Composition and Structure of Fat Globule Surface Layers in Recombined Milk

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ABSTRACT

Protein coverage, composition and structure of surface layers of fat globules in recombined milk were determined. Average protein load was ~6 mg/m² fat surface. Both casein and whey proteins were present in the fat globule surface layer, with casein adsorbed in preference to whey proteins and α_s ($\alpha_{s1}+\alpha_{s2}$)-casein adsorbed in preference to β-casein. Transmission electron microscopy showed that the surface layer of fat globule was made up of casein micelles, fragments of casein micelles and a thin layer of protein, possibly whey proteins. Experiments with surface layers that had been dispersed in EDTA showed that the extent of dissociation of caseins followed the order: β-casein > α_s-casein >> κ-casein, suggesting that most of the κ-casein was probably associated directly with the fat surface.

Key Words: recombined milk, surface layers, fat globules, casein, whey protein

INTRODUCTION

RECOMBINED MILK is obtained by reconstituting nonfat dry milk to produce skim milk and then emulsifying fat into it by homogenization. This process subdivides the fat into minute particles with a very large combined fat surface area. Simultaneously, proteins from skim milk migrate to the newly created fat surface, where they form fat globule surface layers. These layers in recombined milk are almost exclusively made up of milk proteins; the components of natural fat globule membrane are largely absent from such systems (Mulder and Walstra, 1974). The composition and properties of such surface layers directly influence the properties of recombined milk products, especially heat stability and creaming during storage.

To determine the composition of fat globule surface layers in milk, it is necessary to isolate fat globules without selective losses or modifications. Various methods have been used to isolate fat globule surface layers from fresh homogenized milk (Brunner et al., 1953; McPherson et al., 1984; Sharma and Dalgleish, 1993) and recombined homogenized milk (Oortwijn and Walstra, 1979, McCrae et al., 1994). One method involved centrifugation of milk to recover the cream layer, washing to remove entrapped serum, and direct determination of protein composition of the fat globule surface layer (Singh et al., 1993; Sharma and Dalgleish, 1993). Another method involved determination of protein composition of the continuous aqueous phase before and after homogenization, thereby calculating the composition of the fat globule surface layer by difference (Oortwijn and Walstra, 1979). This ''depletion'' method is considered to incur several problems (Hunt and Dalgleish, 1994).

Studies on fresh homogenized whole milk have shown that, in addition to native components of the fat globule membrane, some plasma proteins are transferred to fat globule surfaces during homogenization (McPherson et al., 1984; Sharma and Dalgleish, 1993). McPherson et al. (1984) found both casein and whey proteins (mainly β-lactoglobulin) were major components of the surface layers of fat globules isolated from pasteurized homogenized milk. However, Sharma and Dalgleish (1993) did not find any whey proteins at the surface of fat globules from raw homogenized milk.

Few studies have been reported on the fat globule surface layers in recombined milk and the protein composition and structure of the layers have not been completely established (Oortwijn and Walstra, 1979; McCrae and Muir, 1991; McCrae et al., 1994). Our objective was to conduct a detailed investigation of the protein composition and microstructure of fat globule surface layers in recombined milk.

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MATERIALS & METHODS

Materials

Nonfat dry milk (whey protein nitrogen index, 7.00) and anhydrous milk fat were obtained from the New Zealand Dairy Board, Wellington. All chemicals were analytical grade from either BDH Chemicals (BDH Ltd, Poole, England) or Sigma Chemical Co. (St Louis, MO) unless specified otherwise.

Preparation of recombined milk

Nonfat dry milk was slowly added to distilled water at 40°C with continuous stirring for at least 30 min. Anhydrous milk fat, at 50°C, was then added to produce the required ratio of 9% (w/w) solids-not-fat to 4% (w/w) fat. The dispersion was stirred for 30 min, heated to 52°C and then passed once without applying pressure through a two-stage valve homogenizer (Rannie LAB, Rannie a/s, Albertslund, Denmark) to produce a coarse oil-in-water emulsion. The mixture was then homogenized at 13.8 MPa/3.5 MPa and stored overnight at 5°C. After about 18 hr the recombined milk sample was warmed to 20°C before further analysis.

