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# Interaction of Maize Zein with Wheat Gluten in Composite Dough and Bread as Determined by Confocal Laser Scanning Microscopy

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**Summary:** Protein body-free maize zein, when mixed at 35°C (above its glass transition temperature range), significantly ($p < 0.01$) improved the rheological and leavening properties of sorghum-wheat composite flour dough, resulting in improved loaf volume. Confocal laser scanning microscopy was used to observe the structure of zein fibrils and the interaction between zein and gluten proteins in the composite dough and bread systems. Autofluorescence and immunolocalization techniques were used to locate gluten and zein, respectively. Optical sections were collected every 0.4 µm through the samples and digitally processed to produce reconstructed three-dimensional images. Results showed that zein fibrils form an outer layer that intermittently coats the gluten networks, thereby strengthening them. This type of microstructure is able to withstand the pressure exerted by gas cell expansion during yeast fermentation to increase loaf volume.

**Key words:** composite dough, microstructure, confocal laser scanning microscopy

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## Introduction

Microscopy is a suitable tool for examining structures with dimensions in the order of microns. It is used as an analytical tool for various aspects of food analysis such as locating storage nutrients in processed and unprocessed cereal grains (Yiu 1993) and studying interactions between food components. The microscopic spatial arrangement of components links physicochemical interactions to bulk properties such as texture, appearance, taste perception, and stability of the final product (Autio and Laurikainen 1997, Vodovotz *et al.* 1996). This forms a bridge between the molecular properties of individual components and the desired macroscopic properties of the products.

Dough and bread are primarily composed of proteins, lipids, carbohydrates (mostly starch), water, and air. The main structural components are starch granules, which form 70% of the total dry material, the soluble and insoluble proteins, and air. From the colloidal point of view, dough is largely considered as a continuous protein structure with dispersed phases of starch granules and air cells (Bohlin and Carson 1980). However, the components of dough form a dynamic and complex system that is continuously changing once formed due to various occurring chemical and physical reactions.

Microscopy studies of the breadmaking process have been used to determine optimum dough mixing time, extent of gluten development, and nature of gluten matrix formed (Moss 1974, Parker *et al.* 1990, Sidi and Moss 1991, Zheng 1998). Moss (1974) showed that addition of oxidizing agents such as ascorbic acid, potassium bromate, and potassium iodide, and reducing agents, such as cysteine, affected dough microstructure after mixing. This, in turn, influenced behavior of dough during sheeting. Oxidizing agents prevented development of finer sheets within the optimum mixing time, resulting in an open, coarse, and discontinuous gluten network. On the other end, reducing agents softened the gluten, resulting in more extensible but less elastic dough. Blonk and van Aalst (1993) demonstrated that additives, particularly those interacting with gluten protein, determine final loaf volume by influencing the development of bread dough structure during rise. Microstructural studies also have the potential of elucidating the phenomena underlying handling and baking properties of dough.

Confocal laser scanning microscopy (CLSM) is a relatively new technique in food research offering many advantages for studying the relationships between composition, processing, and final product. One of the main advantages is the minimal degree of sample preparation that is required, because the sample is optically sectioned, which allows use of thicker specimens than conventional microscopy. Optical sectioning also allows imaging of delicate structures within samples that often are distorted by

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physical sectioning. Confocal laser scanning microscopy has been successfully used to follow changes in the structure of the protein phase of dough during development (Zheng 1998), and to follow morphologic changes that take place throughout the starch granules during the process of their digestion by salivary amylase (Lynn *et al.* 1997). Three-dimensional (3-D) imaging has been used to view 3-D images of specimens such as bread (Vodovotz *et al.* 1996) and to visualize dynamic processes such as the growth of air bubbles in dough during yeast fermentation and proofing (Blonk and van Aalst 1993, Heertje *et al.* 1987). Several components can be identified and localized at once with CLSM by using specific fluorescent labels, depending upon laser lines available on the instrument.

In our previous study to determine the effect of added protein body-free maize zein to sorghum-wheat composite flour (Bugusu *et al*. 2001), zein, when mixed above glass transition temperature, was shown to improve the dough-forming properties (mixogram mixing time and peak height), dough extensibility, and resultant loaf volume (Table I). The objective of the present study was to apply CLSM to observe the microstructure of sorghum-wheat-zein composite flour dough and bread so as to establish whether there are interactions between zein proteins and gluten in the composite flour system that are responsible for the stronger dough resulting in increased loaf volume. The study also aimed to establish the mechanism of any observed interactions.

