Effect of Scanning Electron Microscopy Preparation Methods on the Ultrastructure of White Bread[1]

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ABSTRACT

The effects of various microscopy preparation methods on the ultrastructure of white bread were evaluated. Unfixed specimens were air, oven, or freeze dried. Other specimens were fixed with either glutaraldehyde and osmium tetroxide (OsO_4) or OsO_4 alone. After fixation, they were freeze dried directly, air dried at room temperature, or dehydrated with ethanol and freeze dried. The method of drying unfixed bread had little influence on structure. Unfixed bread had air cells coated with a relatively smooth, thin continuous protein layer in which starch granules were embedded. Exposure to buffers, fixatives, and dehydrating agents before drying caused alterations in the protein matrix and the liberation of starch granules from the matrix. The method of specimen preparation profoundly influenced bread structure.

Scanning electron microscopy (SEM) has been used increasingly in recent years in the study of food products. An important advantage of SEM is the relatively simple methods required for sample preparation. Dry materials such as flour need no treatment before they are mounted on a stub and coated with a conductive layer of metal, but materials containing water must be dried before mounting and coating. Freeze drying is a popular method, but others have been used. Varriano-Marston (1977) compared different dough preparation procedures for SEM. Bechtel et al (1978), Burhans and Clapp (1942), Christianson et al (1974), Fleming and Sosulski (1978), Khoo et al (1975), Pomeranz et al (1977), and Sandstedt et al (1958) described the structure of bread but the influence of microscopy preparation techniques on bread structure has not been studied.

Our purpose was to compare various sample preparation procedures for SEM analysis of bread. We evaluated several commonly used methods of drying and of fixation followed by chemical dehydration and compared their effects on the ultrastructure of white bread.

MATERIALS AND METHODS

A commercial white bread prepared by a continuous mix process was used. The bread's moisture content was 40%, determined by drying at 100°C to constant weight. The central regions of slices from the interior of a fresh loaf were cut into uniform cubes (1 cm^3).

In the first series of experiments, the cubes were dried under one of the following conditions: 1) air dried for 24 hr, 2) oven dried at 80°C for 24 hr, 3) frozen by being placed on a freeze dryer shelf (−60°C) and freeze dried for 24 hr, or 4) frozen by immersion in liquid nitrogen (−196°C) and freeze dried for 24 hr. The condenser and shelf temperatures were maintained at −60 to −70°C throughout the freeze-drying process.

In the second series of experiments, bread cubes were treated with buffer or aqueous fixatives before dehydration by chemical or physical procedures. The bread was cut into smaller cubes (approx. 2 × 12 × 12 mm) to achieve penetration of the fixatives. Three procedures were used:

1. Fixation in 5% glutaraldehyde in 0.1 *M* phosphate (pH 7.2) or 0.1 *M* cacodylate buffer (pH 7.1) for 1 hr at room temperature, three rinses in the buffer, postfixation in 1% buffered osmium tetroxide (OsO_4) for 1 hr, and a rinse in water. Specimens were freeze dried directly, air dried at room temperature, or dehydrated through an ethanol series (30, 50, 70, 80, 95, 100, 100%; 15 min each) and freeze dried after freezing in liquid N_2.
2. Fixation in buffered 1% OsO_4 only, a rinse in deionized water, frozen in liquid N_2 and freeze dried or dehydrated through an ethanol series, frozen in liquid N_2, and freeze dried.
3. No fixation, soaking in 0.1 *M* phosphate buffer for 1 hr at room temperature, frozen in liquid N_2, and freeze dried.

After drying, samples were broken into pieces to expose the interior regions. Pieces were mounted with silver paint or double sticky tape randomly on stubs. Stubs were placed in a direct current diode sputtering device and coated with gold/palladium in short bursts for a total of 2–3 min to avoid sample heating. Materials were examined with an AMR-1000 at 5, 10, or 20 kV. Most micrographs were taken at 10 kV.

RESULTS

The method of sample preparation profoundly affected the ultrastructural features of bread. Samples dried directly without fixation and those treated with buffer or fixatives before drying differed the most.

After air, oven, or freeze drying, the general morphology was similar to that reported by Sandstedt et al (1958) and others (Bechtel et al 1978, Burhans and Clapp 1942, Christianson et al 1974, Fleming and Sosulski 1978, Khoo et al 1975, Pomeranz et al 1977). Air cells of variable size were entrapped in a continuous protein matrix. Air cell walls were as thin as 20 μm (Fig. 1). Starch granules were embedded in the matrix but in most cases were disguised by the protein covering them (Figs. 1, 2, and 3a). Small holes were common in the protein layer covering the granules (Figs. 2 and 3).

