

TRANSMISSION ELECTRON MICROSCOPIC IMAGING OF CASEIN SUBMICELLE DISTRIBUTION IN MOZZARELLA CHEESE

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1. INTRODUCTION

The increased demand for reduced-fat dairy products has spurred research into low fat (LF) cheeses, including Mozzarella. The LF Mozzarella procedure developed in our laboratory has been scaled up and tested in schools¹ and is now being served on pizzas in the National School Lunch Program². We have conducted studies with different cooking temperatures and homogenization pressures to determine the effects on casein breakdown, rheological properties, and microstructure in LF Mozzarella^{3,4}, and are using these results to improve the quality of this cheese.

The effects of coagulant type on composition, proteolysis, and physical properties of full fat Mozzarella have been reported recently. Berg *et al.* made Mozzarellas by the direct acid method using calf chymosin, bovine pepsin, porcine pepsin, and *Rhizomucor miehei* (formerly *Mucor miehei*) as coagulants⁵. The calf chymosin cheese had the greatest melt and smallest stretch, the porcine pepsin cheese had the least melt and largest stretch, and the other two cheeses were similar to each other. Coagulant type did not affect pH, moisture, or browning. Yun, Barbano, and Kindstedt prepared full-fat Mozzarellas with *Cryphonectria parasitica* (formerly *Endothia parasitica*), *R. miehei*, and chymosin derived by fermentation, and found no differences in pH, moisture, fat, and protein⁶. The *C. parasitica* cheese underwent more proteolysis of α_{s1} -casein than the others and was the only one in which β -casein was broken down. There were no significant rheological differences among the cheeses, however⁷.

Electron microscopic imaging can be performed on cheese to examine its structure. Previous studies in this laboratory have shown that the microstructure of Mozzarella changes during refrigerated storage^{8,9}. Rearrangement of casein submicelles was observed, and a technique for examining their spacing and distribution was developed. The rear-

Table 1. Coagulants used in preparation of Mozzarella cheeses

| Coagulant | Strength | Enzyme |
|----------------------------|----------|---------------------------------------------------------------------------------|
| Chr. Hansen's calf rennet | single | chymosin |
| Marschall Marzyme Supreme® | double | <i>Rhizomucor miehei</i> (formerly <i>Mucor miehei</i>) protease |
| Pfizer Surecurd® | triple | <i>Cryphonectria parasitica</i> (formerly <i>Endothia parasitica</i>) protease |

arrangement may have been related to proteolytic breakdown of casein. The purpose of this study was to investigate the effect of coagulant type on rheology and microstructure of LF Mozzarella. Electron microscopic imaging was performed to determine if the coagulant type influenced the arrangement of casein submicelles.

2. MATERIALS AND METHODS

2.1. Cheese Preparation

LF Mozzarella cheeses were prepared using the method of Tunick *et al.*⁸ Two batches were prepared each week on different days and three replicates were made from each coagulant shown in Table 1.

2.2. Compositional Analyses

Percentage of moisture was determined by the forced-draft oven method¹⁰ and percentage of fat was measured by the modified Babcock method¹¹. Fat in dry matter (FDM) was calculated as (% fat)/(100 - % moisture). Moisture in nonfat substance (MNFS) was calculated as (% moisture)/(100 - % fat). Three replicates of each sample were analyzed after 1 wk of storage.

2.3. Rheological Analyses

Hardness and springiness were determined by texture profile analyses performed at 22–24°C on an Instron model 4201 Universal Testing Machine (Instron, Inc., Canton, MA)^{3,12}. Elastic modulus (G') and viscous modulus (G'') were measured at 22–24°C at a frequency of 100 rad/s by a Rheometrics Dynamic Analyzer model RDA-700 (Rheometrics, Inc., Piscataway, NJ)^{3,12}. Meltability, which is unitless, was determined by the Schreiber test^{3,12}, in which the increase in diameter of a cheese disk is measured on a target graph of concentric circles after 5 min of heating at 232°C. All tests were performed in triplicate at 1 and 6 wk.

