High-Pressure–Induced Interactions Between Milk Fat Globule Membrane Proteins and Skim Milk Proteins in Whole Milk

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ABSTRACT

The association of β -lactoglobulin (β -LG) and α -lactalbumin (α -LA) with milk fat globule membrane (MFGM), when whole milk was treated by high pressure in the range 100 to 800 MPa, was investigated using sodium dodecyl sulfate (SDS)-PAGE under reducing and nonreducing conditions. In SDS-PAGE under reducing conditions, β -LG was observed in the MFGM material isolated from milk treated at 100 to 800 MPa for 30 min, and small amounts of α -LA and κ -casein were also observed at pressures >600 MPa for 30 min. However, these proteins were not observed in SDS-PAGE under nonreducing conditions. These results indicate that β -LG and α -LA associated with MFGM proteins via disulfide bonds during the high-pressure treatment of whole milk. The amount of β -LG associated with the MFGM increased with an increase in pressure up to 800 MPa and with increasing time of pressure treatment. The maximum value for β -LG association with the MFGM was approximately 0.75 mg/g of fat. Of the major original MFGM proteins, no change in butyrophilin was observed during the high-pressure treatment of whole milk, whereas xanthine oxidase was reduced to some extent beyond 400 MPa. In contrast to the behavior during heat treatment, PAS 6 and PAS 7 were stable during high-pressure treatment, and they remained associated with the MFGM.

(Key words: milk fat globule membrane, β -lactoglobulin, high pressure, sulfhydryl–disulfide interchange)

Abbreviation key: MFGM = milk fat globule membrane, SMUF = simulated milk ultrafiltrate.

INTRODUCTION

High-pressure treatments have been of considerable interest over the last decade as an alternative to thermal treatments for food preservation and processing. The major advantages of high-pressure treatment are

elimination or significant reduction of heating, thus avoiding thermal degradation of food components, and the retention of natural flavors, color, and nutritional value (Huppertz et al., 2002). High-pressure treatments not only inactivate microorganisms and enzymes but also influence the structure of biopolymers. Therefore, these treatments can also be used in the preparation of foods with different functional properties and in the development of new products (Farr, 1990). High-pressure treatment of milk is being studied increasingly to improve the microbiological quality and technological properties of dairy foods, such as cheese and yogurt (Huppertz et al., 2002, 2004).

Bovine milk proteins are sensitive to high-pressure treatments. A number of studies have shown that treatment of milk or skim milk at pressures >200 MPa causes the disruption of casein micelles and unfolding and aggregation of whey proteins (Desobry-Banon et al., 1994; Buchheim et al., 1996; Lopez-Fandino et al., 1996; Gaucheron et al., 1997; Needs et al., 2000; Scollard et al., 2000). However, no study on the effects of high pressure on milk fat globules and the milk fat globule membrane (MFGM) proteins during pressure treatment has been reported.

Previous studies on whole milk show that heat treatment causes a number of changes in the MFGM proteins, including their denaturation and interactions with whey proteins (β -LG and α -LA) via sulfhydryl–disulfide interchange reactions (Dalgleish and Banks, 1991; Houlihan et al., 1992; Sharma and Dalgleish, 1993; Kim and Jimenez-Flores, 1995; Ye et al., 2004). Both β -LG and α -LA associate with the MFGM upon heat treatment and their amounts increase with increasing temperature up to 85°C, but remain constant at higher temperatures (Corredig and Dalgleish, 1996; Ye et al., 2004). In addition, the amounts of PAS 6 and PAS 7, the major MFGM proteins, decrease in the MFGM as the milk is heated (Houlihan et al., 1992; Sharma and Dalgleish, 1993; Kim and Jimenez-Flores, 1995; Ye et al., 2004).

The objective of this study was to determine the behavior of the milk fat globules and the interaction of MFGM proteins during high-pressure treatment of whole milk. The effects of high pressure on the MFGM

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proteins are compared with the heat-induced changes in the MFGM proteins.

