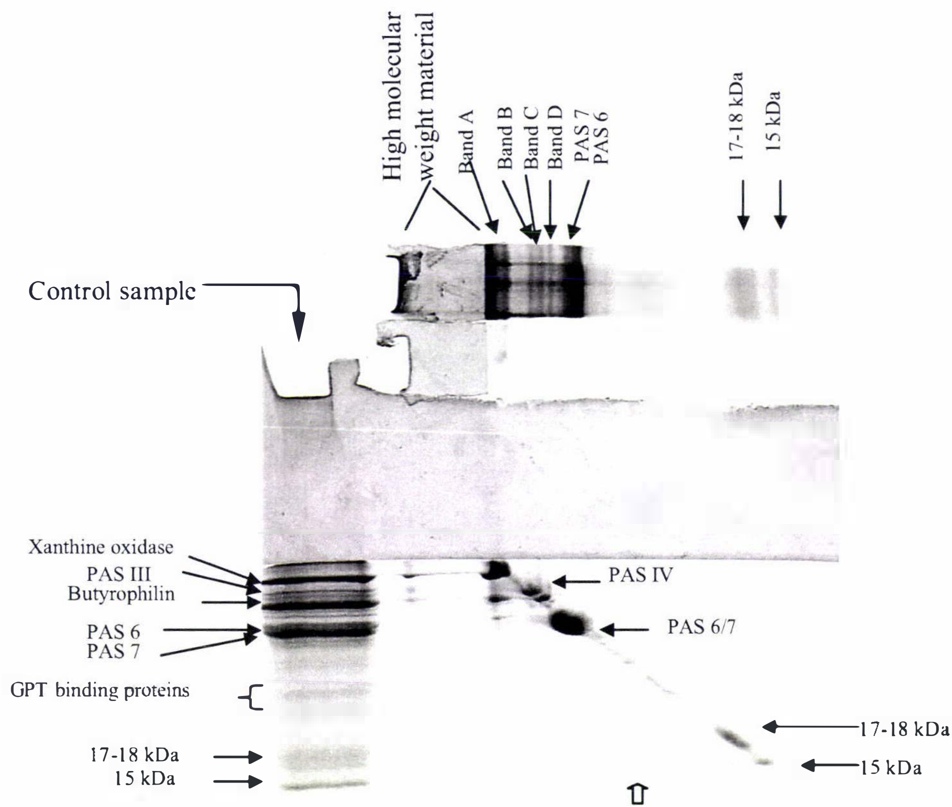


Fig. 4.3. Two dimensional SDS-PAGE patterns (15% acrylamide gel) of MFGM material from mid season whole milk. First (horizontal) dimension: SDS-PAGE run under non-reducing conditions; second (vertical) dimension: SDS-PAGE run under reducing conditions. Membrane polypeptides are named according to Mather (2000).

Previous studies (Valivullah & Keenan, 1989; Mondy & Keenan, 1993; Mather, 2000) indicated that xanthine oxidase and butyrophilin associate in a supermolecular complex, which may involve intermolecular disulfide bonds. Other proteins that may also interact with butyrophilin include the phospholipid-binding protein, MFG-E8 (PAS 6/7), and an unidentified protein of Mr 50.5 kDa. In this study, the possible disulfide-bond cross-linking in MFGM proteins was examined by 2D SDS-PAGE. The results clearly show that xanthine oxidase and butyrophilin were resolved from the high molecular weight material that either remained at the top or had just migrated into the resolving gel, suggesting that xanthine oxidase and butyrophilin are cross-linked through intermolecular disulfide bonds. However, small proportions of xanthine oxidase and butyrophilin could be resolved under non-reducing conditions (corresponding to bands A and D), indicating that some xanthine oxidase and butyrophilin exist in the monomeric state in the MFGM. Besides xanthine oxidase and butyrophilin, the material that was cross-linked through disulfide bonds also involve PAS III and a small proportion of PAS 6. The results indicate that the majority of PAS 6 and all PAS 7 are not involved in the formation of high molecular weight complexes through disulfide bond formation. However, the present study only examined the cross-linking between MFGM proteins via disulfide bonds, whether they associate together via other forces such as hydrophobic or ionic interactions remains unclear.

4.2.2. Characterization of the MFGM proteins from heated washed cream

Cream samples separated from fresh milk samples were washed and heated and then the MFGM proteins were isolated. The polypeptide patterns of heated washed cream from mid season milk, determined by SDS-PAGE under reducing conditions, are shown in Fig. 4.4. Heat treatment at 50 °C for 10 min resulted in the loss of ~50% of total protein from the MFGM, as estimated by the band intensities on the gel. It is not clear why this relatively mild heat treatment resulted in the loss of MFGM proteins; however, this effect was seen even at 45 °C. Perhaps, the melting of lipid phase at > 40 °C results in rearragement of the fat globule surface, with the loss of some MFGM protein components. Despite the loss of proteins, all polypeptides (including minor polypeptides) were present in the heat-treated MFGM in similar proportions as in the

natural MFGM polypeptides (control sample). There was some high molecular weight protein material, which could not be dissociated by β-mercaptoethanol, on top of the stacking gel as the sample was heated at temperature >70 °C for 10 min and the amount of this material increased slightly with an increase in temperature (Fig. 4.4).

Fig. 4.5 shows the polypeptide patterns of heated washed cream, as determined by SDS-PAGE under non-reducing conditions. It was observed that the material that remained on the top or had just migrated into the resolving gel (Band X), which was identified to contain largely disulfide-linked aggregates of xanthine oxidase and butyrophilin (Fig. 4.3), decreased in intensity with heat treatment. There were corresponding increases in the high molecular weight material at the top of stacking gels. These results suggest further polymerization of these proteins as a result of heat treatment. The intensities of bands A and B decreased markedly as the temperature was increased to ≥ 60 °C and disappeared from non-reducing PAGE at 70 °C. Bands C and D disappeared at 60°C. However, PAS 6/7 were relatively stable to heating, although the bands reduced in intensity at 80 °C. Fig. 4.6, in which the washed cream samples were heated at 70 °C for 1 to 40 min, showed that PAS 6/7 were stable at this temperature even after heating for 40 minutes. Generally, similar results of polypeptides patterns were found when washed cream from early or late season milk was heated (results not shown).

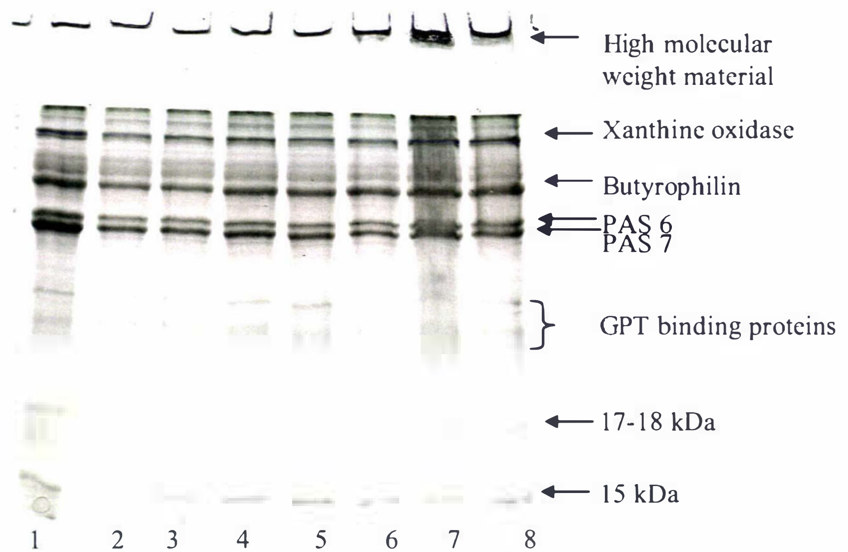


Fig. 4.4. SDS-PAGE patterns (15% acrylamide gel), obtained under reducing conditions, of MFGM material from heated washed cream obtained from mid season whole milk. Lane 1: control (un-heated cream); lane 2–8, membrane material from washed cream heated at 50 to 80 °C, for 10 min, (2) 50 °C, (3) 55 °C, (4) 60 °C, (5) 65 °C, (6) 70 °C, (7) 75 °C or (8) 80 °C. Membrane polypeptides are named according to Mather (2000).

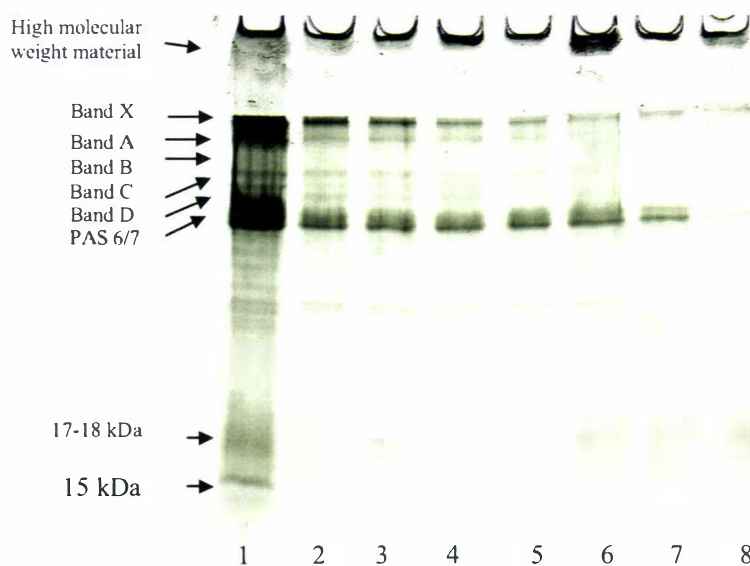


Fig. 4.5. SDS-PAGE patterns (15% acrylamide gel), obtained under non-reducing conditions, of MFGM material from heated washed cream obtained from mid season whole milk. Lane 1: control (unheated cream); lane 2 – 8, membrane material from washed cream heated at 50 to 80 °C, for 10 min, (2) 50 °C, (3) 55 °C, (4) 60 °C, (5) 65 °C, (6) 70 °C, (7) 75 °C, (8) 80 °C.

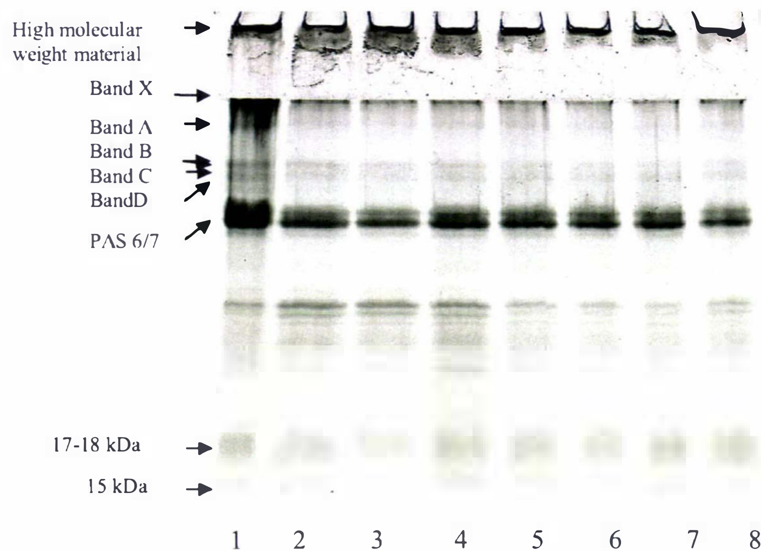


Fig. 4.6. SDS-PAGE patterns (15% acrylamide gel), obtained under non-reducing conditions, of MFGM material from heated washed cream obtained from mid season whole milk. Lane 1: control (unheated cream); lane 2–8, membrane material from washed cream heated at 70 °C, for (2) 1 min, (3) 2.5 min, (4) 5 min, (5) 10 min, (6) 20 min, (7) 30 min or (8) 40 min.

Fig. 4.7 shows two-dimensional SDS-PAGE of the MFGM polypeptides from washed cream heated at 80 °C for 10 min. The high molecular weight protein material that remained at the top of the stacking gel in the first dimension (non-reducing conditions), was resolved into individual polypeptide patterns present on second dimension (reducing condition) (arrow). In this lane, the bands of almost all MFGM polypeptides, including xanthine oxidase, butyrophilin, PAS 6/7 (band 15, 16), GTP-binding proteins, 17-18 kDa and 15 kDa polypeptides were involved (Fig. 4.7 arrow). The spots corresponding to xanthine oxidase, butyrophilin and PAS 6/7 were more intense than the other protein spots. The amount of protein at the top of resolving gel was relatively small. Some of PAS 6/7 migrated into the resolving gel of first dimension, which showed as an intense spot on the second dimension. Although some of the 17-18 kDa and 15 kDa polypeptides were resolved from the high molecular weight material at top of stacking gel of first dimension, more intense spots were from the bands that migrated into the resolving gel of first dimension. In a similar manner to the two dimensional PAGE of the unheated sample (Fig. 4.3), a spot of 15 kDa polypeptide was resolved from the ~25 kDa band of the first dimension (arrow).

The results indicate that the main polypeptides in the MFGM formed aggregates via intermolecular disulfide bonds after heating. In the absence of serum protein, the MFGM polypeptides may form aggregates among molecules of the same or different proteins of the MFGM. The temperature that caused xanthine oxidise and butyrophilin to aggregate was lower (starting from 60 °C, 10 min) (Fig. 4.5) when compared with PAS 6 and 7, which began to aggregate at 80 °C. Appell et al. (1982) reported that butyrophilin denatured thermally and formed intermolecular disulfide bridges at 58 °C. It is obvious that the temperatures at which xanthine oxidase and butyrophilin aggregate are lower than the denaturation temperature of β-lg or α-la. In milk systems, it has been found that MFGM-β-lg or MFGM-α-la complexes are formed through disulfide bonds when the milk was heated (Dalgleish & Banks, 1991; Houlihan et al., 1992; Kim & Jimenz-Flores, 1995). It is suggested that xanthine oxidase and butyrophilin would have already aggregated before they could react with β-lg or α-la.

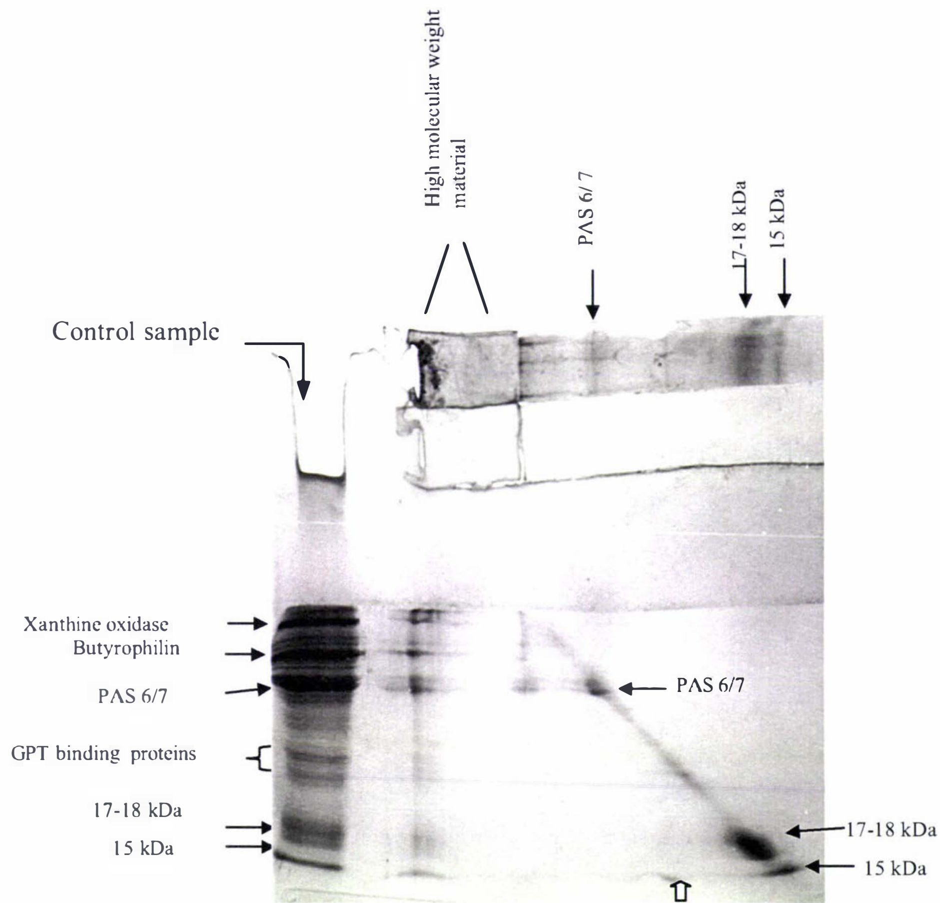


Fig. 4.7 Two dimensional SDS PAGE patterns of MFGM material from heated (80 °C, 10 min) washed cream obtained from mid season whole milk. First (horizontal) dimension: SDS-PAGE run under non-reducing conditions; second (vertical) dimension: SDS-PAGE run under reducing conditions. Membrane polypeptides are named according to Mather (2000).

CHAPTER 5
INTERACTIONS OF WHEY PROTEINS WITH MILK FAT GLOBULE MEMBRANE PROTEINS DURING HEAT TREATMENT OF WHOLE MILK

5.1. Introduction

Heat treatment of milk causes a number of changes in MFGM proteins, which include some denaturation and interactions with serum proteins via sulfydryl–disulfide interchange reactions (Dalgleish & Banks, 1991; Houlihan et al., 1992; Sharma & Dalgleish, 1993; Kim & Jimenez-Flores, 1995). These studies showed that both β-lg and α-la bind to the fat globule when whole milk is heated. However, the mechanism by which these proteins interact with the fat globule is not clear. They may bind to the MFGM via sulfydryl–disulfide interactions (Houlihan et al., 1992). Alternatively, the whey proteins may displace the original membrane material, either by directly competing or because MFGM may break down during heating, leaving gaps through which the whey proteins (which may or may not be denatured at that stage) may adsorb to the newly exposed fat surface (Dalgleish & Banks, 1991). Kim and Jimenez-Flores (1995) also considered that the direct disulfide bonding between whey proteins and MFGM proteins could not completely explain their results and the precise nature of these interactions is not clear.

Houlihan et al. (1992) reported that the quantities of β-lg and α-la on the MFGM increased, whereas the amounts of phospholipids and triacylglycerols decreased, with an increase in the heating time (from 2.5 to 20 min) at 80 °C. α-La was present at low levels in all heated samples. Low levels of κ-casein were also present and this component increased during heating. These results suggest that the MFGM is involved in heat-induced interactions with skim milk components, in particular β-lg and κ-casein, and that the levels of incorporation of these components into the membrane are dependent on the extent of the heat treatments. κ-Casein may interact directly with MFGM components, and may interact with β-lg through sulfydryl–disulfide interchange during heating.

Houlihan et al. (1992) also found that xanthine oxidase, PAS 6 (Band 15) and PAS 7 (Band 16 or 49 kDa) decreased in intensity in the MFGM isolated from milk that was heated at 80 °C. The authors suggested that PAS 6 and PAS 7 might be displaced from the membrane by whey proteins during heating. In contrast, Kim and Jimenez-Flores (1995) suggested that the 49 kDa protein may have been cleaved into smaller molecular weight proteins after heat treatment.

Corredig and Dalgleish (1996) found no significant difference in the amount of β-lg and α-la bound with the fat globules as a function of heating time at 65 and 75 °C in batch heating of milk in water baths. However, at 85 °C the binding of β-lg and α-la increased with heating time. Few studies have determined the kinetics of heat-induced association of whey proteins with fat globules in whole milk. Sharma and Dalgleish (1994) found that the kinetics of β-lg and α-la incorporation into the fat globule membrane fitted a pseudo-first order kinetic equation based on a site filling model. However, their kinetic parameters were limited to only 70 and 75 °C. In addition, the samples were homogenised prior to or after heating, and this would have caused the adsorption of some β-lg and α-la to the interface rather than heating alone. Kinetic parameters for β-lg and α-la association with MFGM during heat treatment of unhomogenised whole milk at a wide temperature range have not been previously reported.

In this chapter, the interactions between milk serum proteins and MFGM proteins, during the heating of whole milk samples, obtained at different times during the New Zealand dairying season have been characterized. SDS-PAGE, under non-reducing and reducing conditions, and two-dimensional SDS-PAGE were used, with a view to understanding the mechanism of binding of whey proteins to fat globules. This chapter also describes the heat-induced reaction kinetics of β-lg and α-la association with the MFGM in whole milks collected on three separate occasions in the New Zealand dairying season. These milks were heated in a UHT plant over a range of temperature/time combinations (65-95 °C, 0 to 40 min).

5.2. Interactions of milk serum proteins with milk fat globule membrane proteins during heat treatment of whole milk

5.2.1. Results

An initial experiment showed that, when the milk was passed through the UHT plant at room temperature, there was no effect on the MFGM protein composition and fat globule size (results not shown). This indicates that the flow conditions in the UHT plant did not damage the milk fat globules and that the MFGM remained intact. Consequently, any change in the composition of the MFGM was due to other factors, such as heating rather than flow conditions in the UHT plant.

The proteins of the MFGM in washed cream, obtained from mid-season whole milk, that was heated at 60–95 °C for 10 min were resolved by SDS-PAGE under both reducing and non-reducing conditions. The results are shown in Figs 5.1A and 5.1B, respectively. Similarly, the SDS-PAGE protein patterns of MFGM material isolated from milk heated at 70 °C for 2.5–60 min are shown in Fig. 5.2 and those of MFGM material isolated from milk heated at 65 °C for 10–40 min are shown in Fig. 5.3.

No bands corresponding to β-lg, α-la or casein proteins were observed in unheated milk samples (Figs 5.1, 5.2 and 5.3), indicating that serum proteins were not involved in the membrane. In SDS-PAGE under reducing conditions, the β-lg band was observed in MFGM material isolated from milk heated at ≥ 60 °C for 10 min; the intensity of this band increased with an increase in heating temperature up to 75°C and then remained almost constant at higher temperatures (Fig. 5.1A). The β-lg band was also found in milks heated at 70 °C for ≥ 2.5 min or 65 °C for ≥ 10 min; under these conditions, the intensity of the β-lg band increased with an increase in heating time (Figs. 5.2A and 5.3A).

The α-la band was also observed in the MFGM material isolated from milk that was heated at ≥ 65 °C for 10 min (Figs 5.1A and 5.3A), whereas the κ-casein band was observed in milks heated at ≥ 75 °C (Fig. 5.1A). In both cases, the bands were faint and fuzzy compared with the β-lg band.

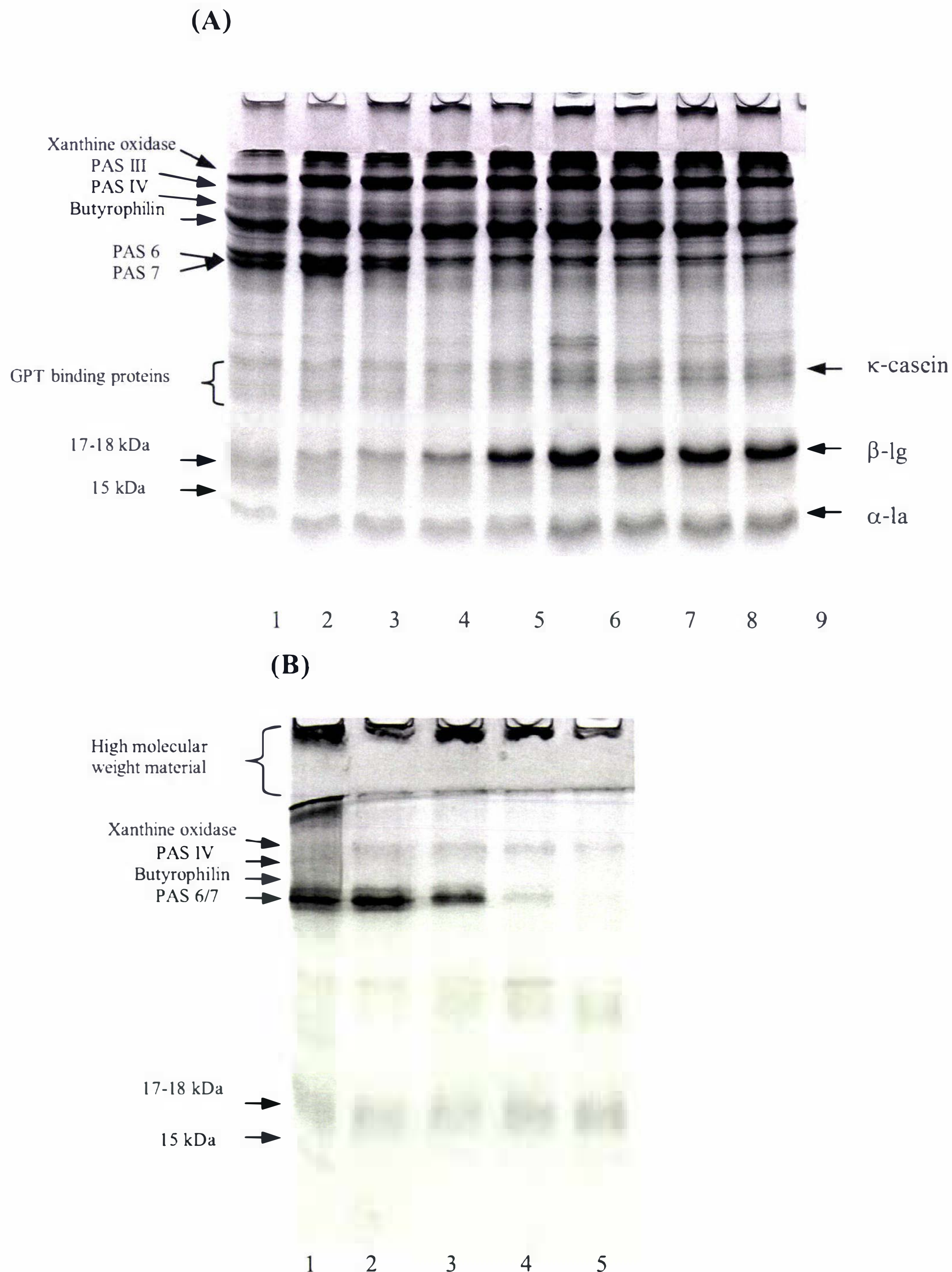


Fig. 5.1. SDS-PAGE patterns (15% acrylamide gel) of membrane material isolated from heated milk under reducing conditions (A) and non-reducing conditions (B). Cream obtained from the heated milks was washed in SMUF containing urea and EDTA. Lane 1: membrane material from unheated milk (control). Lanes 2–9: membrane material from milk heated at 60--95 °C, for 10 min. (2) 60 °C, (3) 65 °C, (4) 70 °C, (5) 75 °C, (6) 80 °C, (7) 85 °C, (8) 90 °C and (9) 95 °C. Membrane polypeptides are named according to Mather (2000).

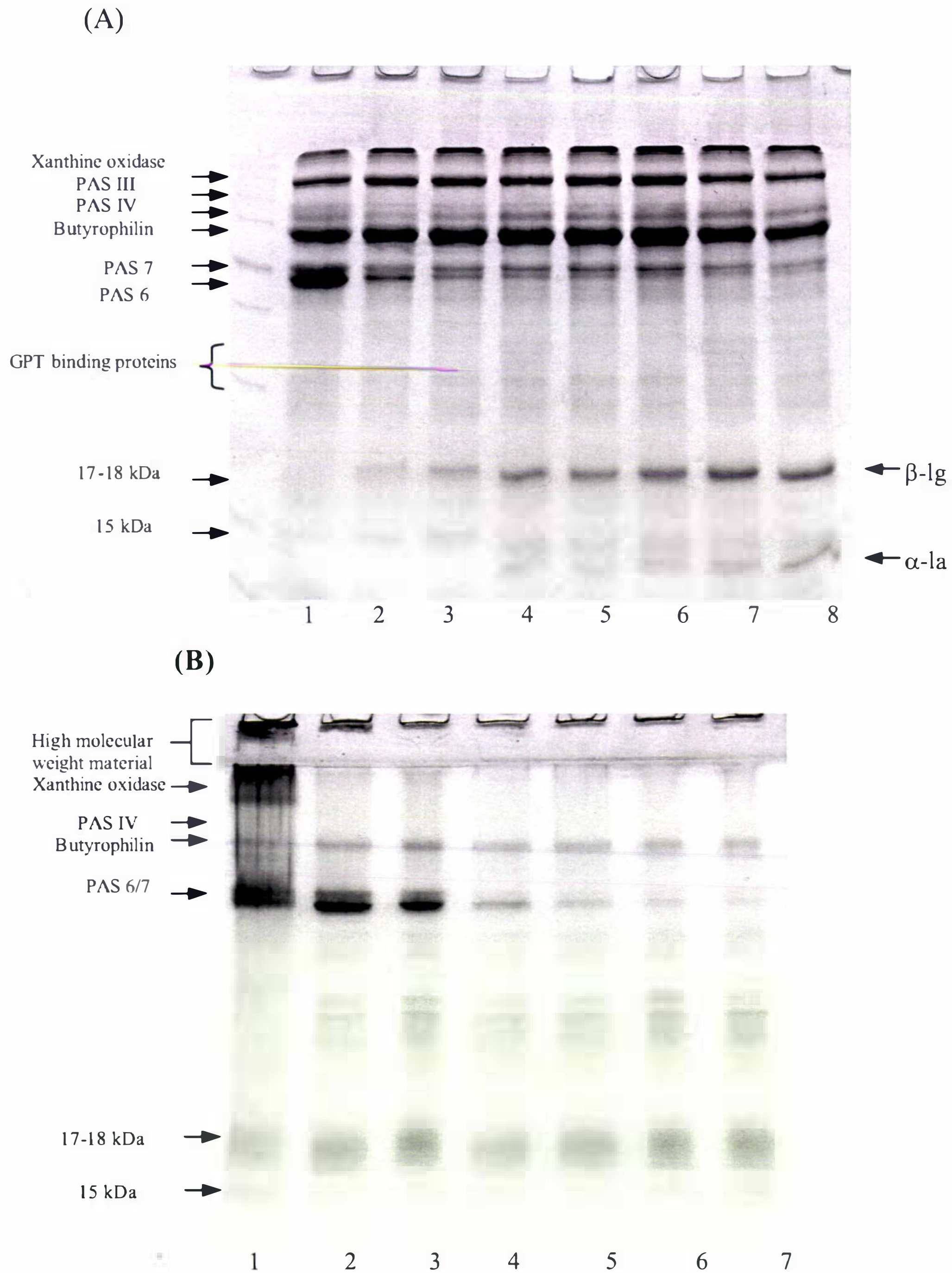


Fig. 5.2. SDS-PAGE patterns (15% acrylamide gel) of membrane material isolated from heated milk under reducing conditions (A) and non-reducing conditions (B). Cream obtained from the heated milks was washed in SMUF containing urea and EDTA. Lane 1: membrane material from unheated milk (control). Lanes 2–8: membrane material from milk heated at 70 °C, for (2) 2.5 min, (3) 5 min, (4) 10 min, (5) 20 min, (6) 30 min, (7) 40 min and (8) 60 min. Membrane polypeptides are named according to Mather (2000).

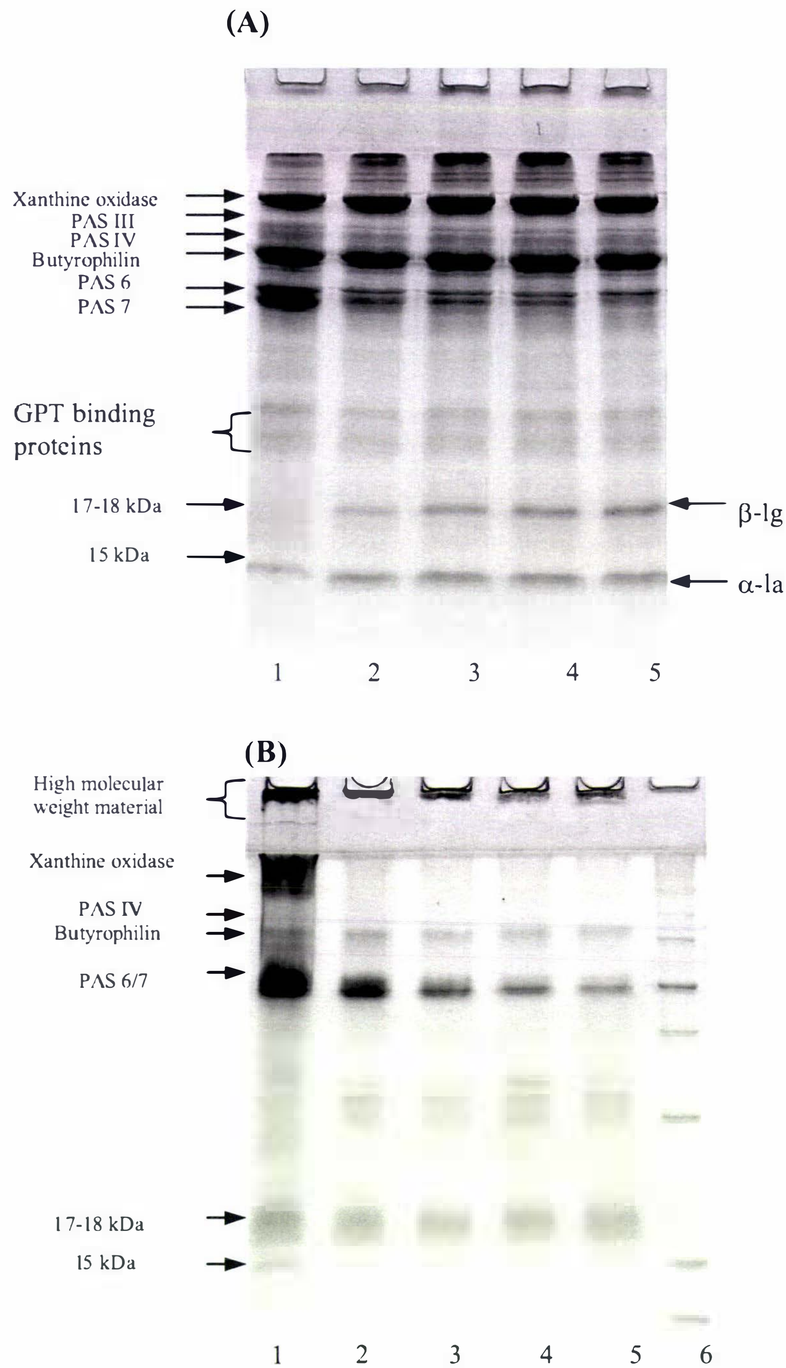


Fig. 5.3. SDS-PAGE patterns (15% acrylamide gel) of membrane material isolated from heated milk under reducing conditions (A) and non-reducing conditions (B). Cream obtained from the heated milks was washed in SMUF containing urea and EDTA. Lane 1: membrane material from unheated milk (control). Lanes 2–5, membrane material from milk heated at 65 °C, for (2) 10 min, (3) 20 min, (4) 30 min and (5) 40 min. Membrane polypeptides are named according to Mather (2000).

Table 5.1 shows that the maximum amounts (obtained by quantitative PAGE) of β-lg and α-la associated with the MFGM in heated mid-season milk were ~ 0.9 mg/g fat and ~ 0.23 mg/g fat, respectively. These saturating β-lg and α-la values were ~ 15% and ~ 3% of the total MFGM proteins. However, this represents only ~ 1% and ~ 0.8% of the total β-lg and α-la in milk (calculated from 0.4% β-lg and 0.16% α-la in 4.5% fat whole milk). The associations of β-lg and α-la with the MFGM in early-season heated milk (~ 1.1 mg/g fat and ~ 0.25 mg/g fat) were slightly higher than those in mid-season milk, whereas the amounts of β-lg and α-la associated with the MFGM in late-season heated milk were intermediate between those in early- and mid-season milks (Table 5.1). For milks heated at 70 °C for various times (Table 5.2), the amounts of β-lg and α-la associated with the MFGM increased with an increase in heating time; larger amounts of β-lg and α-la were associated with the MFGM in the early-season milk compared with the mid- and late-season milks.

A number of authors (Dalgleish & Banks, 1991; Houlihan et al., 1992; Kim & Jimenez-Flores, 1995) have reported the presence of β-lg in the MFGM of heated milk. In studies where quantitative data were obtained (Sharma & Dalgleish, 1993; Corredig & Dalgleish, 1996), the maximum amounts of β-lg associated with the MFGM of heated milk were ~ 0.7 mg/g fat. This value is similar to the results obtained in the present study.

In the case of α-la, the findings of previous studies are contradictory. On the one hand, Corredig and Dalgleish (1996) found a maximum amount for α-la in the MFGM of heated milk of 0.22 mg/g fat. On the other hand, Dalgleish and Banks (1991) did not detect α-la in fat globule membrane preparations from milks that had been heated at 80 °C and, although Houlihan et al. (1992) reported a low level of α-la in all heated samples, they considered that it was an artifact.

Table 5.1. Amounts of β-lg and α-la (mg/g fat) in the fat globule membrane material isolated from early-, mid- and late-season whole milks* that were heated at different temperatures for 10 min.

Temperature	Early-season milk (n = 2)		Mid-season milk (n = 3)		Late-season milk (n = 2)	
(°C)	β-lg	α-la	β-lg	α-la	β-lg	α-la
65	0.10 ± 0.06[#a*]		0.10 ± 0.04[a]		0.10 ± 0.07[a]	
70	0.23 ± 0.05[b]		0.20 ± 0.05[b]		0.21 ± 0.04[b]	
75	0.60 ± 0.04[c]	0.15 ± 0.04[a]	0.39 ± 0.05[f]	0.08 ± 0.03[a]	0.60 ± 0.04[c]	0.10 ± 0.06[a]
80	0.98 ± 0.07[e]	0.20 ± 0.05[c]	0.75 ± 0.08[d]	0.16 ± 0.04[b]	0.81 ± 0.08[d]	0.16 ± 0.04[b]
85	1.05 ± 0.10[e]	0.22 ± 0.02[c]	0.87 ± 0.08[e]	0.20 ± 0.04[c]	0.88 ± 0.10[e]	0.18 ± 0.05[c]
90	1.12 ± 0.08[e]	0.23 ± 0.05[c]	0.90 ± 0.05[e]	0.21 ± 0.03[c]	1.00 ± 0.06[e]	0.25 ± 0.04[c]
95	1.10 ± 0.10[e]	0.25 ± 0.04[c]	0.90 ± 0.04[e]	0.23 ± 0.02[c]	0.98 ± 0.08[e]	0.25 ± 0.04[c]

* Early-, mid- and late-season milks were collected in August, January to March and May, respectively.

[#] Data are means ± sample standard deviations.

* Different superscripts within a row or a column indicate significant differences ($P < 0.05$).

Table 5.2. Amounts of β-lg and α-la (mg/g fat) in the fat globule membrane material isolated from early-, mid- and late-season whole milks* that were heated at 70 °C for different times.

Time (min)	Early-season milk (n = 2)		Mid-season milk (n = 4)		Late-season milk (n = 2)	
	β-lg	α-la	β-lg	α-la	β-lg	α-la
2.5	0.15 ± 0.02[#a*]		0.13 ± 0.04[a]		0.16 ± 0.04[a]	
5	0.18 ± 0.05[b]		0.15 ± 0.02[b]		0.18 ± 0.03[b]	
10	0.23 ± 0.04[c]		0.20 ± 0.03[c]		0.21 ± 0.05[c]	
15	0.37 ± 0.04[d]	0.06 ± 0.02[a]	0.26 ± 0.06[d]	0.06 ± 0.01[a]	0.29 ± 0.06[d]	0.03 ± 0.06[a]
20	0.43 ± 0.05[f]	0.10 ± 0.02[b]	0.32 ± 0.05[e]	0.07 ± 0.01[b]	0.35 ± 0.08[e]	0.06 ± 0.05[b]
30	0.52 ± 0.06[e]	0.12 ± 0.01[b]	0.41 ± 0.06[f]	0.07 ± 0.02[b]	0.40 ± 0.08[f]	0.09 ± 0.05[b]
40	0.67 ± 0.06[h]	0.14 ± 0.03[b]	0.56 ± 0.08[g]	0.10 ± 0.01[b]	0.58 ± 0.07[g]	0.10 ± 0.04[b]

* Early-, mid- and late-season milks were collected in August, January to March and May, respectively.

[#] Data are means ± sample standard deviations.

* Different superscripts within a row or a column indicate significant differences ($P < 0.05$).

Although the amount of κ-casein associated with the MFGM is very small, its occurrence raises the question of whether the casein micelle is present in the MFGM after heating, as κ-casein is one component of the casein micelle. Fig. 5.4 shows the SDS-PAGE protein patterns, under reducing and non-reducing conditions, of MFGM material isolated from milk heated in the range 65–95 °C for 10 min, in which the cream, obtained from the heated milk, was washed using SMUF in the absence of the dissociating agent (urea and EDTA). Under reducing conditions, in addition to the κ-casein band, α_{s1}-, α_{s2}- and β-casein bands were also observed in milk samples heated at temperatures > 80 °C. Bands of α_s- (α_{s1}- + α_{s2}-) and β-casein were also observed in SDS-PAGE under non-reducing conditions, but the κ-casein, β-lg and α-la bands disappeared (Fig. 5.4B). These results suggest that the casein micelles may be directly associated with the MFGM proteins through κ-casein. Alternatively, β-lg could associate with the MFGM via sulfydryl–disulfide interchange with the MFGM proteins, and subsequently interact with the casein micelles through κ-casein.

The intensities of xanthine oxidase, butyrophilin, GPT-binding proteins and 15 kDa polypeptide bands were not affected by heat treatment (Figs 5.1A and 5.2A). However, the intensity of PAS 7 (Band 16) band decreased markedly with increases in temperature and heating time, and this band almost disappeared from the PAGE gels after heating at > 75 °C for 10 min (Fig. 5.1A) or 70 °C for > 20 min (Fig. 5.2A). In addition, there was a slight decrease in the intensity of PAS 6 (band 15) with increases in temperature and heating time (Fig. 5.1A). Fig. 5.5 shows the change in the amounts of individual MFGM proteins in different season milks during heat treatments at 60–95 °C for 10 min. These data show the same trends for xanthine oxidase, butyrophilin, PAS 6 and PAS 7 as described above. It must be pointed out that the values reported in Fig. 5.5 are based on staining intensity, which may vary between protein species. Hence, they may not represent actual protein content, but will highlight differences between samples of the same protein and changes during processing. Houlihan et al. (1992) also found that PAS 6 and PAS 7 were lost from the MFGM of milk heated at 80 °C for 20 min; however, they observed a marked reduction in xanthine oxidase, which is in contrast to the findings of this study.

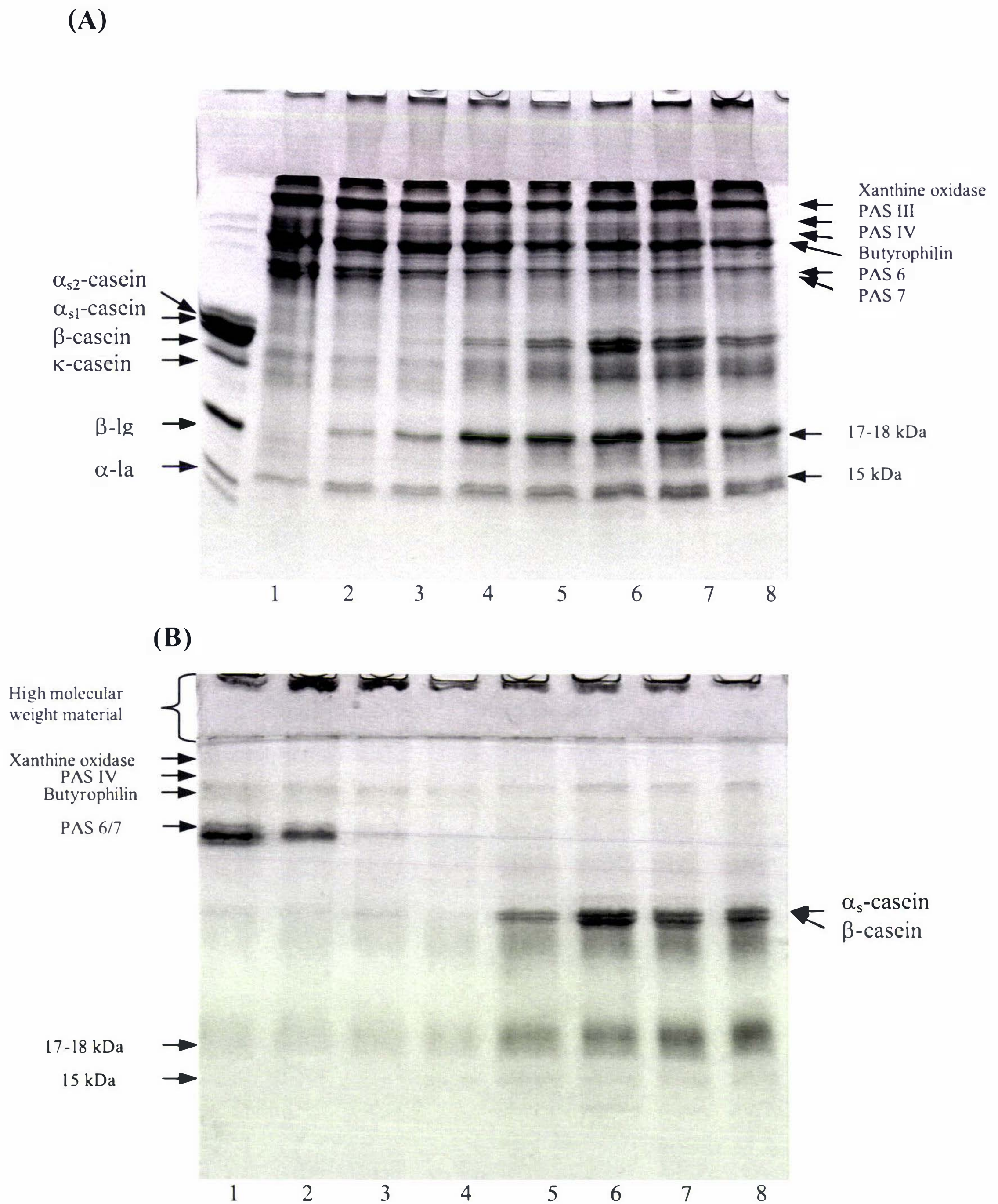


Fig. 5.4. SDS-PAGE patterns (15% acrylamide gel) of membrane material isolated from heated milk under reducing conditions (A) and non-reducing conditions (B). Cream obtained from the heated milks was washed in SMUF only. Lane 1: membrane material from unheated milk (control). Lanes 2–8, membrane material from milk heated at 65–95 °C, for 10 min. (2) 65 °C, (3) 70 °C, (4) 75 °C, (5) 80 °C, (6) 85 °C, (7) 90 °C and (8) 95 °C. Membrane polypeptides are named according to Mather (2000).