Separation and washing of cream layer

Recombined milk was subjected to a series of centrifugal forces in the range 6,000–30,000 × g for 20 min at 20°C (Sorvall RC5C centrifuge, DuPont Company, Newtown, CT), to select a centrifugal force which gave maximum recovery of fat in the cream layer without coalescence of fat globules and with little sedimentation of protein. The centrifugal force, 18,000 × g for 20 min at 20°C, resulted in maximum fat (~85%, w/w) in the cream layer with comparatively small amounts of protein sedimentation (~15%, w/w).

The cream layer was carefully removed, dispersed in simulated milk ultrafiltrate (SMUF) (Jenness and Koops, 1962) for 1 hr and re-centrifuged under the same conditions. The purpose of this washing step was to remove any entrapped and loosely associated protein material from the surface of fat globules. After three washings of the cream layer in SMUF, no further change occurred in protein content. Hence this treatment was considered sufficient for subsequent experiments. The proteins remaining in the cream layer after washing were considered to be adsorbed at the surface of fat globules.

Determination of protein load

The protein load (Γ), as mg protein/m² fat surface area, was calculated from the protein present in the washed cream layers and the fat surface area. Fat surface area was calculated from d_{vs} (volume-surface average diameter), determined using the spectroturbidity method of Walstra (1969).

Table 1—Protein load and compositions of recombined milk and fat globule surface layers

Attribute	Recombined milk	Fat globule surface layer
Protein load (mg/m^2)		5.70 ± 0.42[a]
α_s-Casein (%, w/w)	38.78 ± 2.47	46.24 ± 4.49
β-Casein (%, w/w)	27.70 ± 2.96	26.42 ± 2.77
κ-Casein (%, w/w)	12.69 ± 1.43	16.57 ± 3.32
β-Lg (%, w/w)	15.71 ± 1.21	7.58 ± 1.85
α-La (%, w/w)	5.12 ± 0.94	3.19 ± 1.40
α_s-/β-Casein ratio	1.41 ± 0.17	1.77 ± 0.23
Casein/WP[b] ratio	3.83 ± 0.33	8.83 ± 2.63

[a] Mean ± 2 s.d. (number of samples = 8).
[b] Whey proteins (β-lactoglobulin + α-lactalbumin).

Chemical analyses

Total protein was determined by measuring total N by the macro-Kjeldahl method (AOAC, 1974) and multiplying by 6.38. Samples were digested and distilled using a Kjeltec system (Tecator, Hoganas, Sweden). Total fat in milk and in the cream layer was determined using the Roese-Gottlieb gravimetric method for milk (IDF Standard 1C:1987) and cream (IDF Standard 16C, 1987), respectively.

Quantification of individual proteins

SDS-PAGE was carried out using the method of Laemmli (1970), as described by Singh and Creamer (1991). Cream layer (0.1g) was dispersed in 4.9g SDS buffer (0.5M Tris, 2% SDS, 0.05% mercaptoethanol, adjusted to pH 6.8 with 1M HCl) and heated at 90°C for 5 min. The dispersion was then centrifuged at 800 × g for 5 min to remove the fat. The subnatant (10 μL) was applied to the SDS-gel previously prepared on a Mini-Protean II system (Bio Rad Laboratories, Richmond, CA). Resolving gel was composed of 16% (w/v) acrylamide made up in 1.5M Tris-HCl buffer, pH 8.8. Stacking gel was composed of 4% (w/v) acrylamide in 0.5M Tris-HCl buffer, pH 6.8. The gel was run at 200 V for about 45 min and then stained with Coomassie Blue R in 2.5:1:6.5 isopropanol/acetic acid/water. This was followed by destaining with several changes of 1:1:8 acetic acid/isopropanol/water. After destaining, protein bands were scanned on a laser densitometer (LKB Ultoscan XL, LKB Produkter, Bromma, Sweden). The integrated area under each individual protein band was calculated as percentage of total area for that sample.