2.4. Microscopy

The microstructure of cheese samples after 1 d and 6 wk of storage was examined by scanning electron microscopy (SEM)³. Cubes measuring approximately 5 mm on a side were removed from the interior of the cheese with a razor and fixed in a solution of 1% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.2. The samples were washed in 0.1 M sodium cacodylate buffer, soaked in 2.0 mL of 2% osmium tetroxide-0.1 M sodium cacodylate buffer for 2 h, and rinsed with distilled water. The samples were dehydrated in a graded ethanol series containing 50, 80, 90, and 100% ethanol. After extraction with three

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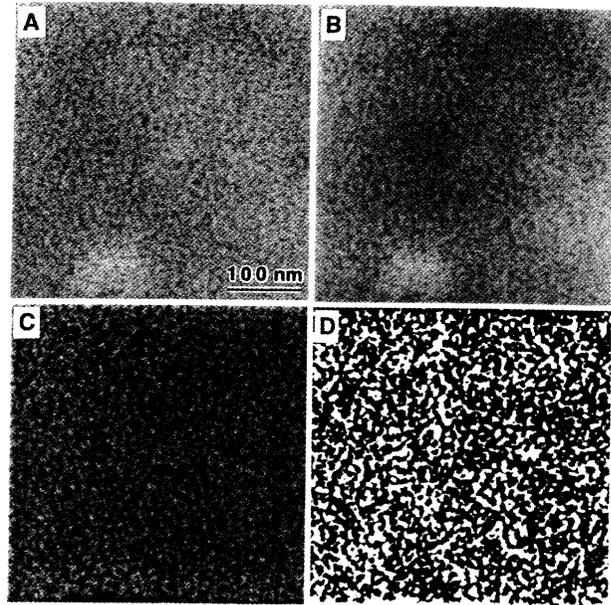


Figure 1. Sequential processing steps on TEM images of Mozzarella. (A) Photographic print. (B) Digitized image of photographic negative. (C) Flattened, brightness-enhanced, and contrast-enhanced. (D) Binary image from gray level segmentation. (Reprinted from reference 8 with kind permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX5 1GB, UK).

changes of chloroform to remove lipids, the samples were transferred into ethanol, freeze fractured into liquid nitrogen, thawed into ethanol, and dried at the critical point in carbon dioxide. The dried blocks were mounted on aluminum stubs, coated with a thin layer of gold in a DSM-5 Cold Sputtering Module (Denton Vacuum, Inc., Cherry Hill, NJ), and examined by secondary electron imaging in a JEOL model 840A scanning electron microscope (JEOL USA, Peabody, MA) at an instrumental magnification of 1000X.

Transmission electron microscopy (TEM) was used to examine the ultrastructure of cheese samples after 1 d and 6 wk of storage^{8,9}. Samples were cut, fixed, washed, and dehydrated as above. 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 with 100% epoxy resin. Thin sections measuring <1 mm on a side and 60–70 nm thick were cut with a microtome, stained with lead citrate and uranyl acetate, and imaged with a Philips model CM12 transmission electron microscope (Philips Electronics, Mahwah, NJ) in the bright field image mode. Images were recorded on photographic film at an instrumental magnification of 60,000X.

Areas of electron microscope negatives, equivalent to one square micrometer, of stained thin sections, were digitized and frame-averaged using a model 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 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 sampled as line profiles, after three cycles of smoothing with a 3 x 3 kernel. Intensity distributions of stained electron density in matched pairs of cheese images were segmented by gray level to represent size and distribution of electron-dense clusters and converted to binary images. Integrated areas of segmented images were calculated from histograms of the binary images.

The effect of the sequence of digital image processing steps on image features is illustrated in Figure 1, which is a Mozzarella made with chymosin and stored for 6 wk. The

Table 2. Effect of coagulant type on composition of Mozzarella cheeses

| | Percent \pm standard deviation | | |
|----------|----------------------------------|------------------|----------------------|
| | Chymosin | <i>R. miehei</i> | <i>C. parasitica</i> |
| Moisture | 57.9 \pm 1.0 | 58.0 \pm 0.5 | 59.7 \pm 1.3 |
| Fat | 8.9 \pm 0.1 | 8.8 \pm 0.3 | 8.5 \pm 0.8 |
| FDM | 21.1 \pm 0.3 | 21.0 \pm 0.4 | 21.0 \pm 1.3 |
| MNFS | 63.6 \pm 1.1 | 63.5 \pm 0.4 | 65.2 \pm 0.8 |

size and organization of discrete stained, electron dense phases in cheese samples remained essentially the same proceeding from (A) photographic prints of the electron microscope negatives to (B) digitized images to (C) flattened, brightness-enhanced and contrast-enhanced images to (D) binary images.

