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Structure-function relationship in wheat dough –

the impact of protein microstructure

on rheology and processing performance

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The future is not what will happen, it is what we do.

PREFACE

Mario Tobias Jekle

Dipl.-LM-Ing.

The results and publications of this thesis were developed at the Technische Universität München, Institute of Brewing and Beverage Technology, Workgroup Cereal Process Engineering from July 2009 to June 2012 and are based on ideas from the work at the Universität Hohenheim, Department of Process Analysis and Cereal Technology from November 2007 to June 2009.

Peer reviewed publications

The following peer reviewed publications (shown in chronological order) were generated in the period of this work (publications which are part of the thesis are indicated in bold).

1. **Jekle, M., Becker, T.: Effects of acidification, sodium chloride, and moisture levels on wheat dough: II. Modeling of bread texture and staling kinetics. Food Biophysics 7 (2012), 200-208.**
2. **Jekle, M., Becker, T.: Effects of acidification, sodium chloride, and moisture levels on wheat dough: I. Modeling of rheological and microstructural properties. Food Biophysics 7 (2012), 190-199.**
3. Schirmer, M., Jekle, M., Arendt, E., Becker, T.: Physicochemical interactions of polydextrose for sucrose replacement in pound cake. Food Research International 48 (2012), 291-298.
4. Beck, M., Jekle, M., Becker, T.: Review: Sodium chloride – sensory, preserving and technological impact on yeast-leavened products. International Journal of Food Science and Technology 47 (2012), 1798-1807.
5. Frei, M., Kohno, Y., Tietze, S., Jekle, M., Hussein, M.A., Becker, T., Becker, K.: The response of rice grain quality to ozone exposure during growth depends on ozone level and genotype. Environmental Pollution 163 (2012), 199-206.
6. **Jekle, M., Becker, T.: Wheat dough microstructure: The relation between visual structure and mechanical behavior. Critical reviews in food science and nutrition, DOI: 10.1080/10408398.2012.656476.**

7. Beck, M., Jekle, M., Becker, T.: Impact of sodium chloride on wheat flour dough for yeast-leavened products. II. Baking quality parameter and their relationship. Journal of the Science of Food and Agriculture 92 (2012), 299-306.
8. Beck, M., Jekle, M., Becker, T.: Impact of sodium chloride on wheat flour dough for yeast-leavened products. I. Rheological parameters. Journal of the Science of Food and Agriculture 92 (2012), 585-592.
9. Jekle, M., Becker, T.: Implementation of a novel tool to quantify dough microstructure. Procedia Food Science 1 (2011), 1-6.
10. Schirmer, M., Jekle, M., Becker, T.: Quantification in starch microstructure as a function of baking time. Procedia Food Science 1 (2011), 154-156.
11. Beck, M., Jekle, M., Becker, T.: Starch re-crystallization kinetics as a function of various cations. Starch/Stärke 63 (2011), 792-800.
12. **Jekle, M., Becker, T.: Dough microstructure: Novel analysis by quantification using confocal laser scanning microscopy. Food Research International 44 (2011), 984-991.**
13. Schirmer, M., Hussein, W. B., Hussein, M. A., Jekle, M., Becker, T.: Impact of air humidity in industrial heating processes on selected quality attributes of bread rolls. Journal of Food Engineering 105 (2011), 647-655.
14. Beck, M., Jekle, M., Selmair, P., Koehler, P., Becker, T.: Rheological properties and baking performance of rye dough as affected by transglutaminase. Journal of Cereal Science 54 (2011) 1, 29-36.
15. Jekle, M., Houben, A., Mitzscherling, M., Becker, T.: Effects of selected lactic acid bacteria on the characteristics of amaranth sourdough. Journal of the Science of Food and Agriculture 90 (2010) 13, 2326-2332.
16. Beck, M., Hofmann, S., Jekle, M., Becker, T.: Novel materials and surface investigations for optimizing dough carrier interactions. European food research and technology 229 (2009), 183-189.

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Margit, your accompaniment gave me the energy for this path. Thank you!

CONTENTS

ABBREVIATIONS

A	area
A_F	area fraction of all objects
A_F^P	pore void fraction
A^K	area under the curve
AR	aspect ratio
A^S	work of adhesion
a_{th}	threshold value
ØA	average size of all objects
$ØA^P$	average pore size
ΣA	total size
BCM	box counting method
BL	baking loss
BI	branching index
BU	brabender units
C	circularity
C^P	pore circularity
CLSM	confocal laser scanning microscopy
C^S	dough cohesiveness
D	resolution
D_F	(Feret's) diameter
D_F^P	pore diameter
D^S	stickiness
E^K	dough extensibility
F	force
F_{96h}-F_{2h}	extent of firming

FD	fractal dimension
F_{lin}	firming rate
F_t	crumb firmness
G	shear modulus
G′	shear storage modulus
G′′	shear loss modulus
G*	complex shear modulus
h	height
H^P	crumb holes
J_{el}	relative elastic part of J_{max}
J_{max}	maximum creep compliance
J_r	recovery compliance
k	rate constant
$\underline{\underline{L}}$	shear rate tensor
LM	light microscopy
LVE	linear viscoelastic region
MAE	mean absolute error
M_r	relative molecular mass
n	Avrami exponent
NA	numerical aperture
NaCl	sodium chloride
ns	no significant effect
N_1	first normal stress difference
N_2	second normal stress difference
$P_h(t)$	hydrostatic pressure
$P_s(t)$	surface tension

$P_v(t)$	viscous forces
P	perimeter
ΣP	(particle) count
ΣP^P	pore count
r	side length of boxes
R	region
R^k_{max}	dough resistance
ROI	region of interest
RSM	response surface methodology
SAOS	small amplitude oscillatory shear measurement
s	distance
S	solidity
t	(storage) time
tan δ	loss factor
TPA	texture profile analysis
V	volume
v	velocity
γ	deformation
γ_e	elastic deformation
γ_v	viscous deformation
$\dot{\gamma}$	shear rate
δ	phase shift
ρ	density
λ	wavelength
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength
λ_{rel}	relaxation time

η	viscosity
τ	stress
$\underline{\underline{\tau}}$	stress tensor
ω	frequency
Θ	uncrystallized material at time t
Λ	retardation time
Ψ_1	first normal stress coefficient
Ψ_2	second normal stress coefficient

SUMMARY

The understanding of food structure enables the engineering of specific nutritional, physical, and sensorial properties. The key factor herein is the relationship between structure and function which is well known for some artificial materials; however, food, and wheat cereal products in particular, consists of complex biopolymer matrixes, complicating the prediction of its behavior. Nevertheless, several structure-function relationships based on the molecular level were established, mainly concerning gluten in wheat dough. Gluten proteins are of particular interest since their unique viscoelastic characteristics predominate the mechanical behavior of wheat dough. The microstructure is the connective link between the molecular level and the mechanical behavior of dough's macrostructure. Since the microstructure can be analyzed in a noninvasive matter, this could be a direct link to developing an enhanced structure-function relationship. So far, much work has been done in investigating dough and dough protein microstructure in particular; however, a quantitative characterization of wheat dough's structural features has yet to be achieved. Therefore, the current thesis presents a methodology developed specifically for dough microstructure quantification DoMiQ. This methodology enabled the extraction of quantitative structural features of the micrographs and thereby delivered the basis for a correlation analysis of the structure and functionality of wheat proteins in the dough matrix. The wheat dough protein microstructure was acquired using a non-destructive confocal laser scanning microscope and processed and analyzed with an image analyzing tool. It was possible to extract highly repeatable results for structural features. Furthermore, modifications due to ingredient variation could be clearly and highly significantly reconstructed. The relationship between the structure and the function was first proven in wheat dough on a micro- to macrostructural scale by the correlation analysis of the structural protein features and rheological properties. Morphologies determining the functionality could be revealed and were discussed. Of special note, the introduced branching index lead to a model which combines the microstructure and its function in a physical approach.

In summary, the DoMiQ method enabled the extraction of the structural features of wheat dough protein microstructure and delivered a novel perspective of the dough system, an extended understanding of the dough matrix, as well as the development of a substantial structure-function relationship in cereal science.

ZUSAMMENFASSUNG

Eine zielgerichtete Lebensmittelproduktion mit spezifischen ernährungsphysiologischen, physikalischen und sensorischen Eigenschaften erfordert ein tiefgreifendes Verständnis über den strukturellen Aufbau der zugrundeliegenden Matrix. Während in den Materialwissenschaften das Verhältnis zwischen Struktur und Funktionalität bereits vielfach als Schlüsselrolle identifiziert wurde, ist dies in biologischen Systemen deutlich erschwert. Struktur-Funktions-Beziehungen sind in Lebensmitteln, im Besonderen auch in weizenbasierten Produkten, aufgrund der Komplexität der Biopolymer-Matrix deutlich weniger entwickelt. Dennoch konnten besonders auf molekularer Ebene Struktur-Funktions-Beziehungen der Glutenproteine des Weizens etabliert werden. Durch seine einzigartigen viskoelastischen Eigenschaften und der daraus resultierenden starken Beeinflussung des mechanischen Verhaltens der Teige tritt Gluten in den Fokus von getreidewissenschaftlichen Arbeiten. Die Mikrostruktur der Weizenproteine wird dabei als Bindeglied zwischen der molekularen Ebene und dem mechanischen Verhalten der Makrostruktur von Teigen angesehen. Durch eine Weiterentwicklung von gängigen mikroskopischen Methoden ist es möglich, eine nicht-invasive Analyse der Teigmikrostruktur zu erhalten. Hierdurch ergibt sich eine neue Ausgangslage zur Erarbeitung von Struktur-Funktions-Beziehungen. In bisherigen Arbeiten konnten umfangreiche Kenntnisse über die Teigstruktur und im Besonderen deren Mikrostruktur erarbeitet werden, jedoch konnte bisher keine quantitative Charakterisierung der Teigmikrostruktur dargestellt werden. Aus dieser Motivation heraus wurde in der vorliegenden Arbeit eine Methodik zur Quantifizierung der Teigmikrostruktur (DoMiQ, dough microstructure quantification) erarbeitet, die eine numerische Strukturcharakterisierung ermöglicht und somit die Grundlage für eine Korrelationsanalyse von Mikrostruktur und Funktionalität von Weizenproteinen in der Teigmatrix darstellt. Die Mikrostruktur der Weizenteigproteine wurde mit einem nicht-destruktiven konfokalen Laser Scanning Mikroskop erfasst und mittels Bildverarbeitung bearbeitet sowie analysiert. Die Strukturmerkmale der Proteine konnten dabei reproduzierbar charakterisiert werden. Eine zielgerichtete Modifikation der Mikrostruktur durch Variation der Teigzusammensetzung konnte statistisch signifikant erfasst und interpretiert werden. Anhand einer Korrelationsanalyse zwischen der Proteinmikrostruktur und einer umfangreichen

rheologischen Charakterisierung konnte erstmalig eine Struktur-Funktions-Beziehung auf mikroskopischer Ebene entworfen und diskutiert werden. Besonders die Einführung der Messgröße „Branching Index" führte zu einem physikalischen Modell der Morphologie der Proteinmikrostruktur.

Zusammenfassend ermöglichte die DoMiQ Methode erstmalig eine quantitative Charakterisierung der mikrostrukturellen Merkmale von Weizenteigproteinen und ermöglichte somit ein erweitertes Verständnis der Struktur-Funktions-Beziehung der Teigmatrix.

1 INTRODUCTION

Wheat products are staple foods in most parts of the world. In particular, the unique viscoelastic properties of hydrated gluten proteins make wheat flour-water systems (dough) a highly interesting object of research. A common purpose of many investigations is the prediction of dough processability and end product quality (Dobraszczyk and Morgenstern, 2003; Miller and Hoseney, 1999; Wang and Sun, 2002). Therefore an in-depth understanding of both the characteristics as well as the chemical and physical changes during the formation (mixing) and processing (fermentation, molding, thermal end treatment) of wheat dough is necessary. Many of these changes, which take place on the molecular level, not only become visible in the resulting dough structure but also influence dough handling, processability, and end product quality. The gluten protein structure, which is alleged to determine the mechanical behavior of the dough, plays a primary role in all of these steps (Lindsay and Skerritt, 1999; Wieser, 2007).

Flour-water systems are a biological complex medium wherein the structure-function relationship represents a predominant position. For a target orientated product design it is essential to know the quantitative structure-function relationship of the matrix. This is valid for material as well as for food and cereal engineering. Further, knowledge of the structure-function relationship plays a particular role in the prediction and specific modification of processing capabilities. This knowledge of the dependency between the structural properties of a component and its derived functional characteristics enables to establish theories and models that move away from qualitative, descriptive science towards a fundamental proposition.

