TECHNISCHE UNIVERSITÄT MÜNCHEN

TUM School of Life Sciences

Lehrstuhl für Brau- und Getränketechnologie

Wheat dough microstructure – Elucidation and prediction of dough functionalities by protein network analysis

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Vollständiger Abdruck der von der Fakultät TUM School of Life Sciences der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

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Prüfer der Dissertation:	1. Prof. Dr. Thomas Becker
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Die Dissertation wurde am 20.05.2020 bei der Technischen Universität München eingereicht und durch die Fakultät TUM School of Life Sciences am 13.10.2020 angenommen.

Acknowledgments

I would like to thank all people who made this thesis possible and a unique experience for me.

First, I would like to express my sincere gratitude to my supervisor Prof. Dr. Thomas Becker for his helpful advice and encouragement throughout the course of this thesis. I thank him for continuously challenging me, for his valuable guidance and scientific training.

I also wish to thank the examining committee, the chairperson Prof. Dr. Michael Rychlik and the examiner Prof. Dr. Jochen Weiss, for accepting this responsibility, as well as for their time and effort in reviewing the thesis.

My deepest gratitude is addressed to Dr. Mario Jekle. He supported me throughout the entire duration of my graduation, gave me constructive advice and encouraged me to critically examine my research in numerous scientific discussions. Thank you for always making time for me and for being such a great group leader and promoter.

I would like to thank all members of the Chair of Brewing and Beverage Technology, in particular all my colleagues of the Research Group Cereal Technology and Process Engineering for supporting me not only as colleagues but also as friends and making the time at the chair unforgettable. Especially Stefanie Hackenberg, thank you for our scientific discussions, fun times in the lab and office and your loyal friendship. Further thanks go to all my students, who have made a valuable contribution to this work.

Special thanks go to my family and friends, especially to my parents and sisters, who encouraged, supported and believed in me all my life.

I cannot begin to express my greatest and most heartfelt thanks to my husband Lars, who supported me through all these years, contributed with his extensive knowledge to this work and motivated me with all his love. I very much appreciate your patience and your profound belief in my abilities. You encourage me to reach my goals and bring joy into my life every day.

Preface

Isabelle Lucas (née Bernklau)

Food Chemist

The results and publications of this thesis were produced at the Technical University of Munich, Chair of Brewing and Beverage Technology, Research Group Cereal Technology & Process Engineering from 2013 to 2017.

Publications

The following peer reviewed publications (shown in chronological order) were generated in the period of this work. Publications, which are part of this thesis, are in bold. The cumulative impact factor for 2018/19 is 22.148 (14.468 for the publications of this thesis).

1. Bernklau, I., Lucas, L., Jekle, M., Becker, T.: **Protein network analysis - A new approach for quantifying wheat dough microstructure.** Food Research International 89 (1) (2016), 812-819.
2. Bernklau, I., Neußer, C., Moroni, A., Gysler, C., Spagnolello, A., Chung, W., Jekle, M., Becker, T.: Structural, textural and sensory impact of sodium reduction on long fermented pizza. Food Chemistry 234 (2017), 398-407.
3. Lucas, I., Stauner, B., Jekle, M., Becker, T.: **Staining methods for dough systems – Impact on microstructure and functionality.** LWT-Food science and technology 88 (2018), 139-145.
4. Lucas, I., Becker, T., Jekle, M.: **Gluten Polymer Networks – A Microstructural Classification in Complex Systems.** Polymers 10 (6) (2018), 617.
5. Lucas I., Petermeier H., Becker T., Jekle, M.: **Definition of network types – Prediction of dough mechanical behaviour under shear by gluten microstructure.** Scientific Reports 9 (2019), 4700.
6. Verbauwhede, A.E., Lambrecht, M.A., Jekle, M., Lucas, I., Fierens, E., Shegay, O., Brijs, K. and Delcour, J.A.: Microscopic investigation of the formation of a thermoset wheat gluten network in a model system relevant for bread making. International Journal of Food Science & Technology 55 (2) (2020), 891-898.

Content

Abbreviations

AACCi	AACC international
ANOVA	one-way analysis of variance
CLSM	Confocal laser scanning microscopy
FITC	Fluorescein isothiocyanate
HMW-GS	High molecular weight glutenin subunits
LMW-GS	Low molecular weight glutenin subunits
M_r	Relative molecular weights
MSM	Mechanical starch modification
MW-GS	Molecular weight glutenin subunits
NaCl	Sodium chloride
PCA	Principal component analysis
PLS	Partial least squares
PNA	Protein network analysis
S	Sulphur
SDF	Soluble dietary fibre
SDS-Page	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TPA	Texture-profile-analyser
λ_{em}	Emission wavelength
λ_{exc}	Excitation wavelength

Summary

The improvement of the quality of wheat-based products requires a distinct understanding of structure-function relationships. Since the haptic and physical quality (e.g., texture, volume, gas-holding capacity) of wheat products depend mainly on properties of the gluten network, its investigation on a microstructural level (the only possibility to cover the spatial arrangement) is crucial to elucidate structure-function relationships. In this thesis, an approach was found to visualise and quantify most appropriately the complex microstructure of gluten network in wheat dough. In this regard, a novel image analysis, called protein network analysis (PNA), was developed which allows characterising the connectivity and strength of gluten microstructure by morphological (lacunarity) and structural network attributes (branching rate, end-point rate, protein width and average length). An additional mandatory requirement for gluten analysis is a most realistic visualisation of the microstructure. This was achieved by confocal laser scanning microscopy (CLSM) and by identifying the staining method "bulk water technique" as the method with no impact on gluten network formation or dough functionality. These developed techniques formed the basis for further studies of different network properties and structure-function relationships. Molecular alterations in gluten polymers were evoked by specific (enzymatic, chemical) and unspecific (plasticising) gluten-modifying agents in various concentrations. These alterations were analysed by microstructural investigations for the first time and detected precisely by PNA. These alterations resulted in a large variety of different gluten network arrangements, which were classified in six characteristic network types by principal component analysis and were associated to their typical rheological behaviour: a cleaved (low viscous), rigid (highly viscous), spread (viscoelastic), strengthened (viscoelastic), particulate and dense (highly viscous) or particulate and loose (low viscous) network. A seventh network type (dense network, low viscous) was proposed later on in the discussion of this thesis. The definition of network types was created as a guiding schema to support the interpretation of any forthcoming microstructural analysis and to enable predictions of dough functionality. Moreover, the mechanical behaviour of specific gluten-modified dough samples was predicted by mathematical formulas based on microstructural PNA attributes with an accuracy up to 90% using partial least square regression. In conclusion, the present thesis provides the basis for upcoming studies of fundamental structure-function relationships. The protein network analysis and the transfer of the defined network types on gluten microstructures provide new possibilities for elucidating and predicting wheat dough functionalities and product quality.

Zusammenfassung

Die Optimierung der Qualität von Weizen-basierten Produkten erfordert ein umfassendes Verständnis von Struktur-Funktionsbeziehungen. Da die haptische und physische Qualität (wie Textur, Volumen, Gashaltevermögen) von Weizenprodukten vor allem von den Eigenschaften des Glutennetzwerks abhängt, ist dessen Untersuchung auf mikrostruktureller Ebene (als einzige Möglichkeit die räumliche Anordnung zu erfassen) zur Aufklärung von Struktur-Funktionsbeziehungen essentiell. Daher wurde in dieser Arbeit eine Möglichkeit geschaffen, die komplexe Mikrostruktur des Glutennetzwerks im Weizenteig am treffendsten zu visualisieren und in neuer Weise zu quantifizieren. Hierfür wurde eine neuartige Bildanalyse – die Proteinnetzwerkanalyse (PNA) – entwickelt, mit der nun die Konnektivität und der Zusammenhalt der Gluten-Mikrostruktur sowohl durch morphologische (Lückenhaftigkeit) als auch durch strukturelle Netzwerkattribute (Verzweigungsrate, Endpunktrate, Proteinbreite und durchschnittliche Länge) charakterisiert werden kann. Eine wichtige Voraussetzung für die Glutennetzwerkanalyse ist eine möglichst reelle Visualisierung der Mikrostruktur. Dies konnte mittels konfokaler Laser-Scanning-Mikroskopie (CLSM) durch die Identifizierung der Färbemethode "Schüttwassertechnik" als Verfahren ohne Einfluss auf die Glutennetzwerkbildung oder auf die Teigfunktionalität erzielt werden. Diese beiden neu entwickelten Methoden bildeten die Grundlage für die weitere Untersuchung verschiedener Netzwerkeigenschaften und Struktur-Funktionsbeziehungen. So konnten molekulare Veränderungen in Glutenpolymeren, welche durch spezifische (enzymatische, chemische) und unspezifische (plastifizierende) Gluten-modifizierende Agentien in verschiedenen Konzentrationen hervorgerufen wurden, anhand mikrostrukturelle Untersuchungen erstmals analysiert und durch die PNA präzise erfasst werden. Diese Veränderungen führten zu einer Vielzahl an verschiedenen Glutennetzwerkanordnungen, die durch die Hauptkomponentenanalyse in sechs charakteristische Netzwerktypen eingeteilt und mit ihrem typischen rheologischen Verhalten in Relation gesetzt werden konnten: ein gespaltenes (niederviskoses), starres (hochviskoses), verteiltes (viskoelastisches), gestärktes (viskoelastisches), partikuläres und dichtes (hochviskoses) oder partikuläres und loses (niederviskoses) Netzwerk. Ein siebter Netzwerktyp (dichtes Netzwerk, niedrigviskos) wurde später in der Diskussion dieser Arbeit vorgeschlagen. Die Definition der Netzwerktypen wurde als Leitschema erstellt, um die Interpretation zukünftiger Mikrostrukturanalysen zu

unterstützen und Vorhersagen über die Teigfunktionalität zu ermöglichen. Darüber hinaus wurde das mechanische Verhalten spezifischer Gluten-modifizierter Weizenteigproben durch mathematische Modelle, die auf den mikrostrukturellen PNA-Attributen basieren, mit einer Genauigkeit von bis zu 90% unter Verwendung der Regression der partiellen kleinsten Quadrate vorhergesagt. Zusammenfassend lässt sich sagen, dass die vorliegende Arbeit die Basis für zukünftige Studien über grundlegende Struktur-Funktionsbeziehungen bildet. Die Proteinnetzwerkanalyse und die Übertragung der Netzwerktypen auf Gluten-Mikrostrukturen eröffnen neue Möglichkeiten zur Aufklärung und Vorhersage von Weizenteigfunktionalitäten und Produktqualität.

Graphical Abstract

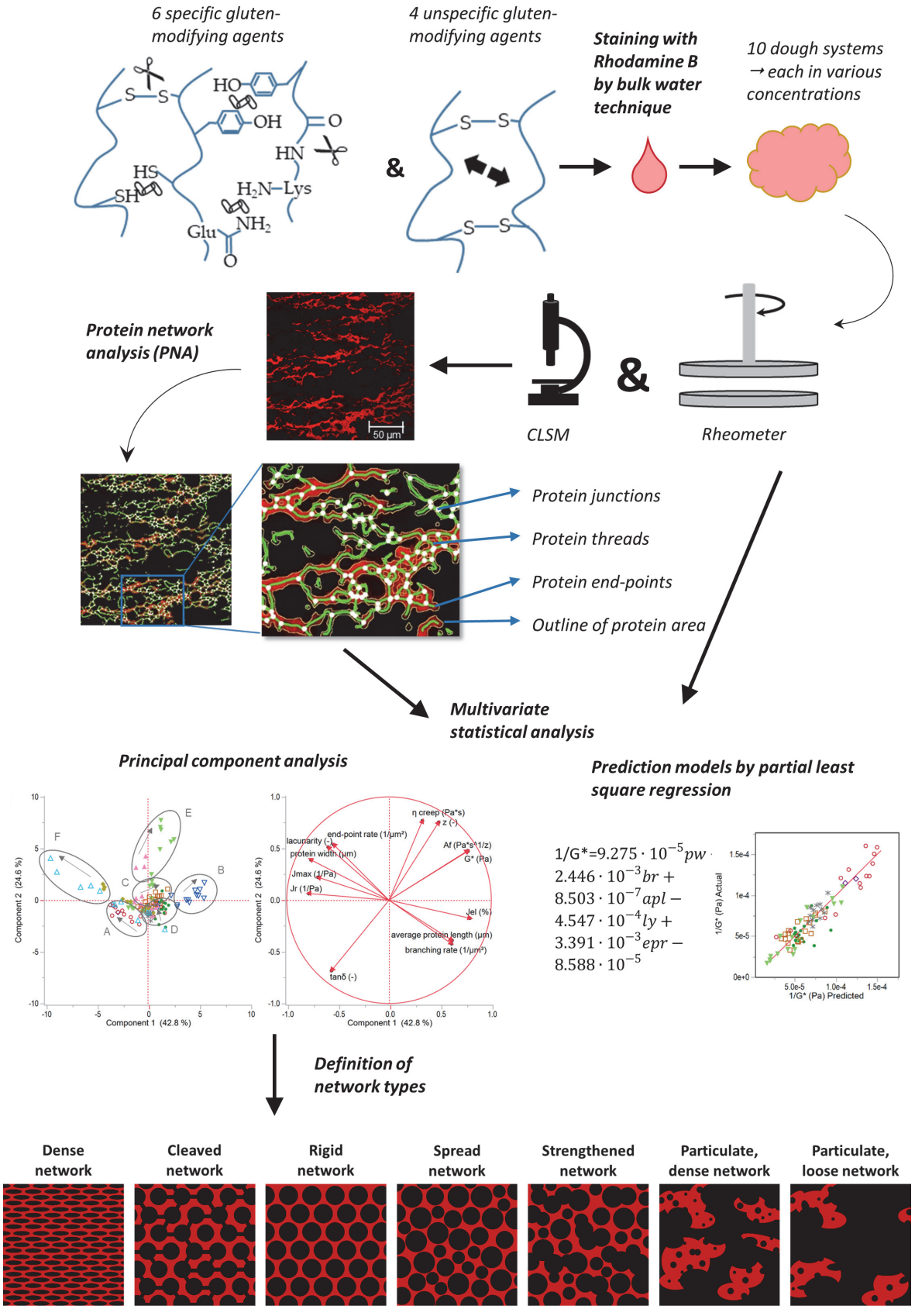

Figure 1: Graphical summary of the main work and findings of this study.

1 Introduction

The improvement of the quality of food products as well as the design of new ones is a steady aim in in the food industry. For wheat-based products, the haptic and physical product quality derives mainly from the unique viscoelastic properties of the gluten proteins (Gianibelli, Larroque, MacRitchie, & Wrigley, 2001). They affect the gas holding properties, dough rheology as well as texture and volume of baked products (Cauvain, 2007; Goesaert et al., 2005; Jekle & Becker, 2015; Wieser, 2007). In order to optimize the product quality, to adapt it to new process conditions or to modify it specifically, it is important to develop and purposefully apply an understanding of structure-function relationships of wheat dough components. In particular, microstructural investigations are required to study structural interactions since most of the elements (e.g., proteins, starch granules, lipid droplets), which affect the mechanical product properties, are in a range below 100 µm (Aguilera, 2005). Due to novel microscopy techniques, such as confocal laser scanning microscopy (CLSM), it is now possible to visualize participating elements non-invasively and specifically by staining them with fluorescent dyes. The evaluation of the produced micrographs can be performed visually and quantitatively by image analysis. For this purpose, it requires a precise analysis of the structural elements. In wheat dough, however, the quantification and interpretation of the gluten proteins is challenging due to their complex network structure and the complex dough matrix. Hence, the development of an appropriate method for the quantification of gluten microstructure would enable determining quantitative relations to dough mechanical behaviour. By means of these correlations, a database used to simplify the interpretation of various network structures could be developed. In consequence, this would provide a prediction of dough functionalities.

In the following section, the molecular characteristics of wheat proteins and formation of the gluten network in dough is explained. Furthermore, possibilities and difficulties of the quantification of gluten network structure as well as the advantages and need of a microstructural investigation to analyse the complex gluten network are shown. Subsequently, influencing factors on gluten microstructure and the link to dough rheology as well as to product quality is discussed before the thesis outline is presented.

1.1 The gluten network in wheat dough

The protein content of commercial wheat flour used for bread making ranges between 10 to 14% (dry base). Although starch is the main component in wheat flour (70-75%), the unique viscoelastic properties of wheat dough derive from the proteins, in particular the gluten-forming proteins of the endosperm (Gianibelli et al., 2001; Goesaert et al., 2005). Gluten proteins comprise about 80-85% of the total wheat proteins and form a continuous network structure during hydration and kneading (Osborne, 1907). The microstructural investigation of the gluten network in wheat dough aimed in this study requires a detailed understanding of the molecular basis of wheat proteins and network formation.

The molecular basis of gluten proteins

According to Osborne (1907), wheat proteins can be classified by their solubility into four groups: a) albumins which are soluble in water; b) globulins which are insoluble in water but soluble in saline solutions; c) prolamins (gliadins) which are soluble in 70-90% ethanol; and d) glutelins (glutenins) which are insoluble in aqueous ethanol but soluble in dilute acid/alkali or after reduction of disulphide bonds (c.f. Figure 2). Albumins and globulins correspond to the non-gluten-forming proteins (15-20%), whereas gliadins and glutenins correspond to the gluten-forming proteins (80-85%)(Osborne, 1907). Gliadins are monomeric proteins with relative molecular weights (M_r) about 30,000 to 60,000, in contrast to glutenins, which are polymeric, aggregative proteins with molecular weights of 80,000 up to 20 million (Veraverbeke & Delcour, 2002).

The classification of wheat proteins by solubility by Osborne was revaluated by P. R. Shewry, Tatham, Forde, Kreis, and Miflin (1986) according to their biological characteristics. Gliadins can be classified according to their amino acid sequences and molecular weights into sulphur (S) -poor ω5- and ω1,2-gliadins (mean molecular weight, MMW) as well as in sulphur-rich α- and γ-Gliadins (low molecular weight, LMW) (c.f. Figure 2). ω-Gliadins have high contents in repetitive sequences of proline and glutamine, whereas the cysteine and methionine content is very low. Since cysteine is essential for the formation of disulphide crosslinks between inter as well as intra molecular chains, ω-gliadins can hardly form disulphide crosslinks (Wieser, 2007). In contrast, α- and γ-gliadins form three and four homologous intra-chain crosslinks due to six cysteines in the α-gliadins and eight in the γ-gliadins of the C-terminal domain,

respectively (Grosch & Wieser, 1999). Both are characterized by high contents of tyrosine. As shown in Figure 2, the proportion of α- and γ-gliadins in the total gluten proteins is much higher than ω-gliadins. The secondary structure of α- and γ-gliadins show a reverse- or β-turns in the repetitive N-terminal domain and α-helices and β-sheet in the non-repetitive C-terminal domain, which are stabilised by disulphide and hydrogen bonds. In contrast, ω-gliadin have no α-helix or β-sheet structure, but are rich in β-turns, which stability derive from hydrophobic interaction (Tatham & Shewry, 1985).

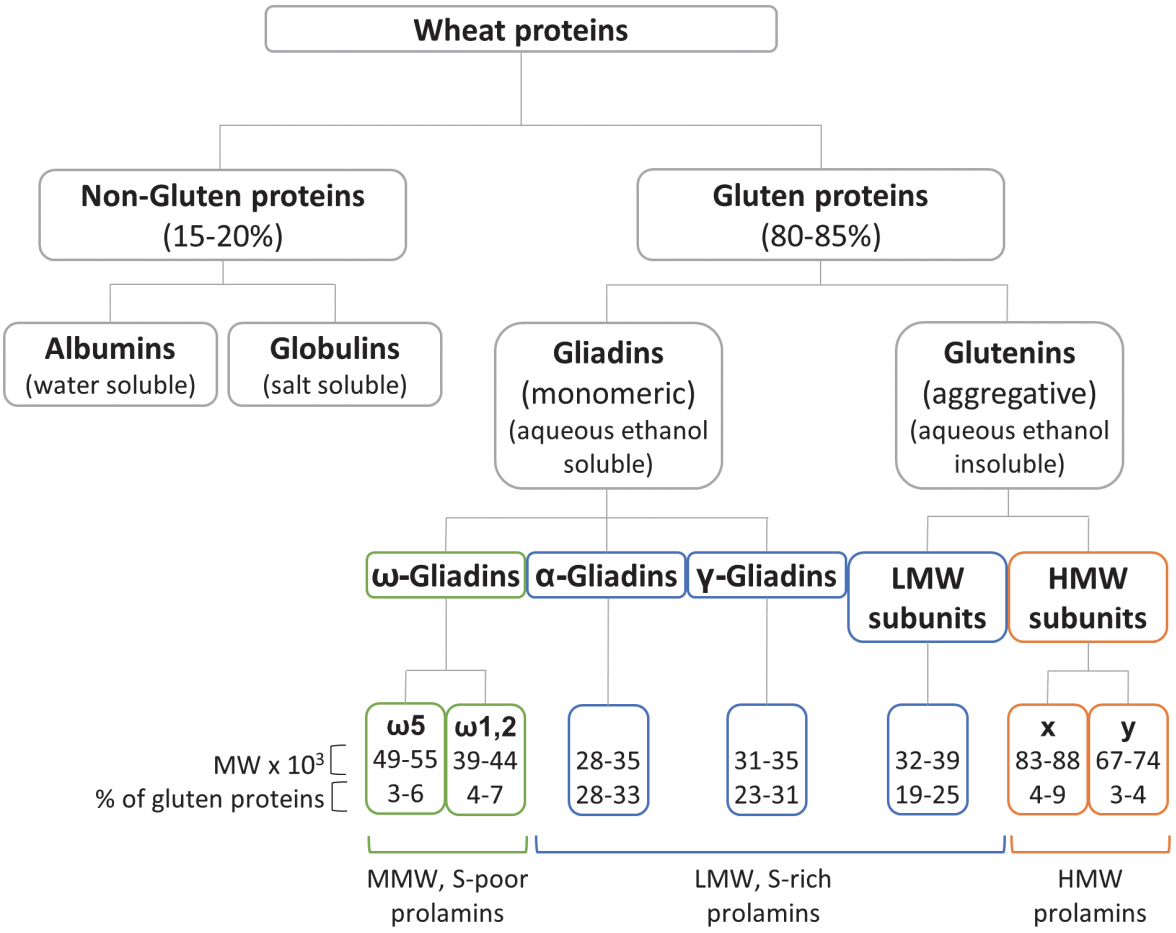

Figure 2: Classification of wheat proteins. The classification was performed according to their solubility (Osborne, 1907) as well as functionality (P. R. Shewry et al., 1986). Molecular weights (MW) and corresponding percentages of gluten proteins were included according to Wieser (2007). Abbreviations: LMW: low molecular weights, HMW: high molecular weights, S: sulphur.

Glutenins are counted to the largest proteins in nature (Wrigley, 1996). However, the analysis of glutenins is difficult due to the large size and poor solubility. Nevertheless, it is known that glutenins are described by subunits since they can be analysed after reduction of disulphide bonds (Goesaert et al., 2005). As a result, glutenins were classified to LMW and HMW glutenin

subunits (GS). LMW-GS can be categorized to B-, C- and D- subunits according to their mobility on SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (Jackson, Holt, & Payne, 1983; Lindsay & Skerritt, 1999). LMW-GS are similar in the primary and secondary structure to α- and γ-gliadins, however, differ from them in one distinct characteristic since LMW-GS form not only intra- but also inter-chain disulphide bonds. This is relevant for the formation of a network structure.

From the gliadins, the HMW-GS differ in the high content of glycine and low content of proline (P. R. Shewry et al., 1986). As visualized in Figure 2, HMW-GS can be distinguished to higher molecular weight x-types (M_r 83,000-88,000) and lower molecular weight y-types (M_r 67,000-74,000) (Wieser, 2007). Even though the proportion of HMW-GS compared to the total gluten content is quite low, they contribute mainly to dough elasticity and functionality. HMW-GS contribute mainly in the gluten network formation since they can form many inter-chain disulphide bonds due to the location of cysteine residuals near the ends. The x-type HMW-GS contain four cysteine residues and the y-types seven, respectively (Grosch & Wieser, 1999). As visualized in Figure 3, the terminal domains of HMW-GS are short with about 80 to 100 amino acid residues in the N-terminal domain and a constant size of 42 amino acid residuals in the C-terminal domain. The central domain varies in length (480-680 amino acid residues). The secondary structure of N- and C-terminal domains comprise mainly of α-helices, whereas the central part consists mainly of repetitive sequences (containing glutamine, proline and glycine) which are believed to form overlapping reversed turns resulting in a β-spiral or helical structure (c.f. Figure 3). Hence, HMW-GS are supposed to have a rigid rod-like confirmation (P. Shewry, Halford, & Tatham, 1992; Veraverbeke & Delcour, 2002).

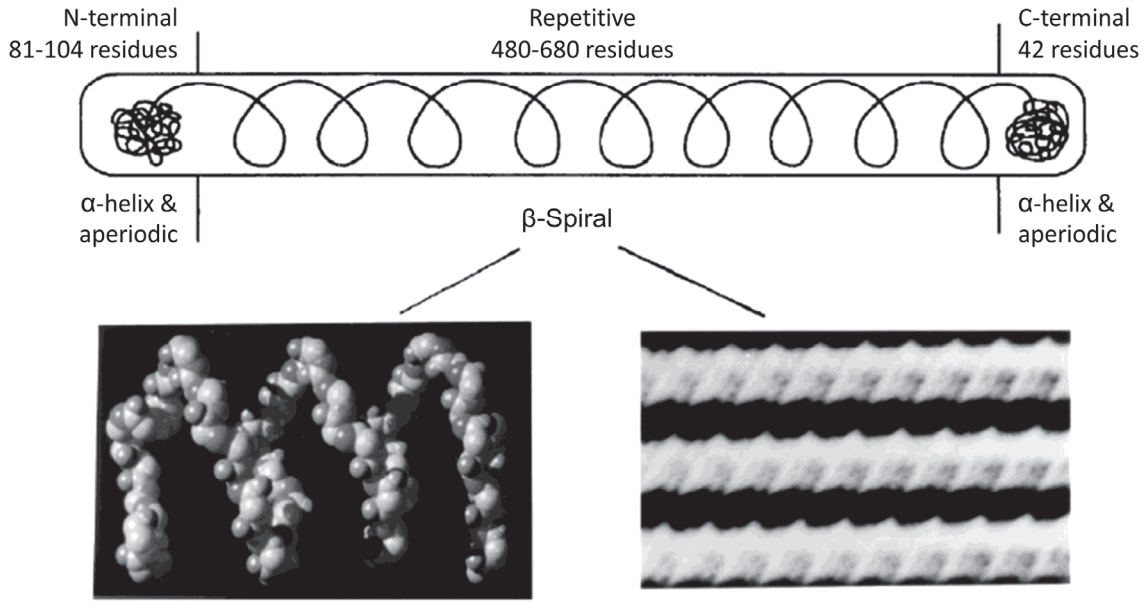

Figure 3: Schematic model of HMW-GS according to P. R. Shewry, Popineau, Lafiandra, and Belton (2000). The N- and C-terminal domain show an α-helix in the secondary structure, whereas the repetitive central domain shows a β-spiral. The β-spiral is visualized by (left) a computer modelling and (right) a scanning probe microscope.

Another structure of the glutenins is discussed in literature, the polymeric glutenin macropolymer (GMP). It consists of large structures of both LMW- and HMW-GS and it is insoluble in aqueous sodium dodecyl sulphate (SDS) (Don, Lichtendonk, Plijter, & Hamer, 2003). The GMP content (about 20-40 mg/g flour) was correlated with quality determinants for elastic properties and bread volume, which makes it to an important contributor for the product quality (P. Weegels, Van de Pijpekamp, Graveland, Hamer, & Schofield, 1996).

The molecular structure of gliadins and glutenins as well as their ability of forming cross-links as described above are relevant for understanding the complex process of gluten network formation during kneading, which is explained in the following section.

Network formation of gluten molecules

When wheat flour is hydrated due to water addition during kneaded, the gluten proteins form a continuous network system determining the viscoelastic properties of wheat dough (Singh & MacRitchie, 2001). The process is described in literature as follows: During kneading, protein aggregates and gluten macrofibrils are formed by covalent linkage of polypeptide chains by intermolecular disulphide bonds. Extended protein threads transform to films, a continuous gluten phase and finally to a homogenous, fine gluten structure (Autio & Salmenkallio-Marttila, 2001; Peighambardoust, Dadpour, & Dokouhaki, 2010). During network formation,

the proteins undergo several depolymerisation and (re-)polymerization processes (P. L. Weegels, Hamer, & Schofield, 1997). Disulphide bonds play a major role in the formation of the three-dimensional gluten network. For understanding these complex processes of network formation as well as the distribution of gluten molecules forming the network structure, various models were established in the past by explaining interactions of glutenin and gliadins as well as the formation of covalent and non-covalent bonds. The model of Ewart (1979) described a linear arrangement of glutenin and suggested gliadins as plasticizer. Graveland et al. (1985) proposed x- and y- type HMW-GS as a backbone in a head-to-tail linear arrangement with LMW-GS as lateral attachments in form of clusters and disulphide bonds as stabilization between both GS types. A development of this model was performed by Lindsay and Skerritt (1999) proposing a branched arrangement of glutenin macropolymer with HMW-GS as a backbone. y-HMW-GS would cause inter-chain disulphide bonds and the branching points contain dimeric structures of LMW-GS. The existence of inter-chain disulphide bonds between LMW-GS and y-HMW-GS was proven by Keck, Köhler, and Wieser (1995). Besides disulphide bonds, Belton (1999) took non-covalent, hydrogen bonds into account for describing gluten elasticity. Thanks to the high concentration of the glutamine, the HMW-GS contain many hydrophilic sequences to form intra- and inter-molecular hydrogen bonds. He pointed out that disulphide bonds explain the resistance to extension but not the elasticity of gluten. Hence, Belton developed the loop and train model stating regions of polymer-surface (train) and polymer-solvent (loop) interactions. The loops increase with hydration level. During dough kneading, loops will deform to trains and hydrogen bonds will be broken because of the stretching of the polymers (c.f. Figure 4a III). The equilibrium configuration of loops and trains will be returning after relaxation (c.f. Figure 4 I) (Belton, 1999).

Later, a further model was developed by Wieser (2007) based on a branched network and the ratio of HMW-GS to LMW-GS (2:1) as well as the molar weight. He proposed that a glutenin polymer double unit contains 2 y-types and 4 x-type of HMW-GS as well as 30 LMW-GS linked by inter-chain disulphide bonds (molecular weight of 1.5 million of one double unit). Furthermore, he assumed that GMP comprises of more than ten of those double units (Wieser, 2007). This model is visualized in Figure 4b.

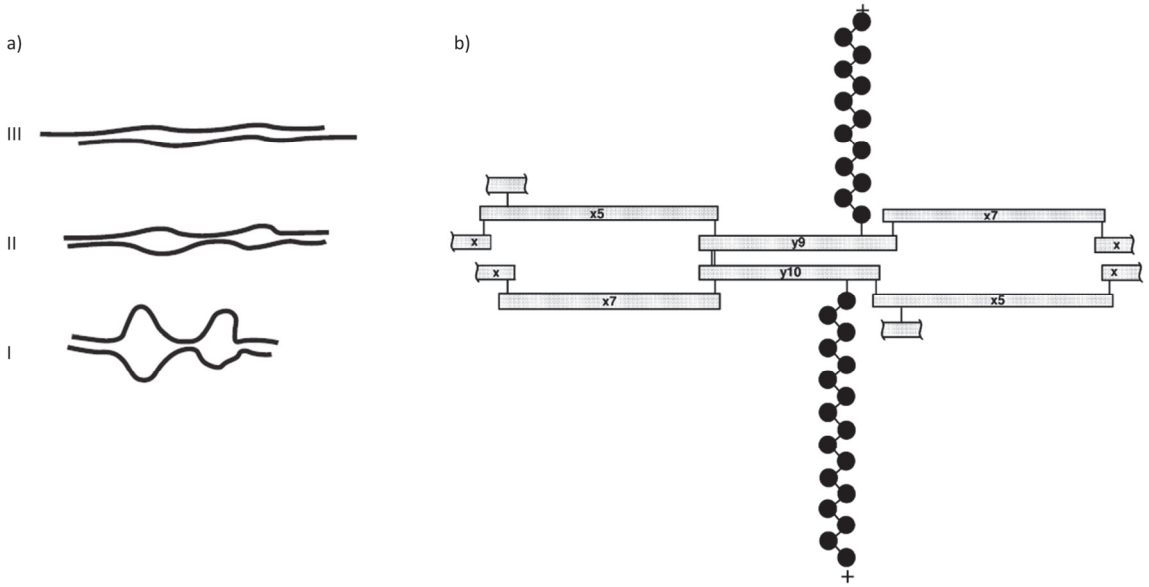

Figure 4: Gluten network models. a) Loop and train model of Belton (1999) showing the deformation of polymers I) at equilibrium, II) under small extension and III) under large extension. **b)** Inter-chain disulphide crosslink model of Wieser (2007) of LMW-GS (•) and HMW-GS (□).

Those models and further research models highlighted that gluten is a complex three-dimensional network, which is highly cross-linked by covalent bonds (disulphide bonds, dityrosine cross-links, (iso)peptide) and non-covalent interactions (hydrogen bonds, hydrophobic and ionic interactions) (Belton, 1999; Tilley et al., 2001; Wieser, 2007). All these bonds and interactions play a major role in formation of the network structure. The most influence on the formation is derived from the number and distribution of disulphide bonds (Wieser, 2007). Recent studies identified disulphide bonds between α- and γ-gliadins, HMW-GS and LMW-GS, eleven of them for the first time (Schmid, Wieser, & Koehler, 2017). Hereby, the network structure is affected by oxidation of SH groups (promoting polymerisation), the presence of terminators (stopping polymerisation) and SH/SS interchange reactions (depolymerising polymers by glutathione or cysteine) (Schmid et al., 2017; Wieser, 2007). This study shows that the elucidation of molecular interactions in gluten network are still ongoing and that the structure of the complex gluten network is not fully investigated yet. The models described in this section are a solid approach for understanding the interactions of gliadins and glutenins and the resulting network structure. However, the models are not entirely validated and the relation of the molecular structure to the spatial network arrangement on a microstructural level is lacking.

