The Ultrastructure of Wheat Gluten: Variations Related to Sample Preparation

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ABSTRACT

Fresh and heated gluten samples were prepared for scanning electron microscopy using a variety of preparation techniques. The ultrastructural characteristics of the wheat glutens were directly correlated with preparation procedures. Dehydration of gluten samples can result in major structural artifacts. Hydrated samples were prepared using a "cryopreparation" technique in which just the surface layer was sublimed. This method greatly reduced the gross artifacts of dehydration, and the hydrated samples showed a three-dimensional spongelike structure similar to that observed in fresh gluten examined with light microscopy.

Electron microscopy is a valuable tool for the study of food products. Ultrastructural studies provide data not available with other techniques. These data are essential to a complete understanding of the changes that occur during food processing. Ultrastructural studies of glutens and doughs are especially difficult because many standard microscopic techniques produce artifacts.

Very little is known about the ultrastructure of wheat gluten. Much of our present knowledge is based on transmission electron microscopic studies of freeze-fractured or freeze-etched specimens that provide only limited information regarding three-dimensional structure. Gluten has been described as a dense protein matrix with no indication of fibrils or protein strands at viewing magnifications as high as 200,000× (Hermansson and Larsson 1986). Fretzdorff et al (1982) described the freeze-fracture gluten surfaces as being a sheetlike matrix. Marion et al (1987), however, found the protein phase of freeze-fractured gluten replicas to exhibit either a fibrillar or granular structure. Using scanning electron microscopy, Cumming and Tung (1975) found the protein matrix to be fibrillar in stressed, defatted gluten, whereas in unstressed gluten the protein matrix was granular or discontinuous. The tendency to form a fibrous network was directly related to the presence of starch.

Many of the reported differences in the structure of gluten and doughs are related to sample preparation. Varriano-Marston (1977) investigated a variety of fixation procedures for doughs and reported that both frozen and nonfrozen samples fixed in solutions of glutaraldehyde and osmium tetroxide, dehydrated in acetone, and critical-point dried, showed structural distortion. Chabot (1981) also reported significant changes in both the protein and starch components of doughs as a result of chemical fixation and dehydration. Hermannson and Buchheim (1981) found no indication that chemical fixation, dehydration, or embedding caused structural artifacts in soy protein gels. They suggested that artifacts could result from improper freezing or critical-point drying. It is obvious that fixation procedures affect ultrastructure, and additional research is required to determine the most suitable techniques, especially for glutens.

This study was undertaken to determine the most artifact-free technique for preparing washed wheat gluten for scanning electron microscopy. The structure of fresh, unfixed gluten observed with light microscopy was compared with gluten ultrastructure following a variety of fixation procedures. Understanding the influence of preparation techniques on the ultrastructure of washed gluten, both fresh and heated, permitted a more accurate interpretation of gluten in complex systems.

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MATERIALS AND METHODS

Gluten was separated from a hard red spring wheat flour using a Glutomatic 2200 system (Falling Number AB, Stockholm, Sweden). Following the final buffer wash, portions of the wet gluten ("fresh gluten") were prepared for light and scanning electron microscopy using the procedures (1-6) outlined in Figure 1. All procedures were replicated at least six times, and the "cryoprocedure" was replicated a minimum of 30 times.

Fresh gluten samples were examined with Nomarski differential interference contrast using a Zeiss laser scanning light microscope equipped with a helium-neon laser (λ = 633 nm) and a 40× Plan Apo oil immersion objective lens. Images were recorded with a Sony UP500 Mavigraph video printer on Sony print paper. Thin slices of gluten were placed in a drop of buffer on a glass slide and compressed by hand with a cover glass to provide a planar viewing surface. This technique allowed us to examine the gluten samples without freezing, fixation, or any form of dehydration, and will be referred to as procedure 1.

All cryoprepared samples were 1 × 1 × 4 mm or smaller and were carefully mounted (to avoid stretching) on specimen stubs using a combination of O.C.T. compound (Ames Co., Elkhart, IN) and Aquadag R (Tousimis, Rockville, MD). The samples had time to relax before being frozen in slushed liquid nitrogen and fractured with a precooled knife in an EMscope SP2000A cryo unit. The frozen, fractured samples were then transferred under vacuum to a cold stage (−170°C) in the JEOL JSM-35 scanning electron microscope. Samples were sublimed on the microscope stage, at 1 × 10^{-6} torr, for 9 min at −70°C. The optimum time and temperature of sublimation were determined in an earlier experiment.

Fresh, unfixed gluten samples 1 × 1 × 4 mm were prepared using the Glutmatic 2200. These samples were frozen in slushed liquid nitrogen, fractured, sublimed, and examined using the cryopreparation technique (procedure 2).

