

ELECTRON-DENSITY PATTERNS IN LOW-FAT MOZZARELLA CHEESES DURING REFRIGERATED STORAGE

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INTRODUCTION

Consumer demand for palatable low-fat foods has prompted the development and production of a wide variety of new foods. Consumer interest in low-fat dairy products has increased dramatically, and among the many new and improved dairy foods are some Mozzarella cheeses that have suitable textural properties yet contain less fat than part-skim varieties (Tunick et al., 1991). We further improved the textural properties of low-fat Mozzarella cheeses by adjusting the proportions of ingredients and processing conditions (Tunick et al., 1993). High levels of moisture in the non-fat substance significantly decreased the hardness, complex viscosity and elastic modulus, and the meltability increased during storage. These improvements in textural properties appeared to be most directly related to increased proteolysis under conditions of high moisture.

The protein matrix of cheeses and other milk products often reveals an extensively uniform pattern composed of two phases, when examined by electron microscopy (deJong, 1978; Knoop and Buchheim, 1980; Schmidt, 1982). One phase consists of electron-dense clusters with dimensions comparable to the average diameters of casein sub-micelles, which are reported to range from 10 to 20 nm. The other phase consists of electron-lucent spaces with similar dimensions, separating the dense clusters. We examined this ultrastructure, related to the molecular organization of the milk proteins, in Mozzarella cheeses before and after refrigerated storage, using stained thin sections. The results bring to focus a clearly detectable, regular structural feature which might link changes in the molecular organization of the matrix to proteolysis and textural properties.

Electron Microscopy of Stained Thin Sections

Full-fat (>20% fat) and low-fat (<11% fat) Mozzarella cheeses were prepared from non-homogenized and homogenized milk at 17.2 MPa as previously described (Tunick et al., 1993). After 0 and 6 wk of storage at 4°C, a 1 cm cube was cut from each block of cheese, and the central part of the cube was diced into 1-mm-sized samples and immersed in 20 mL of 1% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.3 for 2 hr at room temperature, then stored at 4°C. Subsequently, the diced blocks were washed in 0.1 M cacodylate buffer, reacted with 2% osmium tetroxide-cacodylate solution for 2 hr, washed in water, incubated at 55°C for 30 min in 2% uranyl acetate solution, dehydrated in a graded series of ethanol solutions, and finally embedded in an epoxy resin mixture. Thin sections of the cured plastic were cut with diamond knives and stained with uranyl acetate and lead citrate solutions. Images were recorded on photographic film in a transmission electron microscope at an instrumental magnification of 60,000.

Image Processing and Analysis

Areas of photographic negatives, equivalent to 0.25 μm square, were imaged with a Newvicon television camera and digitized. The raw digital images were processed to flatten the background. Brightness was inverted, and contrast was expanded to fill the 256 gray levels. The average spacing between electron dense clusters over the whole area in the images was estimated from the distribution of intensity in fast fourier transforms. Area fractions and size distributions of the electron dense- and electron-lucent phases in images were estimated by integration from binary images, derived from gray level contouring or segmentation of images, using ImagePro Plus software¹ (Media Cybernetics, Silver Spring, MD) or Imageplus software (Dapple Microsystems, Sunnyvale, CA).

RESULTS

Microstructure

Earlier, we found that low- and high-fat versions of Mozzarella cheese displayed topographical evidence of several microstructural compartments by scanning electron microscopy (Tunick et al., 1993). The compartments were an admixture of (1) irregular smooth-surfaced cavities around 10-20 μm wide or larger, (2) a broad range of sizes of circular vesicles from 5 μm to 50 nm or less in diameter, (3) extensive thick and thin fractured faces of a continuous matrix, containing the cavities and vesicles, and finally (4) the discontinuous interfaces between the matrix and cavities and vesicles, where most of the bacteria from the starter culture were apposed. Figure 1 illustrates the topographical structure of high-fat and low-fat Mozzarella cheeses before refrigerated storage.

