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Wheat dough microstructure: The relation between visual structure and mechanical behavior

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Abstract

The microstructure of food matrixes, and specifically that of wheat-flour dough, determines mechanical behavior. Consequently, the analysis of such microstructure is both necessary and useful for understanding the physico-chemical and mechanical alterations during the production of cereal-based products such as breads. Confocal laser scanning microscopy (CLSM) is an established tool for the investigation of these matrix properties due to its methodical advantages such as easy preparation and handling, and the high depth resolution due to the optical sectioning of probes. This review focuses on the microstructure of wheat-flour dough from a mechanical and visual point of view. It provides an overview of the dependencies between the visibly detectable microstructural elements achieved by CLSM and the physical determined rheological properties. Current findings in this field, especially on numerical microstructure features, are described and discussed, and possibilities for enhancing the analytical methodology are presented.

Keywords: CLSM, confocal, gluten, rheology, microscopy, image analysis, structure-function relationship

Abbreviations: A, area; A_F, area fraction of all objects; AR, aspect ratio; ØA, average size of all objects; C, circularity; CLSM, confocal laser scanning microscopy; DATEM, diacetyl tartaric acid esters of monoglyceriedes; DDT, optimal dough development time; D_F, (Feret's) diameter; FD, fractal dimension; FITC, fluorescein isothiocyanate; G', storage modulus; G'', loss modulus; G*, complex shear modulus; LM, light microscopy; NA, numerical aperture; M_r,

relative molecular mass; P, perimeter; ΣP, count; S, solidity; SEM, scanning electron microscopy; SME, specific mechanical energy

1. Introduction

Cereal grains have a distinct microstructure that results from their function as storage units for reproductive components during dry periods. However, processes such as milling, mixing, proofing, and baking lead to great changes in the microstructure of proteins, starch, and minor components. In wheat in particular, distinct structural changes occur from grain kernel to dough. The spatial arrangement of the components and their microstructure determines both the macroscopic behavior of any material, and as well of dough (Bloksma, 1990b). It is well known that microstructure is the link between the components' molecular properties and configuration and the product's macroscopic properties. This has been proven by the fact that the rheological and microstructural properties of wheat dough largely predetermine the quality of the baked goods (Wieser, 2007). A comprehensive knowledge of the components' microstructures would offer the possibility to improve products or design specific properties of novel foods. Microscopic techniques enable the acquisition and the investigation of these microstructural features. A wide range of microscopic techniques have already been used to investigate wheat-flour dough microstructures: light microscopy (Autio and Salmenkallio-Marttila, 2001), (environmental) scanning electron microscopy (E)SEM (Roman-Gutierrez et al., 2002), polarizing microscopy, bright field microscopy (Katina et al., 2006), and fluorescence microscopy (Parkkonen et al., 1997).

However, all of these methodologies have some disadvantages, for example light microscopy is dependent on the sample's depth and therefore has limited resolutions (Blonk and van Aalst, 1993) and SEM requires a complex and destructive sample preparation (Chabot et al., 1979). A good alternative is the use of confocal laser scanning microscopy (CLSM). The easy and non-destructive specimen preparation and selective visualization of constituents have enabled CLSM to become a powerful tool in visualization and therefore useful in the interpretation of microstructures. Furthermore, the distinction between constituents is easily achieved through the multiphase microscopy methodology in combination with fluorescence. Thus, CLSM has already been used in numerous applications in material science (Charcosset et al., 2000), cellular biology (Földes-Papp et al., 2003) and food science.

Several outline works and reviews have been done in the field of CLSM (Blonk and van Aalst, 1993; Dürrenberger et al., 2001; Földes-Papp et al., 2003; Kaláb et al., 1995; Laurent et al., 1994). Therefore, the current article does not have the scope to summarize every study which has utilized CLSM to study the properties of cereal products. Instead, this review specifically discusses the often assumed correlations or dependencies between structural features such as fundamental rheology and the visible microstructure of wheat dough. This would be enabled by image processing and analysis whereby microstructure and rheology could be statistically correlated.

2. Wheat dough microstructure

The characteristic microstructure of wheat dough is developed by the hydration of the ingredients and the input of mixing energy. However, hydration alone is sufficient to develop a

kind of dough microstructure (Unbehend et al., 2004) as the main components of wheat flour (starch and gluten) present complex chemical properties due to their composite interactions with water during hydration. In addition, plasticization takes place which leads to more intermolecular space and the subsequent decrease in viscosity and increase in polymer mobility (Slade and Levine, 1993). This means that the microstructural and rheological properties are determined by a) interaction of the components with water, b) their properties, c) the free water phase, and d) the mobility of water. In general, water mobility in food polymer systems shows a distinct effect on structural properties. Furthermore, hydration itself directly modifies the structure of the components, i.e. gluten (Gras et al., 2000). The distribution of the components in developing dough has already been described in early microscopical studies as a system where starch granules are surrounded by a protein phase (Bechtel et al., 1978). More recent studies describe dough as a bicontinuous protein/water-starch system (Breuillet et al., 2002) or even as a three phase system with a protein/water-starch/gas matrix. Due to the water coated starch surface, the starch granules are fused in the continuous water phase (Dürrenberger et al., 2001) and the proteins are located in the space between the water-fused starch.

The observation of the microstructure takes place in the microscopic resolution (0.1-100 µm). This microscopic scale is based on the molecular scale (with hydrogen bonds, disulfide bonds) and the nanoscopic scale (such as high molecular weight glutenins of 50 nm and its orientation). Further, the microscopic scale covers the resolution of (light or laser based) microscopy (Bloksma, 1990a) and gets detectable in the macroscopic scale, the deformation of rheology. Figure 1 shows a simplified overview of the different scales for the observation levels of materials and of dough in particular. For a better understanding of the microstructure's

formation, changes, and interpretation, the main components (gluten and starch) and their contribution to dough's microstructure are further described in the next part.

2.1. Protein

In addition to albumins and globulins, wheat protein mainly consists of gluten (85%). Gluten is composed of monomeric gliadins and polymeric glutenins and is considered to be the main source of the viscoelastic properties of wheat dough (Lindsay and Skerritt, 1999).

Their mobility in acidic-buffer polyacrylamide gel electrophoresis places the **gliadins** in three groups: ω-gliadins, α/β-gliadins and γ-gliadins. The ω-gliadins have the lowest mobility and lack cysteines, α-gliadins show six cysteine residues, from which three intramolecular disulfide bonds can be formed, and γ-gliadins show eight cysteine residues with four intramolecular disulfide bonds. The relative molecular mass (M_r) of gliadins is within 30,000-75,000 (Lindsay and Skerritt, 1999).

Aggregative **glutenins** consist of low molecular weight subunits (LMWGS, M_r 42,000-70,000) and high molecular weight subunits (HMWGS, M_r 60,000-90,000). Most cysteine residues can be found in terminal domains, but some subunits show also residues in the central domain (Lindsay and Skerritt, 1999). The size of HMWGS are considered to have an approximate length of 50 nm and a diameter of 1.8 nm (Shewry et al., 1989).

The **cohesiveness** of gluten is based on non-covalent and, in particular, covalent bonds. The influence of non-covalent bonds can be indirectly described by the variation of ions (Beck et al., 2011a). The covalent bonds are formed by intramolecular and intermolecular disulfide bonds. The intramolecular bonds can be found in gliadins and glutenin subunits and reveal a molecular

stability in protein conformation. Glutenins additionally form intermolecular bonds which determine gluten structure and behavior.

