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Transmission Electron Microscopy of Mozzarella Cheeses Made from Microfluidized Milk

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The nanostructure of Mozzarella cheeses prepared from microfluidized milk was compared with that of control cheeses made from untreated milk. Milk heated to 10 or 54 °C and containing 1.0 or 3.2% fat was homogenized by microfluidization at 34 or 172 MPa prior to cheesemaking. The effects on the casein particles and fat globules in the cheese were determined by transmission electron microscopy after 1 day and 6 weeks of storage at 4 °C. The micrographs showed that electron-dense regions theorized to be casein submicelles rearranged from a homogeneous configuration to a pattern of clusters during the storage period. The nanostructure of the cheeses made from milk processed under the mildest conditions resembled the controls, but otherwise the fat droplets decreased in size and increased in number as the pressure and temperature were increased. The results indicate that both homogenization temperature and pressure affect the nanostructure of Mozzarella cheese.

KEYWORDS: Homogenization; microfluidization; milk; Mozzarella cheese; TEM; nanostructure

INTRODUCTION

The rheological and melting characteristics of Mozzarella and other cheeses are often considered as important as their flavor. The physical properties of cheese are related to its protein matrix which consists of casein interspersed with fat droplets. Scanning electron microscopy (SEM) has shown that each droplet is enclosed by a fat globule membrane, and that most of the bacteria from the starter culture are found at the fat−protein interface (*1*). Transmission electron microscopy (TEM) revealed that the nanostructure of Mozzarella cheese consists of a discontinuous fat droplet phase and an extensive, continuous phase of small electron-dense areas that have been theorized to be casein submicelles (*2*). The rennet used to coagulate the milk destabilizes the casein micelles by cleaving κ-casein, leading to disappearance of micellar structure and formation of a gel of paracasein. Proteolysis of α_{s1}-casein, the primary structural component of the protein matrix, occurs during storage (*3*), resulting in aggregation of the fat droplets and rearrangement of the micellar material into clusters of casein particles (*2*). These changes increase the meltability and decrease the hardness of the cheese.

Homogenization, in which milk is forced through a constricted orifice, affects the functionality of cheese by reducing the size of the fat droplets (*4*). Moreover, homogenization disrupts the fat globule membrane, which is replaced by membrane fragments complexed with casein particles (*5*, *6*). When low-fat (LF) and full-fat (FF) Mozzarella cheeses were made from milk homogenized at 10.3 or 17.2 MPa, hardness and meltability were related to fat content, and meltability was also dependent on homogenization pressure (*7*, *8*).

Developed by Cook and Lagacé (*9*), microfluidization is a homogenization technique in which milk in a high-pressure chamber is split into two streams which collide with each other at a 180° angle (*10*). This process creates smaller fat particles with a narrower size distribution than those processed in a conventional homogenizer (*11*−*13*). Cavitation resulting from collisions of the fat globules is mostly responsible for the changes in globule size (*14*). Scanning electron microscopy has been used to examine the microstructure of Mozzarella cheeses made from milk which was microfluidized at various temperatures and pressures (*15*). There were no obvious differences between Mozzarellas made from control milk and milk microfluidized at 10 °C because this temperature does not sufficiently liquefy the fat to an extent necessary for its complete microfluidization. When the milk was processed at 54 °C, the fat droplets in the cheese became smaller and more discrete, giving the casein matrix a spongy appearance. Increasing the pressure from 34 MPa (the maximum used for homogenization in dairy plants) to 172 MPa (the limit of the microfluidization equipment) reduced the fat droplet size further, creating an emulsion of fat and protein.

In this work, the effect of microfluidization pressure and temperature on the nanostructure of Mozzarella was investigated.