The structures of low-fat and high-fat versions after six weeks of refrigerated storage are illustrated in Figure 2. The matrix of both the low-fat and high-fat stored samples accounted for more of the surface area in the fractured preparations because

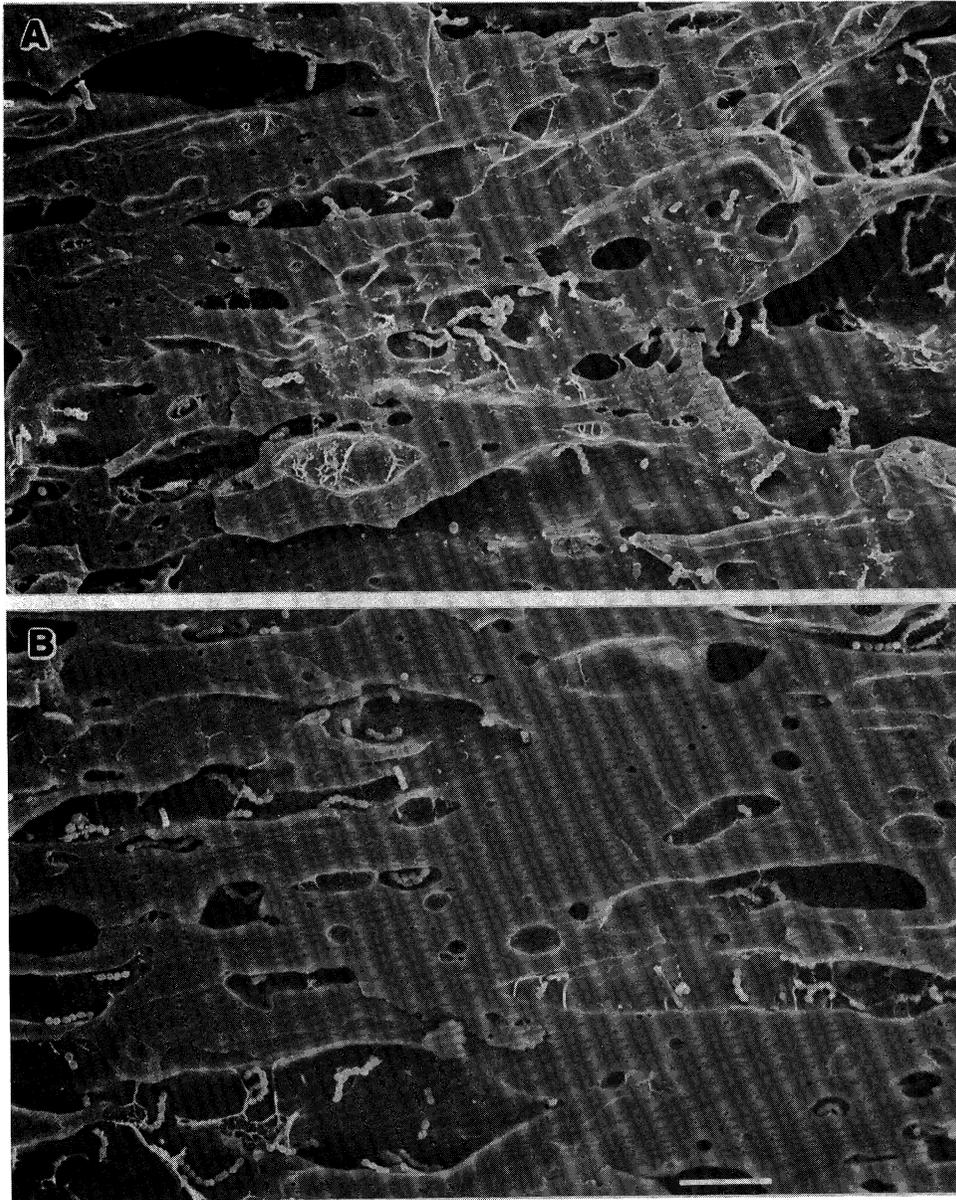


Figure 1. Microstructure of high-fat (A) and low-fat (B) Mozzarella cheese at week 0. Fractured faces of matrix occupied about one-half of the surface area in high-fat, and two-thirds of the area in low-fat cheese samples. Other areas of fracture face were related to surfaces of large irregular cavities, containing starter bacteria, and surfaces of a very broad size range of vesicles. Bar indicates 10 μm .