MATERIALS AND METHODS

Fresh whole milk was collected from the Massey University Dairy Farm, Palmerston North, New Zealand. All the chemicals used were of analytical grade, obtained from BDH Chemicals (BDH Ltd., Poole, England) or Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

High-Pressure Treatment of Whole Milk Samples

Milk samples were transferred to polyethylene terephthalate bottles (330 mL) and tightly closed. Each bottle was then transferred to a polyethylene bag and vacuum-sealed. The samples in the bottles were pressure-treated in a "Food Lab" high-pressure food processor (model: S-FL-065 to 200 to 9-W; Stansted Fluid Power Ltd., Stansted, UK). The high-pressure unit was equilibrated to $20 \pm 1^\circ\text{C}$ by recirculating temperature-adjusted water through the water jacket associated with the unit. The pressure unit and all samples were equilibrated to the desired temperature for at least 1 h before pressurization commenced. The temperature change during pressurization/depressurization cycles was monitored using the thermocouple associated with the unit and standard data logging equipment. After pressure treatment, the unit was automatically depressurized and the samples were processed immediately.

Heat Treatment of Whole Milk Samples

A milk sample (200 mL) in a 250-mL flask was heated to 90°C by immersion in a boiling water bath. The sample was then placed in another water bath maintained at 90°C and held for 15 min. After heating, the sample was rapidly cooled to about 20°C in an ice bath.

Determination of Average Fat Globule Size and Specific Surface Area of Whole Milk

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 the refractive index of fat globules (1.456) to that of the dispersion medium (1.33), was 1.095. The milk sample was dispersed in water or in a 2% SDS/50 mM EDTA solution. The latter was used to dissociate the casein micelles, before fat globule size measurements. Fat globule size measurements are reported as volume-

surface average mean diameters, d_{32} ($= \Sigma n_i d_i^3 / \Sigma n_i d_i^2$, where n_i is the number of fat globules with diameter d_i). Average fat globule diameters were calculated as the average of measurements made on at least 2 freshly prepared samples.

Isolation of MFGM Material

Milk fat globule membrane material was isolated from whole milk, as described by Ye et al. (2002). Milk samples were centrifuged at $15,000 \times g$ for 20 min at 20°C ; the top layer (cream) was removed and dispersed in 10 volumes of simulated milk ultrafiltrate (SMUF) (Jenness and Koops, 1962) containing 6 M urea and 50 mM EDTA or SMUF alone and left at room temperature for 1 h. This dispersion was centrifuged again at $15,000 \times g$ for 20 min at 20°C and the top layer was collected. Washing in SMUF containing urea and EDTA was repeated twice. Washing the cream 3 times was designed to remove all the protein material not associated with the MFGM.

When the isolated MFGM material (cream) was washed in SMUF containing urea and EDTA, the casein micelles adsorbed at the fat globule surface were dissociated and washed away. Only the protein molecules adsorbed directly at the interface of fat globules and the protein molecules bound to the interfacial protein layer via covalent bonds remained on the surface of the fat globules.

Determination of Protein and Fat Contents

The total protein content of the top layer (cream) was determined using the Kjeldahl method (AOAC, 1974). The total fat contents of the whole milk and cream were determined using the Mojonnier method for milk (International Dairy Federation, 1987a) and cream (International Dairy Federation, 1987b), respectively.

Analysis of MFGM Protein Components

The individual proteins in the washed cream were determined by PAGE, as described by Ye et al. (2002). The washed cream was dispersed (1:2 wt/wt) in 0.5 M Tris-HCl buffer, containing 10% glycerol, 2% (wt/vol) SDS, and 0.05% bromophenol blue. For PAGE under nonreducing conditions, the samples were heated at 45°C for 5 min in a waterbath. For reducing conditions, 5% β -mercaptoethanol was added to the samples followed by heating at 95°C for 5 min in a boiling water bath.