The main components in wheat dough are water and starch; however, mechanical properties arise from protein properties, in particular the **glutenin macro polymer** (GMP, M_r 500,000 - 10 million) (Lindsay and Skerritt, 1999; Wieser, 2007). This is one organizational form of glutenin, which is an indicator for wheat flour quality, and can be properly characterized with network models such as the hyperaggregation model (see 5.1.3) (Hamer and van Vliet, 2000). The GMP reveals three important structural elements: 1) The backbone structure consists of glutenin subunits which are able to form at least two intermolecular disulfide bonds. It seems that HMWGS dimers are the main component of the GMP. Studies on mechanical behavior and treatment as well as stepwise reductions indicate this (Skerritt, 1998; Werner et al., 1992). 2) Glutenin subunits have at least two intermolecular bonds (LMW- and HMWGS subunits). These subunits are bound to y-HMWGS which are part of the HMWGS backbone. Therefore, branching points of gluten occur. 3) LMWGS to terminate the chains at the end of the branches. This structure leads to a model of the GMP, wherein either both HMWGS and LMWGS oligomers or HMWGS alone represents a backbone with branches (Lindsay and Skerritt, 1999).

2.2. Starch

Although wheat starch is regarded just as a filler in wheat dough, at least from a rheological perspective (Bloksma, 1990a), it also has a great impact on dough behavior and, in particular, the end product quality of yeast-leavened bread products. Wheat starch is a carbohydrate with the minor components proteins, lipids, dietary fiber, and ash. Proteins, such as friabilin, can be located on the surface of the granules (Morris, 2002) or in granule channels. Using CLSM and

gel electrophoresis, they can be identified as proteins with enzyme activities such as starch synthase and others (Han et al., 2005; Han and Hamaker, 2002). Wheat starch consists of a mixture of amylose and amylopectin at a ratio of 1:4 (Zhang and Simsek, 2009). During heating amylose molecules are mainly responsible for gelling and film-forming behavior and amylopectin determines crystallinity and gelatinization properties (Maningat and Seib, 2010). The morphology of starch granules reveals a bimodal size distribution of lenticular-shaped A-granules and spherical B-granules with sizes gained by image analysis of 29-34 μm and 8-10 μm, respectively (Wilson et al., 2006).

3. Dough microstructure visual acquisition by CLSM

Microscopy is a well-developed technique for studying the microstructure of wheat dough. Light microscopy was primarily used in the early observation of starch and dough (Burhans and Clapp, 1942), is still being used in current studies (Hug-Iten et al., 1999) or with enhanced and simple methodologies such as the epifluorescence light microscopy (Peighambardoust et al., 2010). Furthermore, electron microscopy, and scanning electron microscopy (SEM) in particular, enabled a better understanding of the construction of the components of dough. The resolution of this technique is considerably higher than that of light microscopes. However, specimen preparation is quite complex and can lead to the formation of artifacts (Aguilera and de Vries, 1999). Chabot et al. reported quite early on that sample preparation leads to a modification of the components (Chabot et al., 1979), thereby complicating any interpretation of structural relevance. Despite its lower resolution, light microscopy has several advantages, like the possibility of investigating samples under environmental conditions or following dynamic

mechanisms such as freezing or heating. A further improvement of light microscopy was the development of confocal laser scanning microscopy (CLSM), also called laser scanning confocal fluorescence microscopy (LSCFM or (CF)LSM) (Földes-Papp et al., 2003) and confocal scanning laser microscopy (CSLM) (Heilig et al., 2009; van de Velde et al., 2002), respectively. The technique bypasses another disadvantage of light microscopy, the sectioning of samples, and therefore resolves issues of contamination or destruction. Due to the use of optical instead of physical/mechanical sectioning, in combination with a fine focus, CLSM has greatly enhanced the applications and benefits of light microscopy. The following sections provide a brief overview of the methodology.

3.1. The confocal system

While conventional light microscopy illuminates a whole object field, the confocal microscopy uses an illumination pinhole whereby a disk shaped object field is illuminated (see Figure **2**). This has three main advantages: the light source is focused on a specified position in the x-, y- and z- position, less stray light is produced, and the contrast and focus of the information is increased. A second pinhole in front of the detector (detection pinhole) also prevents light from lower and higher object planes reaching the photomultiplier of the system. The focus of the image is further improved through the reduction of out of focus blur. Thus, confocal microscopy enables an excellent resolution within the focal plane. The confocal principle is based on the simultaneous focus, on the same focal plane of both the light source and the detector.

In confocal laser scanning microscopes (CLSM) a laser is used as light source. The monochromatic light of the laser in combination with the pinholes allows detect on to be limited to a very small region of the specimen. The sample is scanned point by point by moving the laser

in the x- and y-direction. The light detected by the photomultiplier in the detector is transferred to information and an image is created by the software of the system.

The resolution of light microscopes (LM) depends on the magnification power and numerical aperture (NA) of the objective lens. In combination with the light wavelength λ a finite limit exists beyond which it is not possible to distinguish between two separate points in the objective field. The resolution of light microscopes in combination with oil objectives (NA up to 1.5) can reach about 200 nm with conventional lenses. Confocal systems can improve the lateral resolution by a factor of 1.4 (Blonk and van Aalst, 1993). The numerical aperture mainly determines the resolution. With higher NA, the brightness of the image can be extended; however, further magnification cannot enhance the information. In total, the depth discrimination of the CLSM can be less than 1 µm (approximately minimum 500 nm), and depends, next to the NA, on relation of the objective lens to the detection pinhole: The larger the detection pinhole, the smaller the optical sectioning. At the extreme, the confocal microscope can be used as a conventional scanning system. Dürrenberg et al. (Dürrenberger et al., 2001) stated that maximum penetration ranges up to 150 µm in z-direction; this however can be extended with great technical effort. In summary, CLSM has a submicrometer spatial resolution.

3.2. Confocal modes

The CLSM can operate in different modes:

A) light transmission. Due to complex alignments and the limitation of the method by sample thickness this technique is rarely used.

B) light reflection. The usage of light reflection is recommendable for the investigation of topography and surface structures. The reflected laser light from the sample's surface is collected as a signal. Further, the reflection, which is normally avoided by the use of filters in the microscope, can be enhanced by coating the sample with special additives (Dürrenberger et al., 2001).

C) fluorescent light emission. This application is commonly used in biological and food microscopy. The reflective abilities of the structural elements are, with some exceptions, mostly comparable, and therefore well detectable, and will be further discussed.

3.3. Fluorescence

For the fluorescent mode the analyzed constituents have to show fluorescent characteristics. This can be either by inherent fluorescence (auto-fluorescence) or, for clearer distinction and the multiphase detection of different constituents, fluorescent dyes. Auto-fluorescence in cereal products is mainly caused by polyphenolic compounds such as lignin and the ferulic acid of cell walls (Pussayanawin et al., 1988). Every fluorescent dye has its characteristic absorption and emission spectra of photons and forms covalent and non-covalent bindings respectively. For non-covalent bindings (staining), the dye molecules are spread throughout the sample depending on local accessibility and affinity (van de Velde et al., 2003) and are often absorbed by the constituent. Hydrophobicity is a common main affinity effect for many dyes such as fluorescein isothiocyanate (FITC) and Rhodamine B. Non-covalent dyes are usually dissolved and added to the sample. For covalent dyes (labeling), the immune-technique is often used as it enables a highly specific labeling of considered proteins, therefore, antibodies coupled with fluorescent markers are used. These markers can be detected by the fluorescent microscope. For labeling

carbohydrates a methodology based on the specific affinity of lectins is available (Blonk and van Aalst, 1993). The targeted application of covalent labeling has some advantages compared to staining, especially in multi-phase systems. On the other hand, non-covalent staining shows easier sample preparation and is favorable for single-phase systems as well as proteins in multiphase systems. Different dyes for constituents of wheat dough have been used in recent decades. Table 1 lists commonly used dyes, their target components, and their extinction and emission (or rather detector) properties.