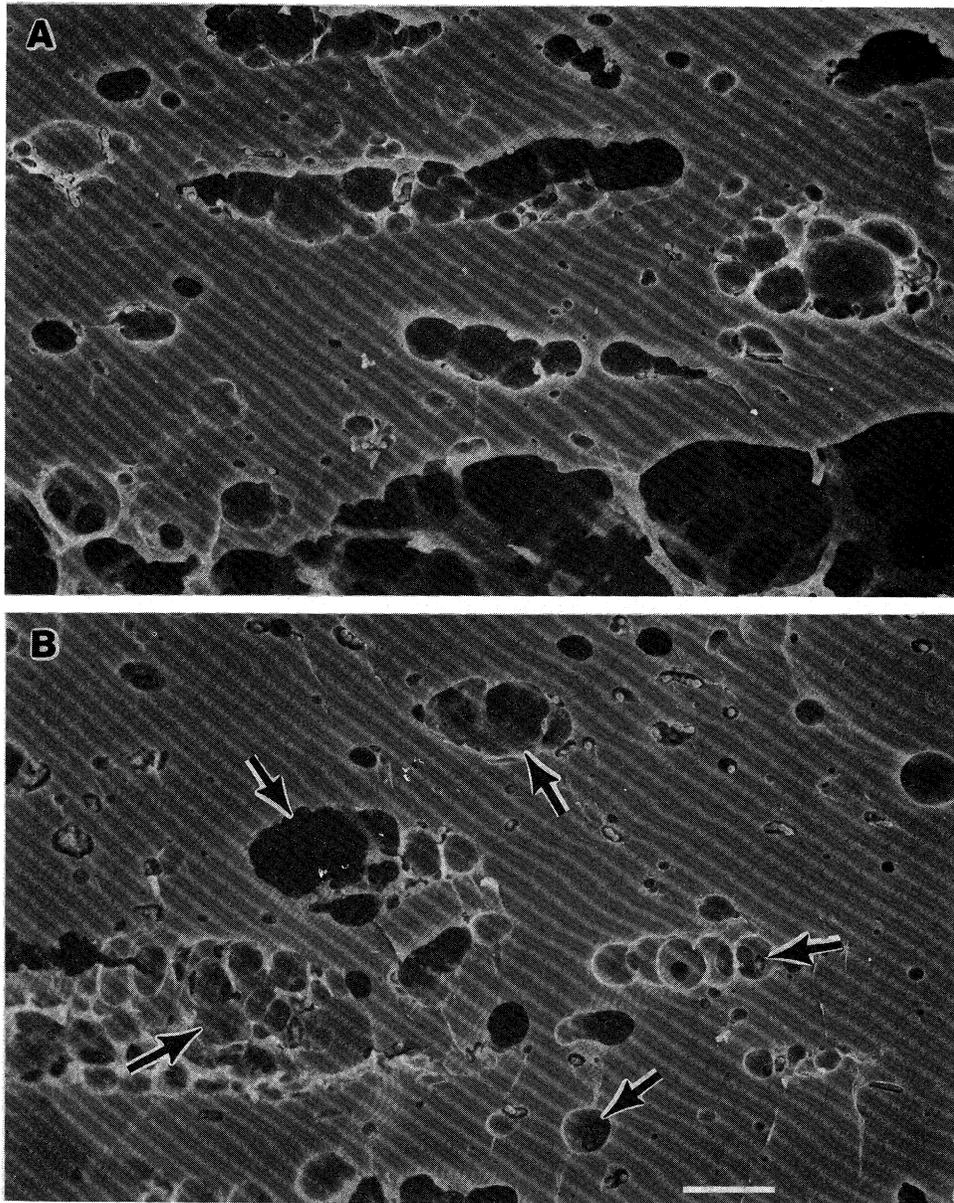


Figure 2. Microstructure of high-fat (A) and low-fat (B) Mozzarella cheese after storage for 6 wk. Smooth fracture faces filled about two-thirds of the surface area in high-fat version and three-fourths in the low-fat sample. Inclusions were not irregular in shape; instead fat was located in aggregated spherical vesicles ranging from 5 to 20 μm in diameter (arrows). Very broad size range of smaller vesicles was similar to 0 wk samples. Bar indicates 10 μm .

of a reduction in the areas of the cavities through changes in their abundance and shape: irregular, smooth-surfaced cavities were completely absent from the stored samples; instead fusiform areas (very large in high-fat samples) or layers composed of aggregated spherical spaces with bacterial cells pressed into the matrix at their surfaces were found. In both the low- and high-fat versions, a population of small vesicles, comparable to those measured in unstored samples, was found throughout the matrix.