Transmission electron microscopy

The general method used was similar to that of Kalab et al. (1976). Two embedding methods were used: (a) water-insoluble Spurr resin and (b) water-soluble Nanoplast resin (Bachhuber and Frosch, 1983). Embedding method using water-soluble resin provided greater details of structure of fat globule surfaces with good contrast between fat and protein particles. Milk (2 mL) was pipetted into a small bottle (45 mm length, 18 mm internal diameter, 1 mm wall thickness, with stopper) and 1 mL of 3% (w/v) low-melting agarose (agarose type IX: ultra-low gelling temp, Sigma Chemical Company) was added. The solution was mixed and allowed to gel at 4°C for 2 hr. The gel was cut into 1-mm^3 blocks and prefixed in 2% (v/v) glutaraldehyde solution overnight at 4°C. The pre-fixed blocks were then washed 4 times in N-2 hydroxyethyl piperazine N'-2-ethane sulfonic acid (HEPES) buffer (0.05 M, pH 6.6, 5 mM $CaCl_2$) and infiltrated with a water-soluble resin (Nanoplast resin, Ted Pella Inc. Redding, CA). The molds were placed in an oven at 60°C for 72 hr for hardening. The hardened blocks, after cooling to room temperature, were trimmed with a block trimmer (Reichert-Jung TM 60, C. Reichert Optische Werke), carefully sectioned (50 nm thickness) with a glass knife using a microtome (Reichert-Jung Ultracut E, C. Reichert Optische Werke, Germany) and collected in a water trough. Sections were then transferred onto a 400-mesh copper grid (Emgrid copper grid 3 mm, HF 36, Emgrid Australia, The Patch, Australia), previously coated with formvar film.

The thin sections on the copper grids were stained by placing the grids in a saturated uranyl acetate solution (made up in 50%, v/v, ethanol). After 3 min, the grids were removed and placed in 50% ethanol for 1 min, then washed with distilled water and dried on filter paper. The grids were finally placed in lead citrate solution for 3 min, washed in water and dried on filter paper. The sections on the grid were viewed under a Philips 201c transmission electron microscope at an accelerating voltage of 70 kV at the Crown Research Institute, Palmerston North.

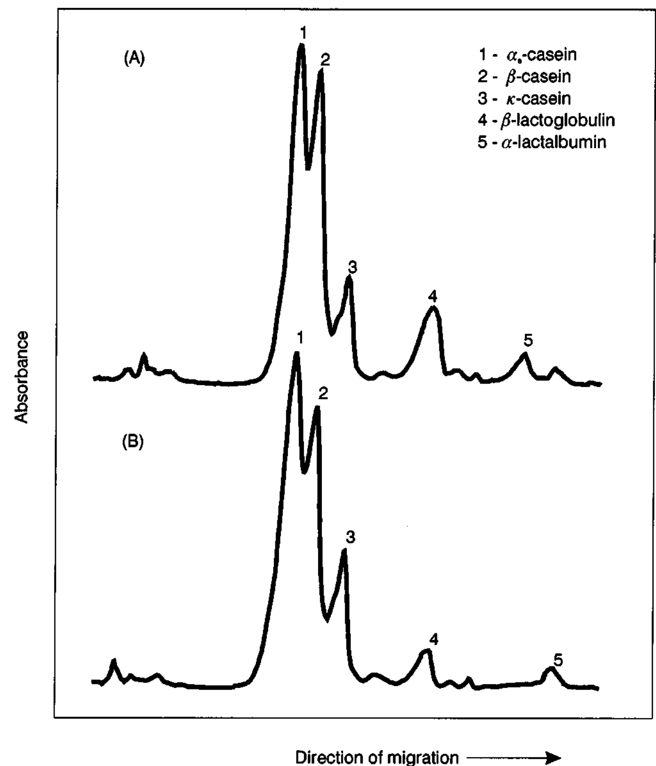

Fig. 1—Densitometric scans of SDS-PAGE obtained from (A) recombined milk and (B) cream layer. Experimental details in Materials & Methods.

