

Imaging of casein submicelles in mozzarella cheeses by transmission electron microscopy

The popularity of pizza has made mozzarella the second most-consumed cheese in the United States. The relationships among the functional properties, protein breakdown, and microstructure of this cheese have been studied by the authors' laboratory,¹⁻⁴ enabling a low-fat mozzarella to be developed for use in the National School Lunch Program.

Casein in milk occurs in the form of micelles. When cheese is made, micelles disassociate into submicelles that can be observed by transmission electron microscopy (TEM). Previous work in the authors' laboratory has shown that submicelles in mozzarella rearrange during refrigerated storage, which may cause changes in the protein matrix.⁴

Cheese manufacturers sometimes find it more economical to replace fresh milk with re-

constituted milk made from nonfat dry milk (NFDM) and cream. Cheese prepared in this manner can now be used in federal feeding programs in the U.S. The relationships among proteolysis, texture, and microstructure of mozzarella made from NFDM have recently been evaluated.⁵ This paper describes the submicellar structure of low-moisture part-

skim mozzarella cheese made from reconstituted NFDM, and compares it to that of mozzarella previously made from fresh milk.

Materials and methods

Each 22.7-kg batch of reconstituted milk was prepared from NFDM (Dairy America, Dublin, CA), municipal tap water,

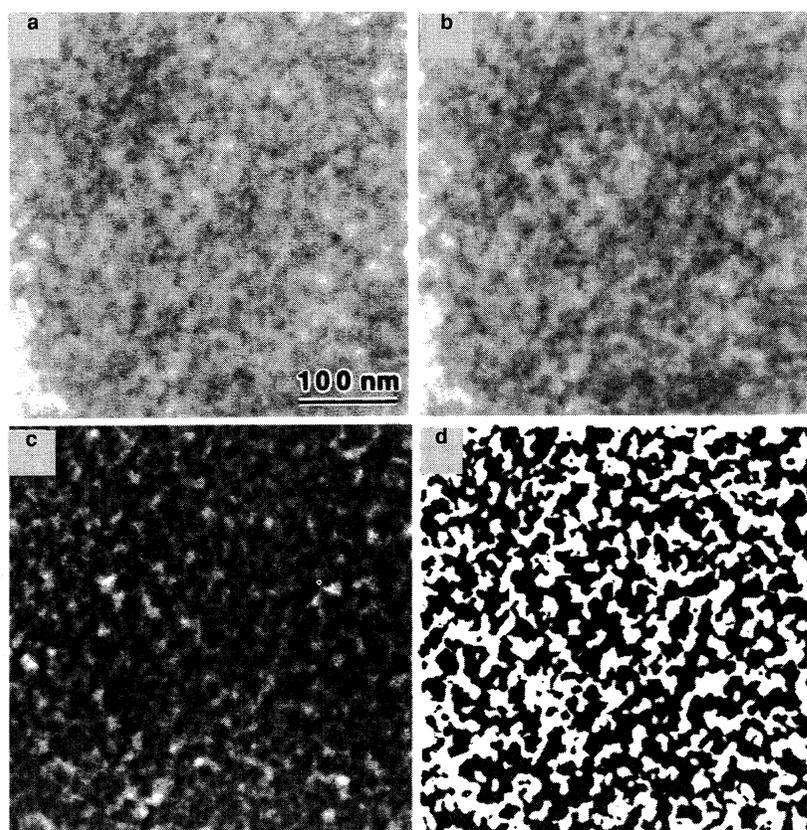


Figure 1 Sequential processing steps on images of low-moisture part-skim mozzarella cheese after six weeks of refrigerated storage. a) Photographic print of electron microscope negative; b) digitized image of photographic negative; c) flattened, brightness-enhanced, and contrast-enhanced image; and d) binary image from gray-level segmentation.