Ultrastructure of the Matrix in Mozzarella Cheeses

Aside from obvious compartmental differences in microstructure related to fat composition, homogenization and redistribution of components during storage that have been recently reported (Kiely et al., 1993; Tunick et al., 1993), no essential structural differences that could be directly related to textural properties were resolved in the protein matrix of these cheeses by scanning electron microscopy. The fracture surfaces of the protein matrices appeared to be a uniform dense continuum, except for scattered profiles of micrometer- and nanometer-size vesicles, thought to contain fat. In thin sections examined by transmission electron microscopy, however, the matrix was clearly resolved into two distinct phases of (stained) electron density, with dimensions and distributions that were different before and after storage. These phases and other forms of structure with similar dimensions have been recognized in a variety of milk protein products, and they are identified with the sub-micellar organization of milk proteins (Schmidt and Buchheim, 1970).

We first measured a regular spacing in the sub-micellar pattern of electron density during a study of rennet-induced gelation of a commercial skimmed milk: we observed the stages of gel formation by optical microscopy and analysed the ultrastructure of the gel by electron microscopy of thin sections. A spacing of 14-16 nm was found throughout the segments of the gel (Figure 3), and similar evidence of a structural subunit was found in thin sections of the separate casein micelles, prepared for electron microscopy before enzymatic digestion. The organization of the electron-

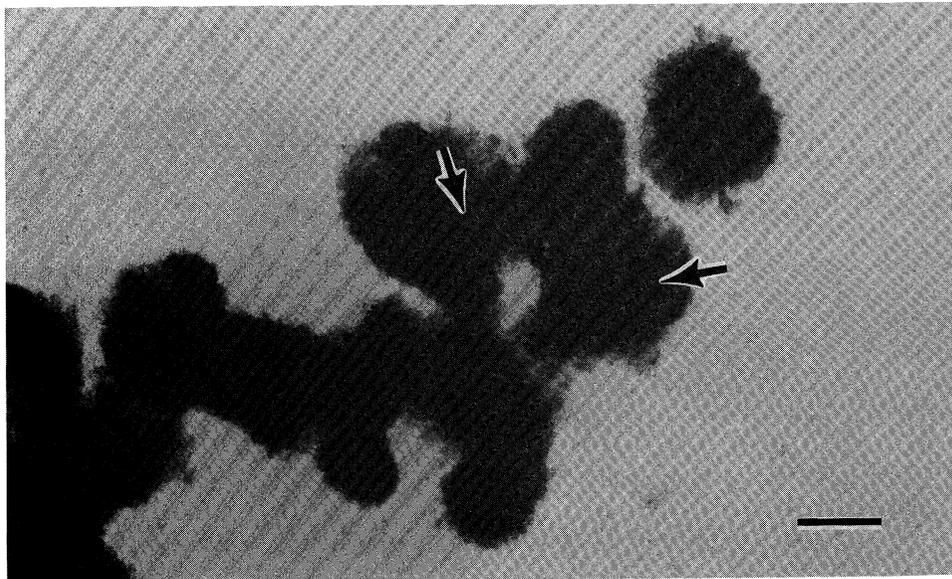


Figure 3. Renneted commercial milk gel in stained thin section showing homogeneous pattern of alternating electron density (arrows). Bar indicates 100 nm.

dense phase was short-range and isotropic, with a spacing that compared with the dimensions usually identified with casein sub-micelles (10-20 nm).

The Matrix before Storage

Thin sections of the matrix in unstored cheese samples contained clear evidence of the homogeneous pattern comprised of two phases of electron density. In digital images of low-fat cheese, the electron dense clusters had an average spacing in the range of 14-17 nm, as estimated from the intensity distribution in fast fourier transforms of the whole image area (Figure 4), and the average circular diameter of clusters estimated from binary images was 12.4 ± 1.1 nm (Table 1). Total integrated areas of electron density measured from whole image areas of unstored low-fat cheese samples were in the range of 61-66%.