RESULTS

Composition of fat globule surface layers

The average protein load in recombined milk was 5.7 ± 0.42 mg/m^2 (Table 1). For recombined milk, prepared from fresh skim milk and anhydrous milk fat, Oortwijn and Walstra (1979) reported a protein load value in the range 8-10 mg/m^2, somewhat higher than our value. However, Oortwijn and Walstra (1979) used a 'depletion method' for estimating protein load, whereas in our study the protein load in the cream layer was directly determined. In the depletion method, the difference between the amount of protein in the aqueous phase before and after emulsification, was considered the amount adsorbed. The application of this method to recombined milk presents several problems. First, centrifugation of recombined milk at relatively high speed causes sedimentation of large casein micelles. These micelles are difficult to re-disperse and hence the accurate measurement of protein in the aqueous phase is difficult. Also, small fat globules are generally not separated by centrifugation and are therefore included in analysis of the aqueous phase. In addition, the amount of protein adsorbed in recombined milk is very small and thus there is very little difference between initial and final protein concentrations in the aqueous phase.

Typical scannning patterns of SDS-gels for recombined milk and cream layers were compared (Fig. 1). Both caseins and whey proteins were present in the cream layer, i.e., at the surface of fat globules (Fig. 1B). The percentage of individual proteins were calculated from the scanning patterns (Table 1). The ratio of casein/whey protein in the cream layer (8.83) was higher than that in recombined milk (3.83), indicating a preference for adsorption of casein. This confirmed earlier observations on fresh homogenized cream and milk (Darling and Butcher, 1978; McPherson et al., 1984) and recombined milk (Oortwijn and Walstra, 1979; McCrae et al., 1994). Furthermore, Sharma and Dalgleish (1993) found no whey proteins at the fat globule surfaces in fresh homogenized milk.

The ratio of α_s-casein/β-casein was also greater in the cream layer than in original recombined milk, suggesting that α_s-casein

was adsorbed preferentially. This was consistent with results for homogenized milk (McPherson et al., 1984).

Microstructure

Electron microscopic examination of recombined milk showed that fat globules and casein micelles were clearly distinguishable (Fig. 2). Fat globule surfaces were covered with some intact casein micelles and a number of smaller particles, which probably resulted from disaggregation of casein micelles. Some casein micelles showed evidence of partial spreading at the fat globule surface. A thin dark layer of protein, probably consisting of whey proteins, was also present. These observations were consistent with those of Oortwijn et al. (1977) who found both whey proteins and casein micelles at the surface of homogenized fat globules.

Surface layers of fat globules of varying sizes

To isolate fat globules of different sizes, recombined milk was centrifuged successively at greater centrifugal forces, 1,000, 5,000, 10,000, and 20,000 × *g* for 20 min at 20°C. After each centrifugation, the cream layer was removed and washed three times with SMUF before analysis. The d_{vs} obtained at different centrifugation speeds ranged from 0.30 to 0.66 μm. In general, larger fat globules had a lower protein load than smaller fat globules (Table 2). The smaller globules also had greater ratios of casein/whey protein and α_s-casein/β-casein than larger fat globules. From electron micrographs, we observed that, on average, smaller fat globules had larger attached casein micelles.

Similar trends have been reported (Walstra and Oortwijn, 1982; Dalgleish and Robson, 1985; McCrae and Muir, 1991), although our protein load values were lower. For example, fat globules of d_{vs} ~0.4 μm had a protein load of ~7 mg/m² compared to a protein load of ~15 mg/m² reported by Walstra and Oortwijn (1982).