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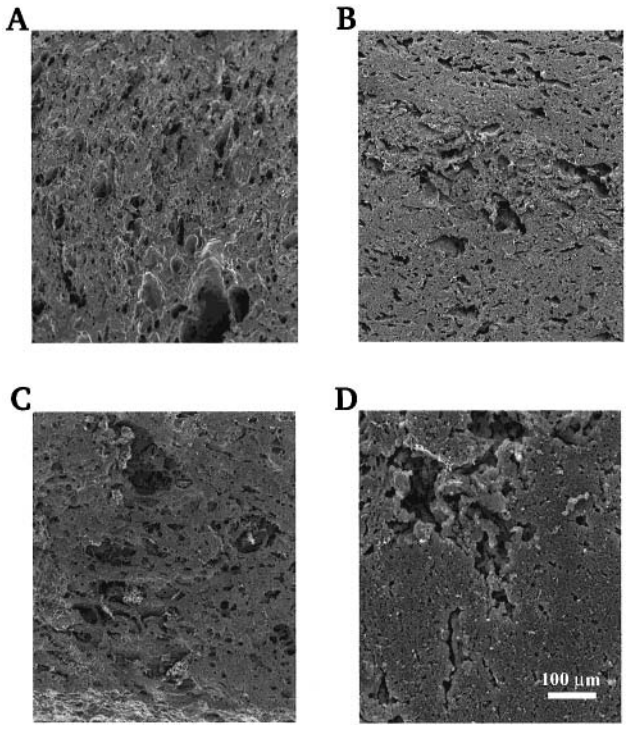

Figure 1. Scanning electron micrographs of Mozzarella cheeses. (A) Low-fat cheese. (B) Low-fat cheese made from milk microfluidized at 10 °C and 34 MPa. (C) Full-fat cheese made from milk microfluidized at 10 °C and 34 MPa. (D) Full-fat cheese made from milk microfluidized at 54 °C and 34 MPa.

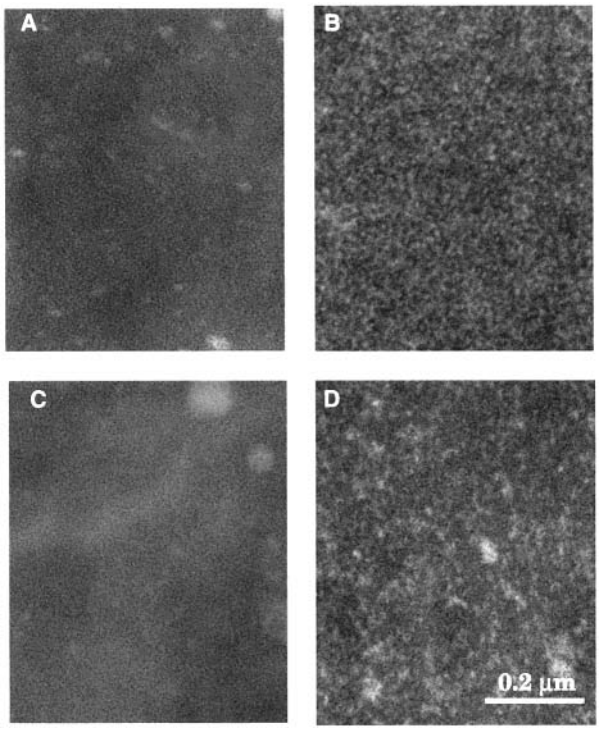

Figure 2. Digitized image areas of electron microscope negatives. Dark areas represent electron-dense clusters. Images were selected to exclude fat droplets. (A) Low-fat Mozzarella cheese after 1 d of refrigerated storage. (B) Same, after 6 wk of refrigerated storage. (C) Full-fat Mozzarella cheese after 1 d of refrigerated storage. (D) Same, after 6 wk of refrigerated storage.

MATERIALS AND METHODS

Milk. Mixed-herd milk from a local agricultural college was standardized to either 1.0% fat (for low-fat cheese) or 3.2% fat (for full-fat cheese), pasteurized at 63 °C for 30 min, and refrigerated overnight. The milk was divided into two equal portions, and each portion was heated to the desired temperature of 10 or 54 °C. The milk was then homogenized at the desired pressure of 34 or 172 MPa using a model 210-EH Microfluidizer (Microfluidics Corporation, Newton, MA). One portion of milk at each fat level was not homogenized or heated and served as a control.