1.2 Challenges in quantifying gluten network structure and the need of microstructural investigation

The models characterising the network structure presented in the section above were mainly based on molecular investigations and possess only model character, which are not fully proven yet. Moreover, the transferability of these molecular models to the formation of the spatial network arrangement (microstructural level) is missing. The challenge of analysing the gluten structure is the large molecular size of gluten polymers up to millions of Daltons and low solubility of gluten (Delcour et al., 2012; Dobraszczyk & Morgenstern, 2003; Gianibelli et al., 2001). Hence, most studies focussed on the determination of disulphide bonds (indirectly by the amount of free sulfhydryl (SH) groups) or the molecular weight of HMW-, LMW-GS or GMP (Hammann & Schmid, 2014; Hanft & Koehler, 2006; Steffolani, Ribotta, Pérez, & León, 2010; Wieser, 2007). For the analysis, disulphide bonds in gluten need to be reduced or gluten proteins are extracted by solvents in the Osborne fractions. Even if Schmid et al. (2017) considered the Osborne fractionation as a kind of mixing of flour and that it is comparable to dough formation during kneading, none of the molecular investigations on gluten are performed non-invasively in a wheat dough system. Hence, all molecular studies are invasive and require a cleavage of the network structure. It became apparent that mainly the primary and secondary structure had been studied until now whereas the quaternary structure (the three-dimensional arrangement of folded or coiling polypeptides/protein complex with all subunits) of gluten is difficult to analyse (Delcour et al., 2012). Hence, it requires an analysis one level up on a microstructural level to gather the spatial network arrangement. Summarising, the effect of various cross-links on the spatial arrangement of gluten proteins as well as the rate of branching within the network cannot be determined on a molecular level making an investigation of the gluten microstructure crucial.

Microstructural description and analysis of gluten network

Microstructural observations show structures in a scale of 0.1 to several 100 µm and cover the spatial arrangement of dough components. Bechtel, Pomeranz, and De Francisco (1978) described the microstructure of wheat dough as a system of starch granules surrounded by a gluten matrix. Don et al. (2003) presented gluten as a colloidal particle system, which forms a gel by shearing. However, this hypothesis was comprehensively discussed by Belton (2005).

Furthermore, the gluten microstructure in a wheat dough was characterized as an interconnected gluten network covering starch granules (Peighambardoust et al., 2010), a homogeneous protein phase with very fine structure (Peighambardoust, van der Goot, van Vliet, Hamer, & Boom, 2006), or a spread network, which is described as a continuous structure with elongated and distributed gluten strands (Jekle & Becker, 2015). Further microstructural network models were discussed in Jekle and Becker (2015) and in Kontogiorgos (2011). Finally, Jekle and Becker (2011) described the microstructure as a system of three phases consisting of the continuous gluten network, a free water phase with starch granules and soluble components, and a dispersed gas phase, as visualised in Figure 5.

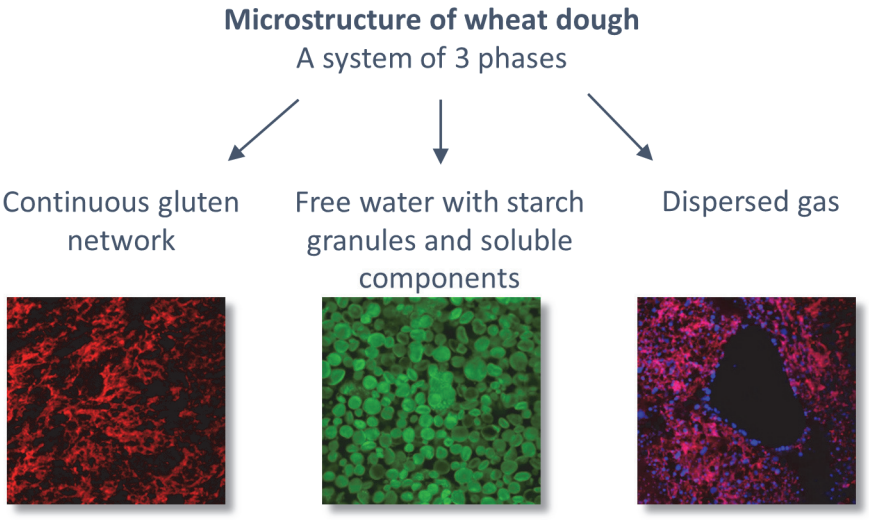

Figure 5: Microstructure of wheat dough. The microstructure is presented by CLSM images of the gluten network (stained by Rhodamine B, λexc = 543 nm, λem = 590/50 nm), starch granules (stained by FITC, λexc = 488 nm, λem = 535 nm) and the gas phase (visualized as black holes; wheat proteins in red, dye Rhodamine B, λexc = 543 nm, λem = 590/50 nm; lipids in blue, dye FatRed, λexc = 635 nm, λem = 650L nm). All images have a size of 686 x 686 µm.

The investigation of gluten microstructure can be performed by light microscopy, epifluorescence light microscopy, electron microscopy, scanning electron microscopy (SEM) or confocal laser scanning microscopy (CLSM) (Bechtel et al., 1978; Dürrenberger, Handschin, Conde-Petit, & Escher, 2001; Hug-Iten, Handschin, Conde-Petit, & Escher, 1999; Peighambardoust et al., 2010; Roman-Gutierrez, Guilbert, & Cuq, 2002). CLSM has some advantages compared to the other methods, which are described in combination with the principle of CLSM in the publication of Jekle and Becker (2015) in detail. In summary, images with a high resolution and a thin optical section can be achieved in CLSM measurements due to the confocal pinhole filtering out of focus light. In addition, several monochromatic lasers

as light sources allow the visualisation of various wheat dough components when staining them with appropriate fluorescent dyes. A further advantage of CLSM is the non-invasive measurement of wheat dough compared to molecular investigations as mentioned above. Hence, the spatial arrangement of the gluten network as it appears in wheat dough can be visualised in CLSM images. However, there are two challenges to consider when using CLSM. Although the microscopy method itself is non-invasive, the visualisation requires a sample preparation by a staining process. Several dyes could be used which bind non-covalently or covalently on the dough components. For gluten proteins, the dye Rhodamine B is commonly used which binds non-covalently on the proteins by hydrophobic interactions (Baier-Schenk et al., 2005; Jekle & Becker, 2011; Maeda et al., 2013). Hereby, the questions rises if those hydrophobic interactions might influence the formation of gluten proteins. Staining was performed in literature either by applying the dye solution on the dough sample's surface or by adding the dye to the bulk water during kneading (Döring, Nuber, Stukenborg, Jekle, & Becker, 2015; McCann & Day, 2013). Another commonly used method was to freeze the dough samples rapidly to fix the structure and to cut thin sections with a microtome for using these sections for microscopy (Bousquieres, Deligny, Riaublanc, & Lucas, 2014; Hesso et al., 2015; Maeda et al., 2013; Peighambardoust et al., 2006). The samples were stained and visualised afterwards. However, freezing is discussed to affect the dough microstructure, especially the protein network formation due to depolymerisation (Berglund, Shelton, & Freeman, 1991; Ribotta, León, & Añón, 2001). Thus, the staining procedure might also have an impact on the gluten network. However, a visualisation of gluten microstructure as realistic as possible is crucial for studying structure-function relationships, and effects of fluorescent dyes as well as staining procedures need to be elucidated.

The second challenge of CSLM and of all other microscopic techniques is that the interpretation of the images is difficult and an objective evaluation of the dough microstructure is required by applying an image analysis. Most of the former studies in literature did not apply an image analysis on their microstructure images and only described the network visually (Baier-Schenk et al., 2005; Dürrenberger et al., 2001). However, qualitative descriptions can cause misunderstandings and thus, an appropriate quantification of the gluten network structures would be crucial. Some studies already used images analyses focussing on the quantification of the protein area or protein particles (number and shape)

(Jekle & Becker, 2011; Lee, Ng, Whallon, & Steffe, 2001; Peighambardoust et al., 2006). Correlations between dough rheology and microstructure were detected underlining the relevance of microstructural quantification methods (Jekle & Becker, 2011, 2012). Since gluten is present as a network structure in wheat dough, a more advanced image analysis evaluating the connectivity and strength of a network would allow an even better elucidation of structure-function relationships. In general, a tree-dimensional network of a polymeric material is described as a structure with several cross-links between long chain molecules (Bardwell & Winkler, 1949a). The relation of cross-links in networks and physical properties of the material had already been reported in early studies of polymeric structures (e.g., rubbers) (Bardwell & Winkler, 1949b; Flory, Rabjohn, & Shaffer, 1949; Gehman, 1952). A further important characteristic of networks are network defects, like terminal chains (Case, 1960). Hence, the image analysis should detect branching points of the gluten network precisely as well as end-points of terminal chains and give quantitative data of those structural network attributes. Since the gluten network does not have a regular grid structure, a description of morphological values might also be useful. However, such an image analysis had not been applied in cereal science, especially not on gluten microstructures, until now.

1.3 Influencing factors on gluten microstructure and the importance for dough and bread quality

The sections above underline the need of studying gluten network on a microstructural level since the complex spatial arrangement can only be covered in this way. It is commonly recognized that gluten proteins define mainly the dough and final product's quality. To improve or even predict dough and final bread quality, an understanding of influencing factors on gluten network is crucial. Figure 6 highlights the influencing factors on gluten network structure.

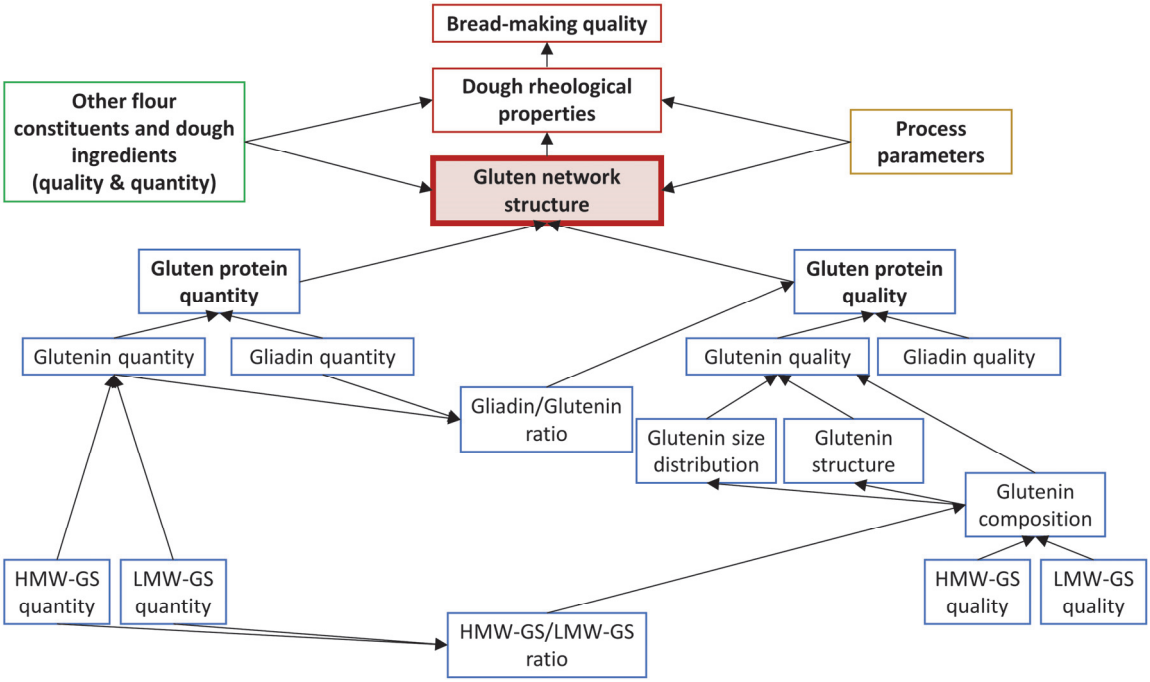

Figure 6: Factors influencing gluten network structure. This matrix highlights influencing factors and relationships of gluten proteins up to bread-making quality, modified and refined according to (Veraverbeke & Delcour, 2002).

The gluten network structure is affected by the gluten quantity and quality. However, the gluten quality is dependent on genetic and environmental factors causing variations in LMW-/HMW-GS and gliadin/glutenin ratio, quality and polymer size distribution (Delcour et al., 2012; Gianibelli et al., 2001; Veraverbeke & Delcour, 2002). Thus, these variations limit an easy prediction of end-product quality. Hence, a lot of research was performed showing correlations of gluten components (as illustrated in Figure 6) and dough rheological properties and/or bread quality. These relations were discussed in detail in Veraverbeke and Delcour (2002) and Goesaert et al. (2005). However, these studies focused mostly on single correlations on a molecular level but the formation of gluten network is dependent of many and interacting influencing factors (c.f. Figure 6). All these factors are reflected in the gluten network structure highlighting the relevance of an investigation of gluten microstructure for structure-function relationships since this is the only method to cover the spatial arrangement of gluten polymers.

Other alterations in gluten network can occur due to process conditions, such as different kneading (time, speed) or fermentation/proofing settings (time, temperature, humidity). There are already many studies presenting microstructural changes of gluten network in under-mixed, optimal mixed and over-mixed dough samples, such as protein rearrangement,

ruptures of gluten threads, air bubbles and starch granules motions (Amend & Belitz, 1991; Auger, Morel, Lefebvre, Dewilde, & Redl, 2008; Boitte, Hayert, & Michon, 2013; Peighambardoust et al., 2010; Peighambardoust et al., 2006; Peressini, Peighambardoust, Hamer, Sensidoni, & Van Der Goot, 2008). Microstructural studies for a fermented wheat dough also exist in literature presenting gluten network alterations caused by gas bubble growth (Autio et al., 2005; Bernklau et al., 2017; Gan et al., 1990; Salmenkallio-Marttila, Katina, & Autio, 2001). It should be noted that changes in process conditions affect not only gluten network structure but also other flour constituents, especially starch granules, and that gluten alterations are not specifically dependent on process variations since they are additionally affected by other ingredients, e.g., yeast. Due to these reasons and because of already investigated effects on microstructure, variations in process conditions will not be the focus of this thesis.

Specific alterations of gluten polymers can be achieved on a molecular level by dough ingredients, such as enzymatic or chemical agents, which cause an effect on dough mechanical behaviour (macroscopic level). Gluten polymers can be ruptured by specific cleavage of disulphide bonds caused by glutathione or of peptide bonds caused by proteases resulting in weakened dough structure (Lagrain, Thewissen, Brijs, & Delcour, 2007; Lindahl & Eliasson, 1992; Lyons, 1982; Verheyen et al., 2015). Oxidizing agents such as ascorbic acid or potassium bromate are said to promote the formation of disulphide bonds and to strengthen the dough (Dong & Hoseney, 1995; Grosch & Wieser, 1999). The enzyme glucose oxidase also improves disulphide formations and additionally dityrosine cross-links (Hanft & Koehler, 2006; Rasiah, Sutton, Low, Lin, & Gerrard, 2005; Steffolani et al., 2010). Further alterations in gluten network can be provoked by the addition of the enzyme transglutaminase, which catalyses the cross-linking of lysine with glutamine residues. Dependent on the concentration of transglutaminase, those new isopeptide bonds improve the resistance to extension and dough strength (Autio et al., 2005; Basman, Köksel, & Ng, 2002; Steffolani et al., 2010). All those studies have in common that they determine and discuss effects of those enzymatic or chemical agents on a molecular level and on dough or bread quality. However, an investigation of gluten microstructure is lacking. Since those enzymatic and chemical agents affect different bonds in gluten network (disulphide, dityrosine, (iso)peptide), the spatial arrangement of the protein network might be completely different. Hence, the questions rises if those alterations

are visible in gluten microstructure and if so, a specific gluten arrangement can be linked to a certain dough mechanical behaviour to conclude on baked product quality. Moreover, other dough ingredients, which interact with proteins such as lipids, or different hydration levels might cause further spatial gluten arrangements. Thus, specific as well as unspecific gluten modifying agents could evoke targeted alterations of gluten network arrangements. To cover gluten quantity and quality variations caused by e.g., environmental and genetic effects as discussed above, different wheat flours should be used for this study.

Consequently, an investigation of gluten microstructure would combine all influencing factors on gluten network structure (c.f. Figure 6). With an advanced image analysis and a realistic visualisation by CLSM as described in section 1.2, quantitative relations between gluten network and dough mechanical behaviour could be developed to predict dough functionality. A challenge hereby could be the interpretation of quantitative results of image analysis and the characterisation of different spatial network arrangements. A possibility of an objective network characterization and a verification would be crucial. Using statistical analyses, like principal component analysis, could verify and support the understanding and interpretation of the results gained by image analysis. Partial least square regressions could be applied for a statistical prediction of dough mechanical behaviour based on network attributes of an image analysis.

1.4 Thesis outline

As expressed in the introduction, the properties, like network connectivity and spatial arrangement of gluten polymers, which form the complex gluten network, cannot be analysed on a molecular level appropriately, but on a microstructural level. For this purpose, microstructure analysis by CLSM is a suitable method to visualise the spatial arrangement of the gluten network. However, a precise image analysis which enables the determination of structural network attributes as well as a realistic visualisation with a suitable staining method for CLSM analysis are required to define structure-function relationships and to accurately forecast dough functionality. Based on these demands, the following hypotheses were identified for this study:

1. A development of a more advanced image analysis for quantifying gluten microstructure in wheat dough based on morphological and structural network attributes enables determining the connectivity and strength of the complex network structure.
2. Existing staining methods with fluorescent dyes and the dye itself (e.g., by hydrophobic interactions) used for CLSM measurement have an impact on protein network formation and dough functionality, which should be considered for a most realistic visualisation of gluten microstructure.
3. Molecular alterations in the gluten polymers caused by specific as well as unspecific gluten-modifying agents influence the gluten arrangement on a microstructural level and the new developed image analysis is precise enough to detect them.
4. Various spatial arrangements of gluten networks can be classified in network types related to dough mechanical behaviour to support the interpretation of the gluten microstructure by image analysis.
5. Dough mechanical behaviour can be predicted by mathematical formulas based on structural and morphological network attributes gained by microstructure image analysis.

Thus, this study aims to develop an appropriate method for the quantification of gluten microstructure and to use it for a definition of quantitative relations to dough mechanical behaviour in order to enable a prediction of dough functionalities.

For this purpose, the first aim of this thesis was to develop and validate an image analysis to detect structural and morphological attributes of the gluten network. This would enable the quantitative description of the connectivity and strength of the complex network structure. In a second step, a staining method for gluten in wheat dough with fluorescent dyes as non-invasive as possible needs to be identified. With fulfilment of these two demands, a most realistic visualization by CLSM and precise quantification of gluten network should be enabled. A further aim of this thesis is to support the interpretation of gained image analysis data by classifying various gluten network arrangements and to relate them to dough mechanical behaviour to find prediction models for dough functionality. Hereby, limits and the robustness of the models should be validated.

2 Methods

The main methods, which were used in this thesis, are summarised in this section.

2.1 Dough preparation

All dough samples used in this thesis were prepared with commercial wheat flour Type 550 and demineralized water. The required kneading time and appropriate water concentration were determined in a Z-kneader (doughLAB; Perten Instruments, Hägersten, Sweden) dependent on the water absorption and moisture (corrected to 14%) of the flour with a targeted resistance of 500 Farinograph units (according to AACCi method 54-70.01). Detailed information about the flours, recipes and dough variations (specific and unspecific gluten-modified samples) can be found in the four publications of this thesis in section 3 (Bernklau, Lucas, Jekle, & Becker, 2016; Lucas, Becker, & Jekle, 2018; Lucas, Petermeier, Becker, & Jekle, 2019; Lucas, Stauner, Jekle, & Becker, 2018).

2.2 Staining of dough samples

Since all dough samples were analysed by confocal laser scanning microscopy, a staining procedure with a fluorescent dye was required. The dye Rhodamine B was used to stain the gluten proteins in wheat dough. Three common staining procedures with increasing dye concentrations were comparatively investigated in the second publication of this thesis (Lucas, Stauner, et al., 2018):

- *Drop technique:* applying a specific drop volume of dye solution on the dough surface with an incubation time of 10 min; previously, dough was placed in an object carrier, cut with a razor blade to achieve a plane surface and sealed with an object slide
- *Bulk water technique:* dye is added into the bulk water during kneading; dough was placed in an object carrier, cut with a razor blade to achieve a plane surface and sealed with an object slide
- *Rapid freezing technique:* bulk water technique followed by a rapid freezing and cutting step with a microtome to achieve a plane surface

After the evaluation of these three methods, the bulk water technique with a Rhodamine B solution of 0.1 g/L water was used in all other three publications of this thesis.

2.3 Microstructure analysis by confocal laser scanning microscopy

Microstructure analysis of gluten network was performed by confocal laser scanning microscopy using an eclipse Ti-U inverted microscope with an e-C1 plus confocal system (Nikon GmbH, Düsseldorf, Germany). Images were recorded with two different objectives (Plan Apo VC 60x/1.40 oil and Plan Apo 20x/0.75) in the first publication of this thesis, whereas only the 20x objective was applied in the second and the 60x objective in the third and fourth publication. Details about the laser wavelengths, resolution, size and number of the recorded images can be found in Bernklau et al. (2016), Lucas, Stauner, et al. (2018) and Lucas, Becker, et al. (2018).

2.4 Method development for image processing and analysis

A main part of this thesis was the method development and validation of an image processing and analysis of CLSM images of gluten networks. First, an appropriate image analysis tool, with which a detailed detection and description of a network is feasible, was required. Since such image analyses of gluten networks had not been applied in cereal science (or even in food systems), it was necessary to search for a suitable software in other fields. For this purpose, similar structures to gluten network of other matrices were evaluated. It was discovered that gluten networks show a similarity to blood vessels on microscopic images. In cancer research, the growth of blood vessels, called angiogenesis, are studied with several images analyses, which detect and determine network attributes. Hence, four tools for angiogenesis were tested on gluten network images: WimTube (Onimagin Technologies, Córdoba, Spain), S.CORE (S.CO LifeScience, Munich, Germany), Angiogenesis Analyzer (plugin for ImageJ, National Institute of Health, Maryland, USA), and AngioTool64 version 0.6a (National Cancer Institute, National Institute of Health, Maryland, USA). The evaluation of these four tools was first performed visually on the precision of the detected gluten network on example images. Afterwards, the two most promising tools Angiogenesis Analyzer and AngioTool were further investigated quantitatively with CLSM images of different dough systems (a standard wheat dough, a standard with additional 10mL of water, and a standard with additional 2% of NaCl). The evaluation was performed according to the precision and correctness of detected junctions, the relevance of determined network attributes for a description of gluten networks and the precision of quantitatively detected differences between the three example dough

systems. Since AngioTool showed the most advantages, it was used for a detailed application test and validation, which was performed in the first publication of this thesis (Bernklau et al., 2016). Furthermore, the determined attributes were adapted to protein networks of wheat dough and further developed as well as new attributes were established. This analysis was finally called “protein network analysis” (PNA).

2.5 Protein network analysis

All produced CLSM images of gluten networks in this thesis were analysed by protein network analysis with the appropriate settings dependent on the applied magnification (20x or 60x objective), as described in the first publication (Bernklau et al., 2016). The attributes protein area, junctions’ density, branching rate, end-point rate, average protein length, protein width and lacunarity were evaluated in the first and second publication (Bernklau et al., 2016; Lucas, Stauner, et al., 2018), whereas the latter five attributes were evaluated in the third and fourth publication (Lucas, Becker, et al., 2018; Lucas et al., 2019).

2.6 Dough rheology measurements

The first, second and fourth publications of this thesis include rheological measurements of the dough samples, performed with an AR-G2 rheometer (TA instruments, New Castle, USA) with parallel crosshatched plates (∅ 4.0 cm). The measurement procedure as well as the settings for oscillatory frequency sweep and creep-recovery tests were applied according to the description in Bernklau et al. (2016). The first two publications contain the evaluation of corresponding attributes for both measurements, e.g., complex shear modulus G* and creep compliance J_{max} (c.f. Bernklau et al. (2016) and Lucas, Stauner, et al. (2018)). In addition, attributes gained by applying the power law on oscillatory frequency sweep test results (network strength A_f, network connectivity z) and by applying the Burgers model on creep-recovery test results (e.g., instantaneous compliance J_0, steady state viscosity η_0) were evaluated in the fourth publication. A comprehensive explanation and description of the measurement, determination and evaluation is summarised in the publication Lucas et al. (2019).

2.7 Statistical analysis

For statistical analysis, results were analysed by a one-way analysis of variance (ANOVA) followed by a Tukey-test with the software GraphPad Prism 6 (version 6.01, GraphPad Software Inc, La Jolla, USA) according to Bernklau et al. (2016), Lucas, Stauner, et al. (2018) and Lucas, Becker, et al. (2018). Multivariate statistics, principal component analysis (PCA) and partial least squares (PLS) analysis were executed with JMP Pro software (version 12.2.0, SAS Institute Inc, Cary, NC, USA) according to Lucas, Becker, et al. (2018) and Lucas et al. (2019).

3 Results (Thesis Publications)

3.1 Summary of thesis publications

Protein network analysis – A new approach for quantifying wheat dough microstructure **Pages 31-39**

The unique viscoelastic properties of wheat dough are derived from their proteins, the gluten proteins, which form a continuous network during kneading. Thus, the physical quality of baked wheat products, like volume and texture, depend mainly on properties of the gluten network. To understand these structure-function relationships, the analysis of the gluten network on a microstructural level is crucial. Hence, the first study of this thesis focused on the quantification of gluten microstructure, which had been challenging due to the complex structure until now. A precise image analysis, called “protein network analysis” (PNA), was developed. For the first time, the connectivity and strength of varying network structures were quantified by absolute values (e.g., protein junctions and average protein length), new developed structural network attributes (branching rate and end-point rate) as well as the morphological attribute lacunarity. The method was validated by applying PNA on CLSM pictures of various network shapes using two different magnifications. For this purpose, wheat dough samples were produced with increasing concentrations of bulk water. In addition, dough rheology by oscillatory frequency and creep-recovery test was measured as a reference method. Correlations of the attributes of both methods confirmed the loss of a network structure with increasing water concentration and thus, the applicability of the image analysis.

Contribution:

The doctoral candidate performed the study conception, method development, data analysis and interpretation, statistical analysis, as well as literature search, and drafted, revised as well as approved the final manuscript.

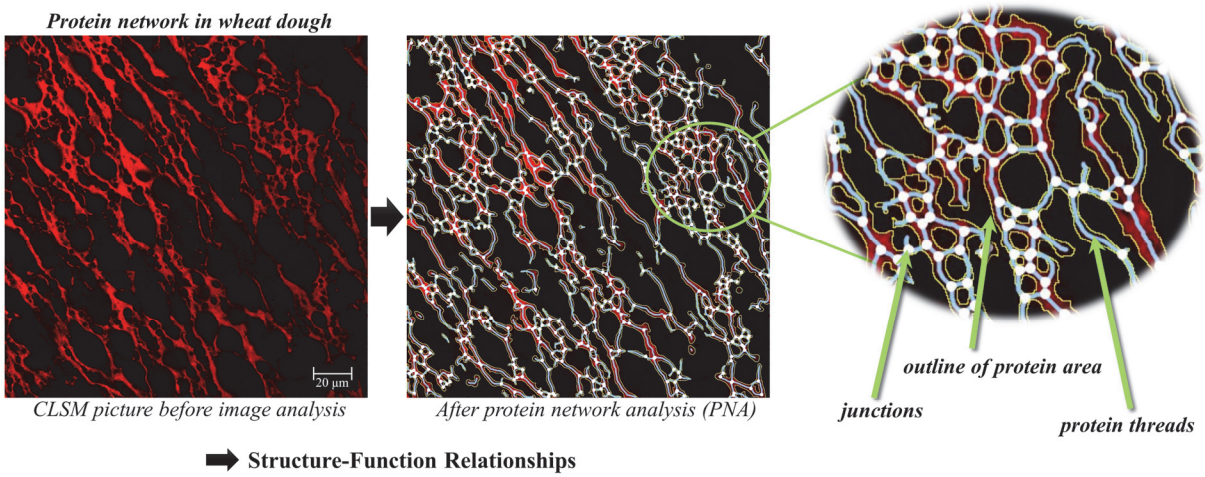

Figure 7: Graphical Abstract of the first publication

Staining methods for dough systems – Impact on microstructure and functionality **Pages 40-46**

The visualisation of gluten microstructure in wheat dough by CLSM is one of the most appropriate methods to analyse the complex network structure in a direct and non-invasive way. However, the measurement requires staining of gluten with fluorescent dyes, which might affect gluten network formation. Thus, the aim of the second study of this thesis was to elucidate the impact of different staining methods on dough microstructure and functionality to find a suitable staining method for a most realistic visualisation of gluten network by CLSM. Tree common staining methods (drop-, bulk water- and rapid freezing technique) were comparatively investigated by CLSM and rheological measurements using increasing concentrations of the dye Rhodamine B. Results showed that the dye itself did not affect structural protein formation or rheological dough properties. Rather the staining procedure had an impact on dough functionality. The drop- and the rapid freezing technique weakened the dough significantly (decreased firmness G* by 12% and 17%, respectively), whereas the bulk water technique did not affect dough rheology. Protein network analysis revealed higher end-point rates (8% and 20%, respectively) and less interconnected protein segments (shorter average protein lengths by 29% and 42%, respectively) when stained with drop- or rapid freezing technique compared to the bulk water technique. The less interconnected protein structure explained the lower dough firmness. Concluding, bulk water technique is recommended for staining in further CLSM studies.

With these first two studies of this thesis, it is now possible to visualize most realistically and to analyse quantitatively the gluten network structure in wheat dough.

Contribution:

The doctoral candidate performed the study conception, data analysis and interpretation, statistical analysis, as well as literature search, and drafted, revised as well as approved the final manuscript.

Gluten polymer networks – A microstructural classification in complex systems **Pages 47-62**

Even though a realistic visualization by CLSM using bulk water technique and a quantification of gluten microstructure by applying PNA is now possible, the interpretation of the attributes for gluten network is challenging due to its various complex, structural arrangements. In order to specify different spatial arrangements of gluten networks in general, the network formation was altered by specific gluten modifying agents (glutathione, ascorbic acid, potassium bromate, glucose oxidase, transglutaminase, bromelain) and analysed by CLSM followed by PNA. Hereby, it was shown that modifications in gluten cross-links provoked by enzymatic and chemical agents could be detected on a microstructural level which had not been published before and that these alterations can be quantified by PNA precisely. The structural network attributes branching rate, end-point rate, protein width and average protein length correlated linearly among each other, which lead to the general assertion: the higher the branching rate, the thinner the protein threads and the larger the interconnected protein aggregate. By means of the morphological attribute lacunarity, a classification of different network arrangements was established. Further investigations were performed by using unspecific gluten-modifying agents (increased and reduced hydration level, addition of rapeseed oil and shortening) in order to provoke additional network arrangements and to extend the classification. Finally, five network types with an interpretation of dough functionality were proposed, which support the understanding of microstructural analysis of the complex gluten network.

Contribution:

The doctoral candidate performed the study conception, data analysis and interpretation, statistical analysis, as well as literature search, and drafted, revised as well as approved the final manuscript.

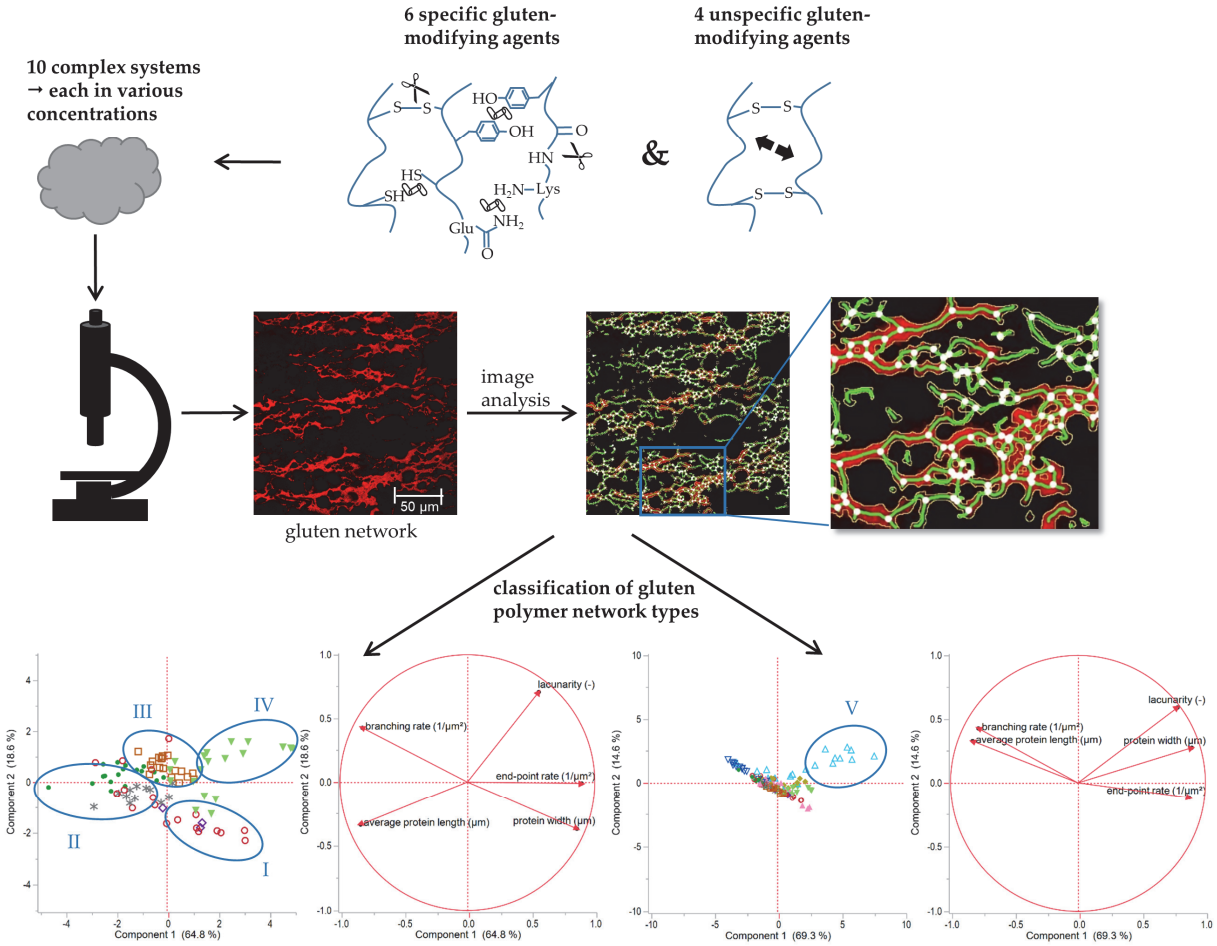

Figure 8: Graphical Abstract of the third publication

Definition of network types – Prediction of dough mechanical behaviour under shear by gluten microstructure **Pages 63-76**

The last study of this thesis shows prediction models of dough mechanical behaviour under shear by gluten microstructure and presents a definition of six protein network types. It is a further development of the network types established in the third publication and they were linked to dough mechanical behaviour. The same dough variants provoked by unspecifically and specifically gluten modifying agents in various concentrations as in the former publication were analysed by rheological measurements in addition to CLSM. Linear, reciprocal and logarithmical relations of dough rheology with gluten network attributes were identified for specific gluten modified samples, and prediction models were defined by PLS regressions with an accuracy up to 90%. Hence, it is now possible to predict dough mechanical behaviour with the five microstructural attributes of gluten network (branching- and end-point rate, protein width and average length, lacunarity). Since prediction models could not be established for every kind of unspecifically gluten-modified dough sample, six network types were defined based on clusters detected with PCA: a cleaved (low viscous), rigid (highly viscous), spread (viscoelastic), strengthened (viscoelastic), particulate and dense (highly viscous) and particulate and loose (low viscous) network. The classification of network types was designed to use it as a guiding scheme supporting the interpretation of gluten network arrangements and the prediction of corresponding dough mechanical behaviour in future research. This study provides the basis of predictions of dough functionality and baking quality as well as new approaches of structure-function relationships and product developments with targeted properties for further studies.