A wide range of flour and dough analyses are commonly used to quantify and forecast structural changes on different scales in wheat dough, with a special focus on the wheat proteins (Popineau et al., 1994; Tronsmo et al., 2003; Wieser and Kieffer, 2001) which are the basis for the development of relationships. For a comprehensive evaluation of the current knowledge and analysis methods, the next chapters review the scales of structure and possibilities of direct and indirect structure analysis methods in the field of cereal science.

1.1 Scales of structures and its relationship with functionality

The structure of a material defines in different scales the construction or the configuration of individually linked components. It describes the spatial arrangement of similar or dissimilar components as well as its interrelationship or morphology, mostly with many-to-many linkages or connections (Bourne, 2002; Heertje, 1993; Raeuber and Nikolaus, 1980). This interrelationship can be defined from a molecular up to a macroscopic level. Therefore the scale of the considered structure has to be specifically mentioned. In general, the characterization of the structure uses simplifications due to the complexity of the topic.

The term structure in combination with an order of magnitude (micro, macro) is often used to describe the structural properties of materials such as food. For instance, often used orders of magnitudes are the molecular level and the microscale. However, the term of the order of magnitudes is often inconsistently used in literature. Therefore, four scales are defined in the current thesis, which are based on the configuration dimensions (in increasing order): molecular, nanoscopic, microscopic, and macroscopic scale or structure (see Figure 1-1).

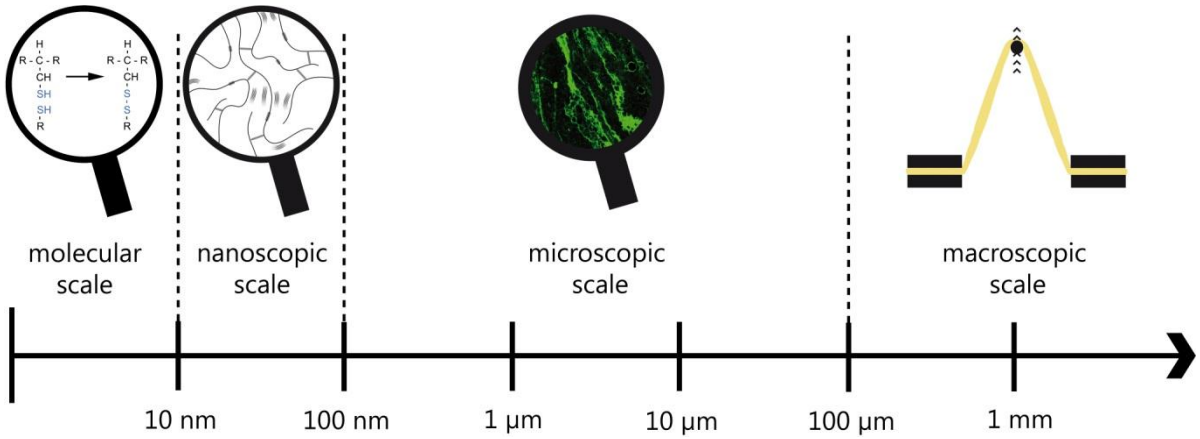

Figure 1-1: Scales of structure, exemplarily presented within a wheat flour-water system. From the molecular (such as disulfide cross-links) up to the nanoscopic (such as polymer entanglements), microscopic (such as the protein network), and macroscopic (such as dough extensibility) scale.

The molecular scale describes the molecules and the monomeric components (< 10 nm). Further, the types of bonds, such as covalent or noncovalent, are defined. The resulting orientation of the oligomers or polymers is characterized on the nanoscopic scale which

involves sizes up to 100 nm. This scale is especially influenced by the number and type of bond, and therefore their physical arrangement, as well as the molecular weight of the resulting molecules. The rather rare term nanoscopic structure is deliberately chosen to distinguish between binding configuration (molecular) and the structure observed through a microscope (microscopic). The scale limitation is based on the fact that strands in this range cannot be distinguished using common microscopy techniques (Pawley, 2006). The mentioned many-to-many connections result in the microstructure of a material, which is characterized in the long-range order such as particles or aggregates within a range of 0.1 up to 100 µm. At this scale microscopic techniques are utilized and the structure can be observed. The term macroscopic scale (>100 µm) defines already the transition to the textural or rheological properties of the material. At this scale, the properties of the molecular, nanoscopic, and microscopic scale are quantifiable in physical and dynamic measures. Hereby, the function of the structures becomes analyzable.

The **structure scales and their relationship with functionality** are exemplarily represented in the form of wheat gluten on the following pages (also see Figure 1-1), since wheat gluten offers unique viscoelastic properties determining most of the macroscopic properties of dough (Beck et al., 2012a; Lindsay and Skerritt, 1999; Thiele et al., 2004; Wrigley and Bietz, 1988). Wheat gluten is a part of wheat flour proteins which are traditionally classified due to their solubility (Osborne fractions): albumins are soluble in water, globulins in dilute sodium chloride solutions, prolamins (gliadins) in 70% ethanol, and glutelins (glutenins) in dilute acid or alkali solutions (Osborne, 1907). This traditional division, being based on technological characteristics, does not clearly separate wheat proteins according to either biochemical/genetical attributes or processing or breadmaking functionality. Therefore, another possibility is classification according to amino acid sequence (Shewry et al., 1986), as shown in Figure 1-2. However, the terms “gliadins” and “glutenins” are still in use. They primarily indicate the functional properties rather than just focusing on the Osborne fractionation (Goesaert et al., 2005), but also refer to their technological properties, which are discussed later on. Together, monomeric gliadins and the polymeric and aggregative glutenins form wheat gluten (see Figure 1-2). They represent around 85% of the total wheat proteins and plays the major role in processing and breadmaking performance (Domenek et al., 2004). The involvement of these and further

subgroups on the different scales of the structures and its functionality are represented and discussed in the following.

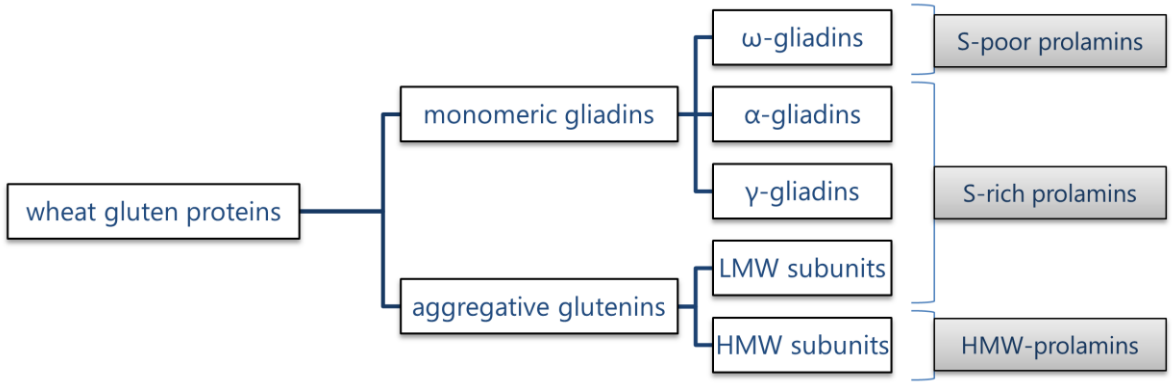

Figure 1-2: Traditional (Osborne, bright background, left) and extended molecular (Shewry et al., gray background, right) classification of gluten proteins (Osborne, 1907; Shewry et al., 1986).

On the **molecular level**, the gliadins can be distinguished in three groups due to their mobility in acidic-buffer polyacrylamide gel electrophoresis: ω-gliadins, α-gliadins and γ-gliadins. Recent methods can even discriminate between more than one hundred components. The ω-gliadins have the lowest mobility and the molecular structure shows a lack of cysteins whereby no disulfide cross-links can be formed (Wieser, 2007). Cysteins have a tremendous effect on the functional properties of proteins because they are the most chemically reactive group found in proteins (de Jongh, 2007). The α-gliadins show six cysteine residues from which three intramolecular disulfide bonds can be formed, while γ-gliadins show eight cysteine residues with four intramolecular disulfide bonds. Thus, no free and reactive sulfide bonds are available for intermolecular cross-links (Lindsay and Skerritt, 1999). The N-terminal domains of the α- and γ-gliadin show β-turn conformations in the secondary structure, which is comparable to the ω-gliadins (Tatham and Shewry, 1985). The relative molecular mass (M_r) of gliadins is within 30,000-75,000.

Glutenins consist of the predominant "low molecular weight (glutenin) subunits" (LMWGS, with the relative molecular mass M_r of 42,000-70,000), with around 20% according to the total gluten proteins amount, and "high molecular weight (glutenin) subunits" (HMWGS, M_r 60,000-90,000), with around 10% according to the total gluten proteins (Lindsay and Skerritt, 1999; Wieser and Kieffer, 2001). On the molecular level, most of the cysteine residues can be found in the terminal domains, but some subunits show also residues in the central domain. The three subgroups of LMWGS (B-, C-, and D-LMWGS, respectively, with

different M_r) revealed some similarities with HMWGS and even gliadins. The HMWGS consist of larger x-type and smaller y-type subunits, which have three and five cysteine residues, respectively. Therefore intermolecular disulfide cross-links can be formed, which is underlined by the fact that HMWGS do not appear in flour or dough as monomers. The secondary structure of the HMWGS seems to be β-reverse turns (Shewry et al., 1992).

In general, gliadins determine the viscous and extensional properties of wheat flour dough and have a low elasticity and cohesiveness (Shewry et al., 1986). Glutenins are responsible for the elastic and cohesive parts of the dough, expressed in the strengthening of dough (MacRitchie, 1980; Wieser, 2007). The elastic functionality of glutenins could be explained by the overlapping turns (secondary structure of the HMWGS) which form a loose spiral (Wieser, 2007) and determine the nanoscopic structure scale. Their technological properties are based on their amino acid sequences, solubility characteristics, and related conformation (Shewry et al., 1997).

Glutenins can achieve M_r from 500,000 up to 10 million (Wieser, 2007), with a hydrodynamic diameter of 5 to 45 nm (in contrast to gliadins with around 8 nm) (Wahlund et al., 1996). Hydrodynamic diameters describe an apparent radius of a hypothetical hard sphere which has the same diffusion properties as the molecule or particle under examination and can be detected by diffusion coefficients of retention times in a field-flow fractionation and a transformation by the Stokes-Einstein equation (Durchschlag and Zipper, 1996). The scale size of these diameters already describes the **nanoscopic scale**. Glutenins are considered the largest protein molecules in nature (Wrigley, 1996). The "real" nanostructure of gluten proteins exhibits due to the interaction of gliadins and glutenins, which determines the physical and mechanical properties of gluten. Next to the covalent bonds, non-covalent bonds based on van der Waals interactions, hydrogen, ionic, and hydrophobic effects play an important role in the level and status of the protein aggregation (Beck et al., 2012a; Belton, 1999). The covalent bonds are formed by cysteine-cysteine (disulfide) bonds which strongly influence the spatial arrangement of the protein structure (de Jongh, 2007) and less important tyrosine-tyrosine bonds (Tilley et al., 2001). A simplified nanostructure of glutenins is shown in Figure 1-3, containing non-covalent bonds, intramolecular covalent disulfide bonds, and intermolecular disulfide cross-links. The important structure determining covalent disulfide bonds are formed by cysteins (intra- or intermolecular),

despite its low concentration of around 2% in gluten proteins (Grosch and Wieser, 1999). Intramolecular disulfide bonds appear within gliadins and glutenin subunits and reveal a molecular stability in the protein conformation. Glutenins additionally form intermolecular bonds (due to free sulfide bonds) which determine gluten structure and behavior. In general, the protein's polymeric character, which is due to cross-linking strongly influences dough formation and processing and breadmaking quality (Chanvrier et al., 2007; Domenek et al., 2004; Weegels et al., 1996a).

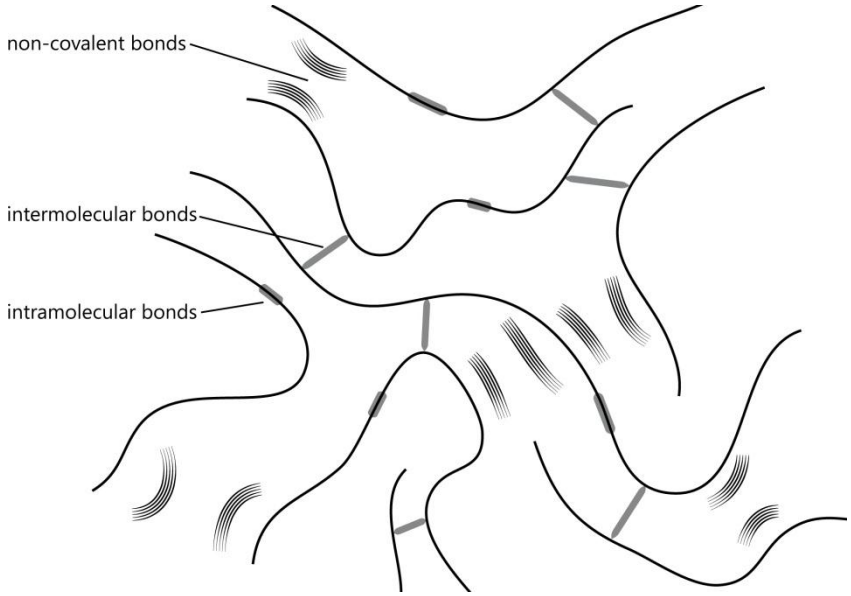

Figure 1-3: Simplified and schematic nanostructure of glutenin proteins in wheat dough. With non-covalent bonds, intramolecular covalent disulfide bonds, and intermolecular disulfide cross-links.