Cheesemaking. Two different batches of cheese were simultaneously prepared on a given day, each from 22.7 kg of milk. Each cheese was made in duplicate over the course of the study. The cheesemilk was heated to 36 °C and inoculated with frozen concentrated CR5 or CR12 starter culture (Marschall-Rhône-Poulenc, Madison, WI), described by the manufacturer as containing equal amounts of *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*. After the pH decreased 0.16 to 0.22 units, 4.4 mL of single-strength calf rennet (Chr. Hansen's Laboratory, Milwaukee, WI) was diluted 1:40 with water and added. The curd was cut after 35 min and held for another 20 min before stirring. The curd was maintained at 36 °C for another 30 min with stirring. Whey was drained in two steps over 60 min and the curd was covered with water at 36 °C for 30 min before draining. The curd was cut into slabs, stacked two slabs high, and flipped over every 15 min until the curd pH reached 5.2. Curd slabs were covered with cheesecloth, packed in ice, and stored overnight at 4 °C.

The next day, the curd was divided into 8 parts and stretched by hand in 77 °C water for 7 min. The samples were pressed into 224-mL polyethylene cups measuring approximately 80 mm in diameter and 55 mm in height, cooled, removed from the cups, brined for 2 h in 23% salt solution at 20 °C, blotted dry with paper towels, and stored in vacuum-sealed pouches at 4 °C for 6 wk. The LF cheeses contained 52.9−57.0% moisture and 8.9−11.0% fat, whereas the FF cheeses contained 43.9−50.5% moisture and 20.9−27.4% fat, as previously reported (*15*).

Electron Microscopy. Specimens for electron microscopy were cut with a stainless steel razor blade from the interior of a cheese sample on the day after the cheese was stretched, and from another cheese from the same batch after 6 wk of refrigerated storage. The specimens were diced into blocks approximately 1 cm on a side, and fixed in a solution of 2.5% glutaraldehyde in 0.1 M imidazole hydrochloride (pH 6.8). The solution was held at room temperature for 1 h and then stored at 4 °C until embedded. The samples were cut into small blocks of 1−2 mm on a side, washed in imidazole buffer for 2 h, and rinsed with distilled water. The samples were fixed in 2% osmium tetroxide in imidazole buffer and dehydrated in a graded series of ethanol solutions (50, 80, 90, and 100%). Samples for SEM were freeze-fractured, defatted, critical-point dried, and imaged as previously described (*16*). Samples for TEM were transferred to propylene oxide, infiltrated overnight with propylene oxide−epoxy resin embedding medium (Electron Microscopy Sciences, Fort Washington, PA), and embedded in 100% epoxy resin. Sections 60-nm thick were cut with a microtome, stained first with uranyl acetate and then with lead citrate, and imaged with a Philips model CM12 transmission electron microscope (FEI, Hillsboro, OR) in the bright field image mode. Images were recorded on 8.3 × 10.2-cm Kodak type 4489 photographic film (Eastman Kodak Co., Rochester, NY) at an instrumental magnification of 60,000×. At least five images of each sample were examined.

RESULTS AND DISCUSSION

The microstructure of the control cheeses consisted of fat droplets of various sizes distributed throughout the protein matrix (Figure 1a). The cheeses processed under the least severe conditions (34 MPa and 10 °C) contained smaller droplets of more uniform size (Figure 1b,c). The droplets were much smaller when the pressure or temperature of microfluidization were increased, and crevices between incompletely fused areas of curd were evident (Figure 1d).