Contribution:

The doctoral candidate performed the study conception, data analysis and interpretation, statistical analysis, as well as literature search and drafted, revised as well as approved the final manuscript.

3.2 Protein network analysis – A new approach for quantifying wheat dough microstructure

Food Research International 89 (2016) 812–819

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Protein network analysis — A new approach for quantifying wheat dough microstructure

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ARTICLE INFO

Article history:
Received 22 July 2016
Received in revised form 9 October 2016
Accepted 10 October 2016
Available online 12 October 2016

Keywords:
Image analysis
CLSM
Gluten network
Rheology
Microscopy

ABSTRACT

Clarification of wheat dough functionalities by visualizing the protein microstructure demands a precise image analysis, which is still challenging. Thus, a novel method for quantifying dough microstructure called protein network analysis (PNA) was established in this study. Hereby, absolute morphological attributes such as junctions' density, branching rate, end-point rate, and lacunarity quantify and characterize the strength of a network. The method was validated in a large range of varying microstructural shapes by increasing the bulk water concentration. In addition, the effect of two different magnifications (objectives with various numerical apparatus) was studied. Resulting values of the branching rate showed a significant linear decrease ($R^2 = 0.97$) by ~40% for both magnifications indicating a decrease in connectivity and cohesion within the network. Rheological measurements, used as reference methods confirmed the loss of a network structure with increasing water addition (e.g. G^* decreased by 89%). Additionally, significant correlations between both methods validated the innovative image analysis PNA. With this new approach of image analysis, effects of additives, varying dough ingredients or changing process conditions on gluten network - the most structure-relevant component in wheat dough - can be quantitatively identified, and targeted functionalities can be controlled.

1. Introduction

The characteristics of wheat dough and products strongly depend on their structure and ingredients' formation. Hereby, the formation of the gluten network is fundamental for rheology and gas retention, which highlights the importance of the structure-function relationship. On a macroscopic scale, dough functionality is usually characterized by rheological measurements. In order to get even better insights, confocal laser scanning microscopy (CLSM) returns detailed results by visualization of main structural factors. For analysing microstructure and elucidating interactions of dough components, a precise method of detecting and quantifying various structural shapes is crucial. Although microscopic images of wheat dough can be evaluated qualitatively, a quantification of the main structure-relevant component, the protein network, is challenging.

In earlier studies, CLSM images of protein network of several cereal products have been analysed visually (Baier-Schenk et al., 2005; Beck, Jekle, & Becker, 2012; Dürrenberger, Handschin, Conde-Petit, & Escher, 2001; Parada & Aguilera, 2011). Peighambardoust, van der Goot, van Vliet, Hamer, and Boom (2006) tried to quantify the area fraction of the protein matrix by producing a binary image, but they stated that computing the area fraction was not sufficient to establish a relation between process parameters and dough development. Another approach of quantifying protein areas was pursued by Lee, Ng, Whallon, and Steffe (2001) by using the histogram function of a CLSM software and computing bright pixels over a grey level image. However, the sole description of the protein area does not give enough information to characterize dough functionalities. Jekle and Becker (2011) established a novel analysis method for protein quantification, besides computing the area fraction, taking into account further characteristics like particle count, average size, perimeter, circularity and fractal dimension. By using this methodology, a more detailed knowledge of the protein formation could be reached. For more information about the network structure, Boitte, Hayert, and Michon (2013) used grayscale morphology as texture analysis to obtain the local protein concentration and orientation. First own studies enabled the development of structure function relationships of proteins in wheat dough based on particle related microstructure properties and simple morphological values (Döring, Nuber, Stukenborg, Jekle, & Becker, 2015; Jekle & Becker, 2012). However, a more advanced and adjusted analysis of the protein microstructure focusing on absolute morphological values (such as branching rates) might distinctly increase the predictive significance of the protein network. Furthermore, a more comprehensive understanding of product properties might be provided, and relationships

Abbreviations: AACCi, American Association of Cereal Chemistry international; CLSM, confocal laser scanning microscopy; ICC, International Association for Cereal Science and Technology; PNA, protein network analysis; rel., relative alteration; ROI, region of interest.

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http://dx.doi.org/10.1016/j.foodres.2016.10.012
0963-9969/

between protein network characteristics and rheological behaviour might be clarified.

In this study, a novel approach of protein network analysis (PNA) was developed to quantify structural alterations. Protein network in wheat dough show a high similarity to other organic structures, like cell systems or blood vessels. Thus, a tool usually used in medical application for characterizing vascular networks was applied on wheat protein networks, and the analysis was adapted to fit the needs of food systems. This method has the advantage of determining not only the protein area, but also the number of junctions and structure regularities for describing the strength of the network. In order to test the capability for detecting protein structures, the applicability of image processing algorithm and their computed attributes were evaluated in detail. For validation of the image analysis, different structure profiles of the gluten network were required. Thus, the bulk water concentration was varied to provoke a large range of microstructural shapes in wheat dough due to a plasticizing effect of water (Jekle & Becker, 2011). As a reference, oscillating rheometry was applied to compare the CLSM results and to combine micro and macrostructural levels. Additionally, different magnifications of CLSM images were compared to study the influence on quantitative results. With this new approach of network analysis, a precise detection of microstructural changes can be provided to elucidate dough functionalities.

2. Materials and methods

2.1. Dough preparation

German commercial wheat flour Type 550 (Rosenmühle, Landshut, Germany) was used for dough preparation. According to methods of the American Association of Cereal Chemistry international (AACCi) and of the International Association for Cereal Science and Technology (ICC), 14.17 ± 0.03 g moisture per 100 g flour (AACCi 44-01), 12.70 ± 0.04 g protein content per 100 g dry flour (AACCi 46-16, N × 5.7), 0.63 ± 0.01 g ash per 100 g dry flour (ICC 104/1), 28.75 ± 0.81 g wet gluten per 100 g flour and a falling number of 407 s (AACCi 56-81) were determined. Dough resistance and water absorption were measured in a Z-kneader (doughLAB; Perten Instruments, Germany) according to AACCi 54-70.01 in order to determine the required kneading time. To reach 500 Farino units, dough consisting of 50.1 g flour and 29.6 g demineralized water were kneaded 180 s at 63 rpm. Bulk water concentration was varied in a range from 59.2 to 89.8 ml per 100 g flour (corrected to 14% moisture) to provoke different microstructural shapes of the protein network. Samples with higher water concentrations should highlight extremes of dough protein structures, like in pre-doughs. To stain the samples for confocal laser scanning microscopy, 5 ml of bulk water was replaced by a Rhodamine B solution (Merck KGaA, Darmstadt, Germany, 0.01 g/100 ml water). All measurements were performed in triplicates.

2.2. Dough rheology measurements

Viscoelastic properties of dough were measured by oscillatory and creep-recovery tests with an AR-G2 rheometer (TA instruments, New Castle, USA) consisting of parallel cross-hatched plates (Ø 4.0 cm) with a constant gap of 2.0 mm and a smart swap Peltier plate temperature system (30 °C constant temperature during measurement). 5 g of dough samples were placed between the plates, the gap was set, the excess of dough was removed and the remaining surface of the dough was treated with paraffin oil to avoid sample drying. After 10 min of resting time for dough structure relaxation, an oscillatory frequency sweep test was performed with a constant deformation of 0.1% (within the linear viscoelastic region) and a frequency ranging from 0.1 to 50 Hz. Fundamental dough rheology properties were characterized by the complex shear modulus G*, the storage modulus G′ and the loss modulus G″ at 1 Hz. Afterwards, a creep-recovery test with a constant shear stress of 250 Pa at 30 °C for 180 s and a relaxation time of 360 s was conducted. The relative elastic part J_{el} was evaluated by the ratio of the creep compliance J_{max} and the creep recovery compliance J_r (c.f. Jekle & Becker, 2011).

2.3. Microstructure analysis by confocal laser scanning microscopy

As described above, dough samples were stained by addition of dye in bulk water with Rhodamine B to visualize proteins. The dough sample (2 g) were transferred to an object carrier with a cylindrical notch (Ø 18 mm, height 7 mm) and sealed with a cover glass. After 10 min of resting time for dough relaxation, samples were analysed by an eclipse Ti-U inverted microscope with an e-C1 plus confocal system (Nikon GmbH, Düsseldorf, Germany) using two different objectives (Plan Apo 20×/0.75 and Plan Apo VC 60×/1.40 Oil). A laser with a wavelength of 543 nm was used for excitation, the emission was detected at 590/50 nm. 10 different images were taken of each dough sample with a resolution of 1024 × 1024 pixel and a size of 215 × 215 µm (for 60× objective) and of 686 × 686 µm (for 20× objective), respectively. Dough samples were produced in triplicates, therefore, 30 images were analysed for one dough type.

2.4. Image processing and analysis

The software-based analysis of CLSM images was performed by AngioTool64 version 0.6a (National cancer Institute, National Institute of Health, Maryland, USA). This tool usually is used for microscopically analysis in medical applications to study changes in vascular networks provoked by angiogenesis (Zudaire, Gambardella, Kurcz, & Vermeren, 2011). AngioTool was applied on CLSM images of gluten network due to high similarities of vascular network.

2.4.1. Implementation details – protein network analysis

Image processing by AngioTool is divided into two main steps; first, the protein network analysis by segmentation and skeleton analysis for identification of protein area, number and density of junctions, protein thread length and number of end-points. Second, the tool analyses the lacunarity for describing irregularities of formation and gaps within the network. Fig. 1 shows a simple network structure before and after segmentation and skeleton analysis by AngioTool. CLSM pictures are converted to grey scale images. Internally, a copy of the image is convoluted with a Gaussian filter to a blurred image. The width of the

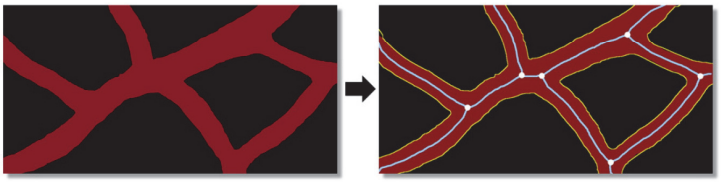

Fig. 1. Simple network structure before and after image processing by AngioTool. Junctions are shown in white, protein skeleton in blue and protein outline in yellow.

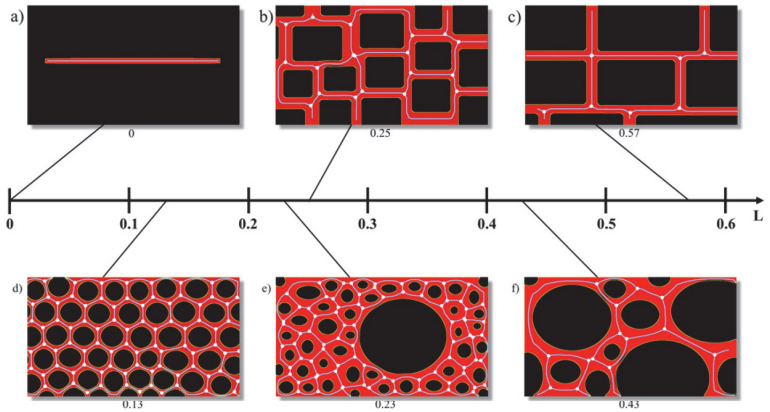

Fig. 2. Images with different lacunarities L. Images with regular shapes have low lacunarities, e.g. a) line with L = 0. Lacunarity increases with increasing irregularities and larger holes. Additionally, network structures after skeleton analysis are shown (white = junctions, blue = protein skeleton, yellow = protein outline/area).

Gaussian filter is set by the user, whereby the protein thread diameter is determined. The blurred image is the base for the shape of the protein network. By blurring, all areas with a high density of bright pixels are enlightened whereby the structure of the protein network is accentuated. By setting the intensity range, the protein area can be limited further (by the operator). Following, the acquired area is visualized in the image by a yellow outline (called region of interest, ROI) and correlates with the computed protein area (Fig. 1). Additionally, holes can be filled and small particles (areas with a low ROI) can be removed automatically to minimize noise.

2.4.2. Evaluation of image analysis on artificial network structures

The software AngioTool, usually used for determining blood vessels, was tested on accuracy by analysing other network structures. For this purpose, artificial images with various shapes (regular/complex shapes; low/high branched networks) were used as illustrated in Figs. 1 and 2. The accuracy of image analysis was evaluated by the precision and correctness of detected junctions. The application of AngioTool on a simple network structure (Fig. 1) could demonstrate that the visual evaluation correspond with the results after image analysis detecting 6 junctions in the network. Likewise, lower branched (e.g. Fig. 2c with 7 junctions) and higher branched (e.g. Fig. 2b with 19 junctions) networks structured as a simple grid could be detected precisely and correctly. Other networks with similar shapes to a protein network in dough (Fig. 2d–f) showed neither over- nor under- detected junctions or structures as well. Thus, AngioTool was applied on CLSM images of protein structures in wheat dough.

2.4.3. Implementation details – lacunarity

The lacunarity is determined independent of the network analysis and specifies the shape of the network. This attribute is not characterized and defined uniformly. Allain and Cloitre (1991) described lacunarity as a lack of rotational or translational invariance in an image. Lacunarity can also be seen as an attribute for non-uniformity of structure or structural variance in an object (Smith, Lange, & Marks, 1996). Another definition was set by Mandelbrot (1994), whereby lacunarity describes images with fractals characterized by large holes, voids or wide gaps. The software AngioTool determines the lacunarity by box counting comparing the similarity of each box. By box counting, images are divided in equal boxes column by column. Pixels with information (white pixel) are counted in each box. This procedure is replicated with 10 different box sizes (edge length 5–60 pixels) for each image and a sliding factor is set to 5 pixels for overlapping. Afterwards, the average of all pixel counts of one box size is computed. Single lacunarity L_S of one box size is calculated by the following equation:

$$L_S = \left(\frac{SD_{PC}}{A_{PC}}\right)^2 \quad (1)$$

with L_S single lacunarity, SD_{PC} standard deviation of pixel count and A_{PC} average pixel count. Mean Lacunarity L_M is computed over all box sizes:

$$L_M = \frac{\sum_{i=1}^{n} L_S}{10} \quad (2)$$

with L_M mean lacunarity, L_S single lacunarity.

Fig. 2 represents various structures and their lacunarities. With increasing values of lacunarity, non-homogeneity increases as well as holes and gaps (c, f). A horizontal line (a) has a lacunarity of zero because of its regular shape without holes. The lacunarity of image e) is higher than d) because of its higher irregularity even if there are smaller gaps than in e). For wheat dough analysis, lacunarity helps describing irregularities and formation of protein network. For example, a dough kneaded to the optimum would have a lower value for lacunarity due to a highly developed network structure resulting in smaller holes within the network than a dough, which is kneaded not long enough.

2.4.4. Used settings for protein network analysis

Each CLSM image was analysed by AngioTool with the same settings and parameters to ensure a reproducible quantification of the protein network. The used parameters were adjusted on the two different magnification of protein structures, appropriately. Vessels diameter (imply protein diameter) was set to 2 and 3 for images recorded with 20× objective (3 and 5 for 60× objective), intensity low and high threshold to 15 and 255 for both magnifications, small particles were removed under 10 (35) and the function "fill holes" was deactivated. Calibration was set to 1.49 pixel/μm for 20× objective and to 4.76 pixel/μm for 60×.

2.5. Statistical analysis

The software GraphPad Prism 6 (version 6.01, GraphPad Software Inc., La Jolla, USA) was used for statistical analysis. For identifying outliners within a series of CLSM measurements, a Rout test with Q = 1% was performed. D'Agostino & Pearson omnibus normality test was applied to ensure a Gaussian distribution. To identify significant differences between samples with various concentrations, a one-way analysis of variance (ANOVA) followed by a Tukey-test was applied due to a normal distribution within all data sets. Correlation tests were

performed (linear regression, exponential growth and one phase decay) within a concentration series and in order to investigate relationships between rheology and microstructural attributes. The determination coefficient R^2 represents the goodness of fit.

3. Results and discussion

3.1. Evaluation and development of image analysis on gluten networks

In wheat dough, the protein network plays an important role for dough properties, such as consistence, cohesion, rheology and gas retention capacity (Beck et al., 2012; He & Hoseney, 1991; Verheyen et al., 2015). By the hydration of flour and the energy input during mixing, the three-dimensional gluten network is developed by cross-linking of protein polymers, especially by forming disulphide bonds, to a continuous and viscoelastic phase (Bache & Donald, 1998; Singh & MacRitchie, 2001). To study changes in gluten network provoked by ingredients, additives or process variations, microstructure analysis by CLSM is a powerful method (Dürrenberger et al., 2001; Hesso et al., 2015; Jekle & Becker, 2012). Therefore, a precise quantification of network attributes by image analysis is crucial. After the evaluation of the applicability of AngioTool on simple network structures (Section 2.4.2), the tool was applied on a CLSM image of a standard wheat dough consisting of flour and water (59.2 ml/100 g flour). A sample picture is shown before and after image processing in Fig. 3.

Having evaluated the resulting image visually, protein network structure could be detected precisely by image processing with AngioTool. Even small holes and thin protein threads were determined, and detected junctions corresponded with the visible branching as well. Quantification of the network is performed by attributes like percentage area, number of junctions and lacunarity. A list of all computed attributes with original terms, terms for protein network characterization and their meanings are shown in Table 1. Although these attributes give a lot of useful information about the network, they are not sufficient for structures with a large range of variation and a dependence of the used image section. When analysing gluten networks in dough systems by microscopy, a visualization of the whole network structure in one CLSM image is impossible. Thus, the position of the visualized structure elements in the image section influences the results of the total number of junctions and the protein area. For example, large gas bubbles in networks would induce varying results depending on the location and size of the bubbles in the image. Hence, additional variables were calculated for a more detailed description of the strength of a protein network (Table 1). For this purpose, the number of junctions was referred to the protein area in order to implement an attribute independent of the used image section. With this attribute called branching rate, the level of network branches can be described. Likewise, the protein end-point rate was established by referring the number of end-points to the protein area. Hereby, the rate of open-ended protein threads are described and can be used for an interpretation of cohesion and consistency of a network. Additionally, the ratio of protein area and total protein length was calculated to describe the thickness of protein threads (protein width). With this developed method called protein network analysis (PNA), the strength of gluten networks can be characterized in detail on a microscopic level considering the level of branching, connectivity, protein length and thickness.

Table 1
Variables determined by AngioTool, their application on protein networks of wheat dough and their explanations. Furthermore, additional variables were calculated for a more precise description of the network strength.

Original terms	Terms for protein network	Content
Explant area		Region of interest embedded by the whole network
Vessels area	Protein area (μm^2)	Area occupied by protein network
Vessels percentage area	Protein percentage area (%)	Vessels area/explant area*100
Total number of junctions	Protein junctions	Total number of junctions in the protein network
Junctions density	Junctions density	Number of junctions/explant area
Total vessel length	Total protein length (μm)	The sum of all protein threads (distance between two junctions)
Average vessel length	Average protein length (μm)	The average length of protein threads
Total number of end-points	Protein end-points	Open-ended protein threads
Mean Lacunarity	Lacunarity	A measure for degree of gaps and irregularities
Additional calculated variables		
	Branching rate	Number of junctions/protein area
	End-point rate	Number of end-points/protein area
	Protein width	Protein area/total length

3.2. Validation of image analysis for quantifying structural changes of protein network by varying bulk water concentration

A method can be validated by e.g. the accuracy, repeatability or precision of the results (Peters, Drummer, & Musshoff, 2007). If a component of a system is varied, especially if changes can be determined visually, significant changes are supposed to be quantified by the used method. Therefore, small standard errors are essential. To validate the application of the developed image analysis method (PNA), bulk water concentration in wheat dough was varied (59.2–89.8 ml/100 g flour) to reach a large range of different protein microstructure shapes. As reported in earlier studies, increasing water addition to wheat dough causes structural changes in protein network due to a plasticizing effect of water (Jekle & Becker, 2011). These effects were to be detected quantitatively and with significant changes by protein network analysis. Evaluating all CLSM images by PNA, all attributes showed significant

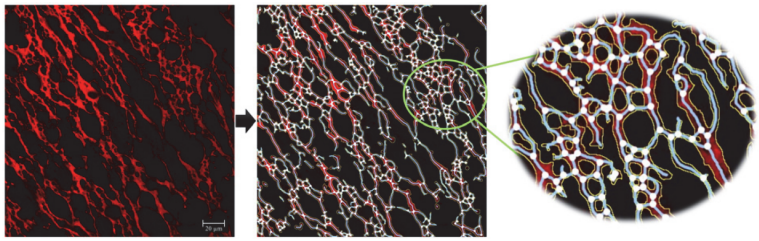

Fig. 3. Protein network analysis of a wheat dough sample. The dough sample based on wheat flour and water (59.2 ml per 100 g flour) were stained with Rhodamine B to visualize proteins and further analysed by CLSM. Left: original CLSM picture (1024 × 1024 pixel, 215 × 215 μm); Middle and Right: picture after image processing with AngioTool (white = junctions, blue = protein skeleton, yellow = protein outline/area).

differences between the standard dough and samples with higher bulk water concentrations as well as partly within the concentration series. Additionally, high precision and repeatability of the results could be pointed out due to small standard errors; this becomes apparent by protein percentage area, lacunarity, junctions' density, protein width, branching- and end-point rate, which are visualized in Fig. 4. In the following part, results are discussed for a magnification with a 60× objective.

In detail, the protein percentage area decreased with increasing bulk water addition (Fig. 4a) by 60% applying an exponential curve fitting on the data set ($R^2 = 0.99$, one phase decay). Similar results could be detected by Jekle and Becker (2011) with a particle based image analysis indicating a linear decrease of protein area with water addition, but in a smaller concentration range. In this range, results of this study would also correlate linearly which confirms the results of the current method. Increasing the water addition to 89.9 ml per 100 g flour, significant differences ($p < 0.001$) could be determined in the range of 59.2–74.9 ml per 100 g flour. At higher concentrations, which represent extremes like pre-doughs, no further significant differences could be detected explaining the exponential decrease to a plateau. By visual evaluation of the CLSM images, an alteration of a highly cross-linked network to clustered proteins with few interconnections was revealed up to a water concentration of 74.9 ml per 100 g flour due to a dilution and a plasticizing effect of water. Above this concentration, proteins were highly aggregated with almost no interconnections. A development of a network might be hindered by the excess of free water resulting in too soft doughs. Therefore, not enough energy can be inserted during kneading which is needed to stretch the proteins. Hence, proteins aggregate due to hydrophobic interactions (Wieser, 2007). These changes in network formation could be quantified by the developed image analysis in absolute values for the first time by the determination of protein junctions. Thus, protein microstructures of wheat dough were characterized by an exponential one phase decrease of junctions density ($R^2 = 0.99$, Fig. 4c) by 75% (relative change, rel.) with rising bulk water concentration confirming the visual study. Furthermore, the agglomeration of proteins could be proven by the linear increasing protein width of up to 126% (rel., $R^2 = 0.95$, Fig. 4d). Another characterization of structural changes could be revealed by the linear rising of lacunarity ($R^2 = 0.98$, Fig. 4b) indicating higher irregular structures and larger holes. Thereby, the large spaces between the protein threads and agglomerates, visible in CLSM images and explained by congregation of starch granules and excess of free water, could be expressed by this novel image analysis. For a precise characterization of the sole protein structure, independent of the used image segment, a calculation of attributes was applied related to the protein area as described in Table 1. Hence, a linear decrease of branching rate was detected ($R^2 = 0.97$, Fig. 4e) as well as a linear increase of end-point rate ($R^2 = 0.98$, Fig. 4f) illustrating a loss of connectivity in protein network with rising amounts of water. When water-binding capacity of flour is exceeded and the excess of free water causes network interruptions as well as protein agglomerations (at concentrations of 79.8 ml per 100 g flour and higher), a branching rate could still be detected due to intramolecular bonds and interactions in protein agglomerates.

In summary, the visual study of structural changes in network formation could be confirmed by quantitative results of the new approach of image analysis in absolute values (protein junctions, thread length or

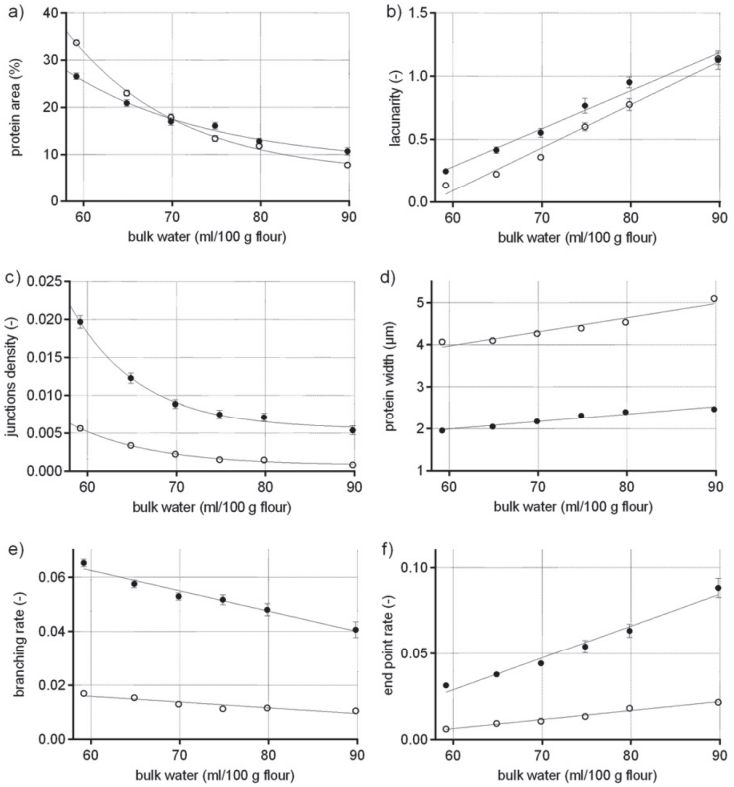

Fig. 4. Influence of different bulk water concentrations on dough microstructure. The dough samples based on wheat flour and water (59.2–89.8 ml/100 g flour) were stained with Rhodamine B to stain proteins, and analysed by CLSM. Two objectives with different magnifications were used (-○- 20× objective; -●- 60× objective). Results are shown as mean ± standard error ($n = 30$).

width) and novel attributes (lacunarity, branching and end-point rate) for the first time.

3.3. Effect of used magnifications on network analysis results

Considering results gained solely by the 60× objective in Chapter 3.2, this part deals with the effect of two different magnifications (objectives with different numerical apertures NA) on image analysis results. As shown in Chapters 3.1 and 3.2, images taken by the 60× objective were analysed precisely by PNA. However, a visualization of dough samples covered by a larger image section might often be helpful to get a better overview of the network. Hence, an objective with a lower magnification would cover more relevant structure fragments of e.g. fermented doughs with large gas bubbles or enlarged protein threads caused by sodium chloride addition. Thus, images of varying bulk water concentration were recorded additionally with a **20×** objective (NA 0.75) and compared with the images of the **60×** oil immersions objective (NA 1.4 oil). Hereby, the aim was to elucidate if network attributes can be detected in the same precise way, even if structures are downsized due to the lower magnification and are recorded by less resolution.

As illustrated in Fig. 4, all results of various protein network attributes were influenced by the two different magnifications. Protein percentage area decreased by 80% (rel.) with a 20× and by 60% (rel.) with a 60× objective, however, both curves showed a similar behaviour (c.f. Fig. 4). Significant differences resulted by the two various magnification were also identified for the attributes junctions' density and branching rate, e.g. the branching rate reached by a 60× objective is four times higher than by a 20×. This effect can be explained by two reasons: first, with a higher magnification, a larger visualization of protein network is reached and complex structures with thin protein threads as well as small holes can be detected more precisely; and second, images taken with the 60× objective have a higher resolution. The resolution depends on the fluorescence wavelength, numerical apparatus, width of the pinhole, pixel dwell and pixel count (Wilson, 2011). The pinhole, pixel count and the wavelength (due to the same fluorescent dye Rhodamine B) was set constantly for both magnifications. The numerical apparatus, therefore, influences the image resolution the most. Thus, the lateral resolution of images taken by the 60× objective is almost twice, the axial almost three times as high. This results in a more precise detection of junctions. Nevertheless, the curve shape and the percentage of the total decrease of junctions' density is similar (83% for 20×, 75% for 60×, rel.) and only the absolute values are higher with the higher magnification and better resolution. Likewise, the linear decrease of branching rate was nearly the same for both magnifications (41% for 20×, 37% for 60×, rel.). However, the change in lacunarity with increasing bulk water concentration acts in almost the same way (nearly the same slopes) for both magnifications due to a network independent determination by box counting (c.f. Section 2.4.1).

Concluding, the precision of detected protein network is higher with a 60× than with a 20× objective. However, significant differences within a concentration series and curve progression (linear, exponential one phase decay, exponential growth) can be detected in a similar way with both magnifications. Nevertheless, absolute values of network characterization are highly influenced by the settings of image recording. Therefore, it is essential to always use the same settings and instrumental equipment for one study and to report all specifications for the indication of results. The decision of an appropriate objective size should be based on either a higher precision of detected network attributes or a visualization of a larger network segment. A higher precision may be crucial when detailed modifications in the protein network must be analysed, provoked by e.g. enzymes. A larger image segment can be useful when analysing doughs with large gaps, which could not be visualized in one image otherwise.

3.4. Dough rheology as a reference method

As a reference method, dough rheology of samples with increasing bulk water concentration was measured by oscillatory and creep-recovery tests. An exponential curve was applied on the data of oscillation tests of doughs and showed a one phase decay by 87% in viscous (G″, $R^2 = 0.97$) and by 89% in elastic (G′, $R^2 = 0.98$) behaviour as well as by 89% in firmness (G*, $R^2 = 0.98$) (see Fig. 5). Similar results could be observed in other studies determining an exponential decrease of G' and G" with increasing water addition in dough (Berland & Launay, 1995; Jekle & Becker, 2011). The relative elastic part J_{el} was determined by creep-recovery-test and revealed an exponential one phase decay ($R^2 = 0.99$) with rising bulk water concentration as well. The decrease of rheological attributes can be traced to the dilution effect the more water is added. However, the asymptotical approach to a plateau can be explained by the limited water absorption capacity of flour and an excess of free water. Above a concentration of 74.9 ml per 100 g flour, a more or less complete decrease (by 98%) of relative elastic behaviour (J_{el}) and no significant differences could be detected any more indicating a watering down of the dough and almost a total loss of a network structure. This effect was also described in literature by adhesion measurements stating that a dough with a distinct network (rubbery state) shifts into a dough without network structures (flow state) with increasing water addition (Heddleson, Hamann, Lineback, & Slade, 1994). The rheological findings correspond with the microstructural characterization of network changes when water addition is increased causing lower cohesion and elasticity in dough, and finally a total loss of a network structure.

3.5. Correlation of microstructural with rheological attributes

To confirm the quantitative description of microstructural changes by network analysis, attributes were correlated with rheological

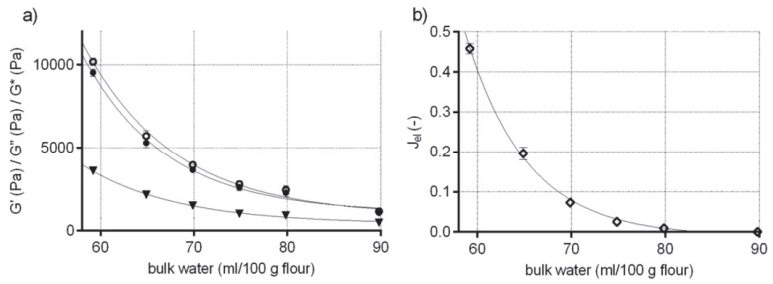

Fig. 5. Influence of different bulk water concentrations on dough rheology. The dough samples based on wheat flour and water (59.2–89.8 ml/100 g flour) were analysed by oscillation (a) and creep-recovery tests (b). Rheological behaviour shows a decrease (exponential one phase decay) for -●- G′ ($R^2 = 0.98$), -▼- G″ ($R^2 = 0.97$), -○- G* ($R^2 = 0.98$) and -◇- J_{el} ($R^2 = 0.99$) with increasing bulk water concentration. Results are shown as mean ± standard error ($n = 5$).

measurements. Protein area, junctions, junctions' density, total and average protein length showed high linear correlations with rheological attributes of oscillation and creep-recovery-test (Table 2). These linear correlations indicate that the rheological behaviour acts in the same way as described by microstructural attributes. In detail, the higher the bulk water concentration, the lower the firmness ($R^2 = 0.993$), viscous ($R^2 = 0.987$) and elastic ($R^2 = 0.993$) behaviour and the lower the number of protein junctions due to a lower cohesion within the network (c.f. Table 2).

When the water holding capacity of dough is exceeded, the proteins agglomerate resulting in shorter thread lengths with a higher width followed by less viscous and elastic doughs. Protein width, branching and end-point rate displayed an exponential growth in correlation to rheological attributes. By rheology measurements, the influence of all components of dough system (starch, protein, lipids) is detected. However, the last three attributes in Table 2 are referred to the protein area describing only changes in the protein network independent of other effects, such as watering of the dough or starch swelling. With increasing bulk water concentration (59.2–74.9 ml/100 g flour), protein network is less interconnected (less branching rate) and viscoelastic behaviour is decreased. At the highest bulk water concentration (89.8 ml/100 g flour), rheological attributes showed almost a non-viscoelastic behaviour whereas in protein agglomerates junctions and end-points were still detected by microstructure analysis due to intramolecular interactions within the aggregated protein particles. This is why the rheological attributes correlate with the branching and end-point rate as well as with the protein width in a non-linear regression.