A further centrifugation at $2500 \times g$ for 30 min was performed before PAGE analysis to remove the fat from the sample after heating in the SDS buffer. Ten microli-

ters of subnatant was then loaded onto the SDS-PAGE gel and the gel was run in a Mini-Protean system (Bio-Rad, Richmond, CA) at 200 V using a Bio-Rad power supply unit (model 1000/500, Bio-Rad). Singh and Creamer (1991) described the SDS-PAGE systems previously. The protein bands were fixed and stained using a solution of Coomassie blue R-250. After the gels had been stained and destained, the MFGM proteins were identified by comparing with molecular weight standard proteins obtained from Bio-Rad, and with the results reported previously (Keenan and Dylewski, 1995; Mather, 2000). The gels were scanned using an Ultrascan XL laser densitometer and the results were analyzed using an LKB 2400 GelScanXL software program (LKB Produkter AB, Bromma, Sweden) to obtain quantitative results.

Under nonreducing conditions, noncovalently linked protein complexes dissociated and migrated into the resolving gel, but disulfide-linked complexes remained at the top of the stacking and resolving gels. In contrast, under reducing conditions, both noncovalently linked and disulfide-linked complexes were dissociated into monomeric proteins and migrated into the resolving gel. Thus, comparison of SDS-PAGE patterns under nonreducing and reducing conditions enabled disulfide-linked complexes to be detected.

A quantitative analysis of β -LG and α -LA was carried out using their respective standard curves. Purified β -LG and α -LA (Sigma Chemical Co., St. Louis, MO) were run on the electrophoresis gels in various amounts from 0.25 to 10 μ g to generate standard curves. 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 MFGM.

Denaturation of Whey Proteins

High-pressure-treated milk samples were adjusted to pH 4.6 by mixing equal quantities of milk with 0.2 M Na acetate and HCl buffer (pH 3.95). The samples were centrifuged at $15,000 \times g$ for 20 min and the supernatants were then removed and filtered through Whatman No. 541 filter paper followed by a 0.2- μ m membrane filter before separation by SDS-PAGE under dissociating (SDS) and nonreducing conditions. The gels were scanned using an Ultrascan XL laser densitometer and the results were analyzed using the LKB 2400 GelScanXL software program (LKB Produkter AB) to obtain quantitative results. A quantitative analysis of the denaturation of β -LG and α -LA was performed by comparing the peak areas of pressure-treated milk samples with those from the untreated sample.

Table 1. Average fat globule sizes (d_{32}) of whole milks subjected to high pressure in the range 100 to 800 MPa for 30 min.

Pressure (MPa)	Average fat globule size (μ m)	
	Milks dispersed in SDS/EDTA buffer	Milks dispersed in water
0 (fresh milk)	1.13	0.86
100	1.10	0.87
200	1.12	0.96
300	1.13	1.04
400	1.13	1.10
500	1.12	1.10
600	1.12	1.15
700	1.13	1.16
800	1.13	1.16

Statistical Analysis

All experiments were performed at least twice. The results were analyzed statistically using the Minitab 12 for Windows package. Differences were considered significant at $P \leq 0.05$.

Reproducibility of Data

Analysis of 16 samples of raw whole milk and 3 samples of pressure treated milk gave the following variations: $\pm 0.03 \mu$ m for d_{32} , ± 0.50 mg/g of fat for total MFGM protein of raw milk, ± 4 mg/g of fat for the total surface protein of concentrated milk, ± 0.25 mg/g of fat for individual MFGM proteins on the surface of fat globules, ± 3.0 mg/g of fat for caseins on the surface of fat globule, ± 0.82 mg/g of fat for β -casein on the surface of fat globule, ± 1.0 mg/g of fat for β -LG on the surface of fat globule, and ± 0.15 mg/g of fat for α -LA on the surface of fat globule.

RESULTS

Effect of Pressure on the Milk Fat Globules

The average fat globule sizes (d_{32}), measured in SDS/EDTA buffer, did not change after the milks were subjected to pressure treatment in the range of 100 to 800 MPa for 30 min (Table 1). This finding indicates that the milk fat globules were not disrupted during the static, high-pressure treatment. When the milks were dispersed in water before particle size measurement, the d_{32} values of the milks increased slightly with increasing pressure up to 700 MPa, but remained constant thereafter (Table 1). This slight increase was considered to be due to disruption of the casein micelles during high-pressure treatment, which reduces their contribution to light scattering (Needs et al., 2000), and therefore gives an apparent increase in the size of the other scattering particles.