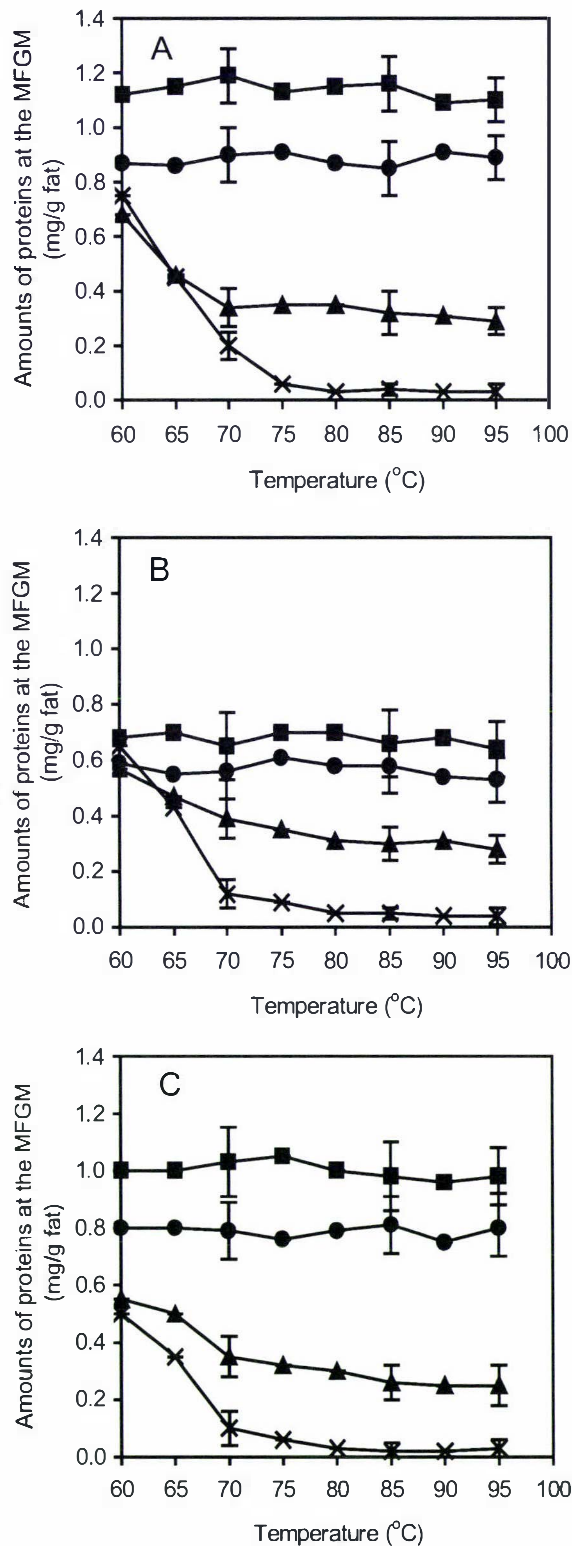


Fig. 5.5. Amounts of xanthine oxidase (●), butyrophilin (■), PAS 6 (▲) and PAS 7 (×) in the fat globule membrane material isolated from early- (A), mid- (B) and late- (C) season milks heated at 60–95 °C for 10 min.

In SDS-PAGE under non-reducing conditions, no bands were observed for β-lg, α-la and κ-casein for both the heated milk samples and the unheated control sample (Figs 5.1B, 5.2B and 5.3B). All the major proteins of the original MFGM decreased in intensity with an increase in temperature or heating time with a corresponding increase in the amounts of high molecular weight material, which remained on the top of the stacking gel. Comparison of the PAGE protein patterns under reducing (Figs 5.1A and 2A) and non-reducing (Figs 5.1B and 5.2B) conditions showed that the changes in PAS 7 band intensity were similar, in that the band intensity markedly decreased with increasing heating time at 65 °C and 70 °C, and the band almost disappeared from the PAGE gels at 75 °C for 10 min (Fig. 5.1) or 70 °C for 20 min (Fig. 5.2). This indicates that PAS 7 is removed from the MFGM before it interacts with other membrane proteins via sulfydryl-disulphide exchange reactions.

The protein complexes observed in the SDS-PAGE gels under non-reducing conditions were further characterized using two-dimensional SDS-PAGE (non-reducing, then reducing). The resultant protein patterns for MFGM material from milk heated at 65 °C for 30 min and at 80 °C for 10 min are shown in Fig. 5.6; a control sample was run on the left of the second dimension gel to assist identification of the proteins. In the milk sample heated at 65 °C for 30 min, almost all the original MFGM polypeptides (including xanthine oxidase, butyrophilin, PAS 6, PAS 7 and GPT-binding proteins) and β-lg were found in the lane resolved from the high molecular weight protein material present at the top of the stacking gel of the first dimension (Fig. 5.6). Some of the PAS 6/7 proteins, which migrated into the resolving gel in the first dimension, were observed as an intense spot in the second dimension. Although some of the 17–18 kDa and 15 kDa polypeptides were resolved from the high molecular weight material at the top of stacking gel of the first dimension, more intense spots were observed from the bands that migrated into the resolving gel of the first dimension. In the milk sample heated at 80 °C for 10 min, the results were similar to those for the sample heated at the lower temperature (65 °C for 30 min), with the exceptions of PAS 7 which was absent from the gel and β-lg which gave a more intense spot when resolved from the high molecular weight protein material at the top of the stacking gel of the first dimension (Fig. 5.7).

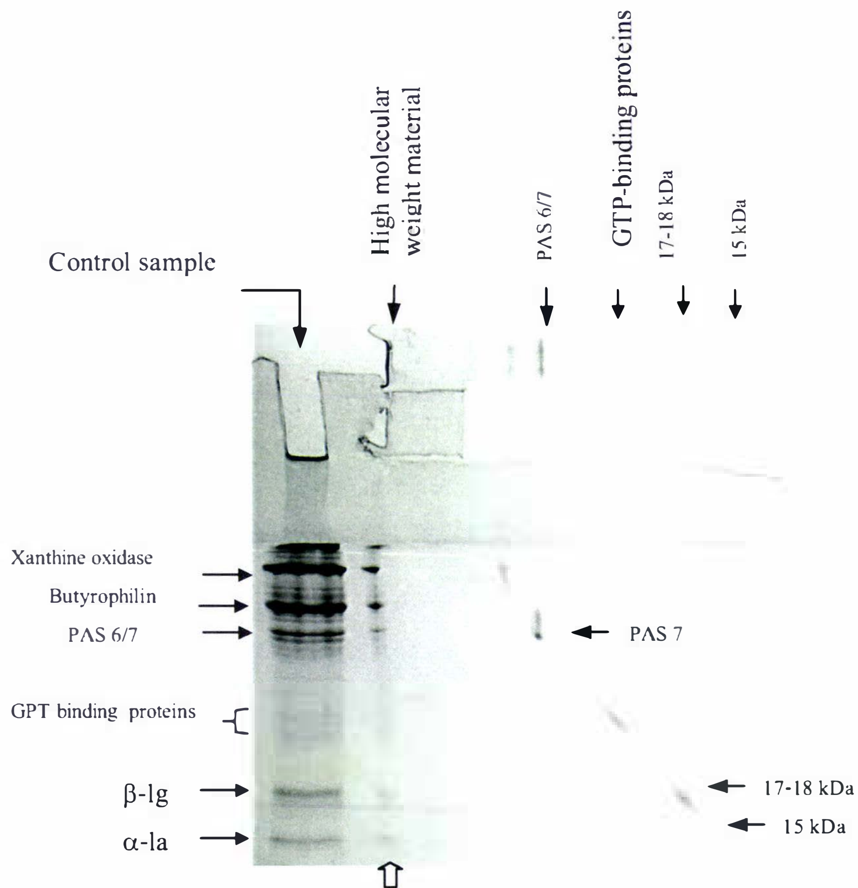


Fig. 5.6. Two-dimensional SDS-PAGE patterns (15% acrylamide gel) of membrane material isolated from milks heated at 65 °C for 30 min. Cream obtained from the heated milks was washed in SMUF containing urea and EDTA. First (horizontal) dimension: SDS-PAGE under non-reducing conditions; second (vertical) dimension: SDS-PAGE under reducing conditions. Membrane polypeptides are named according to Mather (2000).

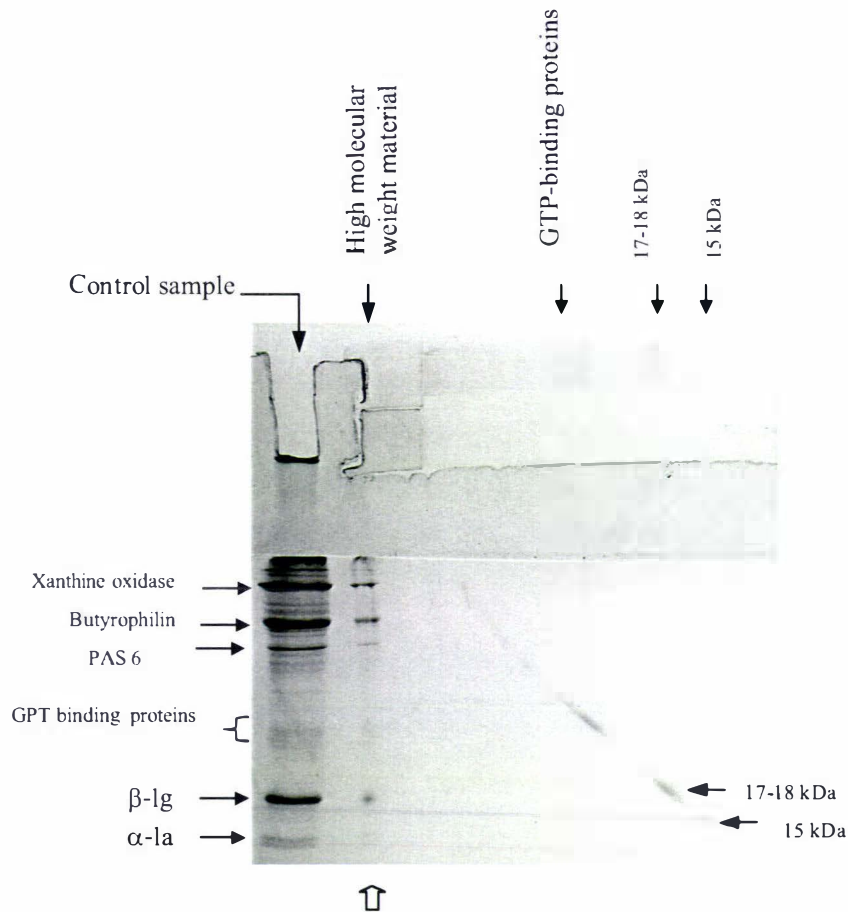


Fig. 5.7. Two-dimensional SDS-PAGE patterns (15% acrylamide gel) of membrane material isolated from milks heated at 80 °C for 10 min. Cream obtained from the heated milks was washed in SMUF containing urea and EDTA. First (horizontal) dimension: SDS-PAGE under non-reducing conditions; second (vertical) dimension: SDS-PAGE under reducing conditions. Membrane polypeptides are named according to Mather (2000).

5.2.2. Discussion

The results clearly show that β-lg was present in the MFGM of milk that was heated at relatively low temperatures, namely 60–65 °C (Figs 5.1A and 5.2A). Furthermore, two-dimensional SDS-PAGE clearly indicated that β-lg was associated with the original MFGM proteins via disulfide bonds in this temperature range (Fig. 5.6). This temperature range is lower than that required to denature this protein; the denaturation temperature of β-lg has been reported to be 78 °C, using differential scanning calorimetry (de Wit & Klarenbeek, 1984; Kinsella & Whitehead, 1989). However, it has been found that some MFGM proteins denature at low temperatures, about 60 °C (Appell, et al., 1982; Chapter 4). Corredig and Dalgleish (1998) found that iron released from the xanthine oxidase when cream was heated at ≥ 62 °C. They suggest that the release of iron would render the cysteines of the membrane protein available for disulfide exchange with other cysteine-containing proteins present in the cream. MFGM proteins contain a large amount of disulfide and sulfydryl groups (Cheng et al., 1988; Mather, 2000). For example, xanthine oxidase, one of the major MFGM proteins, contains 22 disulfide and 38 sulfydryl groups, four of which are detectable in the undenatured protein complex (Cheng et al., 1988). This may imply that the free thiol groups could be provided by the MFGM proteins, for initiating thiol–disulfide interchange between MFGM proteins and β-lg.

The above discussion suggests that the association of β-lg with the MFGM probably occurs after the native β-lg dimer has dissociated to monomers, but before the free thiol grouping of β-lg has been exposed. Dalgleish & Banks (1991) and Corredig & Dalgleish (1996) also found that the association of β-lg with the fat globules occurred at relatively low temperatures (70 °C) and was completed before all of the serum proteins had been denatured. In contrast, the findings of Houlihan et al. (1992) indicate that, when whole milk was heated to 80 °C, the serum proteins were denatured exposing the thiol groups, prior to their incorporation into membrane proteins.

The association of α-la with the MFGM proteins occurs as a result of sulfydryl exchange in a similar manner to that exhibited by β-lg. In both cases, this mechanism

operates at ≥ 65°C although much lower quantities of α-la are incorporated into the membrane compared with β-lg.

Furthermore, small amounts of κ-casein also associate with the MFGM via disulfide bonds as indicated by comparison of SDS-PAGE under reducing and non-reducing conditions. Similar results were reported by Houlihan et al. (1992), who showed that the incorporation of κ-casein into the MFGM increased as heating time at 80 °C increased. However, Dalgleish and Banks (1991) considered that the presence of casein on the surfaces of the fat globules may have been a result of inadvertent partial homogenization during the handling of the milk and they did not support the suggestion that the κ-casein either associated with the MFGM proteins or formed κ-casein/β-lg complexes on the fat globule surface. These authors observed little or no increase in κ-casein in heated milk samples even when substantial increases in β-lg occurred.

In our study, only κ-casein is observed on the SDS-PAGE of the heated MFGM material that had been washed using the micelle dissociating buffer. However, when the MFGM material is washed using the SMUF solution (i.e. no micelle dissociation), other caseins including κ-casein are observed on the SDS-PAGE. Therefore, these observations strongly suggest that the casein micelles associate with the MFGM via the disulfide bonding between κ-casein and MFGM components. In all previous work, only one kind of buffer was used in the washing procedure, which makes it impossible to conclude whether casein micelle or individual casein molecules associate with the MFGM during the heating of whole milk.

The non-reducing SDS-PAGE (Figs 5.1B and 5.2B) and two-dimensional PAGE (Fig. 5.6 and 5.7) results showed that most of the original MFGM proteins except PAS 7 (49 kDa) were involved in interactions with either serum proteins or other MFGM proteins during heating. As for other MFGM proteins, the intensity of the PAS 7 band decreased during heating from SDS-PAGE under non-reducing conditions. However, unlike the others, it also decreased and finally disappeared from the SDS-PAGE under reducing conditions, indicating that this protein migrated into the serum phase during heating. In the absence of serum proteins, PAS 7 does not associate with other MFGM proteins via

disulfide bonds upon heating, but, interestingly, is more heat stable than the other MFGM proteins (Chapter 4). A possible explanation for this difference in behaviour is that the presence of serum proteins, particularly β-lg, results in the formation of β-lg/MFGM complexes, which may alter the membrane structure and environment, resulting in the removal of PAS 7. An alternative suggestion is that PAS 7 could interact directly with β-lg to form β-lg/PAS 7 complexes, which then move from the MFGM to the serum phase. Houlihan et al. (1992) also observed that the loss of components 15 and 16 (PAS 6/7) from membrane material in heated milk was related to the presence of skim milk components during heating. Kim and Jimenez-Flores (1995) also found that this band (PAS 7) decreased in intensity during the heating of milk. However, these authors interpreted this decrease as a breakdown in the structure of this protein with the protein remaining within the MFGM.

The slightly higher amounts of β-lg associated with the MFGM in heated early-season milk (Table 5.1) can probably be attributed to the higher concentrations of total MFGM proteins in early-season milk, as reported in Chapter 4, which might provide more reaction sites for association with β-lg. An alternative explanation is that the higher concentrations of β-lg in early-season milk (Sanderson, 1970) may influence the association of β-lg with the MFGM. However, the amount of β-lg associated with the MFGM in heated milk is only ~ 1% of the total β-lg in milk, and hence it is doubtful that small changes in the concentration of β-lg due to seasonal variation would influence the amount that associates with the MFGM proteins.

The results of this study confirm that heat treatment of whole milk causes the association of whey proteins (β-lg and α-la) with the MFGM. These associations commence at relatively low temperatures (60–65 °C), and increase with increasing temperature and heating time. The maximum amounts of whey proteins associating with the MFGM are small, only 1% and 0.8% of the total β-lg and α-la in the serum, respectively. Most of the original MFGM proteins (e.g. xanthine oxidase and butyrophilin) are not lost from the membrane during heat treatment. However, PAS 7 is heat labile and PAS 6 also declines during heating. This work identifies the heating temperature and time that initially causes the removal of PAS 6/7 from the MFGM. This

temperature is probably a significant index of knowing the intensity of the heat treatment of the whole milk.

5.3. Kinetics of heat-induced association of β-lg and α-la with milk fat globule membrane in whole milk

5.3.1. Results

The amount of β-lg associated with the MFGM of early, mid and late season whole milk after heating at different temperatures as a function of time (min) is shown in Fig. 5.8. In most cases, the values reported are mean values from independent duplicate or triplicate experiments. On heating at < 80 °C, the amount of β-lg associated with the MFGM increased with an increase in temperature and heating time. At temperatures greater than 80 °C, the amount of β-lg associated with the MFGM increased rapidly in the first few minutes to a maximum after which the level remained constant. There were no significant differences in the maximum amount of β-lg associated with MFGM between early, mid and late season heated milks.

The amounts of α-la associated with the MFGM of early, mid and late season milks after heat treatment at different temperature as a function of time (min) are shown in Fig. 5.9. The amount of α-la associated with the MFGM was dependent on both heating time and temperature. Samples heated at ≥ 80 °C for extended time reached a maximum value of ~0.25 ± 0.026 mg α-la/g fat.

The different amounts of β-lg or α-la association with the MFGM were observed in the samples heated at different temperature without holding time (Figs. 5.8 and 5.9). This association amounts of β-lg or α-la may be as a result of heat-induced interactions during the period of the heating samples to required temperature and cooling to room temperature.

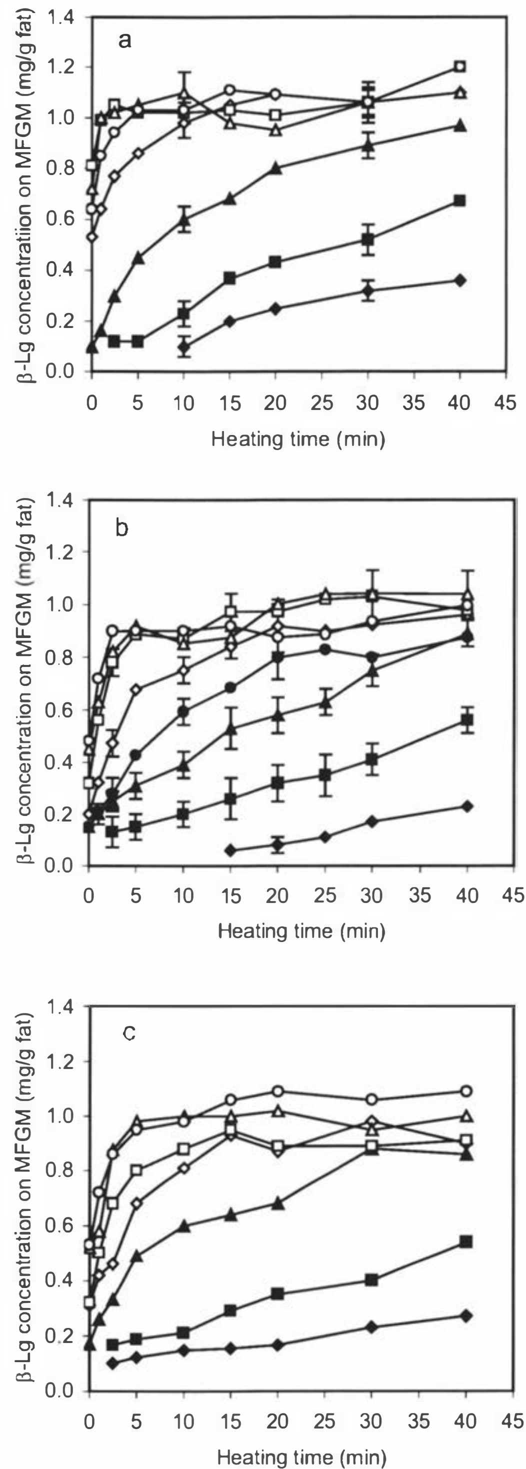


Fig. 5.8. Change in the amount of β-lg associated with the MFGM in early- (a), mid- (b) and late- (c) season whole milks heated at 65 °C (◆), 70 °C (■), 75 °C (▲), 78 °C (●), 80 °C (◇), 85 °C (□), 90 °C (△) and 95 °C (○) for 0-40 min. Bars indicate standard errors.

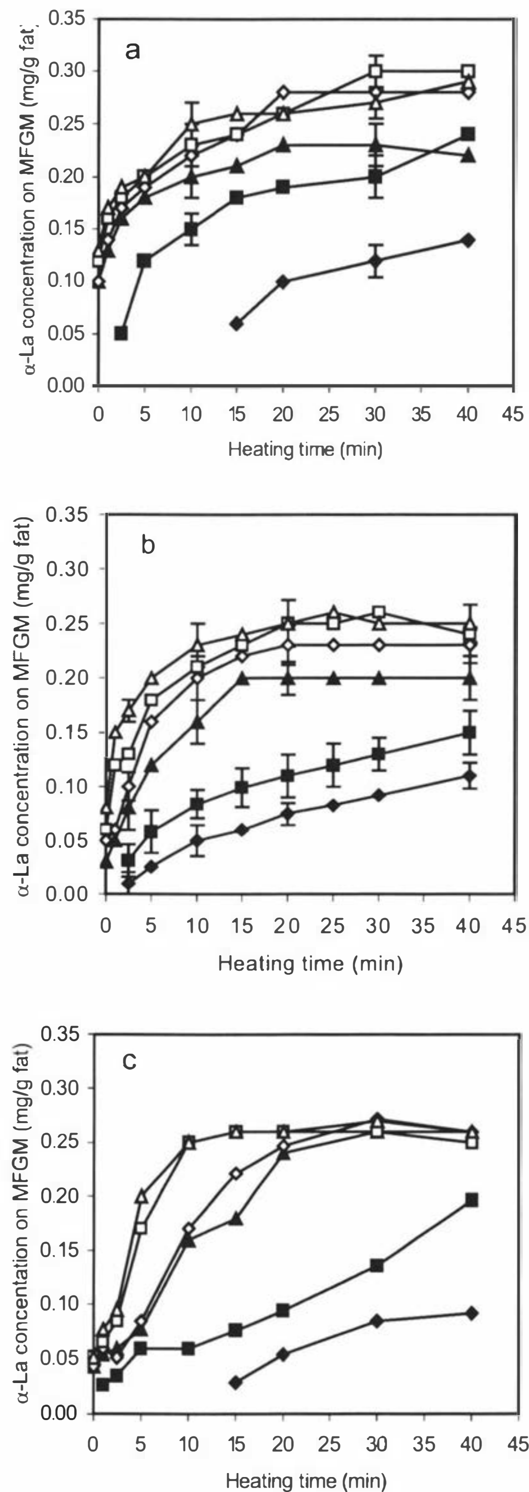


Fig. 5.9. Change in the amount of α-la associated with the MFGM in early- (a), mid- (b) and late- (c) season milks heated at 70 °C (◆), 75 °C (■), 80 °C (▲), 85 °C (◇), 90 °C (□), 95 °C (△) for 0-40 min. Bars indicate standard errors.

5.3.2. Order of reaction and kinetic parameters

Figs. 5.8 and 5.9 showed that the association of β-lg and α-la with the MFGM would arrive at a maximum value, the data before reaching the maximum were used to calculate the rate constant and reaction order. It was assumed that C_{max} (mg/g fat) was the maximum amount of β-lg or α-la that could associate with the MFGM, and C_t (mg/g fat) was the amount of the β-lg or α-la at the MFGM at a given heating time. A site filling model (Sharma and Dalgleish, 1994) (equations 5.1 and 5.2) was used to calculate the kinetic parameters:

$$\left[\left(\frac{C_{\max} - C_t}{C_{\max}}\right)\right]^{1-n} = 1 + (n-1)k_n C_{\max}{}^{n-1} t = 1 + (n-1)kt \qquad (5.1)$$

for $n \neq 1$,

$$\ln\left[\frac{(C_{\max} - C_t)}{C_{\max}}\right] = -k_1 t \qquad (5.2)$$

for $n = 1$.

Where k is the apparent reaction rate constant for β-lg and α-la association with MFGM ($(mg/g\ fat)^{(1-n)}\ s^{-1}$), t is the heating time (*s*), k_1 is the first order rate constant (s^{-1}), and n is the reaction order.

Linear relationships were obtained when $\ln[(C_{max}-C_t)/C_{max}]$ was plotted against t for association of β-lg at the MFGM (mid season milk) in temperature range 65–80 °C, (n = 1). In the temperature range 85 to 95 °C, a plot of equation (5.1) yielded a straight line when n = 2. Results for mid-season milk are shown in Fig. 5.10. The kinetics of the α-la association with the MFGM, followed a first order reaction (equation 5.2) in the lower temperature range (70-80 °C) and second order reaction (n = 2 for equation 5.1) in the higher temperature range (85-95 °C). The results for mid-season milk are shown in Fig. 5.11. The apparent rate constants, k, for the association of β-lg and α-la with the MFGM

at each temperature range were determined by non-linear regression (NLR) as described by Oldfield (1996). The results show that the apparent rate constants (k_1) of β-lg associated with the MFGM were higher than those of α-la at any given temperature, for example, at 75 °C, the values of β-lg and α-la was 0.69 and 0.41 ($s^{-1} \times 10^{-3}$), respectively, for heating of mid-season milk.

These rate constants are considerably lower than those reported by Sharma and Dalgleish (1994) at the same temperature (75 °C). Sharma and Dalgleish (1994) calculated the kinetic parameters using the same model (equation 5.2) and found apparent rate constants of 1.4 ($s^{-1} \times 10^{-3}$) and 1.1 ($s^{-1} \times 10^{-3}$) for β-lg and α-la, respectively. However, Sharma and Dalgleish (1994) used whole milk that was homogenised.

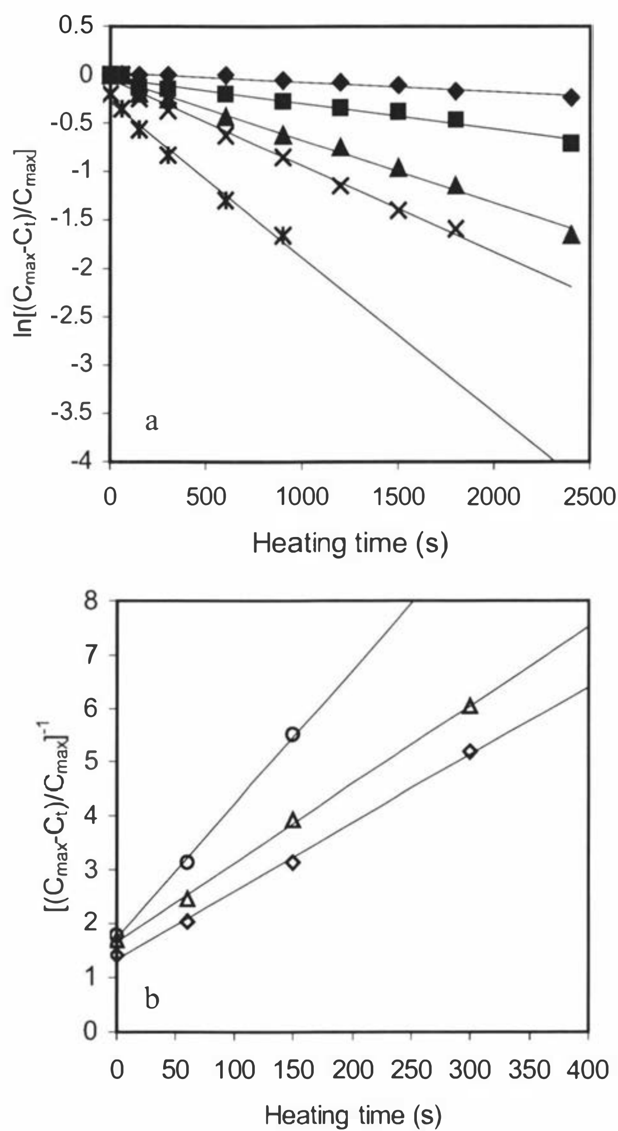


Fig. 5.10. Association of β-lg with the MFGM in heated mid-season milk as a first- (a) and second- (b) order reaction. Heating temperature: 65 °C (◆), 70 °C (■), 75 °C (▲), 78 °C (×), 80 °C (✱), 85 °C (◇), 90 °C (△) and 95 °C (○).

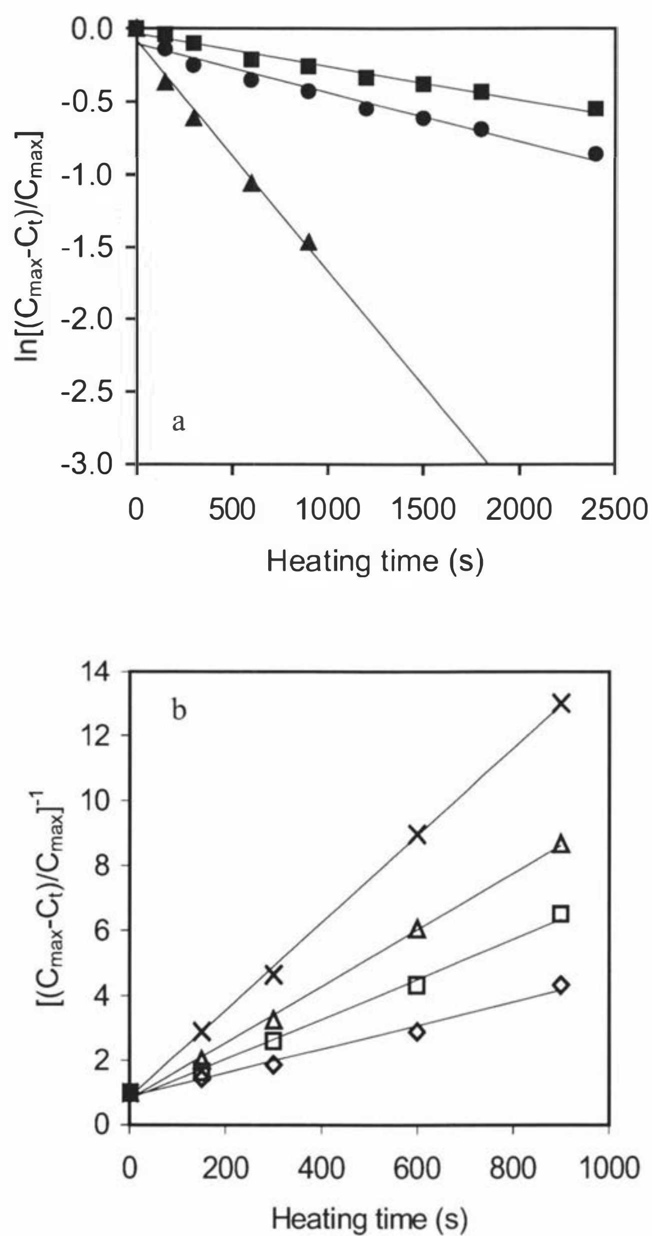


Fig. 5.11. Association of α-la with the MFGM in heated mid-season milk as a first- (a) and second- (b) order reaction. Heating temperature: 70 °C (■), 75 °C (●), 80 °C (▲◇), 85 °C (□), 90 °C (△), 95 °C (×) for 0-40 min.

5.3.3. Effect of temperature on the rate constant

The relationship between the observed rate constants and the temperature of the reaction is given by the Arrhenius equation:

$$k = k_0 \exp\left(-\frac{E_a}{RT}\right) \qquad (5.3)$$

Where E_a is activation energy (kJ/mol), R is universal gas constant (kJ mol^{-1} K^{-1}), k_0 is frequency factor ($((mg/g\ fat)^{(1-n)}\ s^{-1})$), and T is absolute temperature (K).

The plots of ln(k) versus the reciprocal of absolute temperature (Fig. 5.12) were linear within certain temperature ranges. A marked break at about 85 °C for β-lg and 80 °C for α-la associating with the MFGM was observed. The activation energies (E_a) calculated from NLR within the two temperature ranges are shown in Table 5.3.

5.3.4. Thermodynamic considerations

The enthalpy of activation ($\triangle H^{\#}$), the free energy of activation ($\triangle G^{\#}$), and the entropy of activation ($\triangle S^{\#}$) for the thermal association of β-lg and α-la with MFGM proteins were calculated using the Eyring equation.

$$k = \left(\frac{k_b T}{h}\right) \exp\left(-\frac{\Delta G^{\#}}{RT}\right) \qquad (5.4)$$

and the relationships

$$\triangle H^{\#} = E_a - RT \qquad (5.5)$$

$$\triangle G^{\#} = \triangle H^{\#} - T\triangle S^{\#} \qquad (5.6)$$

(k_b = Boltzman's constant and h = Plank's constant).

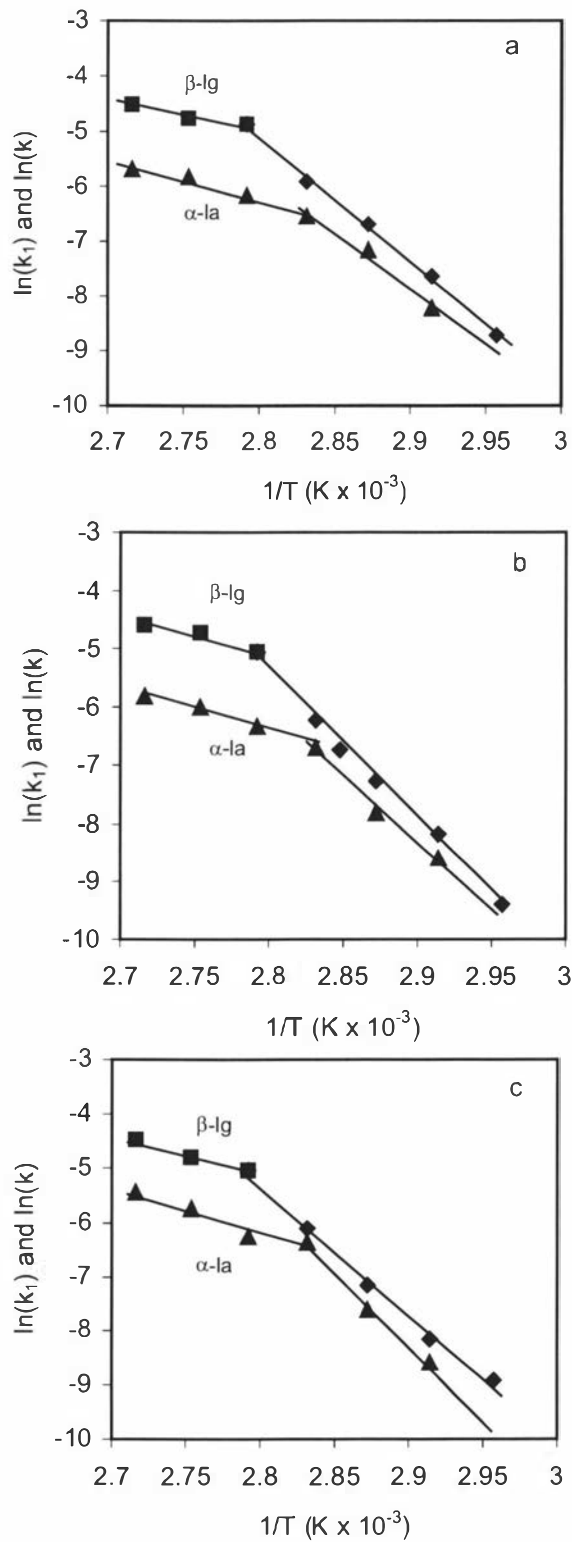


Fig. 5.12. Arrhenius plot for the association of β-lg (■) and α-la (▲) with the MFGM of heated early- (a), mid- (b) and late- (c) season milks.

Table 5.3. Kinetic parameters* for β-lg and α-la association with the MFGM in heated early-, mid- and late-season milks# in 65-95 °C temperature range.

Protein	Temperature (°C)	E_a (kJ/mol)			Ln k_0			R^2		
		Early	Mid	Late	Early	Mid	Late	Early	Mid	Late
β-lg	65-80	190.17 ± 5.27[a★]	206.49 ± 12.80[a]	197.78 ± 8.6[a]	58.96 ± 1.82[b]	64.06 ± 4.42[b]	61.28 ± 2.97[b]	0.997	0.991	0.994
	85-95	38.74 ± 9.35[a]	51.32 ± 12.20[b]	61.63 ± 6.43[b]	8.32 ± 3.10[c]	12.20 ± 4.17[c]	15.64 ± 2.13[d]	0.945	0.943	0.989
α-la	70-80	169.24 ± 23.66[a]	190.19 ± 19.97[a]	224.06 ± 17.32[b]	51.16 ± 8.17[c]	58.00 ± 6.90[c]	69.89 ± 5.98[d]	0.988	0.990	0.995
	80-95	62.62 ± 7.62[a]	64.95 ± 5.60[a]	70.57 ± 11.78[a]	14.83 ± 2.54[c]	15.45 ± 1.87[c]	17.59 ± 3.93[c]	0.977	0.978	0.948

* Parameters with 95% confidence intervals

Early, mid and late season milks were collected in August, January to March and May, respectively.

★ Different superscripts within a row indicate significant differences ($P < 0.05$).

The results are shown in Table 5.4. In the lower temperature range, i.e. below 85 °C, $\triangle H^{\#}$ varied from 190 to 200 kJ/mol for β-lg and from 170 to 220 kJ/mol for α-la, in early, mid and late season milks. $\triangle S^{\#}$ was positive (0.17 to 0.32 kJ/mol K) at < 85 °C and negative (-0.19 - -0.11 kJ/mol K) at high temperature for β-lg and α-la association with the MFGM proteins in early, mid and late season milks. Meanwhile, the value of $\triangle G^{\#}$ remained relatively constant at approximately 105 kJ/mol, over the temperature range studied (65–95 °C). The changes in $\triangle S^{\#}$ and $\triangle G^{\#}$ for β-lg and α-la association with the MFGM proteins were similar to those for the denaturation of β-lg and α-la in heated milks and reconstituted milks reported by Dannenberg and Kessler (1988), Anema and McKenna (1996) and Oldfield et al. (1998 a).

Most studies that have examined reactions involving the irreversible denaturation or aggregation of α-la and β-lg have shown this change in behaviour in the Arrhenius relationship (Fig. 5.12). For α-la, the break was at about 80 °C regardless of the milk protein solution or the denaturation/aggregation reaction under study. However, for β-lg some variation in the position of this break has been observed, and this appeared to be dependent on the aggregation reaction being investigated. Studies on the irreversible denaturation of β-lg in skim milk (Dannenberg & Kessler, 1988; Anema & McKenna, 1996; Oldfield et al., 1998 a) or whey solutions (Park & Lund, 1984) have shown the break to occur at about 90 °C. However, the break was observed at 100 °C when the association of β-lg with the casein micelles was investigated (Oldfield et al., 1998 b), and the current study on the interaction of β-lg with the MFGM observed the break at 85 °C.

Table 5.4. Change in enthalpy of activation, $\triangle H^{\#}$, free energy of activation, $\triangle G^{\#}$, and entropy of activation, $\triangle S^{\#}$, between 65 – 95 °C for association of β-lg and α-la with the MFGM proteins in heated early, mid and late season milks.

Protein	Temperature	$\triangle H^{\#}$ (kJ/mol)			$\triangle S^{\#}$ (kJ/mol K)			$\triangle G^{\#}$ (kJ/mol)		
	(°C)	Early	Mid	Late	Early	Mid	Late	Early	Mid	Late
β-lg	65 - 85	187.32	203.62	194.93	0.236	0.278	0.255	106.30	106.61	106.78
	90 - 95	35.70	48.28	58.59	-0.188	-0.154	-0.125	103.95	104.50	104.38
α-la	70 - 80	166.34	187.29	221.16	0.171	0.228	0.327	106.43	107.88	107.28
	85 - 95	59.60	61.91	67.55	-0.132	-0.127	-0.109	108.14	107.95	106.86

The calculated activation energies and enthalpies for the association of α-la with the MFGM in both temperature ranges (Tables 5.3 and 5.4) were similar to those observed for the irreversible denaturation reactions in milk (Dannenberg & Kesser, 1988; Anema & McKenna, 1996; Oldfield et al., 1998 a). In the low temperature range the activation energy and enthalpy for β-lg association with the MFGM were lower than those for denaturation (Dannenberg & Kesser, 1988; Oldfield et al., 1998 a) but higher than those for the association of this protein with the casein micelles (Oldfield et al., 1998 b). In the high temperature range, the activation energy and enthalpy were similar to literature reports for the denatureation of β-lg (Dannenberg & Kesser, 1988; Anema & McKenna, 1996; Oldfield et al., 1998 a), but higher than those observed for association of β-lg with the casein micelles (Oldfield et al., 1998 b). It should be noted that the comparisons are somewhat complicated by the differences in the temperature ranges for the low and high ranges between the different reactions.

The $\triangle S^{\#}$ was positive in the low temperature range and negative in the high temperature range for both α-la and β-lg, and the $\triangle G^{\#}$ remained relatively constant at all temperatures (Table 5.4). The observed values for $\triangle S^{\#}$ and $\triangle G^{\#}$ are in accord with those observed for the irreversible denaturation reactions (Dannenberg & Kesser, 1988; Anema & McKenna, 1996; Oldfield et al., 1998 a) and for the association of these proteins with the casein micelles (Oldfield et al., 1998 b).

5.3.5. Discussion

The mechanism of association of β-lg and α-la with the MFGM is extremely complex, in which several reactions take place at the same time and a number of intermediate products could be formed. The overall process involves:

(i) denaturation of individual proteins of the MFGM, β-lg and α-la (Appell, Keenan, & Low, 1982; Hillier & Lyster, 1979);

(ii) association of individual polypeptides of MFGM (Appell et al., 1982; Chapter 4) and self-aggregation of β-lg and α-la;

(iii) association of individual protein or associated protein complexes of the MFGM with individual β-lg and α-la or with aggregates of β-lg and α-la;

consequently PAS 7 or the complex of β-lg and PAS 7 possibly leaving the MFGM (Dalgleish & Banks, 1991; Houlihan et al., 1992; Chapter 4).

According to the principle that the slowest process is the rate-determining step, regardless of how many processes are involved, it is possible to deduce this rate determining step from the kinetic and thermodynamic results of this study. The results showed a marked change in temperature dependence at about 85 °C and 80 °C of the rate constant for β-lg and α-la association with the MFGM, respectively (Fig. 5.12). This resulted in marked differences in the E_a, $\triangle H^{\#}$ and $\triangle S^{\#}$ values in the two temperature ranges (Tables 5.3 and 5.4).

The high E_a and $\triangle H^{\#}$ along with a positive $\triangle S^{\#}$ for the low-temperature range were typical of a reaction mechanism, with denaturation (thermal unfolding) of the proteins as the rate-determining process. A denaturation process in which the tertiary structure of the protein is disrupted to give randomly coiled molecules involves the rupture of a large number of (weak) intramolecular bonds. This major conformational change in the protein is expressed as high values for both high E_a, and $\triangle H^{\#}$ and a positive $\triangle S^{\#}$, reflecting the lower state of order in the molecule.