The use of different dyes enables concurrent imaging of different images. The images represent one wavelength intervals of the fluorescent dye and can be combined by overlaying. Therefore, different colors are used to visualize the differently labeled constituents such as protein and starch (Beck et al., 2009; Lynch et al., 2009; Peighambardoust et al., 2006). However, for analyzing the microstructure of the sample, separated image phases and grey scale are preferred.

3.4. 3D structure reconstruction

The most important advantage of CLSM methodology, compared to other microscopic techniques, is the enhanced lateral and axial (in-depth) resolution. Fine stepping controls and the high focus of the microscope enable the acquisition of stacks of images throughout the sample in a single process. The sample is scanned in x-y-direction, and the laser or the sample (microscope table) is moved in z-direction to acquire a z-stack of micrographs. These first vertical virtual-optical sections of the z-direction can be projected upon each other and reconstructed as a three-dimensional (3D) object (Figure 3). This 3D projection delivers a 3D impression of the sample, that can be animated by a specified rotation or can be presented in a slideshow that progresses through the sample. This non-destructive methodology enables insight into the microstructure

and 3D organization of the morphology of materials (Charcosset et al., 2000), biopolymers, including wheat dough in particular (Peighambardoust et al., 2010). In standard wheat dough an acquisition height of around 20-50 µm is realistic.

3.5. Image processing and analysis

Microscopy is a helpful tool for gaining insight into structural features and changes in biopolymers and food matrixes. However, in some cases it is not easy to describe and distinguish between the characteristics. This is especially true if the structural elements are very small, the discrimination between modifications is not possible, or if a number of samples have to be compared. The quantification of the elements has to be enabled to provide information about numerical variations of the microstructure and to obtain objective correlations between the microstructure and other characteristics of the samples. Therefore the images have to be processed and analyzed to establish the relation between the structure and function of biological matrixes. The application of digital image analysis in food systems has been described and discussed recently (Bull, 1993; Flook, 2003; Quevedo et al., 2002; Zheng et al., 2006a, b) and will not be further discussed in this review. The quantified features of objects (polymers or particles) in an image can be: length [m], width [m], (Feret's) diameter D_F [m], perimeter P [m], area A [m^2], average size of all objects ØA [mm^2], area fraction of all objects A_F [%], count ΣP [-], angle (orientation to the x-, y- or z-axis), volume [m^3] as well as shape descriptors such as the aspect ratio AR [-], circularity C [-], solidity S [-] and the fractal dimension FD [-] (see the following equations):

$$\boldsymbol{AR} = \frac{\boldsymbol{major\ axis}}{\boldsymbol{minor\ axis}}\ , \qquad (1)$$

$$C = \frac{4\pi A}{P^2}\ , \qquad (2)$$

$$S = \frac{A}{A_{convex}}\ , \qquad (3)$$

$$FD = \frac{\log(N)}{\log(\frac{1}{r})} \qquad (4)$$

Applications of image analysis in the field of cereal science are discussed later on.

4. Dough microstructure mechanical acquisition

The study of flow and deformation is defined as rheology. With rheological measurements the mechanical properties and the structure of dough can be quantified, the behavior during processing can be characterized and information on the molecular structure and composition can be obtained (Dobraszczyk and Morgenstern, 2003). Rheological tests are accomplished through the application of specified stress or deformation to the dough with subsequent analysis. The reverse method can also be performed. A range of reviews of the rheological behavior and testing possibilities of cereal products is available (Dobraszczyk and Morgenstern, 2003; Faridi and Faubion, 1990; Mirsaeedghazi, 2008; Song and Zheng, 2007). Therefore, only a very brief description of the common rheological dough measurements is provided, divided into empirical (descriptive) and fundamental techniques.

4.1. Empirical rheometry

Empirical measurement systems usually have a poorly defined sample geometry and an uncontrolled or uncontrollable stress and strain status, therefore, the fundamental rheological

parameters such as stress and strain are not applicable (Dobraszczyk and Morgenstern, 2003). The methodology of the tests is normally easy to perform and control, which provides distinct advantages for quality control in industrial applications. However, these techniques reach their limits if stress and strain rates have to be compared in different devices or applications. Furthermore, depending on the fixed device parameters, the output of the test results are often single point variables, whereas the viscoelastic behavior of real dough also depends on strain rates of frequencies of the tests. Therefore, a fundamental acquisition of rheological values is not suitable. Nevertheless, many scientific results have been and being obtained using these methods as they provide easy access to the rheological evaluation of dough. Some empirical methods are Farinograph, doughLab, Mixograph, Extensograph, Kieffer rig devices, as well as starch pasting devices such as Amylograph and Rapid Visco Analyser.

4.2. Fundamental rheometry

Fundamental rheometry uses well-defined physical test parameters which are commonly independent of the size and shape of the sample, and as well of test devices which is the main difference to the empirical rheometry. In addition to the extensional measurements with large deformation often used, fundamental rheological tests also provide both dynamic oscillation measurements and creep and relaxation measurements. The dynamic oscillation test uses sinusoidal oscillating stress or strain with time and measures the response. The test enables the analysis of elastic and viscous moduli as well as complex shear modulus. During the test (in the linear-viscoelastic region) the material or dough is not destroyed and several adaptations of tests (such as strain, frequency, temperature) can be performed. Therefore rheological tests are widely used in the evaluation of cereal dough. However, the oscillation measurements are performed in

deformation conditions which differ from the process conditions of dough handling. Measurement values are typically the shear storage modulus (G'), which defines the elastic part of the sample, the shear loss modulus (G''), which defines the viscous part of the sample, and the complex shear modulus (G*), which defines the rigidity of the sample. The creep and relaxation measurements use constant stress while measuring the following deformation. The deformation is described as compliance (J) and can be interpreted as a model of dampers and springs in series and parallel, and is a combination of the Maxwell and Kelvin model. The combination of these models is represented in the Burger model which is especially used in biological systems to define elastic delay and behavior (Steffe, 1992). During the creep phase a constant stress is applied and the relaxation is subsequently measured without stress. Thereby, a rapid relaxation which is explained with the behavior of small polymer molecules presents in the first few seconds, followed by a slower relaxation which can be associated with the HMW polymers of gluten (Dobraszczyk and Morgenstern, 2003).

The application of small and fundamental (dynamic oscillatory tests and creep and relaxation tests) and large (more or less the empirical tests) deformation measurements to evaluate and predict dough and end product qualities are controversially discussed. Some studies state that small deformation tests are not correlated to dough rheology and end product (Autio et al., 2001; Safari-Ardi and Phan-Thien, 1998) and recommend large deformation tests due to the applied forces during mixing, proofing, and baking (Tronsmo et al., 2003; Van Bockstaele et al., 2008a). However, others found correlations between small deformation tests and dough and product qualities (Miller and Hoseney, 1999; Van Bockstaele et al., 2008b). Taking all issues into account, small deformation tests are more suitable for characterizing the current molecular and

structural status of the network and large deformation tests to describe the mechanical behavior during processing.