Studies of nanoscale of gluten proteins have provided some network models, which describe the functionality under stress (Belton, 2005; Bloksma, 1990a; Létang et al., 1999; Singh and MacRitchie, 2001). Figure 1-4 shows a combination of the models of the relaxed state and one after the impact of tensile or mixing force. Under normal (relaxed) conditions, protein chains form random coils, which have the highest possible entropy level. The elongation and reordering of the chains through tensile or mixing forces leads to a loss of entropy (Aguilera et al., 2001). On the one hand, this could enable the formation of new bonds within the aligned state, which could stabilize the system. On the other hand, the reformation of the random coils reveals the higher entropy condition. As such, the recovery

forces are dominant which becomes evident in the elastic behavior of wheat gluten (Aguilera and Stanley, 1999; Belton, 1999, 2005).

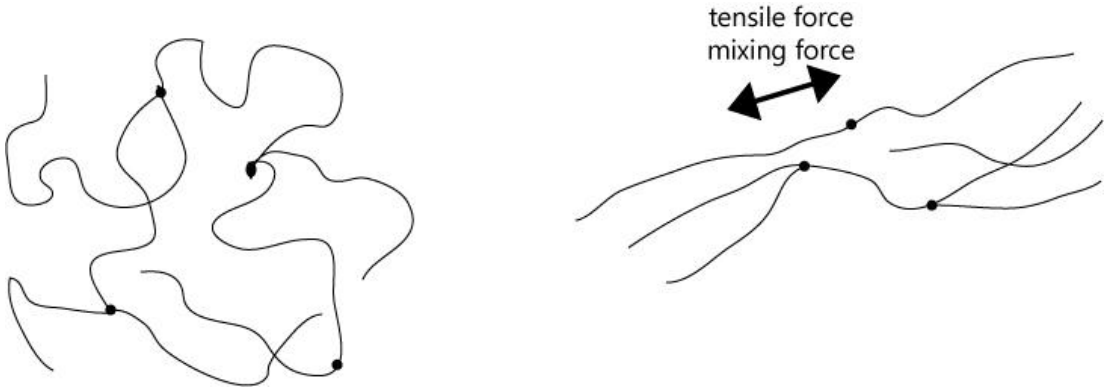

Figure 1-4: Schematic illustration of gluten strands at the nanoscale level (<50 nm) in relaxed and stressed conditions: Relaxed on the left and after elongation or mixing on the right.

The largest organized form of glutenins, the glutenin macro polymer (GMP), reveals an amount of 20 to 40 mg g^{-1} in wheat flour (meaning up to 30% of the proteins in wheat flour with 12% protein) and can be considered a model of a part of the **microstructure**. It has great functionalities on dough's rheological properties (positively correlated with the elastic behavior) as well as the end product quality such as the volume and is therefore regarded as an indicator for wheat flour quality (Don et al., 2005; Shewry et al., 1992; Weegels et al., 1995a; Weegels et al., 1996b). A first structure-function relationship of GMP could be shown by correlating the average particle size of GMP extracted from flour with its intrinsic viscosity (Don et al., 2003a). The use of confocal laser scanning microscopy (CLSM) revealed GMP particles from 5-30 µm with large sphere shapes. The microstructure of GMP is determined by its particle size and shape. Lindsay et al. (Lindsay and Skerritt, 1999) proposed a model of the GMP with three structural elements: 1) A backbone structure of glutenin subunits with HMWGS dimers as the main component (indicated by studies on mechanical behavior and treatment as well as stepwise reductions) (Skerritt, 1998; Werner et al., 1992). 2) Glutenin subunits (LMWGS and HMWGS). These subunits are bound to y-HMWGS, which are part of the HMWGS backbone. Therefore, branching points of gluten occur. 3) LMWGS to terminate the chains at the end of the branches. This structure leads to a model of the GMP wherein either HMWGS and LMWGS oligomers or HMWGS alone represent a backbone with branches. The short branches are supposed to be linear or branched and consist of either LMWGS, or a combination of HMWGS and LMWGS at the

terminal end (Lindsay and Skerritt, 1999). Some HMWGS subunits, such as the "5+10", seem to have a major, positive impact on the viscoelasticity of gluten, such as mixing tolerance, dough strength, and the shear storage modulus G' (as measure of dough elasticity) (Blechl et al., 2007; Don et al., 2006; Lafiandra et al., 1993; Payne et al., 1987). Subsequently, they also affect the bread volume (Popineau et al., 1994). During hydration, a continuous water phase between the gluten particles is formed. Furthermore, the secondary structure of gluten is developed as β-sheets conformation, which influences the connectivity of the network (Khatkar, 2005). During dough processing, the shape of GMP particles is lost and a continuous network of proteins exhibits which can be comprehensively determined by microscopic techniques. The spatial arrangement of the further proteins is mostly aggregated in a kinetic process. The proteins are thereby spontaneously clustered in functional complexes, forming a network driven by a decrease of its energy state (de Jongh, 2007).

The **macrostructure** of a material can be defined as its mechanical behavior and analyzed in a physical and dynamic approach as a measure of its texture and rheology. The gluten proteins are believed to play the dominant role in the macroscopic behavior of wheat dough (Goesaert et al., 2005; Lindsay and Skerritt, 1999; Salvador et al., 2006; Thiele et al., 2004; Wieser, 2007). The macrostructure of wheat gluten represents a viscoelastic behavior which is developed during hydration with solvents. It becomes especially visible during the production of yeast-leavened products such as wheat breads, which is briefly presented in the following:

A) during mixing: characteristic torque curve mostly with a local maximum (Uthayakumaran et al., 1999) due to a reorganization of cross-links (Masi et al., 1998) and the development of a gluten network (Singh and MacRitchie, 2001) accompanied with an drastic increase of gluten extractability (Veraverbeke and Delcour, 2002).

B) during fermentation (proofing): relaxing due to the elastic behavior (Belton, 1999) and gas holding properties that result from an appropriate balance between gliadin's viscosity and glutenin's elasticity (Gan et al., 1995; Goesaert et al., 2005).

C) fixation of crumb structure during the final heat treatment (baking) due to changes in hydrophobicity and a polymerization reaction due to the formation of new disulfide cross-links (Bajd and Serša, 2011; Lefebvre et al., 2000; Rombouts et al., 2011).

In total, the process of dough formation, processing behavior, and transition from a foam system dough to a semi-rigid system crumb is a complex phenomenon in which the macrostructure of the wheat dough undergoes huge structural changes on both the molecular and the microscopic level.

1.2 **Microstructure analysis - microscopy**

The direct analysis of a material's structure can be performed either on a molecular level or by a visual analysis of the microstructure via microscopy. As the structural analysis of flour-water systems on a molecular level has already been investigated in several studies (Koehler et al., 2010; Lindsay et al., 2000; Payne et al., 1987; Popineau et al., 1994; Wieser, 2007) and is mostly invasive, the focus of the current structural analysis is on the microstructure.

In biological and in food materials in particular, many key phenomena appear on the microscopic level. Most food components have sizes on the microscopic scale (such as GMP or starch granules in wheat dough), resulting in a crucial contribution to phenomena such as transport behavior and physical, textural, and rheological properties (José Miguel, 2005). The spatial arrangement of these components, and therefore their microstructure, determines the macroscopic behavior of any material. Also, in the field of cereal science, it is an accepted fact that the microscopic level of dough structure is related to the mechanical behavior of the pre-product dough and the product quality of baked goods (Bajd and Serša, 2011; Bloksma, 1990b; Lefebvre et al., 2000), even if the thermal heating occurs in between. However, the final quantitative proof of the theory is still pending. Therefore, for a comprehensive description of the relation between the structure and the resulting function of the structural properties' analysis is of fundamental importance (Fulcher et al., 1994).

Imaging techniques are helpful tools for examining dynamic changes in the morphology and composition of food's structure (Kaláb et al., 1995). An early microscopic technique was light microscopy (Burhans and Clapp, 1942), followed by (scanning) electron microscopy (SEM) with an enormously increased resolution (Pomeranz, 1976). However, sample preparation of SEM can lead to modifications of the target components (Chabot et al., 1979) and therefore an interpretation of the structure can be complicated or hindered. A further development of light microscopy combined its advantages, such as following dynamic

mechanisms by investigating samples under environmental conditions, with an improved resolution in lateral and axial direction. This was achieved in the technique known as confocal laser scanning microscopy (CLSM). CLSM requires very little sample preparation (Blonk and van Aalst, 1993) and overcomes the disadvantage of light microscopy, namely its destructive sectioning of samples and possible contaminations or damages. It uses optical instead of physical sectioning and a fine focus due to the usage of lasers in combination with fluorescent dyes. Due to these advantages CLSM is already commonly used in cereal science (Beck et al., 2012a; Beck et al., 2011b; Blonk and van Aalst, 1993; Espinosa-Dzib et al., 2012; Jekle and Becker, 2010a; Kenny et al., 2001; Schirmer et al., 2011; Srikaeo et al., 2006; Zarnkow et al., 2007). Figure 1-5 shows an example of the possibilities of the method with micrographs of wheat dough and wheat bread crumb.

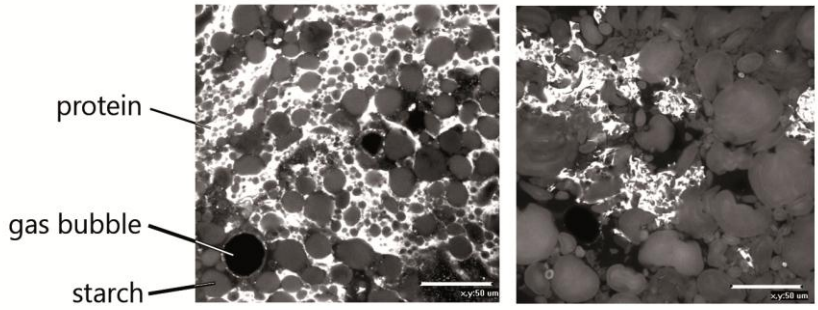

Figure 1-5: CLSM micrograph of a standard wheat dough (left) and a bread crumb (right). Starch granules appear gray, proteins white, and gas bubbles black (Jekle and Becker, 2010a).

As defined in chapter 1.1, the microstructure of dough is located on the scale of 0.1-100 µm. Therefore, the resolution limits of CLSM, which are determined by spatial resolution and noise, are of special interest.

The **spatial resolution** of light microscopes (LM) is determined by the magnification power and numerical aperture (NA) of the objective lens. It comprises the minimal reachable separation distance at which two point signals are imaged and distinguishable. The Rayleigh criterion defines that two points are still resolvable when the first minimum of one Airy disk falls together with the central maximum of the second Airy disk. Airy disks are intensity profiles of a best focused light spot of a perfect lens. Taking the light wavelength λ into account, the (lateral) resolution d is (Aguilera and Stanley, 1999; Corle and Kino, 1996)

$$d_{LM} = \frac{0.61\lambda}{NA}. \quad (1\text{-}1)$$

If air is used as the external medium, the NA is up to 0.95; with oil the NA is up to 1.5. With conventional lenses, the resolution of light microscopes can practically reach around 300 nm. Due to the spatially coherent point source illumination in confocal microscopes, the lateral single-point resolution can theoretically reach

$$d_{CLSM,lateral} = \frac{0.37\lambda}{NA}, \quad (1\text{-}2)$$

and in axial direction

$$d_{CLSM,axial} = \frac{1.4n\lambda}{(NA)^2}, \quad (1\text{-}3)$$

where n is the object refraction index (Corle and Kino, 1996; Földes-Papp et al., 2003).

The resolution is mainly determined by the numerical aperture (NA). The higher the NA, the brighter the image can get. However, further magnification is not able to enlarge the content of visual information. In praxis, the lateral resolution of a CLSM can reach 200 nm. The depth discrimination can be smaller than 1 µm (approximate practical minimum at around 500 nm (Menéndez et al., 2001)) and depends, next to objective lens's NA, on the pinhole: The larger the detector's pinhole, the smaller the optical sectioning becomes. At the extreme, the confocal microscope can be used as a conventional scanning system. Dürrenberg et al. (Dürrenberger et al., 2001) stated that the maximum sample penetration range is up to 150 µm in z-direction; however, this can be extended with great technical effort. In summary, CLSM has a sub-micrometer spatial resolution and is therefore very suitable for the analysis of wheat dough protein.