Interactions Between Serum Proteins and the MFGM Proteins

Sodium dodecyl sulfate-PAGE patterns of MFGM proteins isolated from milks subjected to high-pressure treatments under reducing and nonreducing conditions are shown in Figures 1A and 1B, respectively. No bands corresponding to β -LG, α -LA, or casein proteins were observed in the SDS-PAGE patterns of fresh milk samples, in agreement with our previous studies (Ye et al., 2002). A faint β -LG band was seen in the SDS-PAGE patterns of milk treated at 100 MPa for 30 min; the intensity of the β -LG band increased gradually with an increase in pressure up to 800 MPa (Figure 1A). No α -LA band was observed in the SDS-PAGE patterns until the milks were treated at ≥ 700 MPa (Figure 1A).

A faint κ -casein band was observed in the SDS-PAGE patterns of MFGM obtained from milk treated at pressures ≥ 500 MPa (Figure 1A). However, when the MFGM material was washed, using the SMUF solution alone (i.e., no casein micelle dissociation), other caseins were observed, especially at ≥ 700 MPa (Figure 2). Similar association behavior of κ -casein and other caseins with the MFGM has been observed during the heat treatment of whole milk (Ye et al., 2004).

For the major native MFGM proteins, a decrease in the intensity of the xanthine oxidase band was seen in the SDS-PAGE pattern of milk treated at >400 MPa, but the intensity did not change further with increasing pressure up to 800 MPa. No obvious change in the butyrophilin band was observed. Similarly, no changes in the PAS 6 and PAS 7 bands were seen at up to 700 MPa, but a decrease in the intensity of these bands appeared to occur at 800 MPa (Figure 1A).

In the SDS-PAGE pattern under nonreducing conditions (Figure 1B), no bands for β -LG, α -LA, κ -casein, and xanthine oxidase were observed in the pressure-treated samples. The intensity of the butyrophilin band was much lower than that observed in the SDS-PAGE pattern under reducing conditions. Large amounts of high molecular weight material remained on the top of the stacking gel in the nonreducing SDS-PAGE pattern. Interestingly, unlike that of other MFGM proteins, PAS 6 and PAS 7 band intensities did not decrease in the nonreducing SDS-PAGE, except at 800 MPa (Figure 1B). This result is in contrast with that observed in milk samples during heat treatment, in which the PAS 6 and PAS 7 bands decreased in both reducing and nonreducing gels (Ye et al., 2004).

These results indicate that β -LG associated with the MFGM through interactions with the original MFGM proteins involving intermolecular disulfide bond formation during pressure treatments at ≥ 100 MPa. α -Lactoglobulin also associated with the MFGM via sulfhydryl-

disulfide interchange reactions at pressures ≥ 700 MPa. Xanthine oxidase and butyrophilin, the major original MFGM proteins, appeared to be involved in these interactions with β -LG and α -LA, but PAS 6 and PAS 7 did not appear to interact readily, except at very high pressure.

The amounts of β -LG and α -LA associated with the MFGM (mg/g of fat), estimated from quantitative SDS-PAGE (under reducing conditions), as a function of different pressures are shown in Figure 3A. The amount of β -LG associated with MFGM increased gradually with an increase in pressure, with the increase being more marked between 300 and 800 MPa. The association of β -LG with the MFGM also increased with an increase in the treatment time from 5 to 60 min at 400 MPa (Figure 3B). However, at 800 MPa, the β -LG association reached a plateau (approximately 0.75 mg/g of fat) after 10 min of treatment at 800 MPa. The amount of α -LA increased slightly with increasing treatment time at 800 MPa and the level of association (<0.2 mg/g of fat) was much lower than that for β -LG (Figure 3B).

Changes in the amounts of the original MFGM proteins after pressure treatment are shown in Figure 4. No changes in the amounts of butyrophilin (approximately 1.5 mg/g of fat), PAS 6 (approximately 0.9 mg/g of fat) and PAS 7 (approximately 1.6 mg/g of fat) were observed during the pressure treatment, except a considerable decrease in PAS 6 and PAS 7 at 800 MPa (Figure 4). The amount of xanthine oxidase decreased from about 0.62 to about 0.42 mg/g of fat as the pressure was increased from 400 to 500 MPa, but then remained unchanged as the pressure was increased further to 800 MPa.