The denaturation temperature of the MFGM proteins is low, most of them aggregate after heating at 65°C for 10 min (Appell et al., 1982; Chapter 4). In addition, MFGM proteins contain a large amount of disulfide and sulfhydryl groups (Cheng et al., 1988, Mather, 2000). For example, xanthine oxidase, one of the major MFGM proteins, contains 22 disulfide bonds and 38 sulfhydryl groups, four of the sulfhydryl groups are detectable in the undenatured protein complex (Cheng et al., 1988). This means that there are sufficient sulfhydryl or disulfide groups in MFGM proteins for interactions to take place in the low temperature range.

In the present work the activation energy (E_a) (Table 5.3) below 85 °C for β-lg and α-la association with MFGM was lower than reported values (Dannenberg & Kessler, 1988) of E_a for thermal denaturation of β-lg and α-la in similar temperature range. This difference is possibly because free thiol groups were provided by MFGM proteins, not

β-lg, for initiating thiol-disulfide exchange reactions among MFGM proteins and β-lg or α-la. The reaction probably occurs before the free thiol group of β-lg is exposed.

In the high temperature range, the markedly lower E_a, $\triangle H^\#$ values and the negative $\triangle S^\#$ (Table 5.4) indicate an increase in order as the reactants proceeded to the aggregated complex. The chemical association reactions became rate-determining in this temperature range, although the protein conformation is destroyed as well. In the higher temperature range (85-95 °C), besides the reaction of β-lg and α-la associating to MFGM, aggregation reactions between β-lg itself, β-lg and α-la, and β-lg with casein micelles occur simultaneously. The E_a, $\triangle H^\#$ and $\triangle S^\#$ values for association of β-lg and α-la with the MFGM were not significantly different from the reported values for irreversible denaturation in the higher temperature range.

The results showed the saturating amounts of β-lg and α-la associated with the MFGM were ~1.0 and ~0.25 mg/g fat, respectively. It is not known why the heat-induced association reactions between the MFGM proteins and β-lg or α-la are limited to this value. A possible explanation is that once β-lg and α-la aggregate in the serum or associate with the casein micelle via κ-casein, they are unable to readily further associate with the MFGM proteins. The kinetics of β-lg and α-la denaturation (Dannenberg & Kesser, 1988; Oldfield et al., 1998 a) paralleled the kinetics of β-lg and α-la association with the MFGM proteins in this study. Thus, when most of the serum protein was denatured the maximum association with the MFGM proteins was reached. It appears the denatured serum protein may have their reactive sulfhydryl and disulfide group buried within the interior of the aggregate, and therefore unavailable for sulfhydryl-disulfide interchange reactions with the MFGM protein. Corredig and Dalgleish (1996) also reported similar plateau levels of β-lg and α-la association with the MFGM (~0.7 and ~0.22 mg protein /g fat for β-lg and α-la, respectively) after ~20 min at 85 °C, and considered this was because all the β-lg and α-la available in the skim milk had reacted with casein micelles.

The maximum amount of β-lg and α-la associated with the MFGM as a percentage of the total β-lg and α-la was very low; 0.8-1.0% in this study and < 1% reported by Corredig and Dalgleish (1996). The remaining ~99% β-lg and α-la would have presumably reacted by other competing pathways, including self aggregation and interaction with the casein micelle. Corredig and Dalgleish (1996) conclude that the casein micelle offers the whey proteins a greater surface area for interaction than the fat globule, based on the diameter ranges of casein micelles (50-300 nm) and milk fat globules (0.5-10 μm).

Proposed mechanism for β-lg association with the MFGM proteins:

The results of this study (Arrhenius plot and thermodynamic parameter for the activated complex) suggest that β-lg association with the MFGM proteins, in the temperature region 75-95 °C (above β-lg denaturation temperature), involve the following steps:

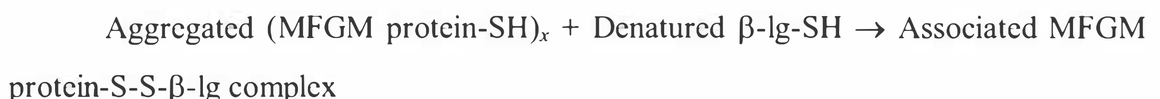
Step 1: Denaturation of β-lg and self aggregation of MFGM protein.

Native β-lg → Denatured β-lg –SH

x Native MFGM proteins → x Denatured (MFGM proteins –SH) → Aggregated (MFGM protein –SH)$_x$

Where x indicates a number of MFGM proteins forming a disulphide-linked aggregated complex.

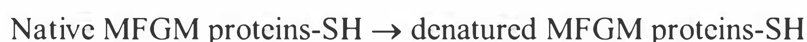
Step 2: Aggregation of denatured β-lg with aggregated MFGM protein complex.


Aggregated (MFGM protein-SH)$_x$ + Denatured β-lg-SH → Associated MFGM protein-S-S-β-lg complex

In the temperature region 60-70 °C, which is below β-lg denaturation temperature, the following reaction steps are proposed:

Step 1: Partial unfolding of β-lg and denaturation of MFGM protein.

Native β-lg → partially unfolded β-lg-S-S-


Native MFGM proteins-SH → denatured MFGM proteins-SH

Step 2: Aggregation of partially unfolded β-lg with denatured MFGM proteins

Partly unfolded β-lg-S-S- + denatured MFGM protein-SH → MFGM proteins-S-S-β-lg-SH.

Heating at temperature > 70 °C results in the denaturation of both β-lg and the MFGM protein. This exposes the reactive thiol groups and allows thiol-disulphide interchange reactions, leading to the formation of disulphide-linked aggregates. However, the mechanism for initial interaction between the β-lg and MFGM proteins may be different in the low temperature region (60-70 °C). Partial unfolding of β-lg may occurs at this temperature range (Relkin & Launay, 1990). The thiol-disulfide interchange between MFGM proteins and β-lg could occur when the free thiol groups are provided by the MFGM proteins which have been denatured in this temperature region (Appell et al., 1982; Chapter 4), but before the free thiol grouping of β-lg has been fully exposed.

There were no significant differences in the kinetic parameters of β-lg and α-la association with the MFGM proteins between the season milks. The higher concentration of β-lg and α-la in early and late season milks or the changes in size of milk fat globules of seasonal milks did not affect the association of β-lg and α-la with the MFGM. Furthermore, the amount of β-lg associated with the MFGM in heated milk is only ~1% of total β-lg in milk. Thus any seasonal variation in β-lg concentration may not influence the amount of β-lg associated with the MFGM proteins because most of the β-lg does not associate with the MFGM.

CHAPTER 6
CHANGES IN FAT GLOBULES AND MEMBRANE PROTEINS DURING CONCENTRATION OF WHOLE MILK IN A PILOT-SCALE MULTIPLE-EFFECT EVAPORATOR

6.1. Introduction

Falling film evaporators, which are predominant in the modern dairy industry, are designed to remove as much water as possible from the milk at a low energy cost and with minimal heat-damage to the milk constituents. In the traditional design, the milk is boiled under vacuum in the first effect through the application of externally supplied heat to the first stage. Subsequent stages (or effects) are heated by the vapour generated from evaporation in the preceding effects.

In addition to concentration, evaporation causes numerous other changes in the milk system. These changes depend, to some extent, on other conditions, such as preheat treatment, temperature during concentration and time elapsed after concentration. The pH of the milk decreases during concentration from an average initial value of 6.7 to approximately 6.1 at 45% total solids (Howat & Wright, 1934). This is partly due to changes in salt equilibria as more calcium phosphate is transferred from the soluble state to the colloidal state, with a concomitant release of hydrogen ions (Fox, 1982).

The viscosity of the milk increases with increasing concentration and the effect is most marked when the concentration of milk solids exceeds 45%. The viscosity increase reflects the increase in the extent of inter- and intramolecular contacts in the milk brought about by concentration. At 45% total solids, the casein micelles alone occupy about 30% of the total volume of milk (Singh & Newstead, 1992).

Treatment of milk in a falling-film evaporator may disrupt the fat globules considerably (Mulder & Walstra, 1974; Mol, 1975). Boiling implies the formation of vapour bubbles while the fat is liquid, and this must disrupt some fat globules. However, it is often

assumed that boiling and evaporation of milk cause the fat globules to coalesce, although data to substantiate this are scarce.

In addition to disruption of the fat globules, the effect of heating during evaporation on the composition and properties of the MFGM should be considered. It has been shown that the native MFGM proteins are very reactive during heating, even at temperatures below 65 °C (Dalgleish & Banks, 1991; Chapters 4 and 5). For normal milk, the residence time in each effect during evaporation is typically 1 min, so that, in the first effect, the milk is held for about 1 min at 70-72 °C, which is a rather more severe heat treatment than pasteurization (typically 72 °C for 15 s). Subsequent stages are at lower temperatures, which would be expected to have less effect (Singh & Newstead, 1992). No data on the effects of heating on the MFGM proteins at intermediate concentrations over this temperature range are available.

A few electron microscopy studies have examined the state of the casein micelles and the fat in concentrated milks (Schmidt et al., 1971; de Felipe et al., 1991; Velez-Ruiz & Barbosa-Canovas, 2000). A thick protein layer, containing casein micelles, was seen on the fat globule surface. However, in these studies, the concentrated milks used were homogenized before examination by electron microscopy, or commercial condensed milks, for which the processing conditions were unknown, were used. No studies on the behaviour of the milk fat globule and the MFGM during the evaporation of whole milk under the conditions used in the commercial manufacture of whole milk powder have been reported.

Hence, the objective of the present chapter was to determine the changes that occur in the milk fat globules and the MFGM proteins as the milk pass through a pilot-scale multiple-effect evaporator. In some experiments, the milk was preheated prior to entry into the first effect of the evaporator.

6.2. Preliminary studies

6.2.1. Milk fat globule size

Milk samples at different stages of evaporation were obtained from the FRC which included the feed milk, flash-vessel preheated milk (about 95 °C for 20 s), 1st, 2nd and the final effect concentrates. The total solid contents of the samples are given in Table 6.1. The average fat globule sizes (d_{32}) of the samples, dispersed in SDS and EDTA buffer, are shown in Fig. 6.1. The d_{32} decreased after preheating and during evaporation, particularly after the final effect (Fig. 6.1). This indicates that the fat globules were disrupted to smaller sizes during evaporation. The d_{32} values decreased almost linearly with an increase in the concentrate total solids from ~11% to ~49% (Fig. 6.2).

Table 6.1. Total solid contents of samples obtained from the FRC pilot plant

Sample	Total solids (%)
Feed milk	11.75
Preheated milk (95 °C for 20 s by flash vessel)	11.72
1st effect milk	15.72
2nd effect milk	24.21
Final concentrate	48.70

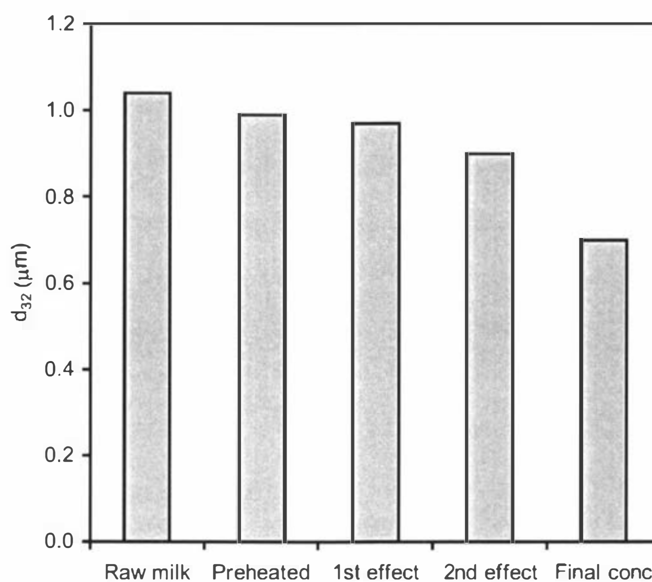


Fig. 6.1. The average size (d_{32}) of the fat globules in milk samples obtained at different stages of evaporation. The fat globule size was determined after dispersion in SDS and EDTA buffer. The samples were obtained from the FRC pilot plant.

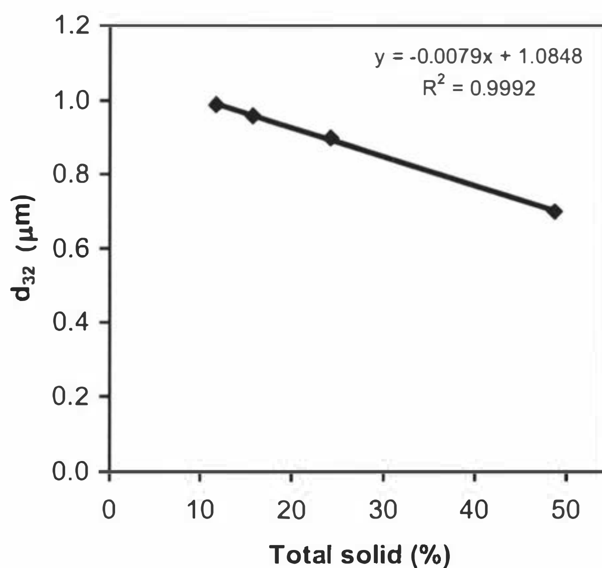


Fig. 6.2. The average size (d_{32}) of the fat globules in milk samples obtained at different stages of evaporation as a function of the total solids content (%). The fat globule size was determined after dispersion in SDS and EDTA buffer. The samples were obtained from the FRC pilot plant.

6.2.2. Total MFGM protein concentration and protein composition

The total MFGM protein concentrations of the samples are shown in Table 6.2. Preheating (95 °C for 20 s) caused an approximately twofold increase in the amount of MFGM protein, which further increased during evaporation. The MFGM protein concentration in the final concentrate was about four times higher than that in the standardized milk.

Table 6.2. The total protein concentration of the MFGM material of samples obtained from the FRC pilot plant

Sample	Total MFGM protein (mg/g fat)	Surface protein coverage of MFG (mg/m^2)
Feed milk	8.52	1.48
Preheated milk (95 °C for 20 s)	14.51	2.42
1st effect milk	16.96	2.74
2nd effect milk	19.62	2.95
Final concentrate	46.58	5.42

The MFGM material isolated from different samples was analysed using SDS-PAGE under reducing conditions. Bands of caseins, β-lg and α-la were observed in the SDS-PAGE pattern of the material isolated from the preheated milk and the intensities of these bands increased in the concentrates after 1st, 2nd and final effects (Fig. 6.3). In the final concentrate, caseins dominated the MFGM proteins. The increase in the amount of total MFGM protein was attributed mainly to the increase in the amounts of the caseins (Fig. 6.4).

The intensities of the xanthine oxidase and butyrophilin bands in the SDS-PAGE patterns were not influenced by preheating and evaporation (Fig. 6.3). However, the intensity of the PAS 6 band markedly decreased and the PAS 7 band almost disappeared from the pattern of the preheated milk. A band with an Mr of ~75 kDa was observed in the patterns of the concentrates and its intensity increased during evaporation (Fig. 6.3). A faint band with an Mr of ~58 kDa, located between butyrophilin (Mr 66 kDa) and PAS 6 (Mr 50 kDa), was also observed in the final concentrate.

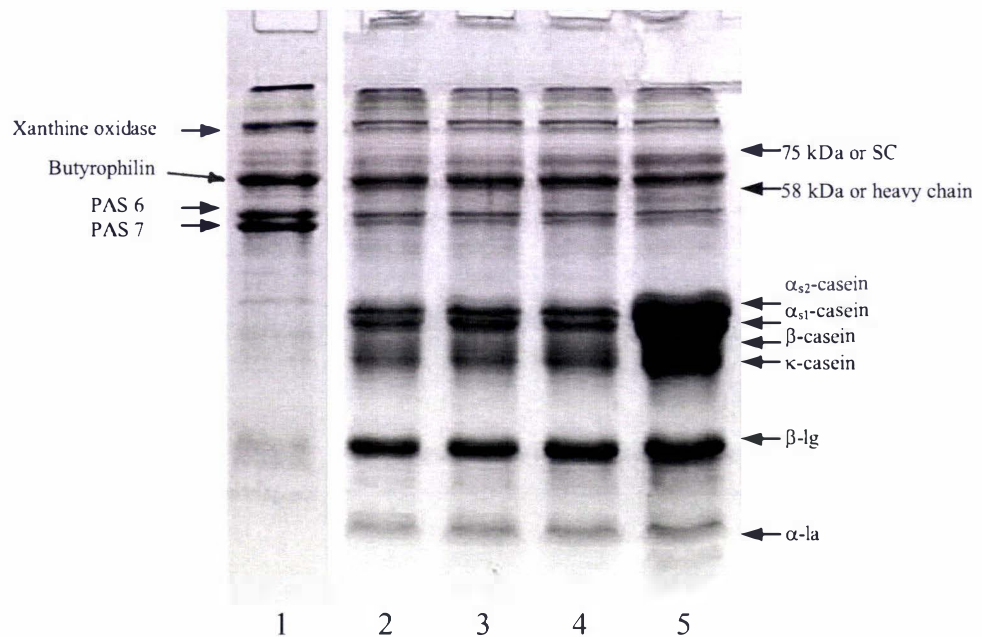


Fig. 6.3. SDS-PAGE patterns (15% acrylamide gel) under reducing conditions of the MFGM material isolated from samples obtained at different stages of evaporation. Lane 1, raw milk; lane 2, preheated milk; lane 3, after the 1st effect; lane 4, after the 2nd effect; lane 5, final concentrate.

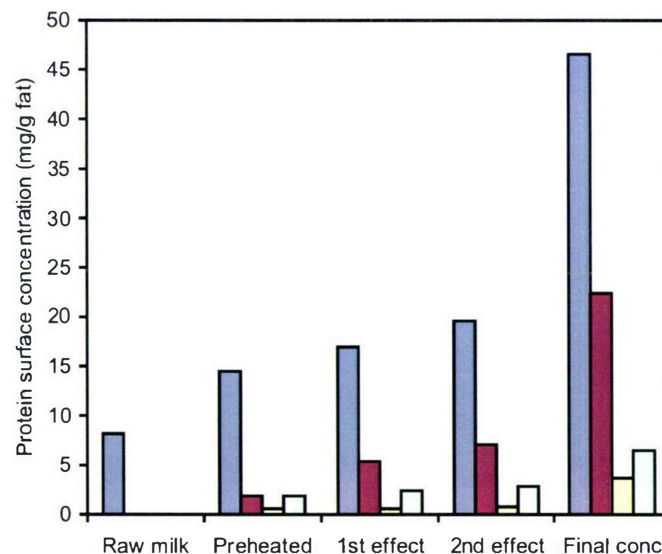


Fig. 6.4. The protein concentrations (mg/g fat) at the MFGM surface of milk samples obtained at different stages of evaporation. The samples were obtained from the FRC pilot plant; ■: total surface protein concentration; ■: caseins concentration; ■: κ-casein concentration; □: β-lg concentration.

6.2.3. Microscopic structures

The milk and the concentrates were examined using CSLM (Fig. 6.5). Although the fat globules were evenly distributed in the raw milk (Fig. 6.5 A), some clusters of fat globules were observed in the micrographs of the preheated and 2nd effect samples (Fig. 6.5 C, arrow). Compared with the raw milk sample, a larger proportion of small globules was apparent in the micrograph of the final concentrate (Fig. 6.5 D), indicating that the average fat globule size decreased in the final concentrate. This is consistent with the fat globule size results obtained using the MasterSizer (Fig. 6.1).

These results strongly suggest that the milk fat globules are disrupted into smaller globules and that a new surface protein layer is formed at the globule surface by adsorbed skim milk proteins during the evaporation of whole milk. In order to explore this further, trials in which controlled non-preheated and preheated milks were evaporated using the multiple-effect evaporator at the FRC pilot plant were carried out.

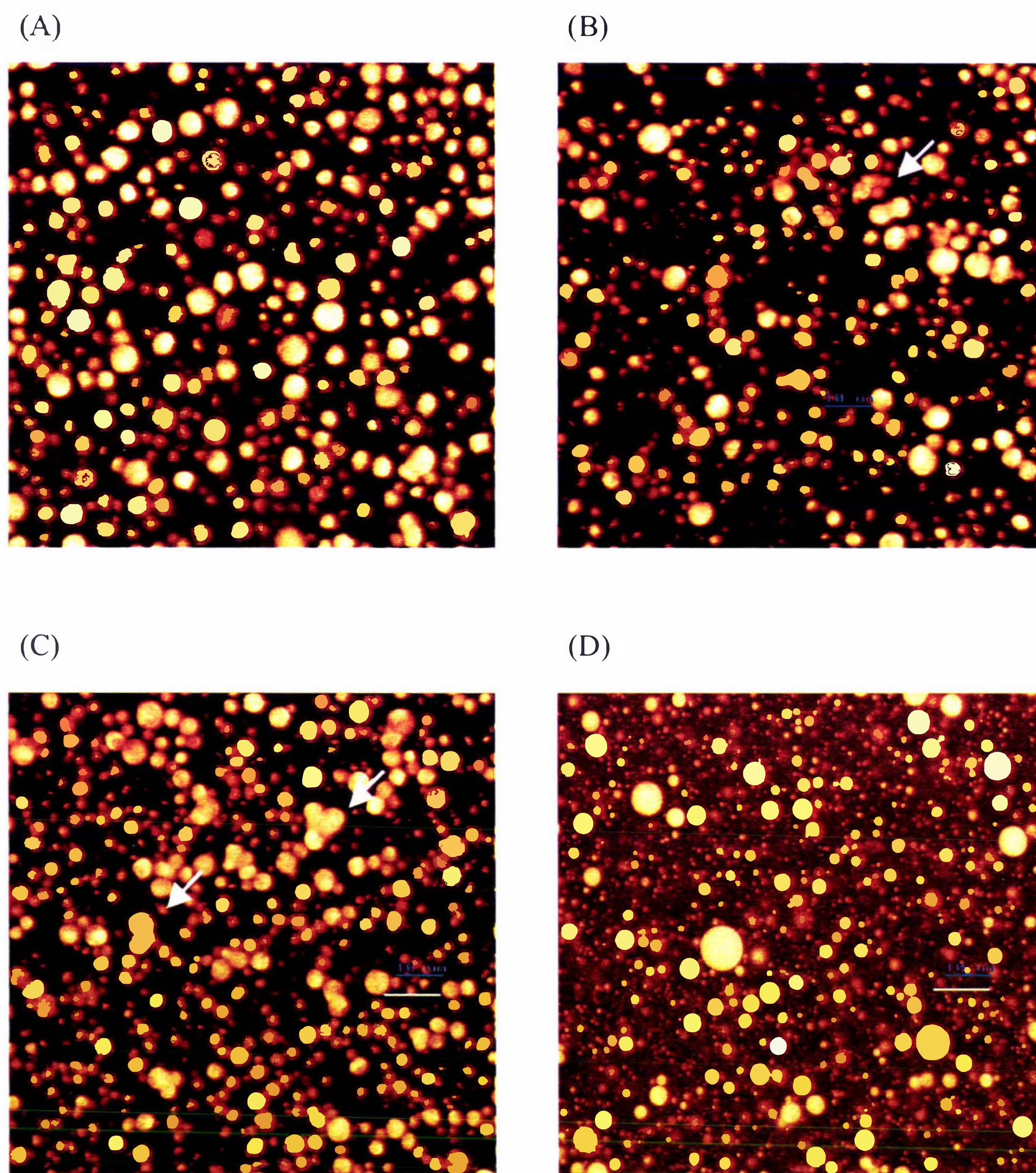


Fig. 6.5. Confocal micrographs of milk samples obtained from multiple-effect evaporation, (A) raw whole milk; (B) preheated milk; (C) concentrate after 2nd effect; (D) final concentrate. Bars indicate 10 μm.

6.3. Main trials

6.3.1. Experimental milk samples

The milk samples obtained were as follows.

Raw whole milk

Standardized milk

Milk preheated milk by DSI (95 °C, 20 s)

Non-preheated or preheated concentrated milks obtained after each effect (Fig. 3.2)

Final concentrate after heating to 79 °C

6.3.2. Change in milk fat globule size distributions

Fig. 6.6 shows the average fat globule diameter (d_{32} and d_{43}) of various milk samples, dispersed in SDS and EDTA buffer. The values of d_{32} and d_{43} decreased during evaporation, particularly after the 2nd and final effects, in both non-preheated and preheated milks. The d_{32} values also decreased slightly with preheating at 95 °C for 20 s by DSI, but heating to 70 °C caused no changes in d_{32} (Fig. 6.6).

When the fat globule sizes were determined after dispersing the milk in water, the d_{32} values decreased slightly during evaporation (Fig. 6.7); this trend was similar to that observed when the milk was dispersed in the SDS and EDTA buffer (Fig. 6.6). However, the d_{43} value increased markedly after the 1st and 2nd effects; further evaporation in the final effect caused a significant decrease in the d_{43} value (Fig. 6.7). Preheating at 95 °C for 20 s by DSI also caused a marked increase in the d_{43} value of the milk sample; d_{43} value increased further after the 1st effect but decreased after the 2nd and final effects (Fig. 6.7 D). The d_{32} and d_{43} values of the final concentrates obtained from both non-preheated milk and preheated milk were similar (Fig. 6.6 and Fig. 6.7). This suggests that the fat globule size of the final concentrate was independent of the preheat treatment used.

The size distribution of the fat globules showed that some large particles, with diameters between 10 and 100 μm (arrow in Fig. 6.8), were formed in the samples in which the d_{43} value was larger than that of the raw milk sample. However, these large particles disappeared from the final concentrate (TS ~49%). These large particles were not found

when the samples were dispersed in SDS and EDTA buffer prior to particle size measurement (Fig. 6.8).

These results confirm that the milk fat globules of whole milk were disrupted to some extent during preheat treatment by DSI and subsequent evaporation in the multiple-effect falling film evaporator.

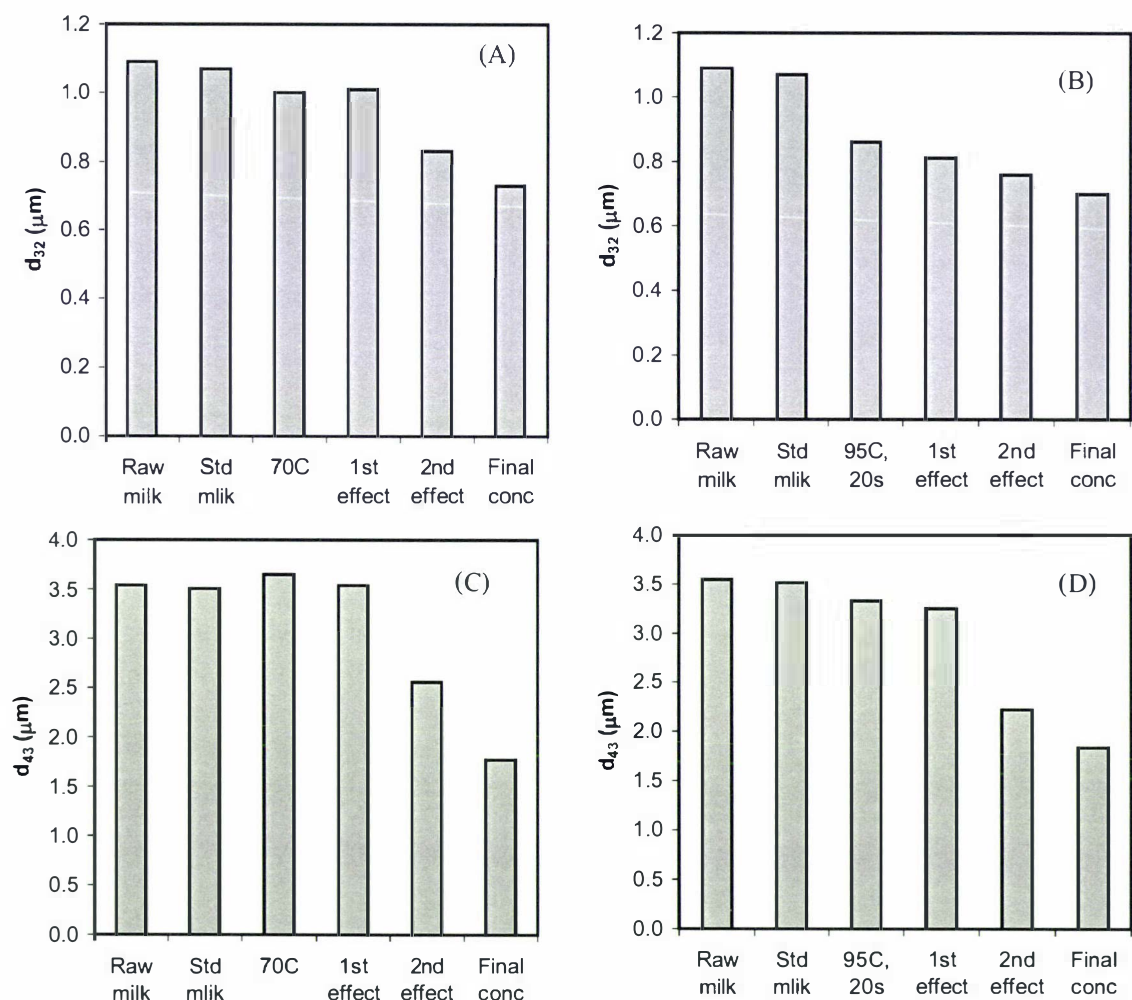


Fig. 6.6. The average size (d_{32}, A, B; d_{43}, C, D) of the fat globules in milk samples obtained at different stages of evaporation. The fat globule size was determined after dispersing the samples in SDS and EDTA buffer. Milk samples (B, D) were preheated at 95 °C for 20 s by DSI prior to evaporation.

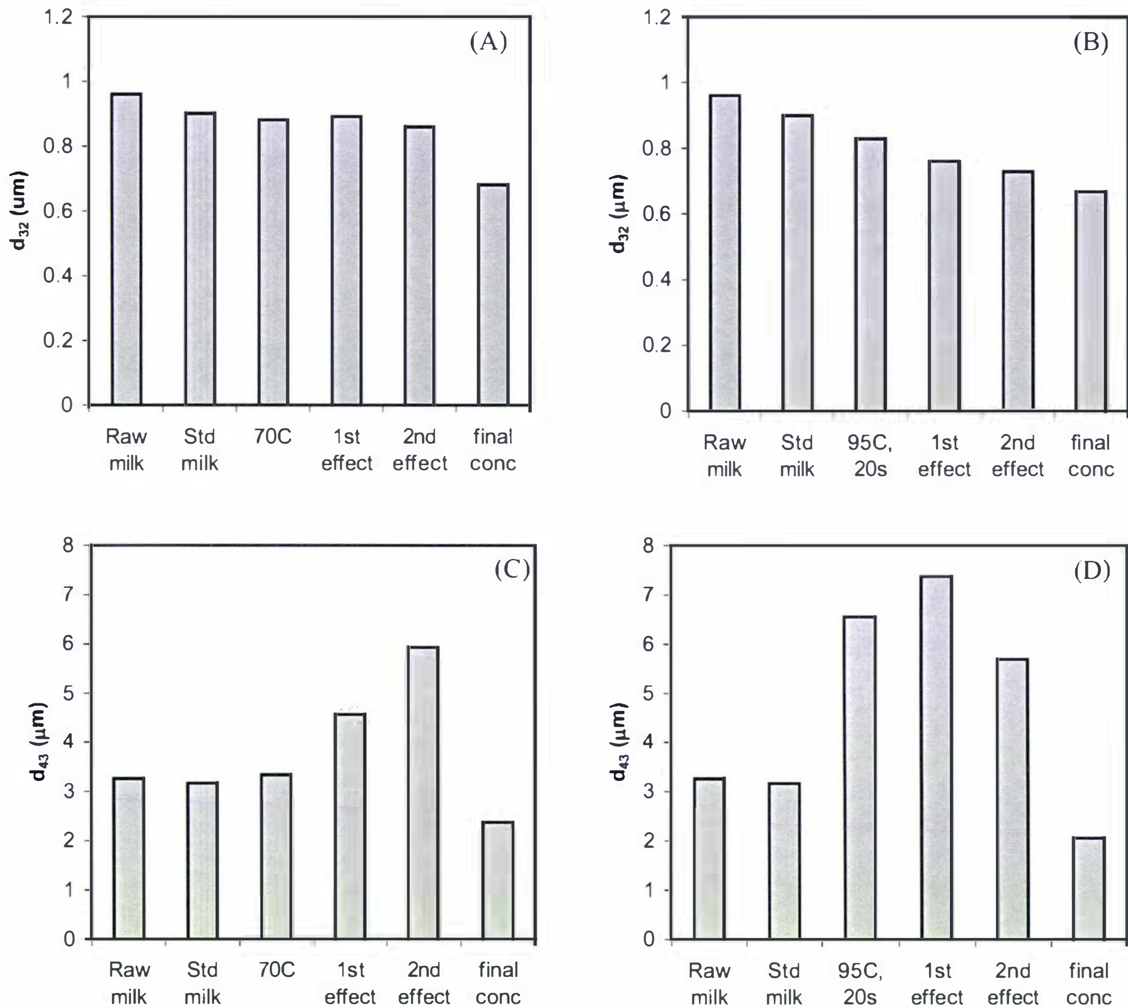


Fig. 6.7. The average size (d_{32}, A, B; d_{43}, C, D) of the fat globules in milk samples obtained at different stages of evaporation. The fat globule size was determined after dispersing the samples in water. Milk samples (B, D) were preheated at 95 °C for 20 s by DSI prior to evaporation.

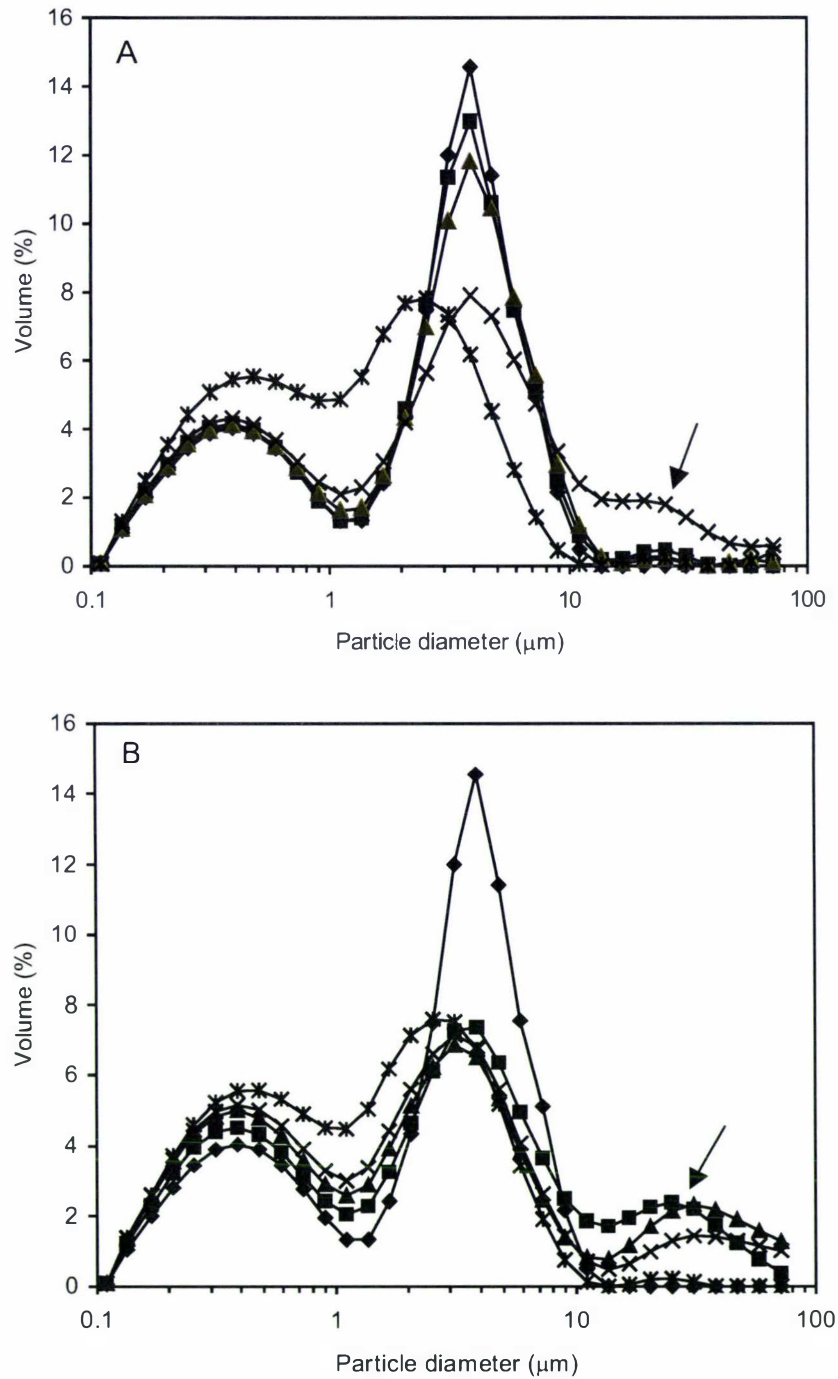


Fig. 6.8. Fat globule size distributions of non-preheated (A) or preheated (B) milk samples at different stages of evaporation. Standardized milk (◆); milk prior to entry into the evaporator (■); after the 1st effect (▲); after the 2nd effect (×); the final concentrate (✳).

6.3.3. Changes in total protein concentration at the milk fat globule surface

The total protein concentration at the milk fat globule surface of various milk samples is given in Table 6.3. The surface protein concentration increased considerably after the 2nd and final effects. The final concentrate (total solids ~49%) had ~approximately four times more surface protein (mg/m^2) than the standardized milk (total solids ~12%) in the non-preheated samples. Preheating at 95 °C for 20 s caused about a two-fold increase in the surface protein concentration. Evaporation further increased the surface protein concentration in the preheated milk. There was about a three-fold increase in the surface protein concentration in the final concentrate compared with the preheated milk; this concentration was about five times higher than that in the standardized milk. The surface protein concentration (~6.0 mg/m^2) in the final concentrate, obtained from milk preheated at 95 °C for 20 s by DSI, was significantly higher than that in the final concentrate obtained from non-preheated milk (~4.0 mg/m^2).

When the final concentrate was heated to 79 °C, the surface protein concentration significantly increased further in both non-preheated milk (from 4.0 to 5.5 mg/m^2) and preheated milk (from 5.9 to 7.2 mg/m^2) (Table 6.3).

Table 6.3. Total protein concentration at the milk fat globule surface of milk samples* obtained at different stages of evaporation of whole milk

Sample	Total solids (%)	Total MFGM protein (mg/g fat)	Surface coverage of MFGM protein (mg/m^2)
Standardized milk	11.50	7.2	1.3
Non-preheated milk			
Prior to evaporation	11.50	6.9	1.1
1st effect milk	15.02	7.1	1.2
2nd effect milk	26.82	13.9	1.9
Final concentrate	49.02	33.4	4.0
Concentrate heated to 79 °C	49.02	41.9	5.5
Preheated milk (95 °C, 20 s)			
Prior to evaporation	11.50	12.5	1.8
1st effect milk	15.61	18.0	2.4
2nd effect milk	24.89	21.8	2.8
Final concentrate	51.55	51.1	5.9
Concentrate heated to 79 °C	51.54	61.8	7.2

* All concentrated samples were diluted with water to ~11.50% total solids before surface protein determination.

6.3.4. Protein composition of the fat globule surface

SDS-PAGE patterns of the fat globule surface material that was isolated from various milks are shown in Fig. 6.9. A marked increase in the intensity of the casein bands was observed after the 2nd and final effects (Fig. 6.9 A), which indicates that the adsorption of caseins at the fat globule surface was the main factor leading to an increase in the total surface protein (Table 6.3). A faint β-lg band was observed in the milk samples prior to evaporation and after the 1st effect. The intensity of the β-lg band increased considerably after the 2nd and final effects. However, this increase was relatively small compared with the increase in the intensity of the casein bands (Fig. 6.9 A). When the final concentrate was heated to 79 °C, there was a further slight increase in the intensity of the β-lg band (Fig. 6.9 A, lane 7).

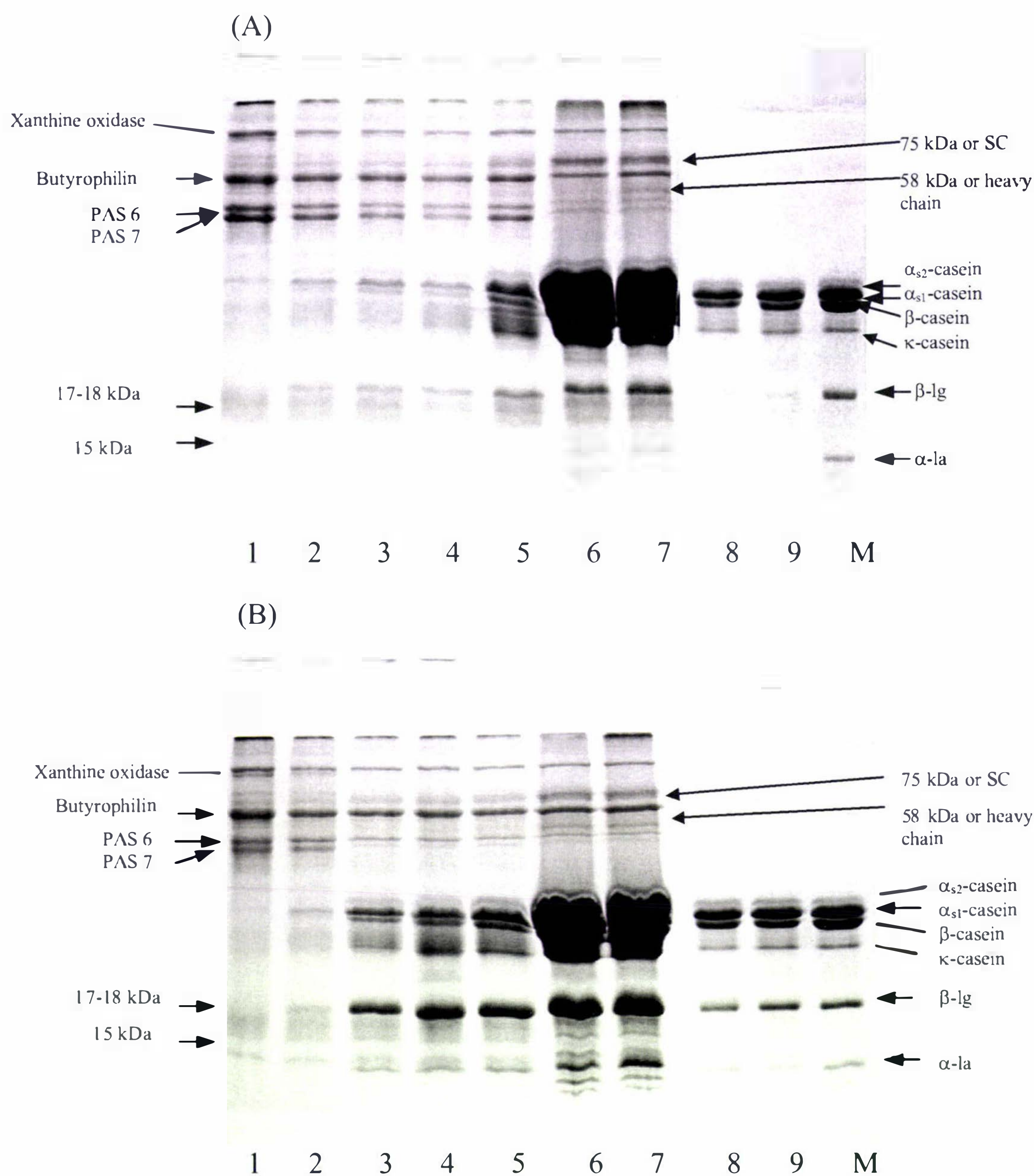


Fig. 6.9. SDS-PAGE patterns (15% acrylamide gel) under reducing conditions of the fat globule surface material isolated from concentrates obtained from non-preheated (A) or preheated (B) milk samples. The surface material was diluted three times with sample buffer (lanes 1 to 7). Lane 1, raw whole milk; lane 2, standardized milk; lane 3, milk prior to entry into the evaporator (70 °C for non-preheated milk or 95 °C for 20 s for preheated milk); lane 4, after 1st effect; lane 5, after 2nd effect; lane 6, final concentrate; lane 7, final concentrate heated to 79 °C. The surface material was diluted 24 times with sample buffer (lanes 8 and 9). Lane 8, final concentrate; lane 9, final concentrate heated to 79 °C; lane M: whole milk (control).

In the preheated samples, casein, β-lg and α-la bands were observed before evaporation (Fig. 6.9 B). The intensities of these bands gradually increased during the subsequent steps of evaporation. Compared with the non-preheated samples, the intensity of the β-lg band was considerably higher in the preheated samples at each effect (Fig. 6.9 A). An α-la band was observed in the preheated samples, but not in the non-preheated samples. Heat treatment (79 °C) of the final concentrate prepared from preheated milk caused an apparent increase in the intensity of both the β-lg band and the α-la band (Fig. 6.9 B).

Fig. 6.10 shows the amounts of caseins and serum proteins on the surface of fat globules in various milk samples. In the non-preheated samples, the caseins predominated the total surface protein in the final concentrate. In the preheated samples and after the 1st effect, the amount of whey proteins was similar to that of caseins. However, the amounts of caseins greatly increased in the final concentrate. The amounts of β-lg and α-la also significantly increased and accounted for about ~15% and ~3% of the total surface protein in the final concentrate. The results showed that the higher total surface protein concentration in the preheated final concentrate, compared with that in the non-preheated sample, could be attributed not only to the larger amount of whey proteins at the surface, but also to the greater amounts of caseins at the surface (Fig. 6.10 B).