Dissociation of adsorbed casein micelles

A proportion of casein adsorbed at the fat globule surface was clearly present in the form of ''intact'' micelles (Fig. 2). Since removal of colloidal calcium phosphate from the micelle caused dissociation of the casein micelle into its sub-units (Lin et al., 1972), it may be possible to characterize those parts of the casein micelle directly involved in interactions with the fat globule surface by dissociating casein micelles using EDTA. Washed cream layers, prepared from recombined milk, were dispersed in 0.05M EDTA and left at 5°C for up to 16 hr. After specified intervals, samples were removed, warmed to 20°C, and centrifuged at 18,000 × *g* for 20 min. Resultant cream layers were analyzed for total protein, fat and individual proteins. The protein load decreased with time in the EDTA solution (Fig. 3). During the first 30 min, there was a dramatic decrease in protein load. In the following 2 hr there was a further small decrease. The final protein load value of 2.0 mg/m² was similar to that reported for sodium caseinate-stabilized emulsions (Oortwijn and Walstra, 1979; Dickinson et al., 1984), suggesting that EDTA treatment caused complete dissociation of adsorbed casein micelles.

The quantity of each casein at the surface decreased with increasing time in EDTA solution (Fig. 4), with a rapid decrease during the first 30 min and thereafter a more gradual decline. The greatest dissociation from the fat globule surface was for β-casein (77%), followed by α_s-casein (58%) and κ-casein (25%). In contrast, β-lactoglobulin and α-lactalbumin did not appear to be affected.

The addition of 0.2% (v/v) mercaptoethanol to the EDTA solution had no effect on the extent of dissociation of α_s- and β-casein from the fat globule surface. However, the dissociation of κ-casein increased from 25 to 50% and there was a slight increase in dissociation of β-lactoglobulin. These observations suggest that a proportion of κ-casein was present as a disulfide-linked polymer at the fat interface. It is also possible that a proportion of β-lactoglobulin was present as a disulfide-linked polymer or as a κ-casein/β-lactoglobulin complex.