Heated gluten was prepared by sealing fresh gluten (1.2 g) in a metal container that was then placed in boiling water for 3 min, according to the procedure described by Ilievska-Chakraborty (1988). The samples were subdivided into 1 × 1 × 4 mm pieces and processed using procedure 2.

Fresh gluten samples 1 × 1 × 4 mm were also attached to the specimen holder and frozen in the freezer compartment of the laboratory refrigerator. After 8 hr at −18°C, these samples were processed according to procedure 2.

Fresh gluten samples were freeze-dried at −60°C for 72 hr (procedure 3) in a laboratory freeze-drier (Virtis Co., Gardiner, NY) and fractured at room temperature before being mounted on stubs and examined.

Fresh gluten samples were processed in an acidified 2,2-dimethoxypropane (DMP) according to the procedure described by Bjerke et al (1979). After dehydration with DMP, the samples were sealed in Parafilm bags filled with absolute ethanol. The samples were then frozen in liquid nitrogen and fractured with a precooled tool. Half of the samples were critical-point dried

using CO_2 as a transitional fluid (procedure 4A), and half were dried using Peldri II (procedure 4B). Peldri II is a proprietary fluorocarbon available from Ted Peila, Inc. (Tustin, CA) and is used as an alternative to critical-point drying. Gluten samples were also prepared using standard fixation techniques including 2.5% glutaraldelhyde (pH 7.4) with postfixation in 2% osmium tetroxide (pH 7.4). Following fixation, half of each sample was chemically dehydrated with DMP (procedure 4) or in ethanol (procedure 5). All samples were then sealed in Parafilm bags containing absolute ethanol, frozen, and fractured in liquid nitrogen. Half of each sample was subsequently critical-point dried (procedures 4A and 5A); the other half was dried with Peldri II (procedures 4B and 5B). Glutens were also fixed in 50% unbuffered glutaraldehyde and processed using procedure 6. The high concentration of glutaraldehyde ensured that there was adequate fixative available for the volume of protein in gluten.

All of the samples were sputter-coated with gold, examined, and photographed using a JEOL JSM-35 scanning electron microscope.

RESULTS

The fresh gluten samples (unfixed and unfrozen) examined with a Zeiss LSM laser scanning light microscope with a differential interference contrast objective lens (procedure 1) revealed gluten with a reticulate pattern (Figs. 2 and 3). This reticulate pattern was most obvious when a series of photographs taken at different depths of focus through the same area was examined. Starch granules were found in open areas surrounded by water. During preparation, portions of some fresh gluten samples were stretched, which resulted in the formation of large protein strands with unidirectional orientation (Fig. 4).

Sublimation for 9 min at −70°C on the cold stage of the scanning electron microscope completely removed the surface ice from the cryoprepared gluten sample. No change in the structural characteristics of the gluten was observed during the sublimation process, indicating that the removal of water from the frozen specimen did not alter its structure. Cryoprepared gluten samples (procedure 2) appeared similar in structure to unfixed, unfrozen gluten observed with light microscopy. At low magnification, the cryoprocessed gluten was spongelike in structure (Fig. 5), with the reticulate network being clearly evident at higher magnifications (Figs. 6–8). The reticulate appearance was directly attributable to the numerous holes within the protein phase. The sublimation process demonstrated that these holes were filled with water in the fresh gluten. Gluten is a complex, three-dimensional structure, with the solid portion representing the protein phase interspersed with holes of a variety of sizes and shapes. Starch granules that remained associated with the gluten following washing were generally associated with the larger holes (Figs. 7 and 8). Elongated protein strands were found only in the cryoprepared samples that were maintained in a stretched condition during the freezing and fracturing.

Gluten samples that were slowly frozen (−18°C for 8 hr) and subsequently processed using procedure 2 were noticeably different ultrastructurally from the rapidly frozen, cryoprepared samples. Slowly frozen samples were characterized by a continuous or solid protein phase and large, angular spaces (Fig. 9). Starch granules appeared to be covered by a layer of protein, and many were completely embedded in the protein matrix.

Freeze-dried samples (procedure 3) were very different ultrastructurally from the slowly frozen gluten, cryoprepared, and fresh samples. Freeze-drying resulted in gluten that had a sheeted appearance with few or no circular holes but rather numerous longitudinal tears or rips in the protein matrix (Fig. 10).

Fresh gluten samples chemically dehydrated with DMP and subsequently critical-point dried (procedure 4A) or dried with Peldri II (procedure 4B) were ultrastructurally different from the same samples processed using the cryotechnique. The protein component of the critical-point-dried and Peldri II-dried samples (Fig. 11) appeared continuous, with numerous very small holes interspersed throughout the protein matrix. Both the size and distribution of the holes were completely different from the holes characteristic of the fresh, unfrozen, unfixed (Figs. 2 and 3) or cryoprocessed sample (Figs. 6–8). Starch granules were apparently completely masked by the continuous protein phase.