When the isolated MFGM material (cream) was washed in urea and 50 mM EDTA buffer, the casein micelles adsorbed at the fat globule surface were dissociated and washed away. The protein molecules adsorbed directly at the interface of the fat globules, and the protein molecules bound to the interfacial protein layer via covalent bonds remained on the surface of the fat globules. SDS-PAGE analysis of these samples showed that only κ-casein remained at the fat globule surface and its intensity increased during evaporation, whereas the other caseins were washed away by the urea and EDTA buffer (Fig. 6.11). The other protein bands, including β-lg, α-la and the original MFGM proteins, were not changed by washing with urea and EDTA buffer. This indicates that only κ-casein in the casein micelle adsorbed directly on to the surface of the fat globules, whereas the other caseins (α_s-caseins, β-casein) did not attach or adsorb at the fat globule surface. Whey proteins associated with the surface of the fat globules could

be attributed mainly to the interaction between the native MFGM proteins and the whey proteins via disulfide bonding during heat treatment.

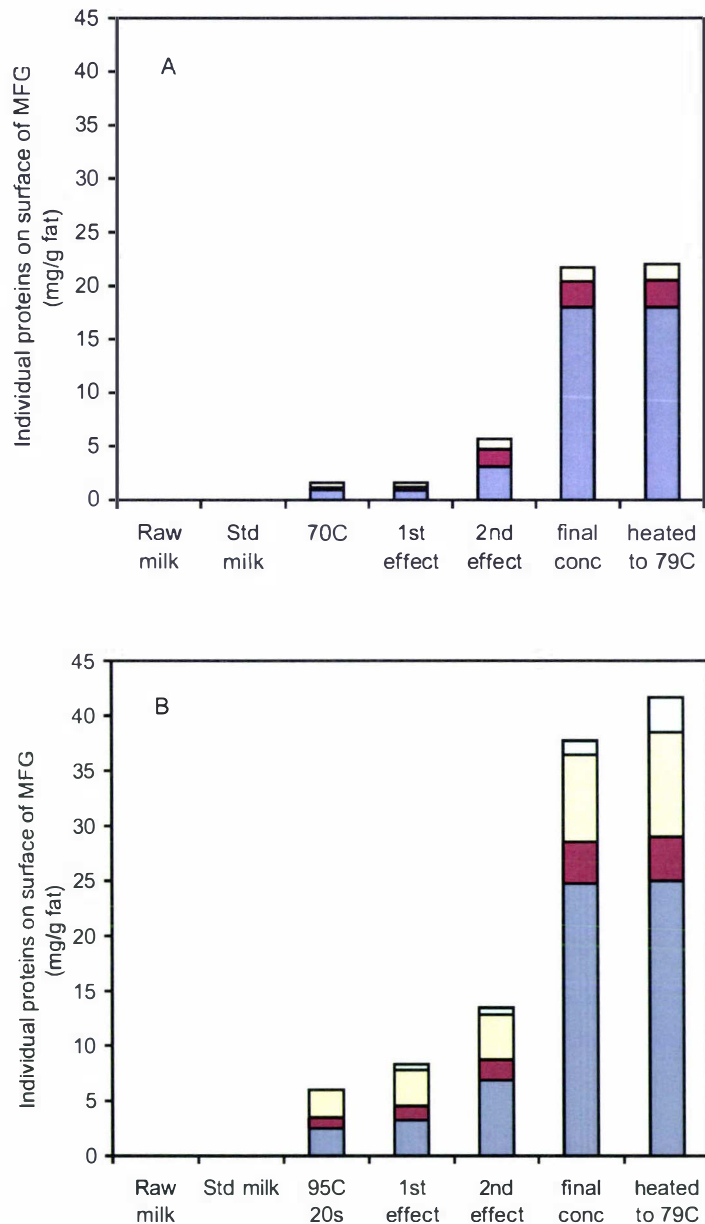


Fig. 6.10. The changes in the concentrations of individual proteins (mg/g fat) at the milk fat globule surface of non-preheated milk (A) and milk preheated at 95 °C for 20 s (B) during evaporation: ■, α_s- and β-casein; ■, κ-casein; ■, β-lg; □, α-la.

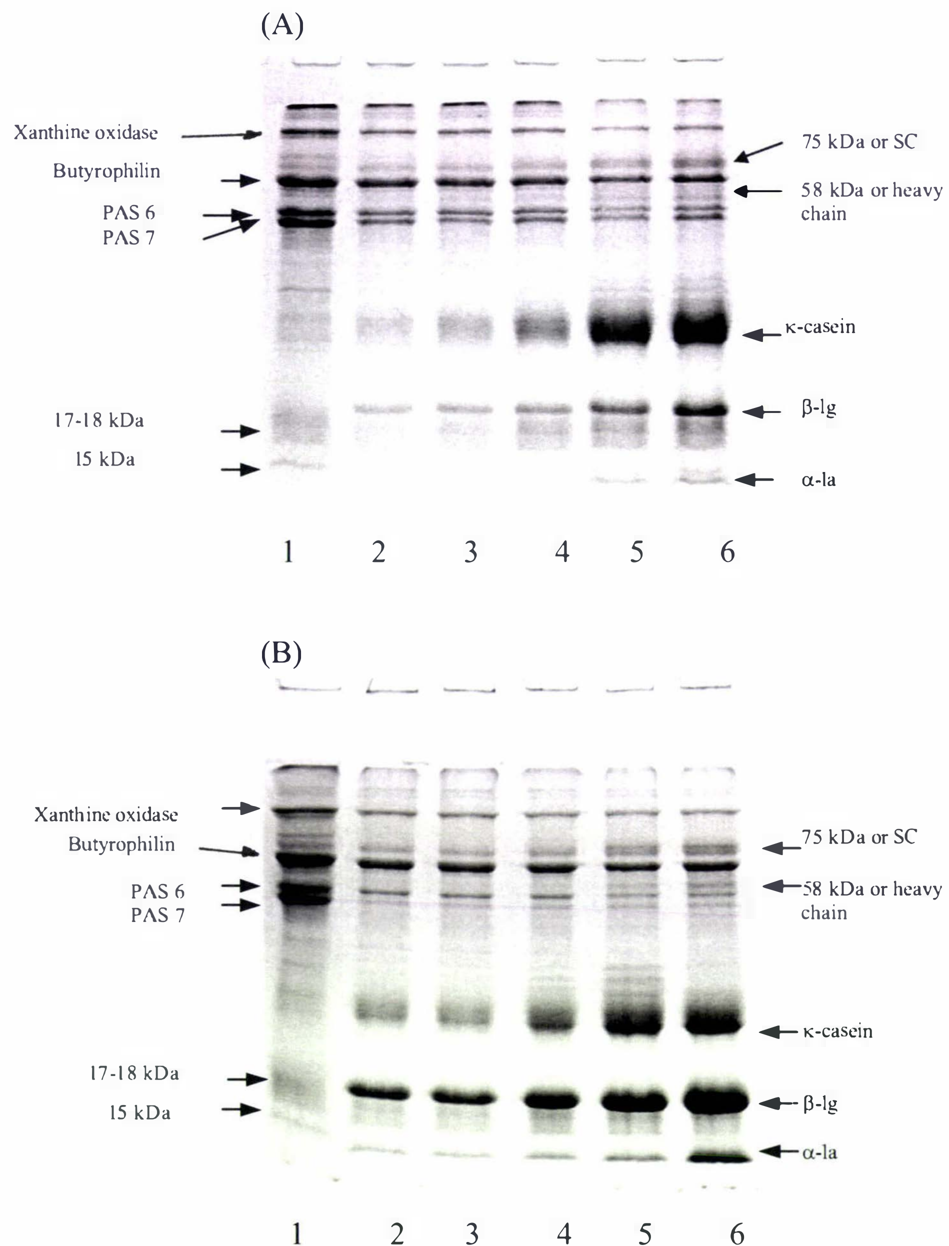


Fig. 6.11. SDS-PAGE patterns (15% acrylamide gel) under reducing conditions of the fat globule surface material isolated from concentrates obtained from non-preheated (A) or preheated (B) milk samples. Cream obtained from the concentrate was washed with SMUF containing urea and EDTA. The surface material was diluted three times with sample buffer. Lane 1, raw whole milk; lane 2, milk prior to entry into the evaporator (70 °C for non-preheated milk or 95 °C for 20 s for preheated milk); lane 3, after 1st effect; lane 4, after 2nd effect; lane 5, final concentrate; lane 6, final concentrate heated to 79 °C.

The intensities of the main original MFGM proteins, including xanthine oxidase, butyrophilin, PAS 6 (Band 15) and PAS 7 (Band 16), did not change during evaporation in the non-preheated samples (Fig. 6.12 A). However, the amounts of PAS 6 and PAS 7 (Bands 15 and 16) significantly decreased when the raw milk was standardized and pasteurized. Preheating at 95 °C for 20 s caused a marked reduction in PAS 7 and PAS 6, with PAS 7 almost completely disappearing from the SDS-PAGE pattern (Fig. 6.12 B). This is in agreement with previous studies, which have reported that these proteins are very sensitive to temperature (Houlihan et al., 1992; Kim & Jimenez-Flores, 1995; Chapter 5).

A protein band with an Mr of ~75 kDa appeared to increase in intensity (in both non-preheated milk and preheated milk) during evaporation (Fig. 6.11). A faint band with an Mr of~58 kDa, located between butyrophilin (Mr 66 kDa) and PAS 6 (Mr 50 kDa), was also observed in the samples of both non-preheated and preheated final concentrates. These proteins, with Mr ~75 kDa and ~58 kDa, are likely to be the secretory component (SC) and heavy chain immunoglobulin component, respectively (Larson, 1992). The protein with an Mr ~75 kDa (probably SC) constituted ~5% of the total surface proteins in the final concentrates. The intensity of this band was not affected by preheat treatment of the milk or washing of the cream using a dissociating agent (Fig. 6.9 and Fig. 6.11). An increase in the intensity of these protein bands was not observed when whole milk was heated at different temperatures (Chapter 5). This suggests that these proteins were directly adsorbed at the fat globule surface as a consequence of fat globule disruption.

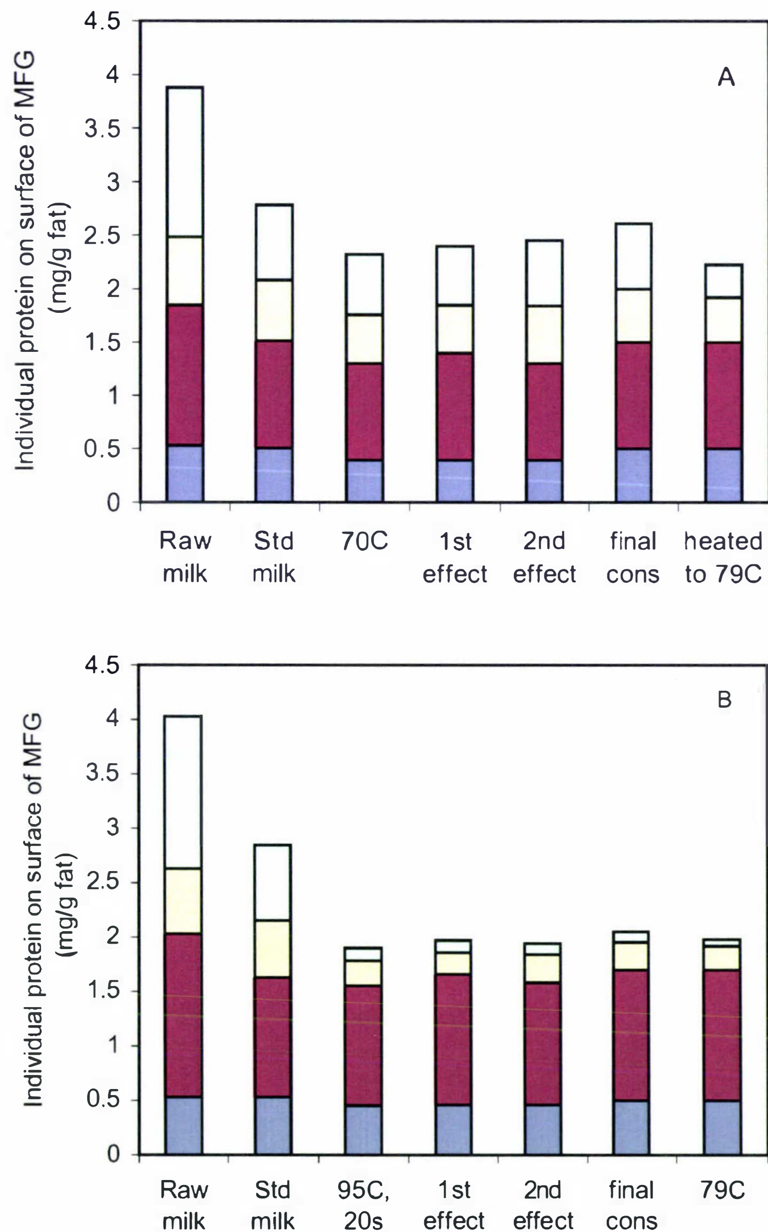


Fig. 6.12. The changes in the concentrations (mg/g fat) of individual major original MFGM proteins during evaporation of milk samples. The milk samples were treated at 70 °C (non-preheated, A) or 95 °C for 20 s (preheated, B) prior to evaporation: ■, xanthine oxidase; ■, butyrophilin; ■, PAS 6; □, PAS 7.

6.3.5. Microscopic structures

CSLM micrographs of raw milk and concentrates obtained from non-preheated milk and preheated milk are shown in Fig. 6.13 and Fig. 6.14, respectively. The fat globules were distributed more uniformly in the raw milk (Fig. 6.13 A), but some clusters of fat globules were observed after the 2nd effect (Fig. 6.13 C, arrow). These clusters of fat globules were also observed in the preheated samples (Fig. 6.14 A), with a greater extent of clustering in the milk samples after 1st and 2nd effects (Fig. 6.14 B and C). Larger proportions of smaller globules were seen in the final concentrate, compared with the raw milk sample (Fig. 6.13 D and Fig. 6.14 D). These observations are in agreement with the light scattering results of the fat globule size, in which large aggregated particles were found in the preheated milk samples after the 1st and 2nd effects. These large particles led to an increase in the d_{43} values of the samples (Fig. 6.7).

Transmission electron micrographs of the preheated milk (95 °C, 20 s) showed that some intact casein micelles were associated with the fat globule surface, and that most of these casein micelles seemed to be linked to the fat globule surface by some hair-like material; most of the surface was still smooth (Fig. 6.15). In the micrographs of the concentrate samples (~50% total solids) (Fig. 6.16 and Fig. 6.17), much greater number of casein micelles appeared to be associated with the fat globules, especially on the surface of smaller fat globules. These micelles appeared to touch or cohere to the surface and form chains from the surface into the serum. Some micelles were shared by two fat globules (Fig. 6.16). These micrographs confirmed that the casein micelles were adsorbed on to the surface of the fat globules during evaporation. The micelles in the preheated samples were joined together by thread-like material into chains (Fig. 6.17).

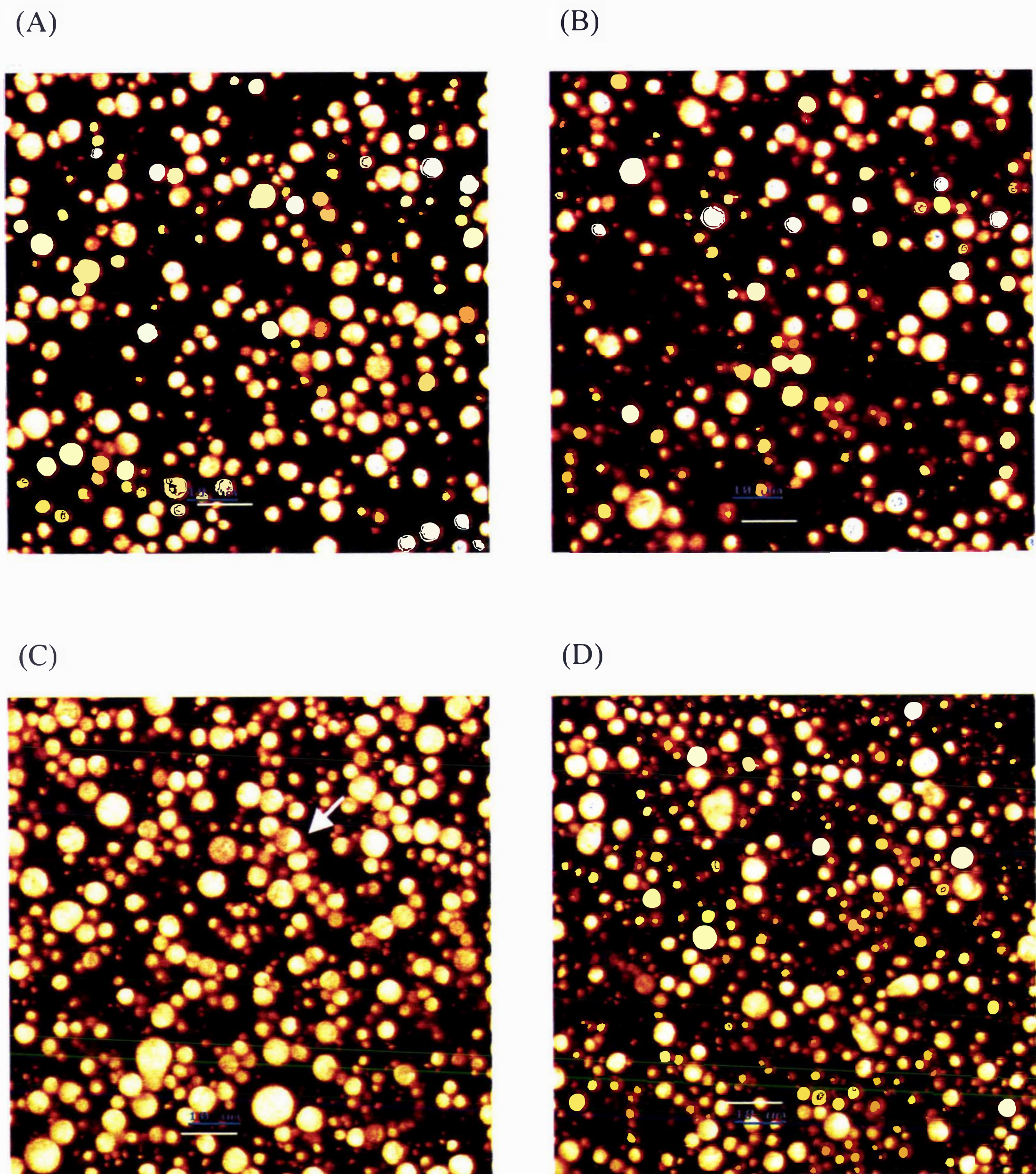


Fig. 6.13. Confocal micrographs of non-preheated milk samples from multiple-effect evaporation: (A) raw whole milk; (B) after the 1st effect; (C) after 2nd effect; (D) final concentrate. The fat globules were stained using Nile Blue solution, indicated by the yellow colour in the micrographs. Bars indicate 10 μm.

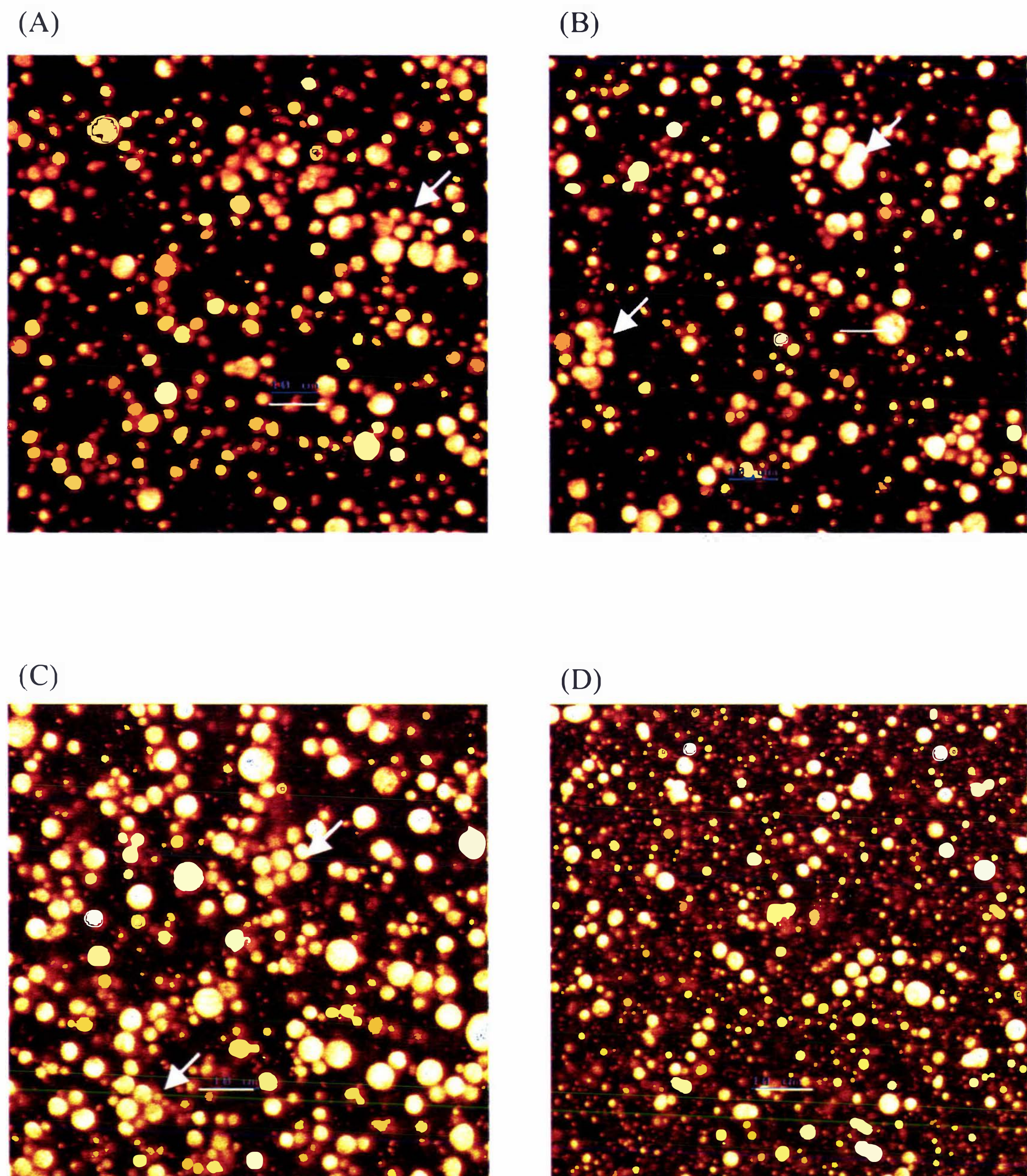


Fig. 6.14. Confocal micrographs of preheated milk samples from multiple-effect evaporation: (A) milk preheated at 95 °C for 20 s by DSI; (B) after 1st effect; (C) after 2nd effect; (D) final concentrate. The fat globules were stained using Nile Blue solution, indicated by the yellow colour in the micrographs. Bars indicate 10 μm.

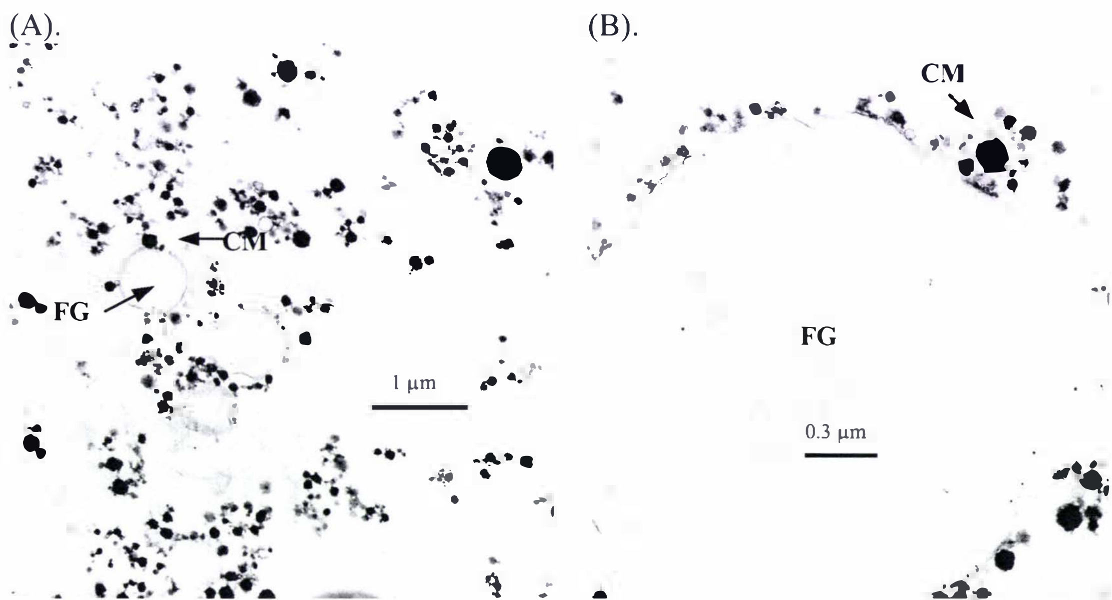


Fig. 6.15. Transmission electron micrographs of milk preheated at 95 °C for 20 s by DSI. Magnification: A, ×15300; B, ×31800.

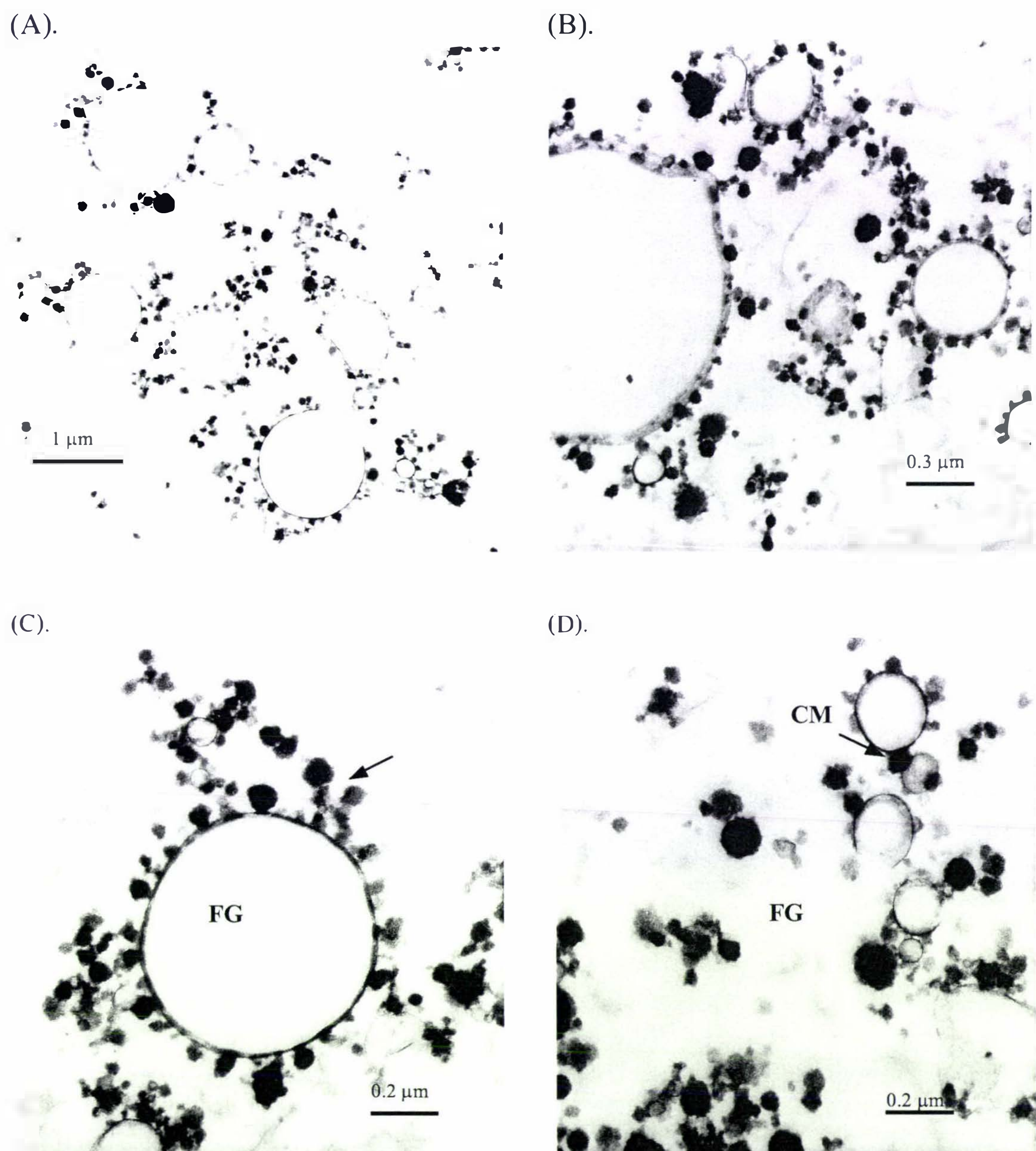


Fig. 6.16. Transmission electron micrographs of non-preheated concentrate (total solids ~49%) evaporated using a multiple-effect falling film evaporator. Magnification: A, ×15300; B, ×31800; C and D, ×48600.

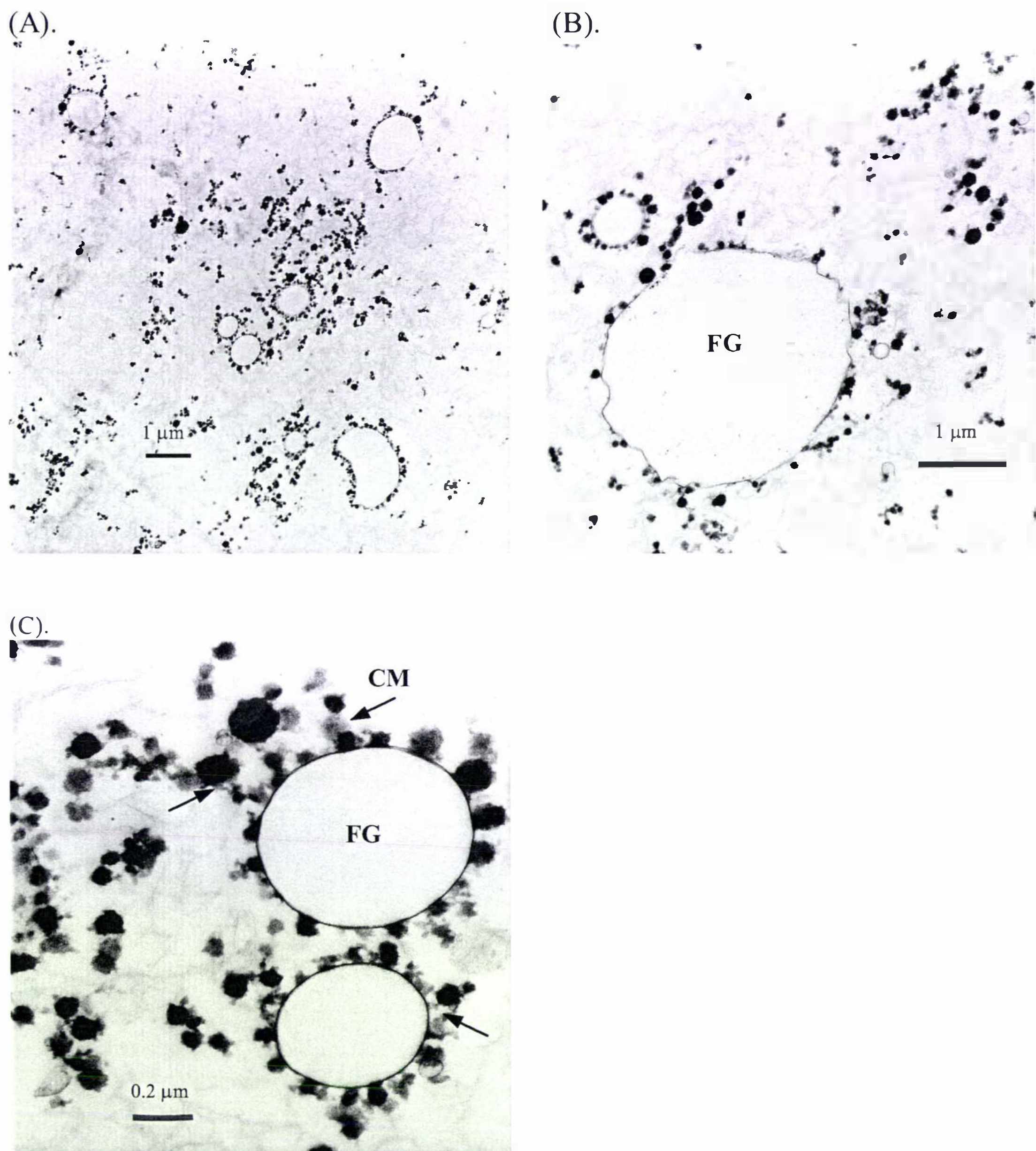


Fig. 6.17. Transmission electron micrographs of preheated (95 °C, 20 s) concentrate (total solids ~51%) evaporated by multiple-effect falling film evaporator. Magnification: A, ×7800; B, ×15300; C, ×48600.

6.4. Analysis of industrial samples

Determinations were carried out on industrial samples obtained from the Fonterra Co-operative Group, Hawera, including standardized milk (Total solids ~10.0%), preheated milk (80 °C), DSI-preheated milk (98 °C), and concentrates after evaporation in the mechanical vapour recompression (MVR) evaporator (Total solids ~34.3%) and the thermal vapour recompression (TVR) evaporator (TS ~45.3%). In agreement with the results of the pilot plant trial, the average size of the fat globules (d_{43}), determined after dispersion in SDS and EDTA buffer, decreased after DSI preheating (98 °C), MVR evaporation and TVR evaporation (Fig. 6.18 A). However, after dispersion in water, the d_{43} values of the preheated and DSI-preheated samples were larger than that of the standardized milk (Fig. 6.18 B).

The results for the MFGM proteins show that a marked increase in the total MFGM protein concentration occurred after evaporation. Preheating increased the MFGM proteins slightly (Table 6.4). This increase involved an increase in the amounts of both caseins and whey proteins (β-lg and α-la) (Fig. 6.19 and Fig. 6.20), whereas the increase in the MFGM protein of the preheated milk samples could be attributed mainly to apparently higher β-lg content, although some caseins were also observed at the MFGM (Fig. 6.19 and Fig. 6.20).

No change was observed in the major native MFGM proteins (xanthine oxidase and butyrophilin) after preheating and evaporation. A decrease in the amounts of PAS 6 and PAS 7 was observed in the preheated samples. An increase in the intensity of the SC band (Mr ~75 kDa) also occurred after evaporation (Fig. 6.19).

It is concluded from these results that the behaviour of the fat globules during preheating and evaporation in industrial manufacture is similar to that seen in the pilot plant trials.

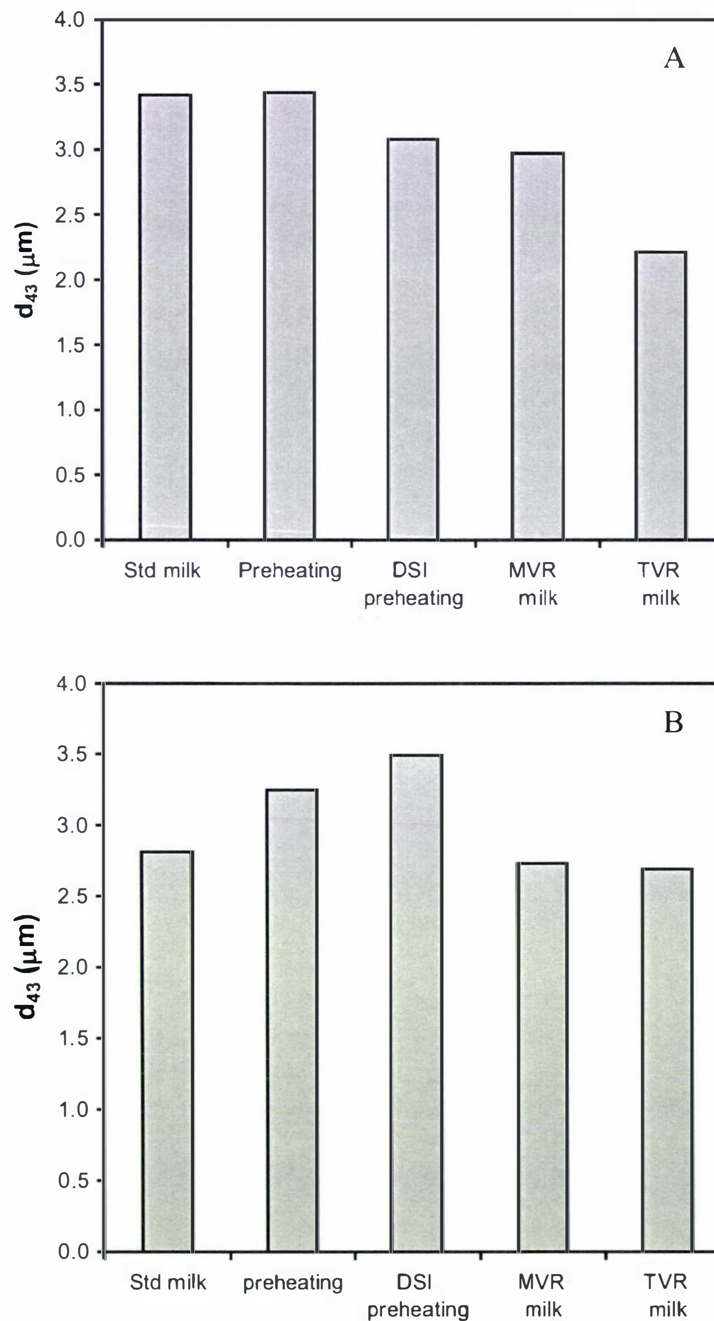


Fig. 6.18. The average size (d_{43}) of the fat globules in milk samples obtained before and after evaporation. The fat globule size was determined after dispersion in SDS and ETDA buffer (A) or in water (B). The samples were obtained from the Fonterra Co-operative Group, Hawera.

Table 6.4. The total protein concentration at the fat globule surface of whole milk before and after evaporation

Sample	Total solids (%)	Total MFGM protein (mg/g fat)	Surface protein coverage of MFG (mg/m^2)
Standardized milk	10.05	7.11	1.34
Pasteurized milk	10.10	7.53	1.38
Preheated milk	10.26	9.68	1.82
DSI preheated milk	10.34	10.33	1.79
MVR concentrate	34.30	23.23	3.42
TVR concentrate	45.32	27.22	3.89

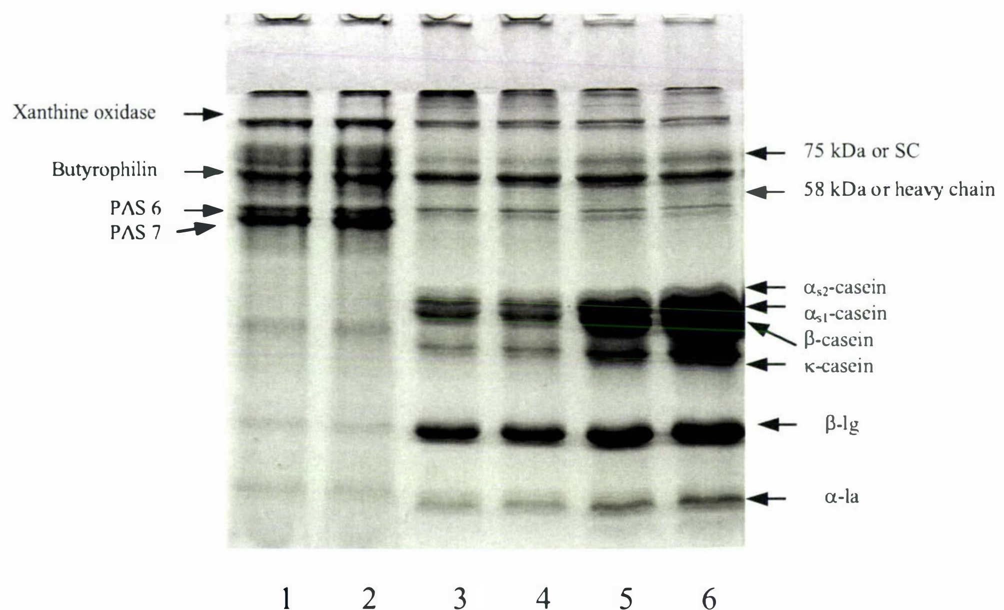


Fig. 6.19. SDS-PAGE patterns (15% acrylamide gel) under reducing conditions of the MFGM material isolated from milk samples obtained before and after evaporation. Lane 1, standardized milk; lane 2, pasteurized milk; lane 3, preheated milk, lane 4, DSI-preheated milk; lane 5, after MVR evaporation; lane 6, after TVR evaporation.

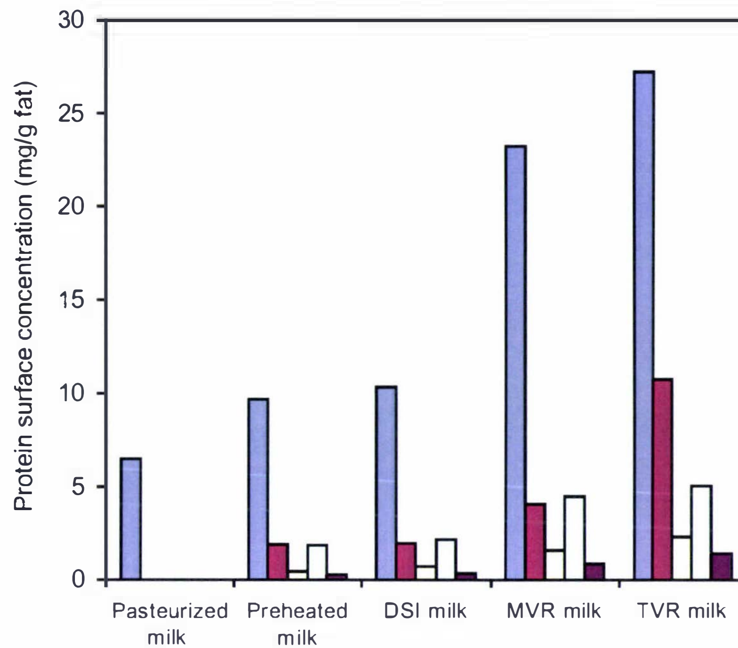


Fig 6.20. The protein concentrations (mg/g fat) at the MFGM of milk samples obtained before and after evaporation. The samples were obtained from the Fonterra Co-operative Group. ■: total surface protein; ■: caseins; ■: κ-casein; : β-lg; ■: α-la.

6.5. Discussion

6.5.1. Disruption of fat globules during evaporation of milk

It is not obvious what caused the disruption of the fat globules during the evaporation of milk. There is a considerable difference in energy input between the evaporation and homogenization processes. However, there may be some similarity between the mechanisms for the disruption of the fat globules during evaporation and homogenization. It is well known that, during homogenization, the fat globules may be disrupted by turbulence, cavitation and the presence of air bubbles (Mulder & Walstra, 1974). In a turbulent flow, according to Kolmogorov, the size of the smallest eddies is given by $d_{vs} \approx$ const. $(\gamma/\rho)^{3/5}\varepsilon^{-2/5}$, where ρ is the mass density of the liquid, ε is the energy density and γ is the interfacial tension. Milk fat globules of 4 μm diameter can be disrupted if ε is at least 10^8 W m^{-3}, which is a very high density (Walstra, 1995). However, when the density of the liquid, ρ, increases (e.g. with an increase in the concentration of the milk during evaporation), the possibility of disruption may effectively be increased.

A large amount of vapour is removed during evaporation; cavitation caused by vapour evaporation is the most likely reason for the disruption of the fat globules. According to Bernoulli's law (local pressure + 1/2 ρv^2 = external pressure), with a high liquid velocity (v) and a high density (ρ), a negative pressure would be produced. Because negative pressure cannot persist under the prevailing circumstances, cavitation sets in, i.e. the formation and collapse of small vapour bubbles. Collapse causes high pressure gradients, which may cause the fat globules to disrupt (Mulder & Walstra, 1974). As the density of milk increases with an increase in the total solids, the cavitation increases. This may explain why disruption of the fat globules increased with an increase in the total solids of milk.

In addition, air bubbles may also play a part in the disruption of the fat globules during the evaporation of milk. When the spreading pressure $P_s = \gamma_{(water\text{-}air)} - [\gamma_{(oil\text{-}water)} + \gamma_{(oil\text{-}air)}] > 0$, γ is the interfacial tension, an oil globule coming into contact with a water-air

interface will spread over that interface. The thin layer of spread oil is easily disrupted if the air bubble is disrupted, as occurs during agitation. The air bubbles may be incorporated when the milk enters the evaporator. However, there is little available information on the influence of milk concentration on the surface tension of milk, and the effect of the concentration or viscosity of milk on the spreading pressure P_s is complicated (Mulder & Walstra, 1974).