The Matrix after Storage

Stored samples of all versions of cheese samples had a different pattern of alternating phases of stained electron density. After six weeks, the matrix was composed of clusters with spacings averaging from 32-36 nm (Figure 5), and the average circular diameter was 23.4 ± 3.4 nm (Table 1). Total integrated areas of electron density in whole images were similar to unstored samples, in the range of 64-67%.

Table 1. Dimensions of electron density features in Figures 4A and 5A.

Feature	Week 0	Week 6
Average spacing (nm)	16	32
Cluster diameter (nm)	12.4 ± 1.1	23.4 ± 3.4
Cluster area (nm ²)	123 ± 24	442 ± 131
Integrated area (%)	62	67

DISCUSSION

The microstructure of cheeses has been described and illustrated in many reports (reviewed in Kalab, 1993). There is general agreement that the dense continuous phase represents a compact form of casein derived from coalescence and fusion of colloidal protein micelles. The inclusions and vesicles represent milk fat trapped in the protein matrix. Differences in cheese texture are usually explained by the size-distribution and arrangement of fat inclusions, and by the organization and porosity of the protein matrix, but since textural properties are complex, no single set of microstructural features has clearly resolved the physical features that could be used to typify cheese texture.

The experimental Mozzarella cheeses that we prepared (Tunick et al., 1993) had structural features that closely resembled preparations shown in earlier published reports (Kalab, 1977; Taranto et al., 1979; Paquet and Kalab, 1988). Sheets, columns and plates of matrix were oriented, and the orientation was reflected also in the

