The Microstructure of Wheat: Its Development and Conversion Into Bread

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Wheat endosperm development has been studied in numerous laboratories. The generalized scheme of protein body formation assembled from these data indicates that storage proteins are initially formed in the rough endoplasmic reticulum (RER). The storage proteins in RER may be processed via the Golgi apparatus into vesicles that enlarge by several mechanisms into membrane-bounded protein bodies. The protein bodies are transported through the cytoplasm to the vacuole where they fuse with the tonoplast and deposit the protein granules into the vacuoles. The protein granules fuse with one another, lose water, and eventually become transformed into the matrix. The framework of bread crumb, however, is of dual composition; the protein network and a newly formed network of gelatinized starch.

Wheat Endosperm Development

The wheat kernel is botanically a fruit (grain or caryopsis) in which the ripened ovary wall adheres to the seed at maturity. Changes during development of the wheat kernel have been independently studied with respect to structure and composition. One aspect of wheat grain development, the formation of storage protein in the endosperm, has been the subject of different interpretations. Graham et al. (1962) showed that the earliest deposited endosperm storage protein was in the form of a single protein granule enclosed by a membrane. Later during development, four or more protein granules were present within a vacuole. The proteins were deposited into vacuoles from the rough endoplasmic reticulum (RER) via an unspecified mechanism called "internal secretion". These results were confirmed by Buttrose (1963), who suggested that the Golgi apparatus was linked to protein deposition by acting in a condensing function. In the same journal issue, Jennings et al. (1963) described the effect of a variety of fixatives on protein body appearance in wheat endosperm. They concluded that the protein body occurred singly and had a slightly appressed membrane, rather than occurring in vacuoles. Later it was concluded that the protein bodies were formed within plastids called proplastids (Morton and Raison, 1963; Morton et al. 1964). The occurrence of single and multiple protein bodies within vacuoles of early developing wheat endosperm was confirmed by Barlow et al. (1974) and Harvey et al. (1974). They also described
the presence of material within the vacuole which was interpreted as ribosomes, the site of the storage protein synthesis. During late stages of development, however, protein bodies formed via a different mechanism (Barlow et al., 1974). This other mechanism involved protein secretion into the RER lumen which resulted in the double membrane being pushed apart and the end then joining to surround a protein body with a single membrane. Barlow et al. (1974) found no evidence for protein transport from the RER and concentration into dictyosomes. Subsequently, it was hypothesized that the protein was synthesized in the cytoplasm and transported to the vacuoles either through the lumen of the RER or by a process similar to pinocytosis (Simmonds, 1978). Briarty and co-workers conducted an extensive stereological analysis on the developing wheat endosperm (Briarty et al., 1979). They concluded that the route followed by newly synthesized storage proteins to the vacuoles was unclear, but the Golgi apparatus was not involved because it was absent 12 days after flowering (DAF) (Briarty, 1978; Briarty et al., 1979). Campbell et al. (1981) suggested that a direct connection exists between the RER and the protein bodies, whereas Parker (1980) observed large amounts of membranous material associated with developing wheat protein bodies. Parker also reported the presence of Golgi apparatus in wheat endosperm up to at least 40 days past anthesis (Parker, 1981, 1982; Parker and Hawes, 1982). We have completed investigations into the presence of Golgi bodies in developing-cereal endosperm during storage protein deposition (Bechtel and Gaines, 1982). We found that the Golgi apparatus was present during protein body formation in five cereals including wheat and that in four of the cereals the dense-stained Golgi secretory vesicles were protease digestible. In the fifth cereal, rice, chemical fixation interfered with protease digestion of storage proteins (Bechtel and Gaines, 1982).

Our light and electron micrographic study of wheat endosperm formation from the day of flowering through the time of harvest (Bechtel et al., 1982a, b) has recently been complemented with a freeze-etch freeze-fracture project on wheat endosperm development (Bechtel and Barnett, 1984a, b; Barnett and Bechtel, 1984). The results of these projects are summarized below.