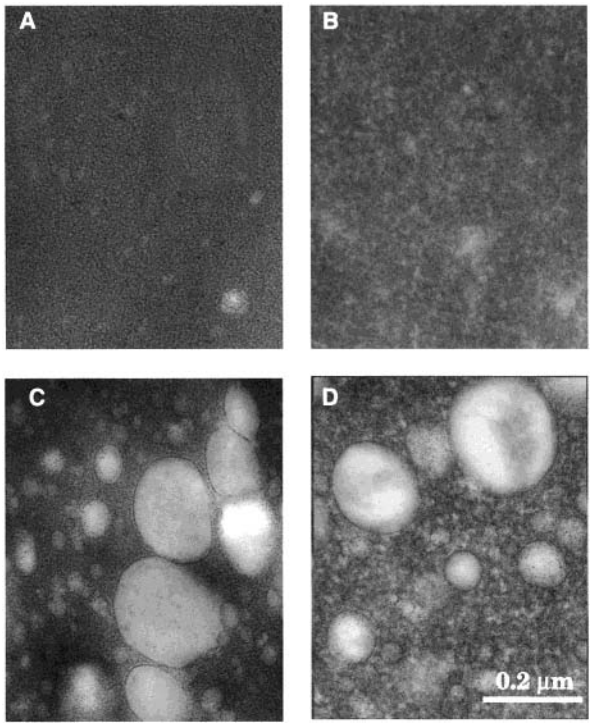

Figure 3. Digitized image areas of electron microscope negatives. Dark areas represent electron-dense clusters, and larger white circular areas represent fat droplets. (A) Low-fat Mozzarella cheese made from milk microfluidized at 10 °C and 34 MPa, after 1 d of refrigerated storage. (B) Same, after 6 wk of refrigerated storage. (C) Low-fat Mozzarella cheese made from milk microfluidized at 54 °C and 34 MPa, after 1 d of refrigerated storage. (D) Same, after 6 wk of refrigerated storage.

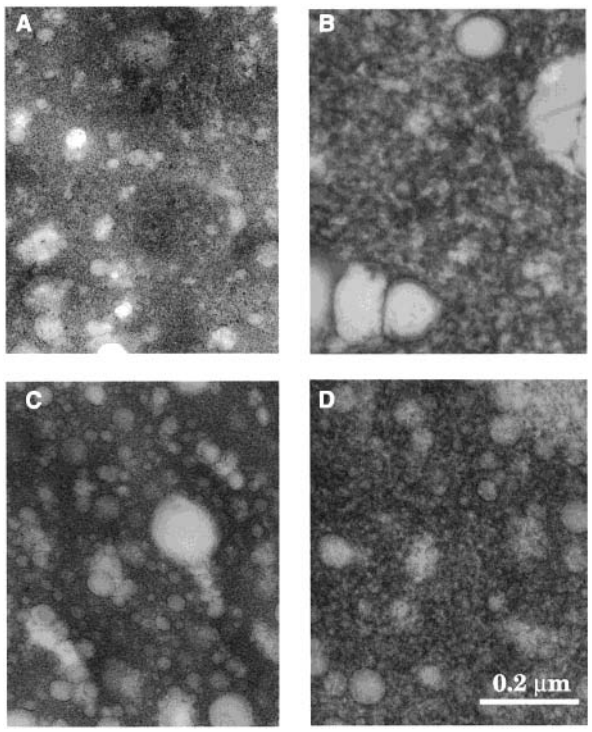

Figure 4. Digitized image areas of electron microscope negatives. (A) Low-fat Mozzarella cheese made from milk microfluidized at 10 °C and 172 MPa, after 1 d of refrigerated storage. (B) Same, after 6 wk of refrigerated storage. (C) Low-fat Mozzarella cheese made from milk microfluidized at 54 °C and 172 MPa, after 1 d of refrigerated storage. (D) Same, after 6 wk of refrigerated storage.