2.5. Statistical Analyses

The data were analyzed by the SAS System-General Linear Models procedure¹³. Means comparisons were performed using the Bonferroni LSD method¹⁴. Comparisons are described as significant only when $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Compositional Analyses

The average percentages of moisture, fat, FDM, and MNFS in the Mozzarella cheeses are shown in Table 2. There were no significant compositional differences among the samples.

3.2. Rheological Results

The hardness, springiness, G' , and G'' decreased during 6 wk of refrigerated storage, and the meltability increased, although many of the differences were not significant (Table 3). These changes with storage were due to proteolysis of the α_{s1} -casein, which is the ma-

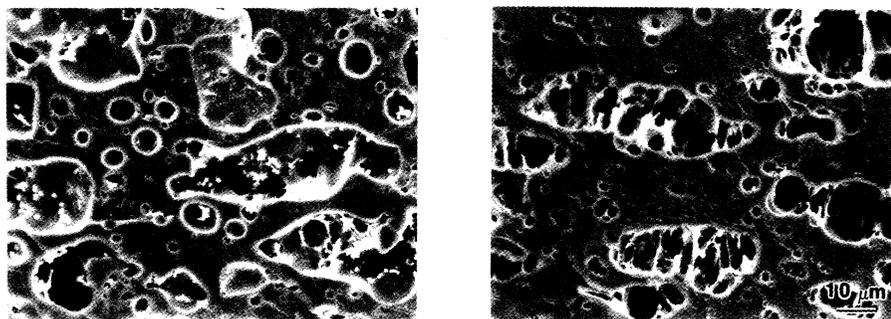


Figure 2. SEM images of Mozzarella made with chymosin after 1 d (left) and 6 wk of refrigerated storage.

Table 3. Effect of coagulant type on rheology of Mozzarella cheeses during storage

| | Chymosin | <i>R. miehei</i> | <i>C. parasitica</i> |
|-----------------|--------------------|--------------------|----------------------|
| Hardness, N | | | |
| 1 wk | 65 ^a | 54 ^a | 41 ^b |
| 6 wk | 44 ^{ab} | 33 ^b | 31 ^b |
| Springiness, mm | | | |
| 1 wk | 8.79 ^a | 8.36 ^{ab} | 8.12 ^b |
| 6 wk | 6.48 ^c | 6.17 ^c | 6.29 ^c |
| G', kPa | | | |
| 1 wk | 52.9 ^a | 47.4 ^a | 43.4 ^a |
| 6 wk | 42.2 ^{ab} | 29.7 ^{ab} | 30.9 ^b |
| G'', kPa | | | |
| 1 wk | 17.4 ^a | 16.2 ^a | 15.3 ^a |
| 6 wk | 15.7 ^{ab} | 11.1 ^{ab} | 11.6 ^b |
| Meltability | | | |
| 1 wk | 1.5 ^a | 1.7 ^{ab} | 2.0 ^b |
| 6 wk | 1.9 ^{ac} | 2.2 ^c | 2.6 ^{bc} |

^{abcd}Within each group, means within the same row or column with no letter in common are significantly ($P < 0.05$) different.

for structural protein in cheese¹⁶. The 1-wk values of hardness, springiness, and meltability for the cheeses made with *C. parasitica* and chymosin were significantly different, but there were no differences attributable to the coagulant type at 6 wk.

3.3. Microstructure

Day-old samples (Figures 2–4 left) contained large irregular smooth-lined cavities, and some isolated spherical spaces, in the protein matrix. These areas were formerly occupied by lipids, and many also contained bacteria from the starter culture. After 6 wk storage, the areas were mostly aggregated and contained relatively few bacteria (Figures 2–4 right). Proteolysis of the casein matrix facilitated coalescence of the fat droplets. There were no apparent differences in microstructure between cheeses made from different coagulants.