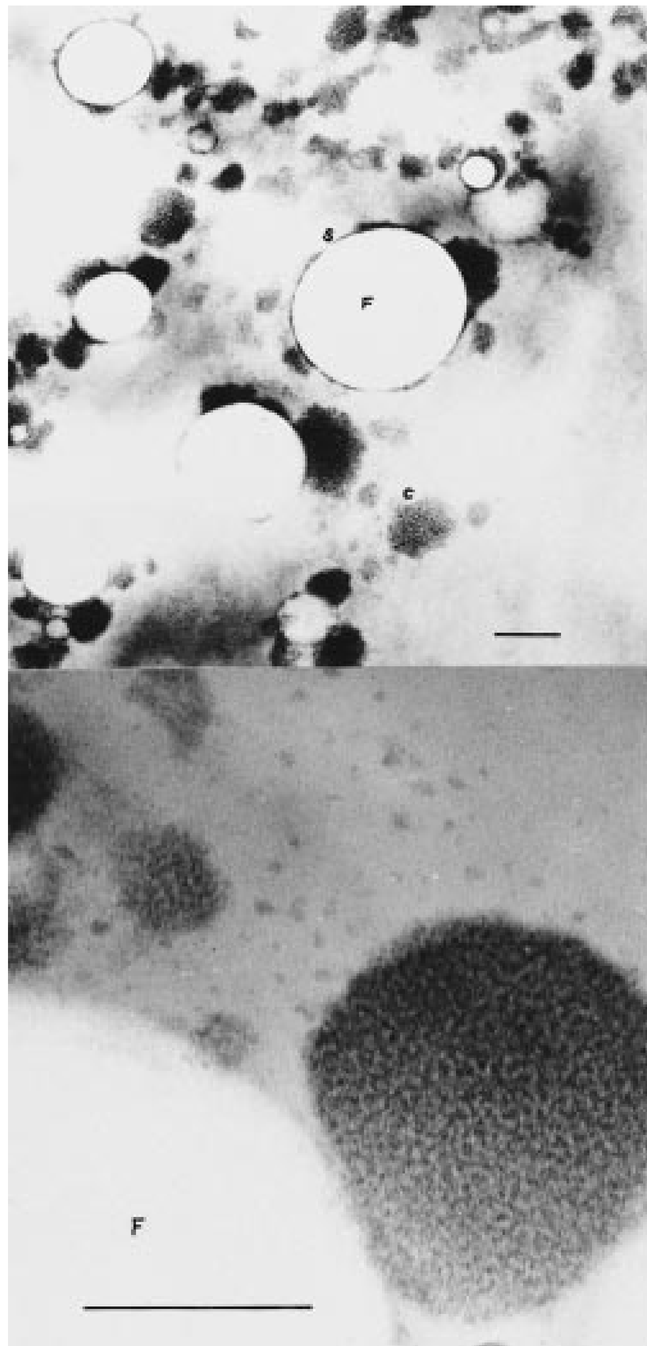

Fig. 2—Transmission electron micrographs of thin sections of recombined milk embedded in water-soluble resin. Fat globules (F), casein micelles (C) and fat globule surface layers (S). Bar = 200 nm.

DISCUSSION

BOTH CASEIN AND WHEY PROTEINS were present at the surface of fat globules but their proportions were different from those in recombined milk (Table 1). The adsorbed whey proteins could possibly have come directly from the serum during homogenization. Alternatively, the whey proteins could be ad-

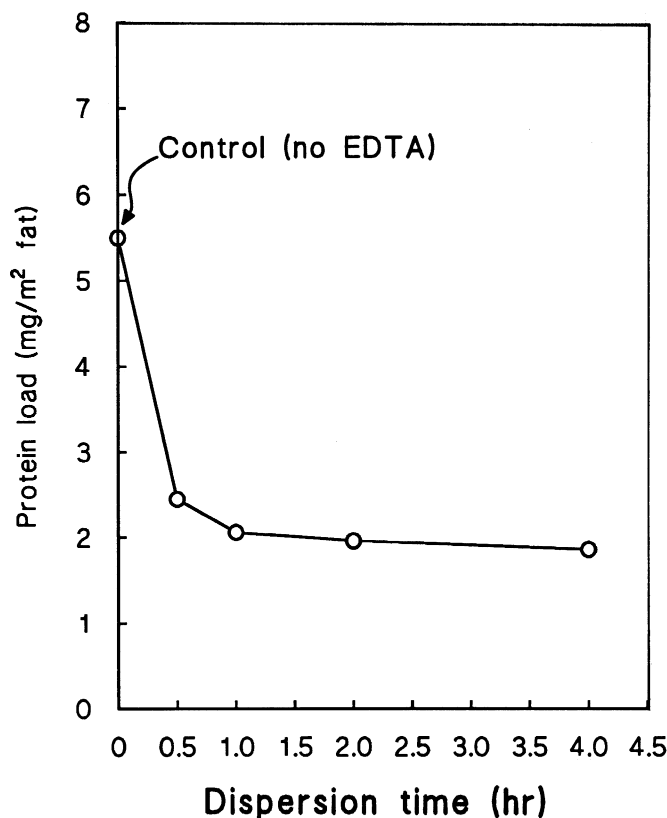

Fig. 3—Changes in protein load after dispersion of washed cream layer in 0.05M EDTA. Experimental details in Materials & Methods.