When examined by TEM, the day-old control cheeses exhibited a homogeneous pattern of electron-dense phases with a regular spacing measuring 15 nm in diameter (Figure 2a,c). These regions corresponded to the dimension of structures theorized to be casein submicelles (*17*). After 6 wk of storage, the nanostructure consisted of numerous electron-dense regions measuring 30−40 nm in diameter (Figure 2b,d), indicating a rearrangement into clusters (*2*, *17*). These results were the same as those seen in our earlier research on Mozzarella made from milk homogenized at 0−17.2 MPa (*17*).

The nanostructure of many of the Mozzarella cheeses made from microfluidized milk was different from that of the controls. Some of the electron-dense regions were distributed on the outside of the fat droplets, acting as a replacement for the fat globule membrane which had been stripped away during microfluidization. This effect has been observed in cheeses made from homogenized milk (*5*, *17*). The LF cheeses made from milk processed at 34 MPa and 10 °C closely resembled the controls after 1 d and 6 wk (Figure 3a,b). The fat in the milk that was processed at 34 MPa and 54 °C was completely liquid during microfluidization and therefore more easily fragmented, resulting in LF cheese that contained many more fat droplets. These droplets ranged from 50 to 350 nm in diameter (Figure 3c,d). The fat droplets were of the same approximate size and number as those in the LF cheese when the highest pressure was used at 10 °C (Figure 4a,b) and 54 °C (Figure 4c,d). The temperature, therefore, did not appear to be a factor in the distribution of fat when the pressure was 172 MPa.

In all of the LF cheeses, a clustering of electron-dense regions during storage was observed, indicating that a rearrangement was taking place as observed at lower homogenization pressures (*2*).

As expected, the FF microfluidized cheeses contained more numerous fat droplets than their LF counterparts. The FF cheeses microfluidized at 10 °C and 34 MPa resembled the FF controls, and their rearrangement of electron-dense regions was evident (Figure 5a,b). When the microfluidization temperature was increased to 54 °C, the fat droplets became far more numerous, with diameters ranging from 30 to 250 nm (Figure 5c,d). In the FF cheeses that were processed at 172 MPa, the extensive and continuous protein phase and the discontinuous fat-droplet phase consisted of small areas of electron-dense regions and numerous fat droplets between 20 and 200 nm in diameter (Figure 6). At 54 °C, a fat−protein emulsion with a very high number of droplets made visualization of discrete electron-dense regions difficult. The reorganization during storage apparently did not take place in this sample because of the physical presence of the fat droplets.

The regular pattern of electron-dense areas in all but one of the samples was converted into a nonhomogeneous arrangement of aggregates. Electrophoretic data indicate that the percentage of α_{s1}-casein in each of the samples decreased by one-third to one-half during the 6-wk storage time (*18*). These results demonstrate that proteolytic breakdown of α_{s1}-casein and the subsequent reorganization of electron-dense regions occurred in most of the samples despite high microfluidization pressures. These pressures apparently did not affect the accessibility of the peptide bonds that are typically attacked by enzymes during ripening.

CONCLUSIONS

High-pressure homogenization of milk caused changes in the macromolecular structure of the resulting cheese. Fat globules

