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SEM Sample Preparation Techniques for Developed and Undeveloped Wheat Doughs

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Abstract

The purpose of this study was to examine five different scanning electron microscopy (SEM) sample preparation methods, and determine which one gave the best visual contrast between starch and protein in undeveloped and developed soft wheat doughs. Samples subjected to freeze drying, vacuum desiccation, and cryo preparation had fewer artifacts than those chemically dehydrated with ethanol. Chemical dehydration caused the protein to compact, producing better contrast. Overall, ethanol dehydration without prior chemical fixation, followed by critical point drying with carbon dioxide, produced the best visual contrast between starch and protein in wheat dough samples.

Key words: scanning electron microscopy, sample preparations, wheat dough

Introduction

Scanning electron microscopy (SEM) is an excellent vehicle for examining the starch and gluten network in wheat dough. Several researchers have used SEM to observe the ultrastructure of bread and bread dough (Aranyi and Hawrylewicz, 1968, 1969; Berglund *et al.*, 1990, 1991; Chabot *et al.*, 1979; Evans *et al.*, 1977, 1981; Khoo *et al.*, 1975; Parades-Lopez and Bushuk, 1982; Pomeranz *et al.*, 1984; Pomeranz and Meyer, 1984; Variano-Marston, 1977). Others have observed the ultrastructure of wheat gluten (Cumming and Tung, 1975; Freeman *et al.*, 1991), wheat flour tortillas (McDonough *et al.*, 1996), and bagels (Umbach *et al.*, 1990). Berglund *et al.* (1990) reported that freeze drying or chemical fixation and dehydration are the most common sample preparation techniques, but these methods may produce artifacts, mask surface detail or cause alterations of the microstructure.

Variano-Marston (1977) explored many SEM preparation techniques for wheat dough specimens including four dehydration procedures: freeze drying at –65°C for 48 hours, vacuum desiccation at room temperature for 24 hours, air drying at room temperature for 24 hours, and acetone dehydration followed by critical point drying. Findings showed freeze drying (frozen dough state) and vacuum desiccation (frozen and unfrozen dough state) gave the best resolution and depth of field. Air drying and chemical fixation, followed by critical point drying produced poor results with wheat dough. Later work by Chabot *et al.* (1979), however, did not conclude that air drying caused more structural distortions than freeze drying in bread samples. Aranyi and Hawrylewicz (1968) used vacuum desiccation for wheat dough specimens and described the observed image as a veil-like network of protein covering an even distribution of starch. Evans *et al.* (1981) used freeze drying to observe optimally developed dough and characterized the dough as having a continuous and strong adhering gluten layer covering the starch granules.

Researchers found that images of dough and bread samples exposed to chemical fixatives did not have a continuous protein covering over the starch granules (Aranyi and Hawrylewicz, 1969), and observed severe

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rupturing in gluten sheets at the starch-protein interface (Evans *et al.*, 1977). Chabot *et al.* (1979) concluded that ethanol dehydration before drying altered specimens by compacting the structure causing it to appear dense. Cumming and Tung (1975) also concluded that fixation and dehydration removed the veil-like protein from the starch, which permitted evaluation of starch morphology. It was suggested by Variano-Marston (1977) that chemical fixatives caused discontinuities in the protein film.

Berglund *et al.* (1990) compared two cryogenic preparation techniques for low temperature SEM on frozen bread dough. Bread dough was frozen at −23°C and sampled using two methods: 1) samples were placed on specimen holders at 22°C to allow partial thawing, and 2) samples were kept frozen with dry ice during mounting on specimen holders. This study showed that thawed specimens produced patterns due to recrystalization of water: samples kept frozen during mounting did not exhibit these patterns. Berglund *et al.* (1990) also found that smaller samples had a greater tendency to thaw during mounting, causing recrystalization upon refreezing in the nitrogen slush.

Extensive research on SEM sample preparation techniques has produced varied results. A poor technique can cause artifacts and produce variable results between images of the same sample. Objectives for the current work were to study five different SEM sample preparation techniques to: 1) Determine which technique minimized sample destruction and artifacts, and maintained the integrity of the starch and gluten matrix in developed and undeveloped dough samples; and 2) Determine which technique produced the best visual contrast between the starch and protein in wheat dough samples. The effect of sample fracturing was also studied.

Materials & Methods

Developed Dough

Developed soft white wheat dough was prepared in a Farinograph following the approved AACC Method 54-21 (AACC, 1995). Fifty grams of flour were placed into the 50g mixing bowl of a Brabender Farinograph (C.W. Brabender Instruments Inc., South Hackensack, New Jersey, USA). The following Farinograph parameters were measured for the flour used in this study: water absorption (50.0 % wt. basis), arrival time (0.25 min), development time (0.50 min), stability time (1.75 min), departure time (2.00 min), and mixing tolerance index (160 BU).