and cream (36.0% fat) from a local dairy. Allowance was made for moisture and fat in the powder in standardizing each batch to 2.3% fat and 9 or 11% solids-not-fat. After pasteurization at 63 °C for 30 min, reconstituted milk was cooled and held overnight at 4 °C. The next day, the cheese milk was heated to 33 °C and inoculated with 125 mL CR5 starter culture (**Marschall-Rhône-Poulenc**, Madison, WI), described by the manufacturer as 50% *Streptococcus thermophilus* and 50% *Lactobacillus delbrueckii* ssp. *bulgaricus*. Calcium chloride (**Sigma Chemical Co.**, St. Louis, MO) was added to a concentration of 0.02%. After the pH decreased 0.16 to 0.22 unit, 4.4 g of #01034 single-strength calf rennet (**Chr. Hansen's Laboratory**, Milwaukee, WI) was added. The curd was cut after 25 min, held for another 20 min, and cooked

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to 43 °C in 60–70 min with stirring. Whey was drained for 25–30 min. The curd was cut into slabs, held until pH reached 5.2, covered with cheesecloth, placed in ice, and stored overnight at 4 °C. The next day, the curd was stretched, brined, and vacuum packaged as previously described.^{1–4} The samples were stored for up to six weeks at 4 °C.

The cheeses contained 49–52% moisture and 16–19% fat, which conforms to the labeling requirements for low-moisture part-skim mozzarella.

TEM was used to examine the ultrastructure of cheese samples after one day and six weeks of storage.⁴ Cubes measuring approx. 5 mm on each

side were removed from the interior of the cheese with a stainless steel razor blade, and fixed in a solution of 1% glutaraldehyde in 0.1 M imidazole-HCl at pH 7.0. The samples were washed in imidazole buffer, immersed in 2.0 mL of 2% osmium tetroxide–0.1 M imidazole buffer for 2 hr, and rinsed with distilled water. The samples were dehydrated in a graded ethanol series containing 50, 80, 90, and 100% ethanol. They were then transferred to propylene oxide, infiltrated overnight with 50% propylene oxide–50% epoxy resin embedding medium (**Electron Microscopy Sciences**, Fort Washington, PA), and embedded

CM12 TEM (**Philips Electronics**, Mahwah, NJ) in the bright-field image mode. Images were recorded on photographic film at an instrumental magnification of 60,000×. Areas of electron microscope negatives, equivalent to one square micrometer, of stained thin sections were digitized and frame-averaged using a Series 68 television camera (**DAGE-MTI**, Michigan City, IN) and DT2853 frame grabber (**Data Translation**, Marlboro, MA) controlled with Image Pro Plus software (**Media Cybernetics**, Silver Spring, MD). Digital images were flattened, and brightness and contrast were inverted and then adjusted to provide matching histograms for