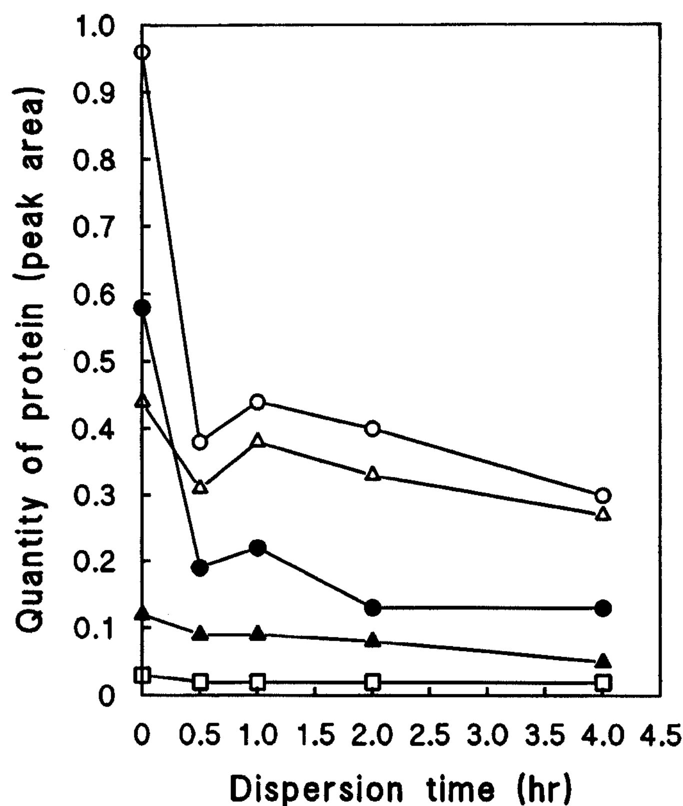

Fig. 4—Amounts of adsorbed (○) α_s-casein, (●) β-casein, (△) κ-casein, (▲) β-lactoglobulin, and (□) α-lactalbumin after dispersion of washed cream layer in 0.05M EDTA. Experimental details in Materials & Methods.

Table 2—Protein load and composition of surface layers of different size fat globules

Fat globule size (d_{vs}, μm)	Protein load (mg/m^2)	Individual protein (%, w/w, of total)					α_s/β-Casein ratio	Casein/WP[a] ratio
		α_s-Casein	β-Casein	κ-Casein	β-Lg	α-La		
0.66	6.02	37.7	24.2	19.5	16.1	2.5	1.56	4.36
0.48	6.56	41.6	24.9	17.1	12.5	3.6	1.67	5.10
0.35	7.88	42.3	24.1	18.4	11.3	3.9	1.75	5.56
0.30	8.14	44.4	23.7	19.2	10.1	2.6	1.87	6.86

[a] WP = Whey protein (β-lactoglobulin + α-lactalbumin).

sorbed as part of a denatured whey protein-casein micelle complex, which may have been formed during the manufacture of non-fat dry milk. Since amounts of adsorbed whey proteins did not change significantly as adsorbed casein micelles were dissociated by EDTA (Fig. 4), the majority of those whey proteins at the fat globule surface must have been adsorbed directly from the serum.

Caseins are the main proteins constituting the surface layer (~90%, w/w) while whey proteins are present in smaller proportions (~10%, w/w). We could calculate the fat surface area covered by casein and whey proteins. The ratio of casein to whey proteins at the surface gave a mass fraction of whey protein of ~0.11. As the average protein load was ~6 mg/m^2, ~0.66 mg/m^2 of whey proteins must have been adsorbed. Milk fat homogenized in an aqueous solution containing only whey proteins results in a protein load of ~2 mg/m^2 (Oortwijn and Walstra, 1979). Hence, in our experiment the surface covered by whey proteins would be $0.66/2 = 0.33$. That is, ~33% of the fat globule surface would be covered by whey proteins while the rest would be covered by casein. Thus, although whey proteins make up only ~10% (w/w) of the total adsorbed proteins, they cover ~33% of the total surface area. In a similar manner, Walstra and Oortwijn (1982) calculated that whey proteins occupied ~25% of the surface of fat globules in recombined milk made from fresh skim milk and anhydrous milk fat.