The aggregation of milk fat globules that occurs during the 1st and 2nd effects of evaporation and by preheat treatment by DSI probably arises from the sharing of casein micelles by fat globules. The presence of casein micelles at the fat globule surface of concentrate was demonstrated by surface protein composition and electron microscopy observations (Fig. 6.9, Fig. 6.16 and Fig 6.17). In principle, when the surface-active material is relatively scarce, two fat globules may share one or more casein micelles at their interfaces. However, there is sufficient surface-active material in the milk system. It is assumed that the disruption of the fat globules under these conditions is due to the cavitation or air bubble mechanisms as discussed above. When fat globules simultaneously penetrate the air-plasma interface, compression of the air-plasma interface due to the decrease in its area pushes the globules together and aggregates of fat globules (clumps) are formed (Mulder & Walstra, 1974). In this situation, the ratio of surface-active material (i.e. casein micelles and whey proteins) to fat is relatively low at the interface; hence sharing of the casein micelles at the surface of fat globules may occur.

In the final effect of the evaporation process, because much smaller amounts of the vapour and air bubbles are produced, the disruption of the fat globules may be caused mainly by the turbulent flow as a result of the high density (ρ) of the concentrated milk. The aggregated fat globules may be disintegrated into individual globules by the forces in the turbulent flow.

Milk fat globules were also disrupted during the preheating by DSI at 95 °C for 20 s (Fig. 6.6), in agreement with the results of previous workers (Ramsey & Swartzel, 1984; Melsen & Walstra, 1989; van Boekel & Folkerts, 1991). Heavy turbulence at the steam

inlet and cavitation caused by flash cooling are considered most likely to disrupt fat globules in DSI processes (van Boekel & Folkerts, 1991).

6.5.2. Change in MFGM during evaporation of milk

In the non-preheated samples, the increase in surface protein concentration (about four-fold) is attributed mainly to the increase in the amount of caseins adsorbed on to the newly formed interface due to disruption of the fat globules during evaporation; only small amounts of β-lg (~3% in total) and no α-la were observed at the surface of the fat globules in the final concentrate (Fig. 6.9). This is similar to a typical surface layer of a fat globule in homogenized milk. Casein micelles are adsorbed preferentially over whey proteins when the plasma proteins are adsorbed on to the fat interface during homogenization (Mulder & Walstra, 1974; Darling & Butcher, 1978). The large increase in surface protein concentration in the final concentrate (Fig. 6.3 and Table 6.2) is probably a result of adsorption of intact casein micelles, whereas sharing of some casein micelles by fat globules occurs in the milk after the 1st and 2nd effects. In addition, the increase in the size of the casein micelles with the concentration of milk (Walstra, 1995) may be responsible for the high surface protein concentration in the final concentrate.

A small amount of β-lg observed at the interface of the fat globules in the non-preheated concentrate samples (Fig. 6.9 and Fig. 6.11) may have resulted from association of β-lg with the native MFGM proteins induced by heating, as the milk was heated to ~70 °C prior to entry into the evaporator. It has been found that the interactions between β-lg and native MFGM proteins may occur at temperatures lower than 70 °C (Chapter 4 and 5).

Considerable association of β-lg (~2 mg/g fat) with the fat globule surface in the preheated milk (95 °C, 20 s) and its increased association during evaporation (Fig. 6.9 and Fig 6.10) may be attributed to two different mechanisms: (1) β-lg interaction with the native MFGM proteins via disulfide bond formation; (2) β-lg adsorption on to the surface of the fat globules due to disruption of the globules. Previous studies (Corredig & Dalgleish, 1996; Chapter 5) have reported that, upon heat treatment of whole milk,

the maximum amount of β-lg that can be associated with the MFGM is about 1 mg/g fat. As the concentration of β-lg adsorbed was over this maximum amount, it is likely that the β-lg was adsorbed on to the newly created surface of the fat globules. This probably arose largely from the adsorption of casein micelles, to which β-lg was associated as a result of heat treatment, although a small amount of β-lg may have been directly adsorbed on to the interface.

Washing experiments (Fig. 6.11) suggested that only the κ-casein in the casein micelle was directly adsorbed at the surface when casein micelles adsorbed to the surface. This implies that the casein micelles are not disintegrated during evaporation and are subsequently adsorbed on to the fat interface as intact micelles.

The increased adsorption of β-lg after heat treatment of the concentrate (Fig. 6.9 and Fig 6.10) may be attributed to the association of β-lg with the casein micelles that had already been adsorbed at the interface of the fat globules during evaporation. Sharma & Dalgleish (1994) reported that the association of β-lg with fat globule membrane in milk that was first homogenized and then heated was greater than that in milk homogenized after preheating. They suggested that β-lg may bind with κ-casein already adsorbed at the fat globule surface as oligomers and that more binding sites for the whey proteins become available as the casein micelles are adsorbed at the fat surface. The observed increase in the amount of α-la on the fat globule surface of the preheated final concentrate after heating at 79 °C indicates that β-lg and α-la can further associate with the surface proteins as β-lg/α-la complexes.

It was found that the amounts of the original MFGM proteins did not change significantly with the decrease in the fat globule size during evaporation. This suggests that the native MFGM proteins are not removed from the fat globules when they are disrupted to smaller globules. Proteins in milk serum are adsorbed on to newly formed surface and they do not displace the MFGM proteins. Changes in the amounts of some MFGM proteins appeared to occur only when the milk was heated. A decrease in the amount of PAS 6 and PAS 7 in the MFGM was observed when milk was heated to over 70 °C (Chapters 4 and 5). Some previous studies have demonstrated that a part of the

original membrane material is released into the serum under conditions, such as agitation and homogenization (Mulder & Walstra, 1974; Mangino & Brunner, 1975; Darling & Butcher, 1978). It was suggested that homogenization would disrupt the membrane and release the components into the serum phase because the protein components with Mr greater than 50 kDa were found in the serum phase after homogenization (Mangino & Brunner, 1975; Darling & Butcher, 1978). However, Keenan et al. (1983 b) reported that xanthine oxidase and butyrophilin remained associated with the lipid globules in homogenized milk.

The presence of Igs components at the surface of the fat globules (~5%) after disruption (Fig. 6.9 and Fig. 6.11) is attributed to its direct adsorption during evaporation. It has been reported that the Igs, particularly IgM and SIgA, are considerably hydrophobic and have high affinity for fat (Frenyo et al., 1987).

CHAPTER 7

CHANGES IN THE FAT GLOBULES AND MEMBRANE PROTEINS DURING HOMOGENIZATION AND HEAT TREATMENT OF MILK CONCENTRATE PRIOR TO SPRAY DRYING

7.1. Introduction

Homogenization of milk reduces the average fat globule size, with a corresponding increase in the surface area by approximately 10 times (Mulder & Walstra, 1974). The creation of a new fat-serum interface results in the adsorption of surface-active material from the serum phase, especially caseins either as semi-intact micelles or as micellar fragments, thus forming a new membrane (Darling & Butcher, 1978; Walstra & Oortwijn, 1982). The composition of this membrane makes a significant contribution to the physical properties of many dairy products (Anderson, et al., 1977; McCrae & Muir, 1991; McKenna et al., 1999).

The composition of this membrane differs largely because of different processing treatments. An increase in the homogenization pressure increases the protein load on the fat globule surface but the protein load decreases as the temperature of homogenization increases (Walstra, 1995; Cano-Ruiz & Richter, 1997). However, the protein load is higher when heated milk (> 80 °C) is homogenized (Walstra, 1995), because casein micelles may coalesce into large micelles during heating (Mohammad & Fox, 1987; Dalgleish et al, 1987; Singh & Creamer, 1991). Sharma & Dalgleish (1994) reported that the protein load and the composition of surface protein were dependent on whether milks were heated before or after homogenization. Their results indicated that the amount of whey proteins present at the fat globule membrane was lower in milk that was heated before homogenization than in milk that had been homogenized and then heated. The whey proteins interact with native MFGM proteins when the milk is heated (Dalgleish & Banks, 1991), but they also interact with the adsorbed caseins when the homogenized milk is heated (Sharma & Dalgleish, 1993). The interaction of denatured β-lg with the fat globules may occur through intermolecular disulfide bond formation with the κ-casein of intact casein micelles and/or large fragments of micelles adsorbed

at the surface of the fat globules (Singh et al., 1996). In addition, the whey proteins may interact with other whey proteins that are already adsorbed at or associated with the fat globule surface (Singh et al., 1996).

In normal casein micelles, κ-casein, located at the surface, exercises a stabilizing effect upon the native micelles and prevents them from coagulating (Dalgleish, 1992). When intact casein micelles and large micellar fragments are adsorbed, the fat globules tend to behave as large micelles (Walstra, 1995). As in the case of normal micelles, the major stabilizing component for the fat globules may still be the κ-casein of the adsorbed casein micelles. Furthermore, any interactions of whey proteins with casein micelles induced by heat treatment are also through the interaction with κ-casein (Sharma & Dalgleish, 1993; Singh et al., 1996; Chapter 6). The observation that, in many instances (Darling & Butcher, 1978; Walstra, 1995; Sharma et al., 1996), the adsorbed casein micelles are partly spread at the fat surface into smaller fragments indicates that κ-casein may redistribute across the surface of the fat globules. There is some evidence (Sharma & Dalgleish, 1993; Sharma et al., 1996) to show that some κ-casein adsorbs directly at the fat-water interface. However, the exact location and the conformation of κ-casein at the fat globule surface are unknown. It is not known clearly whether or not the adsorption of κ-casein at the surface influences the interaction with serum proteins following heat treatment.

Most of the previous work on the influence of homogenization conditions on the fat globules has been carried out on whole milk. No information for highly concentrated milks (> 45%) is available. Homogenization of concentrated milk both before and after heating is used in the manufacture of whole milk powder. Walstra (1995) considered that homogenized evaporated milk may have globules with very thick layers because casein micelles may coalesce into large micelles in concentrated milk. The differences in the fat globule surface proteins, as a result of the differences in the conditions of homogenization, have been shown to influence the reconstitution properties of whole milk powder in water (Mol, 1975; McKenna et al., 1999).

The objective of this chapter was to examine the effects of homogenization conditions (temperature, pressure and placement of the homogenization step, i.e. before or after heat treatment) on the milk fat globules and the MFGM proteins of both non-preheated and preheated concentrates.

7.2. Milk fat globule size

Tables 7.1 and 7.2 show the average fat globule size (d_{43}) of non-preheated and preheated (95 °C, 20 s) concentrates after homogenization at different pressures and temperatures. Homogenization of the concentrates decreased the average fat globule size. An increase in the homogenization pressure caused a further slight decrease in d_{43}. Preheating (95 °C, 20 s) of the milk before evaporation did not significantly influence the fat globule size during homogenization. The homogenization temperature (50, 65 or 79 °C) had no significant effect on the d_{43} of homogenized concentrate.

Slightly higher d_{43} values of the concentrates, when measurements were carried out in the absence of SDS and EDTA, indicate that some fat globules aggregated into clusters by protein bridging after homogenization of the concentrates.

7.3. Changes in surface protein concentration

7.3.1. Heating the concentrate before homogenization

Tables 7.3 and 7.4 show the total MFGM protein (mg/g fat) and the surface protein concentration (mg/m^2) of concentrates before and after homogenization at different pressures (40 and 70 bar) and temperatures (50, 65 and 79 °C). Homogenization of the concentrates caused an increase in the surface protein concentration from ~4 mg/m^2 to ~6 mg/m^2 at 40 bar and to ~7 mg/m^2 at 70 bar in the non-preheated samples (Table 7.3). In the preheated concentrates (Table 7.4), the surface protein concentration increased from ~6 mg/m^2 to ~7 mg/m^2 at 40 bar and to ~8 mg/m^2 at 70 bar depending on the temperature of homogenization. The surface protein concentrations were higher when the concentrate was homogenized at 65 or 79 °C than when it was homogenized at 50 °C at both 40 and 70 bar, but there was no significant difference between 65 and 79 °C.

Table 7.1. Average size (d_{43}) of the milk fat globules in non-preheated concentrate (total solids about 49%) homogenized at different pressures and temperatures

Variable		Milks dispersed in SDS/EDTA buffer	Milks dispersed in water
		d_{43} (μm)	d_{43} (μm)
Concentrate		1.84	2.37
Pressure 40 bar	Temperature (°C)		
	50	1.31	1.53
	65	1.39	1.56
	79	1.18	1.54
Pressure 70 bar			
	50	1.07	1.65
	65	1.16	1.50
	79	1.09	1.51

Table 7.2. Average size (d_{43}) of the milk fat globules in preheated (95 °C for 20 sec) concentrate (total solids about 50%) homogenized at different pressures and temperatures

Variable		Milks dispersed in SDS/EDTA buffer	Milks dispersed in water
		d_{43} (μm)	d_{43} (μm)
Concentrate		1.77	2.06
Pressure 40 bar	Temperature (°C)		
	50	1.34	1.42
	65	1.30	1.32
	79	1.31	1.34
Pressure 70 bar			
	50	1.22	1.33
	65	1.13	1.21
	79	1.07	1.22

Table 7.3. Total surface protein concentration of the milk fat globules in non-preheated concentrate (total solids about 49%) homogenized at different pressures and temperatures

Concentrate	Temperature (°C)	Total MFGM protein (mg/g fat)	Surface protein coverage (mg/m²)
	50	33.4	4.00
	65	31.8	3.91
	79	41.9	5.50
Homogenized at 40 bar	50	44.8	4.96
	65	50.8	5.71
	79	54.2	5.97
Homogenized at 70 bar	50	54.2	5.53
	65	68.2	6.99
	79	65.9	6.60

Table 7.4. Total surface protein concentration of milk fat globules in preheated (95 °C for 20 s) concentrate (total solids about 50%) homogenized at different pressures and temperatures

Concentrate	Temperature (°C)	Total MFGM protein (mg/g fat)	Surface protein coverage (mg/m²)
	50	51.1	5.90
	65	52.0	6.08
	79	61.8	7.20
Homogenized at 40 bar	50	60.5	6.70
	65	61.1	7.01
	79	59.9	7.10
Homogenized at 70 bar	50	64.8	6.89
	65	77.1	7.98
	79	74.1	7.82

The surface protein concentrations of the preheated concentrated milks after homogenization were 1-2 mg/m^2 higher than those of the non-preheated samples in most cases (Tables 7.3 and 7.4).

The higher surface protein concentration in the homogenized preheated concentrates compared with the non-preheated samples is attributed mainly to the association of whey proteins with the MFGM. This association could be through direct interaction of whey proteins with MFGM proteins during preheating (Dalgleish & Banks, 1991); or the whey proteins could first associate with casein micelles during preheating, and then the casein micelle/whey protein complexes could adsorb on to the surface of the fat globules during homogenization.

7.3.2. Heating the concentrate after homogenization

No change in the surface protein concentration was observed when both non-preheated and preheated concentrates were heated to 65 °C after homogenization (Tables 7.5 and 7.6). However, heating at 79 °C caused an increase (about 1 mg/m^2) in the surface protein concentration for both non-preheated and preheated concentrates.

Comparison of the surface protein concentrations in the concentrate that was heated at 79 °C before (Tables 7.3 and 7.4) or after (Tables 7.5 and 7.6) homogenization at 70 bar showed that the surface protein concentrations were significantly higher in concentrates that were heated after homogenization. The higher surface protein concentration in the homogenized samples that were heated to 79 °C was due to the association of whey proteins with casein micelles adsorbed at the surface. Sharma & Dalgleish (1993) also reported that the amounts of whey proteins present in the fat globule membrane were greater in milk that had been homogenized and then heated than in milk that was heated before homogenization.

Table 7.5. Total surface protein concentration of milk fat globules in the non-preheated concentrates (total solids about 49%) that were heated at different temperatures after homogenization.

	Temperature (°C)	Total MFGM protein (mg/g fat)	Surface protein coverage (mg/m^2)
Homogenized concentrate (40 bar)		44.8	4.96
	65	42.9	4.77
	79	55.9	5.76
Homogenized concentrate (70 bar)		54.2	5.53
	65	59.9	6.09
	79	77.3	7.81

Table 7.6. Total surface protein concentration of milk fat globules in preheated (95 °C, 20 s) concentrates (total solids about 50%) that were heated at different temperatures after homogenization

	Temperature (°C)	Total MFGM protein (mg/g fat)	Surface protein coverage (mg/m^2)
Homogenized concentrate (40 bar)		60.5	6.70
	65	61.2	6.96
	79	69.8	7.68
Homogenized concentrate (70 bar)		64.8	6.95
	65	65.4	6.98
	79	76.8	8.15

7.4. Protein composition of the fat globule surface of the concentrate after homogenization

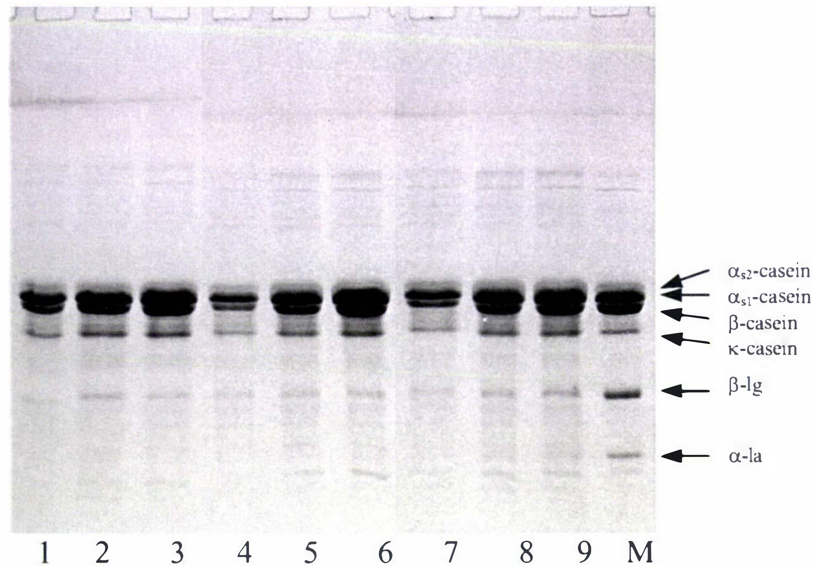
7.4.1. Cream washed with SMUF solution

7.4.1.1. Heating before homogenization

SDS-PAGE of the fat globule surface material isolated from cream washed three times with SMUF showed visible differences in the intensities of the β-lg and α-la bands between the non-preheated and preheated samples (Fig. 7.1). Only a faint β-lg band and no α-la band were observed in the patterns of non-preheated samples, even for samples homogenized at 79 °C (Fig. 7.1 A), whereas a clear β-lg band and a faint α-la band were seen in the patterns of all preheated samples. The intensities of the β-lg and α-la bands were higher in the samples homogenized at 79 °C (Fig. 7.1 B). In addition, the intensity of the α_{s2}-casein band was stronger in the patterns of the preheated samples than in the patterns of the non-preheated samples.

Data (Tables 7.7 and 7.8) on the protein composition of the fat globule surface were obtained from quantitative analysis of the SDS-PAGE patterns of the fat globule membrane material. There were no significant differences in the surface protein composition of non-preheated concentrates before and after homogenization at a given temperature, except for a decrease in the native MFGM proteins. Changes in the homogenization pressure (40 or 70 bar) and temperature (50, 65 or 79 °C) did not significantly affect the relative proportions of the surface proteins. Caseins constituted approximately 85% of the total surface protein, and < 5% of the surface protein was whey proteins when non-preheated concentrate was homogenized (Table 7.7). However, when the preheated concentrates were homogenized, the proportion of whey proteins at the fat globule surface increased to > 20% (Table 7.8). Higher proportions of α-la (~7.0%) were found at the surface of preheated samples that were homogenized at 79 °C than at the surface of preheated samples that were homogenized at 50 and 65 °C (Table 7.8).

(A)



(B)

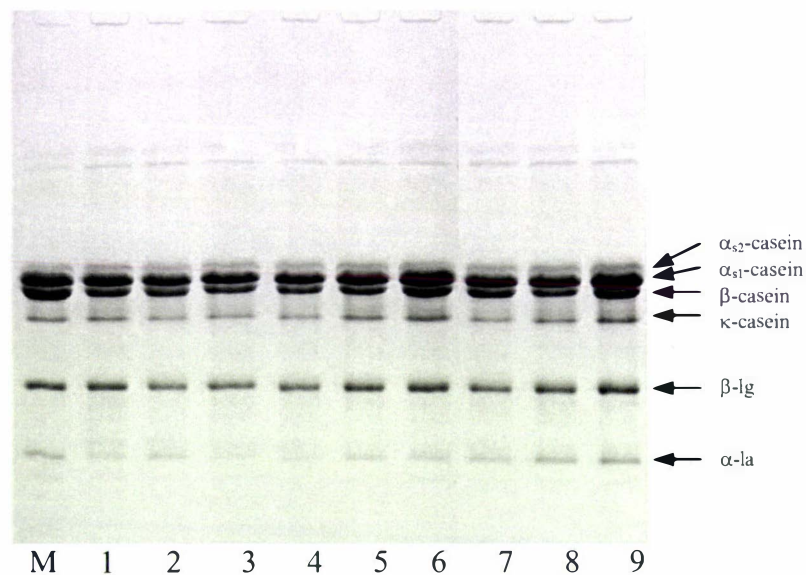


Fig. 7.1. SDS-PAGE patterns (15% acrylamide gel), under reducing conditions, of fat globule surface material, isolated from non-preheated (A) or preheated (B) concentrates before or after homogenization. Cream obtained from the concentrates was washed in SMUF. The surface material was diluted 24 times with sample buffer. Lane 1, concentrates at 50 °C, lane 2, concentrates homogenized at 40 bar and 50 °C; lane 3, concentrate homogenized at 70 bar and 50 °C; lane 4, concentrate at 65 °C; lane 5, concentrate homogenized at 40 bar and 65 °C; lane 6, concentrate homogenized at 70 bar and 65 °C, lane 7, concentrates at 79 °C; lane 8, concentrates homogenized at 40 bar and 79 °C; lane 9, concentrates homogenized at 70 bar and 79 °C; M, whole milk.

Table 7.7. Percentage distribution of proteins at the milk fat globule surface of non-preheated concentrate (total solids about 49%) homogenized at different pressures and temperatures

Concentrate	Temperature (°C)	Others (including native MFGM proteins) (%)	Caseins (%)	β-lg (%)	α-la (%)
	50	17.77	78.39	3.27	0.56
	65	18.26	77.11	3.95	0.68
	79	16.52	78.47	4.33	0.68
Homogenized at 40 bar					
	50	12.13	81.51	5.18	1.18
	65	11.24	84.97	3.21	0.57
	79	10.77	83.56	4.20	1.47
Homogenized at 70 bar					
	50	11.01	84.91	3.30	0.77
	65	10.38	86.54	2.39	0.68
	79	10.57	84.99	3.86	0.57

Table 7.8. Percentage distribution of proteins at the milk fat globule surface of preheated (95 °C, 20 s) concentrate (total solids about 50%) homogenized at different pressures and temperatures.

Concentrate	Temperature (°C)	Others (including native MFGM proteins) (%)	Caseins (%)	β-lg (%)	α-la (%)
	50	14.37	67.69	15.39	2.55
	65	15.54	66.53	14.92	3.11
	79	13.55	65.01	16.40	5.22
Homogenized at 40 bar					
	50	10.12	67.32	19.59	2.97
	65	9.42	69.99	17.31	3.29
	79	9.91	65.08	17.18	7.82
Homogenized at 70 bar					
	50	9.89	68.24	18.68	3.19
	65	9.27	72.2	16.14	2.40
	79	9.32	68.79	15.60	6.28

7.4.1.2. Heating after homogenization

Both non-preheated and preheated concentrates, homogenized at 50 °C, were heated to 65 and 79 °C. Apparent increases in the intensity of the β-lg and α-la bands were observed in the SDS-PAGE patterns of surface material isolated from samples that were heated to 79 °C (Fig. 7.2). Heating the non-preheated homogenized concentrates to 79 °C caused an increase in the percentages of β-lg and α-la (Table 7.9). In preheated homogenized concentrates, heating did not increase the percentage of β-lg, but did increase the percentage of α-la (Table 7.10).

The proportions of β-lg and α-la in the non-preheated concentrates heated at 79 °C after homogenization (Table 7.9) were higher than those in the non-preheated concentrates heated at 79 °C before homogenization (Table 7.7). However, the proportions of β-lg and α-la in the preheated concentrates heated after homogenization (Table 7.10) were similar to those in the preheated concentrates heated before homogenization (Table 7.8).

These results indicate that the increases in the total surface protein by heating could be attributed not only to binding of β-lg to the fat globule surface; casein micelles were also bound to the surface during heating of the preheated samples. During the heat treatment of homogenized samples, β-lg bound to casein micelles might further associate with the β-lg that had already bound at the fat globule surface as a result of heating and adsorption. This also caused the increase in the amount of caseins at the surface. Meanwhile, α-la was associated with the fat globule surface together with the casein micelles that had already associated with β-lg during preheating (95 °C).

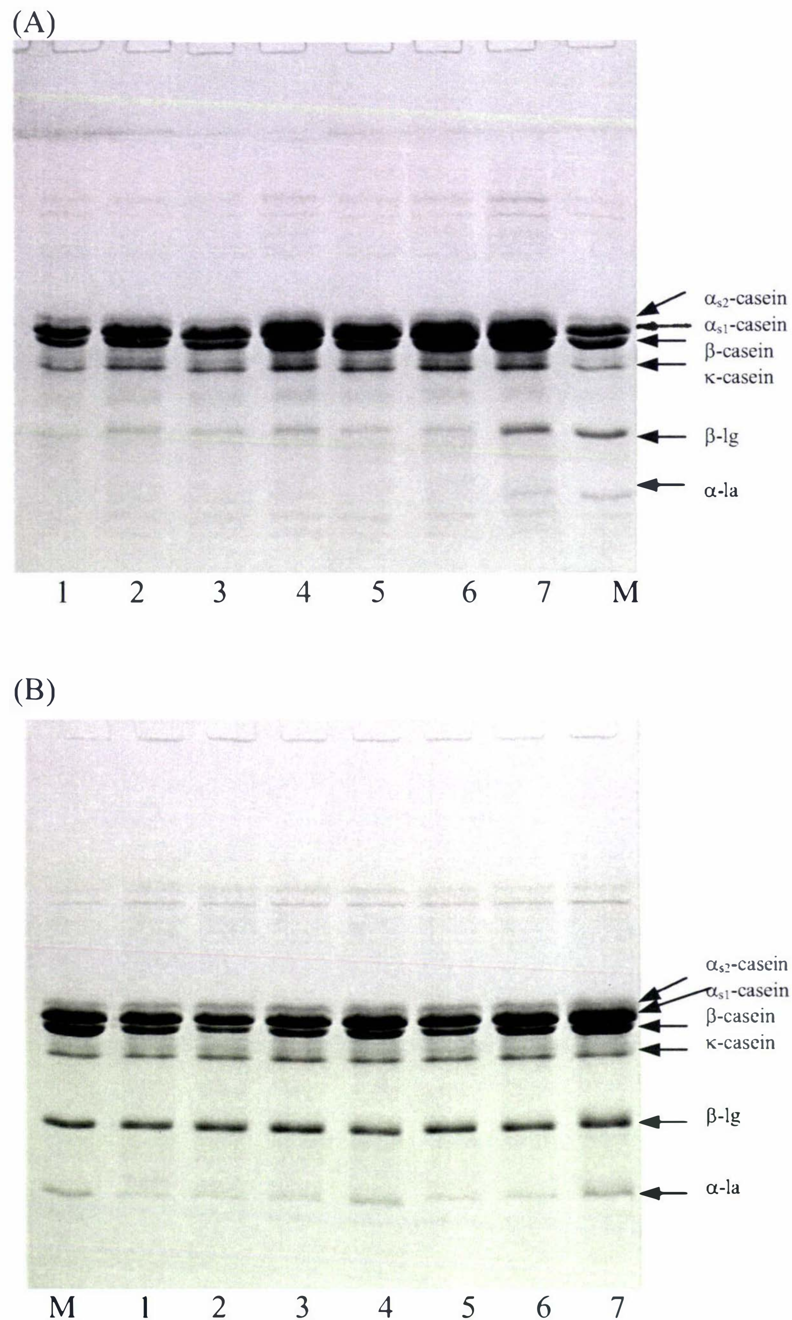


Fig. 7.2. SDS-PAGE patterns (15% acrylamide gel), under reducing conditions, of fat globule surface material, isolated from non-preheated (A) or preheated (B) concentrates before or after homogenization. Cream obtained from the concentrates was washed in SMUF. The surface material was diluted 24 times with sample buffer. Lane 1, concentrate at 50 °C; lane 2, concentrate homogenized at 40 bar and 50 °C; lane 3, concentrate homogenized at 40 bar and then heated to 65 °C; lane 4, concentrate homogenized at 40 bar and then heated to 79 °C; lane 5, concentrate homogenized at 70 bar and 50 °C; lane 6, concentrate homogenized at 70 bar and then heated to 65 °C; lane 7, concentrate homogenized at 70 bar and then heated to 79 °C; M, whole milk.

Table 7.9. Percentage distribution of proteins at the milk fat globule surface of non-preheated concentrate (total solids about 49%) that was heated after homogenization

Pressure (bar)	Temperature (°C)	Others (including native MFGM proteins) (%)	Caseins (%)	β-lg (%)	α-la (%)
40		12.13	81.51	5.18	1.18
	65	11.42	83.08	4.57	0.914
	79	11.93	81.51	5.37	1.19
70		11.01	84.91	3.30	0.77
	65	11.33	85.03	2.95	0.68
	79	11.14	77.18	9.24	2.45

Table 7.10. Percentage distribution of proteins at the milk fat globule surface of preheated (95 °C, 20 s) concentrate (total solids about 50%) which was heated after homogenization

Pressure (bar)	Temperature (°C)	Others (including native MFGM proteins) (%)	Caseins (%)	β-lg (%)	α-la (%)
40		10.12	67.32	19.59	2.97
	65	9.60	69.83	18.02	2.56
	79	9.70	70.36	14.98	4.96
70		9.89	68.24	18.68	3.19
	65	10.05	69.42	17.30	3.24
	79	9.51	66.91	17.02	6.55

7.4.2. Cream washed with a dissociation buffer

When the cream was washed in 6 M urea and 50 mM EDTA buffer, only κ-casein was observed in the SDS-PAGE patterns of homogenized concentrates. The intensity of κ-casein increased in the concentrates homogenized at 70 bar (Fig. 7.3). This indicates that only κ-casein from the casein micelle directly adsorbs on to the fat globules, and that other casein molecules (α_{s1}-, α_{s2}-caseins and β-casein) are not directly adsorbed.

Changes in the homogenization pressure and temperature did not affect the intensity of the bands of the major native MFGM proteins, including xanthine oxidase, butyrophilin, PAS 6 and PAS 7, in the non-preheated samples (Fig. 7.3 A). However, the PAS 6 and PAS 7 bands became very faint in the preheated samples (Fig. 7.3 B). Homogenization at 70 bar at 79 °C enhanced the intensities of the κ-casein, β-lg and α-la bands (Fig. 7.3 B).

Furthermore, the SDS-PAGE patterns showed a protein band with an Mr ~75 kDa and a faint band with an Mr ~58 kDa located between butyrophilin (Mr 66 kDa) and PAS 6 (Mr 50 kDa) in both non-preheated and preheated homogenized samples (Fig. 7.3). The intensity of these two bands in the homogenized samples was higher than that in the concentrate before homogenization and it increased with homogenization pressure from 40 to 70 bar. These proteins were probably the fragments or components of Igs, in which the proteins with Mr ~75 kDa and Mr ~58 kDa are likely to be the secretory component (SC) and heavy chain, respectively (Larson, 1992). The intensities of these bands were fairly similar in the non-preheated and preheated samples (Fig. 7.3). This suggests that these proteins were directly adsorbed at the fat globule surface during homogenization. The bands did not arise from the interactions with MFGM proteins, like the whey proteins, although Igs are the most thermolabile of the major milk proteins (de Wit & Klarenbeek, 1984; Larson, 1992).

The percentage of individual surface proteins after dissociation by urea and EDTA demonstrates the amounts of proteins directly adsorbed at the interface (Table 7.11). It may approximately reflect the percentage of surface area in homogenized milk samples

covered by these proteins. Approximately 40% κ-casein, 30% whey proteins (~7% SC and heavy chain, ~20% β-lg and ~3% α-la) and 30% native MFGM proteins constituted the initial new surface protein layer after homogenization of the non-preheated samples. An increase in the homogenization pressure and temperature caused a decrease in the percentage of total native MFGM proteins and an increase in the percentage of SC and heavy chain at the surface. An increase in the percentage of α-la was observed with an increase in homogenization temperature. However, changes in the homogenization pressure and temperature did not significantly affect the percentages of κ-casein and β-lg at the surface.

When the milk sample was preheated at 95 °C for 20 s, the surface proteins were no longer only in the adsorbed form; markedly higher percentages of β-lg and α-la (~40% and ~10%, respectively) were present at the surface than in the non-preheated samples (Table 7.11). It has been reported that β-lg and α-la are able to associate with the native MFGM proteins via disulfide bonds (Dalgleish & Banks, 1991) during heat treatment. Additional β-lg and α-la at the surface result from the adsorption of κ-casein/β-lg or κ-casein/β-lg/α-la complexes formed during preheating.

7.4.3. Native MFGM proteins after homogenization

The amounts of the major native MFGM proteins in the homogenized non-preheated samples were fairly similar to those in the non-homogenized concentrate (Table 7.12), except that the xanthine oxidase level decreased slightly after homogenization. As in the non-preheated samples, the amounts of these proteins in the preheated samples were not affected by homogenization, although the amounts of PAS 6 (~0.3 mg/g fat) and PAS 7 (~0.1 mg/g fat) were considerably lower than those in the non-preheated samples (Table 7.13).

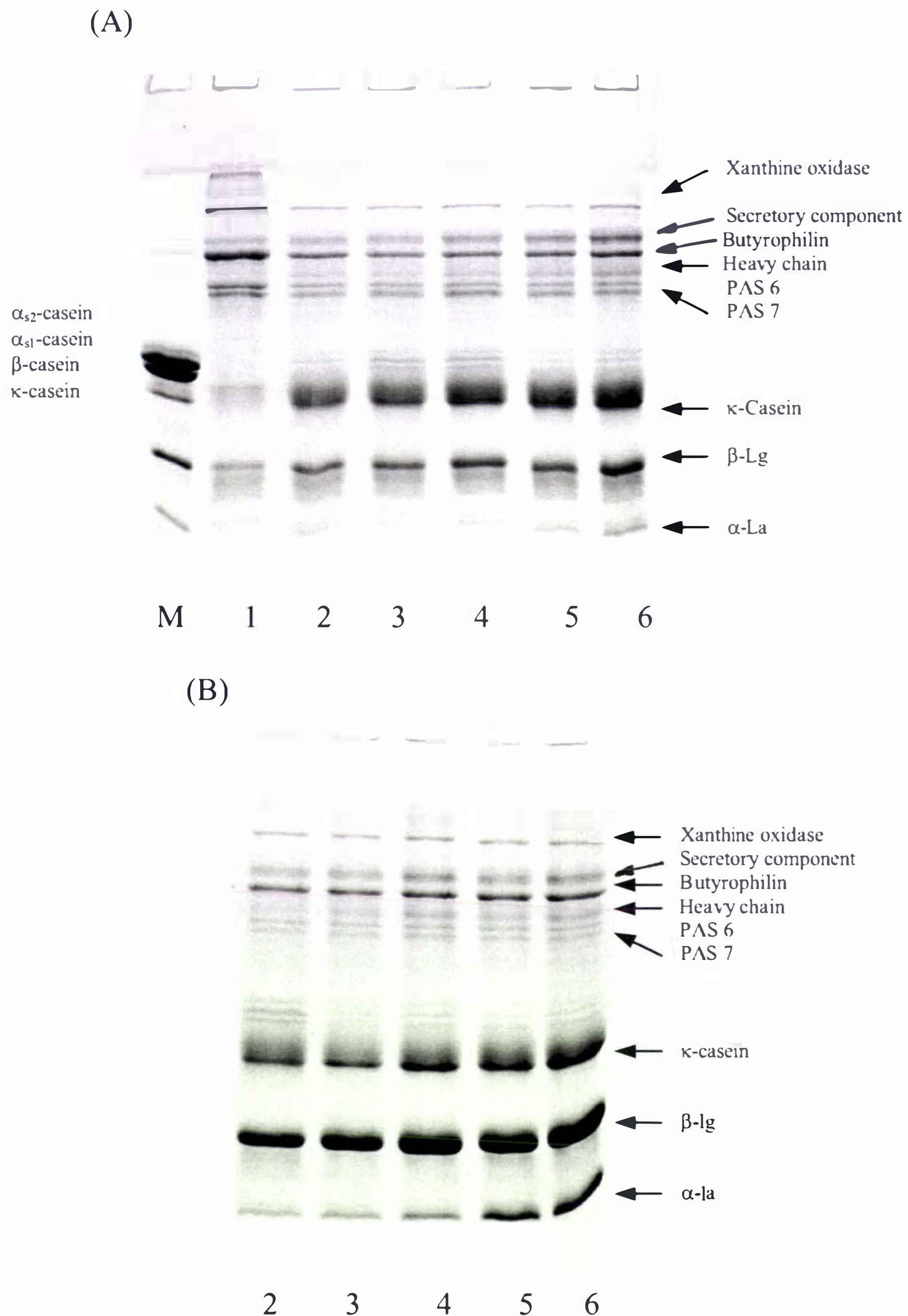


Fig. 7.3. SDS-PAGE patterns (15% acrylamide gel), under reducing conditions, of fat globule surface material isolated from non-preheated (A) or preheated (B) concentrates before or after homogenisation at different pressures and temperatures. Cream obtained from the concentrates was washed in SMUF containing urea and EDTA. The surface material was diluted 6 times with sample buffer. Lane 1, standard milk; lane 2, concentrate; lane 3, concentrate homogenized at 40 bar and 50 °C; lane 4, concentrate homogenized at 70 bar and 50 °C; lane 5, concentrate homogenized at 40 bar and 79 °C; lane 6, concentrate homogenized at 70 bar and 79 °C; M, whole milk.

Table 7.11. Percentage distribution of proteins in the surface protein material isolated from the cream of homogenized concentrated milks, in which the cream was washed with urea and EDTA buffer

Pressure (bar)	Temperature (°C)	Native MFGM proteins (%)	SC and heavy chain (%)	κ-Casein (%)	β-Lg (%)	α-La (%)
Non-preheated samples						
40						
	50	31.3	5.8	43.4	17.3	2.1
	79	25.9	8.5	43.3	18.2	4.1
70						
	50	27.5	6.4	44.5	19.0	2.6
	79	22.7	10.0	43.5	18.9	5.0
Preheated (95 °C, 20 s) samples						
40						
	50	13.9	4.1	27.4	42.8	7.9
	79	11.8	1.8	25.0	44.3	17.0
70						
	50	14.4	5.6	30.5	46.9	8.1
	79	8.1	3.4	26.6	44.3	17.6

7.5. Ratio of individual adsorbed caseins to total caseins at the fat globule surface

The ratios of the individual caseins adsorbed at the fat globule surface to the total adsorbed caseins in the homogenized concentrates are shown in Tables 7.14 and 7.15. The ratios of α_{s1}-casein and κ-casein to total caseins at the fat globule surface were slightly higher than in milk serum, whereas the ratio of β-casein to total caseins was lower than in milk serum. The ratio of α_{s2}-casein to total caseins was not changed after adsorption to the fat globule surface in non-preheated samples, but increased in the preheated samples. The homogenization pressure and temperature did not affect the ratios of individual adsorbed caseins. The differences between the ratios of individual caseins at the surface and in milk plasma indicate that the composition and structure of the casein micelle may have changed after the casein micelles adsorbed on to the fat globules during homogenization.

Table 7.12. The amounts of the major native MFGM proteins in non-preheated concentrate (total solids about 49%) homogenized at different pressures and temperatures

Pressure (bar)	Temperature (°C)	Xanthine oxidase (mg/g fat)	Butyrophilin (mg/g fat)	PAS 6 (mg/g fat)	PAS 7 (mg/g fat)
Concentrate		0.51	1.00	0.50	0.61
40	50	0.39	0.86	0.57	0.68
	65	0.38	0.90	0.55	0.65
	79	0.38	1.00	0.50	0.52
70	50	0.38	0.90	0.53	0.65
	65	0.38	1.1	0.58	0.69
	79	0.39	1.1	0.50	0.65

Table 7.13. The amounts of the major native MFGM proteins in preheated (95 °C, 20 s) concentrate (total solids about 50%) homogenized at different pressures and temperatures

Pressure (bar)	Temperature (°C)	Xanthine oxidase (mg/g fat)	Butyrophilin (mg/g fat)	PAS 6 (mg/g fat)	PAS 7 (mg/g fat)
Concentrate		0.50	1.10	0.25	0.15
40	50	0.43	1.10	0.30	0.10
	65	0.45	1.10	0.31	0.14
	79	0.50	1.25	0.35	0.11
70	50	0.47	1.18	0.30	0.10
	65	0.45	1.20	0.33	0.15
	79	0.50	1.25	0.35	0.11

Table 7.14. The ratios of the individual adsorbed caseins to total caseins at the fat globule surface of non-preheated concentrates homogenized at different pressures and temperatures

Pressure (bar)	Temperature (°C)	α_{s2}-casein /total caseins	α_{s1}-casein /total caseins	β-casein /total caseins	κ-casein /total caseins
40					
	50	0.08	0.46	0.31	0.15
	65	0.07	0.48	0.32	0.13
	79	0.07	0.48	0.32	0.12
70					
	50	0.06	0.49	0.32	0.13
	65	0.06	0.46	0.34	0.13
	79	0.06	0.49	0.33	0.12
Milk		0.07	0.45	0.40	0.09

Table 7.15. The ratios of the individual adsorbed caseins to total caseins at the fat globule surface of preheated (95 °C, 20 s) concentrates homogenized at different pressures and temperatures

Pressure (bar)	Temperature (°C)	α_{s2}-casein /total caseins	α_{s1}-casein /total caseins	β-casein /total caseins	κ-casein /total caseins
40					
	50	0.12	0.49	0.23	0.16
	65	0.11	0.47	0.29	0.14
	79	0.12	0.48	0.26	0.14
70					
	50	0.11	0.48	0.26	0.15
	65	0.10	0.47	0.30	0.13
	79	0.11	0.50	0.26	0.13
Milk		0.07	0.45	0.40	0.09

7.6. CSLM and electron microscopy

CSLM micrographs of the concentrates before and after homogenization at 40 or 70 bar and 50 °C showed that the fat globule size decreased with an increase in the homogenization pressure in both non-preheated and preheated samples (Fig. 7.4). These results are in agreement with the results obtained using the MasterSizer (Tables 7.1 and 7.2). No differences between the samples that were homogenized at 50, 65 and 79 °C were observed in the CSLM micrographs (data not shown).

Transmission electron micrographs of both non-preheated and preheated (95 °C, 20 s) concentrates homogenized at 70 bar showed that both intact and dissociated casein micelles were adsorbed at the fat globule surface (Figs. 7.5, 7.6, 7.7, 7.8 and 7.9), although not all of the fat globule surface was composed of casein micelles. In the concentrates homogenized at 79 °C or heated to 79 °C after homogenization, many adsorbed micelles were joined with the adjacent micelles in the serum to form chains. In the preheated homogenized samples, it was observed that the thread-like or horn-like material between the casein micelles was involved in the formation of these micelle chains (Figs. 7.8 and 7.9). This suggests that the non-micellar material between the casein micelles may be involved in the formation of these casein micelle chains. Hair-like structures or non-micellar materials have been observed on the surface of casein micelles in heated milks (Davies et al., 1978; Mohammed & Fox, 1987) and have been described as κ-casein/β-lg complexes (Singh & Fox, 1987).

In the non-preheated homogenized samples, the amount of thread-like material between the casein micelles was much less although chains of casein micelles were still observed, particularly at the fat globule surface; these chains appeared to be the micelles in contact with each other (Fig. 7.5). McKenna et al. (1999) also reported that the micelles adsorbed at the fat globule surface aggregated with adjacent micelles, and became more electron dense, suggesting that there was some change in the structure of the micelles as they adsorbed to the fat globule surface.