Endosperm did not become discernible until about 2 DAF of a 35-day growing season. The endosperm at that time was a thin coenocytic layer of cytoplasm lining the embryo sac (Fig. 1). By 4 DAF the endosperm had cellularized and completely filled the embryo sac (Fig. 2). Enough differentiation had occurred by 6 DAF to distinguish cells destined to become the aleurone layer, subaleurone region, and central endosperm (Fig. 3). Protein bodies were usually observed in 6-7 DAF endosperm, and were first found near the Golgi apparatus (Fig. 4). Freeze-etch, freeze-fracture replicas of unfixed 7 DAF endosperm showed subaleurone cells containing Golgi bodies and vesicles, vacuoles, mitochondria, and plastids. Traversing the cytoplasm was RER consisting of large sheets (Fig. 5). Protein bodies viewed in cross fracture were membrane bounded and consisted of a homogeneous protein granule that had vesicular material associated with its periphery (Fig. 6). Enlargement of the small protein bodies occurred by several mechanisms: (1) fusion with one to several of the dense Golgi vesicles or fusion with other protein bodies (Fig. 7), (2) fusion with small electron-lucent Golgi-derived vesicles (Fig. 8), (3) pinocytosis of a portion of adjacent cytoplasm into the developing granule (Fig. 9). Direct connections between protein bodies and RER were not observed in any of the numerous TEM or freeze-fractured specimens.

The cytoplasmic protein bodies were transported to the central vacuole(s) where the protein body membrane and tonoplast fused and deposited the granule(s) into the vacuole (Figs. 10-12). During the middle stages of development the RER underwent a dramatic change. Instead of large sheet-like regions, the RER changed to smaller cisternal elements interconnected by tubular ER (Fig. 13). The vacuolar protein deposit became very complex between 12 and 19 DAF. Freeze-fracture showed the periphery of the protein granule to be a mass of vesicular material that seemed to be derived from the tonoplast (Fig. 14). Thin sectioned material revealed three mechanisms in which the protein granules in the vacuoles enlarged: addition of membranous vesicular material of various types (Fig. 15); addition of flocculent material (Fig. 16); and fusion of the granules with other newly deposited protein granules (Fig. 17). The fusion process occurred rapidly after 17 DAF and resulted in the conversion of the spherical protein granules into irregularly shaped protein masses that eventually became the matrix protein. The deposition of storage protein together with starch granule enlargement caused the cytoplasm to be isolated into small regions of the cell. The irregularly shaped protein masses were condensed with cytoplasmic remnants during late stages of development to form the protein matrix. The loss of water during this time certainly plays an important part in the condensation process. Barlow et al. (1973) studied mature wheat with a variety of microscopic techniques and demonstrated that the space surrounding starch granules in thin sectioned material is an artifact of tissue preparation.

**Dough and Bread Structure**

The process of milling is designed to remove as much of the germ and outer layers of the wheat caryopsis as possible, and breaks up the starchy endosperm into small particles. Milling has little effect on the organelles in the starchy endosperm as they are one or two orders of magnitude smaller than the flour particles (Simmonds, 1972). Generally, hard red winter wheat flour consists of particles composed of starch held together by the protein matrix (Aranyi and Hawrylewicz, 1968; Crozet and Guilbot, 1974; Khoo et al., 1975).

The conversion of flour into a dough has proved difficult to study. This is because of...
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Figs. 1-8:  
1. Cross section through 2 DAF wheat caryopsis showing coenocytic endosperm (arrows) lining the embryo sac (Es), surrounded by nucellus (N), seed coats (Sc) and pericarp (P). 
2. Endosperm (E) of 4 DAF caryopsis is completely cellularized. Nucellus (N) and seed coats (Sc) will degenerate by 10 DAF. 
3. Six DAF wheat endosperm shows differentiation into central endosperm (E), subaleurone region (Sb), and young aleurone cells (A). 
4. Protein bodies (Pb) are found near Golgi bodies (G) that secrete dense cored vesicles (*). 
5. Freeze-fracture micrograph from 7 DAF endosperm showing sheet-like RER, Golgi body (G) and cross-fractured mitochondrion (M). 
6. Cross-fractured protein body with associated vesicular material (*). 
7. Fusion of two Golgi derived vesicles (arrow) that will form a protein body. 
8. Fusion of small vesicles (arrows) in 10 DAF endosperm with dense-cored Golgi vesicles.
the dramatic and dynamic changes that occur to flour particles during hydration. Bernardin and Kasarda (1973) have described the "explosive" nature of this hydration. Couple the hydration phenomenon with the mechanical mixing process and one has an extremely difficult system to work with. Our approach to the problem has been to study developing and mature wheat kernels and compare them to doughs. Various microscopic techniques have been applied to dough systems including light microscopy (Sandstedt et al., 1954; Bechtel et al., 1978; Marston and Wannan, 1976), SEM (Chabot et al., 1979; Varianno-Marston, 1977; Khoo et al., 1975; Aranyi and Hawrylewicz, 1968), TEM of thin sections (Bechtel et al., 1978; Khoo et al., 1976; Simmonds, 1972), and TEM of freeze-fractured doughs (Fretzdorff et al., 1982).