5. Dough rheology derived from its visual microstructure

Despite the lower quantity of wheat dough proteins in comparison to starch, proteins play a major role in the mechanical behavior of dough. Furthermore, protein network formation is crucial for gas retention capacity during dough proofing and thus for end product quality. Therefore, this chapter focuses on protein network formation, starting with hydration, the development of GMP, models to describe the network properties, and the ongoing changes during mixing. Following this, changes during processing, and influences due to the addition of ingredients are described. Furthermore, current studies about the possibility of a numerical interpretation of the microstructure are discussed as they are the basis for a comprehensive evaluation and could serve as prediction of properties.

5.1. Formation of dough microstructure

5.1.1. Hydration of flour particles

The formation of a wheat dough protein network is a complex and time dependent process. For a detailed view of the starting point (hydration) of these changes, non-developed dough can be examined. Non-developed dough is prepared by mixing frozen water with flour so that the subsequent melting initiates the hydration process of the flour particles without the input of mechanical energy. Investigations into a non-developed wheat dough revealed large hydrated protein aggregates including some starch granules (Peighambardoust et al., 2005; Peighambardoust et al., 2006). This water-flour mixture mainly exhibited intramolecular

disulfide bonds, which are broken due to shear and extensional deformation in the following mixing process (Lee et al., 2001). The aggregates seems to be distinctly larger (approximately 80 µm, as deduced from a micrograph of the study) than native GMP particles. A study by Unbehend (Unbehend et al., 2004) confirms these results.

5.1.2. Role of the glutenin macro polymer during network formation

The GMP (described in chapter 2.1) are an organization form of glutenins and thus play an important role in the formation and interaction of the protein network. Therefore, the analysis of GMP seems to be a helpful tool for the understanding of the microstructural and mechanical behavior during dough development. The GMP concentration is strongly correlated with the elastic behavior of wheat dough and bread baking performance, specifically volume (Weegels et al., 1996). It is hypothesized that GMP is a gel formed by chemical and physical effects, which exhibits properties of a particle network or aggregated gel (Don et al., 2003a). The changes of GMP structure during mixing and its influence on rheological properties have explored in several studies which are discussed in the following:

The GMP are described as having a diameter of 30-50 µm; however, the mixing process decreases the average size of the GMP particles. At the optimal dough development time (DDT) the particles of different wheat varieties presented the same size. Within the mixing process the particles lost their common shape and became ruptured (Don et al., 2003b). Although the CLSM images showed a distinct particle structure in a water flour system, this structure disappeared and a dispersed system or even a network system appeared after the mixing begun. However, Coulter counter particle size analysis (laser diffraction) showed a clear particle system, with decreasing particle diameters due to mixing. It seems that analysis methodology is an important factor for

the acquisition of results and the finding and interpretation of totally different theories. The particle theory is supported by the study of Lefebvre et al. (Lefebvre et al., 2000) where the gluten network is described as a particle network. Another study presented the theory of soft and deformable colloidal gluten particles which form a network responsible for the mechanical behavior of dough (Lefebvre and van Vliet, 2003). Furthermore, particles which form a network by direct interaction reveal characteristics of a polymeric network during deformations. Therefore it can be concluded that the rheological behavior of dough which follows polymeric theories is based on this network formed by interacting particles. Moreover, Don et al. (Don et al., 2005) investigated an extracted GMP dispersion in a microrheology system in which a probe in a CLSM can be sheared continuously. Spherical glutenin particles were sheared with a low rate whereby the particles became more elliptical and some particle-particle interactions occurred. The effect did not totally disappear after removal of the shear rate. An increased shear rate resulted in a continuous glutenin phase which was stable after removing the shear rate. The relatively elastic behavior of the underlying wheat dough presented low values in an under-mixed dough, the highest values in an optimally mixed dough, and medium values in an over-mixed dough. The optimal dough development time is an important point: particles have already become dissociated and are able to form a continuous network after resting, but still exhibit the ability to re-assemble in their originate states due to the preserved internal chemical structure. The glutenin structure of the under-mixed dough does not form a continuous particle network of which the elasticity remains low. Over-mixing the dough resulted in a changed chemical level of the internal structure and the fragments showed polymer behavior. This means that altered rheological values are determined more by the changes of the internal structure of the particles

and therefore the aggregates formed in level III of the hyperaggregation model (discussed afterwards) (Don et al., 2005). It can be assumed, that this aggregate network in the macroscopic level should be detectable by CLSM.

5.1.3. Network models

Due to the complex properties of the wheat dough protein network several approaches are available to describe these characteristics, and some are presented or extended in the following. The hyperaggregation model defines the network formation of glutenin proteins in three levels (Hamer and van Vliet, 2000): Molecular level (I): only covalent bonds are considered for interactions between HMWGS and LMWGS from which glutenin particles are created. Mesoscopic level (II): Interactions between glutenin particles due to physical aggregations. Macroscopic level (III): particles participate in network formation. These aggregates and the network, respectively, determine the macroscopic dough properties and can be influenced by processing conditions themselves. In the nomenclature of the current review this macroscopic level would be the microscopic scale. Another detailed view on wheat dough properties is by Belton et al. (Belton, 1999). They describe the elasticity in wheat dough using a model of gluten build up using a loop and train structure. Gluten proteins are organized in parallel and are stabilized by non-covalent interactions. If the gluten is stretched firstly the loops are deformed and followed by the trains due to the breaking of the non-covalent bonds. During dough rest, the loop and train equilibrium is restored and the structure of the polymer is enabled to relax.

This is in accordance with a theory of soft and deformable colloidal gluten particles which form a network which is responsible for the mechanical behavior of dough (Lefebvre and van Vliet, 2003). Furthermore, particles which form a network through direct interaction reveal

characteristics of a polymeric network during deformation. Therefore it can be concluded, that the rheological behavior of dough which follows polymeric theories, is based on this network formed by interacting particles.

5.1.4. Further network development during mixing

The monitoring of the microstructure during the development that results from mixing reveals coarse protein domains or unchanged aggregates (Dürrenberger et al., 2001; Peighambardoust et al., 2006). The protein aggregates are formed by physical interaction during dough deformation in the mixing process (Peressini et al., 2008). Gluten macrofibrils are created due to the covalent linkage of polypeptide chains by intermolecular disulphide bonds. This formation of the high molecular weight glutenin polymers leads to the characteristic wheat dough extensibility and gas retention properties (Autio and Salmenkallio-Marttila, 2001). However, especially at the beginning, the protein network is not homogenously distributed, but interfused with clustered areas of mainly starch granules. This effect could be detected in undermixed dough in a z-kneader (Calderón-Domínguez et al., 2003) as well as in a model dough system, composed of potato starch, wheat gluten, and water (Parada and Aguilera, 2011). An increased mixing time reveals a more homogeneous distribution of starch and gluten. This is often described as an extension and thus as protein films. Therefore the visible area of the protein surface increases, the depth decreases and a continuous gluten phase occurs. Dough mixed to its optimum (maximum resistance) exposes a network of interconnected gluten which is evenly interfused by starch granules (Peighambardoust et al., 2010). The described protein matrix with spread starch granules could also be confirmed in the model system mentioned (Parada and Aguilera, 2011). This means that the properties of the single components (gluten and starch) are the most

important factors that form the characteristic microstructure of wheat dough. However, minor components will affect the proportions and the results. In sum, the gluten forms entanglements. Herein, just small areas interact and some separated regions do not show any interaction. This fact explains the elasticity and the possibility of stretching gluten and dough: At the beginning, coiled chains of glutenin subunits are stretched (till breakage of secondary bonds) and afterwards slippage is caused by the entanglement points (Lindsay and Skerritt, 1999).