Denaturation of β -LG and α -LA at Different Pressures

The denaturation of β -LG and α -LA, determined from the supernatant of milk precipitated at pH 4.6, followed by separation by SDS-PAGE under nonreducing conditions from the high-pressure-treated milks, is shown in Figure 5. No denaturation of β -LG occurred at pressures <100 MPa, but the extent of denaturation increased with increasing pressure, with an abrupt and large increase between 300 and 400 MPa. About 90% of the total β -LG was denatured at 800 MPa. The level of α -LA denaturation was much lower than that for β -LG; only about 10% of the α -LA was denatured at 600 MPa, but about 50% of the α -LA was denatured at 800 MPa.

Heat Treatment of Pressure-Treated Milk

Milks treated at 400 or 800 MPa for 15 min were heated at 90°C for 15 min; the SDS-PAGE patterns

Figure 1. Sodium dodecyl sulfate-PAGE patterns (15% acrylamide gel) of milk fat globule membrane (MFGM) material, isolated from the high-pressure-treated milks, under reducing (A) and nonreducing conditions (B). Cream obtained from the high-pressure-treated milks was washed in simulated milk ultrafiltrate containing urea and EDTA. Lane 1: whole milk (control); lanes 2 to 8: MFGM material from untreated milk (2), milk treated at 200 MPa (3), milk treated at 400 MPa (4), milk treated at 500 MPa (5), milk treated at 600 MPa (6), milk treated at 700 MPa (7), and milk treated at 800 MPa (8) for 30 min. Membrane proteins are named according to Mather (2000).

Figure 2. Sodium dodecyl sulfate-PAGE patterns (15% acrylamide gel) of milk fat globule membrane (MFGM) material isolated from the high-pressure-treated milks under reducing conditions. Cream obtained from the high-pressure-treated milks was washed in simulated milk ultrafiltrate. Lane 1: membrane material from untreated milk (control). Lanes 2 to 8: membrane material from milk treated at 100 to 800 MPa for 30 min; (2) 100 MPa, (3) 300 MPa, (4) 400 MPa, (5) 500 MPa, (6) 600 MPa, (7) 700 MPa, and (8) 800 MPa. Membrane proteins are named according to Mather (2000).

of the MFGM materials isolated from these milks are shown in Figure 6. As expected, heat treatment of the control milk at 90°C for 15 min resulted in a marked increase in β -LG association with the MFGM. The milk samples that were first treated at 400 or 800 MPa and then heated showed similar profiles to the control sample (heat-treated only). The amount of β -LG associated with the MFGM in heated milk or milk first treated at high pressure and then heated (approximately 1.5 mg/g of fat) was about twice that in pressure-treated milk without heating (approximately 0.75 mg/g of fat) (Table 2). The intensity of the α -LA and κ -casein bands also increased after heat treatment of the pressure-treated samples at 90°C for 15 min (Figure 6). However, α -LA remained low in the sample treated at 400 MPa, indicating that it was not involved when pressure-treated milk was subsequently heated. This suggests that denatured aggregates were associated with the MFGM, in which the aggregates were mainly β -LG for the 400 MPa sample and were β -LG/ α -LA complexes for the 800 MPa sample. Similar to milk heated alone, PAS 7 decreased after heat treatment of the pressure-

treated milks. However, the decrease in PAS 7 was less than that in the heated milk (Figure 6).