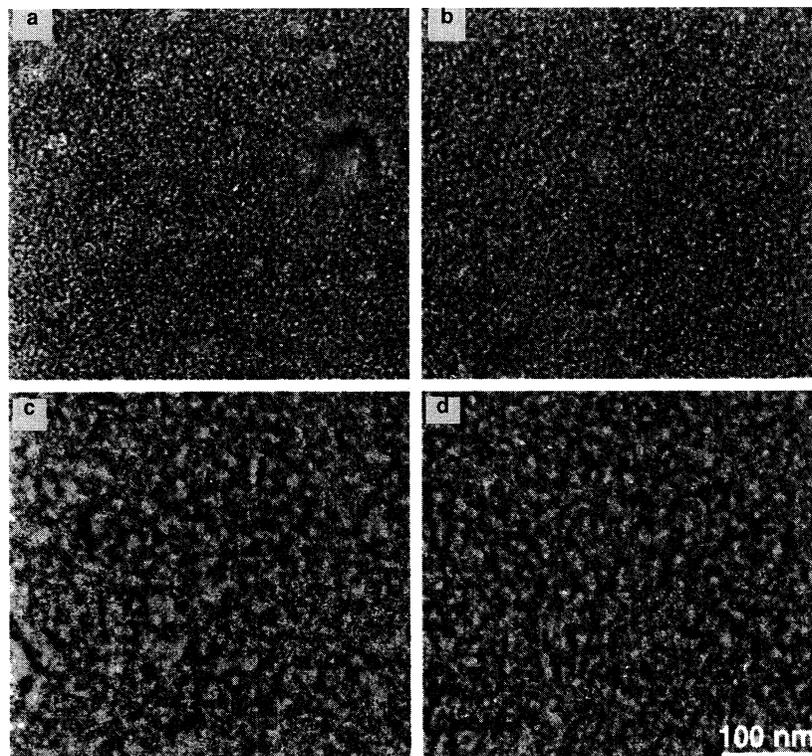


Figure 2 TEM images of mozzarella cheeses made from reconstituted milk: a) 9% NFDM cheese, one day of storage; b) 11% NFDM cheese, one day of storage; c) 9% NFDM cheese, six weeks of storage; and d) 11% NFDM cheese, six weeks of storage.

with 100% epoxy resin. Thin sections measuring <1 mm on a side and 60–70 nm thick were cut with an ultramicrotome, stained with lead citrate and uranyl acetate, and examined with a model

the three paired sets of cheese images. Fast Fourier transforms were computed from whole image areas, and radial distribution plots of intensity in the transforms were sam-

pled as line profiles. Integrated areas of segmented images were calculated from histograms of the binary images.

Scanning electron microscopy (SEM) was also used to examine the cheeses. Sample cubes were cut, fixed, and washed as above. They were then dehydrated by immersing in 50% ethanol solution for 30 min and in absolute ethanol twice for 30 min each. Blocks of cheese were individually frozen in liquid nitrogen for 5 min and freeze fractured with a cold razor blade. The fractured pieces of cheese were thawed into ethanol, and fat was removed by immersing in chloroform twice for 30 min each. After immersing in ethanol for an additional 30 min, the samples were critical point dried in CO₂ and mounted on aluminum stubs with colloidal silver adhesive with fractured faces upwards. The samples were coated with a thin layer of gold by dc sputtering, and digital images in the secondary electron imaging mode were obtained with a JSM 840A SEM (JEOL USA, Peabody, MA) coupled to an Imix workstation (Princeton Gamma-Tech, Princeton, NJ).

Results and discussion

The effect of the sequence of digital image processing steps on TEM image features is illustrated in *Figure 1*, which is a sample of mozzarella cheese stored for six weeks. The size and organization of discrete stained, electron dense phases in cheese samples remained essentially the same proceeding from *a*) a photographic print of the electron microscope negative to *b*) a digitized image to *c*) a flattened, brightness-enhanced and contrast-enhanced image to *d*) a binary image.

Figure 2 shows TEM images of mozzarella made from NFDM reconstituted to 9 or 11% total solids. After one day of storage, both cheeses contained two-phase structures with alternating dark and light areas, and with regular interspacing around 15 nm. The dark (stained) areas corresponded to objects with the di-

mensions of casein submicelles. After six weeks, large irregularly contoured electron-lucent areas were present (*Figure 2c* and *d*), with the spacing between dense and light objects ranging from 30 to 40 nm. The areas occupied by the electron-lucent phases remained constant during storage, an indication that