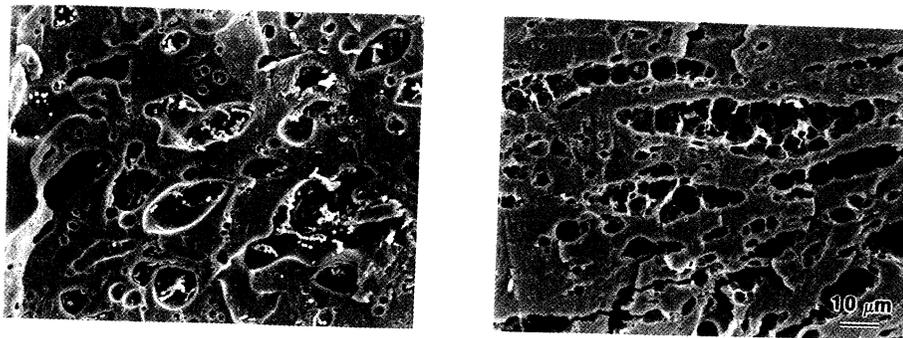
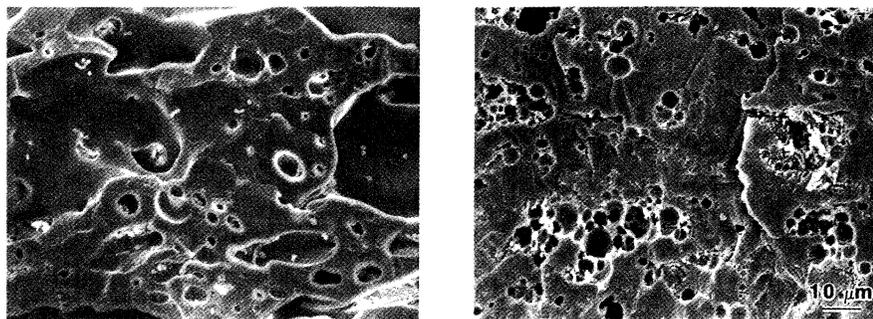
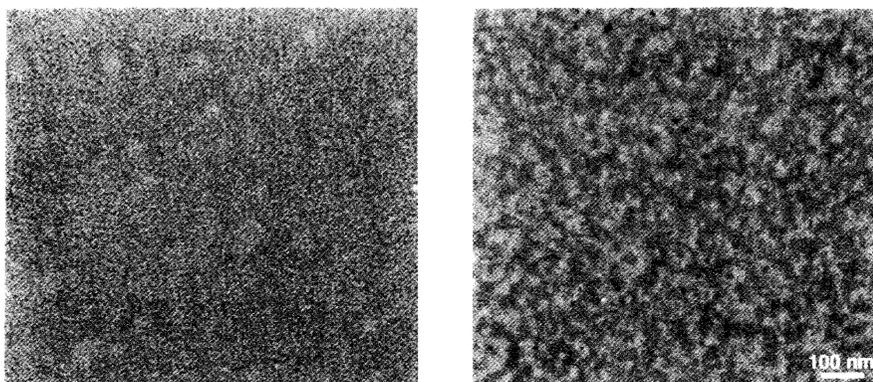
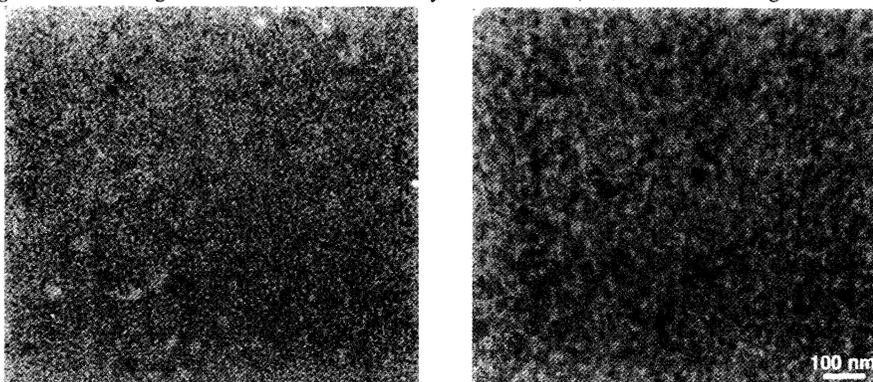


Figure 3. SEM images of Mozzarella made with *R. miehei* after 1 d (left) and 6 wk of refrigerated storage.

