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# Distribution and location of ethanol soluble proteins (Osborne gliadin) as a function of mixing time in strong wheat flour dough using quantum dots as a labeling tool with confocal laser scanning microscopy

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ABSTRACT

Gliadin is the more *fluid* of the two major classes of proteins, gliadins and glutenins, *in wheat flour dough* and flows best among these two protein classes. Glutenins because of their high elasticity and high hydrophobicity are not likely to disperse as well as gliadins especially if gliadins are not well dispersed or are not present in the dough matrix. The role of native gliadin in this networking process is not well understood and has not been studied by prior researchers due to unavailability of molecular tools to specifically probe gliadin behavior until now. Gliadins contribute to the viscosity and extensibility in dough.

In this study, the distribution and location of ethanol soluble proteins (Osborne gliadins) as a function of mixing time in a Brabender Farinograph for model wheat flour dough were investigated for the first time using confocal laser scanning microscopy (*CLSM*). Gliadin proteins were tagged with water soluble, biocompatible amine derivatized polyethylene glycol functionalized quantum dots to increase the clarity and specificity of imaging. The effect of different mixing conditions on the distribution of ethanol soluble proteins (Osborne gliadins) and their role in building dough structure was investigated. The chosen mixing times were arrival time (AT), peak time (PT), departure time (DT) and breakdown time (10 min after departure time).

Location and distribution of gliadins were investigated in AT, PT, DT and 10 min after departure time. Antibody-quantum dot (QD) mixture successfully bonded to gliadins located on dough sections. The specificity of gliadin antibody to ethanol soluble proteins (Osborne gliadins) was shown successfully with a Western Blot experiment excluding binding to all other hard wheat flour proteins. The images obtained from dough sections were bright and clear and allowed to distinguish gliadin easily. Mixing led to considerable changes in the distribution, average particle size, and number of particle count of gliadins in dough microstructure. The QDs were found to be localized not only around the air cells as indicated by higher intensities but also in the bulk dough. Quantum dots can be used as fluorophore probes to tag and track molecules of interest in food microstructure. This combined with immunohistochemistry techniques offers a better understanding of gliadin distribution in dough during mixing.
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## 1. Introduction

Gluten is the main functional protein in the dough and plays a significant role in determining the rheological properties of the dough (Dhanasekharan, Wang, & Kokini, 2001; Tsiami, Bot, Agterof, & Groot, 1997; Wang & Kokini, 1995). Gluten possesses two main sub fractions: monomeric gliadins and polymeric glutenins. Gliadins are responsible for the plasticity in dough matrix while glutenins possesses strong elastic properties (Southan & MacRitchie, 1999). Gliadins, which are highly surface active and hydrophobic proteins, play a significant role in gas holding capacity (Örnebro, Nylander, & Eliasson, 2000).

The bread making process consists of hydration, kneading or mixing, fermentation, dividing and rounding, molding, proofing and baking, leading to important structural changes that affect quality. While the distribution of gluten proteins in bread are well understood, the distribution of gliadins in baked bread has only been recently studied (Ansari, Bozkurt, & Kokini, 2014).

The type of mixing apparatus, energy input, mixing time, and mixing speed all has a lot of influence on the quality of dough (Hoseney, 1985; Janssen, Van Vliet, & Vereijken, 1996). A viscoelastic dough is formed during mixing, by blending of water and flour in which the mechanical energy introduced by mixer blades is transferred to the sample. Mixing leads to the formation of two phases:

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the continuous, three dimensional gluten phase and immiscible aqueous phase consisting of water soluble compounds and starch (Autio & Laurikainen, 1997; Migliori & Correra, 2013). The Farinograph is an instrument that measures the mechanical resistance of dough, which depends on the rheological properties of dough while mixing a mixture of water and flour to form developed dough (Lee, Ng, Whallon, & Steffe, 2001).

Since rheological properties of dough samples are related to the microstructure of dough samples, rheological measurements are coupled with microscopy and imaging techniques in dough samples (Boitte, Hayert, & Michon, 2013; Dürrenberger, Handschin, Conde-Petit, & Escher, 2001; Jekle & Becker, 2011; Jekle & Becker, 2013; Li, Dobraszczyk, & Wilde, 2004). Boitte et al. (2013) developed a novel system called 'RheOptiCAD' which allows the visualization of microstructures under thermo-mechanical treatments. Several microscopic studies have been carried out to assess the effect of mixing on microstructural characteristics of gluten network using light microscopy (LM) (Autio & Salmenkallio-Marttila, 2001; Kuktaite, Larsson, Marttila, & Johansson, 2005), atomic force microscopy (AFM) (McMaster et al., 1999), transmission electron microscopy (TEM) (Amend & Belitz, 1990) epifluorescence light microscopy (EFLM) (Peighambardoust, Dadpour, & Dokouhaki, 2010) scanning electron microscopy (SEM) (Amend & Belitz, 1990; Bache & Donald, 1998; Calderón-Domínguez, Neyra-Guevara, Farrera-Rebollo, Arana-Errasquín, & Mora-Escobedo, 2003; Khatkar, Barak, & Mudgil, 2013; Kuktaite et al., 2005; Létang et al., 1999; Watanabe, Larsson, & Eliasson, 2002), and laser scanning confocal microscopy (CLSM) (Lee et al., 2001; Li et al., 2004; Upadhyay, Ghosal, & Mehra, 2012).