## Materials and Methods

### Materials

Commercial bread flour (13.3% protein, King Arthur flour, Norwich, Vt., USA), maize zein (lot no. 18H0774, Sigma Chemicals Co., St. Louis, Mo.), and sorghum flour (MR732 cultivar, decorticated to remove 20% of original weight and milled in a cyclone laboratory mill to pass through 0.5 mm mesh) were used; 20% sorghum-80% wheat composite flour was used, with 5% added zein; 100% wheat and 80% wheat-20% sorghum were used as control samples.

### Dough Preparation

All flour samples in closed containers and water were kept in a 35°C temperature-controlled room overnight to equilibrate. Flour was weighed and mixed in a 35 g Swanson-Working mixograph (National Mfg. Co., Lincoln, Ne., USA) to optimum dough development.

### Wheat Sample with Fluorescein Isothiocyanate

To determine the appropriate method for identifying gluten, wheat dough samples were prepared with and without fluorescein isothiocyanate (FITC) dye. Flour, water, and five drops of 0.05% FITC were mixed in a 35 g Swanson mixograph (National Mfg. Co.) to optimum dough development. The two samples, with FITC and without FITC (autofluorescence), were compared for visualization of gluten. The results were similar, thus the autofluorescence method was chosen for gluten identification.

### Bread Samples

Bread was made from the different flour combinations using the 100 g basic straight dough breadmaking method (AACC 10-09 1983). The bread formula was 100 g flour, 0.9 g active dry yeast, 5 g sugar, 1 g salt, 64 ml water, and 0.1 g ammonium phosphate, monobasic.

### Sampling, Fixing and Sectioning

Approximately 1 g rectangular pieces were cut from the center of the dough and bread samples and fixed in a 1% glutaraldehyde, 4% paraformaldehyde in 0.05M potassium phosphate buffer, pH 6.8 for 1 h at room temperature. The fixing solution was removed by rinsing with the 0.05 M potassium phosphate buffer, and the samples were dehydrated in a graded ethanol series 15 min each in 10, 30, 50, 70, 90, 95, and 100% ethanol.

The samples were thick sectioned with care to minimize structural damage. Some sections from the center of the dough or bread piece were affixed to the microscope slides for viewing on the microscope, and others for immunolabelling.

TABLE I Effect of added zein on dough-forming properties, extensibility, and loaf volume of sorghum-wheat composite flour

| Flour | Mixing time (min)[1] | ± SD | Peak height (cm)[1] | ± SD | Peak fracture stress [KPa][2] | Loaf vol. (cc) | ± SD |
|---|---|---|---|---|---|---|---|
| 100% wheat | 4.40b | 0.13 | 5.25c | | 14.58 | 883.3d | 4.08 |
| 20% sorghum | 5.24c | 0.25 | 3.70a | 0.25 | 4.75 | 715.8a | 3.76 |
| 20% sorghum + 5% zein | 3.10b | 0.14 | 4.40b | 0.22 | 5.76 | 739.2b | 3.76 |
| 20% sorghum + 10% zein | 2.56a | 0.11 | 5.92d | 0.11 | 10.14 | 809.2c | 9.17 |

[1] Values obtained from mixograph curves.
[2] Peak fracture stress values force per unit area required to extend the dough is a measure of extensibility.
Means followed by different letters within the same column are significantly different using Student-Newman-Keuls, $p<0.01$.

### Immunochemistry

Sections were first soaked in 20 mM Tris, 500 mM sodium chloride, 0.3% Tween-20, pH 8.2 (TBS-T) for 10 min. The sections then were soaked in α-zein specific rabbit polyclonal antiserum (1:500 dilution, obtained from B. Larkins, University of Arizona, Tucson, Ariz., USA) at room temperature for 2 h and rinsed three times with TBS-T to remove excess antibody. They were incubated in several drops of Cy5 fluorescent probe conjugated to goat-antirabbit IgG (Biological Detection Systems, Inc. Pittsburgh, Pa., USA) at a 1:50 dilution in TBS-T for 1 h. The samples were rinsed three times with deionized water and coverslipped for observation.