Undeveloped Dough

Undeveloped dough (which can be used to study the mechanical factors involved in protein development during dough manufacturing) was prepared according to Campos *et al.* (1996) and Schluentz *et al.* (2000). The powder mixture was stored in a −10°C freezer. Samples, when required, were thawed for approximately 3 hours in a moisture proof film at room temperature.

Chemical Fixation & Ethanol Dehydration

Chemical fixation crosslinks proteins in biological samples while ethanol dehydration, followed by critical point drying with CO_2, allows for complete water removal. Developed and undeveloped dough samples, approximately 4 mm^3, were cut from frozen samples. Dough was fixed by submerging it in 4% glutaraldehyde, buffered with 0.1M phosphate buffer (pH 7.4), for 30 minutes at room temperature. Fixed samples were rinsed with phosphate buffer for 10-15 minutes. After buffering, samples were submerged in a graded ethanol series (25%, 50%, 75%, 95%) for 20 minutes at each gradation; then submerged in 100% ethanol for three consecutive 20 minute intervals to ensure full dehydration. Since CO_2 is miscible with ethanol, dough samples were critical point dried using a Balzers Critical Point Dryer (Balzers Union, FL-9496, Furstentum, Liechtenstein). Critical point drying allows ethanol removal in CO_2 without surface tension forces, which may distort the sample.

Ethanol Dehydration Without Chemical Fixation

Previous research efforts included chemical fixation prior to ethanol dehydration. In this research, the same preparation technique mentioned above, without chemical fixation in 4% glutaraldehyde, was followed. No published record of this technique could be found.

Vacuum Desiccation

The sample was allowed to dry in a vacuum desiccator. Small samples (5-7 mm^3), were placed on parafilm in a desiccator containing Drierite (anhydrous calcium sulfate) desiccant, and dehydrated for 24 hours at room temperature.

Freeze Drying

Samples were placed in scintillator vials, previously chilled in an ethanol and dry ice slush at approximately –70°C, for transfer to the freeze dryer (Labconco Corporation, Kansas City, MS). Once the dryer reached –40°C and 12×10^{-3} mbar, samples were freeze dried for 24 hours. After drying, the vials were capped, wrapped in parafilm and placed in a freezer (Forma Scientific Freezer, Marietta, OH) maintained at -86°C for storage.

SEM

Following each preparation technique mentioned above, dehydrated samples were mounted with epoxy resin on standard aluminum stubs. After mounting, samples were sputter coated with gold particles (EMSCOPE SC500, T55-29173, Ashford, Kent) at 20 mA for 4 minutes. Images were created with a JEOL JSM-6400V scanning electron microscope (JEOL, Tokyo, Japan) using an accelerating voltage of 13 kV, 15 mm working distance, and a condenser lens setting of 10. Images, magnified at 2000X, were saved as tagged image files (TIF) on 3.25 inch floppy disks.

Cryo-SEM

Dough samples were mounted on a special cryo stub with Tissue-Tek II O.C.T. (Lab-Tek Products, Naperville, IL) compound. The sample and holder were submerged in a liquid nitrogen slushing chamber and transferred to the JEOL JSM-35CF Cryo-SEM chamber (JEOL, Tokyo, Japan) maintained at approximately –100°C. Etching heated the SEM chamber to -65°C, allowing the water in the sample to sublime over a period of 20-30 minutes. After etching, the sample was transferred back to the EMSCOPE SP2000 (T8-84442, Ashford, Kent) working chamber for sputter coating with gold particles. Samples were viewed in the SEM stage at –90°C since thermal stress caused the sample to crack at –140°C.

Dough Fracturation

Fracturing was accomplished by cutting with a razor blade or shattering a sample with sharp pointed tweezers to expose inner surfaces, and mounting a fractured piece on an aluminum stub for metallic coating.

Results & Discussion

The purpose of testing different specimen preparation methods was to determine which technique gave the best visual distinction between protein (stringy matrix) and starch (discrete granules). The most common prepara-

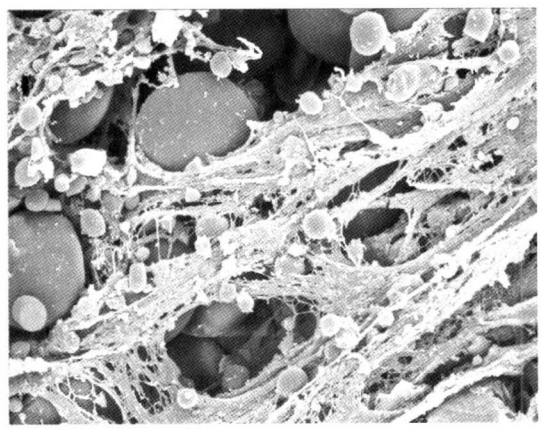

Fig. 1. Developed soft wheat dough not fixed with glutaraldhyde followed by ethanol dehydration and critical point drying with CO_2. (2000X).