These studies have revealed that dough structure has some semblances of mature wheat in that starch granules were surrounded by protein (gluten). The similarities between wheat and dough, particularly with the protein, cease with that simple comparison because dramatic changes have occurred in the protein. Simmonds (1972) compared structural differences between endosperm and dough and suggested that two types of inclusions occurred in the protein phase of the dough. Type I inclusions were irregular, stained densely, and were thought to have been formed from the ER. Type II inclusions were spherical, were not formed in doughs from defatted flours and were believed to be lipid rich. Newly mixed doughs have hydrated protein stretched over starch granules (Khoo et al., 1975).

We have studied the structure of water-flour doughs made from an RBS flour (Regional Baking Standard flour) which is a composite made of many wheat varieties grown in many locations throughout the Great Plains, from good-quality flour with long mixing time, and from poor-quality flour with short mixing time (Bechtel et al., 1978). In addition we have compared the effects of mixing time on dough structure made from these flours. The grossly undermixed composite dough (50% shorter than optimal mixing) had gluten that contained few vacuoles (voids) and was in the form of bulky strands (three dimensionally the strands are sheet-like) with smooth edges, and large groupings of cellular debris (Type I inclusions; Simmonds, 1972) embedded within the protein (Fig. 18). Many starch granules were grouped together and not surrounded by protein. Lipid droplets (Type II inclusions; Simmonds, 1972) were located within the protein strands (Fig. 18). The optimally mixed water-flour composite dough possessed smooth, thin protein strands that contained evenly distributed vacuoles and cellular debris (Fig. 19). Most of the starch granules were surrounded by sheets of protein (Fig. 19). Protein in the grossly overmixed (50% longer than optimally mixed) dough contained the most vacuoles. Broken protein strands surrounded starch granules and had irregular edges (Fig. 20). The dough from the good flour, generally appeared similar to the composite, except that the protein in grossly over-mixed dough from good flour contained fewer vacuoles and fewer broken strands. Protein strands in the undermixed poor flour that surrounded the starch granules were broken and contained many vacuoles and evenly distributed debris. In the optimally mixed poor flour dough there were more broken strands with irregular edges. The overmixed dough, basically, was similar to the optimally mixed one.

Khoo et al. (1975) briefly described various stages of breadmaking using SEM and TEM. They found that during baking, the protein phase changed little microscopically, but the starch granules, particularly the large ones, gelatinized. We have studied samples taken from optimally mixed complete doughs, during fermentation and proofing, and from the top center and bottom center of the loaf, after oven spring (about 5 min.) and final baking (about 24 min.). The ultrastructure of the starch granules and gluten was uniform during mixing, fermentation, and proof. Changes in the starch took place during baking at which time it became gelatinized. Some starch was gelatinized after 5 min. of baking. Gelatinization started from the inside of the starch granules and expanded outwards. Gelatinized starch appeared fibrous, was greatly expanded, and functioned in a structural role in the baked bread (Fig. 21). Gluten in complete doughs that had been mixed, fermented and/or molded had rough edges and was highly vacuolated. Apparently the complete formulation changed the gluten structure from smooth to rough in comparison to water-flour doughs. Shortly after oven spring the proteins had smoother appearing edges with fewer and smaller vacuoles. Proteins from fully baked loaves lacked vacuoles, occurred as very smooth strands and sheets, and did not seem to be denatured by the baking process. Structures (tentatively identified as lipid globules) formed from added shortening were not consistently associated with either the protein matrix or starch granules. Native wheat flour lipids occurred as small droplets integrally associated with the protein matrix. Samples processed for TEM require chemical fixation and dehydration. These procedures have been shown, at least for SEM, to cause profound changes in dough structure (Varianno-Marston, 1977; Chabot et al., 1979). Varianno-Marston (1977) found that freeze- or vacuum-dried dough samples had more intact and continuous gluten surrounding starch granules than samples prepared by any other procedure. Chabot et al. (1979) showed that buffers, fixatives, and dehydrating fluids caused alterations to the protein in doughs and allowed starch liberation.

