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Staining methods for dough systems – Impact on microstructure and functionality

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ABSTRACT

The staining of dough systems with fluorescent dyes is crucial for a specific visualization of structural components by the confocal laser scanning microscopy (CLSM). However, there is no comprehensive study published until now, if the staining method or the dye itself influences the microstructural formation, and if a realistic visualization can be ensured. Therefore, three common staining methods (drop-, bulk water- and rapid freezing technique) were analysed with varying concentrations of the dye Rhodamine B on their effect on protein microstructure and dough rheology. Rheological results showed significant differences ($p < 0.001$) to a standard, unstained dough when using drop- or rapid freezing technique resulting in a lower stiffness (loss of 12% and 17%, respectively). Protein network analysis revealed no significant differences in microstructure with increasing dye concentrations, but between the three staining methods. Thus, the dye itself did not affect dough microstructure or functionality by interactions on the structure. However, a change in micro- and macrostructure of dough was identified for drop- and rapid freezing technique. In contrast, the addition of dye by bulk water had no influence on dough microstructure and rheology. Concluding, microstructures of wheat dough can be analysed in a realistic, non-invasive way by bulk water technique.

1. Introduction

Visualization of wheat dough microstructure is crucial to extend knowledge about structure-function relationships and interactions of various dough ingredients. For this purpose, confocal laser scanning microscopy (CLSM) is an appropriate method for a direct and non-invasive visualization of dough microstructure. For this method, a staining procedure with a fluorescent dye is essential to highlight ingredients specifically. However, many different approaches for the staining of dough samples are carried out in literature without questioning a potential effect of the dye itself or the staining procedure on the formation of structural components in dough.

One of the most common staining methods for the CLSM measurement consists of a freezing step, which is discussed to have an effect on the microstructure of dough (Berglund, Shelton, & Freeman, 1991; Ribotta, Leon, & Anon, 2001). Samples were either rapidly frozen with liquid nitrogen (Hesso et al., 2015; Maeda et al., 2013; Peighambardoust, van der Goot, van Vliet, Hamer, & Boom, 2006) or frozen in a cryo-microtome to fix the structure (Bousquieres, Deligny, Riaublanc, & Lucas, 2014; Dürrenberger, Handschin, Conde-Petit, & Escher, 2001). Thereby, dough samples were sliced with a microtome in order to receive a flat surface, which is needed for microscopy. Afterwards, dough slices were defrosted and incubated with a defined (Bousquieres et al., 2014; Hesso et al., 2015) or non-defined (Lee, Ng, Whallon, & Steffe, 2001; Parada & Aguilera, 2011; Peighambardoust et al., 2006) amount of specific dye solutions to stain proteins, lipids or starch. However, none of the studies dealt with the impact of freezing on dough properties. In this case, microstructures of wheat dough might not be visualized in a realistic way. Especially the protein network formation is discussed to be altered after freezing due to depolymerisation of the proteins (Ribotta et al., 2001). Even if Baier-Schenk et al. (2005); Maeda et al. (2013) and Hesso et al. (2015) mentioned that the impact on the dough structure is kept to a minimum by using rapid freezing, none of the studies published a quantitative proof on a microscopic level.

Another alternative to the freezing method is to analyse fresh doughs by applying a specific drop volume of a dye solution on the samples' surface after the dough preparation (drop technique) (Döring, Nuber, Stukenborg, Jekle, & Becker, 2015). A further staining variety is the bulk water technique. Hereby, the dye solution is added into the bulk water during mixing to ensure a homogeneous distribution in the dough (Jekle & Becker, 2011, 2012; McCann & Day, 2013). Some studies quoted that the interaction of dyes with ingredients (covalent/non-covalent labelling) does not influence the functionality or rheological

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properties (Jekle & Becker, 2012; Tromp, van de Velde, van Riel, & Paques, 2001). However, the dyes may influence the structural formation due to hydrophobic interactions. Furthermore, Dürrenberger et al. (2001) mentioned that the staining methods, in general, may cause swelling or solubilisation of the components. Nevertheless, no comprehensive study has been published about the effect of neither the dye itself nor the staining procedure in detail qualitatively or quantitatively until now.

Therefore, the influence of the dough preparation for three staining methods (rapid freezing-, bulk water- and drop technique) and of the dye itself on the rheological and microstructural properties of the wheat dough were comparatively explored in this study. Analysis were done with different concentrations of the fluorescent dye Rhodamine B. It was selected due to its affinity to proteins, which are the main structural component in wheat dough, and due to its common use in literature (Baier-Schenk et al., 2005; Bernklau et al., 2017; Jekle & Becker, 2011; Maeda et al., 2013). A standard wheat dough was treated with different concentrations of dye solutions by the three staining methods and analysed by rheological measurements in order to elucidate effects on dough functionality. In addition, the microstructure of dough samples were characterized quantitatively by a novel image analysis, called protein network analysis (PNA) (Bernklau, Lucas, Jekle, & Becker, 2016). Thus, a staining procedure for a visualization of dough microstructure as realistic as possible should be evaluated.