Long mixing times (however, in the study still in the stability range) lead to a homogeneous protein phase with a very fine structure and elements larger than 100 µm accross all starch granules (Peighambardoust et al., 2006). Further energy input (overmixing) results in a homogeneous dough microstructure with a finely spread gluten network (Peighambardoust et al., 2010). Although this network is widely spread, the network is weakened due to a disruption of the bonds. This means that the detectable proteins are, to a certain extent, more dispersed that connected. This network weakening could result from the breaking up of disulfide bonds during mixing. Peressini et al. (Peressini et al., 2008) analyzed this with the usage of the SH-Blocker NEMI. First, the conformation of the importance of S-S bonds for the formation of gluten domains in dough was enabled. Afterwards, the disulfide bonds broken due to deformation and without their reformation structures known in the meso-scale, which are detectable by CLSM, could not be reformed. This confirmed that mixing influences the molecular level of dough, and that overmixing results in a weakened dough structure. The covalent disulfide bonds mainly affect the aggregate formation whereas non-covalent interactions play a role in post-mixing dough re-assembly.

Figure 4 and Figure 5 show the results of the author's work (not published). Standard wheat flour (50 g) with 31 g water and 0.9 g NaCl was mixed in a Microfarinograph for 3 min at 63 an 75 rpm. CLSM micrographs of dough proteins were taken using Rhodamine B (λ_{exc}=543 nm, λ_{em}=590/50 nm). 25 individual micrographs were combined to obtain micrographs of 1060x1060 µm. The figures reveal the distinct different protein network described already above. The network with increased energy input is spread more widely and distributed homogeneously over the area.

In summary, a large alteration of microstructural properties takes place during dough development which results in different rheological properties. Confocal microscopy supports the understanding of the formation of the protein network during this process.

5.2. Modification of wheat dough microstructure

For the specification of the microstructure of materials different descriptors are available. Some features of objects are listed in chapter 3.5. In the following, the modification of wheat dough microstructure as analyzed by CLSM and the inferred rheological changes are discussed as a function of addition and variation of ingredients and process conditions.

5.2.1. Ingredients

As described above hydration only starts and the development of dough is only made possible with the addition of water to flour. Observations with CLSM on pretzel dough with three different *water contents* showed that, unlike the control and with high water dough, the protein network in low water dough could not form a continuous structure like in. Thus, dough extensibility tests revealed distinct lower extensible measures for the low water dough which

could be a result of suboptimal protein formation. Pretzel dough is typically produced using limited water addition which affects the development of the gluten network (Seetharaman et al., 2004). However, it is not entirely clear how water modifies the dough microstructure. Further studies on the variation of the water content could help to ascertain better insight into the hydration process of flour in relation to the protein microstructure development.

The density of the gluten network in particular has been described in a study of model systems for *wheat sourdough* (Schober et al., 2003). Sourdough is an important possibility for improving the properties of a wide range of products (Jekle et al., 2010). Schober et al. (Schober et al., 2003) investigate the effects of the acidification and, additionally, of sodium chloride on gluten properties with CLSM and fundamental rheology. Wheat dough was prepared and gluten (washed out) was analyzed. The addition of lactate buffer (pH 3.9) lead to a filmlike gluten microstructure (240x240 µm). Detailed fibrils could not be detected. This film structure is thought to be based on an increased solubility of the gluten molecules and a following increased dissolution. The control, the addition of *sodium chloride* (NaCl), and the combination of lactate buffer and NaCl revealed a gluten network composed of fibrils with some empty space in between. The addition of the ingredients could be detected by the rating of the network density. It seems that in the order control < NaCl addition < NaCl and lactate buffer addition the density of the network increased. The high density is explained with the combination of altered net charge of gluten due to the acidification and the shielding of positive charges due to sodium chloride. The density should not be considered as the real density of the proteins, but rather as an impermeability and evenness of the structure. The complex shear modulus as a measure of firmness increased in the same order as the density of the network (Schober et al., 2003). This

means that the density which is related to the spreading and the junctions of the fibrils affects the rheological properties. If these attributes could be formulated in numerical values, a more fundamental cause and effect relationship could be established.

A further interpretation of the micrographs of frozen wheat dough shown by Kenny et al. (Kenny et al., 2001) was done. A comparable effect of heat treated *whey protein* on the gluten network (similar elongated and distributed as affected by ascorbic acid and DATEM addition) resulted in a comparable effect on the storage modulus G' and loss modulus G'' of fundamental rheological tests. Both showed high values with the addition of these additives; whereas, the protein network of dough containing whey protein appears more fragmented and less organized than the control sample. Also in this case the lowest values of the rheological measures G' and G'' can be associated with these results. In the study the addition of dairy ingredients and dough improvers to frozen wheat dough were investigated by fundamental rheology and CLSM. The protein network was visualized after 10 weeks of storage. In summary, the results of the study can be interpreted as such that a less connected protein network both leads to low storage and loss modulus values, and vice versa, and also underlines the connection between visual microstructure and fundamental rheology.

Further, the effect of the addition of *sodium chloride* on wheat dough has been investigated by two current studies. Lynch et al. (Lynch et al., 2009) published 3D images of wheat dough (stained starch and protein) with relatively low NaCl additions. The protein network was described to show larger diameters with increased uniformity of orientation with increasing NaCl. However, no major structural changes were rated. In the study of Beck et al. (Beck et al.,

2011a) higher additions of NaCl were used and binarized micrographs of the protein network were described. The network radically changed its structure, from spread, uniform, but more isolated protein particles at 0 g NaCl 100 g^{-1} flour to longer and larger protein strands with some junctions and branches. This effect is explained by neutralized repulsing forces of amino acids and therefore a more compact protein structure, however, with increased water phase in between. At the same time a significant increase of the complex shear modulus with increasing NaCl addition was analyzed. This means that in this case the large proteins strands lead to an increased stiffness, as well as increased elasticity. A further image analysis of the processed micrographs could lead to a better understanding of this behavior. First steps in this directions were already showed in rye dough, however, without structural elements (Beck et al., 2009).

5.2.2. Mixing process

The distinct effect of mixing on the microstructure of dough has already been described in chapter 5.1.4. Different forms of this process represent greatly varied types and intensities of mechanical energy input such as shear, rotational and extensional deformations and therefore modify the microstructure. Micrographs of GMP extracted from mixed wheat dough (in a z-blade mixer) and simple sheared dough revealed no detectable particles in the mixed wheat dough. However, the sheared dough showed an aggregation of glutenin particles. An explanation would be that the shearing in the experimental setting (30 min) led to an continuous protein network at the beginning (as shown in the native dough micrograph after 15 min) and subsequently afterwards induced aggregation of the glutenin particles due to the shear forces (Peighambardoust et al., 2005). The specific mechanical energy (SME) showed a distinctly higher value in the shearing process of Spring flour (277 to 181 kJ kg^{-1} after 45 min). In

summary dough shearing affects fewer breaks of particular structures of GMP compared to z-mixing where the particles are disrupted (at the same energy input). Another study (Peighambardoust et al., 2006) analyzed the microstructure of dough by CLSM and compared a z-blade mixer and shear induced dough preparation. The shear induced processing led at the beginning to a heterogeneous protein structure (more than some 100 µm), where protein domains and starch granule rich domains could be detected. The direction of the shear flow led to an orientation of these domains. An increased shearing time (up to 45 min) revealed either a breaking up of the larger proteins into smaller fragments and therefore a more homogeneous protein phase or a similarly sized yet still available large protein domain, depending on the used flour (Soissons/Spring flour). These results are distinctly different to those gained in a z-blade mixer. A further study by Peighambardoust (Peighambardoust et al., 2007) used a special device to apply a specific shear. In particular, the flow profile in a cell affected the gluten structure formation. Furthermore, the breaking up of protein domains into a homogeneous network was defined as a time-independent process in the microscopic domain but determined by the shear and the initial size of the protein domains. The dependency on the shear has been shown in a study using different shear rates (Peressini et al., 2008). The large protein domains gained by dough at low shear rate were not analyzed with increasing shear rates, due to fracturing. It was therefore assumed that these structures are quite weak.