The possibility that our preparation procedures had produced artifacts in our dough samples and that the artifacts had led to wrong interpretations forced us to reevaluate our TEM data. We chose the freeze-etch, freeze-fracture technique to check our TEM results (Fretzdorff et al., 1982). This procedure has a number of advantages; no drying of samples is required, no fixation is required, no exposure to dehydration solutions or buffers, and no embedding in plastics is needed. The only place in the technique where major artifacts can be a problem is in the freezing of the samples and this was not a problem because of the relatively low concentration.
Figs. 9-17. 9. Pinocytotic vesicles (*) pinching off of vacuole membrane and being incorporated into protein granule (Pg). 10. Protein body (Pb) with protein granule (Pg) associated with vacuole (V) at 10 DAF. 11. Membrane of protein body (Pb) and vacuole (V) fusing. 12. Protein granule (Pg) deposited into vacuole (V) and another protein body (Pb) associated with vacuole. 13. Freeze-fracture micrograph showing small cisternal ER (CER) connected to tubular ER (TER) in 12 DAF endosperm. 14. Freeze-fracture micrograph of edge (*) of protein in vacuole. Tonoplast (T) seems to be budding vesicular material in vacuole (V). 15. Vesicular material (*) associated with protein granules (Pg) in vacuole (V). 16. Large amount of flocculent material associated with protein granules (Pg). 17. Fusion of protein granules in a vacuole.
of water (≈62%) and even distribution of water within the dough. Even water-starch slurries showed no sign of ice damage (Fig. 22). The results of this technique produced several unusual findings. The space between the starch granules and gluten proteins seen in LM and traditional TEM was also observed in the freeze-fracture specimens although the space was usually smaller (Fig. 23). In addition, this space is filled with water because it could be deeply etched without showing any internal structure. The addition of soy flour, salt, shortening, and sugar to water-flour doughs altered the dough structure. Sugar in particular aided dough development by reducing the space around starch granules, reducing the number and size of water droplets observed and giving them a smooth appearance, and changing the protein from a network to a sheet-like system (Fretzdorff et al., 1982). In complete doughs protein development was dramatically changed during fermentation when viewed by freeze-fracture. Starch-protein interactions were found in most doughs and in all stages of bread production (Fig. 24). This contrasts with chemically fixed doughs where starch-protein interactions only were observed in the baked bread between protein and gelatinized starch.

Bread development and breadmaking have been difficult systems to study with the microscope. A variety of microscopic techniques have had to be developed and used. While artifacts have not been totally eliminated, we now have a better understanding of what they are. The development and application of new techniques should eliminate many of the problems now encountered.

Acknowledgment

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References


Figs. 18-24: 18. Grossly undermixed composite dough with starch granules (S), large regions of type I inclusions (I) and type II lipid inclusions (L) in protein (Pr). 19. Optimally mixed composite dough with relatively uniform distribution of type I (I) and II (L) inclusions as well as small vacuoles (Va). Protein in thin sheets (Pr) surrounds starch (S). 20. Grossly overmixed composite dough showing large number of vacuoles (Va) and inclusions located inside the vacuoles (*). 21. Crumb from top center of loaf of bread immediately after baking showing condensed protein (Pr) and structural nature of gelatinized starch (S). 22. Freeze-fracture micrograph of water-starch slurry (courtesy of B. Fretzdorff). Note water (W) is amorphous without ice crystal damage to portion of starch granule (S). 23. Freeze-fracture of water-flour dough shows space (*) between starch (S) and protein (Pr). 24. Freeze-fracture micrograph of starch (S) and protein interactions (arrows) in complete dough prior to fermentation.


Discussion with Reviewers

K. Saio: What are the black lines in protein bodies in Fig. 17?

W. J. Wolf: In Fig. 17, what are the clefts in the protein bodies? Are they artifacts of specimen preparation?

Author: The dark irregular lines that occur in many of the wheat protein bodies are folds that probably occur during thin sectioning. It is most likely a hydration phenomenon similar to what occurs in starch. We have never observed them in freeze-fractured material.