2. Experimental

2.1. Dough preparation

Wheat dough was prepared with German commercial wheat flour Type 550 (Rosenmühle, Landshut, Germany). According to methods of the AACC international (AACCi) and of the International Association for Cereal Science and Technology (ICC), moisture content was 13.29 ± 0.02 g/100 g flour (AACCi 44-01), protein content 11.80 ± 0.03 g/100 g dry flour (AACCi 46–16, N x 5.7), ash content 0.62 ± 0.01 g/100 g dry flour (ICC 104/1), wet gluten content 29.11 ± 0.49/100 g flour (ICC 155), and a falling number of 446 s (AACCi 56-81) was determined. The required kneading time for standard wheat dough was estimated by the water absorption and by the targeted resistance of 500 Farinograph units in a Z-kneader (doughLAB; Perten Instruments, Germany) according to AACCi method 54–70.01. To reach these demands, dough was prepared with 49.41 g wheat flour and 29.12 mL demineralized water, and kneaded for 180 s at 63 rpm.

2.2. Staining of dough samples by drop technique

The prepared standard dough was stained with Rhodamine B (Merck KGaA, Darmstadt, Germany, 0.1 g/L water) in order to visualize proteins with the CLSM. The dye was added after the dough preparation by applying one drop of dye solution onto the dough surface (called drop technique). The impact of ten different drop volumes (1, 2, 3, 5, 7, 10, 15, 20, 50 and 100 µL, equals 0.04–4.41 µg Rhodamine B/cm^2 dough surface) on dough rheology and of five different drop volumes (3, 7, 10, 15 and 20 µL, equals 0.13–0.88 µg Rhodamine B/cm^2 dough) on dough microstructure were analysed. For CLSM, a lower dye concentration would not be sufficient to stain the whole protein network; a higher concentration than 0.88 µg/cm^2 dough would not fully seep in the dough and would form a water layer on the dough surface. That would be inappropriate for CLSM measurement. For rheological analysis, a larger range of dye concentration was analysed in order to test limits. The stained dough was placed between the plates of the rheometer and incubated for 10 min. For microscopic analysis, dough was transferred to an object carrier before staining, cut with a razor blade to a plane surface and sealed afterwards with an object slide to prevent the dough from air-drying. After 10 min of incubation time, samples were analysed with CLSM. All measurements were performed in triplicate.

2.3. Staining of dough samples by bulk water technique

The bulk water technique was performed by replacing a part of the bulk water with the Rhodamine B solution (0.1 g/L water) and adding it during kneading to the dough. Thus, a homogeneous distribution of dye in the dough was achieved. Dough was transferred to an object carrier, cut with a razor blade carefully to achieve a plane surface and sealed afterwards with an object slide. The impact of six different dye concentrations (0.1, 0.2, 0.6, 1.0, 2.0 and 3.0 mg/100 g flour) on dough was analysed with CLSM and rheometer in triplicate.

2.4. Staining of dough samples by rapid freezing technique

The staining of dough for the rapid freezing technique was performed as described for the bulk water technique with the same dye concentrations. Afterwards, dough was rapidly frozen with liquid nitrogen and cut with a microtome in order to achieve a plane surface, which is required for microscopy. After defrosting for 1 h, dough samples was analysed with CLSM and rheometer in triplicate.

2.5. Dough rheology measurements

All stained samples were analysed by rheological measurements. An AR-G2 rheometer (TA instruments, New Castle, USA) with parallel cross-hatched plates (Ø 4.0 cm) to prevent slipping, a constant gap of 2.0 mm and a smart swap Peltier plate temperature system (30 °C constant temperature during measurement) were used for the determination of the viscoelastic properties of dough. Oscillatory frequency sweep and creep-recovery test were performed as described in Bernklau et al. (2016). Results were evaluated by the complex shear modulus G* and the creep compliance J_{max}.

2.6. Microstructure analysis

CLSM measurements of the stained dough samples were performed by an eclipse Ti-U inverted microscope with an e-C1 plus confocal system (Nikon GmbH, Düsseldorf, Germany) with a Plan Apo 20×/0.75 objective and a 534 nm laser (emission 590/50 nm). Eight independent images (1024 × 1024 pixel, 686 × 686 µm) recorded on the x-y-plane were taken of each dough sample.

2.7. Protein network analysis

Image analysis was performed with protein network analysis (PNA) according to Bernklau et al. (2016) with AngioTool64 version 0.6a (National cancer Institute, National Institute of Health, Maryland, USA). The calibration was set to 1.49 pixel/µm, vessels diameter (implies protein diameter) to 2 and 3, intensity low and high threshold to 15 and 255, small particles were removed under 10 and the function “fill holes” was deactivated. The protein network was evaluated by the attributes branching rate, end-point rate, lacunarity, protein width, average protein length, protein area and junctions’ density. A detailed description of these attributes can be found in Bernklau et al. (2016).

2.8. Surface tension of dye solution

The surface tension of the Rhodamine B solution (0.1 g/L water) compared to the standard (distilled water) was measured by the pendant-drop-method with a Drop Shape Analyzer (DSA25E, Krüss GmbH, Hamburg, Germany). For this purpose, the density of the Rhodamine B solution was measured with a pycnometer. A needle with a diameter of 1.8 mm was used to achieve a pendant drop. The surface tension was determined of the drop images with the Advance software version 1.3.1.0 (Krüss GmbH, Hamburg, Germany) based on the Young-Laplace equation.