Gluten fixed in glutaraldehyde, postfixed in osmium tetroxide, and then processed according to procedure 4A or 4B appeared continuous and similar to the unfixed samples dried using the same techniques except that there were few circular holes. The protein phase of the gluten was, however, disrupted by many elongated voids (Fig. 12) that appeared as tears or rips. Critical-

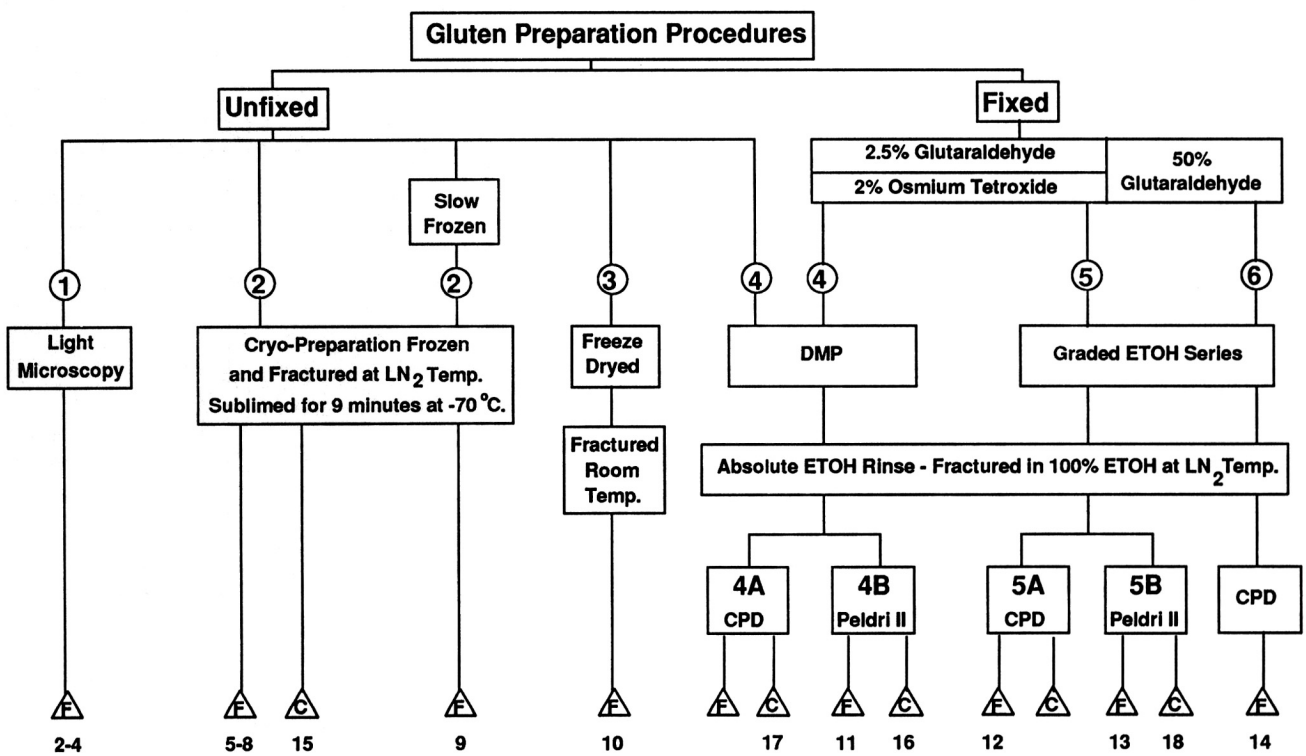
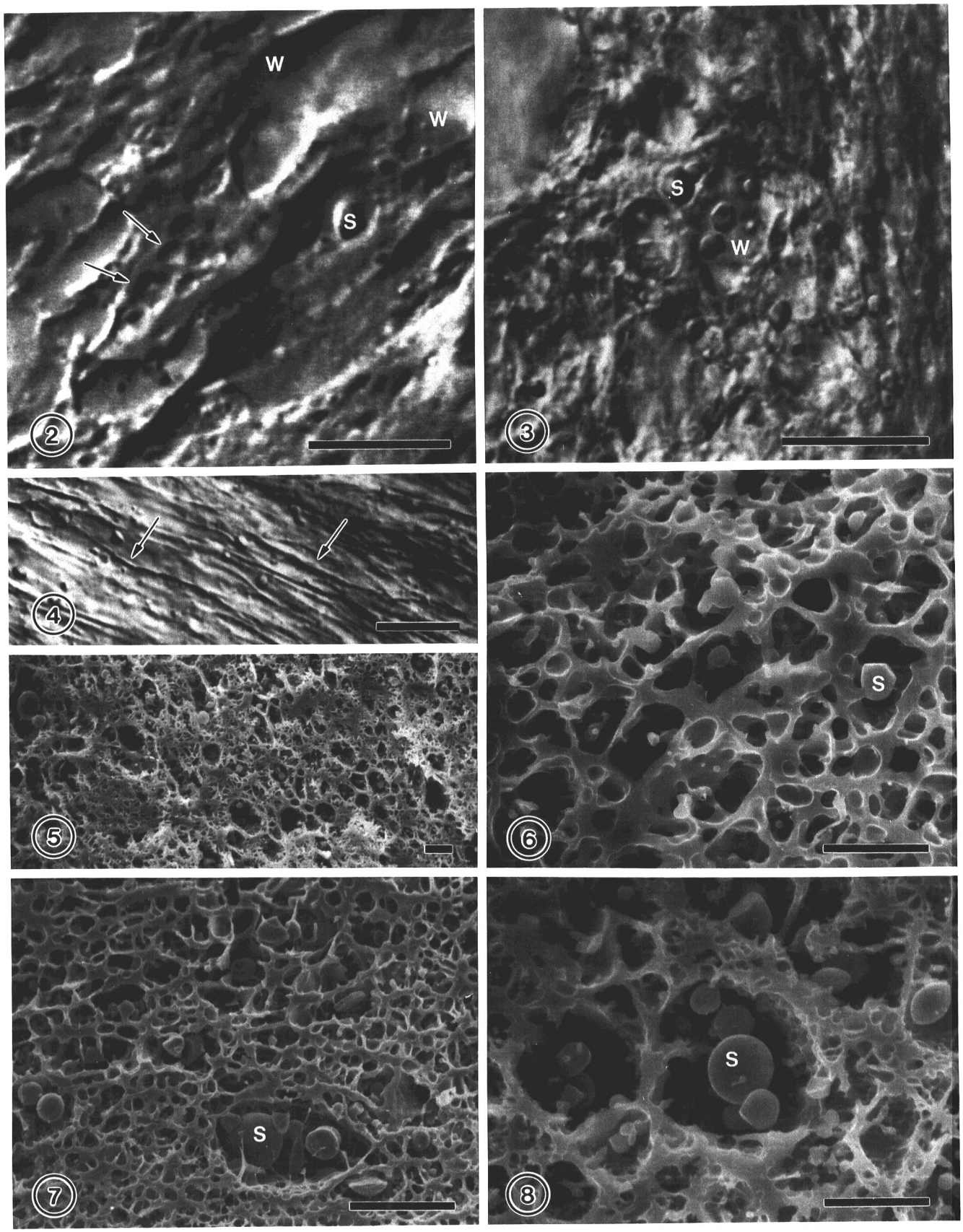
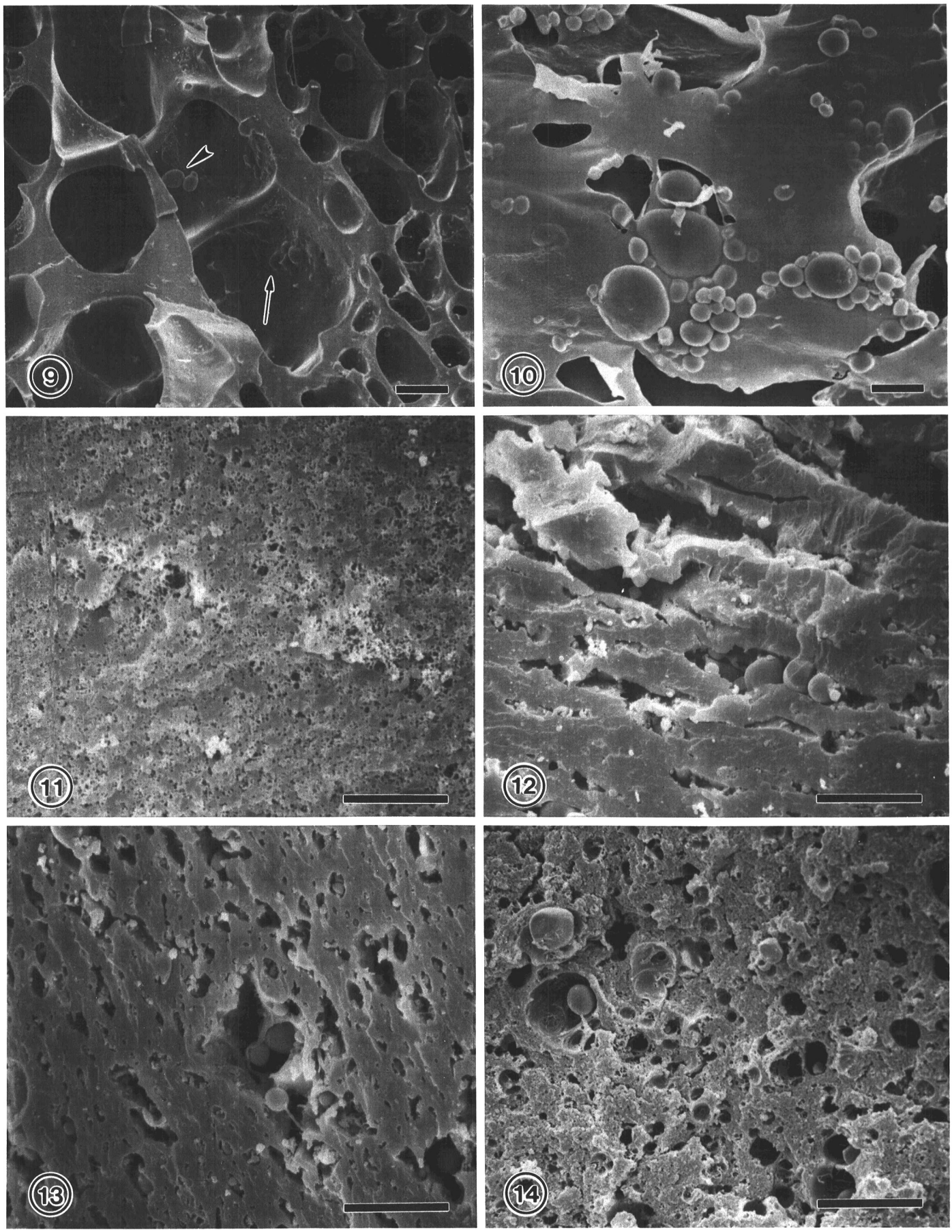