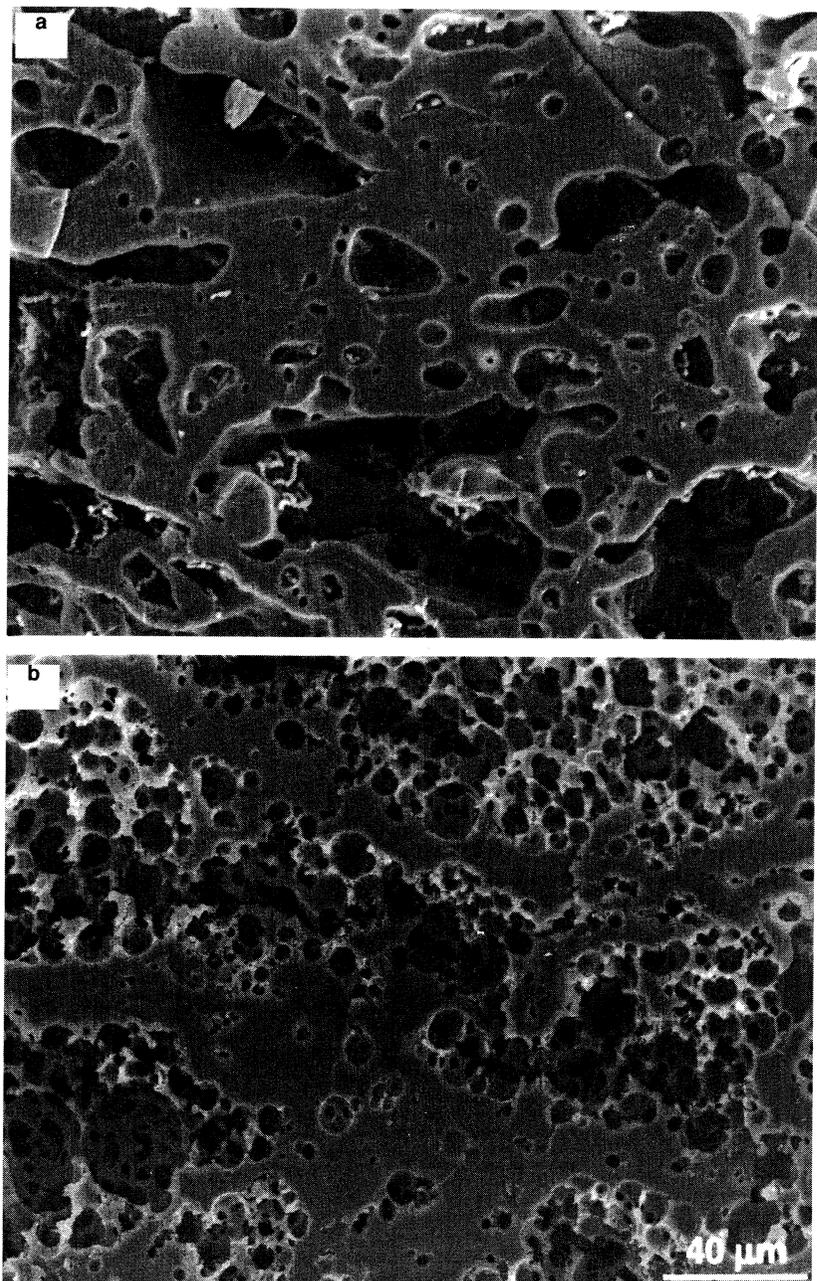


Figure 3 SEM images of mozzarella cheese made from NFDM reconstituted to 9% solids-not-fat, after one day (top) and six weeks (bottom) of refrigerated storage. The irregular cavities correspond to fat globules and the smooth areas to protein matrix. Starter culture bacteria are visible in some of the cavities.

the submicelles did not disintegrate because of proteolysis. Instead, they rearranged into clusters. The same submicellular structure and reorganization was observed in cheeses prepared from fresh milk.⁴ Scanning electron micrographs of mozzarella made with NFDM reconstituted to 9% total solids show that the fat globules were separated by protein matrix at zero weeks, with some of the starter culture bacteria still present (*Figure 3a*). By six weeks, many of the globules had coalesced and the bacteria were no longer evident (*Figure 3b*). The same behavior has been observed in mozzarella cheeses made from fresh milk.¹ The rennet and starter culture bacteria in mozzarella are responsible for proteolytic degradation of α_{s1} -casein, the primary structural protein in cheese. The proteolysis of α_{s1} -casein and con-

current peptide formation may promote a rearrangement of submicelles from a relatively homogeneous distribution to a pattern of clusters and open spaces. The protein matrix thus becomes more porous, which should lead to fewer physical interactions between micelles and a weaker matrix, which in turn allows the fat globules to agglomerate. The matrix becomes less resistant to application of force and provides less protection of the fat globules from heating. The cheese in this study therefore became softer, less elastic, and more meltable with storage. These effects have also been observed in mozzarella cheeses made from fresh milk.¹⁻⁴

Summary

Electron micrographic images suggest that casein submi-

celles reorganize in mozzarella, whether the cheese milk used is fresh or reconstituted from NFDM. Mozzarella cheese made from NFDM can therefore be expected to exhibit functional properties similar to those of mozzarella made from fresh milk.