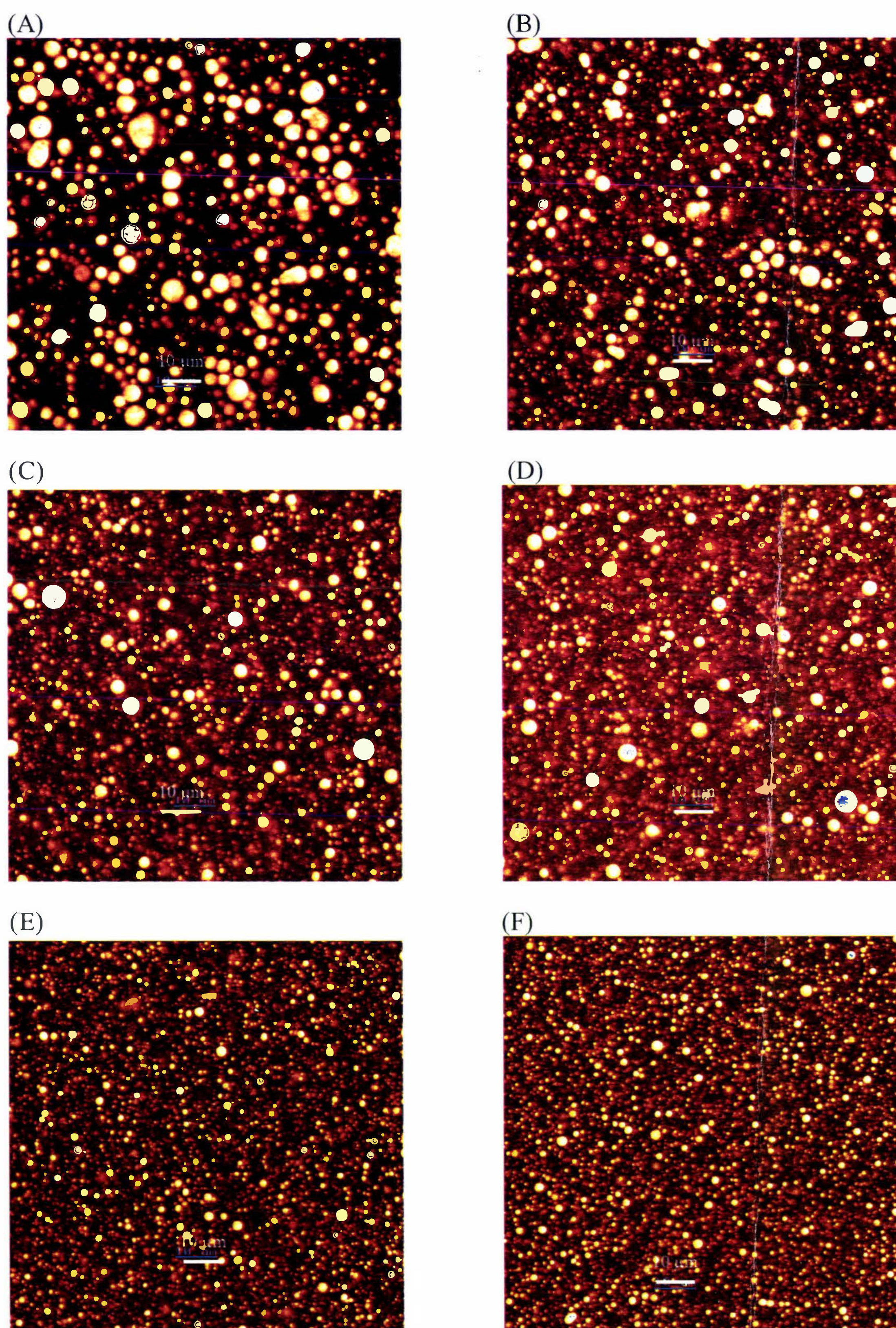


Fig. 7.4. Confocal micrographs of concentrates (50 °C) before (A, B) and after homogenization at 40 bar (C, D) or 70 bar (E, F). Samples A, C and E were non-preheated and the samples B, D and F were preheated at 95 °C for 20 s.

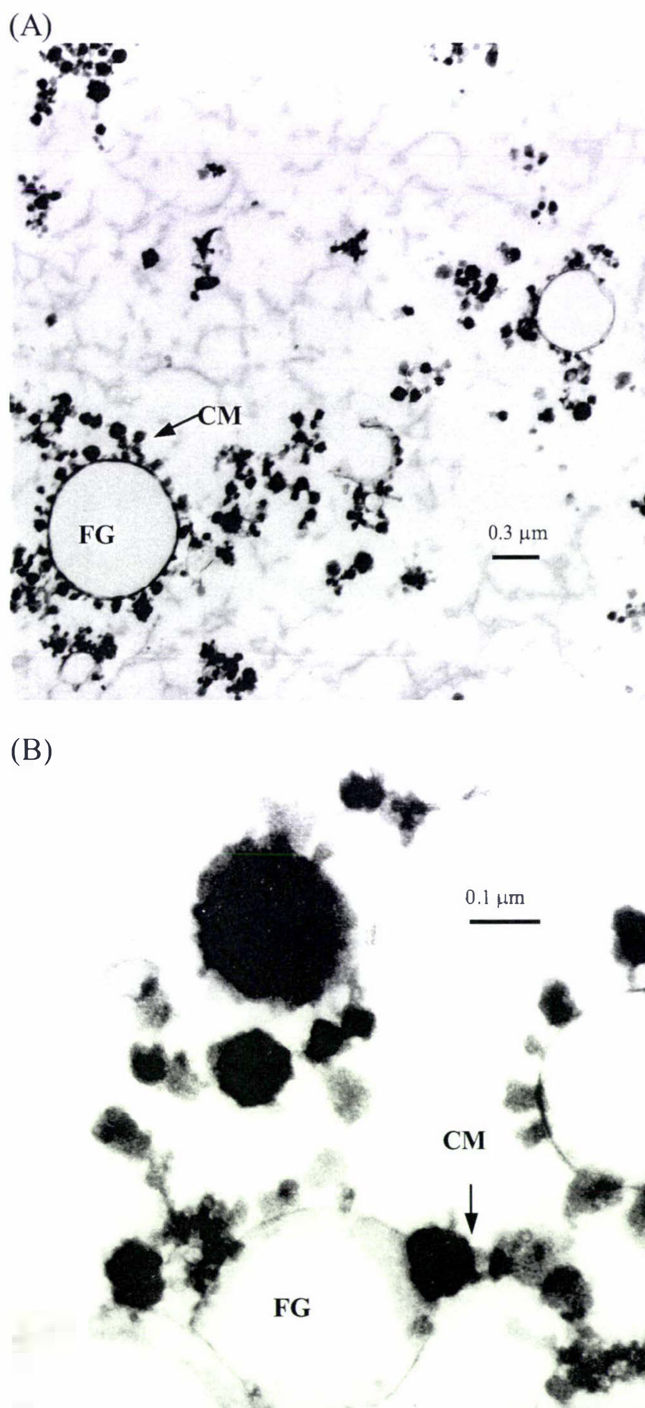


Fig. 7.5. Transmission electron micrographs of non-preheated concentrate, homogenized at 70 bar and 65 °C. FG: fat globule, CM: casein micelle. Magnification: A,×21200; B, ×72100.

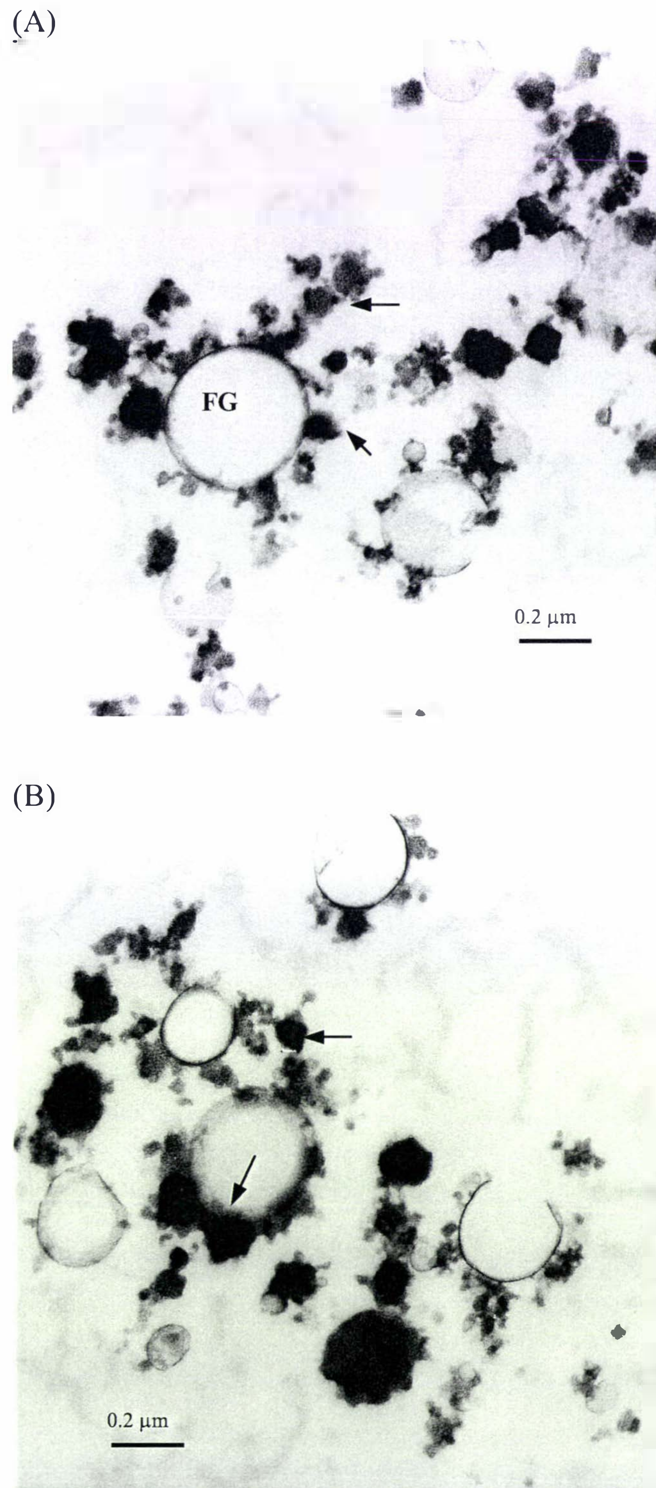


Fig. 7.6. Transmission electron micrographs of non-preheated concentrate, homogenized at 70 bar and 79 °C. FG: fat globule. Magnification: A, B, ×48600.

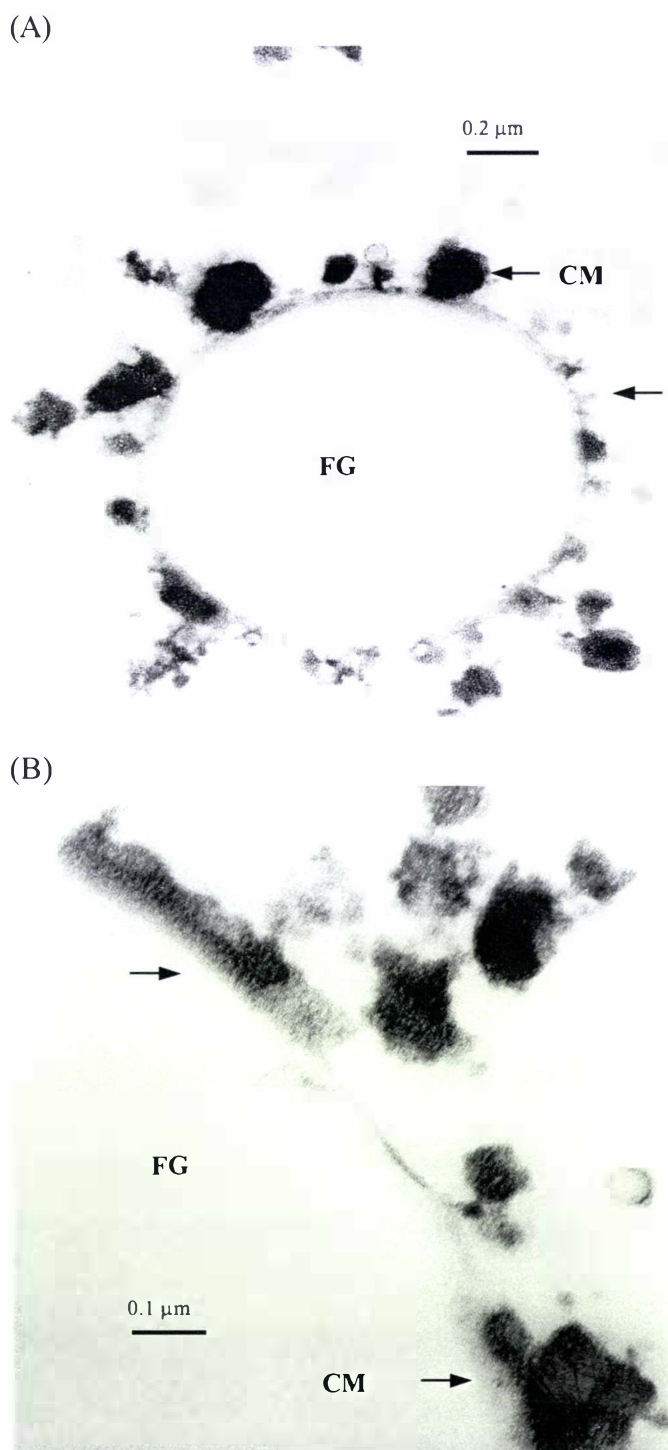
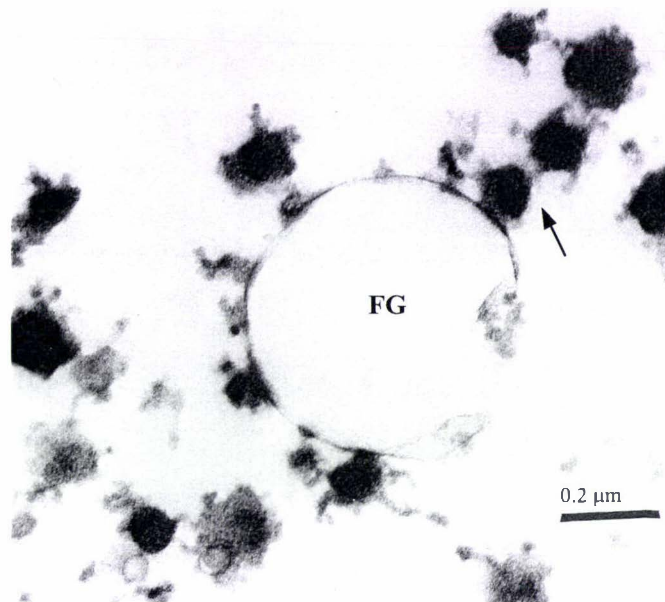


Fig. 7.7. Transmission electron micrographs of non-preheated concentrate, homogenized at 70 bar and then heated to 79 °C. FG: fat globule, CM: casein micelle. Magnification: A,×48600; B, ×103600.

(A)



(B)

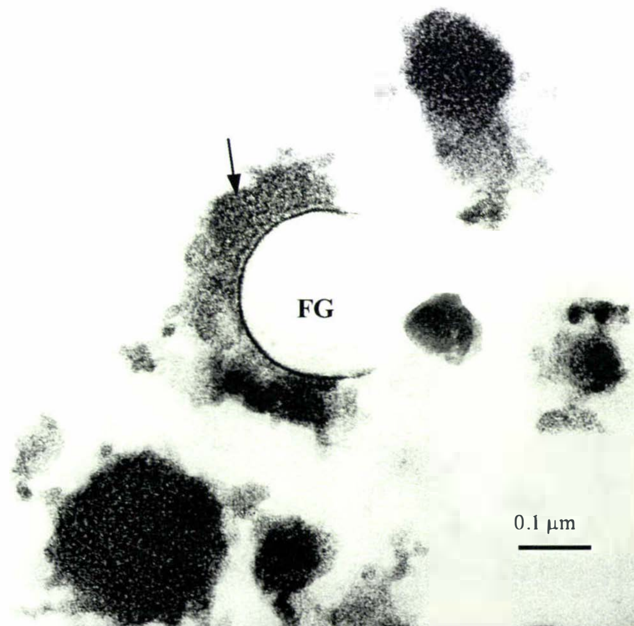


Fig. 7.8. Transmission electron micrographs of preheated (95 °C, 20 s) concentrate, homogenized at 70 bar and 79 °C. FG: fat globule, CM: casein micelle. Magnification: A,×72100, B, ×103600.

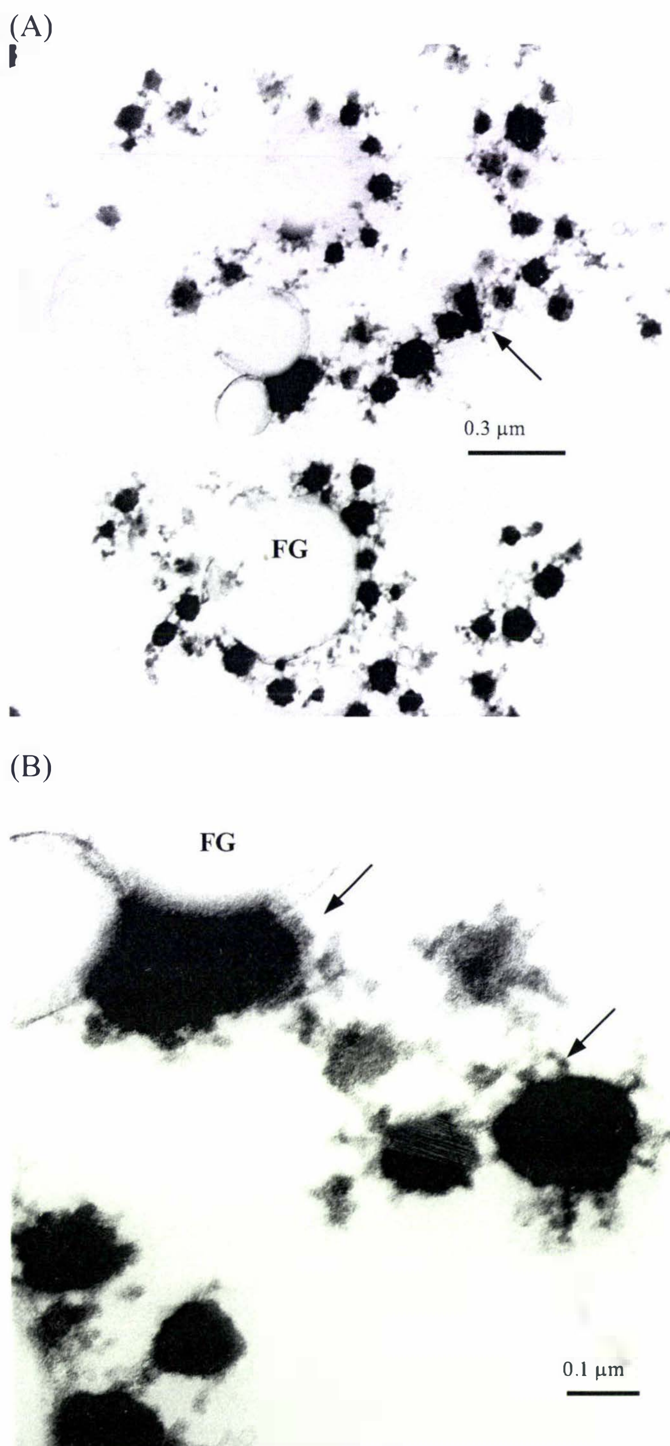


Fig. 7.9. Transmission electron micrographs of preheated (95 °C, 20 s) concentrate, homogenized at 70 bar and then heated to 79 °C. FG: fat globule, CM: casein micelle. Magnification: A,×31800, B, ×103600.

7.7. Discussion

In the non-preheated concentrate, homogenization at low temperature led to a further decrease in the fat globule size and an increase in the surface protein concentration of the fat globules to ~5.5 mg/m^2, although the surface protein concentration in the concentrate before homogenization was already higher than that in the raw milk (Chapter 6). The increase in the surface proteins is attributed mainly to the adsorption of casein micelles (85%); probably the casein micelles aggregate into chains (Figs. 7.5, 7.6, 7.7, 7.8 and 7.9). Some casein micelles were spread over the surface of the fat globules. Other proteins, including whey proteins and native MFGM proteins, constituted approximately 15% of the total surface proteins. This result is fairly consistent with previous reports on the surface proteins of normal milk after homogenization (Oortwijn & Walstra, 1979; Cano-Ruiz & Richter, 1997). These studies obtained protein loads of about 6-9 mg/m^2 for an average fat globule size of 3-5 μm, for milk with 4% milk fat homogenized at 10-30 MPa at temperatures < 60 °C.

A higher surface protein concentration in the concentrate homogenized at high temperature than in the concentrate homogenized at low temperature was observed in the present study. This result is different from the observations on normal milk, in which the protein load decreased upon homogenization at high temperature (Oortwijn & Walstra, 1979) because the adsorbed casein micelles spread over the fat-water interface and the spreading rate appeared to increase with temperature (Walstra, 1995). However, during homogenization of the concentrate, a high temperature may cause more adsorbed casein micelles to aggregate with adjacent micelles in the serum (Fig. 7.5). When the temperature is higher than the temperature of whey protein denaturation, the associations of whey proteins with the surface also result in an increase in the surface protein.

Any heat treatments during processing, including preheating of the raw milk, and heating before and after homogenization, result in the association of whey proteins or whey protein/casein micelle complexes with the surface proteins and subsequently lead to an increase in the total surface protein concentration. During preheating, serum

proteins associate with the native MFGM proteins and the casein micelles via disulfide bonds; these casein micelle/serum protein complexes are subsequently adsorbed on to the surface of the fat globules during homogenisation, resulting in the larger amounts of surface serum proteins and the greater total surface protein concentrations (Tables 7.4 and 7.8). During preheating, only about 1.2 mg whey proteins/g fat associated with the native MFGM (Chapter 5). This amount of whey proteins is relatively small compared with the total surface protein (~60 mg/g fat) (Table 7.4) after homogenization, in which whey protein constitutes about 12 mg/g fat. This effect arises mainly because of the adsorption of the casein micelle/whey protein complexes formed during preheating, including casein micelle aggregate chains linked by whey proteins (Figs. 7.5, 7.6, 7.7, 7.8 and 7.9).

The results (Table 7.11) for the protein composition of the surface material, washed in the dissociation buffer, also show an increase in the amount of surface β-lg and α-la after homogenization, indicating that the directly adsorbed κ-casein is likely to be present as κ-casein/serum protein complexes. This suggests that the association of β-lg and α-la with κ-casein induced by preheating did not influence the adsorption of κ-casein on to the surface during homogenization.

Further increases in the total surface protein concentration and the surface serum proteins (Tables 7.6 and 7.10) were observed when the heat treatment was carried out after homogenization, indicating that further interaction between the adsorbed micelle (non-preheated) or adsorbed micelle/whey protein complexes (preheated) and whey proteins or micelle/whey protein complexes could take place. This result suggests that the association of whey proteins with adsorbed casein micelles via disulfide bonds during heat treatment after homogenization could not be inhibited by the adsorption of casein micelles during homogenization. The amounts of β-lg and α-la associated with adsorbed κ-casein, induced by heating of the concentrates after homogenization, were higher than for the concentrates heated before homogenization (Tables 7.3, 7.4, 7.5 and 7.6). This result is in agreement with observations in normal milk (without concentration) by Sharma & Dalgleish (1993), who showed that casein micelles can associate with more serum proteins after adsorption at the fat globule surface. Sharma &

Dalgleish (1994) suggested that more binding sites for the serum proteins become available as the casein micelles are spread over the fat surface rather than being in their native configuration.

In present work, the SDS-PAGE analysis of the surface protein material washed with urea and EDTA, in which the micelles adsorbed at the fat globule surface were dissociated, showed the proteins adsorbed directly at the surface. Approximately 40% κ-casein, 30% serum proteins (~7% SC, ~20% β-lg and ~3% α-la) and 30% native MFGM proteins make up the initial new surface protein layer after the homogenization of non-preheated concentrates. This percentage of each protein at the surface of the fat globules is approximately equal to the surface area covered by each protein. It changes with changes in the pressure and temperature of homogenization.

Only κ-casein directly adsorbs at the surface when casein micelles are adsorbed at the fat globule surface (Fig. 7.3). This phenomenon does not change when casein micelles spread over the surface at higher homogenization pressure. Homogenization at higher pressure and temperature leads to more κ-casein directly adsorbing at the surface (Figs. 7.2 and 7.3). This may be attributed to more casein micelles adsorbed at the surface and broadly spreading over the surface of the fat globule. This implies that the casein micelles are not disintegrated during homogenization and consequently are adsorbed on to the fat interface as "intact" micelles. However, results reported by previous workers (Anderson et al., 1977; Darling & Butcher, 1978; Sharma et al., 1996) show that a number of casein monomers or sub-units of micelles are also present at the fat interface of homogenized cream or recombined milk.

Differences in the ratios of individual caseins to total caseins between the caseins at the surface and in the milk serum occur possibly because the structure of the casein micelles is altered after adsorption at the surface. The adsorption of κ-casein at the surface probably leads to changes in the interaction forces that link κ-casein with other casein components in the adsorbed micelle. This change in the forces between casein components consequently leads to removal of some of β-casein from the adsorbed micelles and a lesser amount of β-casein in the adsorbed micelles. Sharma et al. (1996)

also observed that the ratio of α_s-casein/β-casein was greater in the cream layer than in the original recombined milk.

The occurrence of Igs components at the interface of the fat globules after homogenization of the concentrate can probably be attributed to the adsorption of IgM and SIgA at the surface during homogenization. It has been reported that IgM and SIgA are considerably hydrophobic and have high affinity for fat (Frenyo et al., 1987). McCrae et al. (1994) reported that the levels of immunoglobulin and serum albumin and/or lactoferrin decreased relative to the total amount of non-absorbed whey protein with increasing pressure of homogenization whereas those of β-lg and α-la increased, and they suggested that immunoglobulin and serum albumin and/or lactoferrin were adsorbed on to the fat globule surface in preference to β-lg and α-la.

CHAPTER 8
EFFECT OF SPRAY DRYING ON THE MILK FAT GLOBULE AND MEMBRANE PROTEINS DURING THE MANUFACTURE OF WHOLE MILK POWDERS

8.1. Introduction

Spray drying is now used almost exclusively in milk powder manufacture. Atomization of the concentrate gives a large surface area over which drying can take place. The droplets are sprayed from the top of the main drying chamber and are intimately mixed with dry heated air (180-220 °C). As the droplet passes through the drier, the moisture evaporates and the temperature of the droplet remains relatively stable (approximately 70 °C).

Before spray drying, milk is usually heated, evaporated and homogenized. These processes cause a decrease in the fat globule size and induce interactions between the proteins and the fat globules. The milk fat globules may be further disrupted by shear during atomization (Mulder & Walstra, 1974). However, little data on the changes in the fat globule size during spray drying are available, and there have been no reports on the specific effects of spray drying on the interactions of milk components, especially the changes in the MFGM.

Various types of atomizers are used in spray drying, but, for the drying of milk, pressure nozzle atomizers and rotary atomizers are used exclusively. The nozzle atomizer may cause a larger disruption of the fat globules than the rotary disc atomizer (Walstra, 1995). The rotary disc atomizer was chosen in the present study for investigating the changes in the milk fat globules and the MFGM during spray drying. The disc is rotated with a peripheral velocity of up to 200 m/s and milk concentrate is fed to the centre of the atomizer disc. The concentrate is carried by centrifugal forces along channels in the disc to exit through ports and then breaks away as a spray of primary particles.

8.2. Changes in fat globule size distributions

The average fat globule sizes (d_{32}) in the powders and the corresponding milk concentrates before spray drying are compared in Table 8.1. The samples were dispersed in SDS/EDTA buffer. Details of the processing conditions used in the manufacture of these powders are shown in Fig. 3.1. The d_{32} values of all powders were lower than those of the corresponding concentrates. The variation in d_{32} among the powders was very small, in spite of large differences in the d_{32} values of the concentrates before drying (0.61-0.71 μm) as a result of the different homogenization pressures used.

The differences in the fat globule size distributions of the powders and the corresponding concentrates before drying are shown in Fig. 8.1. All powders showed a uniform distribution of fat globules, with a majority of the globules in range 0.1-0.4 μm. However, a small proportion of globules in the range from about 4 to 80 μm was also observed. In contrast, the concentrates showed uneven distributions and slightly wider peaks, ranging from 0.1-10 μm. The non-uniform fat globule size distribution of the concentrates was attributed to the different homogenization pressures used. The shift in the size distribution peaks of the concentrates homogenized at 40 or 70 bar after drying is illustrated in Fig. 8.2. This result suggested that the fat globule size distribution of the powder was independent of the fat globule size of the concentrate before drying; the fat globule size distribution was reformed during spray drying. There was a disruption of the fat globules with coalescence of a small amount of the fat globules into large droplets during spray drying.

Table 8.1. Comparison of the average fat globule sizes (d_{32}) in the powders and the concentrates before spray drying. The samples were dispersed in SDS/EDTA buffer. The samples were produced using various processing conditions (see Fig. 3.1)

Sample	Homogenization pressure before drying (bar)	d_{32} (μm)	
		Before drying	Powder
Non-preheated samples			
1	40	0.67	0.51
4	40	0.67	0.48
6	40	0.66	0.50
7	40	0.67	0.52
2	70	0.61	0.51
3	70	0.61	0.51
5	70	0.61	0.51
8	70	0.61	0.50
Preheated samples (95 °C, 20 s)			
9	40	0.68	0.49
12	40	0.68	0.49
14	40	0.71	0.52
15	40	0.69	0.49
10	70	0.64	0.50
11	70	0.64	0.52
13	70	0.63	0.48
16	70	0.62	0.49

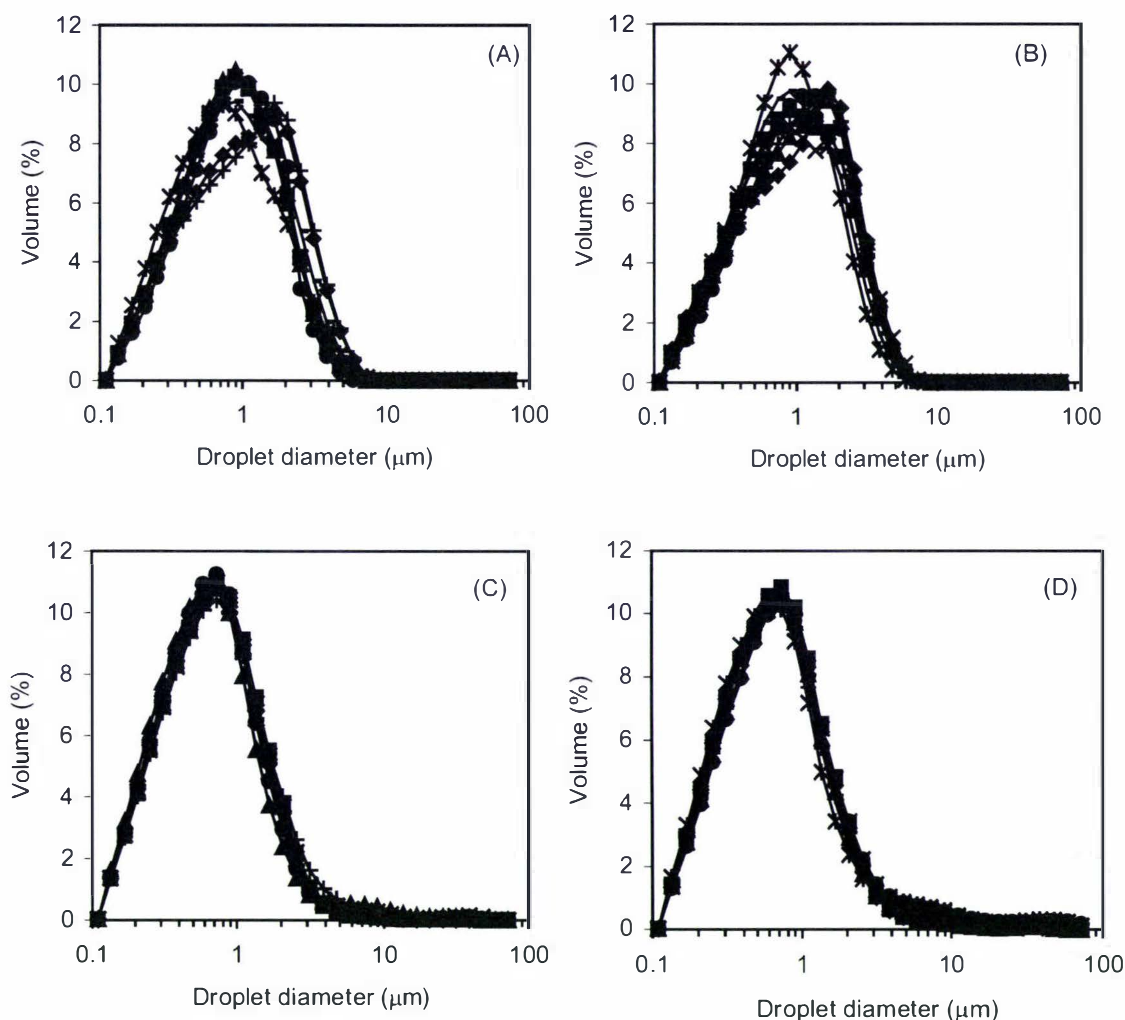


Fig. 8.1. The fat globule size distributions of the concentrates (A, B) and the powders made from the concentrates (C, D). The samples were dispersed in SDS/EDTA buffer before particle size measurements. Non-preheated samples (A, C): powder 1 and corresponding concentrate (◆); powder 2 and corresponding concentrate (■); powder 3 and corresponding concentrate (▲); powder 4 and corresponding concentrate (×); powder 5 and corresponding concentrate (✱); powder 6 and corresponding concentrate (●); powder 7 and corresponding concentrate (+) and powder 8 and corresponding concentrate (-). Preheated samples (B, D): powder 9 and corresponding concentrate (◆); powder 10 and corresponding concentrate (■); powder 11 and corresponding concentrate (▲); powder 12 and corresponding concentrate (×); powder 13 and corresponding concentrate (✱); powder 14 and corresponding concentrate (●); powder 15 and corresponding concentrate (+) and powder 16 and corresponding concentrate (-).

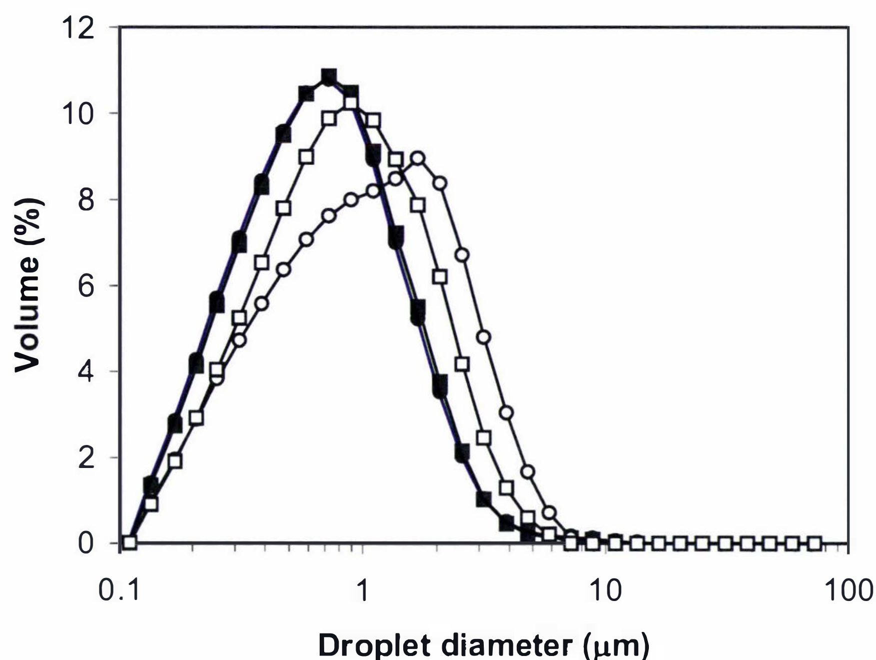


Fig. 8.2. The fat globule size distributions of two powders (●, ■) and the corresponding concentrates, which were homogenized at 40 bar (○) or 70 bar (□).

8.3. Changes in the total protein concentration and the protein composition of the fat globule surface

In general, the surface protein coverage (mg/m^2) decreased slightly during spray drying for both the non-preheated samples and the preheated samples (Table 8.2). Variations in the surface protein coverage (mg/m^2) in both the non-preheated samples and the preheated samples were smaller in the powders than in the concentrates. In contrast, the total MFGM protein concentration (mg/g fat) of the powders was considerably higher than that of the concentrates (Table 8.2). This result indicated that the proteins adsorbed on to the surface of the fat globules during drying, although the surface protein coverage (mg/m^2) was still slightly lower than that in the corresponding concentrates, because of the increase in the fat globule surface area as shown in Table 8.1.

The proportion of caseins was slightly higher in the powders (Table 8.3) than in the corresponding concentrates (Table 8.4), whereas the proportions of the other surface

proteins (β-lg, α-la and the native MFGM proteins) were slightly lower in the powders (Table 8.3) than in the concentrates (Table 8.4) in both the non-preheated samples and the preheated samples. It was also found (Table 8.5) that the percentage of secretory component (SC) in the surface material of the powders (the creams were washed with urea/EDTA buffer), was higher than that in the corresponding concentrates (Chapter 7, Table 7.11). This indicated that the proteins adsorbed at the newly formed surface were casein micelles and Igs components, which caused the decreases in the percentage of whey proteins (β-lg and α-la) and native MFGM protein at the surface. It also indicated that spray drying did not cause further association of whey proteins (β-lg and α-la) with the surface of the fat globules.

8.4. Confocal micrographs of the fat globules in the whole milk powders

The location and the structure of the fat globules on the surface and within the whole milk powders were observed using CLSM (Fig. 8.3 and Fig. 8.4). The confocal micrographs of the powders showed that some larger coalesced fat globules within the powder particles were surrounded by uniform small fat globules in most powders. There was some apparent spreading (shown by arrows) of the fat on the surface of the powder particles in powders 6, 9 and 15 (Fig. 8.3 and Fig. 8.4). The concentrates of these powders were homogenized at 40 bar (Fig. 3.3). However, there were smaller fat globules and a more uniform distribution in powders 2, 3, 5, 8, 10, 11, 13 and 16. The concentrates used in the manufacture of these powders were homogenized at 70 bar (Fig. 8.3 and Fig. 8.4). It appeared that more surface fat was formed in the powders for which the concentrates were homogenized at lower pressure (40 bar).

The confocal micrographs were consistent with the fat globule size distribution results, in which small amounts of large size globules (approximately 4-80 μm) were observed in the powders. The appearance of surface fat in powders produced using lower homogenization pressures is in agreement with the results of previous studies (de Vilder et al., 1979). de Vilder et al. (1979) reported a higher free fat content in whole milk powder produced from non-homogenized concentrated milk and that the free fat content decreased with an increase in the homogenization pressure.

Table 8.2. Comparison of the total surface protein concentrations of the milk fat globules in the powders and the concentrates. The samples were produced using various processing conditions (see Fig. 3.1)

Run	Homogenization pressure before drying (bar)	Total MFGM protein (mg/g fat)		Surface protein coverage (mg/m^2)	
		Before drying	Powder	Before drying	Powder
Non-preheated samples					
1	40	42.9	73.8	5.35	5.55
4	40	55.9	73.5	5.76	5.88
6	40	54.2	70.8	5.97	5.87
7	40	65.2	75.9	7.29	6.64
2	70	59.9	72.5	6.09	6.19
3	70	77.3	72.2	7.81	6.19
5	70	58.9	80.6	5.51	6.83
8	70	68.2	76.8	6.99	6.40
Average		60.31 ± 9.60	74.51 ± 3.14	6.34 ± 0.83	6.19 ± 0.40
Preheated samples (95 ℃, 20 s)					
9	40	61.2	76.2	6.96	6.85
12	40	69.8	71.8	7.68	5.84
14	40	57.9	70.2	6.86	6.28
15	40	61.1	74.1	6.99	6.31
10	70	65.4	77.8	6.98	6.52
11	70	76.8	75.0	8.15	6.54
13	70	74.1	81.3	7.82	6.56
16	70	77.1	79.4	7.98	6.45
Average		67.92 ± 7.10	75.73 ± 3.50	7.43 ± 0.50	6.44 ± 0.29

Table 8.3. Percentage distribution of proteins on the milk fat globule surface of the powders that were produced using various processing conditions (see Fig. 3.1)

Run	Homogenization pressure before drying (bar)	Others (including native MFGM proteins) (%)	Caseins (%)	β-lg (%)	α-la (%)
Non-preheated samples					
1	40	8.2	80.2	1.6	
4	40	7.8	79.8	3.9	
6	40	8.5	74.6	2.4	
7	40	8.0	76.9	2.7	
2	70	8.5	76.5	1.0	
3	70	8.4	74.4	4.6	
5	70	9.0	67.5	10.0	1.2
8	70	9.1	76.8	2.2	
Average		8.38	75.88	3.55	
Preheated samples (95 ℃, 20 s)					
9	40	8.6	67.2	9.1	1.5
12	40	8.0	63.3	13.3	2.5
14	40	8.2	65.4	11.4	3.4
15	40	8.6	66	13.0	2.7
10	70	7.8	67.7	12.1	1.6
11	70	8.3	64.8	13.0	2.6
13	70	7.9	65.2	13.2	3.5
16	70	8.2	66.7	13.0	2.5
Average		8.20	65.79	12.26	2.54

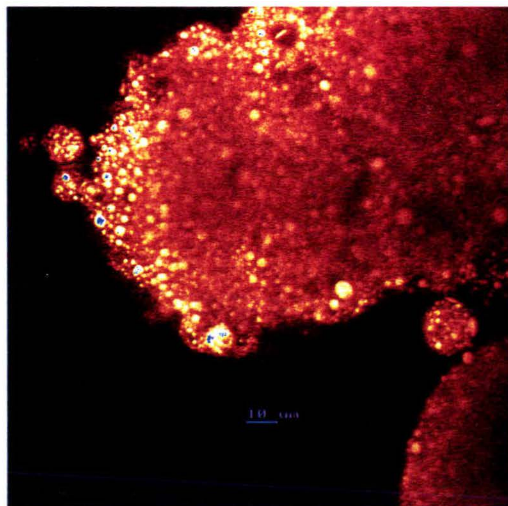
Table 8.4. Percentage distribution of proteins on the milk fat globule surface of the concentrates before spray drying that were produced using various processing conditions (see Fig. 3.1)

Run	Homogenization pressure before drying (bar)	Others (including native MFGM proteins) (%)	Caseins (%)	β-lg (%)	α-la (%)
Non-preheated samples					
1	40	9.2	68.3	4.5	1.0
4	40	9.0	69.2	8.3	2.2
6	40	8.5	73.7	3.7	1.3
7	40	8.6	74.1	2.8	0.5
2	70	8.6	75.0	2.6	0.6
3	70	9.0	72.82	4.0	0.8
5	70	9.0	77.1	3.0	0.7
8	70	10.3	75.9	2.1	0.6
Average		9.0	73.26	3.83	0.96
Preheated samples (95 °C, 20 s)					
9	40	9.8	65.5	16.9	2.4
12	40	10.0	65.3	13.9	4.6
14	40	9.3	59.1	15.6	7.1
15	40	9.6	63.9	15.8	3.0
10	70	9.5	62.2	15.5	2.9
11	70	9.5	63.3	16.1	6.2
13	70	9.1	63.5	14.4	5.8
16	70	9.5	66.2	14.8	2.2
Average		9.54	63.62	15.37	4.28

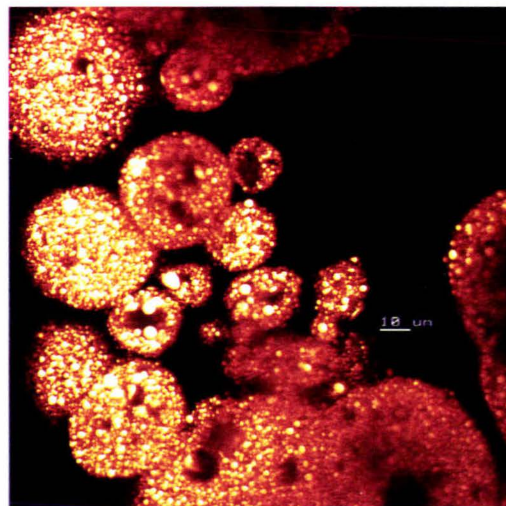
Table 8.5. Percentage distribution of proteins on the milk fat globule surface of the powders that were produced using various processing conditions (see Fig. 3.1), in which the surface material was isolated from creams that were washed with urea/ETDA buffer

Run	Homogenization pressure before drying (bar)	Total native MFGM proteins (%)	Secretory component (%)	κ-casein (%)	β-lg (%)	α-la (%)
Non-preheated samples						
1	40	17.1	11.5	42.8	22.7	5.8
4	40	14.1	9.0	28.3	44.2	4.6
6	40	20.0	12.9	40.2	23.3	4.0
7	40	20.0	13.1	39.5	23.2	4.1
2	70	19.0	14.3	40.0	21.0	5.8
3	70	18.0	12.4	33.9	30.4	5.2
5	70	17.4	12.9	38.4	26.3	5.0
8	70	18.9	11.6	39.2	24.5	7.1
Average		18.1	12.5	38.5	26.0	5.0
Pre-heated samples (95 °C, 20 s)						
9	40	17.8	9.9	29.6	35.4	7.3
12	40	17.1	7.6	26.2	42.0	8.0
14	40	12.7	6.9	27.4	38.6	12.4
15	40	17.3	7.6	24.8	40.6	9.7
10	70	18.7	8.2	27.3	39.0	7.0
11	70	16.5	7.1	25.8	42.7	8.0
13	70	19.6	7.8	27.1	36.6	11.3
16	70	18.4	7.6	25.5	39.3	9.3
Average		17.3	7.8	26.7	39.3	9.1

Powder 1



Powder 2



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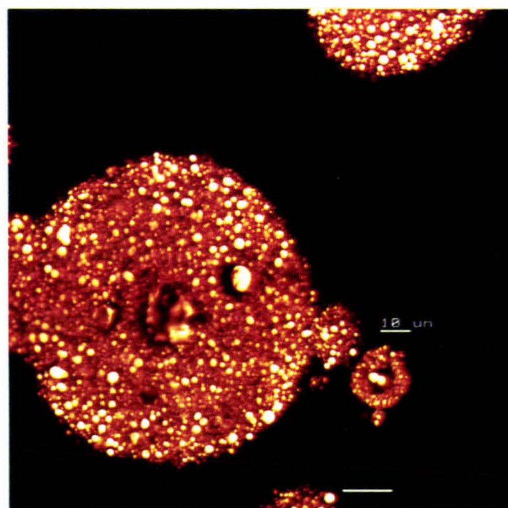

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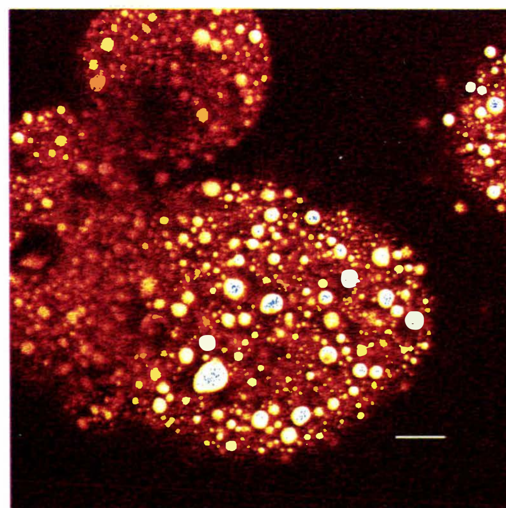
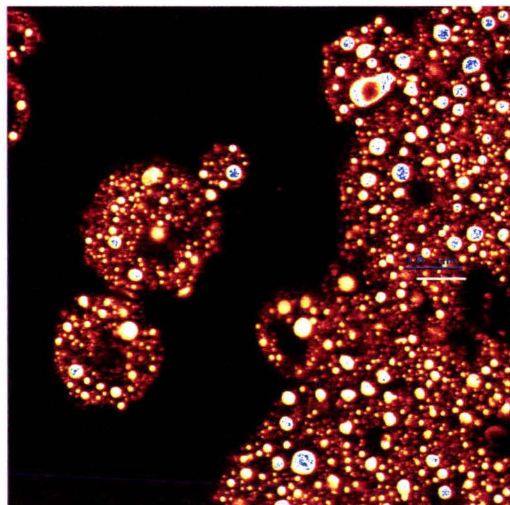


Fig. 8.3. Confocal micrographs of non-preheated milk powder samples, powders 1-8.