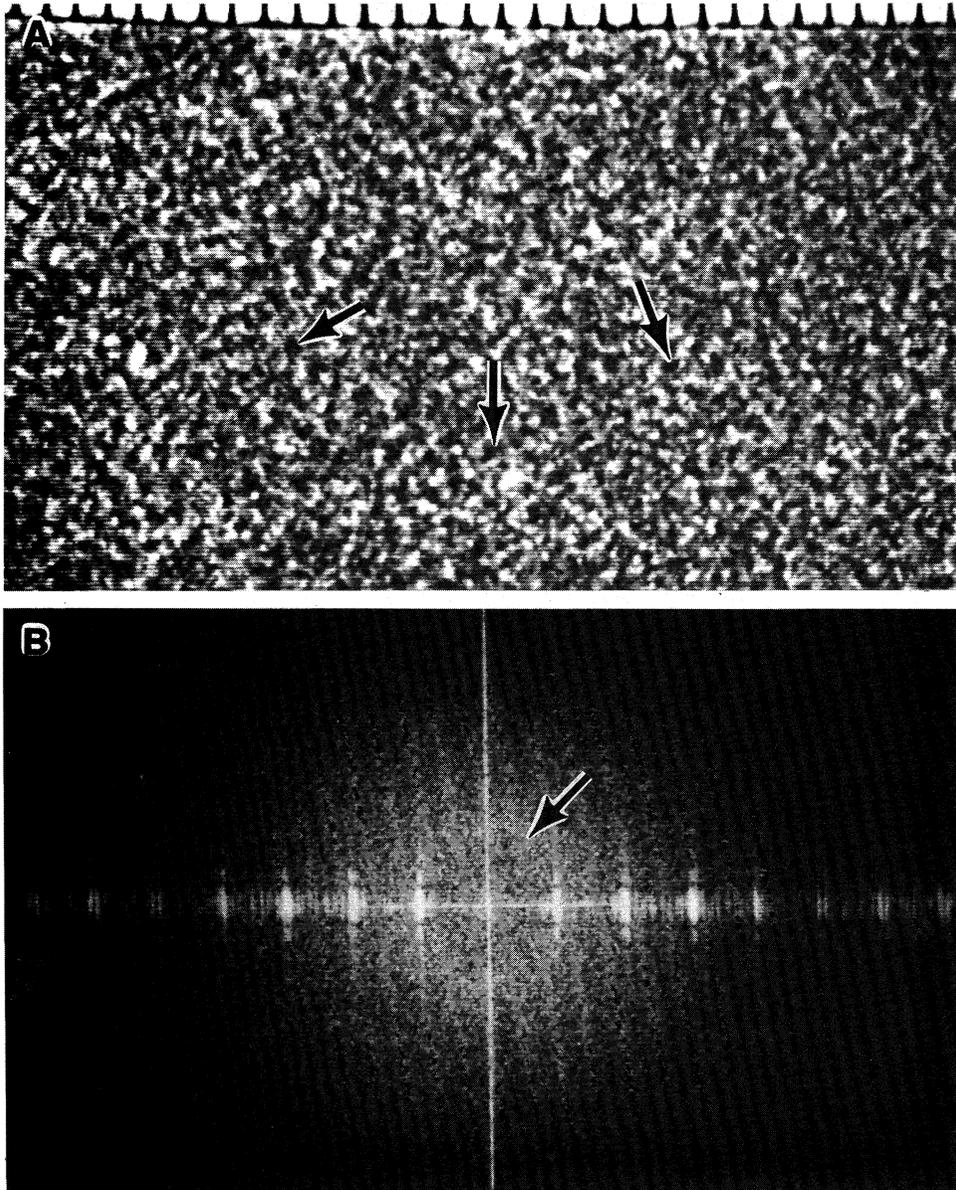


Figure 4. (A) Digitized, processed image of a low-fat sample at week 0. Small electron dense clusters (arrows) are separated by electron-lucent areas with interspaces averaging around 14-16 nm. (B) fast fourier transform of image computed for area in (A) shows a peak of intensity (arrow) corresponding to a reciprocal spacing about 1/16 nm. Spacing of line grating in (A) and reciprocal spacing on the equator in (B) corresponds to 16.7 nm and orders of 1/16.7 nm respectively.