Electron microscopy showed that the fat globule surface contained several apparently intact casein micelles and disaggregated casein micelle particles (Fig. 2). The mechanism of disaggregation of casein particles is uncertain. One possibility is that homogenization caused disruption of the casein micelle structure and susequently the disrupted micelles adsorbed onto the fat globule surface. Another possibility is that disruption of the casein micelles occurred after they were adsorbed onto the fat globule surface. Walstra and Oortwijn (1982) postulated that casein micelles if adsorbed onto a partially covered oil/water interface, may spread to cover the interface. Such spreading would be driven by thermodynamically favorable changes in interfacial free energy which were sufficient to disrupt micelles. Notwithstanding the mechanism of disaggregation, there appeared to be more disaggregated casein particles at the fat globule surface in our recombined milk system than in fresh homogenized milk systems. This could be explained if some structural changes occurred in the casein micelles during manufacture of non-fat dry milk which made them more susceptible to disaggregation. Furthermore, this could account for the lower protein load we found.

Even the apparently ''intact'' micelles on the surface of fat globules were not likely to be in their native state since native micelles do not bind to hydrophobic (polystyrene lattices) surfaces (Dalgleish, 1989). Therefore, the micelle structure must re-arrange before the micelle interacts with the fat surface. Some evidence supports disruption and reformation of micelles during homogenization (Walstra, 1980).

EDTA results (Fig. 3 and 4) suggested that the micellar adsorption at the fat globule surface most likely occurred through interactions with κ-casein molecules, while α_s- and β-casein were less likely to be involved. Observations by Sharma and Dalgleish (1993) on fresh homogenized milk confirmed the effects of κ-casein; however, they also indicated that α_{s2}-casein remained associated with the fat surface. There were possibly rearrangements of surface material during EDTA-induced dissociation which could change the composition of adsorbed material.

The observed preferential adsorption of casein over whey protein (Table 1) may be partly attributed to a considerable proportion of casein adsorbing in the form of intact micelles, compared to whey proteins which adsorb in molecular form. Walstra and Oortwijn (1982) proposed that, since protein adsorption during homogenization occurred via convection, large protein particles adsorbed in preference to small protein particles. The hypothesis also predicts that large protein particles would be preferentially adsorbed onto smaller fat globules, consistent with our data (Table 2).

The greater adsorption of α_s-casein may be due to preferential adsorption of casein micelles that contain larger amounts of α_s-casein. McGann et al. (1980) found that the α_s-casein/β-casein ratios for large, medium and small casein micelles were 1.9, 1.6 and 1.3, respectively. Since the α_s-casein/β-casein ratio in the cream layer was 1.77, preferential adsorption of α_s-casein may be due to the adsorption of larger-sized casein micelles in preference to smaller ones. This is in accord with the mechanism proposed by Walstra and Oortwijn (1982). However, previous studies reported that, as the casein micelle size increased, the κ-casein content diminished (McGann et al., 1980; Donnelly et al., 1984). Therefore, if larger-sized casein micelles were adsorbed in preference to smaller ones, there should have been relatively less adsorption of κ-casein: this was not observed in our results. In fact, there was slightly more adsorbed κ-casein than in the original recombined milk. This indicated that mechanisms of adsorption were more complex than predicted by Walstra and Oortwijn (1982) based on casein micelles differing in size. Such adsorption may involve rearrangement of casein components within micelles and changes in micelle size distribution.

CONCLUSIONS

THE PROTEIN COVERAGE of surface layers of fat globules in recombined milk was ~6 mg/m², lower than reported values for fresh homogenized milk (~10 mg/m²). The protein coverage varied with fat globule size with smaller globules having greater protein coverages. Electron microscopy showed that the adsorbed protein consisted of intact casein micelles, micelle fragments and whey proteins. Caseins adsorbed preferentially and constituted ~90% of the fat surface layer, but covered only ~66% of the total surface area. When adsorbed micelles were dissociated by EDTA results showed that a large proportion of κ-casein was linked directly to the fat surface.

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