In food applications, CLSM is commonly used in the fluorescent light emission mode. Atoms of molecules absorb light from particular wavelengths and emit the light in a slightly longer wavelength. The whole process takes around 10^{-9} s. The reflective power of the structural elements is, with some exceptions, mostly comparable and thus well detectable. The target structures need to show fluorescent characteristics which can be either inherent fluorescence (auto-fluorescence) or obtained by **staining or labeling** with fluorescent dyes. The usage of dyes enables a better distinction and a multiphase detection of different constituents. Each fluorescent dye shows the characteristic absorption and emission spectra of photons and forms covalent and non-covalent bonds, respectively.

Non-covalent dyes (staining) are dissolved and added to the sample. The dye molecules spread in the sample and its accumulation is dependent on accessibility and affinity to other molecules. Hydrophobicity is a common main affinity effect for many dyes such as fluorescein isothiocyanate (FITC) and Rhodamine B. The morphology of the constituents, and therefore the hydrophobic/hydrophilic affinities between the considered phase and the enclosing matrix, considerably affects visualization and imaging. Rhodamine B is exemplarily chosen to explain the way of light in the microscope in the example of Figure 2 presented in chapter 2.2 "Wheat dough microstructure: The relation between visual structure and mechanical behavior": A green HeNe laser emits light at λ_{exc}=543 nm. The laser light transmits the dichromatic filter/mirror and excites the fluorescent dye (Rhodamine B). The emitted light is reflected by the dichromatic filter/mirror and can reach the detector (λ_{em}=590/50 nm).

During labeling a dye is covalently linked to a specific constituent or biopolymer. The technique is highly specific in comparison to the non-covalent staining. For carbohydrates (such as starch) in particular, it represents the best possibility for detection in food matrixes because carbohydrates show disturbing dye affinities (staining) in a certain concentration range (Kukrer and Akkaya, 1999; Linnane et al., 1995; van de Velde et al., 2002). However, carbohydrates are not as reactive as proteins, but some isothiocyanate derivatives for a water-free surrounding are available (Tromp et al., 2001). Nevertheless, the main target compounds of labeling are proteins as these molecules have superficially reactive amino groups (such as glutamine) (van de Velde et al., 2003). Furthermore, "antiwheat gliadin" rabbit polyclonal antibodies are available and used in studies (Urade et al., 2003). The targeted application of covalent labeling has some advantages over staining, especially in multi-phase systems. On the other hand, non-covalent staining offers an easier sample preparation and is favorable for single-phase systems or proteins in multiphase systems. Thus, the usage of Rhodamine B seems to be a beneficial method for staining wheat dough proteins, due to its simple, safe, fast, and appropriate method.

Confocal microscopy is already an approved tool for establishing relationships between microstructure and its derived functional properties. In particular, the method was used to investigate the structure and function of synapses in the fields of neuroscience (Rahamimoff and Melamed, 1993) or casein systems (Auty et al., 2005). The author's own work in the field

of cereal science enabled an explanation of the qualitative behavior of wheat dough as a function of ion addition with descriptive CLSM micrographs (Beck et al., 2012a). However, this descriptive approach reaches its limitations in:

- Comparison of a high number of samples.
- Objective characterization of the micrographs.
- Evaluation of small variations between samples.
- Defining structural features.

Therefore, the extraction of structural measures becomes a necessity in constructing a scientific evaluation of the microstructure that will expose a detailed and high density of information as well as data handling enabling statistical interpretations.

The application of microscopic methods is commonly used for material and engineering science. With the generated micrographs, a visual insight into the microstructural properties of the material is enabled. Although these micrographs consist of numerical data, it is nearly impossible to objectively compare its properties. Furthermore, without quantities, no experimental designs and statistics are usable. Therefore, **image processing and analysis** techniques, which extract morphological properties from the micrographs are required.

Image analysis in food systems regarding fundamental research as well as process monitoring and control has been described, performed, and discussed recently (Bull, 1993; Flook, 2003; Hussein et al., 2011; Quevedo et al., 2002; Schirmer et al., 2011; Zheng et al., 2006a, b). Before the analysis of the microstructural features, the images have to be processed. The micrographs consist of pixels with a defined position at the x- and y-coordinates and a value for the gray scale or color. Colored RGB images have three numeric values, one for each color band (red, green, blue) and are therefore structured as a matrix. Generally, for the extraction of structural features, the following steps have to be performed (also see Figure 1-6): After the acquisition of the micrograph (1) and a pre-processing of the image (2), a segmentation of the structurally relevant information (areas, particles) is necessary (3). Afterwards the analysis of structural features is performed (4), which enables the characterization of the structure.

An example of a possible method is shown in Figure 1-6 where an abstract arrow on a street is processed and analyzed. The colored image is changed to a gray scale image to reduce the level of information. An 8 bit gray level is used which consists of 256 intensity levels between black and white. Afterwards, the image is binarized to apply the segmentation of the arrow (region of interest) to street (background). This thresholding method is simple and the most commonly used segmentation for micrographs (Lorén et al., 2007). It divides the pixels in two classes depending on a threshold value a_{th}. Every pixel is referred to one of two fixed intensity values a_0 (black) and a_1 (white), respectively:

$$f_{th}(a) = \{^{a_0\ for\ a<a_{th}}_{a_1\ for\ a\geq a_{th}}\ . \tag{1-4}$$

This enables a distinction between an object and the background, and thereby the formalization of the characteristic relationship between a pixel and its related region. For instance, the fuzzy thresholding algorithm of Huang et al. (Huang and Wang, 1995) can be used. In Figure 1-6 the arrow is white in the gray level and black in the binarised image.

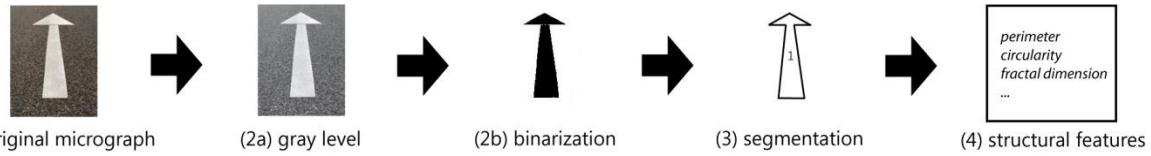

Figure 1-6: Schematic image processing and analysis sequence for the extraction of structural features (exemplarily of an image of an arrow on a street). Numbers in brackets are referred to the steps of the methodology (see text above).

Afterwards, the object is segmented. Subsequently the application of image analysis can be performed. For the description of the structure it is important to define the so called features of a region of interest (ROI). A region (R) of a binary image can be defined as a two dimensional distribution of pixels $x_i=(u_i, v_i)$:

$$R=\{x_1, x_2 \ldots x_N\}=\{(u_1, v_1), (u_2, v_2) \ldots (u_N, v_N)\}\ . \tag{1-5}$$

The features for a description of this region (R) in the micrograph, such as a polymer or particle, can be length [m], width [m], (Feret's) diameter D_F [m], perimeter P [m], area A [m^2], average size of all objects ØA [m^2], area fraction of all objects A_F [%], count ΣP [-], or angle (orientation to the x-, y- or z-axis), as well as shape descriptors such as the aspect ratio AR [-], circularity C [-], or solidity S [-] (see equations (1-6), (1-7), (1-8)) and Figure 1-7):

$$AR = \frac{D_{Fmin}}{D_{Fmax}}, \tag{1-6}$$

$$C = \frac{4\pi A_{ROI}}{P^2}, \tag{1-7}$$

where A_{ROI} is the area of the region of interest (ROI, such as a protein particle). The solidity can be calculated by

$$S = \frac{A_{ROI}}{A_c}, \tag{1-8}$$

where A_C is the convex area of A_{ROI}.

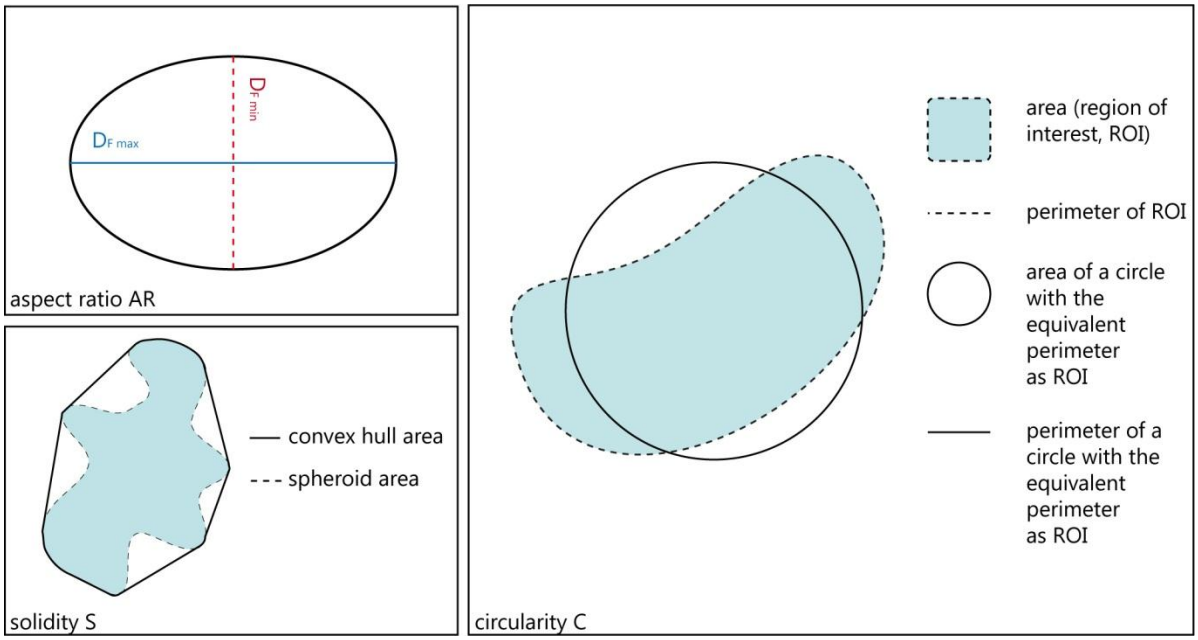

Figure 1-7: Description of the structural features aspect ratio AR, circularity C, and solidity S.

Another structure descriptor is the fractal dimension, which describes the textural features of a micrograph and thereby the arrangement of the basic constituents of the material such as the roughness of lignin polymers with a FD of 1.96 (Quevedo et al., 2002; Radotic et al., 1998). The FD can be calculated using the box counting method (BCM). The method uses cubic boxes with defined and different side lengths (*r*) to cover the border of the target object, such as a protein strand in a micrograph. The FD is calculated by the following equation:

$$FD = \lim_{r \to 0} \frac{\log N_r}{log\frac{1}{r}}, \tag{1-9}$$

where N is the number of boxes and r is the length of the side of the box (2 to 64 pixel size) (Sarkar and Chaudhuri, 1992).

In several biophysical studies, image analysis enabled relationships between the microstructure and functionalities to be established: The application of the quantification of the microstructure allowed, for instance, the comparison with functional properties of collagen (Yang and Kaufman, 2009). Auty et al. (Auty et al., 2005) in a CLSM and image analysis study on micellar casein particles and the functional features of rheology revealed similarities in microstructural and functional behavior. Image analysis of the cross-linking of rye proteins in dough in an author's own research supported the definition of the reasons for an occurring maximum of the corresponding rye bread volume (Beck et al., 2009).

However, a comprehensive acquisition of structural features is not available for dough systems in general. Thus, the described structural features in the current chapter could be applied to micrographs of wheat dough to evaluate and quantify its microstructure. Consequently, the qualitative information from the microscopic methodology could be used to provide quantitative dimensions and following correlations with other structural features and functionalities.

1.3 **Macrostructure analysis - rheometry**

During processing, dough stretches, shears, compresses, and flows as the microstructure's physical network emerges in the macrostructure. The unique properties of wheat dough, and gluten proteins in particular, were discussed in chapter 1.1. Gluten is the main reason for the ability of wheat dough to develop viscoelastic properties, due to its three-dimensional network. Its mechanical characterization can enable an effective prediction of the processing performance of dough (Song and Zheng, 2007). The monomeric gliadins thereby determine the viscous properties; whereas, the polymeric and aggregative glutenins determine the cohesive and elastic part of the dough (Southan and MacRitchie, 1999; Wieser, 2007).

The acquisition of the macrostructure (also termed the dynamic structure) can be performed by rheometric analyses. Rheometry is based on an indirect macroscopic structure characterization and considerably complements direct structure analysis by microscopy. It is a universal technique for explaining dough with its complex viscoelastic structure. With rheometry, a quantification of functional interactions between stresses, deformations, and

the rheological characterization of dough (viscosity, elasticity, viscoelasticity) is enabled (Fischer and Windhab, 2011).