### Confocal Microscopy

Confocal laser scanning microscopy was performed using a BioRad MRC-1024 microscope (Bio-Rad, Hercules, Calif., USA) based on an inverted Nikon Diaphot 300 microscope (Nikon, Japan). The confocal system was equipped with 60× PlanApo 1.4 NA oil immersion objective lens, a water-cooled Innova Enterprise 60 mW output Argon ion laser (Coherent Inc., Santa Clara, Calif., USA), an air-cooled 10 mW output Krypton/Argon laser (ILT Laser, Bio-Rad Laboratories, Hercules, Calif., USA), three fluorescence detection channels, and a three-color transmitted nonconfocal light detector. Laser light 488 and 647 nm attenuated, respectively, to 3 or 10% of the maximum intensity was introduced into the sample. Two photomultipliers were used for simultaneous collection of fluorescence signals from gluten autofluorescence (or FITC) and Cy-5.

Gluten protein autofluorescence was analyzed using 488 nm excitation. Autofluorescence light passed through a 488/568/647 triple dichroic filter (T1 BioRad filter block), then through a 560 nm dichroic long pass filter (E2 Bio-Rad filter block) where longer wavelengths were separated from green autofluorescence. This was then detected in PMT2 after passing through a 522/35 band pass filter (Chroma, Brattleboro, Vt., USA). This setup was used to detect the gluten network both in autofluorescent mode and with FITC dye. An excitation wavelength of 647 nm in combination with a 680DF32 emission filter was used to detect Cy-5 fluorescent probe conjugated to the secondary antibody of zein in PMT3

Dough images were gathered using the Kalman averaging procedure. Three scans of each section were collected in slow acquisition mode. Bread sample images were gathered using five accumulated slow scans to compensate for reduced gluten autofluorescence after baking. Optical sections of the wheat-sorghum-zein samples were collected with z-step of 0.4 µm throughout the dough sample (40 µm thick) and digitally processed using Lasersharp (BioRad) or Voxel View (Vital Images, Inc. Plymouth, Minn., USA) software to produce a reconstructed 3-D image. Presented images were pseudocolored so that gluten appeared green and zein appeared red, and were combined to show the interaction.

## Results and Discussion

### Dough Microstructure

*Fluorescein isothiocyanate-labeled samples:* A preliminary experiment was carried out to determine a suitable method for identifying gluten. Fluorescein isothiocyanate, a fluorescent dye specific for detecting proteins in a sample (Brooker 1995), and natural autofluorescence were used. Fluorescein isothiocyanate excites at about 500 nm, thus the 488 nm line was used. Figures 1 and 2 show the images of FITC-labeled and unlabeled dough samples, respectively. The similarity between the two images suggests that the natural fluorescence from the gluten net-

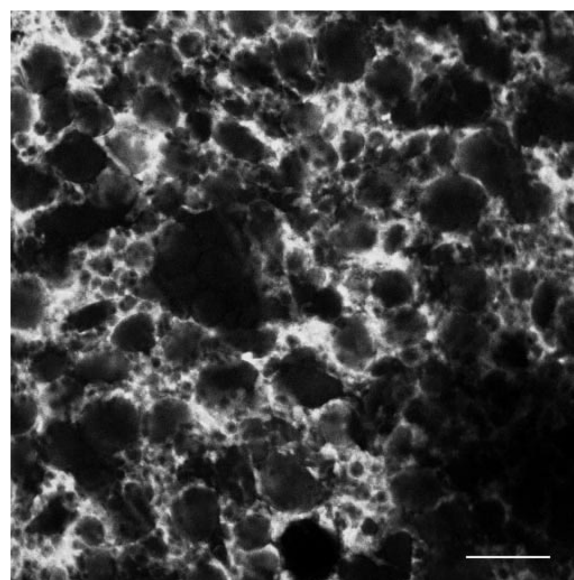

FIG. 1 Confocal micrograph of wheat dough, gluten networks (white) stained with fluorescein isothiocyanate. Size bar = 50 µm.

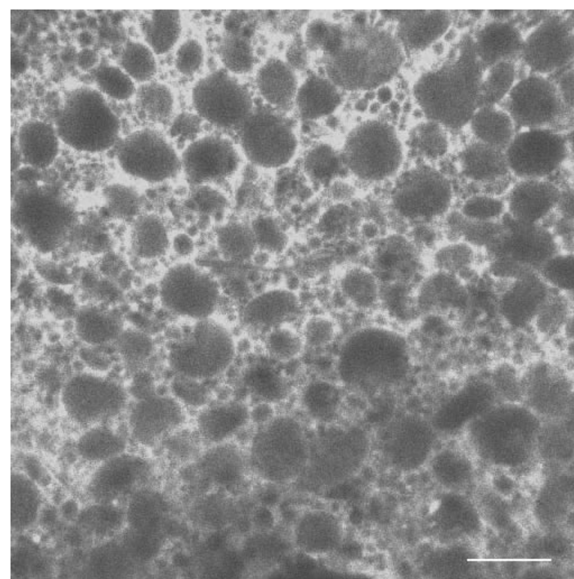

FIG. 2 Confocal micrograph of wheat dough in autofluorescence mode showing gluten networks (white). Size bar = 50 µm.