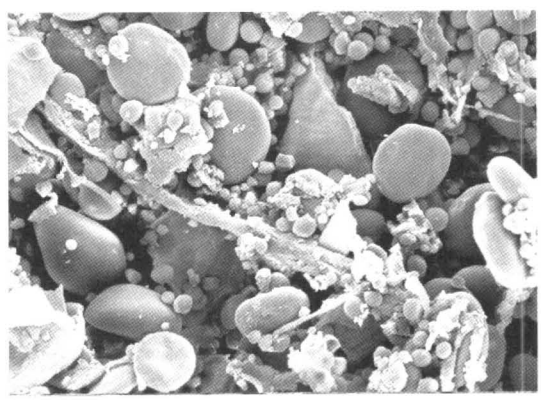

Fig. 2. Developed soft wheat dough chemically fixed with 4% glutaraldhyde followed by ethanol dehydration and critical point drying with CO_2. (2000X).

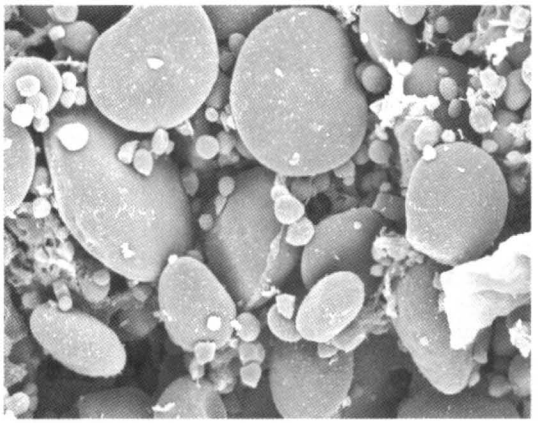

Fig. 3. Undeveloped soft wheat dough not fixed with glutaraldhyde followed by ethanol dehydration and critical point drying with CO_2. (2000X)

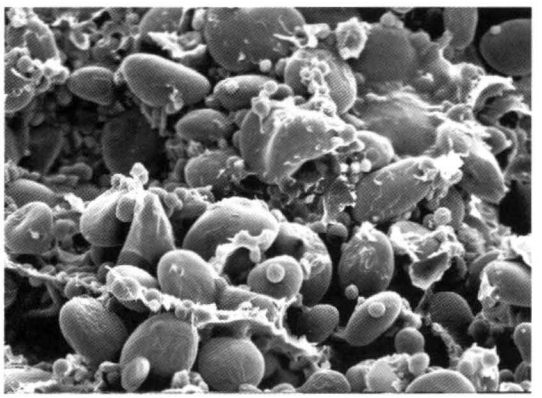

Fig. 4. Developed freeze-dried wheat dough. (2000X).

tion method for biological samples is chemical fixation and dehydration, followed by critical point drying. In the present studies, at least 10 images of each technique were examined, and figures illustrate typical examples. Micrographs of developed dough not fixed, or fixed with 4% buffered glutaraldehyde, followed by ethanol dehydration and critical point drying with CO_2, are shown in Fig. 1 and Fig. 2, respectively. Fig. 1 shows protein continuity and definite contrast between starch and protein. The continuous protein is not evident in Fig. 2, and the contrast between starch and protein is low. Undeveloped dough (Fig. 3) chemically prepared without fixation illustrates a homogenous, hydrated dough that had formed in the absence of mechanical energy input.

It was difficult to distinguish between developed freeze-dried dough samples and undeveloped freeze-dried dough samples, Fig. 4 and 5, respectively. Freeze

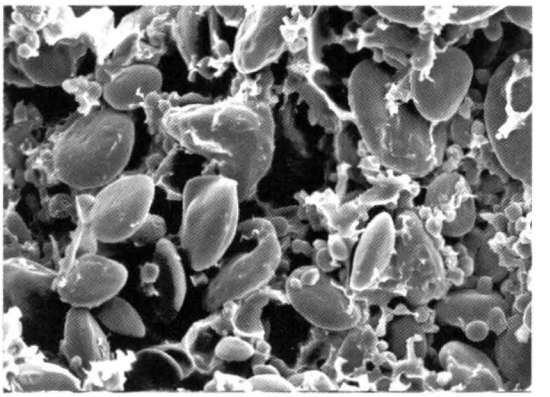

Fig. 5. Undeveloped freeze-dried wheat dough. (2000X).