5.3. Numerical interpretation using image analysis

The quantification of the structural elements of images is necessary to indicate structural alterations based on processing or ingredient variation on any resolution scale (Kaláb et al., 1995). Furthermore, the comparison of a large number of different samples is enabled. Only with

the gained numerical structure parameters is an objective correlation of the microstructure with rheological and other analysis methods possible. Due to the very limited studies in the field of wheat dough in combination with CLSM, a wider range of studies is discussed.

An important application of image analysis has been done by Peighambardoust et al. (Peighambardoust et al., 2006). They investigated the detectable area fraction of *proteins* (visualized by CLSM) *during the mixing process*. The mixing of flour and water initially led to a significant decrease of the protein area fraction compared to non-developed dough. The previously described formation of aggregates can be used to explain in this result. A further increase in mixing time revealed a larger protein area which can be related to a re-distribution of the proteins as the author states, or be an effect of stretching of the protein network and therefore a more filmlike structure. Furthermore, they relate the increase of the area fraction to a more homogeneous protein structure and therefore to a loss of larger gluten structures whereby a reduction of viscoelasticity could occur. However, they evaluate the current use of image analysis in this study as insufficient for comprehensive understanding of the dough development processes.

Schluentz et al. (Schluentz et al., 2000) showed differences in the *protein matrix of developed, partially developed and nondeveloped wheat dough*. The developed dough had the most detectable protein matrix (analyzed with SEM). The disadvantages of SEM (as sample preparation) were avoided by Lee et al. (Lee et al., 2001) who used CLSM for comparable experiments. Additionally, they used partially developed dough, created with shear or extensional deformation in a rheometer. The development of the dough resulted in an increase in

the complex shear modulus G*. Partially developed dough shows higher stiffness due to preparation (in a rheometer) with extensional deformation than with simple shear deformation. Furthermore, they analyzed the detectable protein matrix using the percentage of pixels with high gray scale values and revealed significant differences (nondeveloped dough < shear deformation < extensional deformation < developed dough). The protein matrix varied from around 11 to 40% of the total area. It could be deduced that the strength of the dough is directly correlated with the amount of protein matrix in the dough microstructure. The formation of the protein network is related to the type of deformation and on the total energy addition.

5.4. Numerical interpretation using image analysis in other dough systems

As the current field of application of image analysis in combination with CLSM in wheat dough systems is very limited, image analysis of the microstructure of rye dough systems is briefly described and some other examples summarized.

Rye dough microstructure was studied with the aid of image analysis, whereby rye dough has strongly deviating mechanical behavior and therefore different processing necessities compared to wheat dough (Beck et al., 2011b). Parkkonen et al. (Parkkonen et al., 1997) used fluorescent microscopy to dye cell walls in rye dough, analyzed the cell wall area, and compared these values with the falling number which is related to gelatinization properties during heating and α-amylase activity. It could be shown that dough with a high number of cell walls lead to high falling numbers which was related with rigid, stable dough. Another study on rye analyzed the effect of Transglutaminase, which catalyzes the formation of protein cross-links, on rheological and microstructural properties (Beck et al., 2009). They analyzed rye dough treated with different concentrations of the enzyme by the use of the protein size and the number of protein

fractions. Standard rye dough showed small fragments of proteins without a coherent structure. However, enzyme addition leads to linkages of rye proteins which elongated the protein structures. Decreasing bread volume upon a specified enzyme concentration was explained by a too strong protein aggregation.

Further related methodologies were applied in studies of gelatinization of starch (Srikaeo et al., 2006), starch morphologies (Nagano et al., 2008; van de Velde et al., 2002), correlation of differential scanning calorimetry with starch morphologies (Schirmer et al., 2011), β-Glucan distribution (Fulcher et al., 1994), and noodle proteins (Fardet et al., 1998).

6. Conclusions and future trends

Confocal laser scanning microscopy has shown to be a powerful tool for the apparent investigation of materials in food matrixes. Knowledge about not only the chemical reactions involved but also the structure of dough on a micrometer scale is the basis for a target-orientated adjustment of processes in the baking industry. However, in order to modify and create specific microstructures and product properties it is first necessary to understand structure-function relations. The current studies in this field already promote knowledge about the relationship between microstructure and rheology. A particle-strand based time- and energy dependent network formation is the basis for these properties. A spread gluten network leads to increased stiffness and dough elasticity. This spread network is defined by a continuous structure, with elongated and distributed gluten strands. It can be concluded that gluten is less a throughout or continuous film, but consists of a high number of strands in which other particles (as starch granules) are embedded, which leads to an appearance of a film.

If these attributes could be formulated in numerical values, a more fundamental structure-function relationship could be established. Initial investigations have been undertaken by several authors; however, more work is necessary for the establishment of structural measures and especially the statistical correlation with other measures to reach this aim. Established tools such as threshold algorithms in combination with the application of the analyzing measures described above (chapter 3.5) could serve as the tool to prove this structure-function relationship and investigate the relevance of the protein network for the dough and the end product characteristics.

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Figure captions

Figure 1: Scales of material observations. The molecular scale with disulfide bonds and non-covalent bonds between proteins, the nanoscopic scale with the orientation of the protein strands, the microscopic scale with the protein network, and the macroscopic scale exemplarily represented with an extensional test.

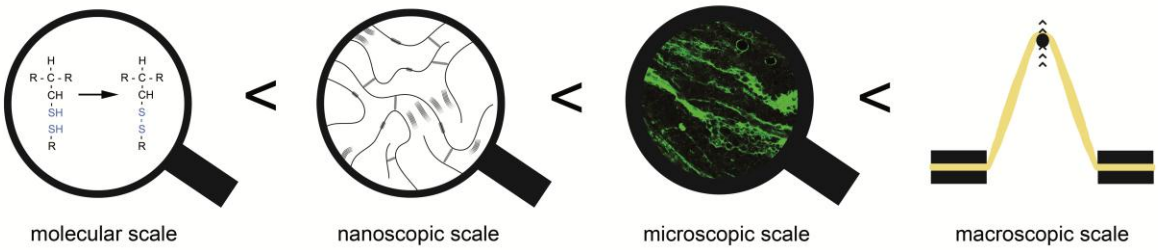

Figure 2: Path of laser beam in a confocal laser scanning microscope. The laser beam is focused on a specific area of the sample and fluorescence is excited. This fluorescent radiation is directed, via the dichromatic mirror, to the detector. Emission coming from planes below or above the focal plane (dashed lines) is out of focus, cannot pass the detection pinhole and is not included in the image.