DISCUSSION

The results clearly show that pressure treatment of whole milk in the range 100 to 800 MPa results in association of β -LG with the MFGM proteins via sulfhydryl-disulfide interchange reactions. An association of β -LG with the MFGM proteins, which increases with increasing pressure or treatment time at a given pressure, appears to reach a maximum of 0.75 mg/g of fat. At very high pressures, α -LA (≥ 700 MPa) and κ -casein (500 MPa) also appear to interact with the MFGM proteins (Figure 1). Associations of β -LG and α -LA with the MFGM appear to follow a similar pattern to that observed for the aggregation of β -LG and α -LA by pressure treatment (Figure 5). An association of α -LA with the MFGM proteins (Figure 3) occurs because of sulfhydryl-disulfide exchange, with the thiol group being provided by β -LG or MFGM proteins. However, this interaction occurs at much higher pressures (≥ 700 MPa),

Figure 3. Amounts of β -LG (◆) and α -LA (▲) associated with the milk fat globule membrane (MFGM) material in (A) milks that were treated at pressures ranging from 100 to 800 MPa for 30 min and (B) milks that were treated at 400 MPa (...) and 800 MPa (—) for 2 to 60 min. Bars indicate the standard errors.

and much lower quantities of α -LA (compared with β -LG) are incorporated into the MFGM. It has been reported that α -LA is much more resistant than β -LG to denaturation during high-pressure treatment, probably because of the more rigid molecular structure of α -LA (Lopez-Fandino et al., 1996; Gaucheron et al., 1997; Needs et al., 2000).

It has been reported that the amount of β -LG associated with the MFGM will reach a maximum (approx-

Figure 4. Amounts of xanthine oxidase (◆), butyrophilin (■), PAS 6 (▲), and PAS 7 (X) in the milk fat globule membrane (MFGM) material isolated from (A) milks that were treated at high pressures from 100 to 800 MPa for 30 min and (B) milks that were treated at 800 MPa for 2 to 60 min.

mately 1.5 mg/g of fat) as whole milk is heated (Corredig and Dalgleish, 1996; Ye et al., 2004). The maximum amount of β -LG associated with the MFGM (approximately 0.75 mg/g of fat) upon high-pressure treatment, observed in the present work, is much lower, although most of the β -LG has already been denatured at 800 MPa. However, further association of β -LG with the MFGM does occur on subsequent heat treatment of pressure-treated milk (800 MPa) (Table 2). This further

Figure 5. Denaturation of β -LG (■) and α -LA (▲) in (A) milks that were treated at high pressures from 100 to 800 MPa for 30 min and (B) milks that were treated at 400 MPa (...) and 800 MPa (—) for 2 to 60 min (B). Bars indicate the standard errors.

association may arise because: (1) β -LG displaces the native MFGM proteins, e.g., PAS 6 and PAS 7; (2) more κ -casein/ β -LG complex associates with the MFGM as the κ -casein content in the MFGM is increased after heating (Figure 6); (3) thiol groups of the MFGM are not exposed on pressure-treatment, but are exposed on subsequent heating, which allows for thiol-disulfide

exchange to occur with the pressure-denatured whey proteins.

Caseins also appear to associate with the MFGM via disulfide bonding between κ -casein and MFGM proteins or β -LG already associated with the MFGM (Figures 1 and 2). It has been shown that treatment of skim milk at 250 to 600 MPa reduces the micelle size by 40 to 50% and that all large micelles are disrupted into smaller fragments at 400 to 600 MPa (Needs et al., 2000). Interactions between β -LG and κ -casein have been observed in mixtures of pure κ -casein and β -LG solution or skim milk treated at 400 MPa (Lopez-Fandino et al., 1997; Schrader and Buchheim, 1998). Hence, it is possible that β -LG/ κ -casein complexes are formed during high-pressure treatment and become incorporated into the MFGM.

Milk fat globule membrane 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 groups and 38 sulfhydryl groups, 4 of which are detectable in the undenatured protein complex (Cheng et al., 1988). This implies that, when the sulfhydryl groups or disulfide bonds of β -LG are exposed, because of unfolding of β -LG during high-pressure treatment, thiol-disulfide interchange between MFGM proteins and β -LG will occur. A similar interaction between the MFGM proteins and β -LG has been found during the heat treatment of whole milk (Dalglish and Banks, 1991; Houlihan et al., 1992; Sharma and Dalglish, 1993; Kim and Jimenez-Flores, 1995; Ye et al., 2004).