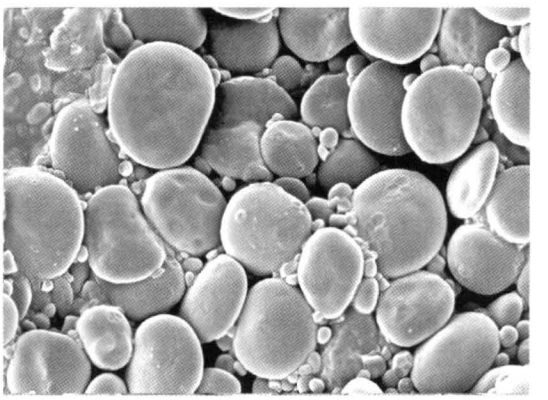

Fig. 6. Developed dough prepared by vacuum desiccation for 24 hours. (2000X).

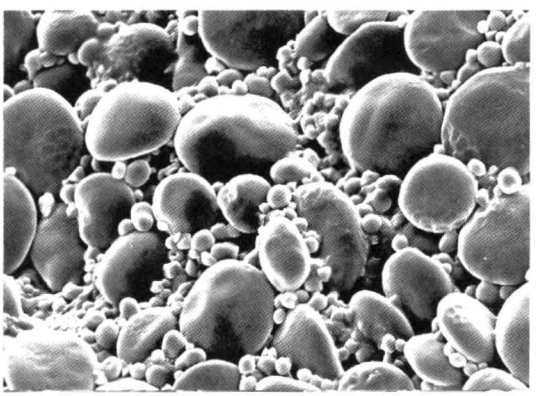

Fig. 7. Undeveloped dough prepared by vacuum desiccation for 24 hours. (2000X).

drying allows a direct change from solid to vapor (sublimation) to occur in the absence of surface tension; however, ice crystal formation may damage specimen morphology.

Fig. 6 shows a developed dough sample dehydrated in a vacuum desiccator for 24 hours, and Fig. 7 is an undeveloped dough sample prepared in the same manner. Protein envelopes the starch particles, covering the starch like a veil making it difficult to visually distinguish starch and protein based on contrast. Furthermore, samples were not fully dehydrated which caused a low vacuum in the SEM, resulting in grainy image texture that lacked good clarity.

It was difficult to distinguish protein and starch based on contrast for both developed and undeveloped samples subjected to cryo preparation (data not shown). Also, microscopic images produced by this time-consuming method could only be obtained with a Polaroid camera and saved as an analog image on a negative, making future numerical digital image analysis difficult.

The effect of sample fracturing to expose the inner surfaces was also studied. Fracturing was done by shattering a dough sample with sharply pointed tweezers, or by cutting the dough sample with a razor blade. The tweezer method distorted the proteinaceous material surrounding the starch, disconnecting the protein network. It appears that the force of fracturing, causes the sample to shatter. This trend was found in both developed and undeveloped dough samples subjected to chemical fixation with glutaraldehyde, freeze drying, vacuum desiccation, and cryogenic preparation techniques. Slicing samples with a razor blade at freezing temperatures also produced unacceptable results because the samples appeared sliced and lacked contrast.

The contrast in SEM images created by chemical dehydration and critical point drying (Fig. 1 and 2) produced the best visual distinction between starch and protein in wheat dough samples. Dough preparation was the least time consuming for freeze drying and vacuum desiccation methods; however, visual distinction between developed and undeveloped dough was difficult. Furthermore, vacuum desiccated samples were not fully dehydrated and produced grainy microscopic images. Cryogenic preparation is advantageous because dough samples were observed as water sublimes; however, this method is the most laborious and only produced analog images.

Our experience with computer-based numerical image analysis shows a strong correlation between visual contrast and our ability to make a quantitative distinction (by examining pixel gray levels) among image elements. Future efforts will involve extensive numerical analysis of SEM image contrast to study the role of deformation type (extensional or shear) and strain level on the development of protein structure in wheat dough.

Conclusion

Five sample preparation methods -- involving fixation, chemical dehydration, vacuum desication, freeze drying and cryogenic methods -- were evaluated for undeveloped and developed wheat dough samples. The best visual contrast between starch and protein was obtained when samples without prior chemical fixation were dehydrated in a graded ethanol series and critical point dried with carbon dioxide before sputter coating with evaporated gold particles.

Acknowledgements

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