Rheology is defined as the study of flow and deformation and of the response of a material to applied stress or strain (Steffe, 1992). Figure 1-8 shows a simple (laminar) shear flow of a material between two plates.

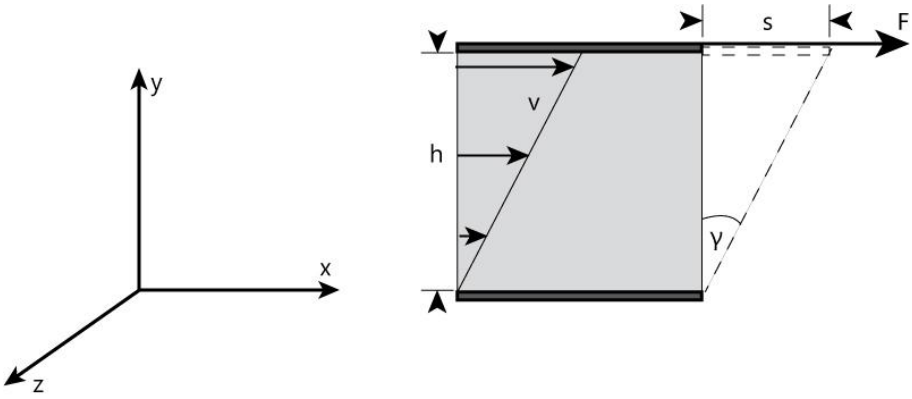

Figure 1-8: Shear flow between two parallel (one fixed and one moving) plates.

The upper plate is displaced with the velocity v in x-direction

$$v = \frac{s}{t}, \tag{1-10}$$

where s is the distance and t the time. The lower plate is fixed. In case of small values of γ, it can be assumed that $tan\gamma = \gamma$, and thus

$$\gamma = \frac{ds}{dh}, \tag{1-11}$$

where h is the distance between the two plates (see Figure 1-8). The deformation γ changes as a function of time, resulting in the shear rate $\dot{\gamma}$

$$\dot{\gamma} = \frac{d\gamma}{dt} = \frac{ds}{dh\,dt}. \tag{1-12}$$

In a representative infinitesimal small element, the resulting stress τ (force (F) per unit area (A)) can be completely described by nine separate quantities. This element might be reflected in terms of Cartesian coordinates (x, y, z) (see Figure 1-9) in which the subscripts of stress (τ_{ij}) symbolize the orientation of the face upon which the force acts (i) and the direction of the force (j) (Steffe, 1992).

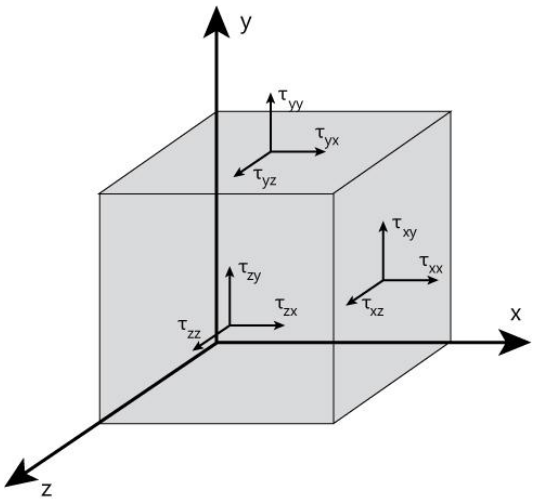

Figure 1-9: Stresses on a representative infinitesimal small material element.

The stress components are summarized in the stress tensor:

$$\underline{\underline{\tau}} = \tau_{ij} = \begin{bmatrix} \tau_{xx} & \tau_{xy} & \tau_{xz} \\ \tau_{yx} & \tau_{yy} & \tau_{yz} \\ \tau_{zx} & \tau_{zy} & \tau_{zz} \end{bmatrix}, \tag{1-13}$$

where the components τ_{xx}, τ_{yy}, and τ_{zz} are normal stresses acting in a perpendicular plane to x, y, and z, respectively, and the other components (τ_{ij} with i ≠ j) are shear stresses acting in the tangential plane. The stress tensor $\underline{\underline{\tau}}$ is symmetrical (considering equilibrium conditions), wherefrom $\tau_{ij} = \tau_{ji}$ ($\tau_{xy} = \tau_{yx}$; $\tau_{xz} = \tau_{zx}$; $\tau_{yz} = \tau_{zy}$). Concluding, only six independent components in the matrix have to be considered (Steffe, 1992).

The shear rate can be characterized analogue to the shear in a Cartesian system of coordinates. Therefore, the tensor of the velocity gradient can be generally presented by

$$\underline{\underline{L}} = \nabla\vec{v} = \begin{bmatrix} \frac{dv_x}{dx} & \frac{dv_x}{dy} & \frac{dv_x}{dz} \\ \frac{dv_y}{dx} & \frac{dv_y}{dy} & \frac{dv_y}{dz} \\ \frac{dv_z}{dx} & \frac{dv_z}{dy} & \frac{dv_z}{dz} \end{bmatrix}. \tag{1-14}$$

However, this tensor is not symmetrical (in case of a shear flow in x direction: $\frac{dv_x}{dy} \neq 0$ and $\frac{dv_y}{dx} = 0$), whereby no reduction of matrix components, as with the stress tensor, is possible. In order to describe and to define complex (process) shear, the matrix has to be simplified so as to reach a symmetrical shear rate tensor:

$$\underline{\underline{\dot{\gamma}}} = \frac{1}{2}\left(\underline{\underline{L}} + \underline{\underline{L}}^T\right) = \begin{bmatrix} \frac{dv_x}{dx} & \frac{1}{2}\left(\frac{dv_x}{dy} + \frac{dv_y}{dx}\right) & \frac{1}{2}\left(\frac{dv_x}{dz} + \frac{dv_z}{dx}\right) \\ \frac{1}{2}\left(\frac{dv_x}{dy} + \frac{dv_y}{dx}\right) & \frac{dv_y}{dy} & \frac{1}{2}\left(\frac{dv_y}{dz} + \frac{dv_z}{dy}\right) \\ \frac{1}{2}\left(\frac{dv_x}{dz} + \frac{dv_z}{dx}\right) & \frac{1}{2}\left(\frac{dv_y}{dz} + \frac{dv_z}{dy}\right) & \frac{dv_z}{dz} \end{bmatrix}, \quad (1\text{-}15)$$

where $\underline{\underline{L}}^T$ is the transposed velocity gradient tensor and $[L^T]_{ij} = [L]_{ji}$. Further, under experimental conditions, a simple shear flow is often applied with a velocity profile $\vec{v} = \begin{bmatrix} v_x(y) \\ 0 \\ 0 \end{bmatrix}$. Taking the generalized (tensorial) Newtonian law $\underline{\underline{\tau}} = 2\eta\, \underline{\underline{\dot{\gamma}}}$ (and $\frac{dv_x}{dy} = \dot{\gamma}$) into account, the stress tensor finally becomes

$$\underline{\underline{\tau}} = 2\eta \begin{bmatrix} 0 & \frac{1}{2}\dot{\gamma} & 0 \\ \frac{1}{2}\dot{\gamma} & 0 & 0 \\ 0 & 0 & 0 \end{bmatrix} = \begin{bmatrix} 0 & \eta\dot{\gamma} & 0 \\ \eta\dot{\gamma} & 0 & 0 \\ 0 & 0 & 0 \end{bmatrix} = \begin{bmatrix} 0 & \tau & 0 \\ \tau & 0 & 0 \\ 0 & 0 & 0 \end{bmatrix}. \quad (1\text{-}16)$$

Thus, the extra stress tensor is described by one element ≠ 0. Therefore, in rheological analysis with simple shear (as in a rheometer), scalar models are valid when describing the mechanical behavior of a material such as dough. The models are used to describe the results of rheological tests and to interpret viscous and elastic components of dough.

Assuming a Newtonian fluid, the laminar shear flow can be defined by the constitutive equation

$$\tau = \eta \cdot \dot{\gamma}\,, \quad (1\text{-}17)$$

where the viscosity η is a material constant.

The elasticity can be described by an ideal solid with linear elasticity and no flow (spring) and is defined by Hook's law

$$\tau = G \cdot \gamma\,, \quad (1\text{-}18)$$

where G is the shear modulus.

In viscoelastic materials, such as wheat gluten or dough, three shear-rate-dependent material functions (viscometric functions) are essential to complete the state of stress (Steffe, 1992). The scalar viscometric functions are: the viscosity function $\eta(\dot{\gamma})$ and the first and second normal stress coefficients $\Psi_1(\dot{\gamma})$ and $\Psi_2(\dot{\gamma})$ (proportionality factors), which are defined as

$$\eta = f(\dot{\gamma}) = \frac{\tau_{yx}}{\dot{\gamma}}\,, \tag{1-19}$$

$$\Psi_1 = f(\dot{\gamma}) = \frac{\tau_{xx}-\tau_{yy}}{\dot{\gamma}^2} = \frac{N_1}{\dot{\gamma}^2}\,, \tag{1-20}$$

$$\Psi_2 = f(\dot{\gamma}) = \frac{\tau_{yy}-\tau_{zz}}{\dot{\gamma}^2} = \frac{N_2}{\dot{\gamma}^2}\,, \tag{1-21}$$

where the first and second normal stress difference are N_1 and N_2 (measures of elasticity). In non-Newtonian materials it is assumed that $|N_2| << N_1$ (Steffe, 1992). In Newtonian fluids N_1 and N_2 are zero which is also valid for viscoelastic materials in the linear region (Kraft, 1996). Therefore, a one-dimensional consideration of the shear stress in viscoelastic materials is a valid approach for rheometric analysis.

Models for describing viscoelastic behavior are, among others, the two parametrical Maxwell, or Kelvin-Voigt model, and the four parametrical Burger model. As an example, the Maxwell model is described in the following. The Maxwell model contains a Newtonian damper in series with a Hookean spring (see left side of Figure 1-13). Thereby, the sum of the strain is

$$\gamma = \gamma_v + \gamma_e\,, \tag{1-22}$$

where γ_v indicates the strain of the damper and γ_e the strain of the spring. Including equations (1-17) and (1-18) and differentiating equation (1-22) with respect to time yields

$$\frac{d\gamma}{dt} = \dot{\gamma} = \dot{\gamma}_v + \dot{\gamma}_e = \frac{\tau}{\eta} + \frac{1}{G}\left(\frac{d\tau}{dt}\right)\,, \tag{1-23}$$

whereby the relaxation time (characteristic time of a Maxwell fluid) is

$$\lambda_{rel} = \frac{\eta}{G}. \tag{1-24}$$

The Maxwell model is then

$$\tau(t) + \lambda_{rel}\left(\frac{d\tau(t)}{dt}\right) = \eta\dot{\gamma}\,. \tag{1-25}$$

Solving the integral and assuming a steady flow with $t < 0: \dot{\gamma} = 0$ and $t \geq 0: \dot{\gamma} = \dot{\gamma}_0$ as well as $t < 0: \tau = 0$, at time $t \geq 0$ this is

$$\tau(t) = G_0 \int_0^t e^{\frac{-t\prime}{\lambda}} dt'\dot{\gamma}_0\,. \tag{1-26}$$

In rotational rheometry, **oscillatory techniques** are especially interesting as they allow (if realized in the linear viscoelastic region (LVE)) a non-destructive, linear, and dynamic characterization of viscous and elastic measures. In oscillatory tests, the material is subjected to an oscillating stress or strain with the frequency ω. Following, an approach based on small amplitude oscillatory shear (SAOS) with applied strain is reviewed. Typically, the sample is placed between two parallel plates. The lower plate is fixed and the upper plate harmonically (sinusoidal) oscillates (Figure 1-10).

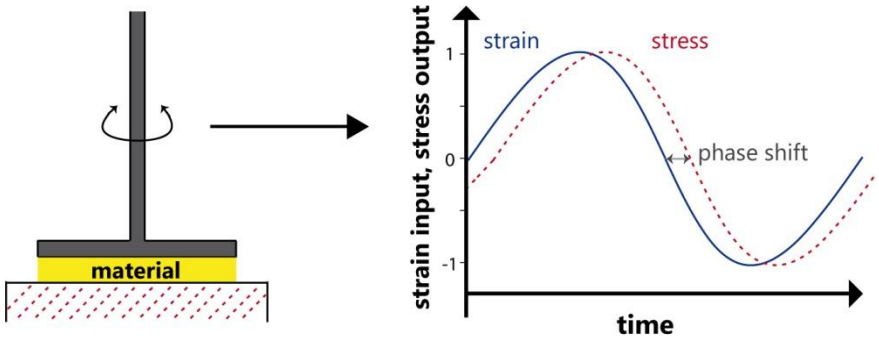

Figure 1-10: Left: System of an oscillatory test with shear in one direction. Right: A normalized strain input with a normalized shear stress output as a function of time.