Fig. 1. Outline of procedures used in preparating washed gluten samples (triangles): F = fresh, C = heated.



Figs. 2–4. Light micrographs of fresh, unfixed, unfrozen gluten. **2,** Light micrograph showing reticulate nature of gluten. Note small circular holes in the protein matrix (arrows), starch granules (S), and pools of water (W). **3,** Light micrograph showing reticulate nature of gluten, starch (S), and pools of water (W). **4,** Light micrograph of gluten stretched during preparation showing protein strands (arrows). A better feeling of the three-dimensional characteristics shown in Figs. 2–4 might be obtained if the photographs are viewed at arm's length. Bars = 10 μm. **Figs. 5–8.** Scanning electron micrographs of cryoprepared gluten samples showing spongelike, three-dimensional, reticulate nature of gluten. Compare the general structure of the cryoprepared samples with the light micrographs (Figs. 2 and 3). Note especially the distribution of starch (S), the voids where water was present, and the similarity of the protein matrix. Bars = 10 μm.



Figs. 9–14. Gluten structure as influenced by sample preparation. **9,** Slowly frozen (procedure 2). Note apparently free starch granules (arrow head) and starch granules covered by a layer of gluten (arrow). **10,** Freeze-dried (procedure 3). **11,** 2,2-Dimethoxypropane (DMP) dehydrated, Peldri II dried (procedure 4B). **12,** Fixed in glutaraldehyde-osmium tetroxide, DMP dehydrated, critical-point dried (procedure 4A). **13,** Fixed in glutaraldehyde-osmium tetroxide, EtOH dehydrated, Peldri II dried (procedure 5B). **14,** Fixed in 50% glutaraldehyde, EtOH dehydrated, critical-point dried (procedure 6). Bars = 10 μm.

point-dried and Peldri II-dried samples appeared similar ultrastructurally. Starch granules were almost exclusively associated with the elongated spaces of the samples fixed in glutaraldehyde-osmium and dehydrated with DMP regardless of the final drying technique used.