Overall, the highly significant correlations between both methods confirm that dough functionalities can be characterized and quantified by PNA microstructure analysis.

4. Conclusion

In this study, a novel image analysis was developed to quantify the microstructure of protein networks in wheat dough. This advanced method called protein network analysis (PNA) was validated for varying dough microstructures provoked by increasing bulk water concentration. For the first time, the connectivity and strength of varying network structures could be quantified in absolute values (protein junctions, thread length or width) and novel attributes (lacunarity, branching and end-point rate). In addition, the effect of two different magnifications on quantitative results was demonstrated. Even if the precision and detection of absolute values is better with a higher magnification, structural changes and curve progression within a concentration series can be determined in a similar way using both magnifications. Nevertheless, a characterization of various network structures in absolute values is merely advisable by reporting all influencing specifications of image recording. For further validation of protein network analysis, the rheological behaviour was measured as a reference method, which confirmed the loss of network structure with increasing bulk water concentration - likewise the image analysis.

Table 2
Correlation-matrix of microstructural with rheological attributes. Coefficient of determination (R^2) describes linear relations besides those marked with [b](exponential growth) and [c](exponential one phase decay).

R^2	G′	G″	G*	J_{el}
Protein area	0.954	0.961	0.956	0.898
Protein junctions	0.993	0.987	0.993	0.993
Junctions density	0.996	0.991	0.996	0.989
Total protein length	0.959	0.973	0.961	0.892
Average protein length	0.933	0.952	0.936	0.875
Lacunarity	0.981[c]	0.986[c]	0.982[c]	0.999[b]
Branching rate	0.991[b]	0.983[b]	0.990[b]	0.971[b]
End-point rate	0.958[c]	0.964[c]	0.958[c]	0.993[b]
Protein width	0.999[c]	0.999[c]	0.999[c]	0.999[b]

Thus, the correlations of rheology and microstructure attributes were highly significant. However, the correlation of both methods does not present a connection of rheology and microstructure in general, it is rather applicable to confirm that dough functionalities can be described and quantified by protein network analysis. Nevertheless, dough rheology can be influenced by other dough ingredients and reactions, like starch gelatinisation, whereas solely the protein microstructure is quantified by image analysis. Hereby, a main advantage is that microstructure analysis is the only direct method to visualize and verify the development and level of branching of the protein network in dough. These effects can now be quantified with PNA. Hence, a combination of rheology measurements and protein network analysis provides a detailed characterization of material properties and offers new opportunities of structure-function elucidation. Thus, effects of additives (e.g. enzymes) or varying dough ingredients (e.g. sodium chloride) on the gluten network - the most structure-relevant component in wheat dough - can quantitatively be elucidated. This knowledge can be used to create doughs with a specific branching rate as well as to predict dough functionalities. Thereby, a better understanding of baked products can be promoted. Hence, the development of new products with targeted structural properties and functionalities can be supported by protein network analysis. Furthermore, changes in dough functionality caused by varying process conditions can be controlled specifically. In summary, a novel method for quantifying microstructures in wheat doughs was established by protein network analysis.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2016.10.012.

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Supplementary data to "Protein network analysis – A new approach for quantifying wheat dough microstructure"

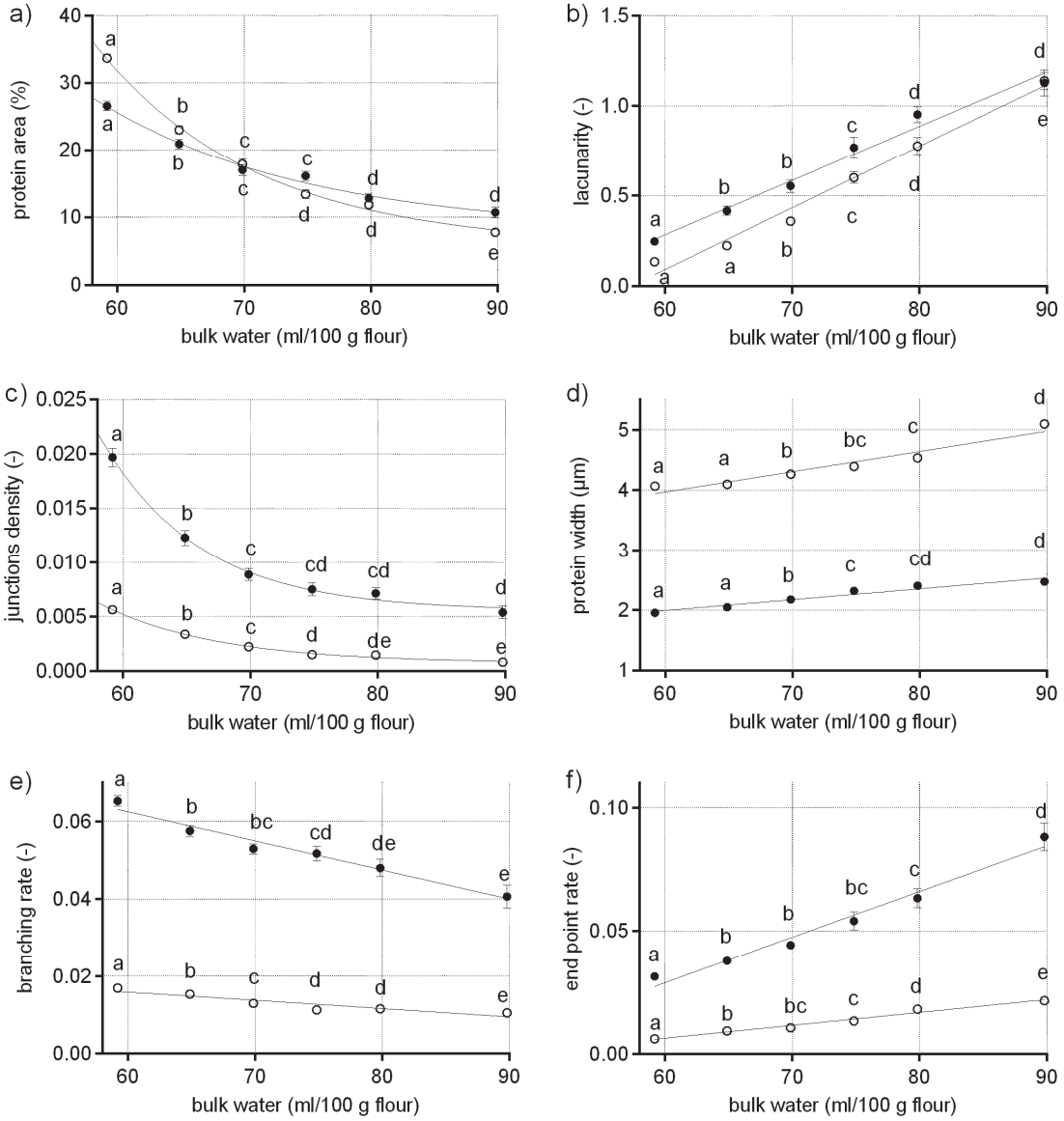

Figure 9 (supplementary data): Influence of different bulk water concentrations on dough microstructure. The dough samples based on wheat flour and water (59.2-89.8 ml/100 g flour) were stained with Rhodamine B to stain proteins and analysed by CLSM. Two objectives with different magnifications were used (-O- 20x objective; -●- 60x objective). Results are shown as mean ± standard error (n=30). Letters a – e represent significant different dough samples of varying water concentrations ($p < 0.01$).

3.3 Staining methods for dough systems – Impact on microstructure and functionality

LWT - Food Science and Technology 88 (2018) 139–145

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Staining methods for dough systems – Impact on microstructure and functionality

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ARTICLE INFO

Keywords:
Fluorescent dye
CLSM
PNA
Protein network
Rhodamine B

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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http://dx.doi.org/10.1016/j.lwt.2017.10.010
Received 16 June 2017; Received in revised form 31 August 2017; Accepted 5 October 2017
Available online 06 October 2017

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.

2.9. Depth of dye diffusion into the dough

The depth of diffusion of Rhodamine B solution was measured by staining dough samples with drop technique. After rapid freezing with liquid nitrogen, dough was cut vertically. Pictures were taken standardized of the sliced samples and the heights of the visible stained area were measured with an in-house software (ImgPro). The measurement was performed in triplicate.

2.10. Statistical analysis

Results were evaluated statistically with GraphPad Prism 6 (version 6.01, GraphPad Software Inc., La Jolla, USA) with one-way analysis of variance (ANOVA) followed by a Tukey-test. All values are represented with the standard error of the mean (SEM). The goodness of fit of nonlinear regressions are represented by R^2.

3. Results and discussion

3.1. Impact of drop technique on dough rheology and microstructure

The impact of three different staining methods for CLSM (drop-, bulk water- and rapid freezing technique) on the rheological dough functionality were comparatively explored in this study. Rheological measurements were chosen as a reference method due to a strong relation of microstructure and rheological behaviour. Moreover, an unstained dough cannot be measured by CLSM, but by rheology. The staining of dough samples by drop technique (applying one drop of dye solution onto dough surface) revealed an impact on dough rheology. Significant differences were detected for G* and J_{max} between the standard (unstained) and the stained dough samples beginning at 3 µL of dye solution (0.13 µg/cm^2 dough) (Fig. 1a). The complex shear modulus G* decreased exponentially (one phase decay, R^2 = 0.74) by 12.1% compared to the standard when dye solution was added up to 100 µL, whereas the creep compliance J_{max} increased (one phase association, R^2 = 0.88) by 56.9%. The broad range of dye volume (1 µL–100 µL) was chosen in order to determine extrema and limits. With increasing drop volume of dye solution (beginning at 3 µL up to 100 µL), no significant differences in dough stiffness were detected within the stained doughs causing an asymptotical approximation to constant values. This effect may be explained by a limited water absorbency of dough resulting in a layer of dye solution on the dough surface. Thus, the influence of the drop technique on dough rheology reaches a limit. Although lower drop volumes of dye solution (1 and 2 µL) did not affect dough rheology significantly, these dye concentrations would be too low to stain the dough sufficiently. Consequently, the drop technique did affect the rheological characteristics of dough in the relevant range of dye drop volume for CLSM measurement. In order to elucidate reasons for this effect, further experiments were done.

In addition to rheology measurements, protein microstructure was analysed by CLSM to elucidate potential effects of drop technique on the structure formation or shape. Protein network analysis revealed that branching rate and lacunarity did not change significantly within the dye concentration range (Fig. 1b). The same outcome were found for other attributes such as end-point rate, junctions' density, protein percentage area, protein width and average protein length (data not shown). The CLSM measurement was done in a concentration range of 3 µL–20 µL of drop volume. Below 3 µL, the volume would be too low for a sufficient staining of the whole protein network as mentioned above. An increasing drop volume (over 20 µL) would cause a too large layer of dye solution due to a limited water absorbency of dough. In this case, it would be impossible to take focussed images of the protein network. In the used range of drop volume for CLSM measurement, there were no significant difference in dough rheology as well. Hence, the results of microstructure by PNA were consistent with the results of rheology.

A reason for significant difference in rheology between stained doughs by drop technique and the non-stained standard dough might not be the dye itself but the addition of water. Therefore, the same tests were repeated with the addition of solely water.

3.2. Comparison of dye solution and water addition by drop technique

To elucidate reasons for the drop technique's influence on dough rheology, the same tests were repeated with addition of solely water instead of the dye solution with representative 7 various drop volumes. Dough rheology changed with increasing drop volume of water in the same way as with addition of dye solution. Both curves assimilated to a constant value (Fig. 2). However, the effect of solely water addition was more distinct displayed in a significant difference between samples of water and dye addition by drop technique. Water addition affected less stiff (19.5% vs. dye/29.2% total at highest drop volume) doughs in comparison to doughs with dye addition. A reason for this effect could be different surface tensions of water and dye solution. In this case, water and dye solution would interact with the dough surface in different intensive ways. Therefore, the surface tension of water and the Rhodamin B solution (0.1 g/L water) were determined. The results showed a significant lower (11.5%) surface tension of dye solution than water ($p < 0.001$). The lower surface tension of Rhodamine B might be explained by a tensioactive effect due to hydrophobic parts in its molecular structure. This may cause a varying surface wettability of dough depending on its surface tension. Thus, the dye solution and water were able to permeate inside the dough to different extents causing various effects on dough functionality (softer/stiffer dough). As shown in previous studies, an increasing bulk water addition causes a

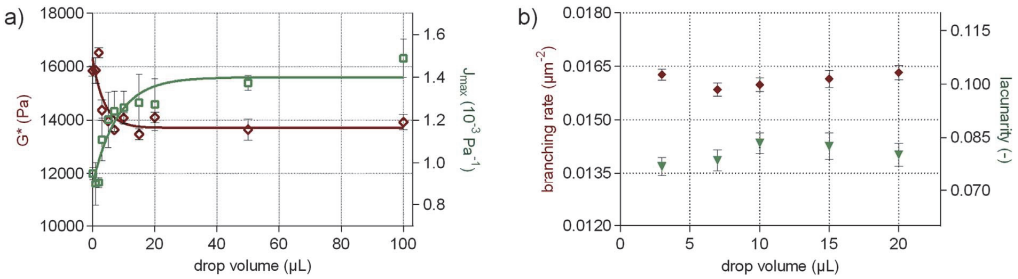

Fig. 1. Influence of dye addition by drop technique on wheat dough rheology and microstructure. The dough samples based on 58.9 g water per 100 g wheat flour were analysed by a) rheometer (-◇- G* and -□- J_{max}, n = 3) and by b) CLSM followed by PNA (-♦- branching rate and -▼- lacunarity, n = 24). Different volumes of a Rhodamine B solution were added on dough samples by drop technique. Means are shown with standard error.

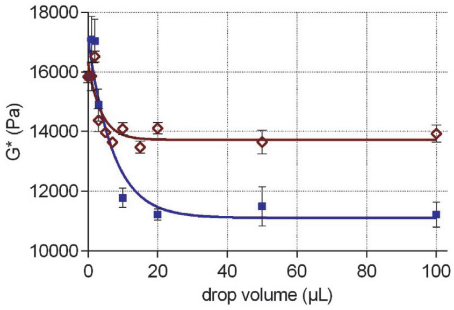

Fig. 2. Influence of dye addition by drop technique on wheat dough rheology. The dough samples based on 58.9 g water per 100 g wheat flour were analysed by rheometer (G*). Different volumes of a Rhodamine B solution and water were added on dough samples by drop technique. Symbols: -◇- dye addition; -■- water addition. Means are shown with standard error (n = 3).

softer dough and weakened protein structure (Berland & Launay, 1995; Bernklau et al., 2016). Even if the water is not kneaded into the dough by drop technique, the study shows that water has a distinct effect on dough microstructure.

To estimate the extent of influence on dough functionality caused by drop technique, the depth of diffusion of the dye solution was measured. The little amount of dye addition was thought to have a negligible effect in relation to the dough sample used for CLSM. However, it was shown that the dye solution diffused only into the first few micrometres (0.53 ± 0.07 mm) of the dough when staining by drop technique with Rhodamine B. Thus, the water concentration is higher in the upper part of the dough sample. Hence, not only the stained part is measured by rheometer, but also an unstained part due to a sample height of 2.0 mm (gap between the parallel plates). A low effect of the dye solution on rheology of the total dough sample would be expected when taking into account that only about a quarter of the dough sample was stained by drop technique. However, the results showed, that even this small part caused a distinct effect on rheology of the whole dough sample. Thus, the effect on dough rheology is even higher in solely the stained part. This section is most important for CLSM measurement because laser light only penetrates into the first few µm under surface. Consequently, the drop technique causes a dilution effect in this area resulting in a structural change of dough rheology.

3.3. Impact of bulk water technique on dough rheology and microstructure

The second staining method, the bulk water technique (adding the dye solution during kneading in the bulk water of the dough), showed no impact on dough rheology. No significant differences were found for G* and J_{max} between all concentrations of stained dough samples and the standard (Fig. 3a). As well, no changes in dough rheology were detectable with increasing dye concentration. Compared to the tensioactive effect of Rhodamine B when staining with drop technique, an effect of dye on dough functionality was also expected for bulk water technique. When dye is kneaded inside the dough, it might influence gluten formation due to hydrophobic interactions of proteins with the hydrophobic parts of Rhodamine B. However, even if there is a tensioactive effect of Rhodamine B, it is too low and not measurable with rheology. In contrast, when staining with drop technique, a higher dye concentrationoccurs on the dough surface resulting in a change of dough rheology. Although this effect can also be explained by the tensioactive behaviour of the dye, the most rheological change caused by drop technique is related to the dilution effect of water. For bulk water technique, neither the dye itself nor the staining method had an impact on dough functionality. These results confirm the statement of Jekle and Becker (2012) that the addition of Rhodamin B did not have an impact on dough rheology. However, the effect of varying dye concentration on dough microstructure had not been studied until now.

The microstructural evaluation by PNA revealed that the protein network is not affected with increasing dye concentration. No significant differences were detected for the attributes branching rate and lacunarity, except of the lowest concentration (0.1 mg/100 g flour), as visible in Fig. 3b. An explanation might be given by the low fluorescence intensity, which is present at that low dye concentration and which caused an out of focus effect. Thus, this concentration was too low to stain the whole protein network adequately, deduced visually of the CLSM images. With a dye concentration of 0.2 mg/100 g flour or higher, dough was stained sufficiently for CLSM measurement, and no significant differences were detected with increasing dye concentrations. Hence, this staining method had no impact on dough functionality and microstructure.

3.4. Impact of rapid freezing technique on dough rheology and microstructure

The rapid freezing technique (freezing dough with liquid nitrogen and cutting in with a microtome to achieve a plane surface) is usually carried out by staining dough slices after rapidly freezing (Bousquieres et al., 2014; Hesso et al., 2015; Lee et al., 2001). However, as displayed above, staining after dough preparation impacts dough microstructure and rheology. Therefore, dye was kneaded into the dough like the bulk water technique, which has no influence on dough rheology, and samples were frozen rapidly with liquid nitrogen afterwards. Hereby, the sole influence of rapid freezing on protein microstructure and rheology could be determined.

Fig. 4 shows the results of rheological and microstructural measurements of defrosted dough samples compared to the ones at RT (room temperature) of bulk water technique. The rheological

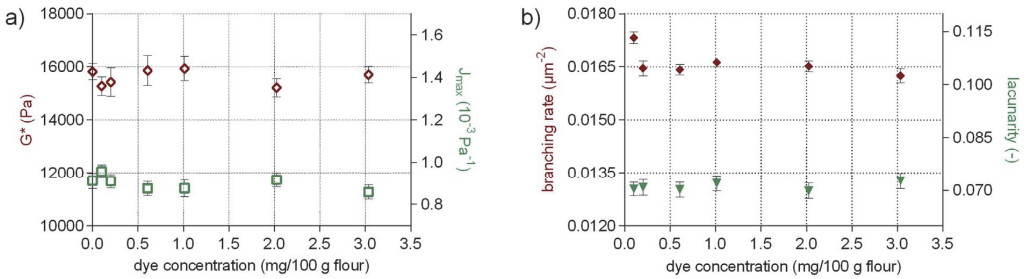

Fig. 3. Influence of dye addition by bulk water technique on wheat dough rheology and microstructure. The dough samples based on 58.9 g water per 100 g wheat flour were analysed by a) rheometer (-◇- G* and -□- J_{max}) and b) by CLSM followed by PNA (-♦- branching rate and -▼- lacunarity). Six different dye concentrations were added in bulk water. Means are shown with standard error (rheometer n = 5; CLSM n = 24).

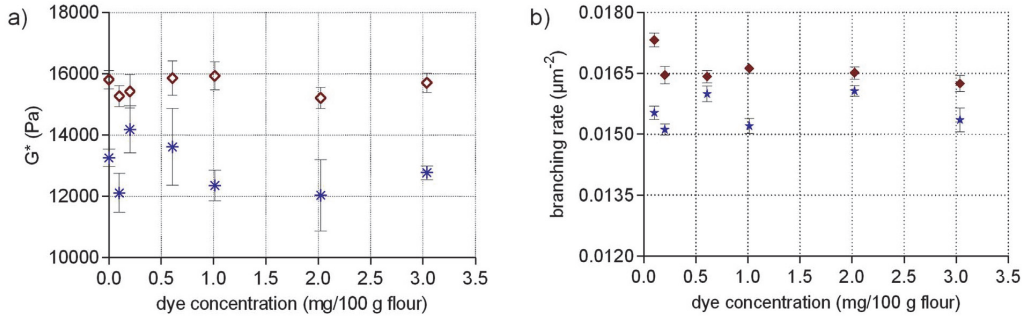

Fig. 4. Influence of rapid freezing technique on wheat dough rheology and microstructure. The dough samples based on 58.9 g water per 100 g wheat flour were analysed by a) rheometer (-◇- G* RT dough (room temperature); -✳- G* defrosted dough) and b) CLSM followed by PNA (-◆- branching rate of RT dough, -★- of defrosted dough). Different dye concentrations were added in bulk water, dough samples were analysed after freezing and thawing (defrosted dough). For comparison, measurements of RT doughs are shown as well. Means are shown with standard error (rheometer n = 3, CLSM n = 21).

measurements revealed that G* was significant lower (by 17%) for the defrosted standard dough (not stained) compared to the RT one (Fig. 4a). The same effect was observed for the stained dough samples compared to their RT equivalents. As identified for the RT dough samples above, no significant differences were detected within the different dye concentrations of defrosted dough samples. This indicates once more, that the addition of dye itself had no influence on dough rheology. However, the preparation of dough samples for CLSM measurement by rapid freezing caused a decrease of dough stiffness by 17%. Most of the former studies on frozen dough dealt with lower freezing rates than freezing with liquid nitrogen. They identified similar results of a softened dough rheology and weakened structure after freezing and thawing (Autio & Sinda, 1992; Ribotta, Pérez, León, & Añón, 2004). The weakening effect is attributed to the ice crystal formation, which impairs the gluten network (Berglund et al., 1991; Havet, Mankai, & Le Bail, 2000). In contrast, higher freezing rates are said to have less impact on rheology and structure, due to smaller ice crystal formation. Meziani et al. (2011) confirmed the influence of freezing rate on gluten formation and rheology of sweet dough, and revealed no significant differences of RT doughs to ones frozen with liquid nitrogen. However, the study was carried out on a more complex dough system with eggs and lipids, which are said to be cryoprotective. In this study, it was proven that rapid freezing influences significantly dough rheology. The weakening effect of the ice crystals and thawing may also explain the variability among the results and the standard error of frozen doughs.

The quantitative results of protein network analysis of the microstructure confirmed the effect on rheology. The branching rate of the protein network was significant lower (6%) for defrosted doughs compared to the RT ones (Fig. 4b). In addition to much higher end-point rates (by 20%, c.f. Table 1), results indicate a weakened network structure caused by rapid freezing and thawing. Concluding, even if other studies assumed that rapid freezing would cause a minimal impact on dough structure (Baier-Schenk et al., 2005; Maeda et al., 2013), it was proven in this study that rapid freezing influences significantly dough functionality.

3.5. Comparison of the three staining methods

The results revealed that, independent of the staining method, the fluorescent dye itself did not influence rheological dough properties. However, dough functionality was affected by drop- and rapid freezing technique confirmed by significant differences between the standard (non-stained) dough and stained doughs of rheology measurements. In contrast, no significant difference was found between standard dough and doughs stained by bulk water technique. Hence, the rheological dough functionality is not affected by bulk water technique, indicating a visualization of the protein microstructure in the most realistic way with this method due to a strong relation of microstructure and rheology. For elucidation of the sole influence of the drop- and rapid freezing technique on the protein microstructure, attributes of the protein network analysis were compared in Table 1 against values of the bulk water technique.

High significant differences ($p < 0.001$) in microstructure were detected between all staining methods (Table 1). Compared to the bulk water technique, the drop technique caused a weakened protein network, indicated by an increased end-point rate by 8%. The decreased average protein length by 29% and decreased junctions' density by 6% indicate a change in structural formation of the network, might explained by a swelling of the components as postulated by Dürrenberger et al. (2001). As displayed in this study, drop technique caused softer doughs due to the dilution effect of water addition. The plasticizing effect of water results in increasing values for lacunarity by 14%.

Table 1
Comparison of the staining methods bulk water-, drop- and rapid freezing technique.

	Bulk water technique	Drop technique	Rapid freezing technique
Protein area (%)	39.33 ± 0.23	38.06 ± 0.27 (–3%)	33.16 ± 0.96 (–17%)
Junctions' density (μm^{-2})	0.00653 ± 0.00006	0.00613 ± 0.00007 (–6%)	0.00519 ± 0.00008 (–21%)
Average protein length (µm)	329.9 ± 7.8	234.9 ± 5.9 (–29%)	191.7 ± 5.3 (–42%)
Lacunarity (−)	0.0712 ± 0.0010	0.0811 ± 0.0014 (+14%)	0.1078 ± 0.0020 (+51%)
Branching rate (μm^{-2})	0.01647 ± 0.00007	0.01612 ± 0.00009 (–2%)	0.01550 ± 0.00010 (–6%)
End-point rate (μm^{-2})	0.00751 ± 0.00007	0.00812 ± 0.00009 (+8%)	0.00901 ± 0.00010 (+20%)
Protein width (µm)	4.021 ± 0.006	4.039 ± 0.007 (n.s.)	4.048 ± 0.009 (+1%)

Mean values of PNA attributes are shown for all dye concentrations (which were not significant different) ± standard error (n = 120).
Percentage values denote significant deviations from the values of the bulk water technique in each row ($p < 0.001$; "n.s." not significant).

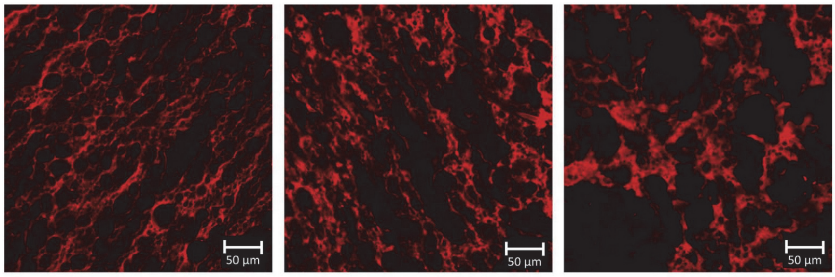

Fig. 5. Microstructure of the protein network stained by bulk water-, drop- and rapid freezing technique. The images of wheat dough were taken by confocal laser scanning microscopy. The protein networks were stained by Rhodamine B with three different staining methods.

The effect of rapid freezing technique on dough microstructure was even more distinct than of the drop technique (c.f. Table 1, Fig. 5). The highly decreased junctions' density (21%) and branching rate (6%) as well as the increased end-point rate (20%) can be attributed to a weakening of network structure caused by freezing and thawing. Intermolecular bonds were ruptured due to ice crystal formation of free water (Berglund et al., 1991). The resulting gaps explain the increasing values for lacunarity (up to 51% higher than for a dough stained by bulk water technique), clearly visible in Fig. 5. Changes in protein formation during frozen storage were also found by Ribotta et al. (2001), who displayed a decrease of high molecular weight proteins with increasing storage time, that could explain the decrease of branching rate in this study. Wang et al. (2014) confirmed the structural change during frozen storage by studies on the secondary structure of glutenin- and gluten-rich fractions. The structure was more disordered, weakened and underwent conformational changes due to an increase of intermolecular β-sheet and β-turn. However, the studies were performed with an extended frozen storage and not with rapid freezing like in this study. Rapid freezing was supposed to preserve the structural properties of dough. However, it was clearly shown in this study that protein network formation was significantly weakened after rapid freezing. However, the effect might be caused more intensively by thawing and release of water than the rapid freezing process itself.

Concluding, rheological and microstructural properties of dough are highly impacted by drop- and rapid freezing techniques, whereas bulk water technique caused no significant effect on dough rheology. Based on this fact and the relation between microstructure and rheology, the bulk water technique can be rated as the method which let microstructures visualize in a most realistic, non-invasive way.

4. Conclusion

This study clarified, that different concentrations of the dye Rhodamine B in the relevant range for CLSM measurement did not change structural formation or rheological dough properties. Rather the staining procedure than the dye itself had an impact on dough functionality. Three different staining methods for CLSM measurements were comparatively investigated. Results revealed that microstructures of wheat dough can be analysed in a most realistic, non-invasive way by bulk water technique, whereas drop- and rapid freezing techniques influenced dough rheology and microstructure significantly. The rapid freezing technique is mostly used in literature. With a microtome, a plane surface is achieved, and dough is often cut to a thin section. However, a thin section is not required due to the confocal system of a CLSM. Moreover, the dough can also be cut with a razor blade and analysed without freezing as demonstrated with the bulk water technique. Taking into account that rapid freezing has a high significant impact on dough rheology and microstructure, this technique is not recommended for further studies.

On the basis of this study, the bulk water technique should be the preferred method for dough staining used for CLSM measurements. Nevertheless, the drop technique cannot be avoided for doughs produced out of the lab scale because the dye cannot be added to dough during kneading in industrial productions due to health risk and cost factors of the dye. However, the operators should be aware when using drop technique that the absolute values of microstructural attributes are changed, pretending a more weakened structure than it really is.

Acknowledgements

The authors would like to thank Kathrin Petersen for her contribution. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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3.4 Gluten polymer networks – A microstructural classification in complex systems

polymers

MDPI

Article

Gluten Polymer Networks—A Microstructural Classification in Complex Systems

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Received: 22 May 2018; Accepted: 3 June 2018; Published: 5 June 2018

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Abstract: A classification of gluten polymer networks would support a better understanding of structure-function relationships of any gluten polymer material and thus, the control of processing properties. However, quantification and interpretation of the gluten network structures is challenging due to their complexity. Thus, the network formation was altered by specific gluten-modifying agents (glutathione, ascorbic acid, potassium bromate, glucose oxidase, transglutaminase, bromelain) in this study in order to clarify if structural alterations can be detected on a microstructural level and to specify different polymer arrangements in general. Microstructure analysis was performed by confocal laser scanning microscopy followed by quantification with protein network analysis. It was shown that alterations in gluten microstructure could be elucidated according to the kind of modification in cross-linking (disulphide, (iso) peptide, dityrosyl). Linear correlations of structural network attributes among each other were found, leading to an assertion in general: the higher the branching rate, the thinner the protein threads and the larger the interconnected protein aggregate. Considering the morphological attribute lacunarity, a quantitative classification of different gluten arrangements was established. These assertions were extended by using unspecific gluten-modifying agents in addition to the specific ones. Ultimately, five network types were proposed based on diverse polymer arrangements.

Keywords: CLSM; protein network analysis; microstructure; wheat; gluten; network type

1. Introduction

Wheat gluten polymer does not only play an important role as a main structural component in wheat dough or bread, it is also applied in biomaterials like films, gels, foams, or bioplastics, due to its unique viscoelasticity and low water solubility [1–4]. Gluten is a complex, highly cross-linked and three-dimensional network formed of intermolecular covalent bonds (disulphide bonds, dityrisine cross-links, (iso) peptide) and non-covalent interactions (hydrogen, hydrophobic, ionic) [5–7]. The structure and properties of the gluten network are determined by both non-covalent and covalent bonds. However, the most influence is exerted by the number and distribution of disulphide bonds (SS), which are dependent on environmental and genetic factors [5]. To compensate these variabilities of gluten properties (e.g., with specific enzymes or chemical agents) as well as to elucidate structure-function relationships in any kind of material (biomaterial or food products), a precise analysis of the network characteristics, especially the degree of cross-linking, is important. However, the network structure is challenging to analyze due to its complexity and low solubility [8,9]. The degree of cross-linking is often characterized by means of the molecular weight of HMW (high molecular weight) and LMW (low molecular weight) glutenin subunits, GMP (glutenin macropolymer) as well as the amount of free sulfhydryl (SH) groups [2,5,10,11]. However, the spatial arrangement of gluten proteins within a complex matrix (present as e.g., clustered agglomerates or homogeneously

distributed network) cannot be elucidated sufficiently with these methods. This would support a better understanding of structure-function relationships and the control of processing properties [4]. An appropriate method to visualize the arrangement of gluten microstructure is the confocal laser scanning microscopy (CLSM). While influencing factors on gluten network formation or modifications in cross-linking, like the effect of enzymes or additives, are investigated in detail on a molecular or macroscopic level [10,12–14], the microstructure is little analyzed.

Thus, the aim of this study is to clarify if structural changes caused by chemical or enzymatic agents can be detected qualitatively as well as quantitatively on a microstructural level with CLSM. Furthermore, gluten network attributes and various types of polymer arrangements should be specified in general. For this purpose, specific gluten-influencing factors were tested in a model flour-water-system by reduction (glutathione, GLU), oxidation (ascorbic acid, ASC) and enzymatic effects (glucose oxidase, GOX, transglutaminase, TG), each variation in different concentrations. In this context, microstructural protein network characteristics dependent on the modification of cross-linking (disulphide, (iso) peptide, dityrosyl) were considered. The flour-water-system was chosen due to its complex matrix structure comparable to a wheat dough and due to its formation to a complex, spatial arranged network in a multiphase system. Furthermore, gained knowledge about network classifications can be derived for other gluten polymer materials (e.g., bioplastics containing of gluten blends with glycerol, water, polycaprolactone or polylactic acid [15]). Structural attributes of the gluten network's microstructure were quantified by means of the method protein network analysis (PNA) established by Bernklau et al. [16]. In order to define network classifications in general, further specific as well as unspecific variations on gluten polymer (reduction (RHL) and increase (IHL) of hydration level; addition of rapeseed oil (ROI), shortening (SHO), potassium bromate ($KBrO_3$), bromelain (BRN)) were analyzed and correlations of structural attributes (lacunarity, branching rate, end-point rate, protein width and length) were performed.

2. Materials and Methods

2.1. Materials

Four different German commercial wheat flours Type 550 were supplied by Rosenmühle (Landshut, Germany). Flour characteristics were analyzed according to methods of the AACC international (AACCi) and of the International Association for Cereal Science and Technology (ICC): the moisture (AACCi 44-01), protein content (AACCi 46-16, N × 5.7), ash (ICC 104/1), and falling number (AACCi 56-81). All specifications and characteristics of the four flours are summarized in Table 1. The use of various flours was intended to detect microstructural changes independent of the raw material. Ascorbic acid was supplied by Carl Roth GmbH + Co. KG (Karlsruhe, Germany), glutathione by VWR International GmbH (Darmstadt, Germany), transglutaminase (≥1000 units/g) and glucose oxidase (≥1100 units/g) by AB Enzymes GmbH (kindly provided by AB Enzymes GmbH, Darmstadt, Germany), potassium bromate ($KBrO_3$) by ThermoFisher GmbH (Karlsruhe, Germany), bromelain (≥3 units/mg protein) and D(+)glucose by Sigma-Aldrich Chemie GmbH (Steiheim, Germany), Rhodamine B by Merck KGaA (Darmstadt, Germany), rapeseed oil by Cargill Oil Packers bvba (Izegem Belgien) and shortening (ingredients: palm fat, coconut fat, rapeseed oil, water, emulsifier, NaCl, citric acid, aroma, carotin) by MeisterMarken (CSM Deutschland GmbH, Bingen, Germany).