The function of the applied strain is

$$\gamma(t) = f(t, \omega, \gamma_0) = \gamma_0 \cdot \sin(\omega t)\,, \tag{1-27}$$

where γ_0 is the amplitude of strain ($\gamma_0 = \frac{s}{h}$, see Figure 1-8), the motion of the upper plate is $s\,sin(\omega t)$, and ω is the frequency (kept constant in the following). The sinusoidal strain input results in a periodical shear rate:

$$\frac{d\gamma}{dt} = \dot{\gamma} = \frac{d(\gamma_0 \sin(\omega t))}{dt} = \gamma_0\, \omega \cos(\omega t)\,. \tag{1-28}$$

If the sample behaves in a linear viscoelastic manner, the subsequent shear stress is defined by the strain input:

$$\tau(t) = f(t, \omega, \delta, \tau_0) = \tau_0 \cdot \sin(\omega\, t + \delta)\,, \tag{1-29}$$

where τ_0 is the amplitude of the shear stress and δ is the phase shift of the answer of the material. Using trigonometrical transformation this is

$$\tau(t) = \tau_0 \cos\delta\, \sin\omega t + \tau_0\, \sin\delta\, \cos\omega t\,, \tag{1-30}$$

or can be written as

$$\tau(t) = G'\gamma_0 \sin\omega t + G''\gamma_0 \cos\omega t \,. \quad (1\text{-}31)$$

The material functions G′ and G′′ are

$$G' = \left(\frac{\tau_0}{\gamma_0}\right)\cos(\delta)\,, \quad (1\text{-}32)$$

$$G'' = \left(\frac{\tau_0}{\gamma_0}\right)\sin(\delta)\,, \quad (1\text{-}33)$$

where G′ is called the shear storage modulus (or elastic modulus) and describes the recoverably stored mechanical energy in the material. Direct deformation occurs if a shear stress is applied, but the original dimensions are recovered after removal of the stress (ideal). In wheat gluten, elasticity is often referred to covalent cross-links; however, it seems that non-covalent bonds of spirals and loops on the nanoscopic scale of HMWGS play a more important role (Belton, 1999; Wieser, 2007). The second material function G′′ is the shear loss modulus (or viscous modulus): a measure for energy loss due to viscous dissipation and permanent displacement of the molecules, mainly globular gliadins (Belton, 2005). This viscous dissipation and elastic recovery determines the viscoelasticity of gluten and wheat dough. The viscoelasticity can be considered by the application of complex numbers, and is visualized by a vector diagram with G′ on the abscissa (real part) and G′′ on the ordinate (imaginary part) in Figure 1-11.

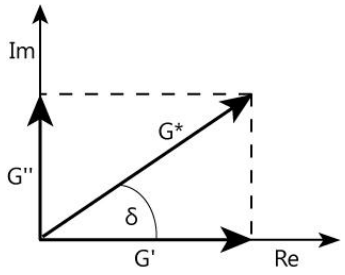

Figure 1-11: Vector diagram with G′ (real part), G′′ (imaginary part), and its resulting vector G*.

The vectorial sum of G′ and G′′ is the complex number G*

$$G^* = G' + iG'' \,, \quad (1\text{-}34)$$

which is also often reported as the complex shear modulus |G*|

$$|G^*| = \sqrt{(G')^2 + (G'')^2} = \frac{\tau_0}{\gamma_0}\,, \quad (1\text{-}35)$$

characterizing the viscoelasticity of a sample (Macosko, 1994). The tangent of the phase shift δ (see Figure 1-11) is called loss factor and is defined as

$$\tan \delta = \frac{G''}{G'} \ . \qquad (1\text{-}36)$$

This is the ratio of the lost energy and stored energy in materials, and is a measure of viscoelasticity. As wheat dough shows a loss factor <0.5, it can be classified as a viscoelastic solid (Beck et al., 2012a; Mezger, 2010). In the literature, the loss factor tan δ is referred to as a proper measure of dough "structure" (Berland and Launay, 1995).

Additional information about the structural behavior of dough can be obtained from non-oscillating large deformation tests such as **creep and recovery tests**, which is a further common rheological analysis. A constant shear stress τ_0 is applied to the sample, and the deformation behavior of the sample is continuously recorded during the shear creep phase as well as after the constant shear stress is removed (see Figure 1-12). The deformation is a measure characterizing the material. For ideal elastic materials (with the absence of viscous properties) the material will return to its original form after the applied shear stress is removed. An ideal viscous material, however, would illustrate a constant flow where a linear response to the shear stress is determined by the inability of the material to recover from the instantaneous stress (also see equations (1-40) and (1-41)).

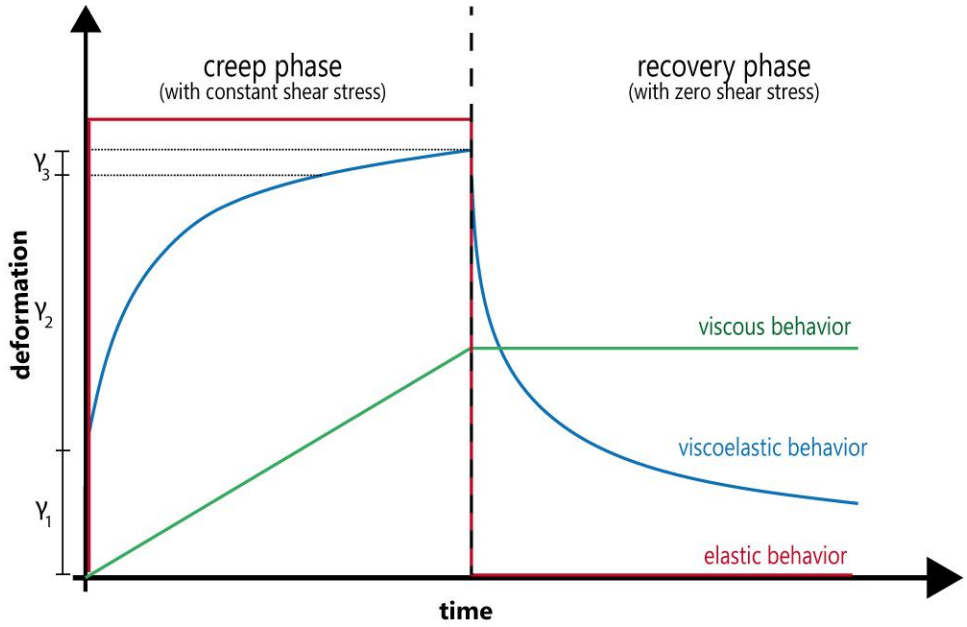

Figure 1-12: Idealized creep and recovery curves of elastic, viscoelastic (such as wheat gluten or dough), and viscous materials.

The deformation occurring during applied shear stress in wheat gluten and dough illustrates a combination of both idealized material characteristics and can be divided into

three effects: After an initial deformation (elasticity), an inconstant shear velocity (shear rate) $\dot{\gamma}$ with $\gamma = f(\tau_0, t)$ takes place (viscoelasticity). Afterwards, a steady state is reached with $\dot{\gamma} = const.$ and $\gamma = f(\tau_0)$ (viscosity). This behavior may be described with the Burger model. The Burger model combines a Maxwell model (see equation (1-25)) and a Kelvin/Voigt model placed in series as shown in the following Figure 1-13.

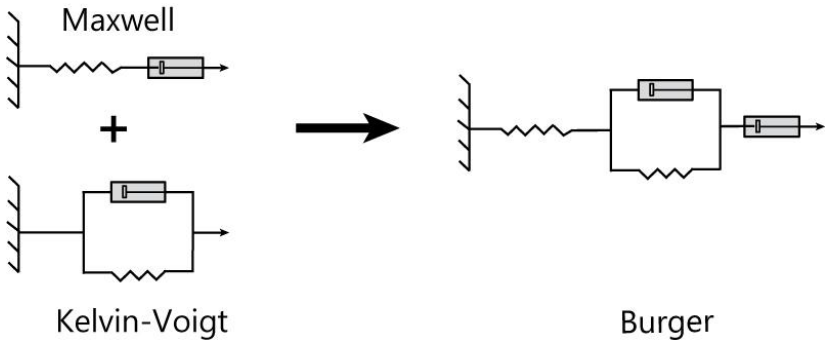

Figure 1-13: Burger model (right) as the sum of a Maxwell and a Kelvin-Voigt model (left) placed in series.

The Kelvin/Voigt model can be described by a spring and a damper in parallel, which can be defined by $\gamma = \gamma_v = \gamma_e$ with

$$\gamma(t) + \Lambda\dot{\gamma}(t) = \frac{\tau}{G} \,, \tag{1-37}$$

where $\Lambda = \frac{\eta}{G}$ is the retardation time, which is a material constant. The differential equation is

$$\tau = G\gamma + \eta\dot{\gamma} \,. \tag{1-38}$$

Analogous to the Maxwell model, the time-dependent deformation of the creep phase (see Figure 1-12) can be calculated from the differential equation (1-37) with the Burger model:

$$\gamma(t) = \gamma_1 + \gamma_2(t) + \gamma_3(t) = \frac{\tau_0}{G_1} + \frac{\tau_0}{G_2}\left(1 - \exp\left(\frac{-t}{\Lambda}\right)\right) + \frac{\tau_0 t}{\eta_0} \,, \tag{1-39}$$

with the shear modulus $G_1 = \tau_0 \gamma_1$ (pure elastic, see equation (1-18)), the shear modulus G_2 (G of Kelvin/Voigt model, see equation (1-37)), and the zero-viscosity η_0.

Practical and easy attributes of the creep recovery measurements can be described in terms of the compliance function

$$J = f(t) = \frac{\gamma(t)}{\tau_0} \,, \tag{1-40}$$

where τ_0 is the applied constant shear stress. In the linear viscoelastic regime of a material J is constant. The compliance at t_{max} is J_{max}, and J_r is the creep recovery of the material or dough after removing τ_0. The ratio of these two values is defined as J_{el}, or the relative elastic part, and describes the elasticity of a material or dough:

$$J_{el} = \frac{J_r}{J_{max}} . \quad (1\text{-}41)$$

However, more **empirical rheological methods** were used to evaluate dough rheology in the first decades of cereal science. Empirical rheological methods such as z-kneaders are well established and commonly used to assure quality in mills and production facilities of baked goods. They are practical, easy to use, and standardized, however, their measure values are company dependent and hardly based on SI units. Furthermore, they are not sufficient to interpret the mechanical behavior of dough independent of the measurement device and its geometries. Some empirical methods are Farinograph, DoughLab, Mixograph, Extensograph, and Kieffer rig devices. The Extensograph method performs uniaxial tension tests, resulting in data about dough extensibility and resistance. However, the data cannot be reliably transferred into material constants (Charalambides et al., 2006) nor scalar viscometric functions in a linear viscoelastic regime since the deformations and stresses have to be described as complex tensors. A smaller sized Kieffer dough and extensibility rig (Kieffer rig) in combination with a texture analyzer uses a similar principle, however, the deformation is not purely uniaxial extension as it also has a shear component (Dunnewind et al., 2003).

A comparison of empirical with SAOS test reveals that in oscillating tests material's polymers retain coiled chains (Dobraszczyk et al., 2003). The interactions between polymer chains remain low. In contrast, in extensional tests (Kieffer rig) the polymers are extended and orientated (aligned) by the deformation. The occurring physical entanglements and interactions between the chains leads to an increase in stiffness – the so called strain hardening, which is associated in cereal science with gluten polymers. Therefore, the SAOS and the extension tests can be used to describe different effects: SAOS for molecular properties of single polymers and changes and large deformation extension tests for the interaction of large polymer aggregates (Dobraszczyk et al., 2003; Padmanabhan, 1995). In summary, it can be assumed, that SAOS are more suitable for describing the current status

of the network without (or with low) deformations, and large deformation tests for analyzing mechanical behavior during processing.

In general, a lot of studies on dough rheology have revealed controversial results about the suitability of fundamental and empirical rheology. Further, some authors stated no or low correlations between oscillatory tests and end product qualities (Autio et al., 2001; Hayman et al., 1998). This could be explained by a calculation of the rate of expansion during proofing and oven rise with 10^{-2} and 10^{-4} sec^{-1}, whereas the magnitude in rheological tests is several times higher (Dobraszczyk et al., 2003). They state, that the strain rates usually used in the SAOS are not relevant to the strain rates which occurs during proofing and baking. Further, the entanglement of the HMWGS is not reflected within these measurements and therefore baking performance cannot be predicted. However, others recommend the use of oscillatory tests to investigate changes in dough characteristics (Khatkar et al., 1995; Miller and Hoseney, 1999) or correlate the measures with end product quality (Khatkar et al., 2002; Van Bockstaele et al., 2008b). Some studies describe large-deformation tests as suitable for the prediction of end product quality (Dobraszczyk and Morgenstern, 2003; Tronsmo et al., 2003; Van Bockstaele et al., 2008a; Wang and Sun, 2002); while, a number of studies found high correlations between empirical tests and end product quality. Due to the controversial literature, and to provide a comprehensive characterization of new methods or approaches, it is necessary to consider the whole range of rheological methodologies.