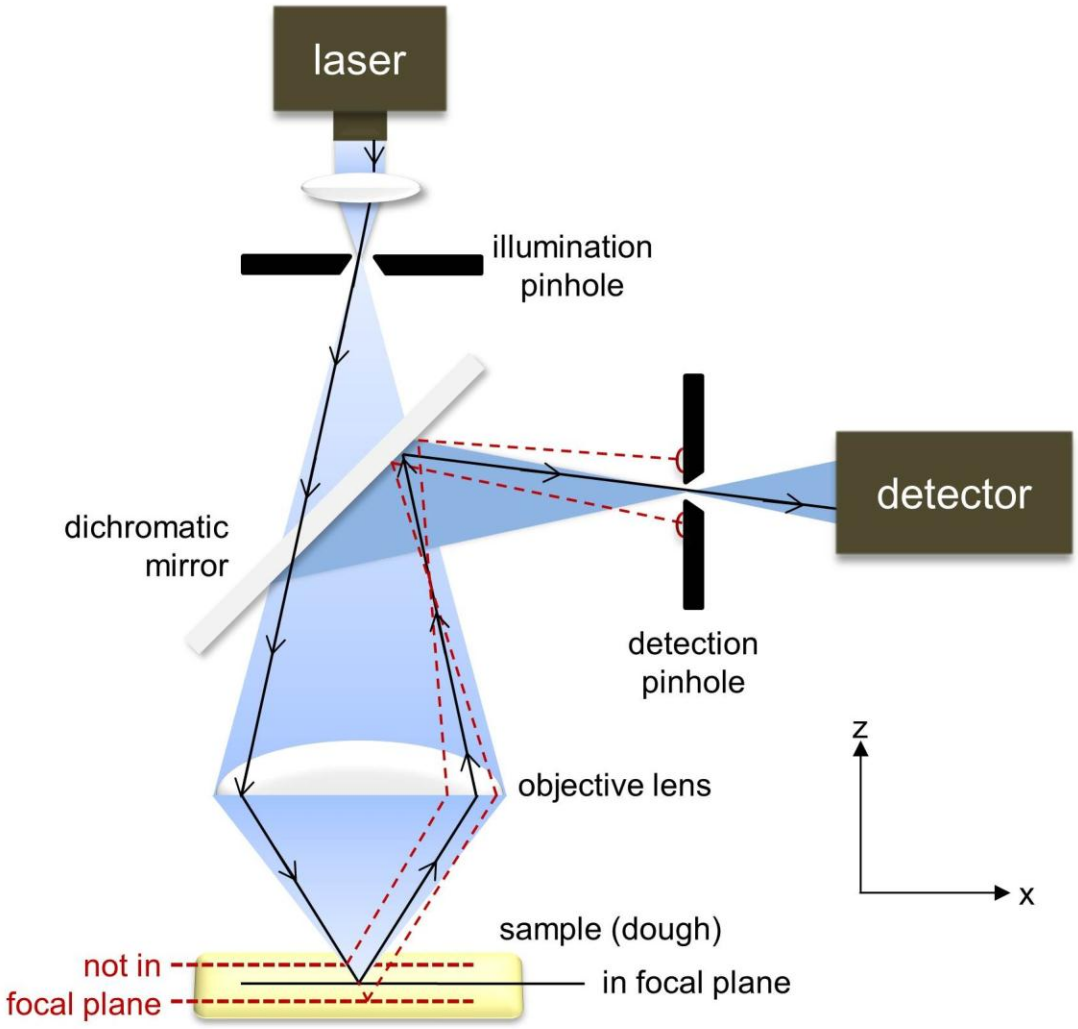

Figure 3: Optical sectioning (z-stack) and 3D reconstruction of a wheat dough sample (protein is white/green).

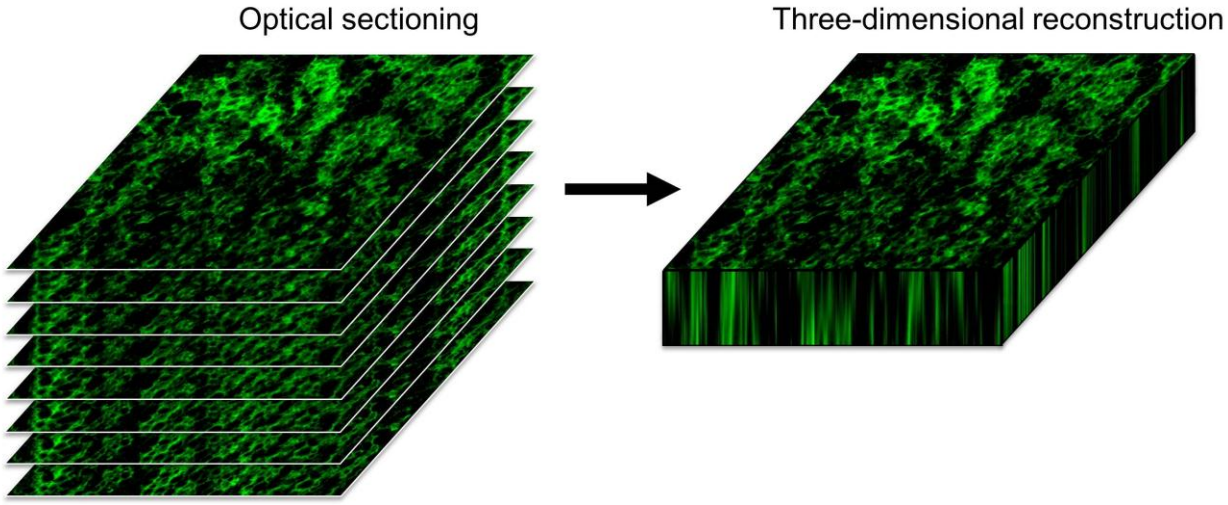

Figure 4: Wheat dough mixed with 63 rpm in a Microfarinograph. Protein was stained with Rhodamine B (gray). The large micrograph has 1060x1060 µm, the zoomed, upper micrograph has 212x212 µm (with increased saturation).