Table 1. Characteristics of the used wheat flours and flour-water-systems. Variations of flour-water-systems were increased hydration level (IHL), addition of rapeseed oil (ROI), shortening (SHO), glutathione (GLU), reduced hydration level (RHL), ascorbic acid (ASC), transglutaminase (TG), glucose oxidase (GOX), potassium bromate ($KBrO_3$) and bromelain (BRN).

	$Flour_a$	$Flour_b$	$Flour_c$	$Flour_d$
Ascorbic acid * (3 mg/ kg flour)	yes	yes	no	no
Protein (g/100 g dry flour)	12.70 ± 0.04	11.49 ± 0.04	11.85 ± 0.04	12.50 ± 0.02
Ash (g/100 g dry flour)	0.63 ± 0.01	0.65 ± 0.01	0.58 ± 0.00	0.64 ± 0.00
Falling number (s)	407.0 ± 14.4	437.3 ± 8.1	434.3 ± 16.8	488.5 ± 18.9
Kneading time ** (s) to 500 FU	180	180	300	240

Flour-Water-Systems										
	$Flour_a$			$Flour_b$		$Flour_c$		$Flour_d$		
Variations	**IHL**	**ROI**	**SHO**	**GLU**	**RHL**	**ASC**	**TG**	**GOX**	$KBrO_3$	**BRN**
Moisture ** (g/100 g flour)	14.17 ± 0.03	13.92 ± 0.01	13.92 ± 0.01	13.91 ± 0.02	14.13 ± 0.3	14.86 ± 0.07	14.28 ± 0.15	14.14 ± 0.08	13.86 ± 0.04	13.86 ± 0.04
Water addition (mL/100 g flour)	59.18	58.32	58.32	57.76	57.83	56.17	60.49	61.06	61.36	61.36
Kneading time *** (s) to 500 FU	180	180	180	180	180	300	250	250	240	240

* Commercial wheat flour usually contains ascorbic acid, ** moisture content of flour varies during storage and was determined for each sample variation, *** rounded to the nearest tens.

2.2. Preparation of Flour-Water-Systems

Each standard flour-water-system (model dough) was produced with commercial wheat flour (c.f. Table 1) and demineralized water. The required kneading time and recipe for the standard flour-water-system of each flour was estimated by the water absorption, moisture (corrected to 14%) and the targeted resistance of 500 Farinograph units in a Z-kneader (doughLAB; Perten Instruments, Hägersten, Sweden) according to AACCi method 54-70.01. The determined kneading times are listed in Table 1. Flour-water-systems were varied by addition of different agents, in each case in increasing concentrations. The unspecific gluten modifications were caused by addition of rapeseed oil (0.0, 5.0, 10.0, 20.0 and 50.0 g/100 g $flour_b$), shortening (0.0, 5.0, 10.0, 15.0, 20.0 and 50.0 g/100 g $flour_b$), increase of hydration level (59.18 (standard), 64.87, 69.86, 74.85, 79.84 and 89.82 mL water/100 g $flour_a$) and reduction of hydration level (reduced water concentration compared to a standard flour-water-system; 57.83 (standard), 53.84, 49.84, 47.58 and 45.85 mL water/100 g $flour_b$). Specific gluten modifications were caused by addition of the chemical agents glutathione (0.0, 7.5, 15.0, 30.0, 45.0, 60.0 and 75.0 mg/100 g $flour_b$), ascorbic acid (0.0, 25.0, 50.0, 100.0, 150.0 and 200.0 mg/kg $flour_c$) as well as potassium bromate (0.0, 60.0, 120.0 and 180.0 mg/kg $flour_d$). Enzymatic agents were represented by bromelain (0.0, 200.0, 1000.0, 2000.0, 3000.0, 6100.0 mg/kg $flour_d$), transglutaminase (0.0, 100.0, 1000.0, 2000.0, 5000.0 and 10000.0 mg/kg $flour_d$) and glucose oxidase (0.0, 20.0, 40.0, 60.0, 100.0 and 150.0 mg/kg $flour_d$, addition of 0.5 g glucose/100 g $flour_d$ to each sample). Each dough variation was produced in triplicate.

2.3. Microstructure Analysis by Confocal Laser Scanning Microscopy

Each sample was stained with Rhodamine B (0.01 g/100 mL water) for CLSM measurement in order to visualize protein microstructure. The dye was kneaded combined with the flour-water-system by replacing 5 mL of bulk water with Rhodamine B solution according to the bulk water technique [17]. An eclipse Ti-U inverted microscope with an e-C1 plus confocal system (Nikon GmbH, Düsseldorf, Germany) with a Plan Apo VC 60x/1.40 oil objective and a 534 nm laser (emission 590/50 nm) were used for visualization. Eight different images were taken of each sample with a resolution of 1024 × 1024 pixel and a size of 215 × 215 µm.

2.4. Image Processing and Analysis

Protein network characteristics were quantified by protein network analysis (PNA) according to the method of Bernklau, Lucas, Jekle and Becker [16]. For this purpose, AngioTool64 version 0.6a (National Cancer Institute, National Institute of Health, Bethesda, MD, USA) was applied. Each CLSM image was analyzed with the same settings to ensure a reproducible quantification of the network attributes. The parameters for vessels diameter (implies protein diameter) were set to 3 and 5, intensity low and high threshold to 15 and 255, small particles were removed under 35 and the function "fill holes" was deactivated. Calibration was set to 4.76 pixel/µm.

At least five attributes are required for a detailed characterization of the protein network [18]: the structural network attributes branching rate (number of junctions/protein area; describes the network connectivity), end-point rate (number of end-points/protein area; describes the weakness of a network), average protein length (length of a continuous protein particle) and protein width (thickness of protein threads) as well as the morphological attribute lacunarity (attribute for the amount and size of network gaps; describes irregularities of a structure). A detailed description of the attributes can be found in the publication of Bernklau, Lucas, Jekle and Becker [16].

2.5. Statistical Analysis

Results were evaluated statistically with GraphPad Prism 6 (version 6.01, GraphPad Software Inc., La Jolla, CA, USA) with one-way analysis of variance (ANOVA) followed by a Tukey-test. All values are represented with the standard error of the mean (SEM). Multivariate statistics and principal

component analysis (PCA) were performed with JMP Pro software (version 12.2.0, SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

Specific as well as unspecific gluten-modifying chemical and enzymatic agents were studied on their effect on the gluten network's microstructure of flour-water-systems, respectively, in order to define network classifications in general. In the following sections, four representative examples of specific gluten-modifying agents are shown in detail, followed by a multivariate statistic of all 10 variations. The gluten polymer investigations were performed by means of a flour-water-system due to its complex matrix comparable to a wheat dough. For that reason, the discussion is based on studies of wheat products.

3.1. Protein Network Formation Modified by Glutathione

The tripeptide glutathione (γ-glutamylcysteinglycine) promote, as a reducing agent, the interchange of SH with SS bonds. Thus, glutathione causes a cleavage of existing disulphide bonds among proteins resulting in a weakening effect of dough structure [19,20]. This effect has been studied comprehensively on a molecular (disulphide bonds, sulfhydryl interchange) and macroscopic (dough rheology, bread volume and texture) level in literature [20–22]. In Figure 1, it is shown that the addition of glutathione had also distinct effect on the microstructure of flour-water-systems. Small gaps are visible in the CLSM micrograph within the protein network due to ruptured protein threads caused by cleaved disulphide bonds (Figure 1f). These findings were also quantified by PNA; the branching rate decreased by 21% at the highest GSH concentration whereas the end-point rate increased significantly by 27% due to a higher rate of open-ended protein threads. The rupture of the disulphide bonds resulted in protein fragments with shorter and thicker protein threads (c.f. Figure 1c,d). The lacunarity decreased by 36% due to the loss of an intact network, which caused closely packed but unconnected protein fragments. These structural changes explain the distinct decrease in dough firmness, which is reported in other studies [20,23].

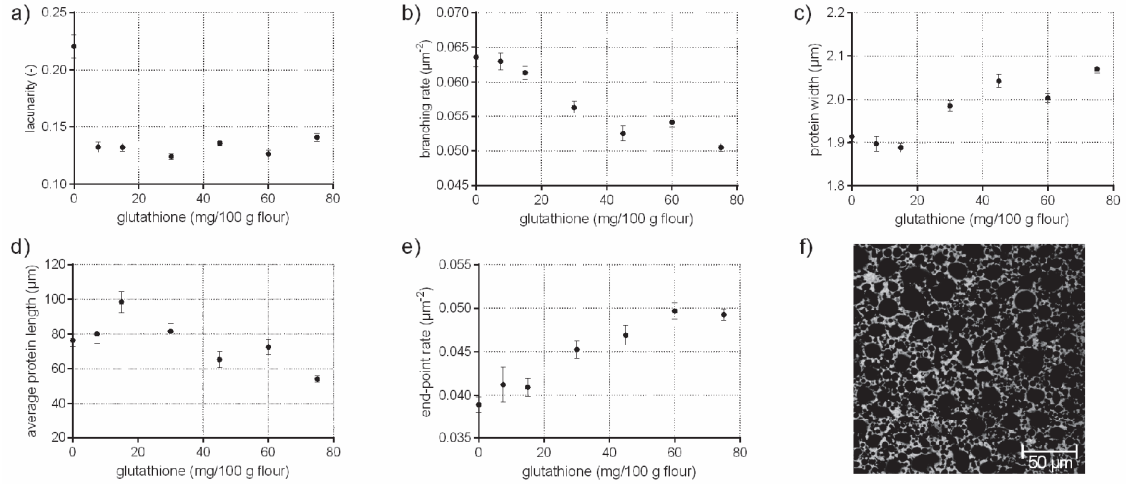

Figure 1. Influence of glutathione on the protein network attributes. Flour-water-systems with increasing concentrations of glutathione were analyzed by confocal laser scanning microscopy (CLSM) followed by protein network analysis (PNA); (**a**) lacunarity; (**b**) branching rate; (**c**) protein width; (**d**) average protein length; (**e**) end-point rate and (**f**) CLSM image (scale 215 × 215 µm, GSH 60 mg/100 g flour). Means are shown with standard error (n = 24).

3.2. Protein Network Formation Modified by Ascorbic Acid

Ascorbic acid rapidly oxidizes by atmospheric oxygen or enzymatically to dehydroascorbic acid, which promote the formation of disulphide bonds among gluten proteins by minimizing the exchange of SH/SS of endogenous GSH with intermolecular SS bonds of gluten [12]. Thus, ascorbic acid leads to stiffer and strengthened doughs [23]. On a microscopic level, ASC influences gluten arrangement, differently as expected, by a decreased branching rate of 12%. The average protein length decreased by 26% with increasing ASC concentrations, whereas the end-point rate increased only by 7%. Usually, higher values for the end-point rate indicate a cleavage of bonds and a weakened structure, as it was shown above for GSH. However, the increase of the end-point rate compared to the standard was much higher for GSH flour-water-systems than for ASC samples. These effects indicate, in addition to an increased protein width and higher values for lacunarity (up to 40%), various locally higher aggregations of gluten proteins with increasing ASC concentrations. Taking the visual evaluation of CLSM micrographs into account (Figure 2f), it seems that ASC causes a contraction and inhomogeneous distribution of the proteins, leading to starch accumulations (black areas in Figure 2f). This would explain the high values for lacunarity, the increased protein widths and the locally higher aggregation of proteins. Thus, the contracted and thicker proteins might characterize a highly strengthened network. The influence of ASC on the protein formation is discussed contradictory in literature focused on gluten polymers in wheat dough. A strengthened effect of ASC on dough rheology is reported and an increase of cross-links is expected [12,23]. In contrast, an increase of free thiol groups was determined in the glutenins in other studies [11,24], indicating a decrease of disulphide bonds. In addition, Hanft and Koehler [25] found a decrease in dityrosine cross-links with ASC addition. Even if the degree of dityrosine in wheat dough is quite low, it might have an effect on the gluten polymer. Both studies indicated a decrease in gluten cross-links on a molecular level, which could confirm the decrease in the branching rate of gluten network's microstructure in the present study. Thus, ASC might not influence gluten cross-linking (or at least not on a microscopic level), but affects gluten arrangement and causes strengthened protein threads.

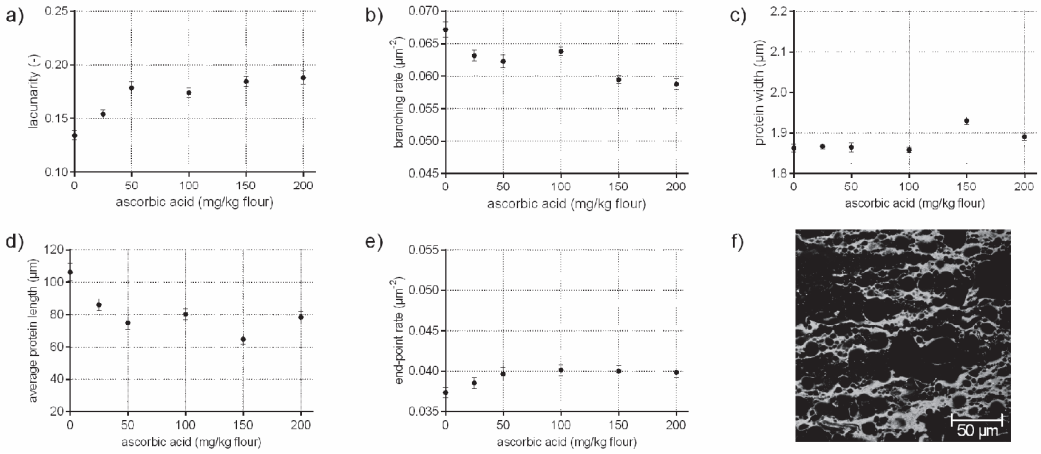

Figure 2. Influence on ascorbic acid on the protein network attributes. Flour-water-systems with increasing concentrations of ascorbic acid were analyzed by CLSM followed by PNA; (**a**) lacunarity; (**b**) branching rate; (**c**) protein width; (**d**) average protein length; (**e**) end-point rate and (**f**) CLSM image (scale 215 × 215 µm, ASC 150 mg/kg flour). Means are shown with standard error (n = 24).

3.3. Protein Network Formation Modified by Glucose Oxidase

The enzyme glucose oxidase (EC 1.1.3.4) catalyzes, in the presence of oxygen, the oxidation of D-glucose to D-gluconolactone/D-gluconic acid. The by-product hydrogen peroxide (H_2O_2) is responsible for the increase in gluten cross-linking by oxidizing thiol groups to disulphide bonds [11,26].

In addition, H_2O_2 causes in combination with endogenous wheat peroxidase dityrosine cross-links in gluten significantly [11]. Thus, an increase of the branching rate of gluten on a microstructural level was assumed in this study, which was confirmed quantitatively by PNA (Figure 3b). Furthermore, the significant decrease in end-point rate (9%), higher average protein lengths (14%) as well as the constant low value for lacunarity indicate the presence of a continuously interconnected gluten network with increasing GOX concentrations (c.f. Figure 3d,e). Likewise, the visual evaluation of the CLSM micrograph revealed a homogeneous, highly branched gluten network (Figure 3f). This is in accordance with Steffolani, Ribotta, Pérez and León [10], who determined a decrease of thiol groups, an increase of the glutenin macropolymer and large protein aggregates. Niu et al. [27] reported also a strengthened and stable gluten network with GOX addition due to an increase of β–sheet content and α–helix proportion, measured by FT-IR.

Compared to the addition of ascorbic acid (Section 3.2), which has also an oxidative effect, the gluten network was modified to a completely different extent due to other mechanisms of disulphide cross-linking and dityrosine formation. These results show, that different degree and type of cross-linking cause diverse gluten polymer arrangement.

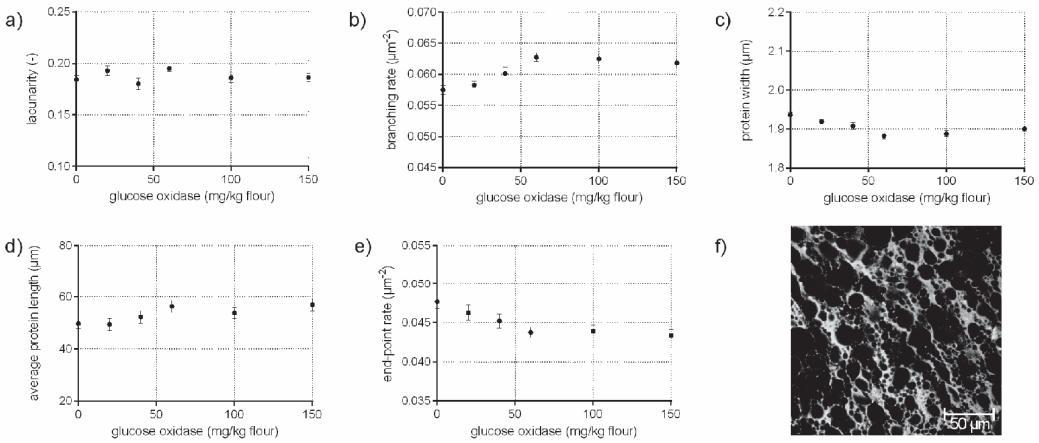

Figure 3. Influence of glucose oxidase on the protein network attributes. Flour-water-systems with 0.5 g glucose/100 g flour and increasing concentrations of glucose oxidase were analyzed by CLSM followed by PNA; (**a**) lacunarity; (**b**) branching rate; (**c**) protein width; (**d**) average protein length; (**e**) end-point rate and (**f**) CLSM image (scale 215 × 215 µm, GOX 100 mg/kg flour). Means are shown with standard error (n = 24).

3.4. Protein Network Formation Modified by Transglutaminase

The enzyme transglutaminase (EC 2.3.2.13) catalyzes the cross-linking between lysine and glutamine residues of gluten (the impact on high molecular weight, HMW, is most pronounced). Thus, the development of isopeptide bonds improves dough strength and resistance to extension [28]. This modification in the gluten network can be identified by an increased branching rate of 6% when transglutaminase is added up to 1000 mg/kg flour, quantified with PNA of the CLSM micrographs (Figure 4b). Furthermore, the flour-water-system with TG is characterized of a larger, interconnected gluten network (higher average protein length, lower end-point rate) with thinner protein threads compared to the standard and similar to GOX samples (c.f. Section 3.3). However, the effect of TG varies depending on the concentration, resulting in less elastic doughs at higher TG concentrations [10,29]. The alteration cannot only be observed in rheology, but also in the microstructure. The structure of gluten network changed from a homogeneous, highly branched one to a clustered, agglomerate formation of protein fragments at TG concentrations over 2000 mg/kg flour, visible in Figure 4f. These alterations were also detected quantitatively by PNA. The branching rate decreased by 11% for the highest TG concentration compared to the standard, whereas the end-point rate increased

significantly by 9%. Moreover, the agglomerate formation was quantitatively expressed by thicker protein threads (5%) and an enormously decreased average protein length (by 37%). The lacunarity was 2.5 times higher compared to the standard due to larger gaps between the individual agglomerate fragments. An uneven distribution of the proteins at high TG concentrations were also detected visually in other microscopic studies [28]. The agglomerate arrangement of the proteins at high TG levels explain also the findings on a molecular level that the GMP particle size increased, whereas GMP content did not increase [30]. This indicates a rearrangement of the proteins to a clustered agglomerate formation at high TG concentrations caused by new isopeptide bonds. This alterations in the gluten arrangement resulted in rigid and less extensible doughs [28].

Overall, a precise and quantitatively determination of the gluten formation under enzymatic or chemical influences has not been published on a microscopic level before. With the research of this study, not only a quantitative description of different kinds of gluten arrangements could be established; but also microstructural alterations dependent on the modification of cross-linking (disulphide, (iso) peptide, dityrosyl) were elucidated.

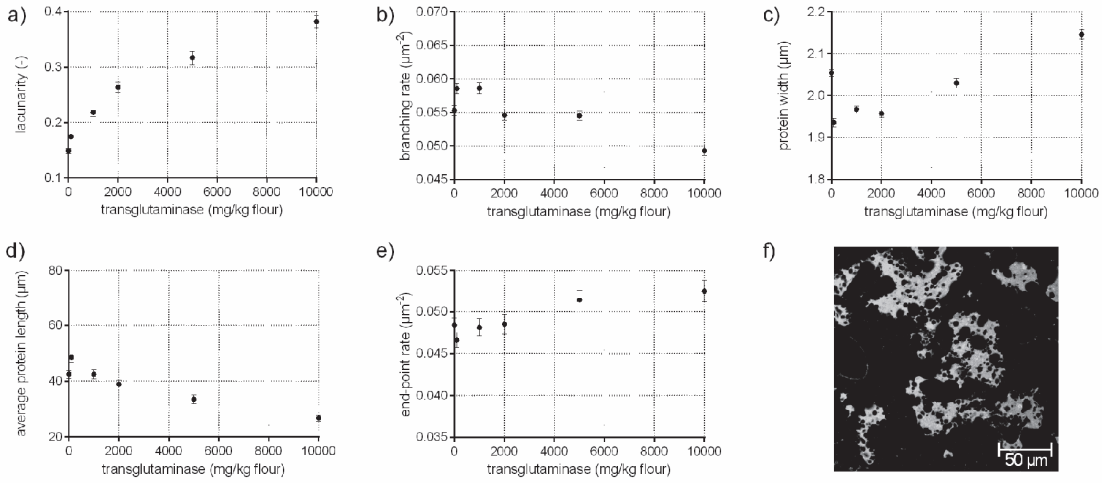

Figure 4. Influence of transglutaminase on the protein network attributes. Flour-water-systems with increasing concentrations of transglutaminase were analyzed by CLSM followed by PNA; (**a**) lacunarity; (**b**) branching rate; (**c**) protein width; (**d**) average protein length; (**e**) end-point rate and (**f**) CLSM image (scale 215 × 215 µm, TG 5000 mg/kg flour). Means are shown with standard error (n = 24).

3.5. Classification of Gluten Polymer Networks

Based on the results of the sections above, it became apparent that various modifications and types of cross-links cause different arrangements of the proteins. However, the interpretation of the five quantitative attributes of PNA, especially the meaning of changes in these attributes in regard of the spatial arrangement of the whole network, is challenging. In order to specify general assertions about structural dependencies and to define different polymer arrangements, correlations of the network attributes were performed.

3.5.1. Effect of Specific Gluten-Modifying Agents

In addition to the four specific gluten-modifying agents of Sections 3.1–3.4, further specific agents, bromelain and potassium bromate, were studied on their effect on gluten microstructure. Bromelain, at low concentrations (<1000 mg/kg flour), showed similar effects as glutathione (c.f. supplementary data 1). At higher concentrations, the protein network was destroyed entirely due to the proteolytic activity of bromelain. Thus, the proteins formed a liquid, homogenous mass with embedded starch granules instead of a polymeric network system. This impact caused misleading quantitatively results

of the microscopic images. Hence, the results of bromelain concentrations higher than 1000 mg/kg flour were not included for further studies (correlation matrix, PCA). The influence of potassium bromate on gluten polymer was similar to ascorbic acid. Even if most of the activity of potassium bromate takes place during fermentation and baking [31], results showed, that dough microstructure was slightly affected by potassium bromate after kneading. However, the effect of potassium bromate was not as distinct as with ascorbic acid. Results can be found in the supplementary data 2.

When correlating each PNA attribute of all specifically gluten-modified samples, some distinct relation were identified. As visible in Figure 5a, strong linear correlation can be observed for e.g., end-point rate and average protein length (Pearson correlation coefficient $r = -0.82$) or branching rate and protein width ($r = -0.84$). Furthermore, the end-pointe rate shows linear correlations with the protein width ($r = 0.69$) and with the branching rate ($r = 0.65$). This indicates that the more open-ended protein threads occur, the lower the length of an interconnected protein aggregate, the thicker the proteins threads and the lower the branching rate (up to a weak network structure with contracted protein threads). Inversely, the higher the branching rate, the thinner the protein threads and the larger the interconnected protein aggregate (up to a fully developed network with stretched proteins). These structural relations became also apparent by means of the PCA loading plot in Figure 5c, whereby the correlation of the branching rate and the protein width was most pronounced. While the first component is mainly defined by the structural network attributes (almost 65%), the second component (almost 19%) is dominated by the morphological attribute lacunarity. For this attribute, no linear correlations with the structural network attributes were found. Even if PCA is based on linear correlations, it gives distinct indications that a network classification, in general, is highly influenced by the attribute lacunarity (second component). Hence, the morphological attribute lacunarity separates further network properties. For example, if the branching rate is very low, the lacunarity can be either characterized by very low (0–0.16) or by very high (>0.25) values. The correlation matrix (Figure 5a) of lacunarity vs. branching rate, protein width or end-point rate revealed about four main network types (an example of the network type identification is visualized in Figure 5b):

I. A network with a low lacunarity (0–0.16), a very low branching rate, very high end-point rate as well as a high protein width, such as for flour-water-systems with glutathione (c.f. Section 3.1) or bromelain addition (c.f. supplementary data 1).

II. A network with a median lacunarity (0.17–0.26), very high branching rate, low end-point rate and a low protein width, such as for a standard flour-water-systems or with glucose oxidase addition (c.f. Section 3.3).

III. A network with a median lacunarity (0.17–0.26), low/median branching rate, high end-point rate as well as a high protein width, such as for flour-water-systems with high ascorbic acid (c.f. Section 3.2) or potassium bromate (c.f. supplementary data 2) concentrations.

IV. A network with a high lacunarity (>0.27), low branching rate, very high end-point rate as well as a high protein width, such as for flour-water-systems with high transglutaminase concentration (<1000 mg transglutaminase/kg four; c.f. Section 3.4).

The terms low or high in the description of the network types are related to each standard flour-water-system. As an interpretation, network type I can be defined as a weak network with only few cross-links, such as disulphide bonds, and as a soft material with a low viscoelasticity. In contrast, network type II describes a highly cross-linked (disulphide and dityrosine bonds), strengthened and homogenously distributed polymer network, perhaps leading to a pronounced viscoelastic material. Network type III is characterized by thicker protein threads, which can be an indication for a strengthened network system even though the degree of cross-links is decreased. However, this only functions in terms of a low or median lacunarity. Compared to network type III, network type IV has a much higher value for lacunarity, a very high end-point rate and protein width. These attributes indicate a clustered agglomerate formation of the protein polymers. Thus, network type IV is more

like a particular system than a network. Material properties of a network type IV might be of a high firmness and low viscoelasticity.

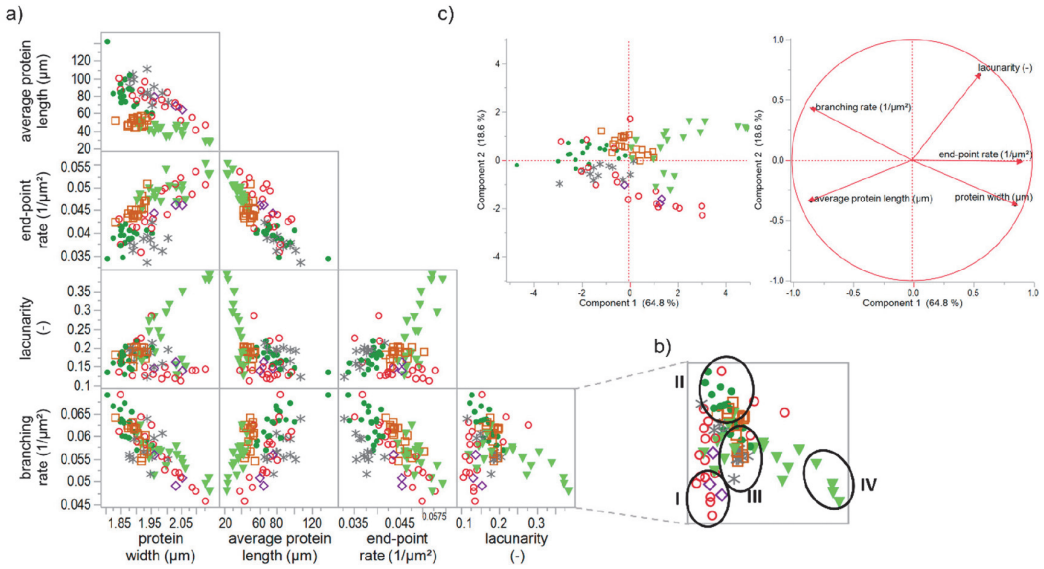

Figure 5. Correlation matrix and principal component analysis of specific gluten modifications. (**a**) Correlation matrix of microstructural attributes of solely gluten-modified flour-water-systems; (**b**) Identification of network types I–IV in the correlation graph of lacunarity vs. branching rate, as an example; (**c**) PCA score and loading plot for the first and second principal component. Symbols: -●- ASC, -✳- $KBrO_3$, -◊- BRN, -○- GSH, -□- GOX, -▼- TG.

3.5.2. Effect of Unspecific and Specific Gluten-Modifying Agents

The effect of unspecific gluten-modifying agents were investigated in combination with the specific ones, in order to extend the assertions about the four network types above. For this purpose, the increase and decrease of hydration level as well as the addition of rapeseed oil and shortening were studied combined with the specific gluten-modifying agents. The increase of hydration level (IHL) caused a weakened gluten network structure due to a dilution effect of water. Detailed results can be found in the manuscript of Bernklau, Lucas, Jekle and Becker [16]. The addition of rapeseed oil (supplementary data 3-ROI) and shortening (supplementary data 4-SHO) caused a similar weakened effect and a formation of clustered protein particles at higher concentrations. However, the lacunarity was much higher for IHL samples and highest concentration of ROI than for SHO ones. This can be explained by a more scattered arrangement of the agglomerates caused by a dilution effect and higher mobility of the particles in IHL and ROI samples. In that case, proteins cannot be stretched during kneading, and a development of a network formation is inhibited, leading to clustered protein particles. This pronounced form of a modified structure is also clearly evident in the correlation matrix and PCA score plot of PNA attributes of all specifically and unspecifically gluten modified samples (Figure 6). The reduction (detailed results visualized in supplementary data 5-RHL) and increase of hydration level mainly caused a shift of a linear correlation for solely specifically gluten-modified samples to an exponential relation of several PNA attributes for specifically plus unspecifically gluten-modified samples. The correlation of e.g., average protein length with the end-point rate (R^2 = 0.77) or with lacunarity (R^2 = 0.75) resulted in a non-linear, exponential decay. However, there were also some unclear relations between the attributes or indications for linear relations, but with inconsistencies of IHL values (e.g., protein width vs. branching rate). The PCA score and loading plots revealed that almost 84% of the data could be expressed by the first two components (Figure 6b). As visible in the score plot, most of the IHL data as well as the highest concentrations of ROI and TG were

divergent from all remaining data. These data were mainly influenced by increasing lacunarity values, which can be explained by an agglomerate formation or clustered protein particles as described above. This influence was even more distinct for increasing concentrations of IHL samples. Due to the strong dilution effect, the flour-water-systems had a very low viscosity and almost no viscoelastic behavior [16]. Consequently, based on these results, a further network type, V, could be identified, which is an intensified form of network type IV (c.f. Section 3.5.1). Both types, IV and V, describe a particular network formed of protein agglomerates. The difference of both types is, that the protein particles are scattered densely in network type IV and loose in type V, which is confirmed by much higher values for lacunarity. Moreover, the agglomerates of type V are formed due to a plasticizing and dilution effect of the unspecific gluten-modifying agents resulting in unstreched, clustered protein particles, whereas the agglomerate formation of type IV is caused by isopeptide bonds. Based on literature findings for each example (TG for type IV; IHL for type V), network type IV might has a rigid structure, whereas type V is expressed by a very low viscosity.

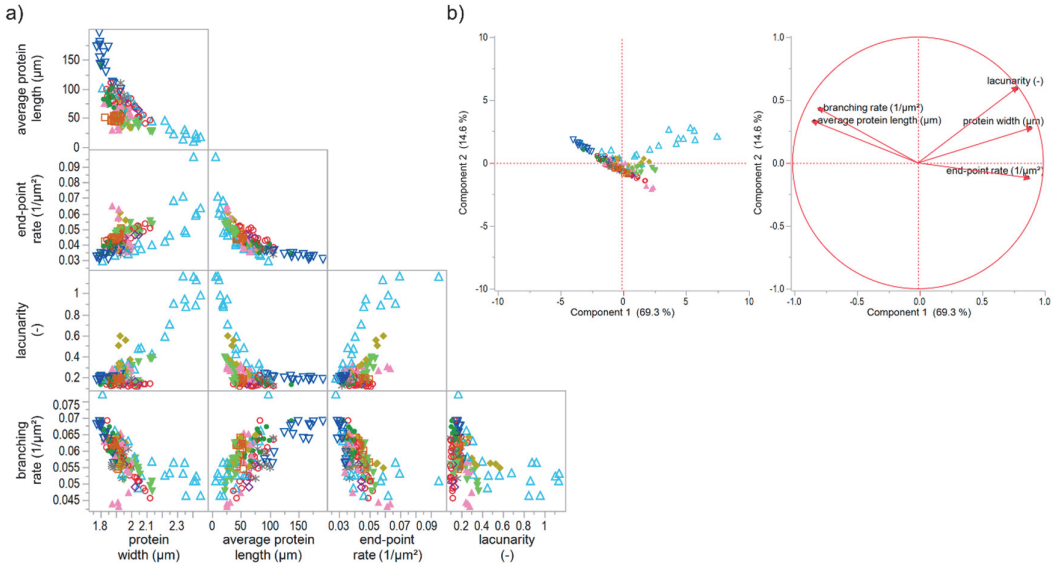

Figure 6. Correlation matrix and principal component analysis of unspecific and specific gluten modifications. (**a**) Correlation matrix of microstructural attributes of all flour-water-system variations; (**b**) PCA score and loading plot for the first and second principal component. Symbols: -●- ASC, -✳- $KBrO_3$, -◊- BRN, -▲- SHO, -○- GSH, -□- GOX, -◆- ROI, -△- IHL, -▽- RHL, -▼- TG.

4. Conclusions

In the present study, it was not only clarified that modifications in cross-links (disulphide, (iso) peptide, dityrosyl) of gluten polymer network by enzymatic or chemical agents can be detected precisely and quantitatively on a microstructural level, which has not been published before. It was also shown that findings on a molecular level can be elucidated by microstructural alterations, and that various polymer arrangements can be determined quantitatively by protein network analysis.