work is strong enough to examine the microstructure of the dough without addition of fluorochromes. This reduces the possibility of artifacts that result from extensive sample preparation. Figures 1 and 2 represent a typical microstructure of optimally developed wheat dough, which consists of gluten protein stretched out into fibrils that form a continuous and extensive network. This agrees with studies done by Parker *et al.* (1990) and Moss (1974) using scanning electron microscopy, which showed that during the process of mixing in breadmaking, the hydrated gluten forms a continuous matrix in which starch, lipid, yeast cells, and wall fragments are evenly distributed. In these studies, gluten was also shown to form thin film walls around the gas cells.

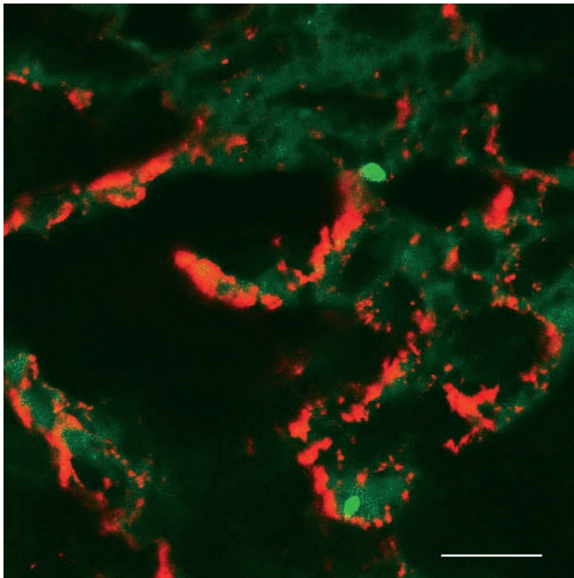

FIG. 3 Confocal micrograph of sorghum-wheat-zein dough treated with α-zein primary antibody. Green is the gluten network and red is zein fibrils. Size bar = 50 μm.

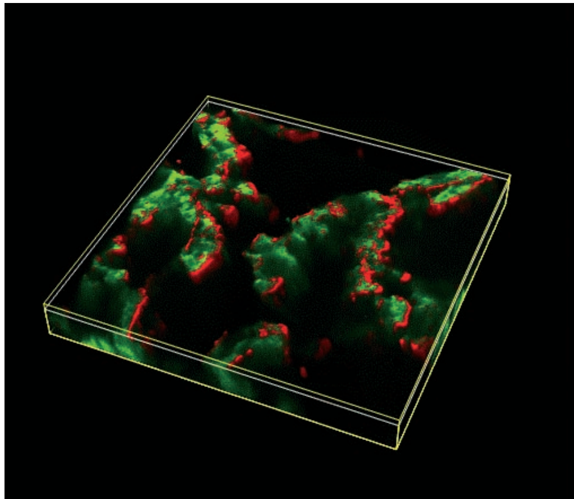

FIG. 4 Confocal micrograph of reconstructed three–dimensional image shown in Figure 3, observed at 10 μm depth of the dough sample.

*Identification of zein protein in dough:* Specific localization of the zein component was achieved using α-zein specific rabbit polyclonal antiserum as the primary antibody and the Cy5 fluorescent probe conjugated to the secondary antibody goat antirabbit IgG. The α fraction of zein constitutes 75–85% of the total zein protein in maize and is the principal storage protein (Larkins 1989). To determine specificity, all samples were reacted with the zein antibody, excited at 647 nm, and red-colored fluorescent signals were collected. The ability of the CLSM to visualize the gluten in autofluorescence mode (colored green) and immunolabeled zein (colored red) made it possible to determine the interaction between the two proteins. All control samples (samples without zein) showed little to no red-colored fluorescence (figures not shown); however, further investigation revealed that the fluorescence was only on the surface of the sample. This could have been due to inadequate rinsing of excess antibody during sample preparation. These results indicate that the antibody did not crossreact with other proteins in the sample.