In general, it was difficult to distinguish among specimens that were air, oven, or freeze dried (Fig. 2). Furthermore, freezing

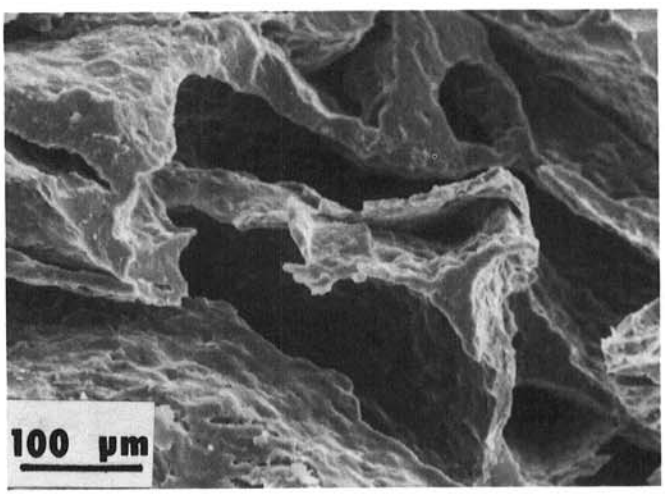

Fig. 1. Cross section of air cells in unfixed freeze-dried bread.

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temperature before freeze drying had no effect on ultrastructure. Varriano-Marston (1977) found that air drying caused more structural distortions in doughs than freeze drying. We did not observe this effect in bread.

Bread samples that were exposed to aqueous solutions before dehydration had a very different appearance (Fig. 3). The buffer treatment caused the protein to separate from the starch granules and to become somewhat disrupted (Fig. 3b). Crozet (1977) and Varriano-Marston (1977) suggested that the disruption or disappearance of the continuous protein film was related to chemical fixatives. Our results indicate that buffers without chemical fixatives can produce the same effect. Large and small granules were evident and intact but had the typical folded and deformed shapes characteristic of those gelatinized in a limited water system (Derby et al 1975; Hoseney et al 1977, 1978).

Fixation with glutaraldehyde and OsO_4 followed by freeze drying without ethanol dehydration resulted in a different image (Fig. 4). Starch granules were loosened from the protein, as were the buffer-treated freeze-dried specimens (Fig. 3b); however, fine strands were present between the small round granules. The protein matrix appeared more filamentous especially over the starch granules. If the fixed specimens were dehydrated with ethanol before freeze drying, the morphology of the fixed bread was altered (Fig. 4b). The structure was not as disrupted in the ethanol-dehydrated samples, and the fine filamentous protein network was not as prominent. Ethanol caused a general compacting of the structure so that it appeared more dense. Specimens that were subjected to critical point drying after fixation did not differ morphologically from those that were dehydrated with ethanol and freeze dried.

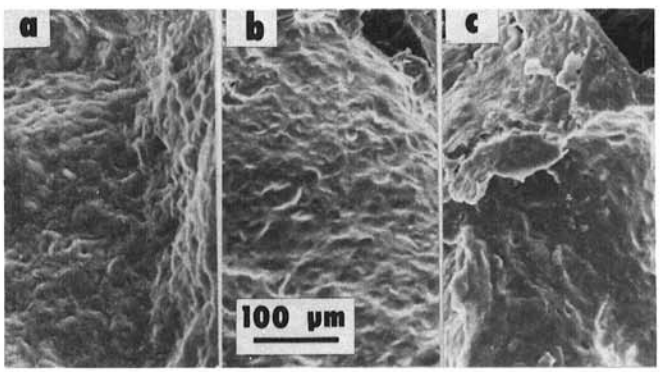

Fig. 2. Effect of drying method on the structure of unfixed bread: **a**, air dried, **b**, oven dried, and **c**, freeze dried.

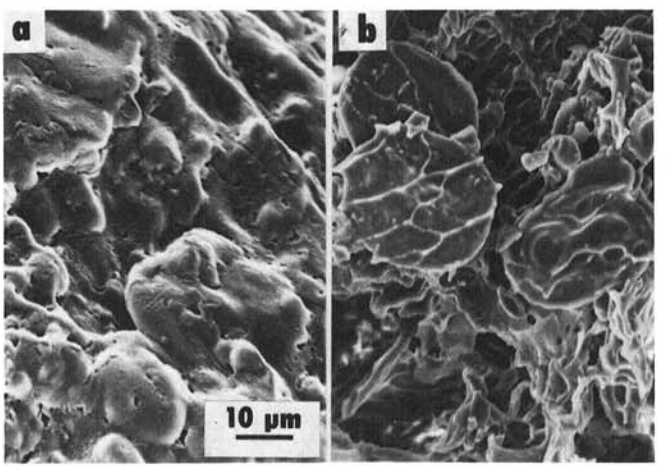

Fig. 3. Effect of hydration in buffer before freeze drying unfixed bread: **a**, unhydrated; **b**, hydrated.

Fixation with only OsO_4 (without glutaraldehyde) and subsequent freeze drying resulted in slightly different morphology (Fig. 5). The protein matrix appeared to be more disrupted when glutaraldehyde was omitted as a fixative. This difference was especially evident in specimens dehydrated through an ethanol series (Fig. 5b). Fixation with OsO_4 alone also resulted in greater liberation of starch granules from the matrix. Glutaraldehyde and OsO_4, and OsO_4 alone, seemed not to influence the size or shape of the starch granules, but starch granules that were freeze dried after buffer treatment (Fig. 3b) had more irregular and folded surfaces than those fixed before drying (Figs. 4 and 5). Apparently chemical fixation affects the protein matrix and also influences starch granule morphology.