Furthermore, a classification of five different types of gluten polymer networks were established on a microstructural level. When solely the gluten network is modified in a complex system (here: flour-water-system) by specific gluten-modifying agents, like protein related enzymes, the structural network attributes branching rate, end-point rate, protein width and average protein length correlated linearly among each other. This revealed the general assertion: the higher the branching rate, the thinner the protein threads and the larger the interconnected protein aggregate. Furthermore, the morphological attribute lacunarity separates the attributes in four main network types: (I) a weak network structure with cleaved and only a few cross-links; (II) a strengthened, highly

cross-linked and homogeneously distributed polymer network; (III) a strengthened, less cross-linked network, but with thick protein threads; (IV) a rigid, particular network with clustered agglomerates. These assertions were extended by investigating unspecific gluten-modifying agents, additionally to the specific ones. Thus, a fifth network type was defined: (V) a low viscous, particular network with widely scattered agglomerates. The classification of gluten networks enables a detailed characterization and interpretation of material properties. Gained knowledge about the classifications in a flour-water-system can be derived for other gluten polymer materials, like films, gels, foams or bioplastics. However, individual effects on materials' mechanical properties should be characterized in the future. Thus, combined with rheological or textural studies, a precise definition of structure-function relationships for gluten polymer materials could be developed.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4360/10/6/617/s1, Figure S1: Influence of bromelain on the protein network attributes, Figure S2: Influence of potassium bromate on the protein network attributes, Figure S3: Influence of rapeseed oil on the protein network attributes, Figure S4: Influence of shortening on the protein network attributes, Figure S5: Influence of a reduced hydration level on the protein network attributes.

Author Contributions: Conceptualization, methodology, data curation, writing—I.L.; supervision—T.B. and M.J.

Funding: This work was supported by the German Research Foundation (DFG) and the Technical University of Munich (TUM) in the framework of the Open Access Publishing Program.

Acknowledgments: The authors would like to thank Katerina Kiousi, Tamar Bar-Zeev, Mariam Bapir, Ksenia Egginger and Ludmilla Adolf for their contribution.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary data

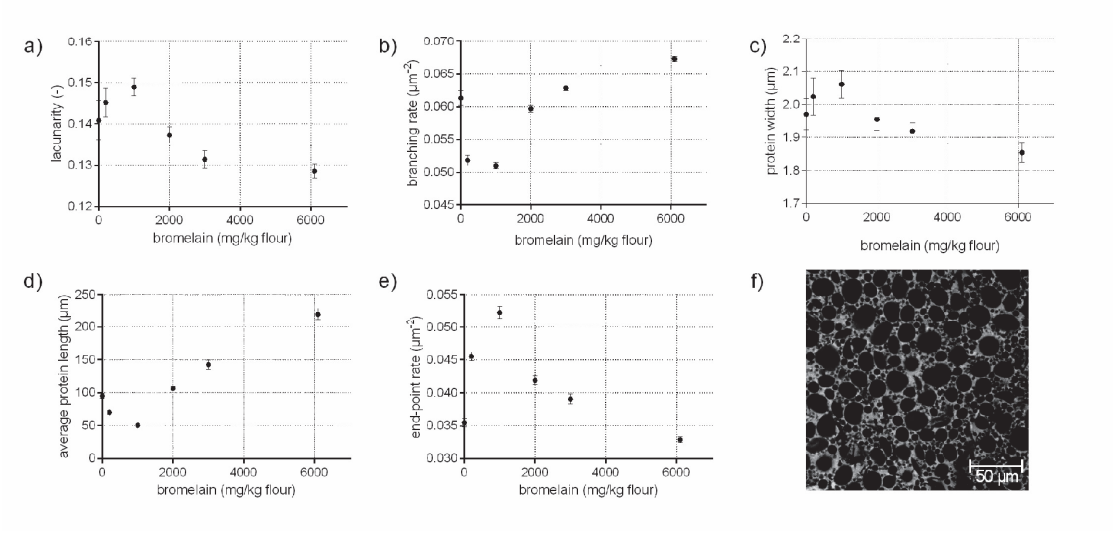

Figure S1. Influence of bromelain on the protein network attributes. Flour-water-systems with increasing concentrations of bromelain were analysed by CLSM followed by PNA; a) lacunarity, b) branching rate, c) protein width, d) average protein length, e) end-point rate and f) CLSM image of an entirely destroyed protein polymer structure by proteolysis (scale 215x215 µm, BRN 6100 mg/kg flour). Means are shown with standard error (n=24).

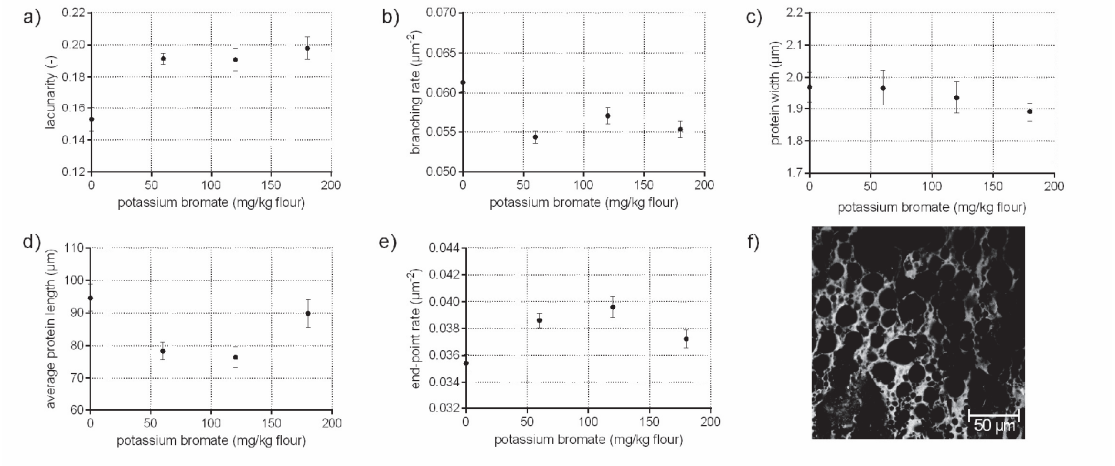

Figure S2. Influence of potassium bromate on the protein network attributes. Flour-water-systems with increasing concentrations of potassium bromate were analysed by CLSM followed by PNA; a) lacunarity, b) branching rate, c) protein width, d) average protein length, e) end-point rate and f) CLSM image (scale 215x215 µm, KBrO3 120 mg/kg flour). Means are shown with standard error (n=24).

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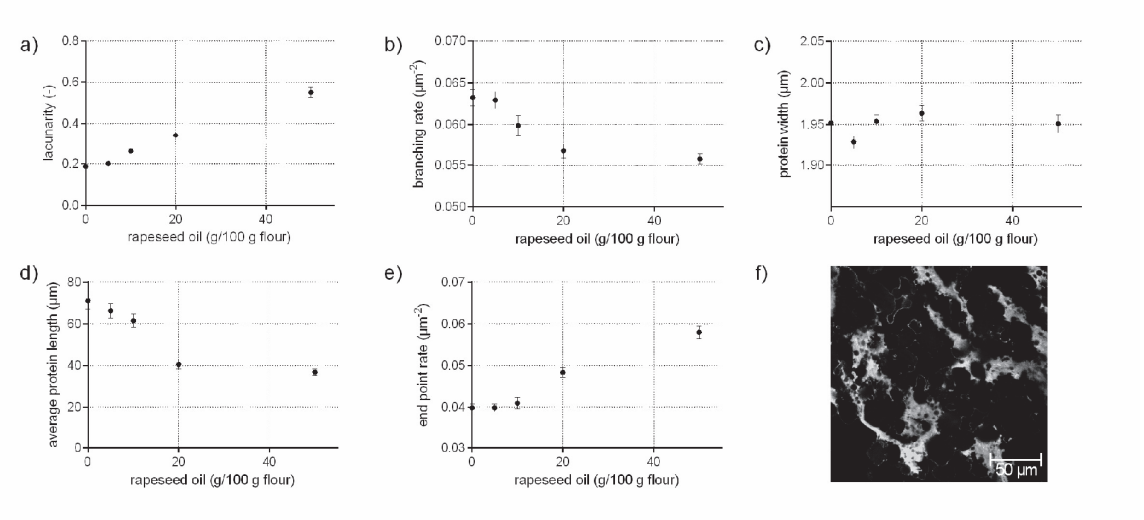

Figure S3. Influence of rapeseed oil on the protein network attributes. Flour-water-systems with increasing concentrations of rapeseed oil were analysed by CLSM followed by PNA; a) lacunarity, b) branching rate, c) protein width, d) average protein length, e) end-point rate and f) CLSM image (scale 215x215 µm, ROI 50 g/100 g flour). Means are shown with standard error (n=24).

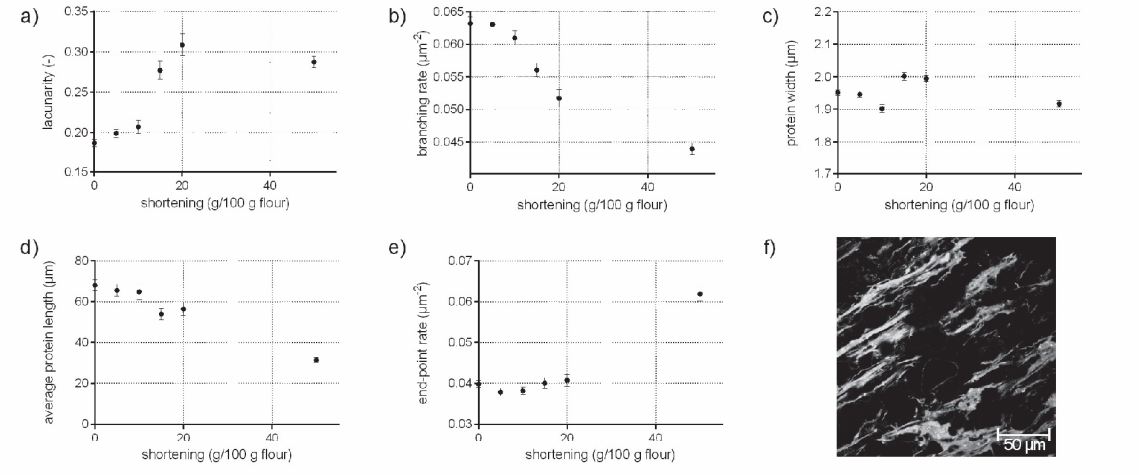

Figure S4. Influence of shortening on the protein network attributes. Flour-water-systems with increasing concentrations of shortening were analysed by CLSM followed by PNA; a) lacunarity, b) branching rate, c) protein width, d) average protein length, e) end-point rate and f) CLSM image (scale 215x215 µm, SHO 50 g/100 g flour). Means are shown with standard error (n=24).

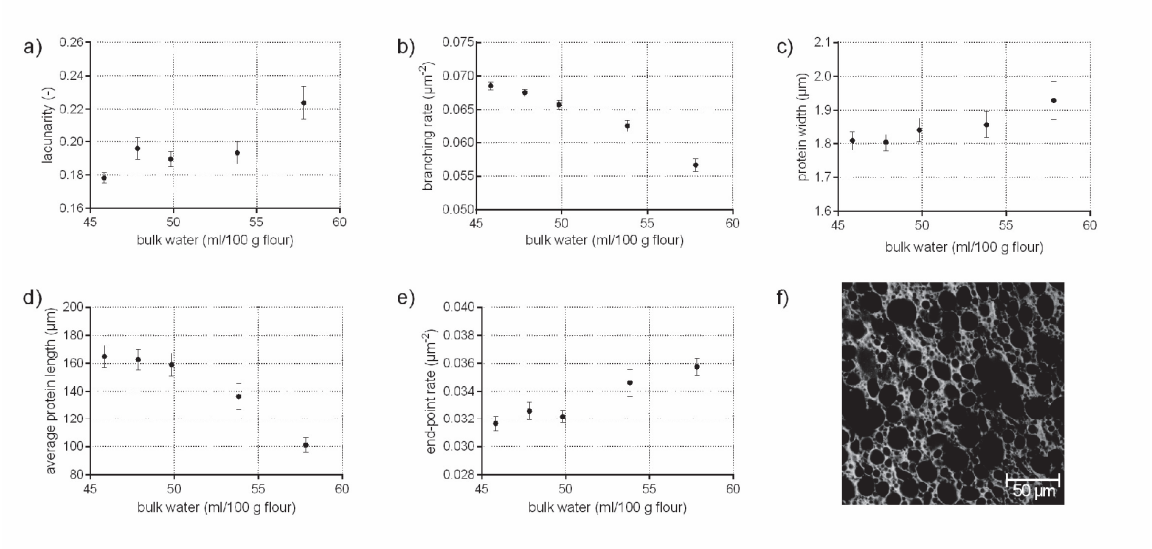

Figure S5. Influence of a reduced hydration level on the protein network attributes. Flour-water-systems with decreasing water hydration levels were analysed by CLSM followed by PNA; a) lacunarity, b) branching rate, c) protein width, d) average protein length, e) end-point rate and f) CLSM image (scale 215x215 µm, RHL 45.85 ml/100 g flour). Means are shown with standard error (n=24).

3.5 Definition of network types – Prediction of dough mechanical behaviour under shear by gluten microstructure

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Definition of network types – Prediction of dough mechanical behaviour under shear by gluten microstructure

Received: 13 August 2018
Accepted: 1 March 2019
Published online: 18 March 2019

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This study defines network types of wheat gluten to describe spatial arrangements of gluten networks in relation to dough mechanical behaviour. To achieve a high variety in gluten arrangements, ten specific and unspecific gluten-modifying agents in increasing concentrations were added to wheat dough. Gluten microstructure was visualized by confocal laser scanning microscopy and quantified by protein network analysis. Dough rheological behaviour was determined by both oscillatory and creep-recovery tests. Based on correlation matrices and principal component analysis, six different network types were identified and associated to their rheological characteristics: a cleaved (low viscous), rigid (highly viscous), spread (viscoelastic), strengthened (viscoelastic), particulate and dense (highly viscous) or particulate and loose (low viscous) network. Furthermore, rheological dough properties of specifically gluten-modified samples were predicted with five microstructural gluten attributes (lacunarity, branching rate, end-point rate, protein width, average protein length) and assigned properly by the obtained partial least square model with an accuracy up to 90% (e.g., $R^2Y = 0.84$ for G*, 0.85 for $tan\delta$, 0.90 for J_{max}). As a result, rheological properties of wheat doughs were predicted from microstructural investigations. This novel, quantitative definition of the relation between structure and mechanical behaviour can be used for developments of new wheat products with targeted properties.

Due to its unique viscoelastic properties, the gluten network is the most relevant physicochemical structuring component in wheat products. It is responsible for the mechanical behaviour of dough, and it affects the gas holding properties, texture and final bread quality[1,2]. Thus, understanding the relation between gluten network structure and dough functionality makes an optimization of existing products and design of new ones feasible. A lot of research dealt with structure-function relationships of dough so far investigating the gluten network on a microstructural level[1,3–7] since the analysis of the microstructure is most appropriate to cover the spatial arrangement of gluten proteins. However, relations between dough mechanical behaviour and microstructure were not analysed sufficiently. The reason might be the complex structure of the gluten network and the difficulties in its quantification and interpretation due to a lack of suitable describing attributes.

To overcome this, protein network analysis (PNA), an image analysis for a precise quantification of structural and morphological network attributes of the complex gluten microstructure, was established[8]. Using this method, several studies showed that dough mechanical properties could be elucidated by means of the microstructural attributes of PNA[9–11]. In addition, correlations between protein microstructure and dough mechanical behaviour were demonstrated. Even though the studies mentioned above presented correlations between dough microstructure and rheological properties, these correlations included only individual dough systems, but no universal relationship. Furthermore, the interpretation of the quantitative results of microstructural attributes, especially the effect of changes in these attributes with regard to the spatial arrangement of the whole network, remained challenging. For that reason, we specified microstructural relations as well as network characteristics in general and classified various spatial arrangements in Lucas, *et al.*[12]. Based on this, we proposed five different types of protein networks. An implementation of these results combined with rheological studies would support a precise and

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universal definition of the relation between structure and mechanical dough behaviour. Moreover, the protein network attributes could be used to predict dough properties.

Thus, the aim of the study was to identify quantitative relations between gluten microstructure and dough rheology in general. To gain universal assertions, a high variety of gluten spatial arrangements was provoked by specific (glutathione, ascorbic acid, potassium bromate, glucose oxidase, transglutaminase, bromelain) as well as unspecific gluten-modifying agents (reduction and increase of hydration level, addition of rapeseed oil and shortening). By applying oscillatory frequency test within the linear-viscoelastic region (LVE) and creep-recovery test in the non-LVE, the elucidation of fundamental rheological relationships as well as relationships between microstructure and rheology in this wide range of different types of dough systems from very low to high viscosity were aspired. To study the dependencies, correlation matrices and principal component analysis (PCA) were used. Prediction models for dough rheology by using microstructural attributes were obtained by partial least square regression (PLS). While our previous research[12] focused solely on the protein microstructure and the discovery of network arrangements, the current study aims to confirm and further develop the definitions of proposed network types by rheological investigations as well as to define quantitative relations between structure and mechanical dough behaviour by multivariate statistical evaluations.

Materials and Methods

Materials. Four different wheat flours (Type 550, Rosenmühle, Landshut, Germany) were used in order to detect microstructural changes independent of the raw material. Flour characteristics were analysed according to AACC international and AACCi methods as well as to the International Association for Cereal Science and Technology (ICC): the moisture (AACCi 44-01), protein content (AACCi 46-16, N × 5.7), ash (ICC 104/1), and falling number (AACCi 56-81). All specifications and characteristics of the four flours (A, B, C, D) are summarized in the previous publication[12]. Commercial wheat flours usually contain 3 mg/kg flour of ascorbic acid, like the flours A and B, whereas flours C and D did not. Glutathione was supplied by VWR International GmbH (Darmstadt, Germany), ascorbic acid by Carl Roth GmbH + Co. KG (Karlsruhe, Germany), transglutaminase (≥1000 units/g) and glucose oxidase (≥1100 units/g) by AB Enzymes GmbH (kindly provided by AB Enzymes GmbH, Darmstadt, Germany), potassium bromate ($KBrO_3$) by ThermoFisher GmbH (Karlsruhe, Germany), bromelain (≥3 units/mg protein) and D(+)glucose by Sigma-Aldrich Chemie GmbH (Steiheim, Germany), Rhodamine B by Merck KGaA (Darmstadt, Germany), rapeseed oil by Cargill Oil Packers bvba (Izegem, Belgien) and shortening (ingredients: palm fat, coconut fat, rapeseed oil, water, emulsifier, NaCl, citric acid, aroma, carotin) by MeisterMarken (CSM Deutschland GmbH, Bingen, Germany).

Dough preparation. Dough preparation was already described in[12] and is summarized in the following section. All dough samples consisted of wheat flour and demineralized water produced with the required kneading time, water concentration and recipe for each standard wheat dough according to AACCi method 54-70.01, see Table 1. Specific as well as unspecific gluten-modifying agents were added separately to a standard wheat dough in varying concentrations. The targeted resistance of 500 Farinograph units was determined in a Z-kneader (doughLAB; Perten Instruments, Hägersten, Sweden) in reliance on the water absorption and the moisture (corrected to 14%), which is dependent on the flour type (A, B, C, D) as well as on the storage time of each flour. All dough variations were produced in triplicate.

Rheological analysis. 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 to determine the viscoelastic properties of each dough sample. For this purpose, oscillatory frequency sweep and creep-recovery tests were performed as reported by Bernklau, *et al.*[8].

Oscillatory frequency sweep test. The oscillatory frequency sweep was performed within the LVE, which was determined before with an amplitude sweep test. Oscillatory frequency tests were carried out at a constant deformation of 0.1% in a frequency range of 0.1 to 50 Hz. Results were evaluated by the complex shear modulus G^* (Pa) at 1 Hz and the loss factor $tan\delta$ (−) (the ratio of the loss modulus G'' and storage modulus G'). In addition, the complex shear moduli (at 0.1–50 Hz) were fitted according to the power law equation in order to obtain distinct information about network characteristics:

$$G^*(\omega) = A_f \omega^{1/z} \tag{1}$$

Where ω (s^{-1}) is the angular frequency. A_f (Pa $s^{1/z}$) is calculated by the model power law, G* values over the whole frequency period 0.1–50 Hz. A_f is interpreted as the network strength (strength of interactions between the network components) and z (−) as the network connectivity (extend of interactions)[13,14].

Creep-recovery test. During dough processing, dough is exposed to large strains and shear rates, which represent a mostly nonlinear viscoelastic behaviour. This is why the creep-recovery test was performed in the non-LVE region, as recommended by Van Bockstaele, *et al.*[15]. A constant shear stress of 250 Pa for 180 s and a relaxation time of 360 s was applied. Results were evaluated by the creep compliance J_{max} (Pa^{-1}) (maximal compliance at the end of the creep phase), creep-recovery compliance J_r (Pa^{-1}) (minimal compliance at the end of the recovery phase) and the relative elastic part J_{el} (%) (J_r/J_{max} * 100). In order to gain further information about the dough rheological behaviour, especially the instantaneous change in compliance and viscous deformation, creep data were analysed with a four parameter (Eq. 2) and recovery data with a five parameter Burgers model (Eq. 3) according to Van Bockstaele, *et al.*[15]:

	Abbreviation	Variation	Concentrations	Flour type	Water addition (mL/100 g flour)	Kneading time* (s) to 500 FU
Specific gluten-modifying agents	ASC	Ascorbic acid	0.0, 25.0, 50.0, 100.0, 150.0 and 200.0 mg/kg flour	C	56.17	300
	BRN	Bromelain	0.0, 200.0 mg/kg flour	D	61.36	240
	GSH	Glutathione	0.0, 7.5, 15.0, 30.0, 45.0, 60.0 and 75.0 mg/100 g flour	B	57.76	180
	GOX	Glucose oxidase	0.0, 20.0, 40.0, 60.0, 100.0 and 150.0 mg/kg flour, addition of 0.5 g glucose/100 g to each sample	D	61.06	250
	$KBrO_3$	Potassium bromate	0.0, 60.0, 120.0 and 180.0 mg/kg flour	D	61.36	240
	TG	Transglutaminase	0.0, 100.0, 1000.0, 2000.0, 5000.0 and 10000.0 mg/kg flour	D	60.49	250
Unspecific gluten-modifying agents	IHL	Increase of hydration level (compared to the standard)	59.18 (standard), 64.87, 69.86, 74.85, 79.84 and 89.82 mL water/100 g flour	A	59.18	180
	RHL	Reduced hydration level (compared to the standard)	57.83 (standard), 53.84, 49.84, 47.58 and 45.85 mL water/100 g flour	B	57.83	180
	ROI	Rapeseed oil	0.0, 5.0, 10.0, 20.0 and 50.0 g/100 g flour	B	58.32	180
	SHO	Shortening	0.0, 5.0, 10.0, 15.0, 20.0 and 50.0 g/100 g flour	B	58.32	180

Table 1. Variations of produced dough samples. Specific as well as unspecific gluten-modifying agents were added to standard wheat doughs in varying concentrations. A standard wheat dough consists of flour and demineralized water. *Kneading times were rounded to the nearest tens.

$$J_{creep}(t) = J_{0creep} + J_{1creep}[1 - \exp(-t/\lambda_{creep})] + t/\eta_0 \quad (2)$$

$$J_{rec}(t) = J_{0rec} + J_{1rec}[1 - \exp(-t/\lambda_{1\,rec})] + J_{2\,rec}[1 - \exp(-t/\lambda_{2\,rec})] \quad (3)$$

With the time t, J_0 (Pa^{-1}) the instantaneous compliance (pure elastic part), J_1 (Pa^{-1}) and J_2 (Pa^{-1}) the retarded elastic compliances, λ_1 (s) and λ_2 (s) the retardation times and η_0 (Pa s) the steady state viscosity. A five parameter Burgers model with two Kelvin elements was chosen for the recovery phase due to a better representation of the experimental data[16].

Microstructure analysis. Microstructure analysis has been carried out in Lucas, *et al.*[12] and summarized as follows.

Confocal laser scanning microscopy (CLSM). For the visualization of the gluten proteins by CLSM, all dough samples were stained with Rhodamine B (0.01 g/100 mL water) according to the bulk water technique[17]. An eclipse Ti-U inverted microscope with an e-C1 plus confocal system (Nikon GmbH, Düsseldorf, Germany) with a Plan Apo VC 60x/1.40 oil objective and a 534 nm laser (emission 590/50 nm) were used for microstructure analysis. Eight independent (non-overlapping) images were taken of each sample with a resolution of 1024 × 1024 pixel and a size of 215 × 215 µm.

Protein network analysis. On CLSM micrographs, image analysis quantified network characteristics using the method protein network analysis (PNA)[8]. For this purpose, the software AngioTool64 version 0.6a (National Cancer Institute, National Institute of Health, Maryland, USA) was used[18]. The settings and calibrations were adjusted as described by Lucas, *et al.*[12]. The following network attributes were evaluated: the structural network attributes branching rate (describes the network connectivity), end-point rate (describes the weakness of a network), average protein length (length of a continuous protein particle) and protein width (thickness of protein threads) as well as the morphological attribute lacunarity (attribute for the amount and size of network gaps; describes irregularities of a structure).

Statistical analysis. Multivariate statistics, principal component analysis (PCA) and partial least squares (PLS) analysis were performed with JMP Pro software (version 12.2.0, SAS Institute Inc., Cary, NC, USA). The goodness of fit of linear and nonlinear regressions were represented by R^2.

PCA was used to identify structural similarities or clusters based on rheological and microstructural characteristics. PCA was performed for solely rheological data as well as for rheological combined with microstructural data (for all samples as well as for solely gluten-modified samples). Evaluation was performed by score (matrix of scatterplots of the scores for pairs of principal components) and loading plots (matrix of two-dimensional representation of factor loadings)[19].

For PLS model, the response variable Y (here: rheological attributes) was explained by several predictor variables X characterizing the microstructural attributes to determine and quantify the relation between them. By introducing so-called latent variables based on linear combinations of the original data, PLS identifies the factors, which explain the highest variance between the predictors and the response. PLS can deal with multicollinearity amongst the data as well as a large number of X variables[20–22]. PLS models were estimated with the NIPALS (nonlinear iterative partial least squares) algorithm with "leave-one-out" cross-validation for model validation. The appropriate number of factors were identified by the minimum Root Mean PRESS (predicted residual error sum of squares). Variable importance for projection (VIP) scores greater 0.8 defined those variables which are relevant indicators and important predictors for the model[19]. VIP were used to point out the microstructural variables with the highest contribution for the projection model of each rheological attribute.

Since some of the data sets showed nonlinear relationships between X and Y values and PLS is based on linear dependency, the response (Y) variables (J_{max}, J_r, η_0, G^*, A_f) were linearized as suggested by Wold, *et al.*[23]. For J_{max}, J_r and η_0 the logarithm and for G^* as well as A_f the reciprocals were taken.

Results and Discussion

All ten dough variations, specifically as well as unspecifically gluten-modified, were evaluated collectively to identify relations in general. Initially, appropriate rheological tests and attributes were evaluated for further investigations and correlation to microstructure. Subsequently, the relation between dough rheology and gluten microstructure was investigated first with both unspecifically and specifically-gluten modified samples and second, with solely the specific modified samples. Concluding, the definition of network types is presented. Discussion focusses on different network arrangements and the effect on dough rheology considering all data collectively. For a detailed discussion about the single chemical or technological effects of each specific or unspecific-gluten modifying agents, the authors refer to Lucas, *et al.*[12].

Rheological consideration. Generally, the mechanical behaviour is often determined with rheological methods assuming a linear dependency of the viscoelasticity. However, dough mechanical properties during dough processing should be determined in large strains, i.e. out of the LVE[15,24]. Indeed, it is discussed in literature that shear tests within the LVE, like oscillatory measurements, are not sufficient to describe dough properties during processing or to detect differences in doughs of various wheat flour types[24,25]. On the contrary, such methods are widely used to describe dough mechanical behaviour and their suitability is reported as well[26–28]. Thus, shear methods for both measurement ranges, namely oscillatory frequency test in small strain (measured in the LVE) and creep-recovery test in large strains (measured in the non-LVE), were applied in order to evaluate dependencies among both as well as the applicability to characterize dough properties.

Figure 1a,c,d presents the correlation of selected rheological attributes. The correlation matrix containing all attributes can be found in the Supplementary Data 1. Results revealed that most attributes of oscillatory frequency test (G^*, G', G'', A_f) correlated nonlinearly (exponential, R^2 between 0.66 and 0.81) with those of the creep-recovery test (J_{max}, J_r, J_{el}, J_{0creep}, J_{1creep}, J_{0rec}, J_{1rec}, J_{2rec}). This implicates, that despite the large deformations, there are intrinsic properties of the dough matrix, which correspond to the dough behaviour under small strain. Similar results were reported by Van Bockstaele, *et al.*[28] between creep-recovery and dynamic oscillation characteristics of 17 wheat cultivars highlighting that rheological properties of small deformation tests can be related with nonlinear correlations to large deformation ones. In the current study, also a linear relation between results of the creep-recovery and oscillatory frequency test was found. The steady state viscosity η_0 correlated linearly with the stiffness G^* ($R^2 = 0.79$) for almost all samples, excluding samples with higher transglutaminase (TG) concentrations than 5000 mg/kg flour (c.f. Fig. 1a) which can be explained by the fact that highly concentrated TG samples showed a much higher viscosity than stiffness, maybe due to the densely clustered protein agglomerates[12]. Hence, for samples with low and moderate TG concentration, the proportionality – the higher the viscosity, the higher dough stiffness – holds. Relations between the different attributes of creep-recovery and oscillatory frequency test were also visible in the loading and score plots of the principal component analysis (Fig. 1b). The score plot confirms the indication of non-linear relations and the loading plot highlights the detected linear relations of some rheological attributes of the correlation matrix (η_0 with attributes of oscillatory frequency test). The score plot showed a separation of the data to stiffer and softer doughs. Oscillatory frequency attributes as well as η_0 characterized RHL (reduced hydration level) and TG samples (stiffer doughs). Conversely, softer doughs, such as samples with increasing water (IHL) or rapeseed oil (ROI) concentration, were characterized by creep-recovery attributes due to a higher deformation.

Focusing on the oscillatory frequency test, the network strength A_f (recorded over whole frequency period 0.1–50 Hz) gives no more distinct information than the variable G^* (single value recorded at 1 Hz). Both correlated highly linearly with a R^2 of nearly 1 (c.f. Supplementary Data). The correlation of the network strength A_f and network connectivity z showed that for larger z values (>5), A_f can take low (SHO), medium (TG) and large values (RHL). This indicates that the network connectivity separates stiff dough samples with different protein network formations. This important fact for the relationship between the rheology and the microstructure will be discussed in detail later (following chapter). Interestingly, the network connectivity factor z correlated inversely proportional with $tan\delta$ ($R^2 = 0.71$), meaning, the lower the network connectivity, the more does the samples act as an (ideal) viscous liquid (c.f. Fig. 1c).

Considering the various variables of the creep-recovery test, it became apparent that most attributes of the Burgers Model (for creep as well as recovery) provide no additional information about rheological behaviour in this experimental setup. The reason is that the common attributes J_{max}, J_r and J_{el} as well as J_r, J_{1rec} and J_{2rec} are correlated linearly among each other (R^2 between 0.90 and 0.97) as well as J_{max} with J_{1creep} ($R^2 = 0.68$) (c.f. Supplementary Data). However, the Burgers attributes J_{0creep} and η_0 were able to discriminate microstructures. The instantaneous compliance J_{0creep}, which describes the pure elastic part of a dough sample, increases with higher

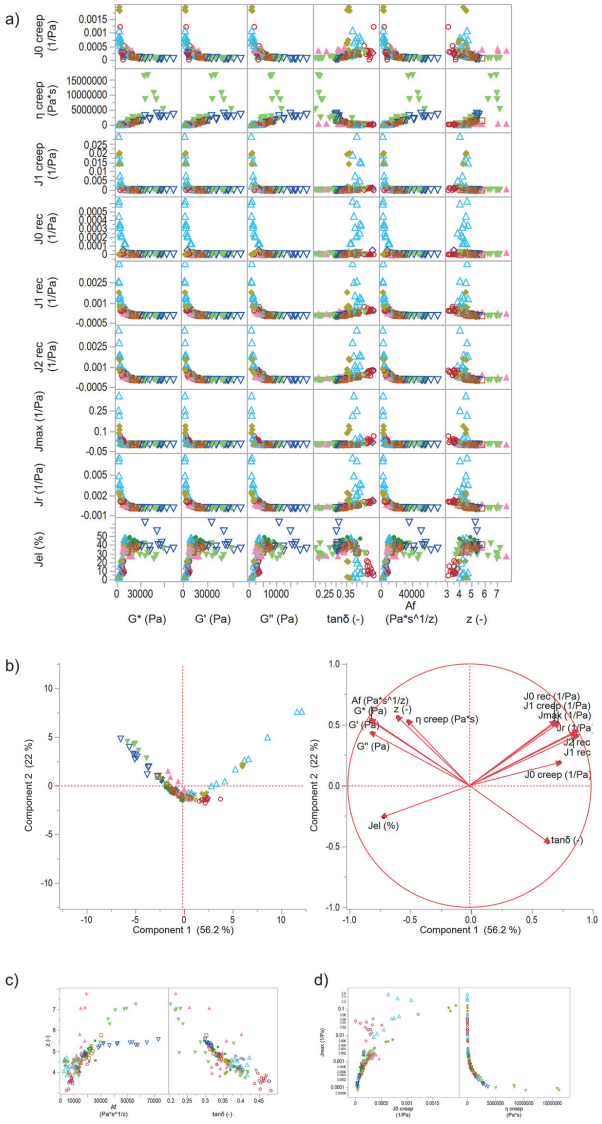

Figure 1. Correlation matrix and principal component analysis of the rheological data of unspecifically as well as specifically gluten-modified samples. (**a**) Correlation matrix of selected rheological attributes (oscillatory frequency test and creep-recovery test) of all gluten-modified samples. (**b**) PCA score and loading plot for the first and second principal component. (**c**) Correlations between z and A_f as well as tanδ. (**d**) Correlations between J_{max} and $J_{0\ creep}$ as well as η_{l0} (J_{max} is presented with log scale). Symbols: -●- ASC, -*- $KBrO_3$, -◊- BRN, -▲- SHO, -○- GSH, -□- GOX, -◆- ROI, -△- IHL, -▽- RHL, -▼- TG.

dough deformation (J_{max}). However, there is a shift to a nonlinear extent for dough samples with a high creep compliance J_{max} (GLU, high IHL samples) to comparatively very low J_{0creep} values. Very low values for the relative elastic part J_{el} characterized these samples. As illustrated in Fig. 1d, η_{l0} correlated nonlinearly (exponential) with all compliances (e.g., $R^2 = 0.97$ for J_{max}). This indicates that dough samples with an extremely high deformation

J_{max} (GLU, ROI or IHL samples) correspond with a low steady state viscosity η_0, whereas low deformed doughs (TG > 1000 mg/kg flour, RHL) are characterized by a very high viscosity.