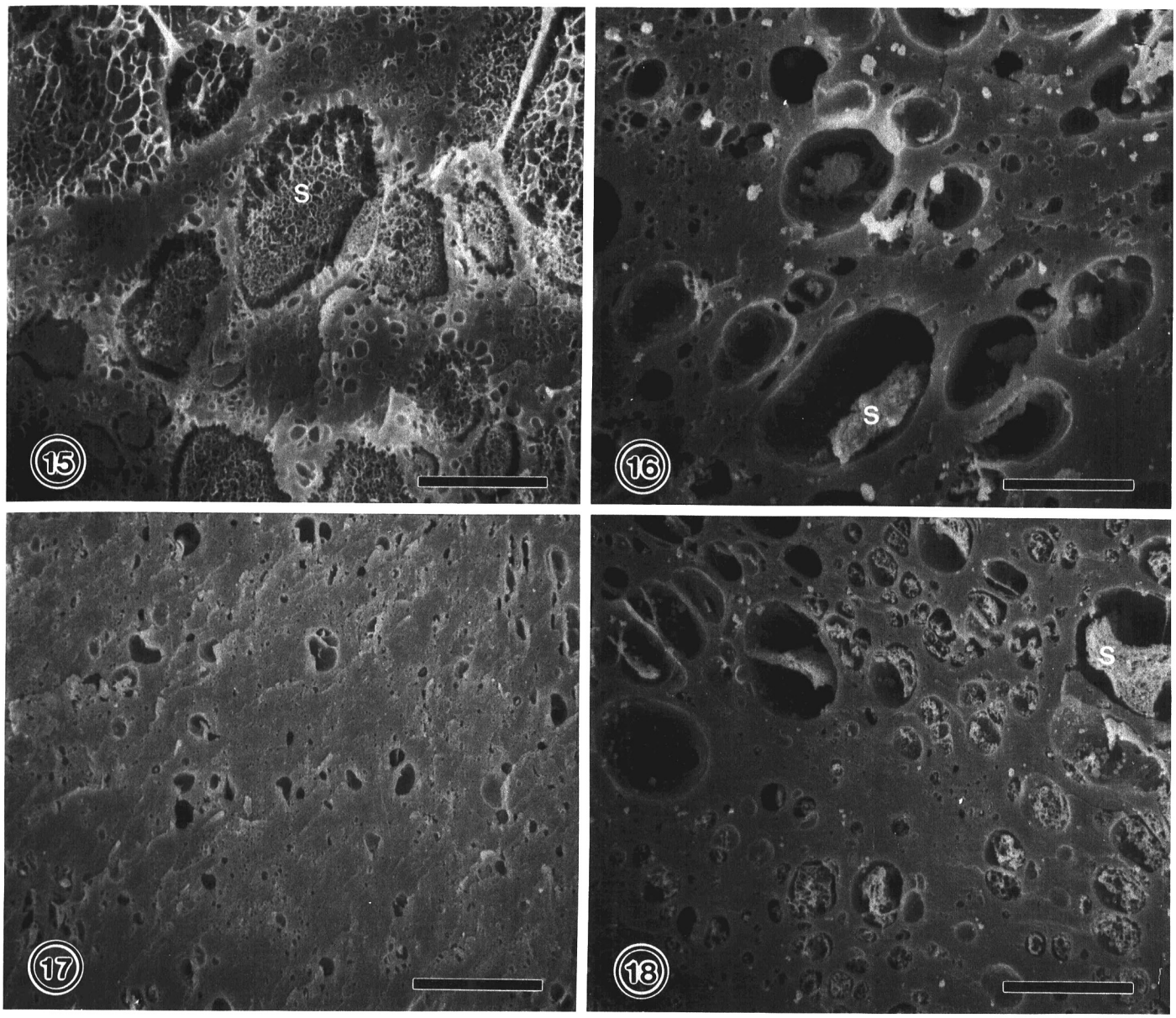
Gluten samples fixed in glutaraldehyde-osmium, dehydrated in ethanol, and either critical-point dried (procedure 5A) or dried with Peldri II (procedure 5B) also had a protein matrix that appeared relatively continuous, with numerous tears or fractures as well as more holes of various dimensions dispersed throughout the protein matrix (Fig. 13). Starch granules again were most commonly associated with the larger holes or pores. These samples were quite similar in appearance to the fixed samples that were chemically dehydrated in DMP.

Fixation in 50% unbuffered glutaraldehyde, followed by dehydration in a graded ethanol series, and critical-point drying (procedure 6), resulted in a granular and discontinuous protein phase (Fig. 14). Gluten prepared using this procedure appeared much more porous or open than samples fixed in glutaraldehyde-osmium. Starch granules were generally evident only in the large holes of the protein matrix. This gluten more closely resembled that of the cryoprepared or fresh, unfixed samples but still lacked the spongelike, three-dimensional characteristic.

Heated gluten samples processed using the cryopreparation technique (procedure 2) were distinctly different from the unheated samples processed using the same technique (Fig. 15). The reticulate pattern that was evident in the fresh, unfrozen, unfixed samples (Figs. 2 and 3) or the cryoprepared samples (Figs. 6–8) was lost in heated samples (Fig. 15). The protein phase of this heated gluten was somewhat similar to the chemically fixed samples. Large spaces were still evident and contained starch gelatinized by the heating process.

Heated gluten samples processed in DMP, fractured in absolute ethanol, and critical-point dried (procedure 4A), or dried with Peldri II (procedure 4B), were generally characterized by a continuous protein phase with numerous large holes containing gelatinized starch (Fig. 16) that apparently shrank during the dehydration process. A few small round holes were also apparent throughout the protein matrix.