DISCUSSION

Light, transmission, and scanning electron microscopy have been used to study the changes that occur when flour is hydrated, mixed into a dough, and baked. Bechtel et al (1978), Khoo et al (1975), and Sandstedt et al (1958) showed that preparation of the

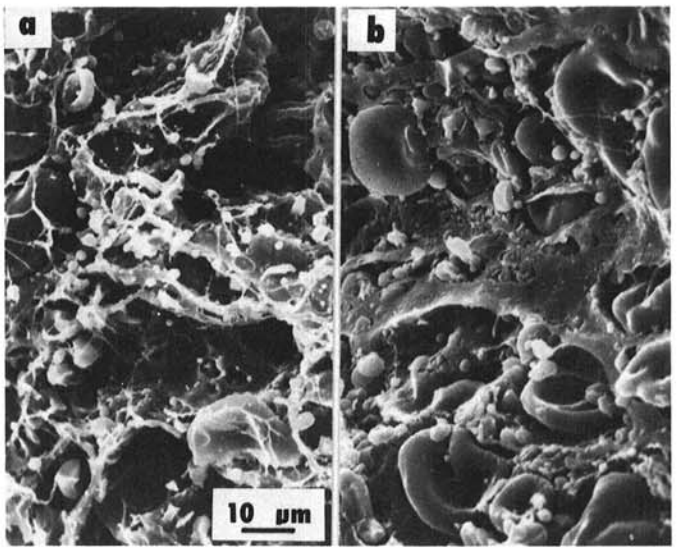

Fig. 4. Effect of ethanol dehydration on freeze-dried, glutaraldehyde, and osmium tetroxide-fixed bread: **a**, without ethanol; **b**, with ethanol.

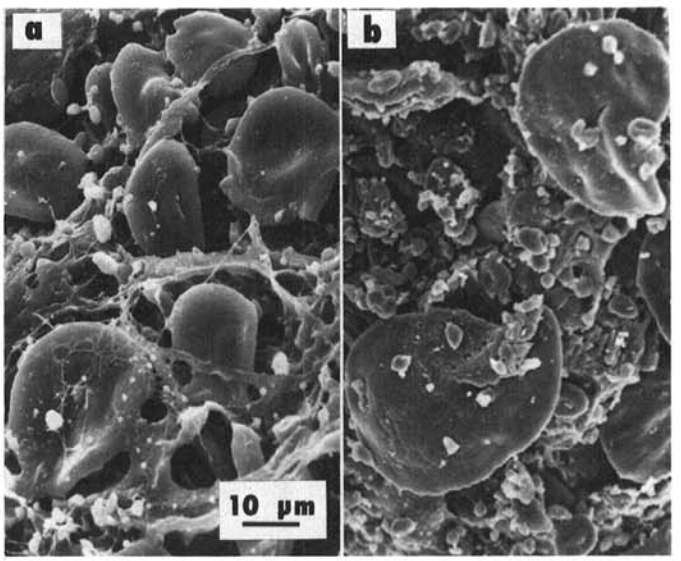

Fig. 5. Effect of ethanol dehydration on freeze-dried, osmium tetroxide-fixed bread: **a**, without ethanol; **b**, with ethanol.

dough and mixing result in formation of a continuous gluten network in which starch granules are embedded. During mixing, starch-protein masses are stretched into sheets. Heating during baking causes an expansion of gas cells and further stretching and thinning of the sheets. Starch granules are enrobed by protein. The protein covering is so complete that it prevents iodine staining of starch granules (Sandstedt et al 1958).During baking, starch granules gelatinize and flexibly fit around the air cells. The granules remain intact and identifiable, however, because limited water is available during gelatinization.

We have shown that freeze-dried white bread has a veil of protein over the starch granules and that the walls of the gas cells are very thin. Small droplets near the granules may be lipid. Crozet and Guilbot (1974) demonstrated that fixation of wheat flour with OsO_4 induces aggregation of some lipid components into spheres up to 0.5 μm in diameter.

Water washes starch out of baked goods, so it is not surprising that aqueous fixatives cause separation of starch and protein. Sandstedt et al (1958) suggested that, under conditions of limited water such as during baking, a strong bond is formed between starch and protein. Fixatives may alter these bonds and result in release of starch granules.

Evans et al (1977) fixed dough samples in buffered glutaraldehyde for 24 hr followed by dehydration in alcohol and critical point drying. They found ruptures in the gluten sheet at starch/protein interfaces that were thought to have occurred during mixing. A space separating the granules and protein was also noted. We have not observed any differences in ultrastructure between bread specimens dehydrated with alcohol and freeze dried and those that were critical point dried; but fixatives do alter the protein matrix in bread.

Fixation and dehydration procedures such as those used for transmission electron microscopy clearly cause profound changes in the ultrastructure of bread. Nevertheless, useful information can be derived from applying these techniques. Fixation and dehydration remove the protein veil from the starch and therefore permit evaluation of starch granule morphology in situ.

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