Table 4. Total integrated areas of electron dense regions in Mozzarella cheeses during storage

| Storage time | Area of electron density, % | | |
|--------------|-----------------------------|------------------|----------------------|
| | Chymosin | <i>R. miehei</i> | <i>C. parasitica</i> |
| 1 d | 47.8 | 47.9 | 48.4 |
| 6 wk | 48.1 | 46.4 | 48.3 |

**Figure 4.** SEM images of Mozzarella made with *C. parasitica* after 1 d (left) and 6 wk of refrigerated storage.**Figure 5.** TEM images of Mozzarella made with chymosin after 1 d (left) and 6 wk of refrigerated storage.**Figure 6.** TEM images of Mozzarella made with *R. miehei* after 1 d (left) and 6 wk of refrigerated storage.

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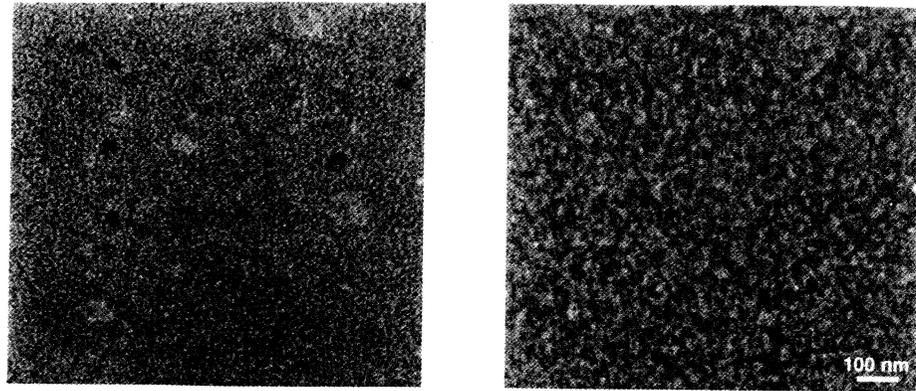


Figure 7. TEM images of LF Mozzarella made with *C. parasitica* after 1 d (left) and 6 wk of refrigerated storage.

3.4. Ultrastructure

Fresh samples (Figures 5–7 left) contained a two-phase structure with alternating dark and light areas, and interspacing around 15 nm. The dark areas corresponded to casein submicelles. After 6 wk, large irregularly contoured electron-lucent areas were present (Figures 5–7 right), with the spacing between dense and light ranging from 30 to 40 nm (Figure 8). The area occupied by the electron-lucent phase remained constant during storage (Table 4), an indication that the submicelles did not disintegrate because of proteolysis. Instead, they rearranged into clusters. All three types of cheese exhibited the same submicellular structure.

The loss of α_{s1} -casein and concurrent peptide formation may facilitate reorganization of submicelle particles from a relatively consistent distribution into a pattern of clusters and open spaces⁸. The increase in porosity of the protein should lead to fewer physical interactions between micelles and result in a weaker casein matrix. Moreover, molecular modeling indicates that the peptides formed from cleavage of α_{s1} -casein have a strong ten-

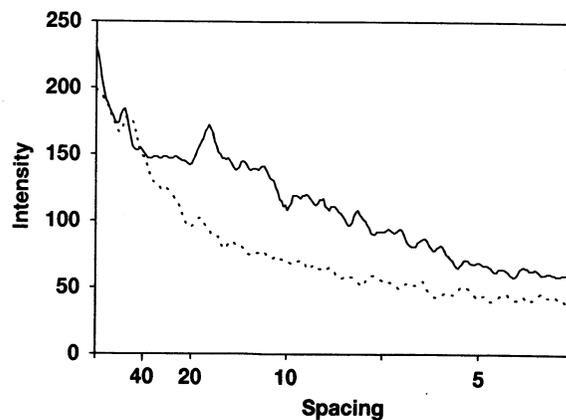


Figure 8. Radial distribution profiles of reciprocal spacings in electron density in Fast Fourier Transforms of protein matrix images of Mozzarella. Cheeses made with chymosin and stored for 1 d (solid line) and 6 wk (dashed line). Relative intensity is measured in gray levels. Spacings are measured in nm^{-1} .