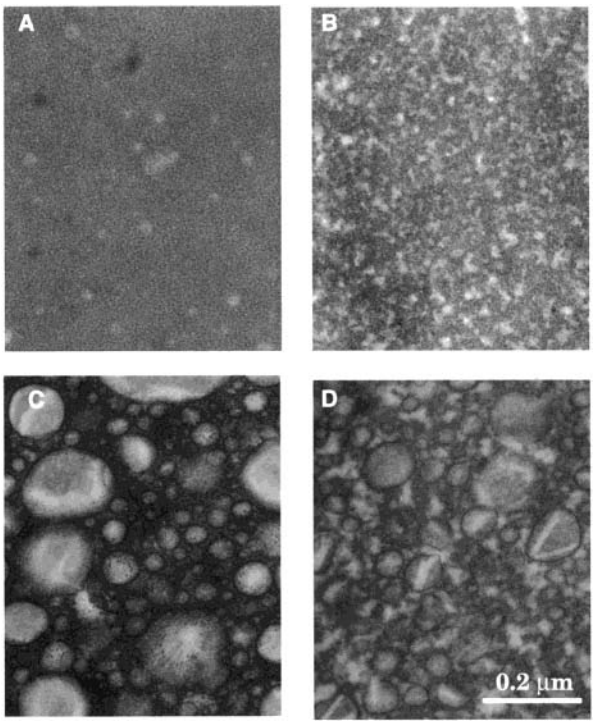

Figure 5. Digitized image areas of electron microscope negatives. (A) Full-fat Mozzarella cheese made from milk microfluidized at 10 °C and 34 MPa, after 1 d of refrigerated storage. (B) Same, after 6 wk of refrigerated storage. (C) Full-fat Mozzarella cheese made from milk microfluidized at 54 °C and 34 MPa, after 1 d of refrigerated storage. (D) Same, after 6 wk of refrigerated storage.

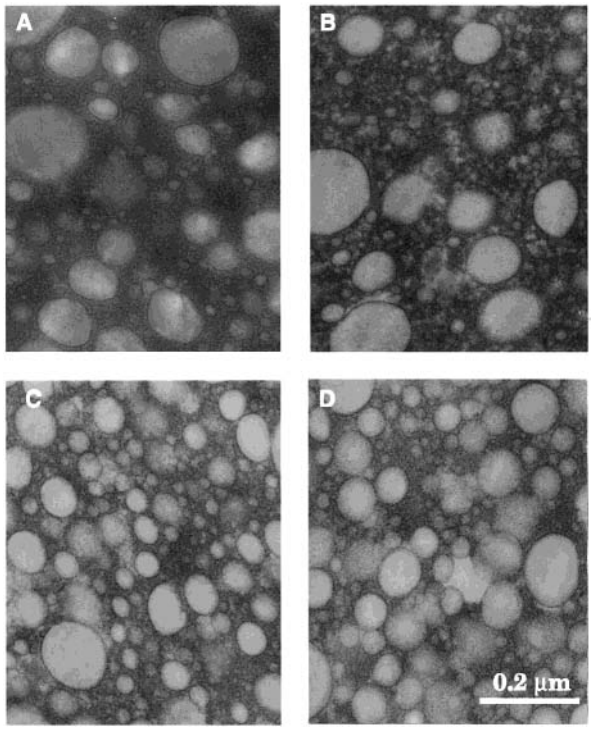

Figure 6. Digitized image areas of electron microscope negatives. (A) Full-fat Mozzarella cheese made from milk microfluidized at 10 °C and 172 MPa, after 1 d of refrigerated storage. (B) Same, after 6 wk of refrigerated storage. (C) Full-fat Mozzarella cheese made from milk microfluidized at 54 °C and 172 MPa, after 1 d of refrigerated storage. (D) Same, after 6 wk of refrigerated storage.

were smaller and more numerous when the milk was processed at a temperature above the melting point of milk fat, and at the highest microfluidization pressure available. During storage, a rearrangement of electron-dense areas was observed in all but one of the microfluidized cheeses, as well as in the controls, indicating that high homogenization pressure does not inhibit reorganization of material.

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LITERATURE CITED

(1) Dean, M. R.; Berridge, N. J.; Mabbit, L. A. Microscopical Observations on Cheddar Cheese and Curd. *J. Dairy Res.* **1959**, *26*, 77−86.

(2) Tunick, M. H.; Cooke, P. H.; Malin, E. L.; Smith, P. W.; Holsinger, V. H. Reorganization of Casein Submicelles in Mozzarella Cheese During Storage. *Int. Dairy J.* **1997**, *7*, 149−155.

(3) Fox, P. F. Proteolysis During Cheese Manufacture and Ripening. *J. Dairy Sci.* **1989**, *72*, 1379−1400.

(4) Peters, I. I. Homogenized Milk in Cheesemaking. *Dairy Sci. Abstr.* **1964**, *26*, 457−461.