Powder 5



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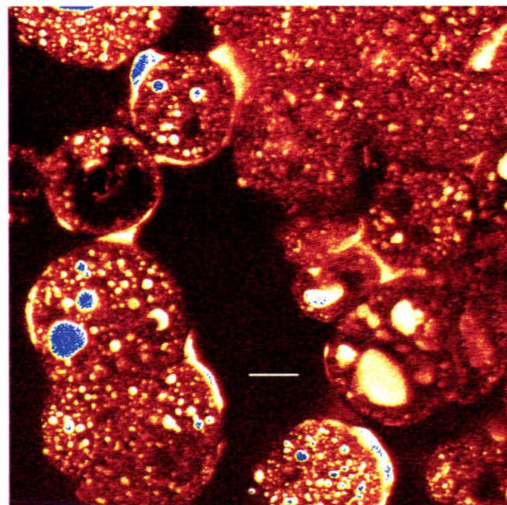
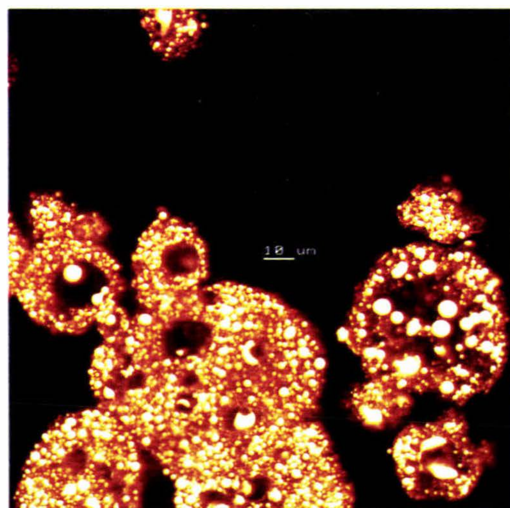


Powder 7



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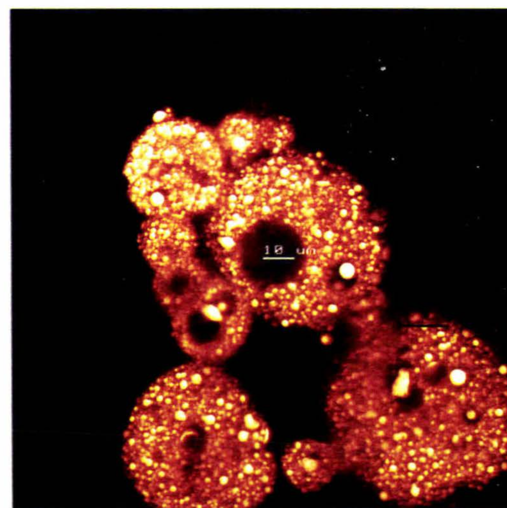
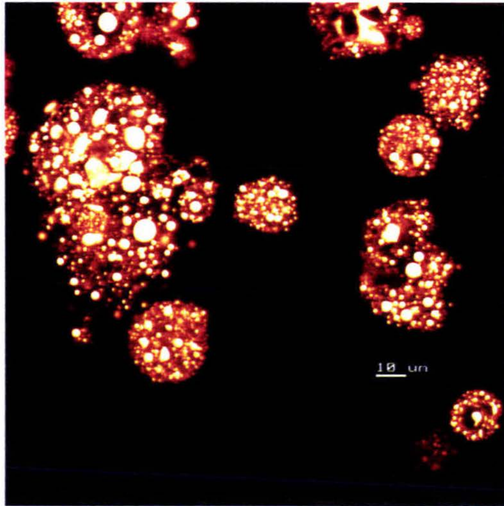
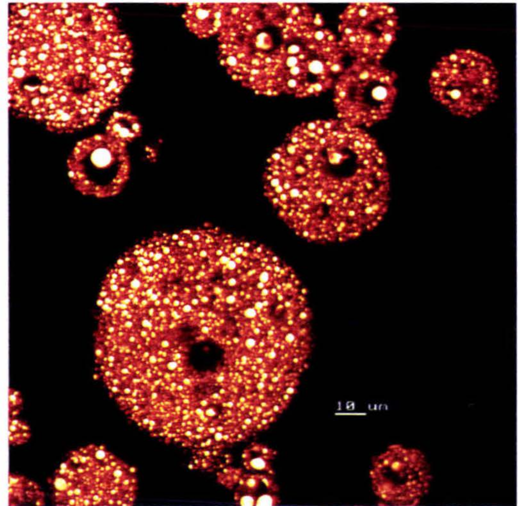


Fig. 8.3. Continued.

Powder 9



Powder 10



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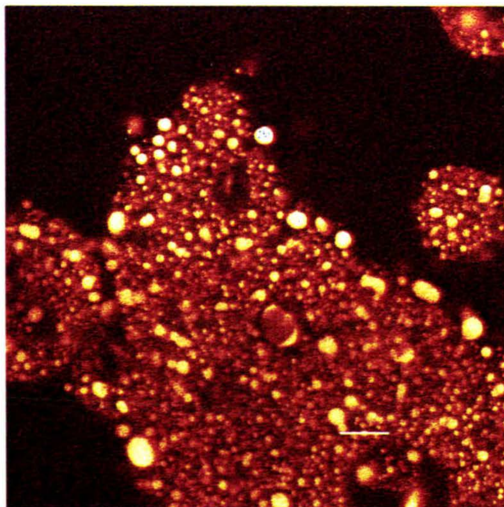


Powder 12

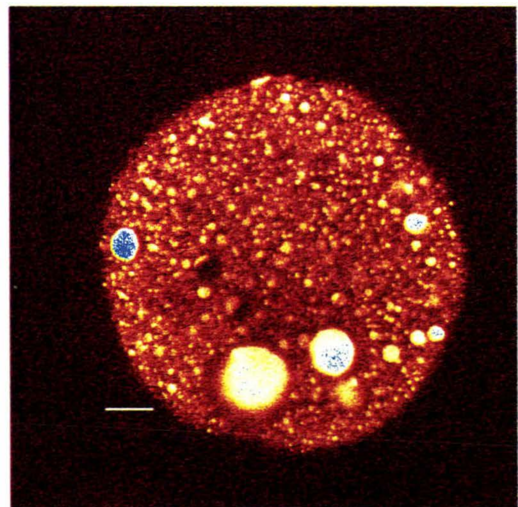
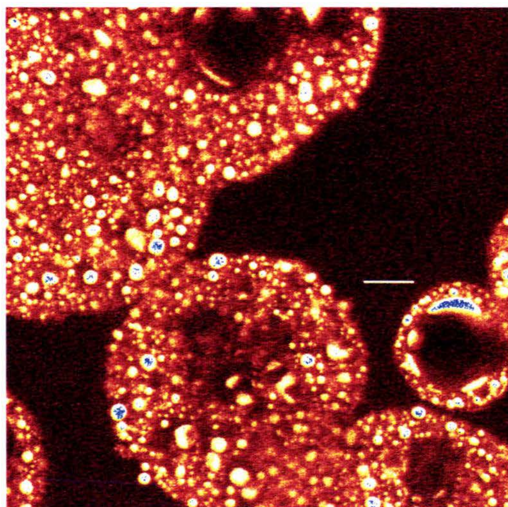
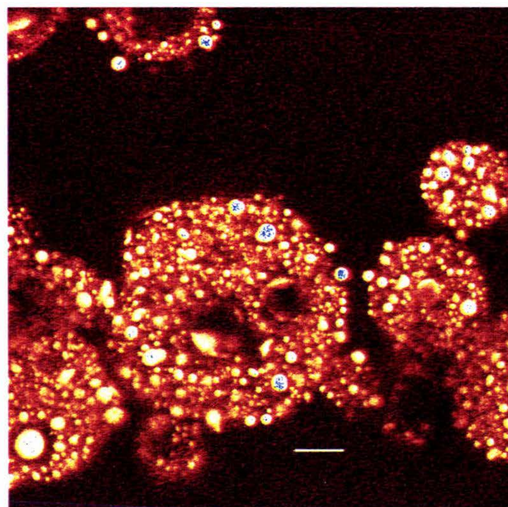


Fig. 8.4. Confocal micrographs of preheated milk powder samples, powders 9-16.

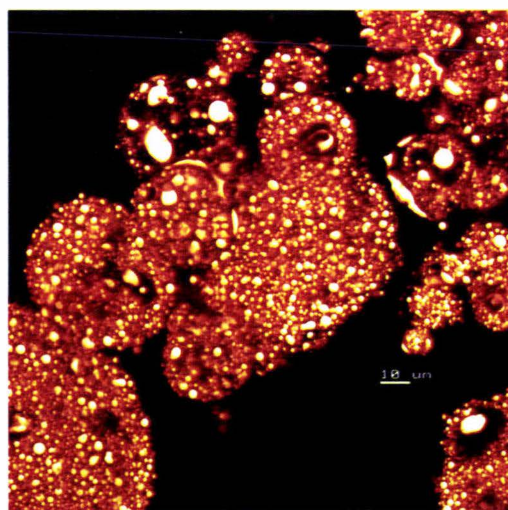
Powder 13



Powder 14



Powder 15



Powder 16

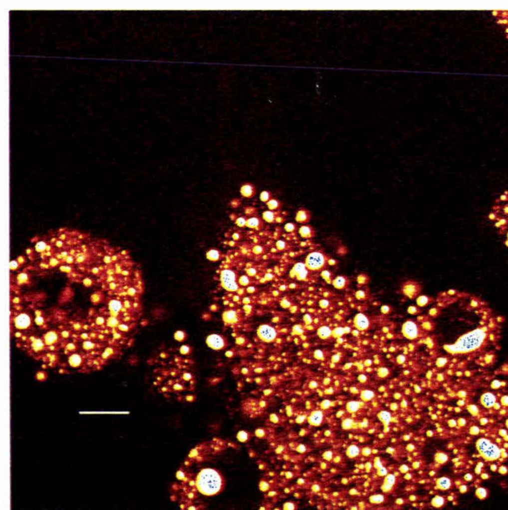


Fig. 8.4. Continued.

8.5. Discussion

The average size of the milk fat globules was lower in the powders than in their concentrates, indicating that the fat globules were disrupted during spray drying. The atomizer disc speed and diameter, the air temperature and the total solids of the milk concentrate used were kept constant for the drying of all concentrates. It is interesting to note that the final fat globule sizes in the powders were similar to each other despite obvious differences between the concentrates. This suggests that the disruption of the fat globules during spray drying was quite severe and overrode any differences between the fat globule sizes in the concentrates.

The increase in total surface protein (mg/g fat) during spray drying (Table 8.2) occurred because proteins were adsorbed on to the newly formed fat surface as a result of the disruption of the fat globules. However, the surface protein coverage (mg/m^2) of most powders was slightly lower than that of the concentrates before spray drying (Table 8.2). It is unknown at which step of the drying process the disruption of the fat globules occurs. If it occurs when the concentrate is sprayed into the drying air, where the concentrated milk comes immediately into contact with the drying air after leaving the atomizer disc, evaporation from the surface of the primary particle begins, making the adsorption of protein to the fat surface difficult, particularly at the newly uncovered surface of the fat globules located at the surface of the primary particle. In addition, the fat globules located at the surface of the powder particles come into contact with the air-water surface of the drying droplet, which may lead to the fat globules losing membrane and subsequently spreading over the surface of the powder particles (Walstra, 1995).

Obviously, the intensity of the heat treatment (approximately 70 °C) during drying was not sufficient to cause association of the whey proteins (β-lg and α-la) with the fat globule surface. The whey proteins associated with the fat globule surface of the powders were those that had already associated during processing prior to spray drying. Singh & Creamer (1991) noted that spray drying had no significant effect on the denaturation of the major whey proteins in the manufacture of low heat (72 °C for 15 s) and high heat (110-120 °C for 2-3 min) powders. The denaturation of whey proteins

during spray drying was minimal, with no apparent loss of IgG and only a small loss of bovine serum albumin (3-7%) (Oldfield, 1996).

The values of "free fat" in the powder were not determined in the present study, because the method for "free fat" or "uncovered fat" determination based on solvent extraction does not seem to be accurate (Buma, 1971; Evers et al., 2001). Extractable fat does not just consist of "uncovered fat" (Buma, 1971), but also includes the surface fat of particles and fat in globules that are in contact with the particle surface, and is dependent on the conditions of extraction (time, concentration and the size of particle). Buma (1971) considered that only the surface fat and the size distribution of the fat globules are of practical importance. Often, surface fat or some fat spread over (part of) the surface of the particles of whole milk powder can be observed by microscopic examination in the reconstituted milk or powder (Buma, 1971; Buchheim & Dejmek, 1990; McKenna et al., 1999). However, whether the surface fat is a "free fat" or an "uncovered fat" is still doubtful (Buma, 1971; Walstra, 1995). Several workers have explained the occurrence of a free fat in milk powder by assuming that such fat consists of fat globules in which the fat globule membranes, which protect the fat globules in fresh milk, are damaged during the processing steps used in milk powder manufacture. However, Walstra (1995) believes that the term "free fat" or "uncovered fat" is a loose term; the "free fat" or "uncovered fat" does not normally occur in liquid milk products. He considers that, as soon as uncovered milk fat comes into contact with milk serum, proteins will adsorb and thus cover the fat in a very short time (~10 ms). In fact, the fat globules in milk powder particles have been subjected to a number of processing steps that would have damaged all or the major part of the native membranes. These steps include pasteurization, concentration and spray drying. In particular, the homogenization step causes significant changes in the fat globules and the surface protein layer.

From the present study, it appears that the surface fat is the "uncovered fat" that is probably produced during spray drying as discussed above. This surface fat is not related to the disruption of the fat globules during the processing steps prior to spray drying, which is in agreement with Walstra's theory that "uncovered fat" cannot be produced in liquid milk. The fat globule sizes of milk samples before spray drying are

expected to influence the amount of surface fat of the powder particles. Concentrates that have larger fat globule sizes have larger decreases in size during spray drying and subsequently form more "uncovered fat".

As "uncovered" surface fat exists in the powder, these uncovered fat globules may join together with each other into larger fat globules in the later processing steps such as fluid bed drying, agglomeration and storage after spray drying. Further systematic studies are required to further understand this phenomenon.

CHAPTER 9

ROLE OF FAT GLOBULES AND MEMBRANE PROTEINS IN INFLUENCING THE RECONSTITUTION PROPERTIES OF WHOLE MILK POWDERS

9.1. Introduction

Milk powders possess various functional properties that are important to both industrial users and consumers. Of the functional properties, solubility of milk powders is important when the powders are used for recombining or in the manufacture of various food products. Solubility is a measure of the final condition to which the constituents of the powder can be brought in solution or stable suspension. The powder must disperse rapidly in water (hot and cold) or hot coffee and be quickly and completely dissolve, i.e. form a stable colloidal suspension of fat and casein micelles without leaving a visible residue on the surface of the milk or on the sides and the bottom of the container. Protein-protein and protein-fat interactions have been shown to influence the reconstitution properties of powder in water (Mol, 1975; Caric & Kalab, 1987; Ohba et al., 1989; de Ruyck, 1991).

The interactions of the specific components in milk, i.e. casein and whey proteins, fat globules and minerals, during milk powder processing play a major role in determining the functional properties when the powder is reconstituted in water. It is known that these interactions arise from the processing steps during the manufacture of the milk powder. Pasteurization, homogenization, concentration, heating and drying will influence the interactions among various components of the milk, specifically the milk proteins and the fat. It has been shown that an increase in protein adsorption on to the fat globules and an increase in protein aggregation will result in more sediment after powder reconstitution in water and coffee (Mol, 1975; McKenna et al., 1999). Mol (1975) considered that the adsorption of casein on the fat globule membrane is one of the most important reasons for the poor solubility of whole milk powder. However, little work on the effect of component interactions on the MFGM and on the structure and functional properties of powder products has been published (Singh & Newstead, 1992).

Further knowledge about the effect of these interactions on functional properties may lead to improved processes and better performing milk powder products.

Previous chapters examined the size distribution of fat globules, the amounts of adsorbed protein and the composition of the MFGM under different conditions, e.g. temperature and pressure, during preheating, evaporation, homogenization and spray drying. How these differences in the fat globule size and the composition of the MFGM in the powders are related to the solubility properties of powders is discussed in this chapter.

9.2. Functional properties of whole milk powders

The functional properties of the powders are shown in Table 9.1. As the powders in the present study were collected after spray drying and the agglomeration process was not carried out, their instant functional properties were poor (Wettability > 60 s, Dispersibility > 6 and cold SDP E) and out of the acceptable range (i.e. for IWMPs Wettability ranges from 9 to 60 s, Dispersibility ranges from 1 to 6 and the cold SDP ranges from C to E).

The solubility index (SI), hot sediment and coffee sediment test results (Table 9.1) showed that the differences in the solubility between the individual powders were related to the various processing conditions (Fig. 3.1). In general, there were small differences in the solubility properties between the non-preheated powders and the preheated powders (Table 9.1). In the non-preheated powder samples, powder 3 had the highest SI, hot sediment and coffee sediment and powder 5 also had higher SI and hot sediment compared to other non-preheated powder samples (Table 9.1). In the preheated powder samples, powder 11 had a higher hot sediment and powder 13 had a higher SI. These powders (powders 3, 5, 11 and 13) were produced from concentrates that were homogenized at 70 bar and heated to 79 °C (Fig. 3.1). The concentrates of powders 3 and 11 were heated after homogenization, whereas the concentrates of powders 5 and 13 were heated before homogenization. The results indicate that the poor solubility properties may be related to the higher homogenization pressure and the higher heating temperature to which the concentrate was subjected to before drying.

Table 9.1. Functional properties of whole milk powders

*Sample	Solubility index 24°C (ml)	Hot sediment (ml)	Coffee sediment (ml)	Dispersibility	Wettability (s)	Cold SDP
Non-preheated samples						
1	0.23	0.43	1.1	7	> 60	E
2	0.20	0.44	0.95	7	> 60	E
3	1.36	1.96	2.1	7	> 60	E
4	0.25	0.4	0.65	7	> 60	E
5	0.44	1.05	0.83	7	> 60	E
6	0.28	0.55	0.5	7	> 60	E
7	0.25	0.5	0.9	7	> 60	E
8	0.22	0.56	0.95	7	> 60	E
Preheated samples						
9	0.23	0.82	0.95	7	> 60	E
10	0.28	0.35	0.95	7	> 60	E
11	0.18	1.17	0.85	7	> 60	E
12	0.19	0.62	0.9	7	> 60	E
13	0.9	0.62	0.88	7	> 60	E
14	0.28	0.68	0.9	7	> 60	E
15	0.18	0.46	0.96	7	> 60	E
16	0.17	0.63	1.15	7	> 60	E

* see Fig. 3.1 for processing conditions used in the manufacture of these powders.

9.3. Fat globule size distributions of reconstituted whole milk powders

The average fat globule sizes (d_{43}) of the powders dispersed in water or dissociating buffer are shown in Table 9.2. When the powders were dispersed in a dissociating buffer, the d_{43} of all the powders was fairly similar (approximately 1.0 μm) and was lower than those of powders reconstituted in water. There were larger variations in the d_{43} values (from 1.24 to 7.77 μm) of these powders when reconstituted in water. The differences in the d_{43} values between the powders dispersed in dissociating buffer and dispersed in water indicate that the fat globules in these powders were aggregated, to some extent, by protein bridging.

The particle size distributions of the reconstituted powders (Fig. 9.1) showed that there were more particles in the size range > 10 μm in the powders dispersed in water (Fig. 9.1 A and B) than in the powders dispersed in dissociating buffer (Fig. 9.1 C and D). The results showed that the proportions of fat globule aggregates were higher in powder 3 and powder 5 than in the other powders. Powders 3, 5 and 7 had d_{43} values greater than 2 μm, whereas the other powders had smaller d_{43} values (< 2 μm). Similar results were also observed in the preheated samples. Powder 13 had a large amount of fat globule aggregates and the largest d_{43} values (approximately 8 μm).

9.4. Confocal micrographs of reconstituted powders

Confocal micrographs of the powders reconstituted in water for 24 h are shown in Fig. 9.2 and Fig. 9.3. All powders showed fairly similar fat globule sizes with some powders showing clusters (arrow) formed by aggregation of a number of small fat globules. Powders 3, 5 and 7 appeared to show greater amounts of these large clusters. These clusters were also observed in the preheated samples, e.g. powders 13 and 15. These fat globule clusters were probably related to the larger particles observed in the size determination and the insoluble material in the functionality test.

Table 9.2. The average fat globule sizes (d_{43}) of the reconstituted powders. The powders were dispersed in water or in a dissociating buffer. The samples were produced using various processing conditions (see Fig. 3.1)

Run	d_{43} (μm)	
	Powders dispersed in water	Powders dispersed in a dissociating buffer
Non-preheated samples		
1	1.41	0.88
2	1.24	0.88
3	3.80	1.06
4	1.59	1.05
5	2.05	0.95
6	1.80	0.95
7	2.01	1.00
8	1.62	0.88
Preheated samples		
9	1.84	1.33
10	2.01	0.98
11	1.48	1.28
12	1.31	1.05
13	7.77	1.02
14	2.36	1.03
15	2.10	1.16
16	1.43	1.03

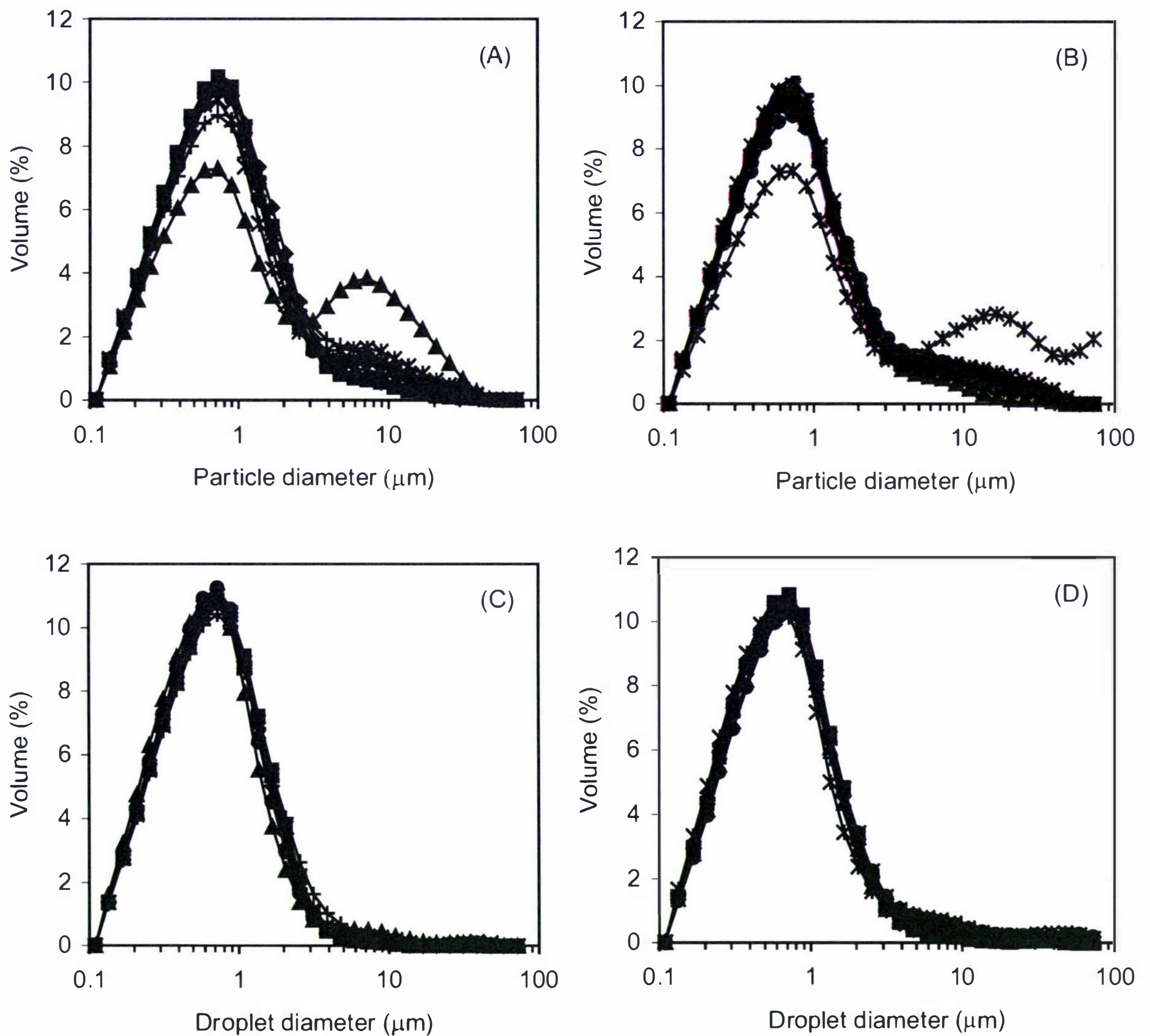
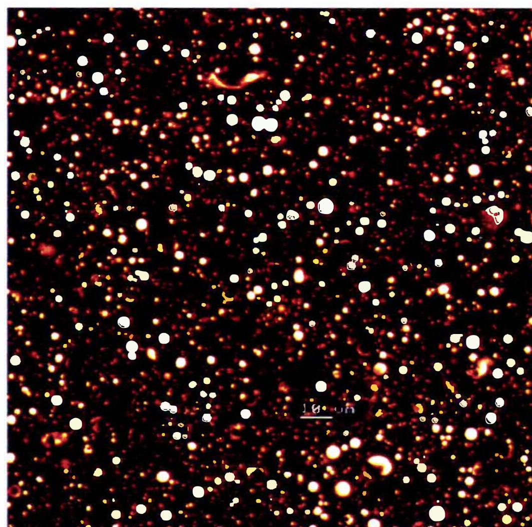
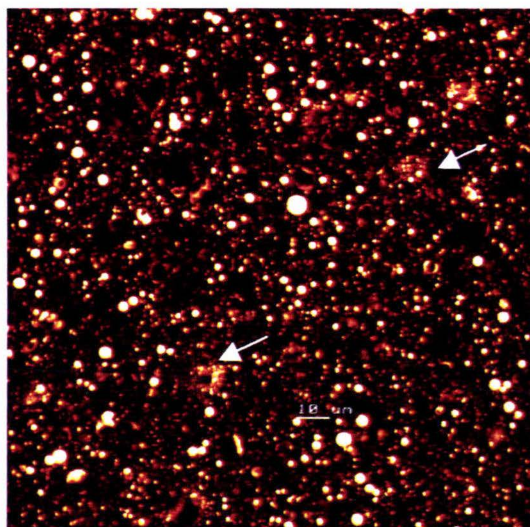


Fig. 9.1. The particle size distributions of the powders after reconstitution in water (A, B) or dispersion in a dissociating buffer (C, D). Non-preheated samples (A, C): Powder 1 (◆); powder 2 (■); powder 3 (▲); powder 4 (×); powder 5 (✱); powder 6 (●); powder 7 (+); powder 8 (-). Preheated samples (B, D): powder 9 (◆); powder 10 (■); powder 11 (▲); powder 12 (×); powder 13 (✱); powder 14 (●); powder 15 (+); powder 16 (-).

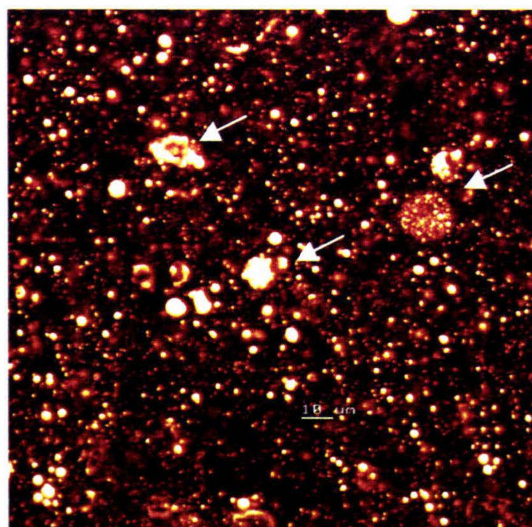
Powder 1



Powder 2



Powder 3



Powder 4

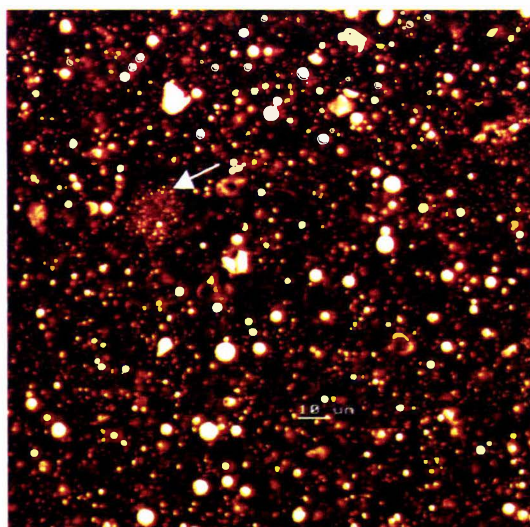
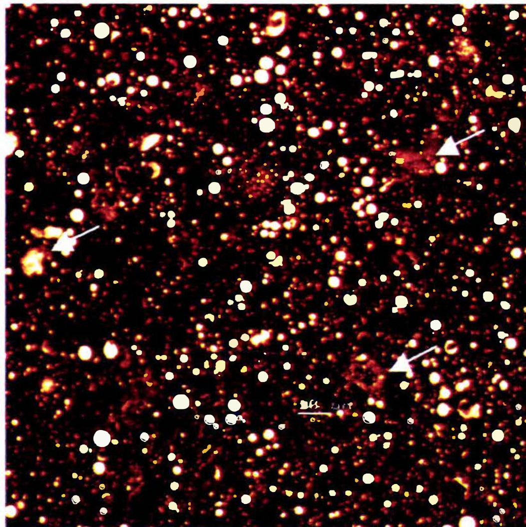
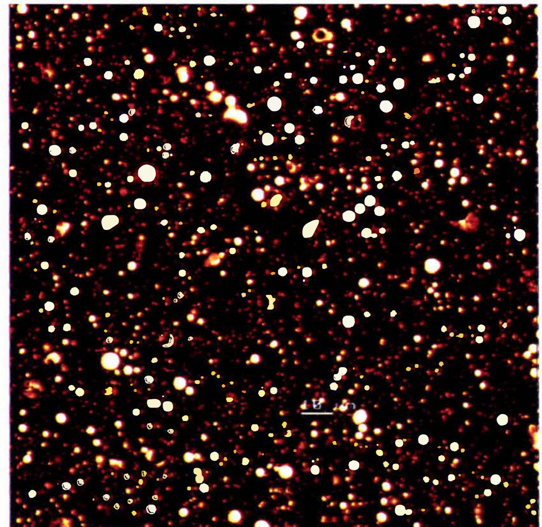


Fig. 9.2. Confocal micrographs of non-preheated milk powder samples after reconstitution in water for 24 h, powders 1-8. Powder numbers are as shown in Fig. 3.1.

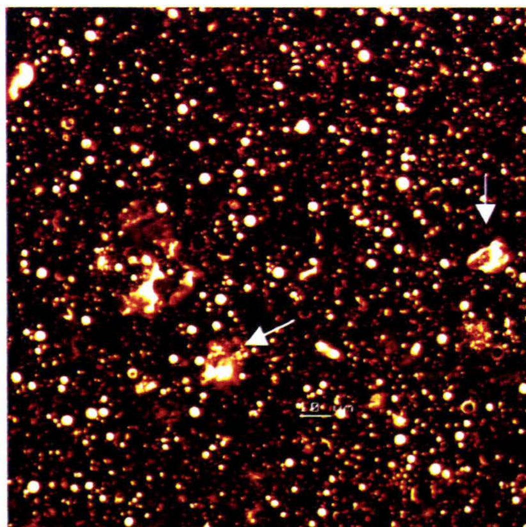
Powder 5



Powder 6



Powder 7



Powder 8

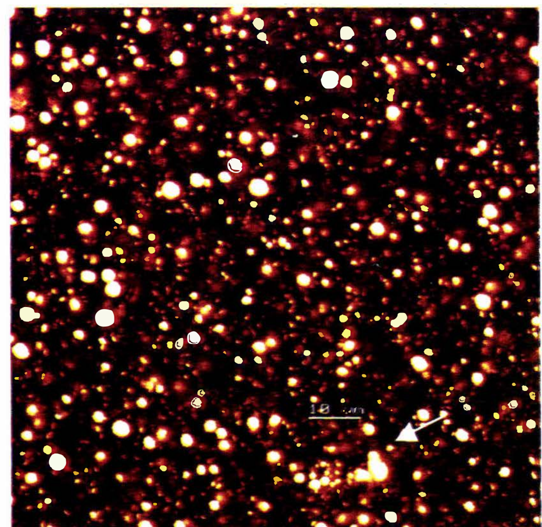
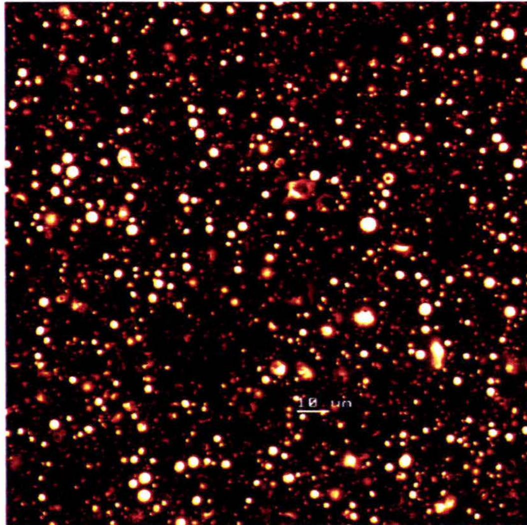
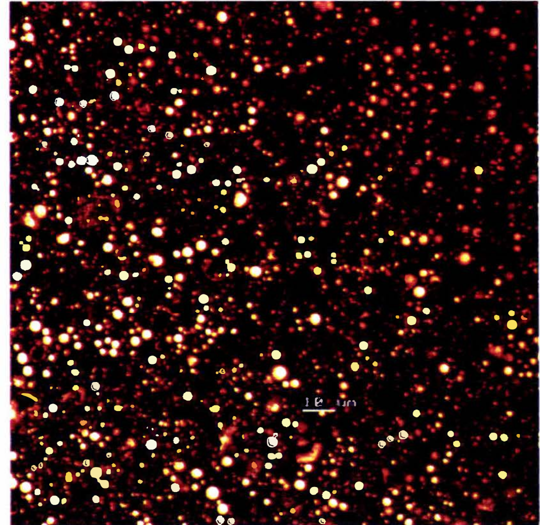


Fig. 9.2. Continued.

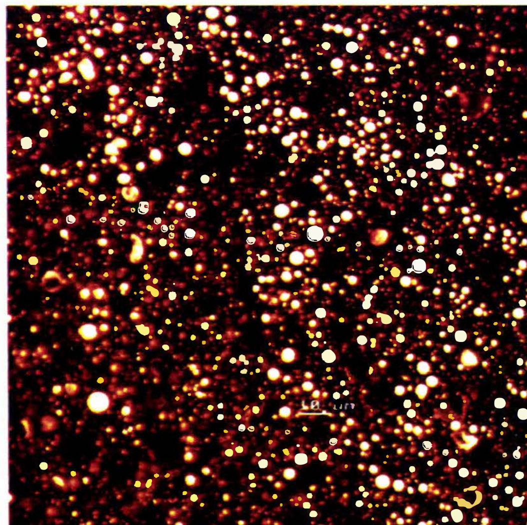
Powder 9



Powder 10



Powder 11



Powder 12

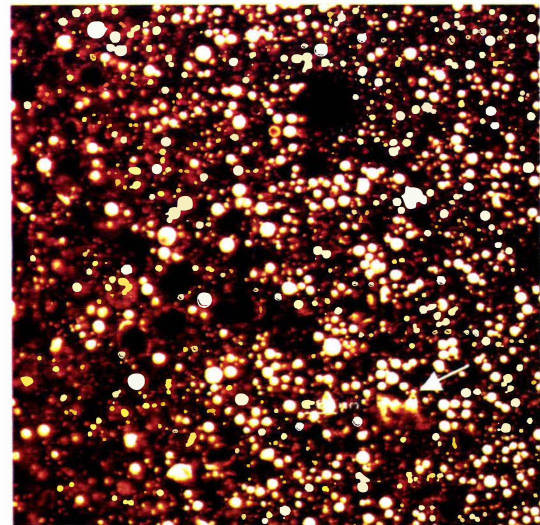
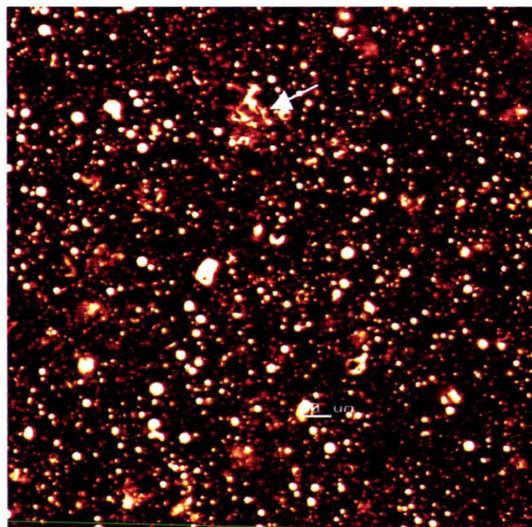
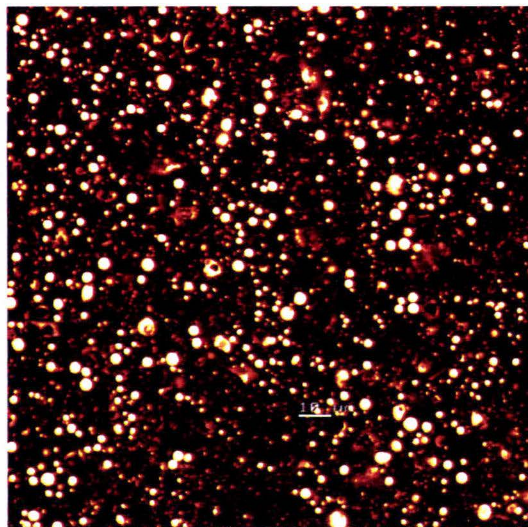


Fig. 9.3. Confocal micrographs of preheated milk powder samples after reconstitution in water for 24 h, powders 9-16. Powder numbers are as shown in Fig. 3.1.

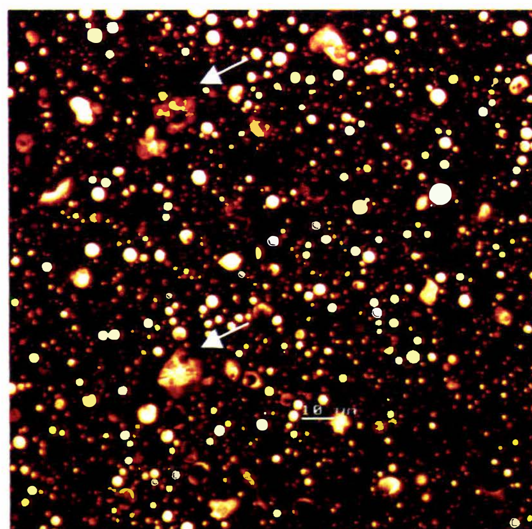
Powder 13



Powder 14



Powder 15



Powder 16

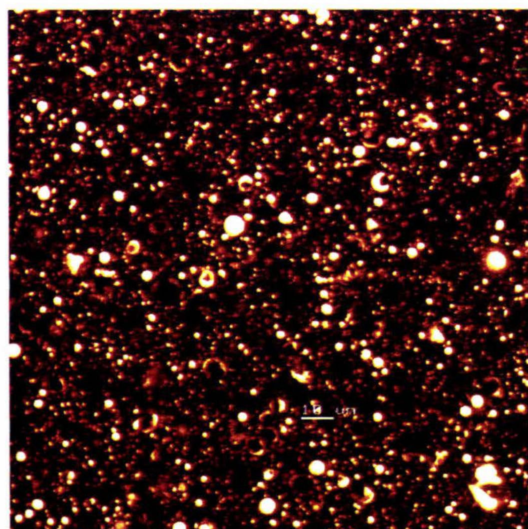


Fig. 9.3. Continued.

9.5. Surface protein coverage and composition of the fat globules in the powders

There were relatively small differences between the fat globule surface protein coverages (~5.9 to ~6.8 mg/m^2) of the powders samples for both the non-preheated samples and the preheated samples (Table 9.3), although the milk concentrates used in their manufacture were homogenized at different pressures (40 bar or 70 bar) and had larger differences in surface protein coverage. Possible mechanisms causing this effect have been discussed in Chapter 8. The powders (3, 5, 11 and 13) that had poorer solubility did not have higher surface protein coverage than the other powders. There was a slightly higher surface protein coverage in the preheated powders than in the non-preheated powders. It appeared that the difference in the solubility of the powders was not related to the surface protein coverage of these powders.

SDS-PAGE patterns of the fat globule surface material isolated from cream washed three times with SMUF are shown in Fig. 9.4. Caseins apparently dominated the surface proteins of both the non-preheated powder samples and the preheated powder samples, whereas the intensities of the whey protein (β-lg and α-la) bands were stronger in the preheated samples than in the non-preheated samples. The intensity of the β-casein band was slightly weaker than the intensity of the α_{s1}-casein band in all powders when the bands of the two caseins in the whole milk powder samples were compared. The intensity of whey proteins (β-lg and α-la) in the surface material of powder 3 (~11%) was apparently stronger than that of the other non-preheated powders, this was because its concentrate was heated to 79 °C after homogenization.

When the surface material was washed with the dissociating buffer, essentially only κ-casein and almost no other caseins (α_{s1}-, α_{s2}- and β-caseins) were observed in the SDS-PAGE patterns of all powders (Fig. 9.5). Bands of native MFGM proteins (xanthine oxidase, butyrophilin, PAS 6 and PAS 7), β-lg and α-la were present in the patterns for all powder samples. Furthermore, SC (Mr ~75 kDa) and heavy chain (Mr ~58 kDa) (the fragments or components of Igs) were also resolved from the surface material (Fig. 9.5). The native MFGM proteins, SC, heavy chain and κ-casein were fairly similar in intensity for both the non-preheated powders and the preheated powders. However, the

intensities of the whey proteins (β-lg and α-la) were different between the individual powders.