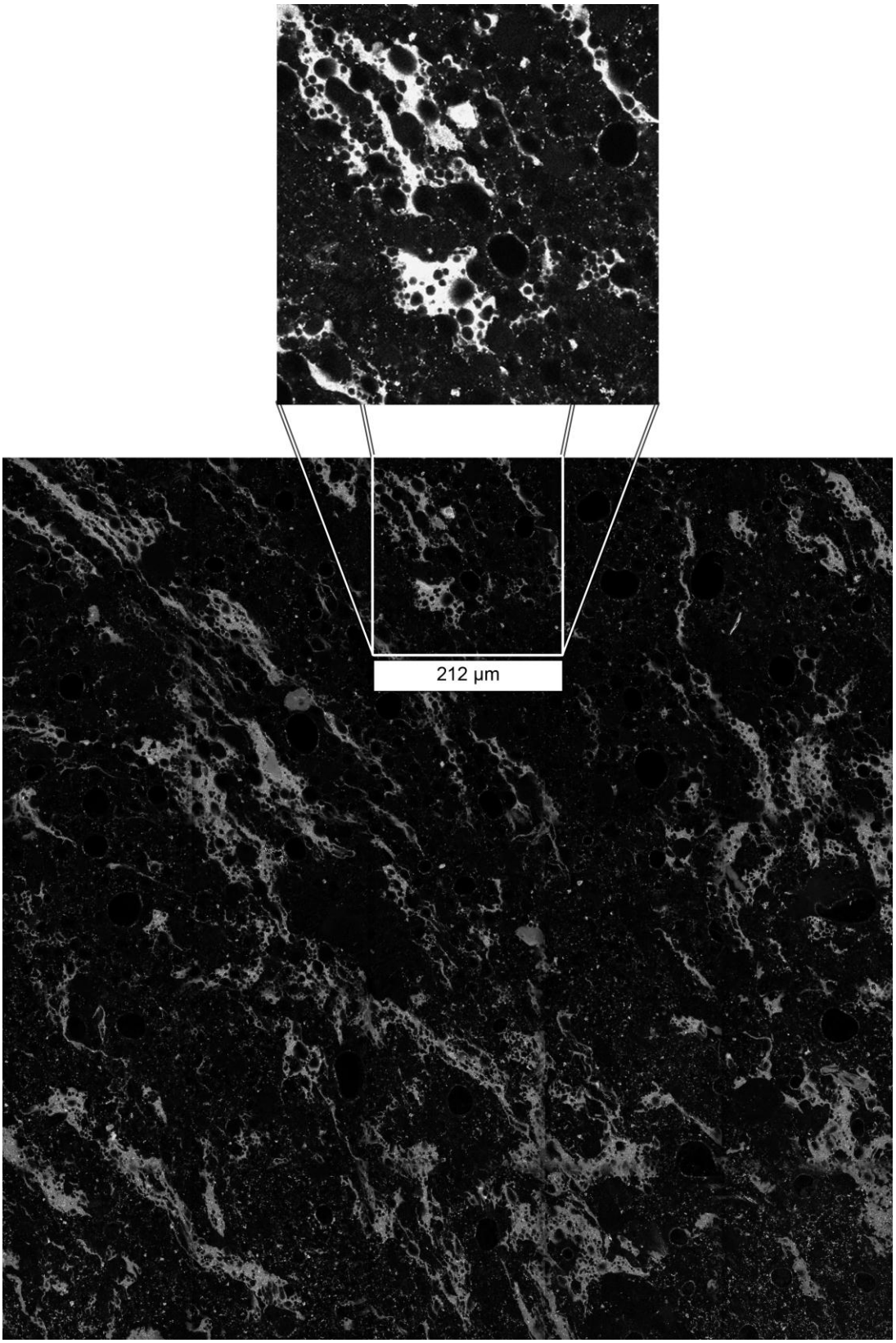

Figure 5: Wheat dough mixed with 75 rpm in a Microfarinograph. Protein was stained with Rhodamine B (gray). The large micrograph has 1060x1060 µm, the zoomed, upper micrograph has 212x212 µm (with increased saturation).

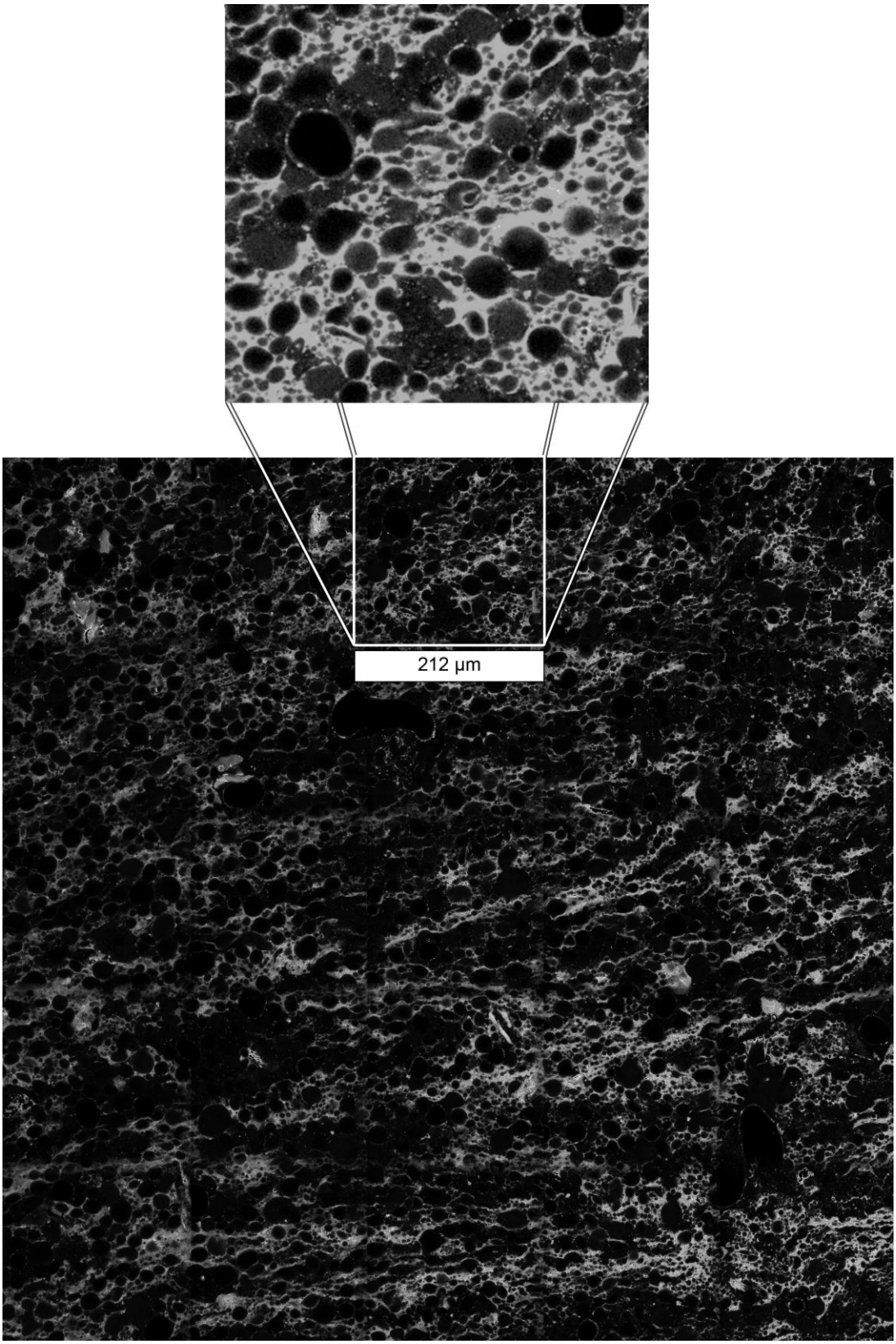

Table captions

Table 1: Commonly used dyes with their properties (as specified in the study) and target components in wheat dough.

Table 1

dye	*target component*	*laser wavelength [nm]*	*detector wavelength [nm]*	*Parallel use with other dye*	*study*
Acid Fuchsin	protein	568	600-620	no	(Dürrenberger et al., 2001)
8-aminopyrene-1,3,6-trisulfonic acid, trisodium salt (APTS)	starch	488	490-560	no	(Naguleswaran et al., 2011)
fluorescein isothiocyanate (FITC)	starch	488	518	Rhodamine B for protein	(Peighambardoust et al., 2006)
	starch	488		Rhodamine B for protein	(Parada and Aguilera, 2011)
	protein	488	520-560	no	(Lee et al., 2001)
	protein			no	(Don et al., 2005)
Nile Blue	protein and starch	633	670-810	no	(Kenny et al., 2001)
Rhodamine B	protein	568	625	FITC for starch	(Peighambardoust et al., 2006)
	protein	543	590/50	no	(Beck et al., 2011a)
	protein	568		FITC for starch	(Parada and Aguilera, 2011)
	protein	568	585 LP[a]	no	(Weegels et al., 2003)
Safranin O	starch	488	530-550	no	(Dürrenberger et al., 2001)

[a] LP= long pass filter