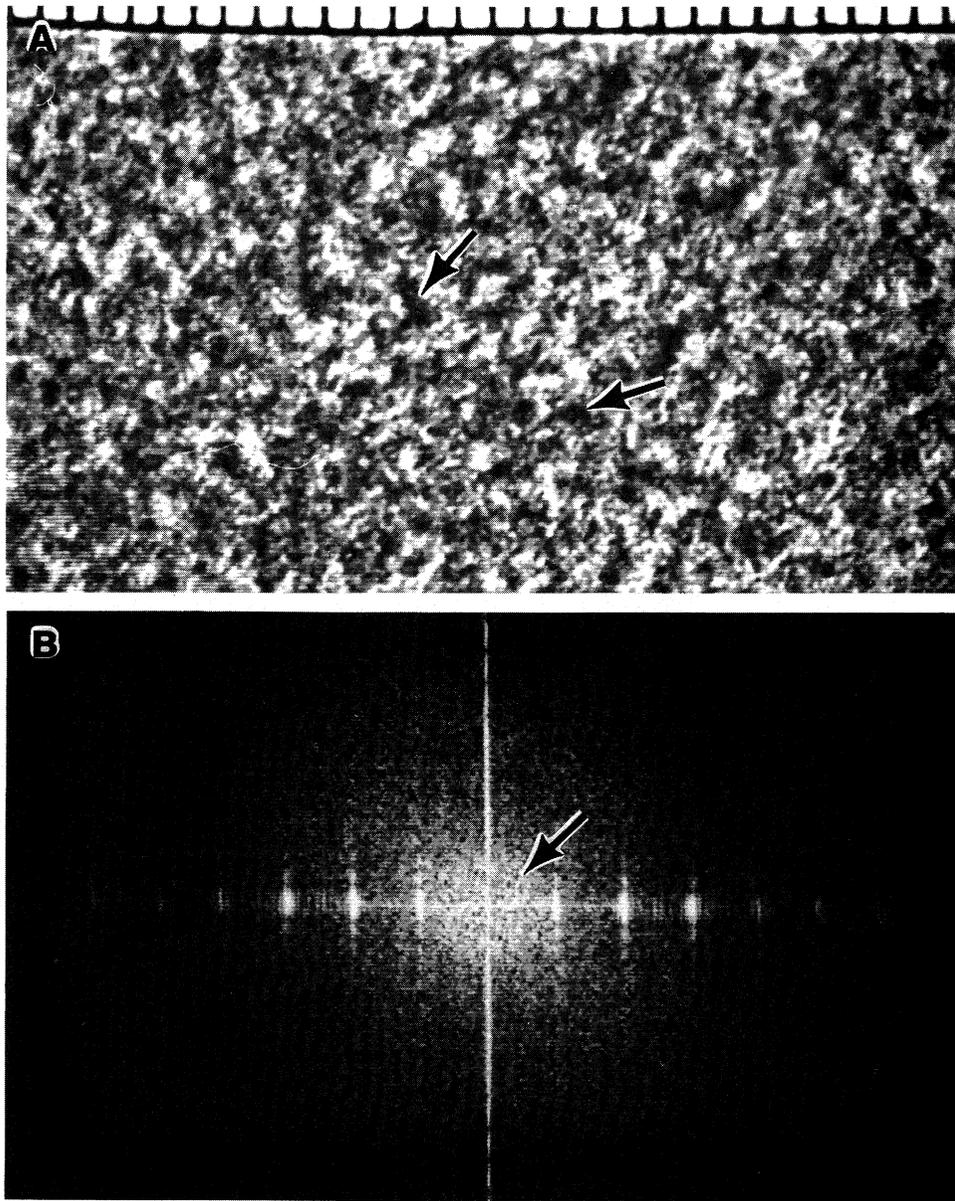


Figure 5. (A) Digitized, processed image of a low-fat sample after 6 wk of refrigerated storage. Electron dense clusters (arrows) are larger than week 0 and separated by electron lucent areas with interspaces ranging from 32-36 nm. (B) fast fourier transform of image computed from area in (A) shows intensity in a central disk with a diameter corresponding to a reciprocal spacing about 1/32 nm (arrow). Spacing of line grating in (A) and reciprocal spacing on equator in (B) corresponds to 16.7 nm and orders of 1/16.7 nm respectively.

irregular shapes of the large (fat) inclusions, or in the case of stored samples, fusiform aggregations of smaller spherical inclusions. The most obvious difference between fresh and stored cheeses we observed was related to the shape of lipid inclusions: during storage, large fat inclusions were transformed into aggregations of small spherical inclusions, resulting in a consolidation of the protein matrix. The mechanism of this rearrangement is not known. Kiely et al., (1993) suggested that increased porosity of the protein matrix resulting from enzymatic activity during storage, allowing confluence of lipid inclusions. Time lapse motion pictures through an optical microscope during storage might reveal more about the microstructural basis of this transformation.

Complex chemical and physical interactions within the protein matrix are usually regarded as the most likely factors effecting texture. Proteolysis of α_{s1} -casein, protein hydration and solvation of calcium phosphate bridges linking casein are among the possible key mechanisms controlling cheese texture. We extended our structural studies of low-fat Mozzarella cheeses in order to probe for possible quantitative correlates between molecular structures and functional properties. Refrigerated storage for 6 wk produced desirable texture profile features in cheeses containing high moisture levels and low fat. Increased softness and reduced elasticity were related to increased enzymatic degradation of α_{s1} -casein (Tunick et al., 1993). When examined by transmission electron microscopy, we found that storage affected the organization of the matrix at dimensions that have been identified with casein sub-micelles in ripened cheeses (Knoop and Buchheim, 1980). Fresh Mozzarella cheeses, regardless of fat content or homogenization, contained a homogeneous two-phase pattern of electron density in the protein matrix, with a uniform spacing around 15 nm. This spacing of electron density probably represents the residual structure of sub-micelles in the curd, since similar structures are found in casein micelles and milk gels. Following storage, the 15-nm spacing was not found; instead the electron-dense phase consisted of large clusters with an average interspace around 35 nm. Since the total areas occupied by the electron-dense phases before and after storage were about the same (60-70%), the difference in spacing must result from a reorganization of material on a molecular scale during storage. Enzymatic cleavage of α_{s1} -casein might lead to changes in net charge or other molecular properties of sub-micelles, resulting in aggregation of near neighbors, or some other form of reorganization within or between sub-micelles and proteolytic fragments, to produce the change in projected electron density. The larger spacing after storage could effect texture by reducing the mechanical interactions among proteins in the matrix, increasing softness and meltability. The change in dimensions of the subunits that we have detected throughout the matrix may be a useful quantitative index to correlate with texture properties. Further study of the electron density patterns of the matrix, resolved under native conditions, are needed to improve the accuracy of the observed changes in structure.

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