Heated gluten fixed in glutaraldehyde-osmium, dehydrated in DMP, and processed using procedures 4A and 4B was characterized by an extremely continuous protein phase. Large holes were absent, and the size and number of small holes were greatly reduced (Fig. 17). Starch was less common in these specimens,



Figs. 15–18. Heated gluten structure as influenced by sample preparation. **15,** Cryoprepared gluten (procedure 2). **16,** 2,2-Dimethoxypropane (DMP) dehydrated, Peldri II dried (procedure 4B). **17,** Fixed in glutaraldehyde-osmium tetroxide, DMP dehydrated, critical-point dried (procedure 4A). **18,** Fixed in glutaraldehyde-osmium tetroxide, EtOH dehydrated, Peldri II dried (procedure 5B). S = gelatinized starch. Bars = 10 μm.

but when it was identified, it was found only in the largest holes present.

Ethanol-dehydrated samples of gluten fixed in glutaraldehyde-osmium and heated (procedures 5A and 5B) were more porous than the samples dehydrated with DMP. The protein component of the gluten was continuous and characterized by numerous, large holes that were filled with gelatinized starch (Fig. 18). No appreciable differences were noted between the samples that were critical-point dried and those processed with Peldri II.

DISCUSSION

Gluten is extremely difficult to examine using light microscopy techniques. Surface reflection, specimen thickness, depth of focus, and resolution limit bright-field and phase-contrast images. However, the Zeiss laser scanning light microscope with a differential interference contrast objective allowed us to obtain valuable information regarding the structure of gluten. The limited depth of focus was at least partially overcome by obtaining a through-focus series of micrographs. This technique clearly demonstrated the spongelike nature of the gluten. The presence of a complex, reticulate network in fresh, unfixed, unfrozen gluten confirmed that the spongelike characteristic of gluten is real and not an artifact associated with cryopreparation.

Cryoprepared and fresh, unfrozen, unfixed gluten samples (procedure 2) were similar in structure to the fresh gluten examined with light microscopy in that both were characterized by a complex, reticulate network of protein that can be described as spongelike in appearance. In both procedures, starch granules were dispersed in large cavities surrounded by water. Discrete protein fibrils described by other researchers (Bechtel et al 1978, Marion et al 1987) were not observed in fresh or cryoprepared gluten samples, probably because of the limits of magnification and resolution of scanning electron microscopy. Even the large protein strands we observed were lost if the gluten was allowed to relax prior to processing.

The gluten components of chemically fixed and dehydrated doughs have been described as a network covering starch granules (Aranyi and Hawrylewicz 1969), as irregularly shaped sheets (Moss 1974), as a thin sheet with crepelike texture (Evans et al 1977), as sheets and fibrils with inclusions (Bechtel et al 1978), and as an amorphous mass when relaxed and fibrous when stressed (Cumming and Tung 1975). Moss (1974) indicated the gluten protein in dough could be stretched to form an open network that is characterized by the presence of many discontinuities. Evans et al (1977) concluded that the severe ruptures found in gluten sheets at starch-protein interfaces may result from stress during dough manipulation. In overmixed doughs, Bechtel et al (1978) found discontinuous protein in the form of a network associated with large vacuoles. When the overmixed dough was allowed to relax, the vacuoles decreased in both size and number. It is very possible that some of the reported structural variations of gluten in doughs may be directly related to artifacts of sample preparation.

Our light microscopy and sublimation studies clearly demonstrated that most of the spaces in the protein matrix of gluten contained water. Water accumulation was previously described in gluten samples prepared using freeze-fracture techniques (Fretzdorff et al 1982, Hermansson and Larsson 1986, Marion et al 1987). The three-dimensional characteristics of gluten are not apparent when using either freeze-fracture or cryofixation techniques because of the small sample size, specimen thickness, shallow depth of sublimation, and limited depth of focus available. The freeze-fractured samples of gluten reported by Hermansson and Larsson (1986) and Fretzdorf et al (1982) reveal a very dense, continuous structure somewhat similar to that observed in our freeze-dried or chemically dehydrated samples. Such comparisons are at best difficult because of the differences in magnification, resolution, and diameter of the field of view. Transmission electron micrographs of freeze-fractured specimens generally show only small areas, usually less than 4 μm in diameter, at magnifications as high as 50,000×; scanning electron micrographs of cryoprepared specimens may be larger than 90 μm in diameter and photographed at magnifications as low as 500×. Cryopreparation and scanning electron microscopy provide three-dimensional information not available using other techniques.

The spaces we have described in both fresh and cryoprepared samples are not air pockets incorporated into the gluten during development or washing. Pockets of trapped gases were easily identified, because they were many times larger than the spaces associated with the starch granules. The gluten surrounding the air pockets was continuous in structure without holes or pores. The gluten forming the limits of the gas pockets had structural characteristics similar to those of air-dried or dehydrated gluten.