1.4 **Motivation**

Designing processed food to meet consumers' requirements and expectations is a great challenge. Because the structure-function relationship of the food matrix greatly influences a product's nutritional, physical, and sensorial property, it is essential to understand this relation in order to control both the processing and quality of the product (Kaufmann and Palzer, 2011). Thereby, a target-orientated production process following structural properties is enabled. This requires knowledge of structural features from a molecular up to a microscopic scale as well as macroscopic behavior and its influence on processing conditions. For some materials, such as metals and artificial polymers, these relationships are well-known due to the homogenous and simple construction of the materials. However, food comprises complex biopolymer matrixes; the establishment of these dependencies is complicated and thus knowledge about structure-function relationships is still minimal (José Miguel, 2005).

Nevertheless, some structure-function relationships have been successfully established in cereal science. Most of the studies considered relations between the molecular or nanoscopic scale and the macrostructure of wheat proteins, such as rheological properties (Belton, 1999; Gupta et al., 1992; Hamer and van Vliet, 2000; MacRitchie, 1999; Marchetti et al., 2012; Shewry et al., 1992; Southan and MacRitchie, 1999). Herein, the key role of wheat proteins in dough functionality seems to be an accepted fact (Beck et al., 2012a; Goesaert et al., 2005; Lindsay and Skerritt, 1999; Wieser, 2007). Since the end product quality of baked goods is closely related to structural dough properties, studies have also attempted to define relations between the molecular level and consumer quality attributes (Khatkar, 2005; Létang et al., 1999; Li et al., 2003; Weegels et al., 1995b; Wieser and Kieffer, 2001; Zörb et al., 2009). Although the molecular analysis of gluten fractions strongly contributed to the clarification of the structural development of dough, these studies mostly employed an invasive treatment of the system which partly destroyed the matrix. In contrast, new methodologies for the acquisition of the microstructure, such as confocal laser scanning microscopy (CLSM), enable a noninvasive way to obtain structural properties.

Although some basic relations between the microstructural scale of wheat proteins and their functionality in dough are described, they are still rare and of a descriptive nature. For

instance, micrographs of wheat proteins were used to explain rheological properties (Domenek et al., 2004; Li et al., 2010; Peighambardoust et al., 2006; Peressini et al., 2008). However, a quantitative characterization of the structural features of wheat dough has not yet to be achieved. It is evident that the numerical classification of the microstructure would enable the establishment of a fundamental and significant structure-function relationship. The development of an appropriate methodology using CLSM in combination with image processing and analysis would enable the extraction of the quantitative structural features of the micrographs and therefore deliver the basis for a correlation analysis of the structure and functionality of wheat proteins in the dough matrix. Furthermore, the real impact and the significance of the protein network in wheat dough on processing and breadmaking performance could be investigated and re-evaluated on this novel basis.

The thesis is therefore organized in four parts:

1. Critical review of the fundamentals of wheat dough microstructure development and currently known relations with functionalities
2. New approach for wheat dough protein quantification, with
    a. Establishment of a methodology to quantify wheat dough protein microstructure by structural features
    b. Possibility and traceability of modifications of structural features
    c. First correlation with dough macrostructure and processing performance
3. Evaluation of a global description of dough macrostructure (rheology) with structural protein features by variation of several matrix components
4. Evaluation of the prediction of breadmaking performance by protein microstructure

A novel methodology to extract the structural features of wheat dough protein microstructure would enable innovative perspectives on the dough system, and a renewed understanding of the dough matrix as well as the development of a substantial structure-function relationship in cereal science.

2 RESULTS (THESIS PUBLICATIONS)

2.1 Summary of results

The thesis publications are summarized in this chapter, followed by full copies of the papers.

PART 1 Wheat dough microstructure: The relation between visual structure and mechanical behavior.

PAGE 45

The microstructure of food matrixes determines its mechanical behavior. Consequently, the analysis of the microstructure is both necessary and useful for understanding physico-chemical and mechanical alterations during the production of cereal-based products such as bread. Confocal laser scanning microscopy (CLSM) is an established tool for the investigation of these matrix properties due to its methodical advantages such as easy preparation and handling, and high depth resolution due to the optical sectioning of probes. Therefore, the author provides, in the first thesis publication, an overview of current knowledge on dependencies between the visibly detectable microstructural elements achieved by CLSM and physically determined rheological properties. Current findings in this field, especially on possible numerical microstructural features are described and discussed and possibilities for enhancing the analytical methodology are presented. The necessity of understanding the formation of wheat dough from the molecular to the microstructural scale in order to establish a structure-function relationship is discussed. It is concluded that gluten is less a continuous film throughout the dough, but rather consists of a high number of strands, in which other particles (such as starch granules) are embedded, which leads to an appearance of a film. If these attributes were formulated in quantitative values, a more fundamental structure-function relationship could be established. In combination with the application of the analyzing measures, established tools such as threshold algorithms could serve as a tool to prove a quantitative structure-function relationship and emphasize the relevance of the protein network for both the dough and the end product characteristics.

PART 2 Dough microstructure: Novel analysis by quantification using confocal laser scanning microscopy.

PAGE 55

The aim of this study was to establish a new methodology for quantifying wheat dough protein microstructure. To evaluate the method the effect varied water addition has on the microstructural and viscoelastic properties of wheat flour dough was investigated using CLSM and a spectrum of rheological methods. Dough with water addition in the range of 52.5-70.0 g water 100 g^{-1} flour was investigated using the stickiness test, the uniaxial elongation test, and fundamental rheology like small amplitude oscillatory shear measurement and the creep recovery test. Micrographs of dough protein gained by CLSM were processed and analyzed. The method developed to quantify dough microstructure using CLSM in combination with image processing and analysis is described in this study. The methodology was highly repeatable and dependencies between microstructural features and macroscopic properties were found. The usability of protein microstructure quantification was proven. In summary, the author showed that a) most of the rheological methods had high significant correlations among the methods, at least due to the variation of water addition to wheat flour, and b) that there were high linear correlations between the protein microstructure of dough and its rheological properties. Therefore, the dependency between protein microstructure and its viscoelastic behavior that has been assumed for decades was validated. The DoMiQ (dough microstructure quantification) method has proven to be a powerful tool for examining dough protein microstructure.

PART 3 Effects of acidification, sodium chloride, and moisture levels on wheat dough: I. Modeling of rheological and microstructural properties.

PAGE 63

To validate the method to quantify dough microstructure, the effect of three experimental factors pH (addition of lactic acid and sodium hydroxide), water addition, and sodium chloride (NaCl) addition, on the microstructural properties of wheat dough protein were investigated. Further, the processing performance of the dough was studied by empirical and fundamental rheological properties. The results were modeled through the application of a response surface methodology. It was possible to determine microstructural attributes such as the area fraction (R^2=0.88) and Feret's diameter (R^2=0.86) as a function of pH, water addition, and NaCl addition. This provided further evidence of the significance and usability of the DoMiQ method. Rheological measures of the dough, especially the dough resistance R^k_{max}, revealed highly significant correlations with the protein microstructure such as the branching index (r=0.79). The experimental design and response surface methodology enabled the determination of significant effects on empirical and fundamental as well as structural attributes. Almost all experimental factors (pH, water addition, and NaCl addition) significantly affected the properties of dough. In summary, the study confirmed the influence of the protein microstructure on the rheological attributes of wheat dough and the dependency of the pH, sodium chloride, and water addition on this microstructure. Additionally, influences of the experimental factors on the starch behavior are assumed to play another important role.

PART 4 Effects of acidification, sodium chloride, and moisture levels on wheat dough: II. Modeling of bread texture and staling kinetics.

PAGE 73

In addition to the prediction that wheat dough macrostructural behavior is a function of the protein microstructure investigated in the previous study, the prediction that baking performance and end product quality are related to dough micro- and macrostructure was investigated. The effect of three experimental factors pH (addition of lactic acid and sodium hydroxide), water addition, and sodium chloride (NaCl) addition, on wheat bread baking performance (volume, baking loss, crumb firmness, crumb grain features) and crumb staling during storage was studied. Staling behavior was modeled using the Avrami equation and linear regressions. All experimental values showed significant effects on end product characteristics. A correlation analysis revealed significant linear dependencies of the dough macrostructure on the firmness of the crumb and can thus be used to study bread making potential. In particular, small amplitude oscillatory shear measurements (complex shear modulus) showed significant correlations with bread crumb firmness after 24 h of storage ($r=0.73$). Due to a dependency on the initial crumb firmness values, the complex shear modulus also correlated with the linear firming behavior of bread crumbs. The microstructural dough attributes acquired in the previous study, showed significant but low correlation with the bread making performance attributes. To predict these attributes, a microstructural investigation of the protein microstructure, the starch granules, and their morphological alteration due to the heating process, from the foam system dough to the sponge system of the bread crumb has to be proposed.

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

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

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Abstract

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

1. Introduction

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

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

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

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

2. Wheat dough microstructure

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

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

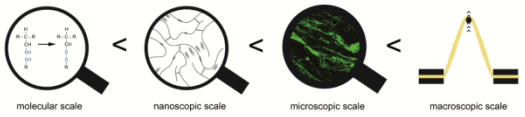

Figure 1: Scales of material observations. The molecular scale with disulfide bonds and non-covalent bonds between proteins, the nanoscopic scale with the orientation of the protein strands, the microscopic scale with the protein network, and the macroscopic scale exemplarily represented with an extensional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

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

2.1 Protein

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

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

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

The **cohesiveness** of gluten is based on non-covalent and, in particular, covalent bonds. The influence of non-covalent bonds can be indirectly described by the variation of ions (Beck et al., 2012). The covalent bonds are formed by intramolecular and intermolecular disulfide bonds. The intramolecular bonds can be found in gliadins and glutenin subunits and reveal a molecular stability in protein conformation. Glutenins additionally form intermolecular bonds which determine gluten structure and behavior.

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

2.2 Starch

Although wheat starch is regarded just as a filler in wheat dough, at least from a rheological perspective (Bloksma, 1990a), it also has a great impact on dough behavior and, in particular, the end product quality of yeast-leavened bread products.

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

3. Dough microstructure visual acquisition by CLSM

Microscopy is a well-developed technique for studying the microstructure of wheat dough. Light microscopy was primarily used in the early observation of starch and dough (Burhans and Clapp, 1942), is still being used in current studies (Hug-Iten et al., 1999) or with enhanced and simple methodologies such as the epifluorescence light microscopy (Peighambardoust et al., 2010). Furthermore, electron microscopy, and scanning electron microscopy (SEM) in particular, enabled a better understanding of the construction of the components of dough. The resolution of this technique is considerably higher than that of light microscopes. However, specimen preparation is quite complex and can lead to the formation of artifacts (Aguilera and de Vries, 1999). Chabot et al. reported quite early on that sample preparation leads to a modification of the components (Chabot et al., 1979), thereby complicating any interpretation of structural relevance. Despite its lower resolution, light microscopy has several advantages, like the possibility of investigating samples under environmental conditions or following dynamic mechanisms such as freezing or heating. A further improvement of light microscopy was the development of confocal laser scanning microscopy (CLSM), also called laser scanning confocal fluorescence microscopy (LSCFM or (CF)LSM) (Földes-Papp et al., 2003) and confocal scanning laser microscopy (CSLM) (Heilig et al., 2009; van de Velde et al., 2002), respectively. The technique bypasses another disadvantage of light microscopy, the sectioning of samples, and therefore resolves issues of contamination or destruction. Due to the use of optical instead of physical/mechanical sectioning, in combination with a fine focus, CLSM has greatly enhanced the applications and benefits of light microscopy. The following sections provide a brief overview of the methodology.

3.1 The confocal system

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

In confocal laser scanning microscopes (CLSM) a laser is used as light source. The monochromatic light of the laser in combination with the pinholes allows detect on to be limited to a very small region of the specimen. The sample is scanned point by point by moving the laser in the x- and y-direction. The light detected by the photomultiplier in the detector is transferred to information and an image is created by the software of the system.