As clearly shown, correlations revealed strong dependencies between rheological properties measured with small and large deformation tests. Since most relations were exponential, an evaluation of both methods is important when taking into account that dough rheological behaviour is related to the final product properties and for getting insights of structure-function relationships. Although the relevance of those measurements for conclusions on dough functionality and final product properties is discussed contradictory in literature[24], there are studies showing that e.g., viscoelastic data gained by creep-recovery measurements correlate linearly with empirical rheological parameters (dough extensibility, mixing time) and bread volume[29]. As well, other studies reported that G^* correlated nonlinearly[28] and J_{max}[30] as well as J_r[31] linearly with bread volume. For a comprehensive characterisation of wheat dough mechanical behaviour related to processing performance and product properties, further rheological analysis such as biaxial extensional measurements or even under hyperbolic contraction flow is recommended, since biaxial forces occur during proofing and baking[24,32,33]. However, the main focus of this study was to define relations between gluten microstructure and mechanical behaviour of dough at all and to start with shear measurements. Thus, biaxial investigations should be considered in future studies. In the current study, the mechanical behaviour under shear was focussed.

Hence, most relevant information about dough rheological behaviour regarding the aims of this study are provided by the attributes J_{max}, J_r, J_{ei}, η_0, G^*, $tan\delta$, A_f and z. Thus, these attributes were considered for further investigations in the following sections.

The link between rheology and microstructure – Unspecifically- combined with specifically-gluten modified samples. *Correlation matrix and PCA.* Figure 2a shows the correlation matrix of microstructural versus rheological attributes. At first, no clear linear relations between those attributes were found. A closer observation of the data revealed some exponential relations of G^*, A_f or J_{ei} with the five microstructural attributes on some subsets of the data (except TG samples), especially the IHL and RHL samples, which represent unspecifically gluten-modified samples by reducing or increasing water concentrations. Therefore, principal component analysis was performed to identify relations or groupings within the data set. While PCA of solely the rheological properties (section "Rheological consideration") separated the data to softer and firmer doughs, PCA of rheological combined with microstructural attributes (Fig. 2b) highlighted several more clustered data. One grouping, labelled with "E", was represented by samples with a TG concentration of 2000 mg/kg flour and above, and by SHO samples ≥50 g/100 g flour. These samples were most represented by the second component, which is dominated by the steady state viscosity η_0 (increase) and inversely by the loss factor $tan\delta$ (decrease). These are very stiff doughs with a behaviour like an elastic solid of a high resistance to deformation. Note that according to microstructure analysis[12], these samples were characterized by clustered protein particles, which were scattered densely. These unevenly distributed protein networks with high TG concentrations were confirmed by Autio, *et al.*[34]. Since even highly different sample types, modified specifically with the enzyme transglutaminase (TG) and unspecifically with shortening (SHO), showed the same microstructural and rheological characteristics, it can be concluded: gluten network arrangement of densely clustered agglomerates are related to highly viscous and stiff wheat doughs.

Dough samples with reduced water concentrations compared to a standard dough (RHL samples) formed a further cluster in PCA score plot (labelled with "B" in Fig. 2b). Especially the high dough firmness, defined by G* and the network strength A_f, affected the separation in the score plot. Furthermore, as already indicated in section "Rheological consideration", network connectivity z separated wheat doughs with a high network strength A_f. The network connectivity, which describes the extent of interactions of involved units, was 17–37% lower than for cluster E for almost the same network strength. In contrast to cluster E (high SHO and TG samples), samples of cluster B had a larger relative elastic recovery by an average of 25%. These effects led to the conclusion that doughs of cluster B show higher elastic behaviour than those of cluster E even if the remaining rheological attributes seemed to be quite similar. However, gluten network of cluster B samples were completely different. These samples had the highest branching rate as well as average protein lengths and lowest end-point rates compared to all other dough samples, forming a continuous and uniform network structure.

As visualized in the PCA score plot (Fig. 2b) and the discussion from the rheological point of view already indicated, samples with increasing water (IHL) or rapeseed oil (ROI) concentration formed a cluster (labelled with "F"). Those doughs can be characterized by clustered agglomerates, but in contrast to doughs of the cluster E, widely scattered (very high values for lacunarity) due to a dilution and plasticizing effect of the additives. This kind of network results in a low resistance to deformation (high J_{max}, low η_0) and a very low relative elastic recovery (J_{ei}) of dough. For a better understanding, schematic illustrations of different network types based on the detected PCA clusters are visualized in the section "Definition of gluten network types".

Residual data were accumulated around the centre of the PCA score plot. Further clusters can be detected within the data (A, C, D), however, mainly represented by specifically gluten-modified samples. Hence, a further investigation about microstructural and dough rheological relationships was done with solely specifically gluten-modified samples in the following sections.

Prediction models by means of PLS analysis. An aim of this study was to determine statistical models to predict dough rheology by microstructural attributes. Since no distinct relations were detected in the correlation matrix above, also no significant prediction models were found with PLS analysis. Although the rate of explained variation of Y by J_{max} or J_r were about 60%, IHL values dominated the predictions pretending an appropriate fit. Residual by predicted plot revealed clearly that the model did not fit the data (data not shown).

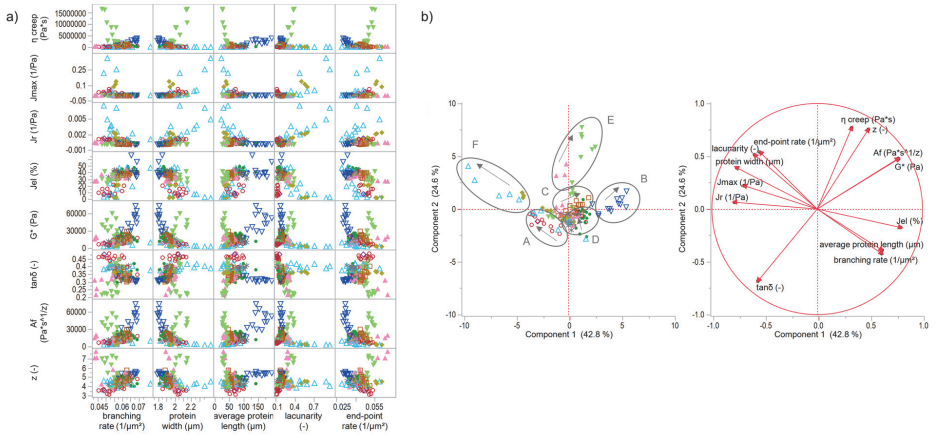

Figure 2. Correlation matrix and principal component analysis of unspecifically as well as specifically gluten-modified samples. (**a**) Correlation matrix of rheological and microstructural attributes of all gluten-modified samples. (**b**) PCA score and loading plot for the first and second principal component. Labelled clusters were used for the identification of different network types. Arrows within the clusters denote increasing concentrations. Symbols: -●- ASC, -*- $KBrO_3$, -◊- BRN, -▲- SHO, -○- GSH, -□- GOX, -◆- ROI, -△- IHL, -▽- RHL, -▼- TG.

The link between rheology and microstructure – Specifically gluten-modified samples. Concluded from the results above, the correlations between dough rheological and gluten microstructural attributes were highly predominant of unspecifically gluten-modified samples, especially IHL and RHL samples. Furthermore, not only the gluten network was affected by unspecific gluten-modifying agents, but also the whole dough system was influenced by plasticizing and dilution effects and thus, the dough rheology. Hence, a sole elucidation of the relation between dough rheology and gluten microstructure can only be determined with correlation and PCA analysis of solely specifically gluten-modified samples.

Correlation matrix and PCA. In contrast to the correlation matrix of all samples, the correlation of specifically gluten-modified samples revealed distinct correlations between the morphological attribute lacunarity with the rheological attributes. As visualized in Fig. 3a, the lacunarity showed exponential dependency $y = a + be^{cx}$ (e.g., $R^2 = 0.91$ for η_0) with the attributes of creep-recovery test and linear correlations (e.g., $R^2 = 0.70$ for G^*) with those of the oscillatory frequency test. The network attribute average protein length showed some nonlinear (exponential) correlations as well ($R^2 = 0.93$ for η_0), although some correlations were not significant (e.g., $R^2 = 0.47$ for A_f). These correlations indicate, that the higher the lacunarity and the lower the average protein length (length of an interconnected protein particle), the higher the dough stiffness (G^*) and the lower dough deformation under strain (J_{max}). However, no direct link between the other network attributes and rheological behaviour was found. Therefore, PCA was performed to detect relations between the attributes or groupings. Figure 3b shows a clear separation between the different clusters in accordance to the protein network classifications of Lucas, *et al.*[12]. Cluster E, predominant of high TG samples, was already detected and described in the previous section. A further cluster is represented by GLU and BRN samples (cluster A), which were specified by a very low lacunarity, low branching-rates and very high end-point rates. These structural characteristics of a weak network are linked to a high dough deformation against strain (J_{max}), a very low relative elastic recovery (J_{el}) and a low dough stiffness (G^*). The decrease of dough firmness with GSH addition is in accordance with other studies[27,35]. Cluster D (mainly ASC and $KBrO_3$ samples) is mainly influenced by the first principal component, especially high average protein lengths and high protein widths. This kind of network with strengthened protein threads goes along with high values for J_{el} and $tan\delta$. With increasing concentration of the added agents (highlighted with arrows in Fig. 3b), the protein width increased whereas the branching rate decreased. In contrast, gluten networks of cluster C altered conversely with increasing concentration (GOX samples, TG $\leq$ 1000 mg/kg flour). These networks consist of highly cross-linked and homogenously distributed proteins[12]. Steffolani, *et al.*[36] reported similar microstructural properties of a continuous, closed and stronger network structure with GOX and TG addition. Rheological properties of cluster C samples showed a viscoelastic behaviour similar to those of cluster D. However, doughs of cluster C were a bit stiffer (G^*) and had a higher viscosity (η_0). Since cluster C is allocated around the centre of the PCA score plot, those samples had no extrema in rheological or microstructural properties and can thus be described as regular (standard) up to strengthened doughs.

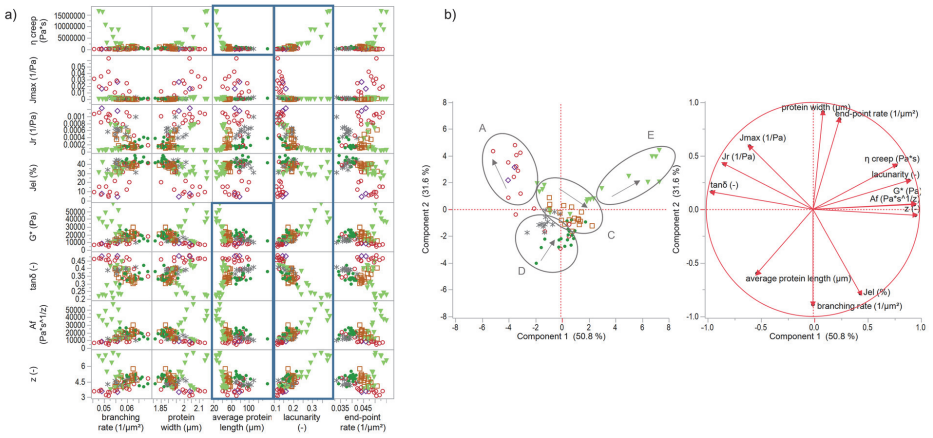

Figure 3. Correlation matrix and principal component analysis of specifically gluten-modified samples. (**a**) Correlation matrix of rheological and microstructural attributes of specific gluten-modified samples. Key correlations are denoted with a blue rectangle. (**b**) PCA score and loading plot for the first and second principal component. Labelled clusters were used for the identification of different network types. Arrows within the clusters denote increasing concentrations. Symbols -●- ASC, -*- $KBrO_3$, -◊- BRN, -○- GSH, -□- GOX, -▼- TG.

Prediction models by means of PLS analysis. Partial least square regressions were applied to determine prediction models for dough rheology by using microstructural attributes. Since scatterplots suggested some nonlinear dependencies between rheological and microstructural attributes, the suitable transformations of the data resulted in linear relations as described in the section "Statistical analysis". Table 2 summarizes prediction formula for each rheological attribute, the percentage, which explains the variation for predicted values (R^2Y) and Fig. 4 displays the parity-plot. The prediction models clearly show a mathematical relation between dough rheology and gluten microstructure. As the correlation analysis above suggested, rheological attributes of the creep-recovery test depend logarithmically on protein network attributes in the prediction models, whereas those of the oscillatory frequency test showed linear or reciprocal behaviour. R^2Y shows, that the logarithmic values of J_{max} and η_0 explain almost 90% of the variation (c.f. Table 2). The residual rheological attributes were predicted with an R^2Y between 72 to 85%. This is remarkably high, since the models are derived from microscopic images, which usually have a higher variance due to operator influences. Furthermore, the models listed in Table 2 predicted rheological properties of standard doughs as well as of the lowest concentration of the unspecific gluten-modified samples ROI, SHO, and RHL. Standard errors of the original value accumulated between 8 and 16% for J_{el}, $tan\delta$ and z values, between 10 to 16% for the logarithmic attributes J_{max}, J_r and η_0, and around 26% for the reciprocal attributes G^* and A_f.

Considering the VIPs, prediction models were mainly affected by the morphological attribute lacunarity. In addition, the network attributes average protein length and branching rate had a high impact on the prediction models as well. However, all five network attributes were required for a precise prediction model.

Therefore, in case of wheat doughs with specifically modifying gluten protein additives, the models predict the rheological properties by gluten network attributes very well. This holds too for additives, which modify unspecifically or to a low extent. Hence, it can be deduced that gluten proteins dominate rheological dough properties, even though there are several further influencing factors. It should be noted that some of the prediction models are based on logarithmic relations as well as the deviations (J_{max}, J_r, η_0). The predicted values of the sole rheological values remained a higher variation due to an increase of the deviations. Nevertheless, plots of predicted vs. actual values showed promising linear correlations of J_{max} ($R^2 = 0.8$), J_r ($R^2 = 0.71$) and η_0 ($R^2 = 0.77$).

Definition of gluten network types. Based on the findings of the sections above, protein microstructure can be classified into six main network types, as summarized in Table 3. Network types correspond to the clusters visualized in Fig. 2 as follows: A – cleaved network, B – rigid network, C – spread network, D – strengthened network, E – particulate, dense network, and D – particulate, loose network. Microstructural evaluation already indicated five network types[12]. Combined with rheological considerations, a sixth network type (rigid network) was identified and an improvement as well as further development of network characteristics related to dough mechanical behaviour for all network types was established in this study. The classification of the network types was performed according to the attribute lacunarity. In contrast to the structural network attributes, lacunarity is a morphological attribute, which pointed out distinct gluten network properties. Furthermore, Gao, *et al.*[11] proposed the attribute lacunarity as an indicator for gluten network formation and thus, for dough mixing properties and wheat quality, highlighting the importance of this attribute. The lacunarity values are dependent on the resolution of the images. In this study, images were recorded with 1024 × 1024 pixel, which is a commonly used

Rheological Attribute	Prediction Formula	PLS factors	VIP	R^2Y (%)
$Log_{10}(J_{max})$	$1.598pw - 48.694br + 0.016apl - 9.188ly + 52.705epr - 4.874$	4	apl ly	89.65
$log_{10}(J_r)$	$0.950pw - 29.975br + 0.005apl - 6.651ly + 3.072epr - 2.905$	3	apl ly	81.56
J_{el}	$-33.843pw + 689.778br - 0.265apl + 72.428ly - 1591.011epr + 132.938$	4	pw br apl ly epr	71.85
$Log_{10}(\eta_0)$	$57.529br - 1.567pw - 0.018apl + 11.776ly - 54.449epr + 6.519$	4	apl ly	89.68
1/G*	$9.275 \cdot 10^{-5}pw - 2.446 \cdot 10^{-3}br + 8.503 \cdot 10^{-7}apl - 4.547 \cdot 10^{-4}ly + 3.391 \cdot 10^{-3}epr - 8.588 \cdot 10^{-5}$	5	br ly	84.26
tanδ	$0.151pw - 4.067br + 7.868 \cdot 10^{-4}apl - 1.010ly + 1.501epr + 0.377$	3	aplly	84.83
$1/A_f$	$9.161 \cdot 10^{-5}pw - 2.491 \cdot 10^{-3}br + 8.597 \cdot 10^{-7}apl - 4.586 \cdot 10^{-4}ly + 3.489 \cdot 10^{-3}epr - 8.525 \cdot 10^{-5}$	5	pw br ly	83.81
z	$2.222pw + 81.507br + -5.997 \cdot 10^{-3}apl + 13.389ly - 29.568epr - 5.274$	4	apl ly	83.26

Table 2. Prediction models for selected rheological attributes. Equations for rheology prediction were determined by PLS models of the microstructural attributes protein width (pw), branching rate (br), average protein length (apl), lacunarity (ly) and end-point rate (epr). The models' dominated attributes are given by VIP (variable importance for projection) values greater than 0.8. R^2Y describes the percentage explained for cumulative Y.

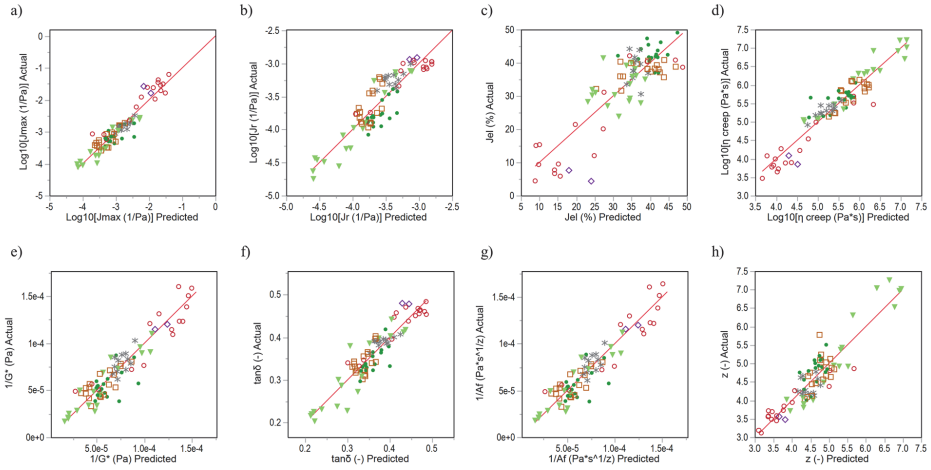

Figure 4. Actual by predicted plots. Rheological attributes were predicted with PLS models of microstructural attributes. Actual by predicted plots are shown for (**a**) $log_{10}(J_{max})$, (**b**) $log_{10}(J_r)$, (**c**) J_{el}, (**d**) $log_{10}(\eta_0)$, (**e**) 1/G*, (**f**) tanδ, (**g**) $1/A_f$ and (**h**) z. Symbols -●- ASC, -*- $KBrO_3$, -◇- BRN, -○- GSH, -□- GOX, -▼- TG.

resolution. Since lacunarity is barely dependent on the used magnification in contrast to the residual network attributes[8], it is an appropriate value for the classification of most dough samples prepared without yeast. Please note that the lacunarity ranges shown in Table 3 should give an indication and are no fixed values. The lacunarity values (which describe holes and gaps within a network) will increase if another component, e.g., gas due to yeast addition, is present. In this case, Table 3 is still applicable for network classifications when comparing the alterations of values to a standard dough as explained subsequently.

Table 3 supports the interpretation of protein network micrographs in future studies. Evaluation needs to be performed in comparison to a standard dough (without any additive) to distinguish structural alterations. It is created to use it as a guiding classification scheme according to the following procedure:

	Cleaved network	Rigid network	Spread network	Strengthened network	Particulate, dense network	Particulate, loose network
Classification by Lacunarity (−)	↓ 0–0.16	↓ 0.17–0.26	→ 0.17–0.26	↑ 0.17–0.26	↑ 0.27–0.5	↑↑ >0.5
Branching rate (μm^{-2})	● ↓↓	●●●● ↑↑	●●● ↑↑	●● ↓	● ↓	● ↓↓
Protein width (μm)	●●● ↑	● ↓	●● ↓	●●● ↑	●●● ↑	●●●● ↑↑
Average protein length (μm)	●● ↓	●●●● ↑↑	●● ↑	●●● ↓	● ↓↓	● ↓↓
End-point rate (μm^{-2})	●●●● ↑↑	● ↓↓	●●● ↓	●● ↑	●●●● ↑↑	●●●● ↑↑
Network description	Ruptured protein threads, short protein segments	Uniform, dense and continuous structure, highly branched	Homogenous, very branched, elongated and distributed protein threads	Locally strengthened protein threads, continuous	Clustered agglomerates, densely arranged	Clustered agglomerates, widely scattered
Rheological characteristics	Low viscous	Highly viscous	Viscoelastic	Viscoelastic	Highly viscous	Low viscous
η_0 (Pa*s)	●	●●●	●●●	●●	●●●●	●
G* (Pa)	●	●●●●	●●●	●●	●●●●	●
z (−)	●	●●●	●●●	●●	●●●●	●●
tanδ (−)	●●●●	●●	●●	●●●	●	●●
J_{el} (−)	●	●●●	●●●	●●●	●●	●
J_{0creep} (1/Pa)	●●	●	●	●●	●	●●●
J_{1creep}, J_{1rec}, J_{2rec}, J_{max}, J_r (1/Pa)	●●●	●	●	●●	●	●●●●
Correspond to labels in Fig. 2	A	B	C	D	E	F
Correspond to network type of Lucas, *et al.*[12]	I		II	III	IV	V
Examples	GLU ≥ 30 mg/100 g flour, BRN ≤ 200 mg/kg flour	RHL ≤ 49.8 ml/100 g flour	GOX ≥ 40 mg/kg flour, TG ≤ 1000 mg/kg flour	ASC ≥ 50 mg/kg flour, $KBrO_3$ ≥ 60 mg/kg flour SHO ≤ 15 g/100 g flour	TG > 1000 mg/kg flour, SHO ≥ 50 g/100 g flour	IHL ≥ 69.9 ml/100 g flour, ROI ≥ 50 g/100 g flour

Table 3. Definition of gluten network types. The classification is performed according to the microstructural attribute lacunarity (determined on images with a resolution of 1024 × 1024 pixel and size of 215 × 2015 μm). Network attributes are described by their strength ● (low) - ●●●● (very high). Arrows (↓→↑) denote the development of microstructural attributes of a network type's typical sample compared to its corresponding standard wheat dough.

- Determination of lacunarity range
- Comparison of PNA results of the sample with corresponding standard dough (without any additive) regarding to an increase (↑) or decrease (↓) of the values
- Identification of the corresponding network type

More detailed, to identify the corresponding network type for a dough sample, the range of lacunarity should be determined first. Second, results of protein network analysis of the sample should be compared to its corresponding standard dough (without any additive) regarding to an increase or decrease of the values (c.f. arrows in Table 3). Hence, the corresponding network type, its interpretation, and its related dough rheological behaviour should have been identified. The strength of each microstructural or rheological attribute in general and in comparison to all network types is expressed by bold dots. Schematic illustrations of the network types as well as examples underline each network arrangement (c.f. Fig. 5).

When solely comparing the categorized strengths of network attributes (bold dots), it seems that the cleaved, particulate dense and particulate loose network types are quite similar (Table 3). However, the main difference between those three network types is the morphological characteristic lacunarity, which is very different for each type. Consequently, this results in completely different rheological properties of wheat dough. This example shows that sole evaluation of quantitative values of network attributes is challenging and that interpretation of the results is now supported due to the classification of network types, which allows conclusions on protein network arrangements and rheological behaviour of dough. In turn, this makes the mapping of different protein network types and similar rheological dough properties feasible and contributes therefore to the understanding of structure-function relationships. For example, a rigid as well as particulate, dense network can be linked to different protein network types.

Considering the nano- and microscale of gluten proteins, there are some network models combining structural properties with functionalities and product properties[37–41]. As this study shows that quantitative relations and predictions models can be performed between dough mechanical behavior and gluten microstructure, these findings can be used as a basis for future research. Hence, the network types can be linked to dough functionality with further investigations, especially using further rheological tests in the non-LVE, using large deformation extensional measurement, and under hyperbolic contraction flow[24,32,33].

It should be noted, that the network types express distinctive forms of gluten arrangements and that gluten networks can also occur in an attenuated form of the six types. A standard dough can also be categorized into one of the six network types but to a lower extent. A standard dough had been described in literature as an interconnected gluten network covering starch granules[42], a homogeneous protein phase with very fine structure[4], or a

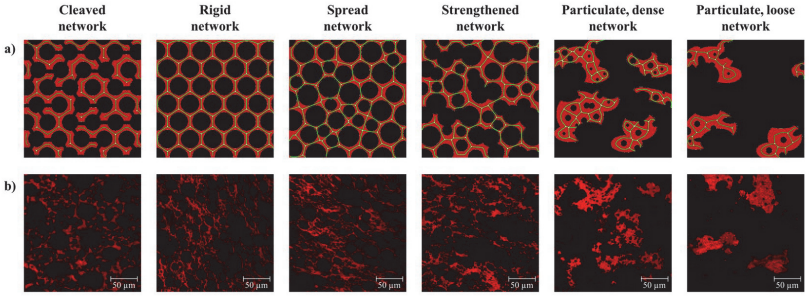

Figure 5. Illustrations of gluten network types. (**a**) Schematic illustrations of network types, (**b**) Original CLSM micrographs of appropriate examples (215 × 215 µm, 1024 × 1024 px).

spread network, which is defined as a continuous structure with elongated and distributed gluten strands[1]. These descriptions may be categorized to the network type "spread network". However, Autio, *et al.*[34] reported a looser protein network structure for a standard dough from an organic flour compared to regular flours. Comparing the descriptions of standard dough mentioned in literature, standard doughs might be categorized mostly to a spread or strengthened network as well as rarely to a cleaved or rigid network but to a lower extent. However, no universal standard dough exist in general due to variations in raw materials, different flour qualities and no standardized production process. Consequently, no standard protein network type exist and thus, no individual network type for a standard dough was established. Hence, individual standard doughs always need to be defined in relation to doughs within a measurement series or experiment.

Conclusion

In this study, empirical relations of gluten microstructure and dough rheology were presented. Using specifically gluten-modified dough samples, linear (J_{el}, $tan\delta$, z), reciprocal (G^*, A_j) as well as logarithmic (J_{max}, J_r, η_0) relations of dough rheology with gluten network attributes were found, and prediction models were defined by PLS regressions with an accuracy up to 90%. Furthermore, these prediction models provided reliable predicted rheological values for dough samples of low concentrations of unspecific gluten-modifying agents. Thus, with these mathematical models, it is possible to predict rheological properties via the five microstructural attributes lacunarity, branching rate, end-point rate, and average protein length as well as protein width. It should be noted that estimated linear models are based on values gained by micrographs of the specific magnification (60x objective) and resolution (215 × 215 µm) used in this study. Since microstructural results always depend on the applied setting and equipment, no absolute rheological values can be determined with prediction models in general. Nevertheless, comparative values can be determined with micrographs of other magnifications or resolutions, and estimations of rheological properties can be proposed with the models of the current study.

It was shown that prediction models could not be established for every kind of gluten network arrangements, especially for doughs effected by unspecific gluten-modifying agents due to additional impacts, like plasticizing or starch swelling effects. In spite of this, relations between dough rheology and gluten microstructure were determined based on cluster detections with principal component analysis. Hence, six network types were defined, which represent various distinctive gluten network arrangements. The definition of the network types is the first, which allows both quantitative definitions of gluten networks and the characterization of the relation of spatial arrangement of proteins to dough rheology. The classification of network types was designed to use it as a guiding scheme for the interpretation of protein network arrangements and prediction of corresponding dough rheological behaviour for further studies. Since rheological behaviour is related to bread quality, this study provides the basis for predictions and new approaches of structure-function relationships. Moreover, additional network types can be defined for more complex dough systems (e.g., with yeast or sodium chloride) or for doughs under mechanical forces (e.g., mixing, kneading, or sheeting) in the future. With the knowledge acquired in this study that quantitative relations and predictions between dough mechanical behaviour and gluten microstructure can be determined, further investigations and additional prediction models about dough functionality, processing behaviour and baking quality can be performed.

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Acknowledgements
This work was supported by the German Research Foundation (DFG) and the Technical University of Munich (TUM) in the framework of the Open Access Publishing Program. The authors would like to thank Katerina Kiousi, Tamar Bar-Zeev, Mariam Bapir, Ksenia Egginger and Ludmilla Adolf for their contribution.

Author Contributions
I.L. planned and coordinate the experiments, interpreted the results, carried out the statistical analyses and wrote the main manuscript. H.P. participated in the statistical analysis and contributed to write the manuscript. T.B. and M.J. supervised the work, participated to the interpretation of the results and contributed to write the manuscript. All authors review and approved the manuscript before submission.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-41072-w.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Definition of network types – Prediction of dough mechanical behaviour under shear by gluten microstructure

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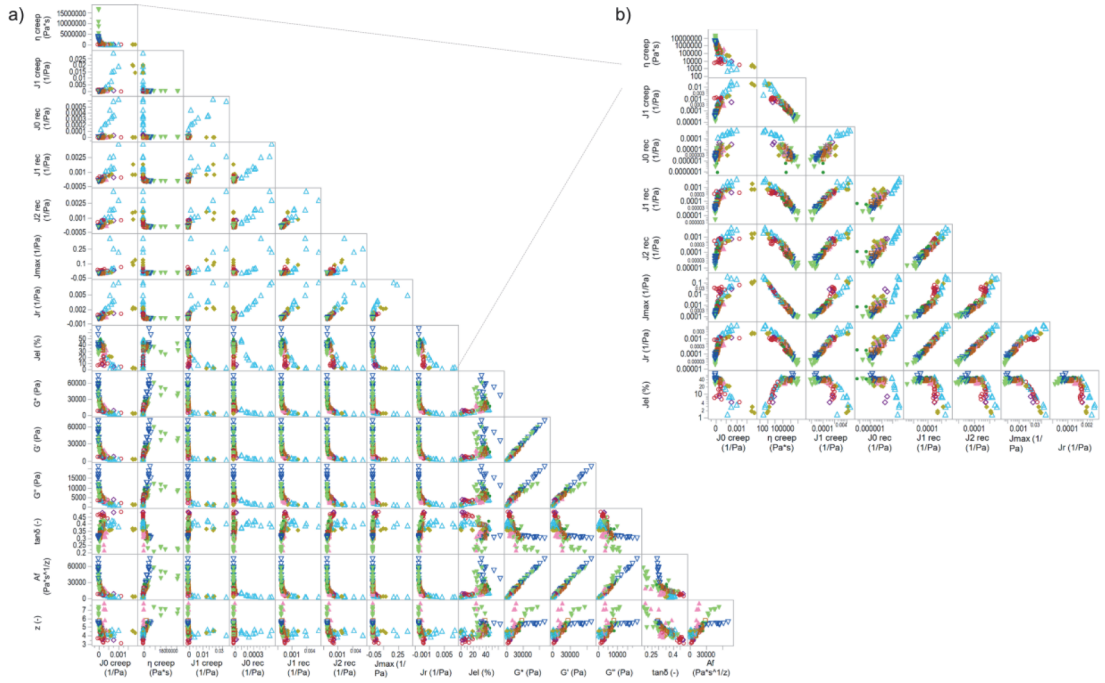
Supplementary data



Supplementary Figure S1. Correlation matrix of the rheological data of unspecifically as well as specifically gluten-modified samples. (a) Correlation matrix of rheological attributes (oscillatory frequency test and creep-recovery test) of all gluten-modified samples. (b) Correlation matrix of attributes of creep-recovery test with logarithmic scales. Symbols: -●- ASC, -*- $KBrO_3$, -◊- BRN, -▲- SHO, -○- GSH, -□- GOX, -◆- ROI, -△- IHL, -▽- RHL, -▼- TG.

4 Discussion, conclusion and outlook

The improvement of the quality of wheat-based products requires a distinct understanding of structure-function relationships. In this thesis, an approach was developed to visualise and quantify most appropriately the complex structure of gluten network in wheat dough on a microstructural level. These developed techniques formed the basis for the investigation of different network properties and structure-function relationships. Based on the hypotheses stated in the thesis outline (1.4), the following findings were identified in this study:

- ✓ The protein network analysis (PNA), a more advanced image analysis for quantifying gluten microstructure in wheat dough based on morphological and structural network attributes, was developed and enables now defining the connectivity and strength of the complex network structure
- ✓ The staining methods drop- and rapid freezing techniques have an impact on protein network formation and dough functionality whereas the dye itself has not
- ✓ A staining method for gluten in wheat dough with fluorescent dyes as non-invasive as possible was identified (bulk water technique), which enables a most realistic visualization of gluten network when using confocal laser scanning microscopy and without interfering microstructural changes
- ✓ Molecular alterations in the gluten polymers caused by specific (enzymatic, chemical) as well as unspecific (plasticising) gluten-modifying agents influenced the gluten arrangement on a microstructural level and the new developed image analysis PNA was precise enough to detect them
- ✓ Various spatial arrangements of gluten networks were classified in six network types to support the interpretation of the gluten microstructure by image analysis
- ✓ Those network types were related to dough mechanical behaviour which enables the prediction of dough functionalities by PNA attributes
- ✓ Dough mechanical behaviour was predicted by mathematical formulas based on structural and morphological network attributes gained by PNA for specific gluten-modified samples with an accuracy of up to 90%

These summarised findings are discussed based on the hypotheses, subsequently.

Hypothesis 1: A development of a more advanced image analysis for quantifying gluten microstructure in wheat dough based on morphological and structural network attributes enables determining the connectivity and strength of the complex network structure.

The **first hypothesis** of section 1.4 was confirmed in the first study (Bernklau et al. (2016), c.f. section 3.2). Most of the former studies in literature did not apply an image analysis on their microstructure images (Baier-Schenk et al., 2005; Dürrenberger et al., 2001) or used methods focussed on the analysis of protein area or protein particles (Jekle & Becker, 2011; Lee et al., 2001; Peighambardoust et al., 2006). The protein network analysis developed in this study is the first method, which enables a quantification of the connectivity and strength of network structures in absolute values (protein junctions, protein thread length or width), ratio values (branching- and end-point rate) and the morphological attribute lacunarity (Bernklau et al., 2016). By validating the method and comparing to dough rheology, it was highlighted that PNA is a suitable method to characterise the complex gluten network formation detecting structural differences in various dough systems and explaining changes in mechanical dough behaviour. The characterization of protein threads would be even more precise, if the length distribution of single protein threads between two junctions was returned by the tool. However, only the average length of an interconnected and continuous protein particle can be determined.