The micrograph of dough with added zein shows that zein tends to form thinner and finer networks compared with gluten that coat the gluten fibrils from the outside (Fig. 3). This finding is clearly seen in the reconstructed 3-D image starting at 10 μm depth from the sample surface (Fig. 4). Although small amounts of zein were detected within the gluten fibrils, the vast majority of the protein was formed on the outside of the gluten matrix. Further observation of the 3-D image at depth of 20 μm (figure not shown) showed the same. The observed improvement in rheological properties of composite dough by zein addition can, therefore, be attributed to this microstructure in which zein apparently strengthens the gluten matrix by coating them in a loose network.

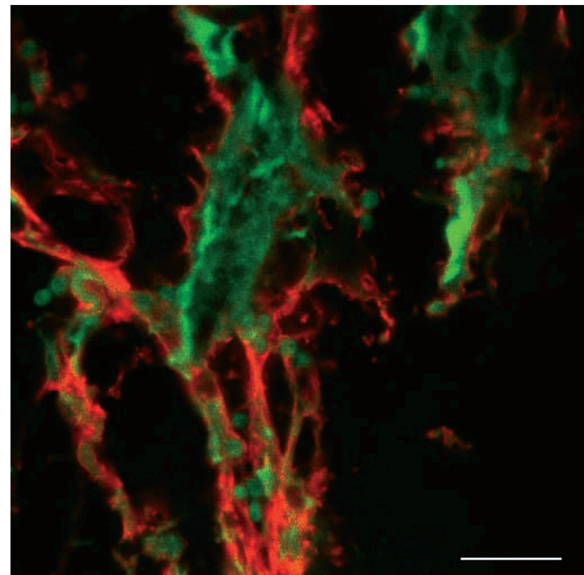

FIG. 5 Confocal micrograph of wheat-sorghum-zein bread treated with α-zein primary antibody. Size bar = 50 μm.

*Bread microstructure:* There was a reduction in gluten autofluorescence after baking. Bread images were collected by accumulating five slow scans. The microstructure of wheat bread fixed in 1% glutaraldehyde, 4% paraformaldehyde in 0.05M potassium phosphate buffer at pH 6.8 (figure not shown), showed discontinuities in gluten network that could be due to several factors, including expansion of air cells during fermentation and baking. Gan *et al.* (1990) using scanning electron microscopy showed similar results. In the wheat-sorghum bread (figure not shown), the gluten network appeared more continuous, likely due to the fact that the composite flour dough expands less during fermentation and baking, resulting in lower loaf volume (Table I).

In the wheat-sorghum-zein bread microstructure (Fig. 5), the gluten network was shown to be coated by the zein fibrils. There appears to be some intermingling between zein and gluten, in the sense that zein seems to fill up the discontinuities observed in the gluten networks in the wheat bread. Zein appears to strengthen the forming bread structure to result in improved loaf volume (Bugusu *et al.* 2001). High loaf volume positively correlates with a number of consumer-preferred quality characteristics of bread. The insoluble protein component, gluten, forms the viscoelastic networks, which retain carbon dioxide gas produced by yeast fermentation during dough rise. The changes in dough structure during rising determine the final loaf volume and are influenced by additives interacting with gluten proteins. Blonk and van Aalst (1993) successfully used CLSM to view the influence of additives and gluten network in dough on loaf volume.

Observable changes take place in dough microstructure during baking. They include expansion of gas cells (in early-oven stage), starch gelatinization (Dreese *et al.* 1988), protein crosslinking (Schofield *et al.* 1983), and melting of fat crystals and their incorporation onto the surface of air cells (Brooker 1995). Starch gelatinization results in watering of the gluten phase. Using an electron microscope, Parker *et al.* (1990) showed a typical bread microstructure to consist of continuous gluten networks surrounding gelatinized starch, yeast, and wall fragments.

## Conclusion

Addition of zein appears to enhance dough-forming properties and extensibility by forming thin fibrils that coat gluten networks. This microstructure is able to withstand pressure exerted by gas cell expansion during fermentation and proofing to increase loaf volume. A possible explanation for this observation is that, during fermentation and proofing, the expanding inner gluten networks are strengthened by the outer zein fibrils, making them more extensible and strong to hold gas. It is clear from this study that CLSM offers an innovative alternative approach to conventional physical techniques for investigating the interaction and location of food components and new food additives in a solid food system.

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