W. J. Wolf: Does Fig. 17 actually illustrate fusion or are the protein bodies merely in close contact with each other? There still appear to be distinct boundaries between the large body on the right and the one at 12 o'clock.

Author: I feel the protein granule at 12 o'clock is very close to being fused while the one at 8 o'clock is even closer. In freeze-fracture, closely associated protein granules have a distinct boundary where they are touching while those that have fused look like budding yeast cells with their surfaces continuous.

W. J. Wolf: In Fig. 21, what is the structure towards the upper left directly above the area labeled S? The "fibrous" starch appears to surround this entire structure.

Author: There are only portions of two protein strands in Fig. 21, the one labeled Pr and one in the lower right hand corner. The rest of the material in the figure is gelatinized starch. We have found that gelatinized starch is very difficult to prepare for TEM. Part of the problem is that solubility increases resulting in loss of material. As a result, we obtain some strange images of the gelatinized starch.

K. Saio: In Fig. 6, what are the cracks located on the right side of the protein body? Can they be considered tubular ER or are they simply artifacts?

Author: These structures are just changes in the plane of fracture through the cytoplasm and do not represent any organelles.

K. Saio: The space between the starch granule and protein matrix is bigger in Fig. 19 than that in Fig. 23. Does it happen because of some artifact in the sample preparation of TEM (like shrinking of starch granules which are difficult to fix)?

Author: Barlow et al. (1973) showed that in mature wheat the space surrounding starch granules is artifact, probably due to starch shrinkage. This shrinkage in doughs also occurs and results in the large spaces seen in Fig. 19. Freeze-fracture of untreated doughs (Fig. 23), however, shows that a small area does surround starch granules and most likely represents the real situation.

R. Moss: The grossly overmixed doughs possessed protein strands with irregular edges, yet the baking experiments showed rough protein from all stages of dough development. Are these changes due to dividing, molding or proofing? In the reviewer's experience they can totally alter microstructure ex mixer and give rise to large differences in bread quality.

Author: One cannot directly compare the mixing results with those of the baking experiments because the mixing studies were done with water-flour doughs. This was done to eliminate the effects of ingredients. Use of conventional thin sectioning and TEM (Bechtel et al., 1978) did not reveal significant differences during fermentation--probably due to the fixatives--dehydrating fluids and embedding materials. Freeze-fracture of untreated doughs (Fretzdorff et al., 1982), however, showed significant changes during bread production.

J. N. A. Lott: Because of the fixation quality, I have some difficulty being less than skeptical about Fig. 9 showing that material pinches off the vacuole membrane.

Author: I myself have been as skeptical, but freeze-fracture of both fresh-frozen and glycerol
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Imbibed endosperm exhibit these invaginations (see Fig. 25). The B-face (convex surface) has pits which correspond to pinocytotic vesicles whereas the A-face (concave) has evaginations which correspond to the vesicles invaginating into the vacuole.

Fig. 25: Freeze-fracture micrograph of endosperm.

J. N. A. Lott: You mention that you think enlargement of small protein bodies occurs by several mechanisms. Do you have any idea as to what proportion of the enlargement is Golgi mediated as opposed to involving pinocytosis of the adjacent cytoplasm?

Author: Based on our traditionally fixed TEM samples I had concluded that not much of the storage proteins passed through the Golgi apparatus (Bechtel et al., 1982a). The freeze-fracture technique has allowed us a three-dimensional view of protein body enlargement and gave very different results. The freeze-fracture micrographs showed many more vesicles and fused vesicles than thin sections. This was probably due to the fact that freezing fixed the samples better and faster than chemical fixation, thus preserving fusion intermediates. This coupled with the 3-D views suggest that the Golgi apparatus "processes" much more storage protein than we had thought. Of course we cannot quantify the amount yet but are working on some cell fractionation studies to assist us in quantifying the amount.

K. Salo: How can you distinguish between fusion and pinching off of vesicles from static micrographs?

Author: Obviously, we cannot observe movement in the electron microscope, but by using developmental studies we can determine which structures are enlarging, staying the same size or becoming smaller. Thus, if we compare several stages of development and protein granules are larger in the older stage, something has been added to the granule and if small vesicles are fused with the protein body, we assume that the vesicles have added material. While this may not always hold true, the dense-cored vesicles we observed were good markers to determine direction of transport.