Table 9.3. The total surface protein and the surface protein coverage of the milk fat globules in the milk powders. The samples were produced using various processing conditions (see Fig. 3.1)

Run	Total MFGM protein (mg/g fat)	Surface protein coverage (mg/m^2)
Non-preheated samples		
1	73.8	6.21
2	72.5	6.19
3	72.2	6.19
4	73.5	6.21
5	80.6	6.83
6	70.8	5.87
7	75.9	6.64
8	76.8	6.40
Average	74.51 ± 3.14	6.19 ± 0.40
Preheated samples		
9	76.2	6.85
10	77.8	6.52
11	75.0	6.54
12	71.8	6.04
13	81.3	6.56
14	70.2	6.28
15	74.1	6.31
16	79.4	6.45
Average	75.73 ± 3.50	6.44 ± 0.29

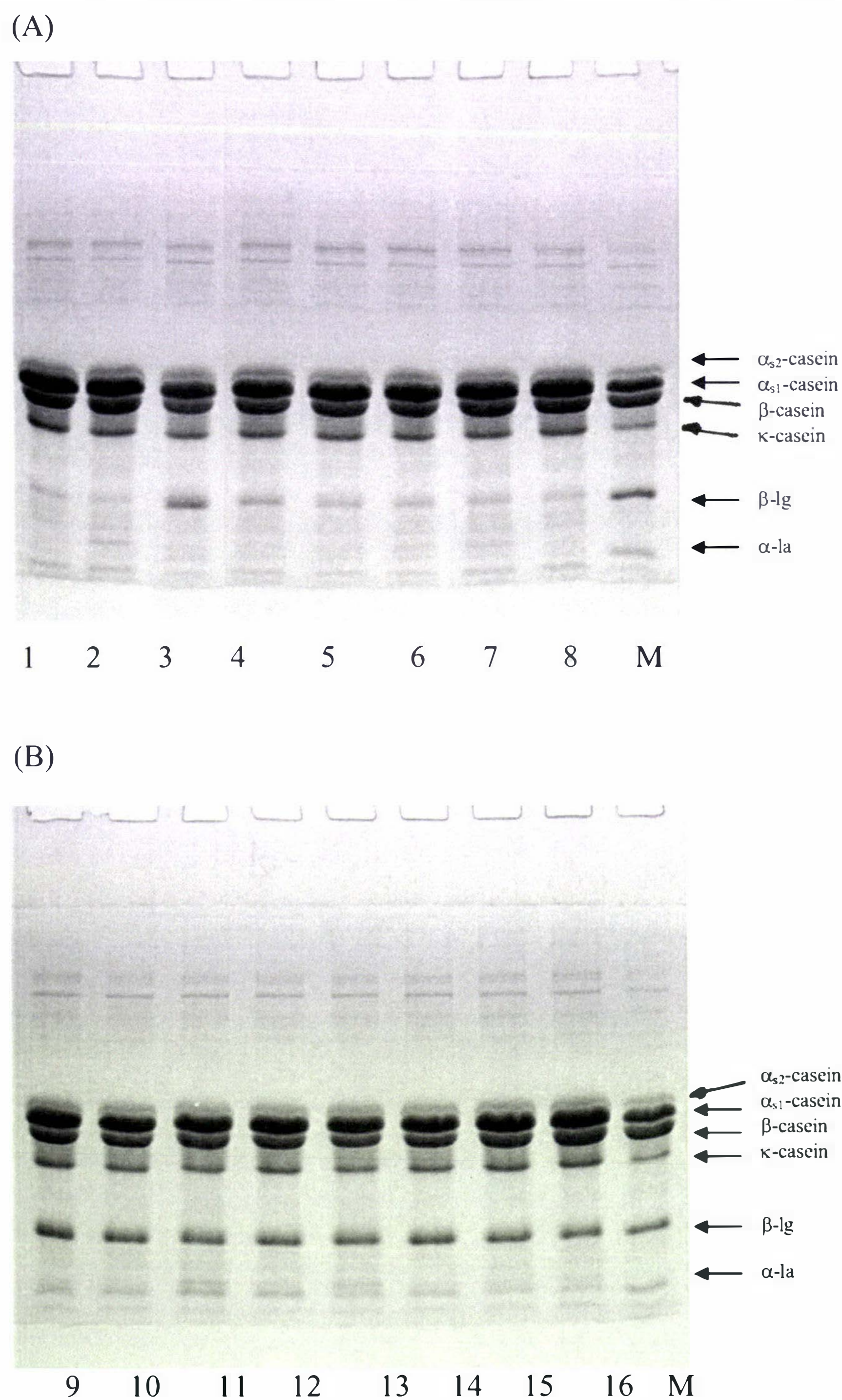


Fig. 9.4. SDS-PAGE patterns (15% acrylamide gel) of fat globule surface material, under reducing conditions, isolated from non-preheated (A) or preheated (B) powders reconstituted in water to approximately 12% total solids. Cream obtained from the reconstituted powders was washed in SMUF. M, whole milk.

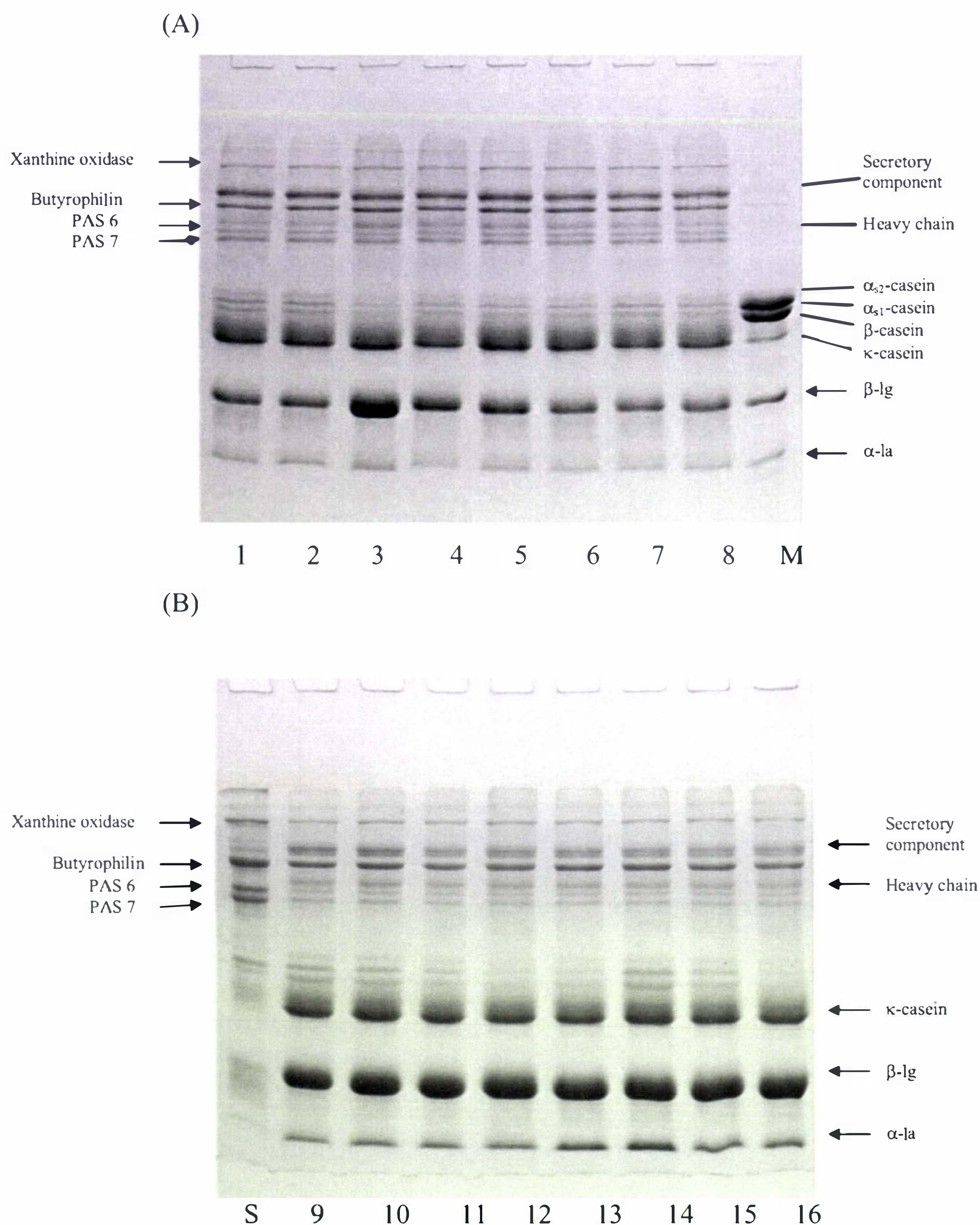


Fig. 9.5. SDS-PAGE patterns (15% acrylamide gel) of fat globule surface material, under reducing conditions, isolated from non-preheated (A) or preheated (B) powders reconstituted in water to approximately 12% total solids. Cream obtained from the reconstituted powders was washed with urea and EDTA buffer. M, whole milk; S, surface material isolated from the standardized milk.

9.6. Discussion

The present results showed that clusters of aggregated fat globules were present in some powders, which also showed poor reconstitution properties. These large aggregated fat particles were attributed largely to the linkages of interfacial proteins among the fat globules. McKenna et al. (1999) reported that the "insoluble" material in hot water and coffee is a mixture of fat globules and proteins (casein micelles and whey proteins), using microscopy (CLSM and TEM).

It was observed in the present study that there was little difference between the reconstitution properties of the non-preheated powder samples and the preheated powder samples (Table 9.1), although larger amounts of whey proteins were present at the fat globule surfaces of the preheated powders. This suggests that the whey proteins that were associated with the MFGM during preheating were not related to the solubility of the powders. In other words, these whey proteins were not involved in the formation of the fat globule aggregates.

Of the non-preheated powders, powder 3, that had the poorest solubility-related functional properties (Table 9.1), i.e. coffee sediment and hot sediment, had a large amount of whey proteins (~11%) at the fat globule surface. Another poor solubility powder, powder 5, also had a higher amount of whey proteins (~4%). These larger amounts of whey protein at the fat globule surface of these powders were attributed to the fact that the concentrates of these powders were homogenized at high pressure (70 bar) with heating to 79 °C. The concentrate of powder 3 was heated after homogenization, whereas the concentrate of powder 5 was heated before homogenization. These conditions, i.e. heating to 79 °C and homogenization at 70 bar, may have also led to the poor solubility of preheated powders 11 and 13. In contrast, high homogenization pressure (70 bar) with low temperature (65 °C) heating or high temperature (79 °C) heating with low pressure (40 bar) homogenization did not result in poor solubility in the other powders (Table 9.1). These results indicate that the combination of high homogenization pressure and high temperature results in the poorer solubility, particularly when heating is carried out after homogenization, probably

because more whey proteins are associated with the surface after the concentrate is homogenized. The results in Chapter 7 showed that more whey proteins associated with the fat globule surface when the concentrates were heated after homogenization than when the concentrates were heated before homogenization, largely because there are more sites for whey protein binding after the adsorption of casein micelles at the surface (Sharma & Dalgleish, 1994). The poor solubility of the powders is also probably a result of the adsorbed casein micelles aggregating with other casein micelles (adsorbed micelles or micelles in the serum) during heating to form thicker surface or protein chains, as observed in Chapter 7. It was suggested in Chapter 7 that the structure of the casein micelles may change after adsorption at the surface. This change may make aggregation of the micelles easier during subsequent heating and consequently may result in more whey proteins at the surface.

Thus, homogenization is the first condition that changes the structure of the proteins adsorbed at the surface; heating is the second condition that causes the interactions between the adsorbed proteins and the proteins present in the milk plasma. These interactions occur (i) between adsorbed casein micelles of the same fat globule or between fat globules; (ii) between the adsorbed casein micelles and casein micelles presented in the skim milk; (iii) between the adsorbed casein micelles and whey proteins including associated whey proteins and proteins present in the skim milk.

Mol (1975) reported that increasing the homogenization pressure of the concentrate increased the number of insoluble particles in the resulting whole milk powder, and suggested that the casein micelles adsorbed on the fat globules may be less heat stable during subsequent heating processes (e.g. drying) than the casein micelles normally present in the milk serum. The heat stability of milk is reduced as a result of homogenization and this effect is enhanced by increasing homogenization pressure, fat content and concentration (Sweetsur & Muir, 1983; Walstra & Jenness, 1984; Singh et al., 1996). It was also concluded by McKenna et al. (1999) that the increased number of casein micelles adsorbed on to the fat globules after homogenization had a detrimental effect on powder solubility. McKenna et al. (1999) considered that this effect is due mainly to the adsorption of casein micelles on to the fat globules and subsequent

clustering of the fat globules, possibly caused by the sharing of adsorbed casein micelles between two or more fat globules.

From this study, it appears that the clusters of aggregated fat globules formed through linkages of interfacial proteins in powder are related to the solubility properties of the powder. Preheating of the milk appears not to influence the solubility of powders. A combination of high homogenization pressure and high heating temperature of the concentrate results in the poor solubility. Heat treatment after homogenization causes the largest protein interactions at the surface, which leads to the poor solubility of the powder.

CHAPTER 10
OVERALL DISCUSSION AND CONCLUSIONS

The goal of this thesis was to further our knowledge about the influence of processing factors on component interactions during the manufacture of whole milk powder and to understand the effects of these interactions on the reconstitution properties of whole milk powders. The major focus was on developing an understanding of the behaviour of fat globules and their interactions during preheating, evaporation, homogenization and spray drying.

This chapter provides a summary of the work carried out in the thesis and how the results have contributed towards this goal.

10.1. Changes in the fat globule size and the MFGM proteins during the manufacture of whole milk powder

The changes in the fat globule size and the MFGM proteins during the different stages of whole milk powder manufacture i.e. from raw milk to whole milk powder are summarised in Table 10.1.

The results of the present study show that the disruption of fat globules during the processing treatments, such as standardization of fat content, pumping and heating (indirect heat exchange) was very minor, as indicated by a slight change in the size distribution of fat globules. Almost no caseins adsorbed at the surface of fat globules during these processing treatments. By contrast, DSI heating and subsequent processing treatments, such as evaporation, homogenisation and drying, markedly alter the fat globule sizes and MFGM composition. The changes in the average fat globule size (d_{32}) and fat globule size distribution of samples that were obtained at each processing step in the manufacture of a typical whole milk powder are shown in Fig. 10.1.

Table 10.1. Summary of the changes in fat globule size and the MFGM proteins that occur during the manufacture of whole milk powder.

Stage of process	**Fat globule size (d_{32})**	**MFGM proteins**
Raw milk	• ~1.2 μm	• Total MFGM protein: ~6.5 mg/g fat • Surface coverage: 1.3 mg/m^2 • Variations in seasonal milks
Pasteurisation	• No change	• Small amount of whey proteins associating with the MFGM
Standardization	• Minor change	• Small amount of caseins adsorbing at the fat globule surface
Preheating by indirect heating	• No change	• Increase in the MFGM proteins (~8 mg/g fat, 1.6 mg/m^2) • Association of serum proteins (~1.0 and ~0.25mg/g fat for β-lg and α-la, respectively) • Decrease in PAS 6 and 7 levels
Preheating by DSI	• Decrease in size to ~0.8 μm	• Increase in the MFGM protein concentration (~1.8 mg/m^2) • Casein micelles adsorbing at the fat globule surface • Whey proteins associating with the MFGM
Evaporation	• Decrease in size after each effect to ~0.7 μm in final concentrate • The higher the total solids in the concentrate, the lower the fat globule size	• Increase in the MFGM protein concentration after each effect to ~4 and ~6 mg/m^2 for the final non-preheated and preheated concentrates, respectively • Casein micelles adsorbing at the fat globule surface • Small amounts of β-lg, α-la and Igs components (SC and heavy chain) adsorbing at the fat globule surface
Homogenization	• Further decrease in the size to ~0.66 μm and ~0.60 μm at 40 bar and 70 bar homogenisation pressures, respectively	• Increase in the surface protein concentration to ~7 mg/m^2 • Higher surface protein concentration in preheated samples compared to the non-preheated samples due to the involvement of whey proteins • Casein micelles and whey proteins adsorbing at the fat

		globule surface • Original MFGM proteins remaining at the surface
Concentrate heating	• No change	• Whey proteins further associating with the fat globule surface • In samples heated after homogenisation, more whey proteins associated with the surface compared with the samples heated before homogenisation • Further association of casein micelle with the fat globule surface together with whey proteins via interactions
Spray drying	• Decrease in the size to a similar size in all powder samples, over-riding the difference between the fat globule sizes in the concentrates • Some fat globules coalescing into large "spreading fat" at the surface of powder particles	• Surface protein coverage generally decreased compared to that of concentrates, despite total surface protein increased due to casein micelles adsorbing to fat globule surface during spray drying • Some "uncovered fat" may form at the surface of powder during spray drying
Whole milk powder	• ~0.5 μm • Some fat globules aggregated into larger clusters	• At the fat globule surface of powders produced from non-preheated milk, surface protein concentration: ~6.2 mg/m^2, composition: ~73% caseins, ~8% original MFGM proteins, ~4% β-lg and ~1% α-la • At the fat globule surface of powders produced from preheated milk, surface protein concentration: ~6.5 mg/m^2, composition: ~64% caseins, ~8% native MFGM proteins, ~15% β-lg and ~4% α-la

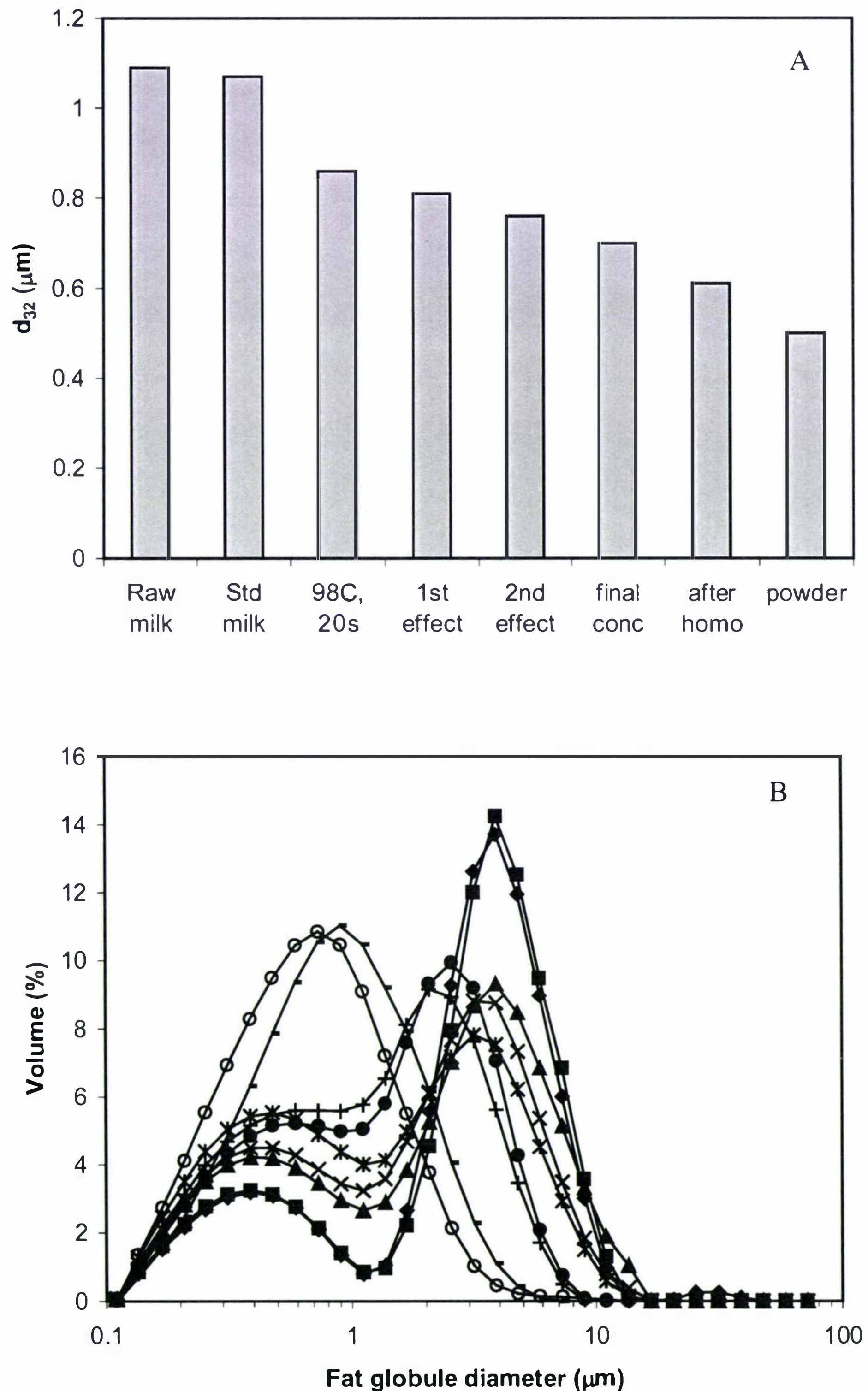


Fig. 10.1. Changes in the average fat globule size (d_{32}, A) and size distribution (B) of samples that were obtained at each processing step in the manufacture of a typical whole milk powder. Raw milk (♦), standardized milk (■), preheated by DSI (▲), after the 1st effect (✱), after the 2nd effect (×), the final concentrate (●), heating concentrate at 79 °C (+), after homogenisation of concentrate at 70 bar (▬) and powder (○).

10.2. Adsorption behaviour of casein micelles at the fat globule surface during processing

The TEM micrographs of homogenized concentrated milk showed that most of the adsorbed casein micelles were relatively intact with some micelles spreading over the fat surface. Some of the micelles were shared by two or more fat globules. SDS-PAGE of the surface material showed that the composition of adsorbed casein micelles was slightly different from that of the normal casein micelles. When the surface material was washed by a dissociating buffer, it was found that only κ-casein directly adsorbed at the fat surface (Chapter 7). Other caseins (α_{s1}-, α_{s2}-caseins, β-casein) were not directly adsorbed, despite some casein micelles apparently spreading over the fat surface during/after adsorption. Furthermore, greater spreading of casein micelles over the fat surface at high homogenisation pressures and temperatures caused more κ-casein to be directly adsorbed at the surface. These observation indicate that the casein micelles are not disintegrated during homogenization and are adsorbed onto the fat interface as intact micelles. If the casein micelles were disrupted before or after adsorption, it would be expected that some α_{s1}-, α_{s2}-caseins and β-casein would be directly adsorbed at the fat surface.

A number of casein monomers or sub-units of micelles at the fat globule surface of homogenized unconcentrated milk, cream or recombined milk were observed by previous workers (Anderson et al., 1977; Darling and Butcher, 1978; Sharma et al., 1996; Cano-Ruiz and Richter, 1997). However, the mechanism of formation of these casein sub-micelles or fragments that were observed at the fat globule surface is uncertain. One possibility is that the disruption of the casein micelle structure is caused by the act of homogenisation and subsequently the disrupted micelles adsorb on to the fat globule surface (Henstra and Schmidt, 1970). Another possibility is that the disruption of the casein micelles occurs after they are adsorbed on to the fat globule surface. Walstra & Oortwijn (1982) suggested that casein micelles if adsorbed on to a partially covered oil-water interface, may spread to cover the interface. This spreading is driven by thermodynamically favourable changes in the interfacial free energy which are sufficient to disrupt micelles.

Dalgleish (1989) showed that native micelles do not bind to hydrophobic (polystyrene lattices) surfaces, suggesting that the apparently "intact" micelles on the surface of fat globules were not likely in their native state. Therefore, micelle structure must re-arrange before the micelle interacts with the fat surface.

In the present study, because only κ-casein was found to be adsorbed directly at the fat surface when casein micelles adsorbed on to the fat surface, changes in the structure and composition of the adsorbed casein micelles appear to occur during the adsorption of casein micelles. It is likely that κ-casein, located at the surface of micelles, interacts with the fat surface through hydrophobic interactions, whereas the internal α_{s1}-, α_{s2}-caseins and β-caseins do not interact with the surface of fat globules. The adsorption of κ-casein at the fat globule surface via hydrophobic interactions probably leads to some changes in the interaction forces between κ-casein and other casein components within the micelles.

In its normal position on the surface of casein micelles, κ-casein is probably linked to the remainder of the micelles via the hydrophobic para-κ-casein part of the molecule, allowing the hydrophilic and charged glycomacropeptide to protrude from the surface into the surrounding solution and interact with the solvent to stabilize the micelles (Dalgleish and Holt, 1988; Walstra, 1990; Dalgleish, 1992). A strongly hydrophobic bonding of the N-terminal region of κ-casein to the rest of the micelle is also suggested in the model of Holt (1992). Horne (1998) also proposed a model of casein micelle, in which the attractive interactions between the hydrophobic regions of the caseins play an important role in maintaining micelle integrity and internal structure. κ-Casein links into the hydrophobic region through its hydrophobic N-terminal block but its C-terminal block cannot extend the polymer chain through the phosphoserine cluster. Therefore, chain and network growth are terminated, leaving the casein micelle network with a surface layer of κ-casein molecules (Horne, 1998).

It is proposed that as κ-casein adsorbs on to the fat globule surface during homogenization, its hydrophobic part is shifted to the fat surface from the micelle. This change in the orientation of κ-casein may consequently diminish its hydrophobic

linkages with the other caseins, particularly β-casein. This change in the interaction forces between casein components may consequently lead to the removal of some β-casein from the adsorbed micelles (Chapter 7). This may explain the higher ratio of α_s-casein/β-casein in the adsorbed micelles as compared with normal micelles.

It is also likely that spreading of casein micelles at the fat globule surface would result in a lesser or insufficient amount of κ-casein being present at the side of micelle surface facing toward the serum phase. In other words, these adsorbed casein micelles may be partly depleted in κ-casein at the micelle surface facing towards the serum phase, especially the adsorbed micelles that have spread extensively over the fat globule surface. A schematic representation of the surface protein layer at the fat globules of homogenized concentrate is shown in Fig. 10.2.

If it is true that the adsorbed casein micelles are partly depleted in κ-casein, the adsorbed casein micelles could react more readily than the casein micelles in the milk serum. The fat globules with adsorbed casein micelles may not only behave like a normal casein micelle, but their colloidal stability will be lower than that of the casein micelles in the milk serum. Fat globules that have adsorbed casein micelles, which are already deficient in κ-casein, would readily take part in fat globule-fat globule and/or fat globule-micelle interactions.

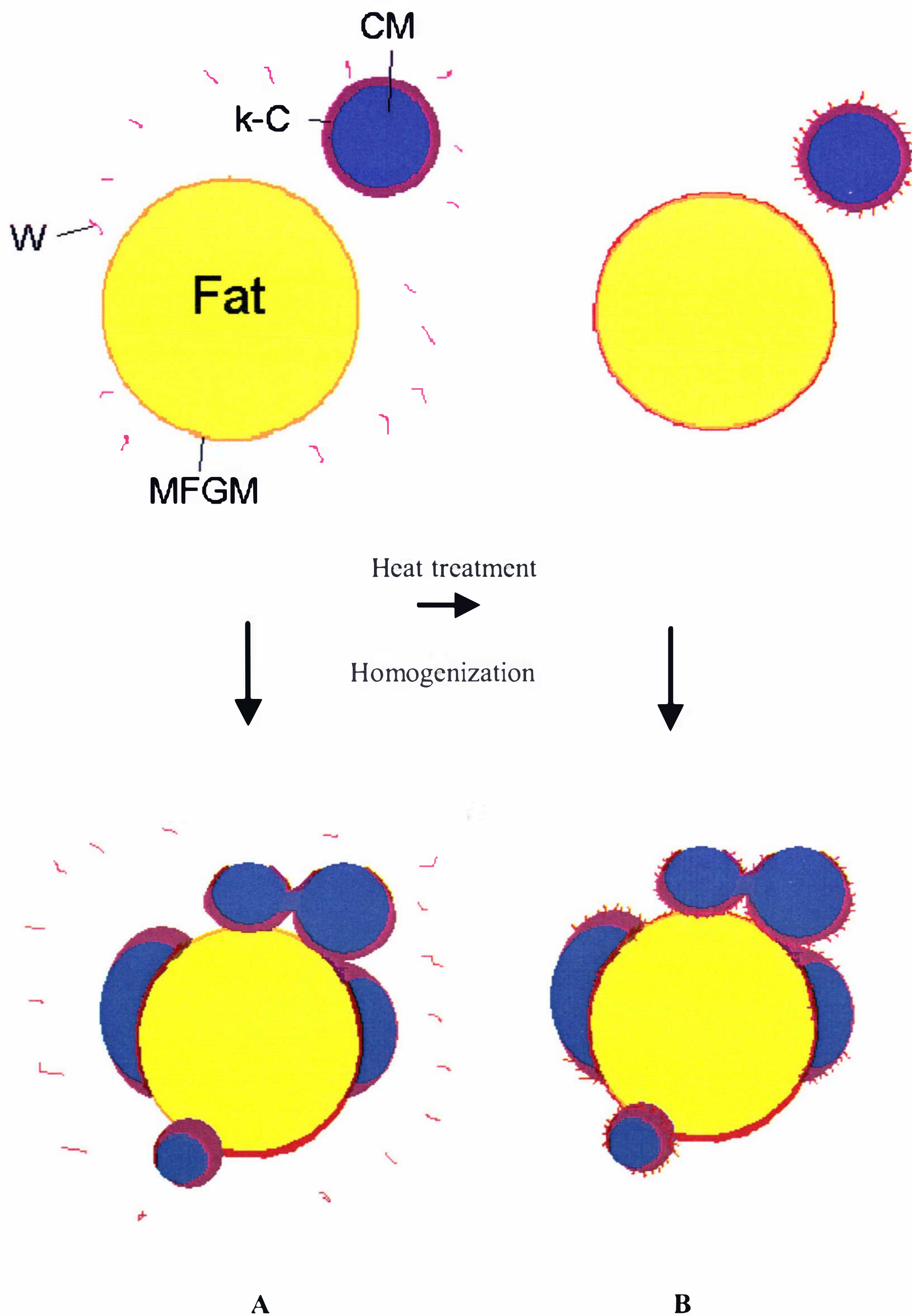


Fig. 10.2. Schematic representation of a fat globule surface layer in the non-preheated (A) or preheated (B) concentrate after homogenisation. CM: casein micelle, W: whey protein, k-C: κ-casein, MFGM: milk fat globule membrane.

10.3. Association of whey proteins with the MFGM during heat treatment

β-Lactoglobulin

The results of the present study clearly showed that β-lg was present in the MFGM of milk that was heated at relatively low temperatures (60–65 °C). Dalgleish & Banks (1991) and Corredig & Dalgleish (1996) also found that β-lg was present at the MFGM of the whole milk heated at 65 °C. However, the mechanism by which the serum proteins bind to the fat globule at this relatively low temperature was not identified. These authors considered that there are two ways by which the binding of serum proteins at the MFGM may occur. Whey proteins may bind to the proteins which are already present in the natural fat globule membrane system. Alternatively, whey proteins may displace the original membrane material, either by directly competing, or because the heating causes the original membrane to break down, leaving gaps through which the serum proteins (which may or may not be denatured at that stage) adsorb to the newly exposed fat surface (Dalgleish & Banks, 1991). However their results could not distinguish between these two mechanisms unambiguously. In the present study, using SDS-PAGE under non-reducing conditions and two-dimensional SDS-PAGE, it is shown clearly that β-lg is associated with the original MFGM proteins via disulfide bonds in this temperature range.

The temperature range of 60-65 °C is lower than that required to denature this protein; the denaturation temperature of β-lg has been reported to be 78 °C, using differential scanning calorimetry (de Wit & Klarenbeek, 1984; Kinsella & Whitehead, 1989). However, it has been found that some MFGM proteins denature at lower temperatures, about 60 °C (Appell et al., 1982; Chapter 4). For example, xanthine oxidase, one of the major MFGM proteins, contains 22 disulfide and 38 sulfydryl groups, four of which are detectable in the undenatured protein complex (Cheng et al., 1988). This may imply that the free thiol groups could be provided by the MFGM proteins, for initiating thiol–disulfide interchange between MFGM proteins and β-lg. In this temperature range, the native β-lg dimer is dissociated to monomers and partial unfolding of β-lg monomers may occur (Relkin & Launay, 1990). Although, the free thiol grouping of β-lg has not been exposed yet, the disulfide bonds are probably exposed at the surface of the

monomer. The thiol--disulfide interchange between MFGM proteins and β-lg could occur in which the free thiol groups are provided by the MFGM proteins which have been denatured in this temperature region (Appell et al., 1982; Chapter 4).

The results of the present study suggest that β-lg association with the MFGM proteins, in the temperature region 75-95 °C (i.e. above β-lg denaturation temperature), involve the denaturation of β-lg, self aggregation of MFGM protein and aggregation of denatured β-lg with aggregated MFGM protein complex through the thiol--disulfide interchange. The kinetic behaviour and the E_a, $\triangle H^{\#}$ and $\triangle S^{\#}$ values for association of β-lg with the MFGM were similar to the reported values for denaturation of β-lg in the higher temperature range, strongly suggests that the interaction between the MFGM proteins and β-lg is dependent on the denaturation reaction, and as a consequence its reaction pathway closely follows that of denaturation. As the amount of β-lg association with the MFGM do not further increase when the temperature is > 80 °C, it is likely that the aggregated β-lg complexes formed at the high temperature could not interact further with the MFGM proteins.

α-Lactalbumin

The mechanism of α-la association with the MFGM is not clear from the previous work (Dalgleish & Banks, 1991; Houlihan et al., 1992; Corredig & Dalgleish, 1996). The amount of α-la present at the fat globule surface was very low in all samples (including the unheated control sample) (Dalgleish & Banks, 1991; Houlihan et al., 1992) and did not increase with increasing temperature (Corredig & Dalgleish, 1996), suggesting that α-la may be present as an artefact (Houlihan et al., 1992). Kim & Jimenez-Flores (1995) and Lee & Sherbon (2002) suggested that the direct disulfide bonding between α-la and the MFGM proteins is the major force in the association of α-la with the membrane.

In the present study, it is suggested that α-la could associate with the MFGM through the thiol--disulfide interchange during heating, because the MFGM proteins have the abundant free sulfydryl groups. In contrast to previous findings, the amount of α-la association with the MFGM increased with increasing the temperature to 80 °C, then

reached a plateau. The kinetic behaviour of α-la interactions with the MFGM protein was similar to that of the heat-induced denaturation of α-la.

BSA and Igs

BSA and Igs are more heat sensitive than β-lg and α-la (Dannengerg & Kessler, 1988; Singh & Creamer, 1991). However, the heat-induced associations of BSA and Igs with the MFGM were not reported by previous workers. Also, BSA and Igs were not observed at the MFGM after heating in the present study. The reason is unknown.

Heat-induced association of whey proteins with the fat globule surface of the homogenized milk

Heat treatment of the homogenized milk causes the association of whey proteins with the fat globule surface (Sharma & Dalgleish, 1994). Denatured serum proteins interact with both the κ-casein of the adsorbed caseins, either as semi-intact micelles or as micellar fragments (Sharma & Dalgleish, 1994), and the native fat globule membrane (Dalgleish & Banks, 1991). If whole milk is heated before homogenisation, the denatured serum proteins interact with both the κ-casein of the casein micelle and the native fat globule membrane. During subsequent homogenisation, the micellar complex of casein and serum proteins will adsorb on the newly formed fat surfaces. Sharma & Dalgleish (1994) reported that the amounts of serum proteins associated with the MFGM are smaller in milk that was heated before homogenisation than in milk that had been homogenized and then heated. They suggest that more binding sites for the whey proteins became available as the casein micelles were spread over the fat surface rather than being in their native configuration. However, Lee and Sherbon (2002) did not agree with the results of Sharma & Dalgleish (1994). They found no difference in the amount of whey proteins associated with the fat globule surface between milks that were heated before or after homogenisation.

In the present study, larger amounts of β-lg and α-la were associated with the fat globule surface of the concentrated milks heated after homogenisation than the concentrated milks that were heated and then homogenised. This may be related to the change in the structure of casein micelles after adsorption. The spreading of adsorbed micelles may allow greater binding of whey proteins. In addition, the adsorbed casein

micelles may aggregate with the casein micelles in milk serum during heating (discussed in Section 10.2), bringing more whey proteins to the fat globule surface.

10.4. Behaviour of the native MFGM proteins during processing

The MFGM proteins are known to be highly reactive. They contain large number of disulfide and sulfydryl groups (Cheng et al., 1988; Mather, 2000). Heat treatment causes the denaturation and aggregation of the MFGM proteins. Butyrophilin forms disulphide-stabilized complexes on heating at 58 °C (Appell et al., 1982). The present study shows that, in the absence of skim milk proteins, the xanthine oxidase and butyrophilin further aggregate on heating ≥ 60 °C, whereas the PAS 6 and PAS 7 are more stable to heating and do not aggregate until the temperature is up to 80 °C. However, when whole milk is heated, xanthine oxidase and butyrophilin interact with the serum proteins, whereas PAS 6 and PAS 7 are removed from the MFGM at ~70 °C. This indicates that the loss of PAS 6 and PAS 7 from the MFGM is related to the presence of the serum proteins. However, the mechanism is not clear. A possible explanation is that the formation of β-lg/MFGM protein complexes may alter the membrane structure and interaction forces within the MFGM, leading to the removal of PAS 6 and PAS 7. PAS 7 probably is a peripheral protein within the MFGM (Keenan & Dylewski, 1995). An alternative suggestion is that PAS 7 could interact directly with β-lg to form β-lg/PAS 7 complexes, which may then move from the MFGM to the serum phase. Houlihan et al. (1992) also observed that the loss of components 15 and 16 (PAS 6/7) from the membrane material in heated milk is related to the presence of skim milk components during heating. However, Kim and Jimenez-Flores (1995) interpreted this decrease as a breakdown in the structure of this protein with the protein remaining within the MFGM.

10.5. Relationship between the reconstituted properties of powder and the MFGM proteins`

It was observed in the present study that the whole milk powders with poor reconstitution properties have greater numbers of large aggregated particles which are mixtures of fat globules and proteins than whole milk powders that are easy to

reconstitute. This result indicates that the nature of surface proteins after homogenisation play a key role in the reconstituted properties of powder.

It was shown by McKenna et al. (1999) that the increased number of casein micelles adsorbed on to the fat globules after homogenization had a detrimental effect on the reconstituted properties of instant whole milk powder. McKenna et al. (1999) reported that when milk powder is reconstituted in hot water or coffee, the "insoluble" material formed a mixture of fat globules and proteins (casein micelles and whey proteins). They considered that this effect is due mainly to the adsorption of casein micelles on to the fat globules and subsequent clustering of the fat globules, possibly caused by the sharing of adsorbed casein micelles between two or more fat globules.

The fat globule surface protein of the whole milk powder is composed of three kinds of proteins: (1) the original MFGM proteins, (2) proteins adsorbed from the milk serum due to the disruption of the fat globules and (3) proteins associated with the original MFGM proteins and the adsorbed proteins, due to heating.

The present study found that the combination of high homogenization pressure and high temperature treatment of the concentrate before spray drying results in poor reconstitution properties of the powder, particularly when the heating is carried out after homogenization. This suggests that the poor reconstitution properties of the powder are related to the proteins directly adsorbed at the fat globule surface during homogenisation plus those proteins associated with adsorbed proteins.

Adsorption of casein micelles on to the fat globule surface could cause changes in the structure of micelle, e.g. casein micelles may be partly depleted in κ-casein at the micelle surface facing towards the serum phase (discussed in Section 10.2), allowing more site for the binding of the whey proteins and thus resulting in a decrease in the stability during subsequent heating. Heating after homogenisation may induce interactions of the adsorbed caseins on the fat globule surface with other adsorbed caseins or casein micelles in milk serum. This would enhance the linkages between the adsorbed proteins on different fat globules. Hence, the clusters of fat globules and the

"insoluble" materials increase in the powder when heating is carried out after homogenization.

Results of the present study show that the powders that have poor reconstitution properties also have amounts of relatively high whey proteins at the fat globule surface. This indicates that the concentrate before drying was heated at high temperature and whey proteins were involved to the formation of "insoluble" materials. When the concentrate is heated, whey proteins interact with the adsorbed casein micelles to form thread-like or horn-like material. TEM results (Chapter 7) show that these whey protein aggregates are partially responsible for the linkages between the adsorbed casein micelles and other micelles in the serum, causing fat globules to aggregate and form a cluster. McKenna (2000) showed that the milk powders that were manufactured from the whey-protein-depleted whole milk have lesser aggregated structures after drying and upon reconstitution in water, using the TEM.

A summary of the interactions occurring during the manufacture of whole milk powder, as observed in the present study, is represented schematically in Fig. 10.2.

10.6. Further studies

Based on the present investigation, the following subjects are recommended for further studies.

Differences in the properties of the native MFGM and adsorbed protein layer

Although there have been many studies on the characterization of the MFGM, the structure of the native MFGM, which consists of a complex mixture of proteins, phospholipids, glycoproteins, triglycerides, cholesterol, enzymes and other minor components, is still unclear. After the disruption of fat globules, a much thicker protein surface layer is formed at the new formed surface. The differences between the native MFGM and the new surface protein layer and their impact on the properties of dairy products, such as heat stability and deteriorative reactions, such as lipolysis and autoxidation of the fat, need to be further investigated and understood.

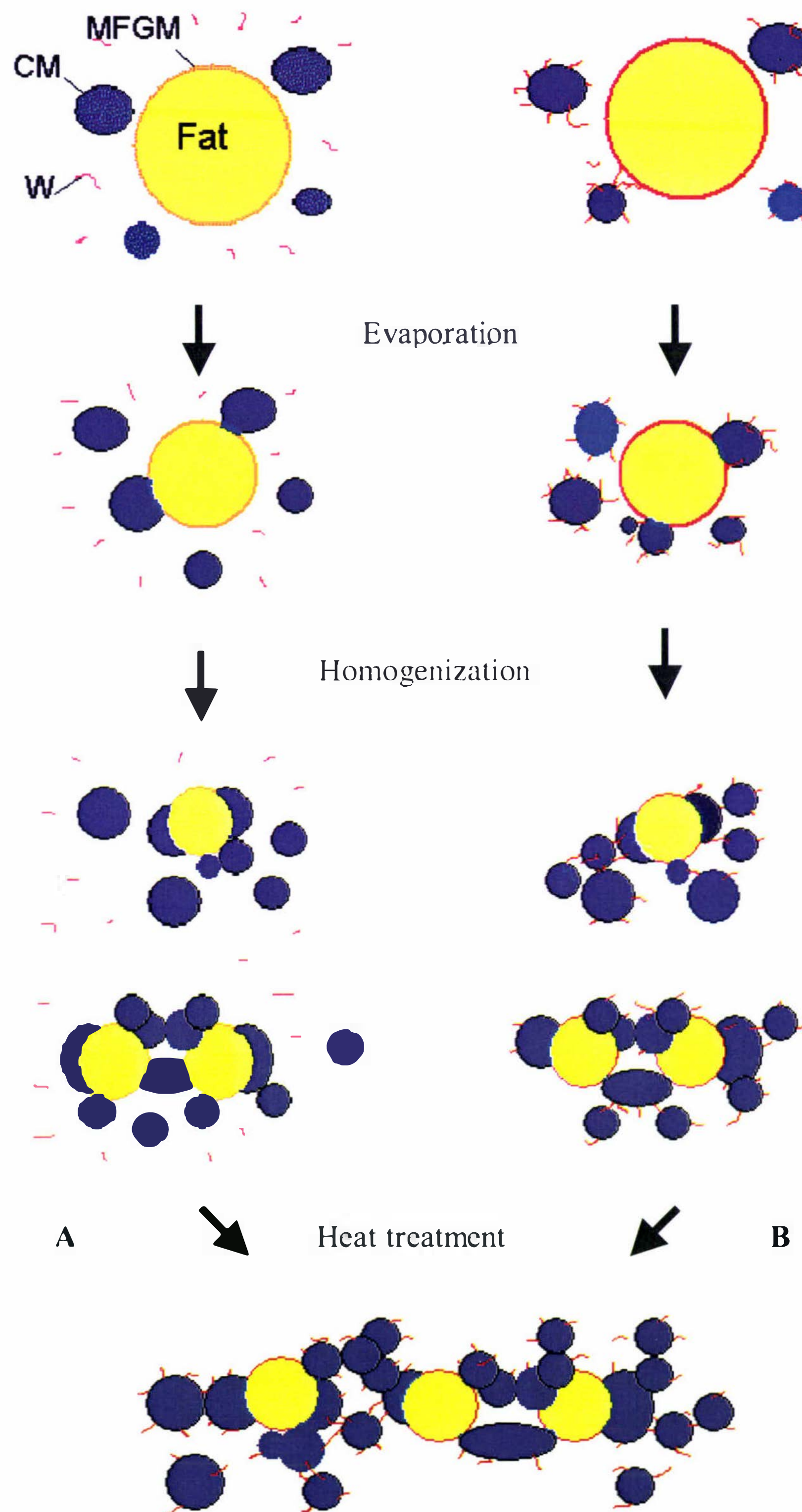


Fig. 10.3. Schematic representation of changes in the fat globules and the surface proteins during the evaporation, homogenisation and heat treatment. A: non-preheated milk; B: preheated milk, CM: casein micelle, W: whey protein, MFGM: milk fat globule membrane.

Differences between the adsorbed casein micelle and normal casein micelle

The structure of casein micelles appears to be changed after adsorption at the fat globule surface. Corresponding chemical and physical properties of adsorbed casein micelle probably are different from that of the normal casein micelles. The fat globules with a thick adsorbed casein micelle layer may or may not only behave like a casein micelles within a dairy product. A further study on the adsorbed casein micelle including its structure, composition and corresponding chemical and physical properties would be useful in better understanding the functional properties of dairy products produced through homogenisation.

Relationship between the fat globule or the MFGM and flow properties of milk or concentrate during unit operations

This thesis has analysed the change in the fat globules and the MFGM proteins during processing treatments and discussed the influence on the functional properties of powder. However, there is very little information available on the relationship between the milk or concentrate flow behaviour and the state of the fat globules or the MFGM during processing treatment. It would be interesting to investigate the influence of the changes in the fat globules and the MFGM on the rheological properties of milk or concentrate during the unit operations in the manufacture of whole milk powder.

Influence of spray drying conditions on the fat globule and 'uncovered fat'

The 'uncovered fat' may be formed during the spray drying. The amount of the 'uncovered fat' formed during the spray drying is related to the fat globule size of concentrated milk and methods and conditions of spray drying. Further investigations on the influence of spray methods, temperatures and equipments on the formation of 'uncovered fat' in the powder are recommended.

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