Isotopic tracers, organic dyes, quantum dots, and other tools have been employed in biolabeling of living tissues for a long time. Currently, organic dyes have been widely used for biolabeling of biological molecules. In food science research, organic dyes such as Rhodamine (Rh), fluoresceinisothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC), light green, and acid fuchsin, are commonly used. Quantum dots are highly luminescent, semiconductor nanocrystals which vary in size from 2 nm to 10 nm and possess roughly 200–10,000 atoms (Sozer & Kokini, 2014). Several drawbacks of using organic dyes as a fluorescent probe are observed such as relatively low quantum yield when coupled with affinity molecules, and considerable photo bleaching resulting in a loss of color brightness. Novel combinations of covalent and non-covalent labeling techniques were advanced to concurrently envision the various components of interest located in phase separated blends of food biopolymers (Van de Velde et al., 2003). Therefore, fluorescent labeling techniques using quantum dots with superior sensitivity and selectivity have been developed for analyzing biomolecules.

The use of polyclonal antibodies conjugated to fluorescent probes offers enormous potential for tracing individual proteins of interest at the ultrastructural level. Wheat storage proteins found in wheat endosperm and in baked and unbaked wheat products were identified using immunohistochemistry techniques (fluorescent dyes labeled polyclonal and monoclonal antibodies) by light microscopy (Ariss, 1986). Confocal laser scanning microscopy (CLSM) delivers several benefits for examining relationships between composition, processing and the final product quality (Blonk & van Aalst, 1993; Kaláb, Allan-Wojtas, & Miller, 1995). It does not require long sample preparation time (Autio & Laurikainen, 1997) and allows scanning of thicker sections (5–10 μm and even more) (Lee et al., 2001). CLSM has the ability to generate blur-free images of thick specimens at various depths (three-dimensional specimen). Jekle and Becker (2011) brought a novel approach in which CLSM combined with image processing and analyzing of micrographs of dough samples using a fuzzy thresholding algorithm was employed to quantitatively determine the microstructure of dough samples to investigate the effects of water addition to flour on microstructure of wheat flour dough CLSM coupled with conjugated quantum dots was recently used in our lab to understand the effect of baking on distribution of gliadins (Ansari et al., 2014).

Even though a number of publications examined structural changes in dough during the mixing process, they focused on the gluten matrix. No study has been documented to investigate the relationships between specific sub fractions of gluten such as gliadin during the dough mixing process. The overall objective of this research was to investigate the distribution and location of gliadin in model wheat flour dough using confocal laser scanning microscopy. In our study, we used an immunohistochemistry approach to visualize gliadins using quantum dots as a result of mixing. Location and distribution of gliadins were investigated. Our study offers a more detailed understanding of the development and networking of gliadin during dough mixing, a very important and critical unit operation to for the cereal industry.

## 2. Materials

Polyclonal anti-gliadin antibody, acetone, phosphate buffered saline (PBS), HiTrap protein G high performance affinity column and prolong media were purchased from Sigma Aldrich (St Louis, MO). Qdot 625 was purchased from Life Technologies and Invitrogen Company. Wheat flour was purchased from a local market in Champaign, IL. (Gold Medal brand, manufactured by General Mills, Minneapolis, MN, approximate protein content 10.5% and approximate carbohydrate content of 79%) Tissue-Tek™ CRYO-OCT Compound, Tissue-Tek cryomolds and TRUBOND 380 microscope slides were acquired from Fisher Scientific (Pittsburgh, PA) through the University of Illinois Institute Of Genomic Biology. Sodium dodecyl sulfate (SDS), coomassie blue, Tris buffer pH 6.8 (1 M), glycine, bromophenol blue, glycerol, glacial acetic acid and methanol were supplied from Fisher Scientific (Pittsburgh, PA). Precision plus protein, Tris–Cl pH 8.8 (1.5 M), Tris–Cl pH 6.8 (0.5 M), 30% acrylamide/Bis solution, immun-Blot PVDF membrane, criterion cell for gel electrophoresis, and criterion blotter with plate electrodes were purchased from Bio-Rad (Hercules, CA). Anti-rabbit HRP secondary antibody, tetramethylethylenediamine (TEMED), and ammonium persulfate were obtained from Thermo-scientific (Pittsburgh, PA). All other chemicals used in experiments were of analytical grade.

## 3. Methods

### *3.1. Preparation of dough samples*

A Farinograph (C.W. Brabender® Instruments, Inc., South Hackensack, NJ) with a 300 g bowl was utilized to prepare the dough samples, and constant flour weight procedure was used (AACC International, 2011a). Dough samples consisted of wheat flour and deionized water. Arrival time (AT), where the curve reached 500 Brabender Units, the peak time (PT), after the hydration peak, departure time (DT) where the curve went below the 500 BU line, and 10 min after departure time were recorded from the Farinogram. The water absorption of wheat flour samples, which shows the amount of water required to fully develop the dough to a Brabender consistency of 500 BU was recorded at peak time of the curve. Additionally AT, PT, DT and 10 min after DT which are characteristic times of a Farinograph mixing profile were also determined. Dough samples were obtained from the Farinograph at each specific mixing time for further studies.

### *3.2. Sectioning of samples for microscopy imaging*

Small pieces of dough samples (0.5 $cm^{3)}$ were taken from the Farinograph bowl at four different mixing times which included arrival time, peak time, departure time and 10 min after departure time and were placed one at a time into a beaker containing 20 ml of heparin solution (500 IU/ml) for 10 min to remove auto fluorescence of dough. The samples were then rinsed with deionized water (DI) and placed into disposable plastic Tissue-Tek cryomolds after their surfaces

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