(5) Henstra, S.; Schmidt, D. G. On the Structure of the Fat-Protein Complex in Homogenized Cow's Milk. *Neth. Milk Dairy J.* **1970**, *24*, 45−51.

(6) Schmidt, D. G.; Buchheim, W. Electronmikroskopische Untersuchung der Feinstruktur von Casein-Micellen in Kuhmilch. *Milchwissenschaft* **1970**, *25*, 596−600.

(7) Tunick, M. H.; Malin, E. L.; Smith, P. W.; Shieh, J. J.; Sullivan, B. C.; Mackey, K. L.; Holsinger, V. H. Proteolysis and Rheology of Low Fat and Full Fat Mozzarella Cheeses Prepared from Homogenized Milk. *J. Dairy Sci.* **1993**, *76*, 3621−3628.

(8) Tunick, M. H.; Malin, E. L.; Smith, P. W.; Holsinger, V. H. Effects of Skim Milk Homogenization on Proteolysis and Rheology of Mozzarella Cheese. *Int. Dairy J.* **1995**, *5*, 483−491.

(9) Cook, E. J.; Lagacé, A. P. Apparatus for Forming Emulsions. U.S. Patent 4,533,254, 1985.

(10) Lemay, A.; Paquin, P.; Lacroix, C. Influence of Microfluidization of Milk on Cheddar Cheese Composition, Color, Texture, and Yield. *J. Dairy Sci.* **1994**, *77*, 2870−2879.

(11) Pouliot, Y.; Paquin, P.; Robin, O.; Giasson, J. Étude Comparative de L′effet de la Microfluidization et de L′homogénéisation sur la Distribution de la Taille des Globules de Gras du Lait de Vache. *Int. Dairy J.* **1991**, *1*, 39−49.

(12) Strawbridge, K. B.; Ray, E.; Hallett, F. R.; Tosh, S. M.; Dalgleish, D. G. Measurement of Particle Size Distributions in Milk Homogenized by a Microfluidizer: Estimation of Populations of Particles with Radii Less than 100 nm. *J. Colloid Interface Sci.* **1995**, *171*, 392−398.

(13) Dalgleish, D. G.; Tosh, S. M.; West, S. Beyond Homogenization: The Formation of Very Small Emulsion Droplets During the Processing of Milk by a Microfluidizer. *Neth. Milk Dairy J.* **1996**, *50*, 135−148.

(14) Paquin, P. Technological Properties of High-Pressure Homogenizers: The Effect of Fat Globules, Milk Proteins, and Polysaccharides. *Int. Dairy J.* **1999**, *9*, 329−335.

(15) Tunick, M. H.; Van Hekken, D. L.; Smith, P. W.; Malin, E. L. Effect of High-Pressure Microfluidization on Microstructure of Mozzarella Cheese. *Lebensm.-Wiss. Technol.* **2000**, *33*, 538−544.

(16) Tunick, M. H.; Mackey, K. L.; Shieh, J. J.; Smith, P. W.; Cooke, P.; Malin, E. L. Rheology and Microstructure of Low-Fat Mozzarella Cheese. *Int. Dairy J.* **1993**, *3*, 649−662.
(17) Cooke, P. H.; Tunick, M. H.; Malin, E. L.; Smith, P. W.; Holsinger, V. H. Electron Density Patterns in Low-Fat Mozzarella Cheeses During Refrigerated Storage. In *Chemistry of Structure/Function Relationships in Cheese*; Malin, E. L., Tunick, M. H., Eds.; Plenum Press: New York, 1995; pp 311−320.
(18) Malin, E. L.; Tunick, M. H.; Van Hekken, D. L.; Smith, P. W.; Holsinger, V. H. Proteolysis and Casein-Whey Complex Formation in Mozzarella Cheeses Made From Microfluidized Milk. *J. Agric. Food Chem.*, submitted for publication, 2001.

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