PNA is based on a tool (AngioTool), which was originally intended for use in cancer research of angiogenesis. The idea of using this tool for gluten network structures occurred when recognizing its similar shape to blood vessels. For this purpose, the resulting attributes needed to be adapted for the evaluation of gluten networks. Moreover, the attributes branching rate and end-point rate were developed, since they are independent of the recorded image section and give information about the connectivity of a network. Furthermore, the protein width was calculated for a detailed description of protein threads. The adaption of AngioTool results on gluten microstructure worked very well, and indicate that PNA could be used not only in other dough systems, but also in baked products, like for gluten-free (e.g. rice, buckwheat) or rye proteins. Rye proteins (Döring, Hussein, Jekle, & Becker, 2017; Döring et al., 2015) as well as rice proteins (Moore, Heinbockel, Dockery, Ulmer, & Arendt, 2006) form network structures with the addition of transglutaminase. Applying PNA on those microstructures would support the interpretation of the mechanism of network formation as well as on their impact on dough

rheology. Moreover, many network structures occur in food systems and most of their microstructures are not quantified by a network analysis as well. The proteins in milk and cheese form complex network structures, which were only little investigated quantitatively and not with structural network attributes (Auty, Fenelon, Guinee, Mullins, & Mulvihill, 1999; Buffa, Trujillo, Pavia, & Guamis, 2001; Lopez, Camier, & Gassi, 2007; Ong, Dagastine, Kentish, & Gras, 2011). Hence, PNA would be a useful tool for the characterisation of any kind of network microstructure and it is suggested for a comprehensive investigation in future food studies. Nevertheless, the link to mechanical properties of those other food systems or gluten free products must be investigated when applying PNA, since the network types of this thesis might not be applicable on non-gluten systems.

Hypothesis 2: Existing staining methods with fluorescent dyes and the dye itself (e.g., by hydrophobic interactions) used for CLSM measurement have an impact on protein network formation and dough functionality, which should be considered for a most realistic visualisation of gluten microstructure.

The **second hypothesis** was partially debunked with the second publication of this thesis (Lucas, Stauner, et al. (2018), c.f. section 3.3). The dye Rhodamine B did not have an impact on protein network formation due to the potential hydrophobic interactions as expected. Thus, the network formation was not impaired by the dye. In contrast, the staining procedure by drop- and rapid freezing technique affected dough microstructure and mechanical behaviour. For investigating structure-function relationships by CLSM, a realistic visualisation of the microstructure is crucial. CLSM is a non-invasive method. However, the components need to be stained with fluorescent dyes for visualising with CLSM. Even if the CLSM measurement itself is non-invasive, this study shows that the required staining procedure has an invasive effect when using drop- or rapid freezing technique. Considering the fact that both methods were commonly used in literature, it can be concluded that the microstructure of gluten had not been visualised as realistic as possible very often in the past. Nevertheless, the staining procedure “bulk water technique” was identified as the method for a visualisation as realistically as possible since it has no impact on dough mechanical behaviour and it is non-invasive. Hence, the bulk water technique is recommended for further studies. Since it is not always practical to add the dye during dough preparation, the drop technique is suggested as a second option. Moreover, the bulk water technique is mostly feasible in lab-scale, whereas

the application of the drop technique is required for up-scale processes, as it was used for e.g., the analysis of pizza dough (Bernklau et al., 2017). Although the drop technique affects dough rheology, the effect is much smaller than using the rapid freezing technique.

The stated hypothesis was related to the CLSM analysis of gluten network. An investigation of other fluorescent dyes for further dough components should be considered in future studies. Likewise, specific dyes with covalent linkages to dough components should be evaluated prospectively. The dye Rhodamine B, which was used in this study, stains all gluten proteins unspecifically. If subunits of glutenins or gliadins could be visualized specifically, it would support a detailed structure-function analysis.

The first two studies of this thesis formed the basis for following microstructure studies of the gluten network in dough. Now, it is possible to visualise gluten microstructure most realistically by CLSM and to quantify the complex structure of gluten network by specific network attributes.

Hypothesis 3: Molecular alterations in the gluten polymers caused by specific as well as unspecific gluten-modifying agents influence the gluten arrangement on a microstructural level and the new developed image analysis is precise enough to detect them.

The **third hypothesis** was confirmed in the publication Lucas, Becker, et al. (2018) (c.f. section 3.4). In the first study of this thesis (Bernklau et al., 2016), PNA was only validated on gluten network structures affected by different hydration levels. Those network structures showed distinct differences with increasing hydration levels, which was expected due to the plasticising effect of water. The third hypothesis of this thesis focused on the precision and accuracy of PNA. It states that even little effects and specific modifications on gluten network formation, caused by e.g., enzymes or chemical agents, modify the gluten network arrangement and that these effects can be detected by PNA. In the past, those effects on protein network formation were analysed on a molecular level in detail, but rarely on a microstructural level. In the present study, it was shown that first, specific modifications in cross-links (disulphide, (iso)peptide, dityrosyl) caused by reduction, oxidation or enzymes (glutathione, bromelain, ascorbic acid, potassium bromate, glucose oxidase and transglutaminase) are visible on a microstructural level in form of altered network arrangements and second, PNA detected those alterations precisely. Moreover, structural

alterations within all concentration series of both specifically as well as unspecifically gluten-modified structures were quantified by PNA, which confirms the hypothesis 3.

However, microstructure analysis has its limits as highlighted by the effect of bromelain on gluten network in Lucas, Becker, et al. (2018). As in the case of high bromelain dosages, the protein network was entirely cleaved due to the proteolytic activity of the enzyme. The remaining protein fragments formed a homogenous and liquid mass with embedded starch granules, as illustrated in Figure 10c. Thus, PNA did not detect real cross-links or gluten threads, which are formed and stretched during kneading (c.f. image of a standard wheat dough in Figure 10a). At low bromelain concentrations (e.g., 200 mg/kg flour, Figure 10b), a skeleton of a protein network as well as protein fragments are still present, and ruptured protein threads were detected with PNA by increased end-point rates similar as with glutathione addition (c.f. section 3.4, third publication). In contrast to glutathione, which only reduces disulphide bonds, bromelain breaks peptide bonds, which leads to a collapse of the whole protein network structure at high bromelain concentrations. The remaining liquid, homogenous mass looked like a network on CLSM images due to the black gaps caused by the starch granules. Hence, applying PNA would cause misleading results by detecting junctions. Consequently, applying PNA on microstructure images is only suitable if a real network structure is still present. This decision requires a distinct knowledge of cereal microstructures and expertise of the operator, which are anyway a prerequisite when using CLSM. Nevertheless, it can be stated that hypothesis number 3 was confirmed in this study.

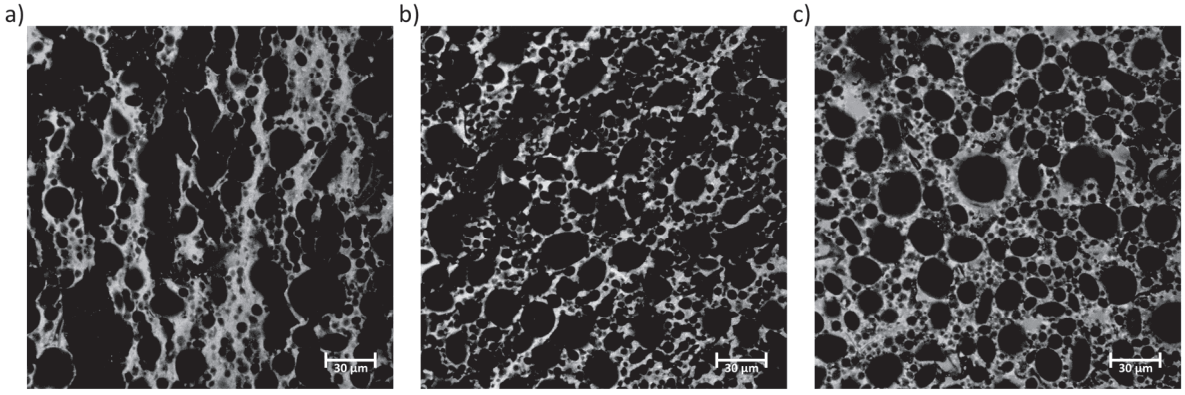


Figure 10: CLSM images of **a)** a standard wheat dough, **b)** a dough with 200 mg bromelain/kg flour and **c)** 6100 mg bromelain/kg flour. Gluten proteins are stained with Rhodamine B and visualized as grey colour in the images (215x215 µm).

Hypothesis 4: Various spatial arrangements of gluten networks can be classified in network types related to dough mechanical behaviour to support the interpretation of the gluten microstructure by image analysis.

The **fourth hypothesis** was confirmed in the third publication (Lucas, Becker, et al., 2018) as well as in the fourth publication (Lucas et al., 2019) (c.f. sections 3.4 and 3.5). Even though a method for the quantification of the gluten network was provided in the first publication (Bernklau et al., 2016), the meaning of the network attributes remained challenging for an interpretation of the whole network structure or its effect on dough functionality. When confirming hypothesis 3 by different modifications in gluten network, it became apparent that the evaluation of single network attributes is not enough to understand the kind of network arrangement and to draw conclusions on mechanical behaviour. At least five network attributes and their combined interpretation were required to describe the protein networks (branching rate, end-point rate, average protein length, protein width and lacunarity). Moreover, based on the findings of hypothesis 3 that different modifications in cross-links (disulphide, (iso)peptide, dityrosyl) lead to various network arrangements, this fact led to the assumption that there must exist various network arrangements with specific characteristics. Consequently, these spatial network arrangements must be classifiable in network types for a general interpretation of PNA results based on network attributes. Those various network arrangements were provoked by using specific as well as unspecific gluten-modifying agents in order to gain as much as different network arrangements and quantitative values of the five network attributes in a large range. Using principal component analysis, five typical gluten network arrangements were identified and verified in Lucas, Becker, et al. (2018). The inclusion of rheological attributes in the principal component analysis enabled the detection of a sixth network type (Lucas et al., 2019). Finally, six network types were classified according to the morphological attribute lacunarity and related to dough mechanical behaviour. Hence, the plain data of PNA of microstructural images compared to the data of a standard dough are enough to identify the network type (arrangement of gluten polymers) and to predict dough mechanical behaviour in future studies.

Hypothesis 5: Dough mechanical behaviour can be predicted by mathematical formulas based on structural and morphological network attributes gained by microstructure image analysis.

The **fifth hypothesis** was partly confirmed in the last study of this thesis (Lucas et al. (2019), c.f. section 3.5). Prediction formulas were developed for specifically gluten-modified samples using PLS analysis (Table 2 of Lucas et al. (2019)). With these formulas, it is now possible to predict dough mechanical behaviour with the microstructural network attributes branching rate, end-point rate, average protein length and protein width as well as the morphological attribute lacunarity. The accuracy was remarkably high (up to 90%), since results are based on microstructure images which are more dependent on the operator (e.g., individual decisions which images of a dough sections are taken) than other measurement methods which have no subjective influence. A further step could be a development of prediction models for final product characteristics (e.g., texture attributes, volume) that a prediction of the product quality would be feasible solely based on gluten microstructure of the dough. This could give an opportunity to design products with targeted textural properties. For this purpose, the knowledge to modify the gluten network specifically by using chemical or enzymatic agents is crucial, which can be developed by protein network analysis.

However, it was not possible to determine prediction formulas for unspecifically gluten modified samples due to a lack of distinct relations within the data. The influence of unspecific gluten-modifying agents on other flour ingredients than gluten outweighed that no significant mathematical relation could be detected. On the contrary, this highlights the determining, single influence of gluten and all other dough constituents on the functionality of wheat-based products and underline the importance of the investigation of structure-function relationships. Nevertheless, the development of the network types provides a good compromise to predict dough mechanical behaviour related to gluten microstructure even for unspecifically gluten-modified samples.

Transfer of network types on literature examples

There are already few studies published, which applied protein network analysis on CLSM images of wheat protein microstructure. In the following sections it is discussed, if the networks described in these literature studies can be classified also to one of the six network types of Lucas et al. (2019).

Before the establishment of the six network types, the effect of sodium chloride and fermentation/proofing on gluten networks in pizza dough was studied by protein network analysis (Bernklau et al., 2017). Pizza dough is a more complex system compared to the wheat flour-water systems of this thesis because of the addition of yeast, sodium chloride and sunflower oil in the recipe. Another difference to the studies of this thesis is the application of a 20x objective to cover a larger network section due to increased gas bubbles caused by fermentation. Nevertheless, a classification of the network structures to the network types was tested. As mentioned in the fourth publication of this thesis, the classification of network types is based on lacunarity because it is less dependent on the used magnification compared to the structural network attributes (Lucas et al., 2019). It is also emphasised that the given lacunarity values are solely a range and no fixed values which can vary dependent on the dough system and measurement settings. In the first publication, a comparison of both magnifications using the 20x and 60x objective was presented (Bernklau et al., 2016). Lacunarity values were very similar for both magnifications, but a mean multiplying factor of 1.5 could be applied on lacunarity results of the 20x magnification to achieve similar values of the 60x objective. A classification of network types could be performed only by comparing the results to a standard dough by evaluating the value decrease or increase of each attribute even if the lacunarity values do not fit exactly in the given ranges of Table 3 of Lucas et al. (2019). After multiplying the lacunarity results of the pizza study of Bernklau et al. (2017), the lacunarity values were even more in accordance with the ranges. The addition of sodium chloride caused a decrease of lacunarity compared to the standard dough (≈0.11 or after multiplying ≈0.17 at highest NaCl level). Furthermore, sodium chloride lead to a dense gluten network expressed by very high branching rates, high average protein lengths and very low end-point rates. Hence, dough with sodium chloride addition can be classified to a **rigid network**. It should be noted that the addition of sodium chloride affects also the water absorption and that it was not adapted in this study. Hence, the influence on gluten network

is not only derived from sodium but also from various hydration levels. The rigid network is characterized by a high firmness (G*) and an increased relative elastic part (J_{el}). Rheological measurements by texture-profile-analyser (TPA) revealed a higher resistance to extension and extensibility confirming the properties of a rigid network. Moreover, the rheological investigation by elongation measurements extend the knowledge of the classification of this network type. Fermentation and proofing of dough caused an increase in lacunarity values (up to ≈0.23) compared to the unfermented standard dough (Bernklau et al., 2017). Since the branching rate and average protein length decreased slightly and the end-point rate as well as the protein width increased, the network of fermented and proofed pizza dough can be classified to a **strengthened network**. The lacunarity value of fermented and proofed dough fitted in the range of a strengthened network without multiplying with a factor of 1.5 even if the magnification with a 20x objective was applied. If multiplying with 1.5, a lacunarity value of about 0.33 resulted, which would fall into the classification of a particulate, dense network. However, the average protein length is too high to fit to a particulate, dense network. Furthermore, the visual evaluation does not show clustered protein agglomerates, but a branched network with locally strengthened protein threads, which fits more the characteristics of a strengthened network. Moreover, the rheological evaluation indicated still a viscoelastic behaviour even if the extensibility was lower than the standard dough. Likewise, the gas holding capacity indicated an intact and strong network because no CO_2 loss was detected. Hence, the classification of the fermented and proofed pizza dough is proposed as a strengthened network type. Taking into account that usually higher yeast concentrations are used as in this study of pizza dough, larger gas bubbles and thus, even higher lacunarity values (≙ larger gaps, higher irregularities) and a weakening of the protein network would be expected. Studying fermented doughs could be a topic for future research, which might also lead to a definition of a new network type. Another option could be to extent the lacunarity range of the strengthened network to cover also doughs produced with yeast. Nevertheless, the lacunarity ranges of network types of Table 3 in Lucas et al. (2019) are just suggestions and no fixed values.

There are further publications, which used the protein network analysis. Q. Li, Liu, Wu, and Zhang (2017) produced wheat doughs with different soluble dietary fibre (SDF) fractions of wheat bran and studied the effect on gluten network formation using CLSM and PNA.

Compared to the standard dough (without SDF), lacunarity values and end-point rate decreased whereas the branching rate increased for dough with soluble dietary fibre fractions. Those characteristics can be classified to a **rigid network**, which was confirmed by an increased hardness and resistance to extension measured by TPA. However, the absolute values of lacunarity did not fit in the range of a rigid network. An explanation for this might be the poor quality of the recorded CLSM images which were illustrated in the publication of Q. Li et al. (2017). The proteins appeared pixelated and partly oversaturated affecting lacunarity results since the algorithm of PNA is based on box counting (pixel counting) (Bernklau et al., 2016).

PNA was also applied by Gao et al. (2018) to study the absence and presence of Ax1 and Dx2 subunits on glutenin polymerisation. The analysis was performed without a standard, only on gluten samples and not in a wheat dough system and the average protein length as well as protein width were not evaluated, which is why a categorisation to network types is not possible. However, the authors found significant differences in gluten networks and correlated the attributes with dough mixing properties confirming the applicability of PNA. Moreover, they proposed lacunarity and end-point rate as an indicator for dough properties. In a subsequent study of Gao's co-workers, a linear correlation of dough mixing properties (dough development and stability time) with lacunarity values was demonstrated (S. Li et al., 2019). However, correlations to further dough mechanical properties (e.g., resistance to extension, creep compliance, viscosity) were not analysed. Since the classification of network types in this thesis is also based on lacunarity values, the attribute lacunarity can indeed be a good indicator for dough properties. However, it can give only indications and a reference to a standard dough is always required for an accurate classification.

The present thesis focussed on network types affected by various dough ingredients (specific and unspecific gluten modifying agents). In contract, the research of Hackenberg et al. deals with mechanical effects on wheat flour ingredients (gluten, starch) caused by various process condition during milling and its effect on gluten network formation during kneading (Hackenberg, Jekle, & Becker, 2018; Hackenberg, Vogel, Scherf, Jekle, & Becker, 2019). At the highest mechanical starch modification (MSM) level and at optimum kneading time ($Peak_{time}$) according to AACCi method 54.21, the gluten network appeared as clustered protein agglomerates with decreased branching rates and average protein lengths as well as highly

increased end-point rates and protein widths compared to a standard dough. The lacunarity increased up to a value of about 0.4 and it can be assumed as higher (0.58 with the multiplying factor of 1.5), since a 20x objective was used. Hence, this network can be classified as a **particulate, loose network**. The reduced resistance to extension and extensibility compared to a standard dough fit to the weakened structural properties of this kind of network type. Hackenberg et al. (2019) found out that with increasing kneading times, a fully developed protein network comparable to a standard dough could be achieved at a second peak (2.$Peak_{time}$). With a too long kneading time and thus a too high energy input (>2.$Peak_{time}$), the network appeared as a dense and highly branched structure with accumulated protein threads (highly increased branching rates and average protein lengths, decreased end-point rates and protein width compared to the standard). Since the lacunarity decreased compared to the standard, this network could fall into the network type "rigid network". However, the lacunarity value is very low (≈0.03) and lower than the corresponding range of Table 3 in Lucas et al. (2019). Furthermore, the rigid network is characterized by a highly viscous, stiff dough, which does not comply with the measured decreased resistance to extension and extensibility in the study of Hackenberg et al. (2019). Other studies with overmixed dough showed the same structural changes to a homogenous, finely spread and closely interconnected gluten microstructure with a weakening effect on dough (Amend & Belitz, 1991; Peighambardoust et al., 2010; Peressini et al., 2008). Thus, it can be concluded that this network cannot be categorized in one of the existing six network types. Based on this and on additional further studies, a **seventh network type** could be established in the future. A proposed classification (called e.g., **"dense network")** is given in Table 1 and a visualisation in Figure 11. Additionally, all new findings to extend the definition of network types related to dough mechanical behaviour are summarised in Table 1.

Table 1: Extension of the definition of gluten network types based on Lucas et al. (2019). The strength of network and rheological attributes is given by dots ● (low) up to ●●●● (very high). The network characteristics compared to its corresponding standard wheat dough are denoted by arrows (↑→↓). All new findings and extension of network types are highlighted in blue. The classification of a "dense network" is not fully proven and just a proposal (lacunarity range is estimated in this case).

	Dense network	***Cleaved network***	***Rigid network***	***Spread network***	***Strengthened network***	***Particulate, dense network***	***Particulate, loose network***
Classification by Lacunarity (-)	↓↓ 0 – 0.1	↓ 0 – 0.16	↓ 0.17 – 0.26	→ 0.17 – 0.26	↑ 0.17 – 0.4	↑ 0.27 - 0.5	↑↑ > 0.5
Branching rate (μm^{-2})	●●●● ↑↑	● ↓↓	●●●● ↑↑	●●● ↑↑	●● ↓	● ↓	● ↓↓
Protein width (µm)	● ↓	●●● ↑	● ↓	●● ↓	●●● ↑	●●● ↑	●●●● ↑↑
Average protein length (µm)	●●●● ↑↑	●● ↓	●●●● ↑↑	●● ↑	●●● ↓	● ↓↓	● ↓↓
End-point rate (μm^{-2})	● ↓	●●●● ↑↑	● ↓↓	●●● ↓	●● ↑	●●●● ↑↑	●●●● ↑↑
Network description	Densly accumulated protein threads, highly branched, homogenous	Ruptured protein threads, short protein segments	Uniform, dense and continuous structure, highly branched	Homogenous, very branched, elongated and distributed protein threads	Locally strengthened protein threads, continuous	Clustered agglomerates, densely arranged	Clustered agglomerates, widely scattered
Rheological characteristics	Low viscous	Low viscous	Highly viscous	Viscoelastic	Viscoelastic	Highly viscous	Low viscous
η_0 (Pa*s)		●	●●●	●●●	●●	●●●●	●
G* (Pa)		●	●●●●	●●●	●●	●●●●	●
z (-)		●	●●●	●●●	●●	●●●●	●●
tanδ (-)		●●●●	●●	●●	●●●	●	●●
J_{el} (-)		●	●●●	●●●	●●●	●●	●
$J_{0\ creep}$ (1/Pa)		●●	●	●	●●	●	●●●
$J_{1\ creep}$, $J_{1\ rec}$, $J_{2\ rec}$, J_{max}, J_r (1/Pa)		●●●	●	●	●●	●	●●●●
Extensibility (mm)	●		●●●		●●		●
Resistance to extension (N)	●		●●●●		●●		●
Examples	MSM 8.15 g/100 g flour +dough development >$Peak_{time}$ (c.f. Hackenberg et al. (2019))	GLU ≥30 mg/100 g flour, BRN ≤200 mg/kg flour	RHL ≤49.8 ml/100 g flour, NaCl ≥1.1 g/100g dough (c.f. Bernklau et al. (2017)), various SDF fractions (c.f. Q. Li et al. (2017))	GOX ≥40 mg/ kg flour, TG ≤1000 mg /kg flour	ASC ≥50 mg/ kg flour, $KBrO_3$ ≥60 mg/kg flour SHO ≤15 g/ 100g flour, 0.13 g yeast /100g dough +proofing (c.f. Bernklau et al. (2017))	TG >1000 mg /kg flour, SHO ≥50 g /100 g flour	IHL ≥69.9 ml/ 100 g flour, ROI ≥50 g /100 g flour, MSM 8.15 g/100 g/flour+dough development <$Peak_{time}$ (c.f. Hackenberg et al. (2019))

These examples highlighted that protein microstructures analysed by PNA can be classified to the network types according to Table 3 in Lucas et al. (2019). Moreover, CLSM images recorded with other settings can still be classified according to this scheme (summarised in Table 1). In this thesis, only effects of specific as well as unspecific gluten-modifying agents on

gluten structure in flour-water dough were investigated. The application of classifying network types on literature examples as discussed above showed that even more complex dough systems as well as dough affected by process conditions can still be classified in the six network types. This proves the existence of the network types and their applicability. The network types were developed on dough systems modified by chemical, enzymatic or plasticising (and thus hydrophobic/hydrophilic) effects on gluten network with low effects on other components of flour, such as starch. If other effects, such as mechanical (kneading, stretching, sheeting), temperature dependent or specific effects on other flour components (especially starch) modify gluten network formation, the six network types might not be sufficient. As the last literature example indicated, further network types probably exist, which could be elucidated in forthcoming studies. However, there is a high probability that the influence of those unspecific effects on gluten will predominate and that a prediction of dough functionality by solely the analysis of gluten network is not practicable in these cases. Hence, the "dense network" is just a proposed classification, since it is not fully proven.

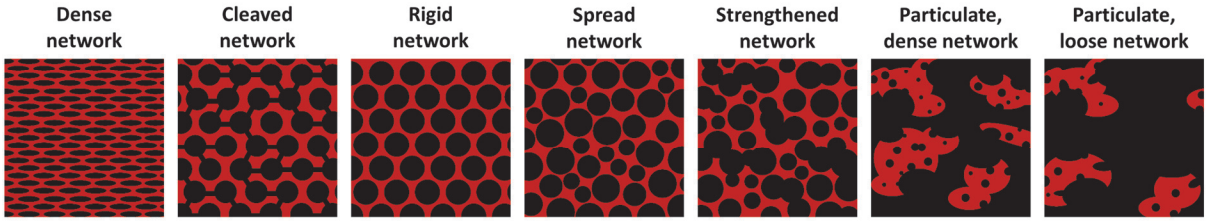


Figure 11: Schematic illustrations of network types. A seventh network type, called "dense network", was proposed in addition to the six network types of Lucas et al. (2019).

As demonstrated above, the classification of the literature examples to the network types extended the knowledge of dough mechanical behaviour related to the network types by including the resistance to extension and extensibility (c.f. Table 1). To summarize, the seven gluten network types cause characteristic **dough mechanical properties** as described in following bulleted list. In addition, further likely dough handling properties as well as the potential effect on specific volume and crumb texture of **baked products** were discussed based on literature results corresponding to the applied modifying agent on the network type:

- **Dense network:** Low viscous, but still elastic dough with poor elongation properties (very low extensibility and resistance to extension). It is more elastic than a dough of a cleaved network.

- **Cleaved network:** Low viscous dough with a very low firmness (complex shear modulus) and a very high deformation against strains (e.g., creep compliance) resulting in a very soft or even liquid (very high loss factor) dough system with almost no elastic properties (very low relative elastic recovery). If the microstructural properties reach extremes, the elongation properties could not be measured since this kind of dough system would be too liquid.

  Comparing literature results of rheology and texture studies for the used examples of this network type (increased glutathione concentrations, low concentrations of bromelain), it can be assumed that a dough of a cleaved network has a decreased elasticity resulting in lower specific bread volumes and an impaired crumb texture (Dong & Hoseney, 1995; Every, Morrison, Simmons, & Ross, 2006; Verheyen, Albrecht, Becker, & Jekle, 2016).
- **Rigid network:** Highly viscous dough with a very high firmness and relative elastic recovery as well as strong elongation properties (very high extensibility and resistance to extension), resulting in a stiff but still elastic dough.

  Comparing literature results of rheology and texture studies for the used examples of this network type (reduced hydration level/low concentrations of water addition), it can be assumed that a dough of a rigid network has a high resistance to deformation and shows good elastic properties, resulting in a compact crumb with small gas bubbles and higher crust firmness (Mastromatteo et al., 2013).
- **Spread network:** Viscoelastic dough with a high resistance to deformation. It has a higher firmness and viscosity than a dough of a strengthened network, both are very elastic (relative elastic recovery).

  Comparing literature results of rheology and texture studies for the used examples of this network type (low concentrations of transglutaminase, or glucose oxidase addition), it can be assumed that a dough of a spread network has an improved resistance to extension (Autio et al., 2005; Steffolani et al., 2010). Final products' properties were reported contrarily in literature, which is why no estimated assertions can be given and further studies are required (Altınel & Ünal, 2017; Basman et al., 2002; Rasiah et al., 2005; Steffolani et al., 2010; Vemulapalli, Miller, & Hoseney, 1998).

- **Strengthened network:** Viscoelastic dough with median elongation properties, a median resistance to deformation and a high relative elastic recovery. It is similar to a dough of a spread network, but softer and less viscous.

  Comparing literature results of rheology and texture studies for the used examples of this network type (ascorbic acid or potassium bromate addition, or low concentrations of shortening), it can be assumed that a dough of a strengthened network has an improved expansion during proofing, an increased resistance to extension and dough strength (Aamodt, Magnus, & Faergestad, 2003; Gandikota & MacRitchie, 2005; Joye, Lagrain, & Delcour, 2009). The specific volume and crumb texture of baked products might be enhanced compared to a standard bread (Goesaert et al., 2005; Vemulapalli et al., 1998; Yamada & Preston, 1994).
- **Particulate, dense network:** Highly viscous and very stiff dough with a high resistance to deformation. It is less elastic and even higher viscous than dough of a rigid network. Comparing literature results of rheology and texture studies for the used examples of this network type (high concentrations of transglutaminase or shortening), it can be assumed that a dough of a particulate, dense network has an increased resistance to extension, but a decreased extensibility resulting in a rigid and less extensible dough. Furthermore, the specific volume of final bread will likely be decreased compared to a standard bread (Autio et al., 2005; Steffolani et al., 2010).
- **Particulate, loose network:** Low viscous dough with a low resistance to deformation, a very low relative elastic recovery and poor elongation properties resulting in a very soft to even liquid dough. If the microstructural properties reach extremes, the elongation properties could not be measured since this kind of dough system would be too liquid. The deformation against strains is even higher than of a dough of a cleaved network, whereas the loss factor is lower.

  Comparing literature results of rheology and texture studies for the used examples of this network type (increased hydration level/very high concentrations of water addition), it can be assumed that a dough of a particulate, loose network shows poor elasticity and high softness, resulting in irregular, large gas bubbles in the crumb and a poor loaf volume of baked bread (Mastromatteo et al., 2013).

As an outlook, the investigation of relations between gluten microstructure and modifications on a molecular level as well as the relation to final product quality based on the findings of the present thesis and a further extension of the network types including product characteristics could be an interesting topic as prospective research. The potential effect of the various network types on baked products' properties were discussed above and give good impressions. However, these assumptions must be investigated and proven in future studies. Furthermore, the microstructure of baked products should be studied as well. First attempts to determine the microstructure of wheat dough during heating with PNA was performed by Verbauwhede et al. (2020), but without an analysis of texture properties. Further investigations of the microstructure of baked products analysed by PNA were already performed on pizza crust by Bernklau et al. (2017) and related to the product's texture. However, detailed research needs to be done for a classification of network types related to product quality. Then, this knowledge can be used to design targeted dough and product functionalities. An even more specific texture design could be achieved if the relations between molecular and microstructural level would be investigated in more detail (e.g., modifications in covalent/non-covalent gluten cross-links, environmental influences on gluten quality, and alterations in gliadin/glutenin quantity). A recent study published linear correlations between lacunarity and the protein content, the SDS-unextractable polymeric protein as well as with the glutenin/gliadin ratio (S. Li et al., 2019), presenting first trials of relations between molecular and microstructural level using PNA. In the present thesis, all wheat proteins were stained non-covalently with Rhodamine B to analyse on a microstructural level the overall effect on gluten caused by modifications on a molecular level. These and further modifications on a molecular level could be elucidated more precisely in the future by specific staining of protein subgroups by covalent linked fluorescent dyes. There are already possibilities to stain specifically gluten, gliadins or even HMW subunits (Ansari et al., 2015; Blomfeldt, Kuktaite, Johansson, & Hedenqvist, 2011; Bozkurt et al., 2014; Sozer & Kokini, 2014). However, the sample preparation requires mostly an invasive staining process. When optimizing these staining processes combined with the PNA analysis and using the prediction formulas developed in this thesis as well as further develop those formulas, a huge database of relations on all levels (molecular, microstructure and macrostructure) could be achieved in upcoming research studies to provide a distinct knowledge of structure-function relationships.

In conclusion, the present thesis provides the basis for upcoming studies of fundamental structure-function relationships. The main work and most important findings are summarised in the graphical abstract in Figure 1. The protein network analysis and the transfer of the defined network types on gluten microstructures provide new possibilities for elucidating and predicting wheat dough functionalities and prospectively, the product quality.

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6 Appendix

6.1 Reviewed paper

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6.2 Non-reviewed paper

Bernklau, I.: Interview – Liegt die Zukunft im 3D-Druck? Brot + Backwaren 6 (2015), 26.

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Bernklau, I., Jekle, M., Becker, T.: Die Zukunft druckt das Brot. Brot + Backwaren 6 (2014), 40-41.

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6.3 Oral presentations

Bernklau, I., Jekle, M., Becker, T.: Elucidation of wheat dough functionalities by network image analysis of protein microstructure. Innovations in Food Science & Technology, Erding, Germany, 2017-05-10

Bernklau, I., Jekle, M., Becker, T.: Proteinnetzwerkanalyse – Ein neuartiges Bildanalyseverfahren zur Quantifizierung von Mikrostrukturen im Weizenteig. Innovation Award am 5. DLG-Forum FoodTec, Frankfurt, Germany, 2016-11-23.

Bernklau, I., Jekle, M., Becker, T.: Vom Unsichtbaren zum Sichtbaren – Mikrostrukturen von Backwaren effizient und praxisorientiert interpretieren, 5. WIG Frühjahrstagung, Freising, Germany, 2016-04-06.

Bernklau, I., Jekle, M., Becker, T.: Enhanced protein network analysis as a new approach for quantifying dough microstructure by CLSM. 2nd Food Structure and Functionality Forum Symposium, Singapore, 2016-02-28.

Bernklau, I., Jekle, M., Becker, T.: Einfluss der Anfärbemethode zur Charakterisierung der Mikrostruktur mittels konfokaler Laser Scanning Mikroskopie auf die Funktionalität der Teige, 1. D-A-CH-Tagung für angewandte Getreidewissenschaften, Wien, Austria, 2015-10-02.

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Bernklau, I., Jekle, M., Becker, T.: Visualisierung der Mikrostruktur der Stärke-Protein-Matrix mittels CLSM: Vom Modell zum Teig, 3. WIG Frühjahrstagung, Freising, Germany, 2014-04-02.

6.4 Poster presentations

Lucas, I., Jekle, M., Becker, T.: Gluten microstructure – Definition of network types and the link to dough rheology, Cereals & Grains AACCi Annual Meeting, London, UK, 2018-10-22.

Bernklau, I., Jekle M., Becker, T.: A visualizing method for microstructural analysis of dough – The Confocal Laser Scanning Microscopy, 13th European Young Cereal Scientists and Technologists Workshop, Freising, Germany, 2014-05-14.