Of the original MFGM proteins, PAS 6 and PAS 7 do not appear to associate with either the whey proteins or other MFGM proteins, as their concentrations in the MFGM remain constant during pressure treatments (Figures 1A and 1B). This result suggests that β -LG can associate with other MFGM proteins without affecting PAS 6 and PAS 7, (Keenan and Dylewski, 1995). In other words, the peripheral proteins, PAS 6 and PAS 7, do not hinder the interactions between β -LG and other MFGM proteins via disulfide bond formation. This behavior is different from that observed during the heat treatment of milk in previous studies (Houlihan et al., 1992; Ye et al., 2004), in which PAS 6 and PAS 7 are removed from the MFGM at temperatures $\geq 65^\circ\text{C}$, when β -LG begins to associate with MFGM (Houlihan et al., 1992; Ye et al., 2004). It has been suggested that the formation of β -LG/MFGM protein complexes, which may alter the membrane structure and environment, results in the removal of PAS 6 and PAS 7. Alternatively, PAS 6 and PAS 7 may interact directly with β -LG to form β -LG/PAS 6 or PAS 7 complexes, which then move from the MFGM to the serum phase (Houlihan et al., 1992; Ye et al., 2004). This may imply that a

Figure 6. Sodium dodecyl sulfate-PAGE patterns (15% acrylamide gel) of milk fat globule membrane (MFGM) material isolated from milks that were treated at high pressures or first treated at high pressure and then heated at 90°C for 15 min under reducing conditions. Cream obtained from the high-pressure-treated milks was washed in simulated milk ultrafiltrate containing urea and EDTA. Lanes 1 to 5: membrane material from milks treated at 800 MPa for 2 to 60 min; (1) 2 min, (2) 5 min, (3) 15 min, (4) 30 min, and (5) 60 min; lane 6: milk heated at 90°C for 15 min; lane 7: milk treated at 400 MPa for 15 min and then heated at 90°C for 15 min; lane 8: milk treated at 800 MPa for 15 min and then heated at 90°C for 15 min. Membrane proteins are named according to Mather (2000).

different mechanism is involved in the high-pressure-induced association of β -LG, compared with heat-induced association. Hydrophobic interactions may be more important in the high-pressure-induced association between β -LG and the MFGM.

Furthermore, the reason for the loss of xanthine oxidase from the MFGM at pressures >400 MPa is unknown. It is probably not related to the association of whey proteins with the MFGM, but may be due to a change in the structure of the membrane under high

pressure. The loss of xanthine oxidase from the membrane was not observed during the heat treatment of milk (Ye et al., 2004).

CONCLUSIONS

β -Lactoglobulin, α -LA, and κ -casein associate with the MFGM when whole milk is treated at high pressure, although the size of the milk fat globules does not change. The association of β -LG with the MFGM begins at pressures >100 MPa, with the amount increasing with increases in pressure and in treatment time at a given pressure. The associations of α -LA and κ -casein occur at pressures ≥ 700 and 500 MPa, respectively, but the amounts are lower than observed for β -LG. The associations of these proteins with the MFGM at high pressure are attributed to interactions via sulfhydryl-disulfide interchange between β -LG, α -LA, and κ -casein and the MFGM proteins. The high-pressure-induced interactions between whey proteins (β -LG and α -LA) and the MFGM proteins appear to follow the denaturation pattern of β -LG and α -LA upon the high-pressure treatment of milk. Xanthine oxidase and butyrophilin

Table 2. Amounts of β -LG and α -LA associated with the milk fat globule membrane (MFGM) of milk samples undergoing high-pressure or thermal treatment, or both.

Milk treatment	β -LG	α -LA
	— (mg/g of fat) —	
Milk treated at 400 MPa for 15 min	0.30	...
Milk treated at 800 MPa for 15 min	0.75	0.11
Milk heated at 90°C for 15 min	1.53	0.32
Milk treated at 400 MPa for 15 min and then heated at 90°C for 15 min	1.55	0.17
Milk treated at 800 MPa for 15 min and then heated at 90°C for 15 min	1.50	0.33

are the major MFGM proteins involved in the high-pressure-induced interaction with β -LG, whereas PAS 6 and PAS 7 do not appear to react with β -LG. Peripheral membrane proteins PAS 6 and PAS 7 remain in the MFGM, unlike their behavior during heat treatment, when they are removed from the membrane.

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