The possibility of ice crystal damage in the cryoprepared samples must be considered. The size and basic circularity of the small holes in the gluten are not in keeping with the angular, six-sided shapes expected from ice crystal formation. The similarity in the size and shape of the holes within the protein matrix of both fresh and cryoprepared samples demonstrated that their presence was not the result of ice crystal formation but rather that they were a normal part of washed gluten. Most of the holes or spaces within the protein matrix were lost or reduced as a result of chemical fixation or dehydration. Our data demonstrated that freezing in liquid nitrogen did not result in changes in either the size or the shape of the holes nor did it result in changes in the three-dimensional structure. We detected no noticeable changes in the size or distribution of the holes from outside to inside of the cryoprepared samples as would be expected if they were related to ice-crystal damage. However, fresh gluten samples that were slowly frozen showed what we interpreted to be ice crystal damage (Fig. 9). Slowly frozen gluten samples were characterized by very large, angular holes within a very continuous protein phase that were undoubtedly related to ice crystal formation.

Heated gluten samples were markedly different from the fresh, cryoprepared gluten in regard to the number, size, and distribution of holes. This would not be expected if the spaces resulted from ice formation, as both samples contained approximately the same volume of free water and were processed using identical techniques. There were two major differences between the fresh and heated gluten samples. The starch was gelatinized as a result of heating, and the protein matrix was more continuous as a result of protein alteration.

Results obtained using chemical fixation were similar to those observed by other researchers (Varriano-Marston 1977, Chabot 1981, Hermansson and Buchheim 1981) and confirm their conclusions that chemical fixation of gluten samples for electron microscopy is inadequate in preserving structural morphology.

Our research showed more specifically that removal of water from the gluten during fixation and dehydration resulted in formation of structural artifacts. The removal of water and its structural support resulted in the formation of sheets or continuous dense layers of protein and the loss of the three-dimensional reticulate characteristics. The relationship between starch and gluten protein was also significantly altered. Cryopreparation utilizing rapid freezing in liquid nitrogen slush and sublimation on a cold stage of a scanning electron microscope was shown to be a suitable technique for the study of wheat glutens with minimal loss or alteration of the native structure. Only the cryoprepared samples had a structure similar to that observed in unfixed specimens examined with light microscopy.

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LITERATURE CITED

ARANYI, C., and HAWRYLEWICZ, E. J. 1969. Application of scanning electron microscopy to cereal specimens. Cereal Sci. Today 14:230-

233, 253.
BECHTEL, D. B., POMERANZ, Y., and DE FRANCISCO, A. 1978. Breadmaking studied by light and transmission electron microscopy. Cereal Chem. 55:392-401.
BJERKE, J. M., FREEMAN, T. P., and ANDERSON, A. W. 1979. A new method of preparing insects for scanning electron microscopy. Stain Technol. 54:29-31.
CHABOT, J. F. 1981. Studies of food microstructure. Scanning Electron Microsc. pp. 10–13.
CUMMING, D. B., and TUNG, M. A. 1975. The ultrastructure of commercial wheat gluten. J. Inst. Can Sci. Technol. Aliment. 8(2):67-73.
EVANS, L. G., VOLPE, T., and ZABIK, M. E. 1977. Ultrastructure of bread dough with yeast single cell protein and/or emulsifier. J. Food Sci. 42:70-74.
FRETZDORFF, B., BECHTEL, D. B., and POMERANZ, Y. 1982. Freeze-fracture ultrastructure of wheat flour ingredients, dough, and bread. Cereal Chem. 59:113-120.
HERMANSSON, A. M., and BUCHHEIM, W. 1981. Characterization of protein gels by scanning and transmission electron microscopy. J. Colloid Interface Sci. 81:519-530.
HERMANSSON, A. M., and LARSSON, K. 1986. The structure of gluten gels. Food Microstruct. 5:233-239.
ILIEVSKA-CHAKRABORTY, T. I. 1988. Physical and chemical approach to the study of hardness in hard red spring and hard red winter wheat. M.S. Thesis. North Dakota State University, Fargo.
MARION, D., LE ROUX, C., AKOKA, S., TELLIER, C., and GALLANT, D. 1987. Lipid-protein interactions in wheat gluten: A phosphorus nuclear magnetic resonance spectroscopy and freeze-frature electron microscopy study. J. Cereal Sci. 5:101-115.
MOSS, R. 1974. Dough microstructure as affected by the addition of cysteine, potassium bromate, and ascorbic acid. Cereal Sci. Today 19:557-561.
VARRIANO-MARSTON, E. 1977. A comparison of dough preparation procedures for scanning electron microscopy. Food Technol. 31:32-36.

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