Table 4. Total integrated areas of electron dense regions in Mozzarella cheeses during storage

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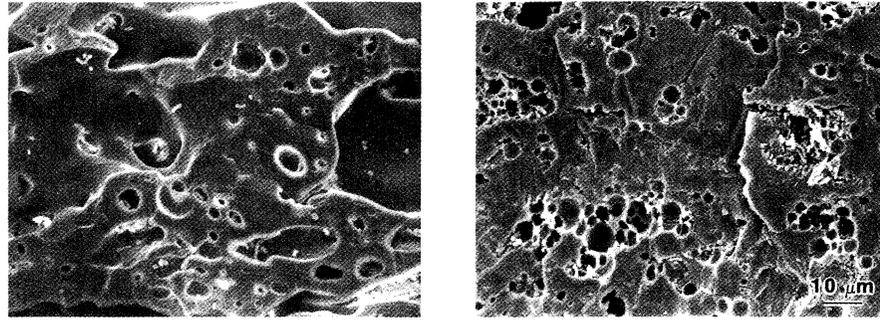


Figure 4. SEM images of Mozzarella made with *C. parasitica* after 1 d (left) and 6 wk of refrigerated storage.

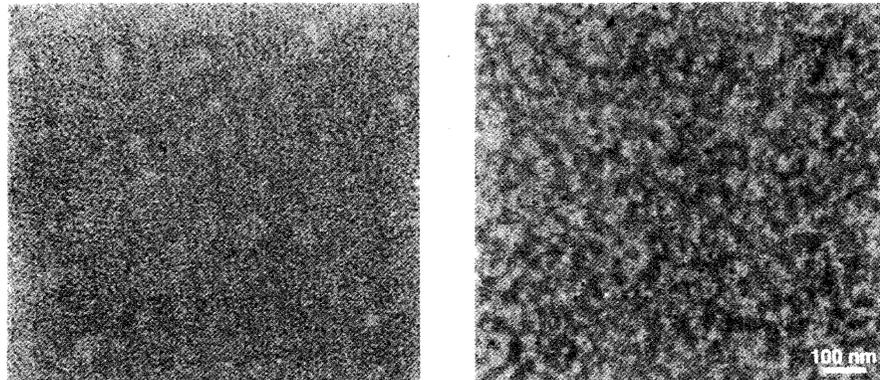


Figure 5. TEM images of Mozzarella made with chymosin after 1 d (left) and 6 wk of refrigerated storage.

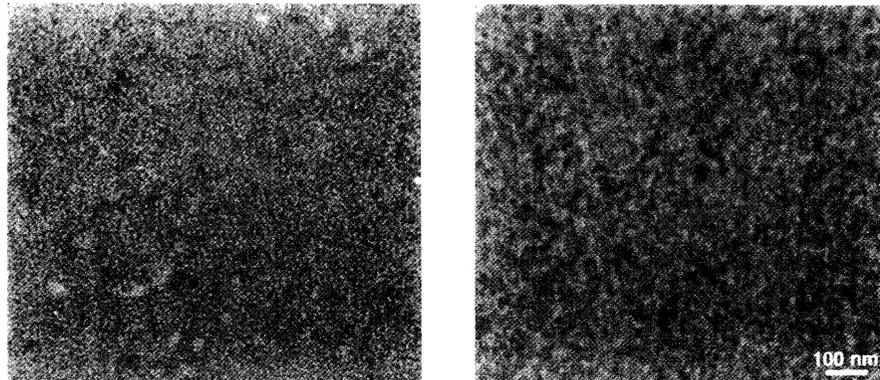


Figure 6. TEM images of Mozzarella made with *R. miehei* after 1 d (left) and 6 wk of refrigerated storage.

dency to become more compact and stable¹⁷. The smaller structures provide less resistance to application of force and less protection of the fat globules from heating. The cheese in this study therefore became softer, less elastic, and more meltable with storage.

4. CONCLUSIONS

The source of rennet does not affect the rheological properties of low-fat Mozzarella cheeses. SEM and TEM micrographs indicate rearrangements of fat globules and casein submicelles, but there are no differences attributable to the coagulant.

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