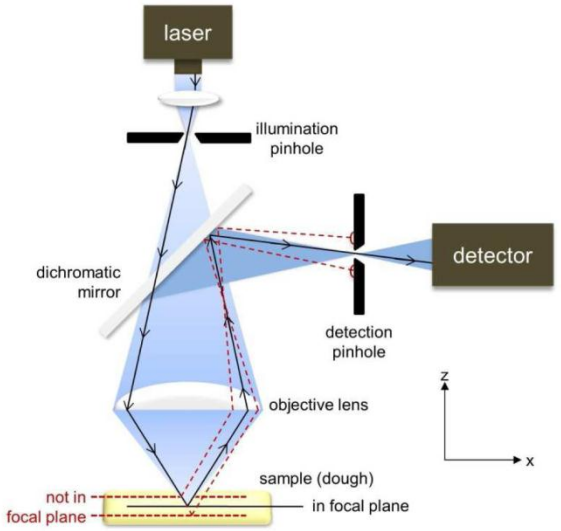

Figure 2: Path of laser beam in a confocal laser scanning microscope. The laser beam is focused on a specific area of the sample and fluorescence is excited. This fluorescent radiation is directed, via the dichromatic mirror, to the detector. Emission coming from planes below or above the focal plane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

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

3.2 Confocal modes

The CLSM can operate in different modes:

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

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

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

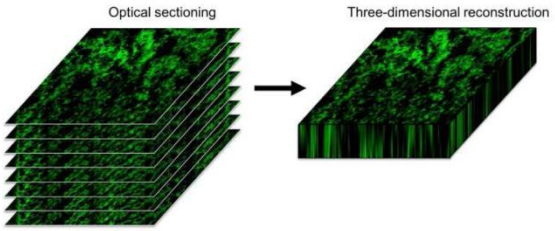

Figure 3: Optical sectioning (z-stack) and 3D reconstruction of a wheat dough sample (protein is white/green).

3.3 Fluorescence

For the fluorescent mode the analyzed constituents have to show fluorescent characteristics. This can be either by inherent fluorescence (auto-fluorescence) or, for clearer distinction and the multiphase detection of different constituents, fluorescent dyes. Auto-fluorescence in cereal products is mainly caused by polyphenolic compounds such as lignin and the ferulic acid of cell walls (Pussayanawin et al., 1988). Every fluorescent dye has its characteristic absorption and emission spectra of photons and forms covalent and non-covalent bindings respectively. For non-covalent bindings (staining), the dye molecules are spread throughout the sample depending on local accessibility and affinity (van de Velde et al., 2003) and are often absorbed by the constituent. Hydrophobicity is a common main affinity effect for many dyes such as fluorescein isothiocyanate (FITC) and Rhodamine B. Non-covalent dyes are usually dissolved and added to the sample. For covalent dyes (labeling), the immune-technique is often used as it enables a highly specific labeling of considered proteins, therefore, antibodies coupled with fluorescent markers are used. These markers can be detected by the fluorescent microscope. For labeling carbohydrates a methodology based on the specific affinity of lectins is available (Blonk and van Aalst, 1993). The targeted application of covalent labeling has some advantages compared to staining, especially in multi-phase systems. On the other hand, non-covalent staining shows easier sample preparation and is favorable for single-phase systems as well as proteins in multiphase systems. Different dyes for constituents of wheat dough have been used in recent decades. Table 1 lists commonly used dyes, their target components, and their extinction and emission (or rather detector) properties.

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

3.4 3D structure reconstruction

The most important advantage of CLSM methodology, compared to other microscopic techniques, is the enhanced lateral and axial (in-depth) resolution. Fine stepping controls and the high focus of the microscope enable the acquisition of stacks of images throughout the sample in a single process. The sample is scanned in x-y-direction, and the laser or the sample (microscope table) is moved in z-direction to acquire a z-stack of micrographs. These first vertical virtual-optical sections of the z-direction can be projected upon each other and reconstructed as a three-dimensional (3D) object (Figure 3). This 3D projection delivers a 3D impression of the sample, that can be animated by a specified rotation or can be presented in a slideshow that progresses through the sample. This non-destructive methodology enables insight into the microstructure and 3D organization of the morphology of materials (Charcosset et al., 2000), biopolymers, including wheat dough in particular (Peighambardoust et al., 2010). In

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

dye	*target component*	*laser wavelength [nm]*	*detector wavelength [nm]*	*parallel use with other dye*	*study*
Acid Fuchsin	protein	568	600-620	no	(Dürrenberger et al., 2001)
8-aminopyrene-1,3,6-trisulfonic acid, trisodium salt (APTS)	starch	488	490-560	no	(Naguleswaran et al., 2011)
fluorescein isothiocyanate (FITC)	starch	488	518	rhodamine B for protein	(Peighambardoust et al., 2006)
	starch	488		rhodamine B for protein	(Parada and Aguilera, 2011)
	protein	488	520-560	no	(Lee et al., 2001)
	protein			no	(Don et al., 2005)
Nile Blue	protein and starch	633	670-810	no	(Kenny et al., 2001)
Rhodamine B	protein	568	625	FITC for starch	(Peighambardoust et al., 2006)
	protein	543	590/50	no	(Beck et al., 2012)
	protein	568		FITC for starch	(Parada and Aguilera, 2011)
	protein	568	585 LP[a]	no	(Weegels et al., 2003)
Safranin O	starch	488	530-550	no	(Dürrenberger et al., 2001)

[a] LP= long pass filter

standard wheat dough an acquisition height of around 20-50 µm is realistic.

3.5 Image processing and analysis

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

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

$$\boldsymbol{C} = \frac{\boldsymbol{4\pi A}}{\boldsymbol{P^2}} \quad (2)$$

$$\boldsymbol{S} = \frac{\boldsymbol{A}}{\boldsymbol{A_{convex}}} \quad (3)$$

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

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

4. Dough microstructure mechanical acquisition

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

4.1 Empirical rheometry

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

4.2 Fundamental rheometry

Fundamental rheometry uses well-defined physical test parameters which are commonly independent of the size and shape of the sample, and as well of test devices which is the main difference to the empirical rheometry. In addition to the extensional measurements with large deformation often used, fundamental rheological tests also provide both dynamic oscillation measurements and creep and relaxation measurements. The dynamic oscillation test uses sinusoidal oscillating stress or strain with time and measures the response. The test enables the analysis of elastic and viscous moduli as well as complex shear modulus. During the test (in the linear-viscoelastic region) the material or dough is not destroyed and several adaptations of tests (such as strain, frequency, temperature) can be performed. Therefore rheological tests are widely used in the evaluation of cereal dough. However, the oscillation measurements are performed in deformation conditions which differ from the process conditions of dough handling. Measurement values are typically the shear storage modulus (G'), which defines the elastic part of the sample, the shear loss modulus (G''), which defines the viscous part of the sample, and the complex shear modulus (G*), which defines the rigidity of the sample. The creep and relaxation measurements use constant stress while measuring the following deformation. The deformation is described as compliance (J) and can be interpreted as a model of dampers and springs in series and parallel, and is a combination of the Maxwell and Kelvin model. The combination of these models is represented in the Burger model which is especially used in biological systems to define elastic delay and behavior (Steffe, 1992). During the creep phase a constant stress is applied and the relaxation is subsequently measured without stress. Thereby, a rapid relaxation which is explained with the behavior of small polymer molecules presents in the first few seconds, followed by a slower relaxation which can be associated with the HMW polymers of gluten (Dobraszczyk and Morgenstern, 2003).

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

account, small deformation tests are more suitable for characterizing the current molecular and structural status of the network and large deformation tests to describe the mechanical behavior during processing.

5. Dough rheology derived from its visual microstructure

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

5.1 Formation of dough microstructure

5.1.1 Hydration of flour particles

The formation of a wheat dough protein network is a complex and time dependent process. For a detailed view of the starting point (hydration) of these changes, non-developed dough can be examined. Non-developed dough is prepared by mixing frozen water with flour so that the subsequent melting initiates the hydration process of the flour particles without the input of mechanical energy. Investigations into a non-developed wheat dough revealed large hydrated protein aggregates including some starch granules (Peighambardoust et al., 2005; Peighambardoust et al., 2006). This water-flour mixture mainly exhibited intramolecular disulfide bonds, which are broken due to shear and extensional deformation in the following mixing process (Lee et al., 2001). The aggregates seems to be distinctly larger (approximately 80 µm, as deduced from a micrograph of the study) than native GMP particles. A study by Unbehend (Unbehend et al., 2004) confirms these results.

5.1.2 Role of the glutenin macro polymer during network formation

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

The GMP are described as having a diameter of 30-50 µm; however, the mixing process decreases the average size of the GMP particles. At the optimal dough development time (DDT) the particles of different wheat varieties presented the same size. Within the mixing process the particles lost their common shape and became ruptured (Don et al., 2003b). Although the CLSM images showed a distinct particle structure in a water flour system, this structure disappeared and a dispersed system or even a network system appeared after the mixing begun. However, Coulter counter particle size analysis (laser diffraction) showed a clear particle system, with decreasing particle diameters due to mixing. It seems that analysis methodology is an important factor for the acquisition of results and the finding and interpretation of totally different theories. The particle theory is supported by the study of Lefebvre et al. (Lefebvre et al., 2000) where the gluten network is described as a particle network. Another study presented the theory of soft and deformable colloidal gluten particles which form a network responsible for the mechanical behavior of dough (Lefebvre and van Vliet, 2003). Furthermore, particles which form a network by direct interaction reveal characteristics of a polymeric network during deformations. Therefore it can be concluded that the rheological behavior of dough which follows polymeric theories is based on this network formed by interacting particles. Moreover, Don et al. (Don et al., 2005) investigated an extracted GMP dispersion in a microrheology system in which a probe in a CLSM can be sheared continuously. Spherical glutenin particles were sheared with a low rate whereby the particles became more elliptical and some particle-particle interactions occurred. The effect did not totally disappear after removal of the shear rate. An increased shear rate resulted in a continuous glutenin phase which was stable after removing the shear rate. The relatively elastic behavior of the underlying wheat dough presented low values in an under-mixed dough, the highest values in an optimally mixed dough, and medium values in an over-mixed dough. The optimal dough development time is an important point: particles have already become dissociated and are able to form a continuous network after resting, but still exhibit the ability to re-assemble in their originate states due to the preserved internal chemical structure. The glutenin structure of the under-mixed dough does not form a continuous particle network of which the elasticity remains low. Over-mixing the dough resulted in a changed chemical level of the internal structure and the fragments showed polymer behavior. This means that altered rheological values are determined more by the changes of the internal structure of the particles and therefore the aggregates formed in level Ⅲ of the hyperaggregation model (discussed afterwards) (Don et al., 2005). It can be assumed, that this aggregate network in the macroscopic level should be detectable by CLSM.

5.1.3 Network models

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

This is in accordance with a theory of soft and deformable colloidal gluten particles which form a network which is responsible for the mechanical behavior of dough (Lefebvre and van Vliet, 2003). Furthermore, particles which form a network through direct interaction reveal characteristics of a polymeric network during deformation. Therefore it can be concluded, that the rheological behavior of dough which follows polymeric theories, is based on this network formed by interacting particles.

5.1.4 Further network development during mixing

The monitoring of the microstructure during the development that results from mixing reveals coarse protein domains or unchanged aggregates (Dürrenberger et al., 2001; Peighambardoust et al., 2006). The protein aggregates are formed by physical interaction during dough deformation in the mixing process (Peressini et al., 2008). Gluten macrofibrils are created due to the covalent linkage of polypeptide chains by intermolecular disulphide bonds. This formation of the high

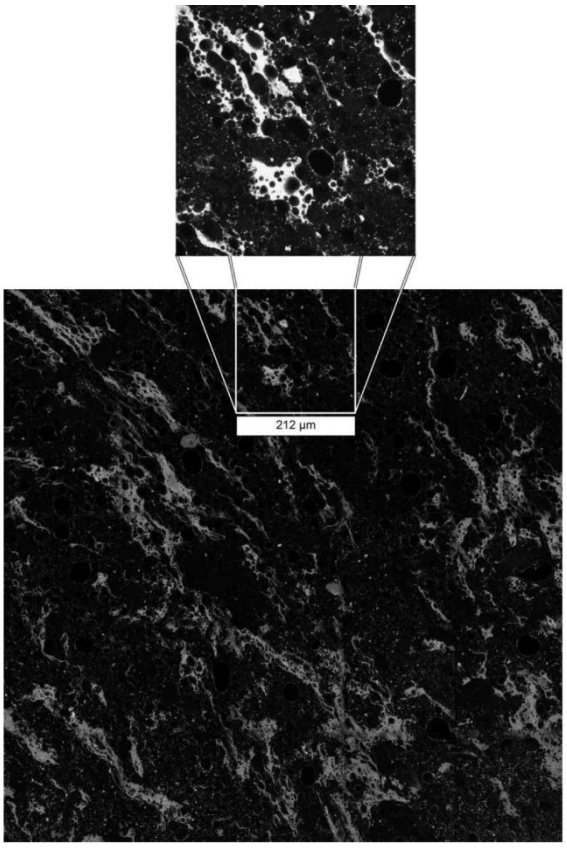

Figure 4: Wheat dough mixed with 63 rpm in a Microfarinograph. Protein was stained with Rhodamine B (gray). The large micrograph has 1060x1060 µm, the zoomed, upper micrograph has 212x212 µm (with increased saturation).

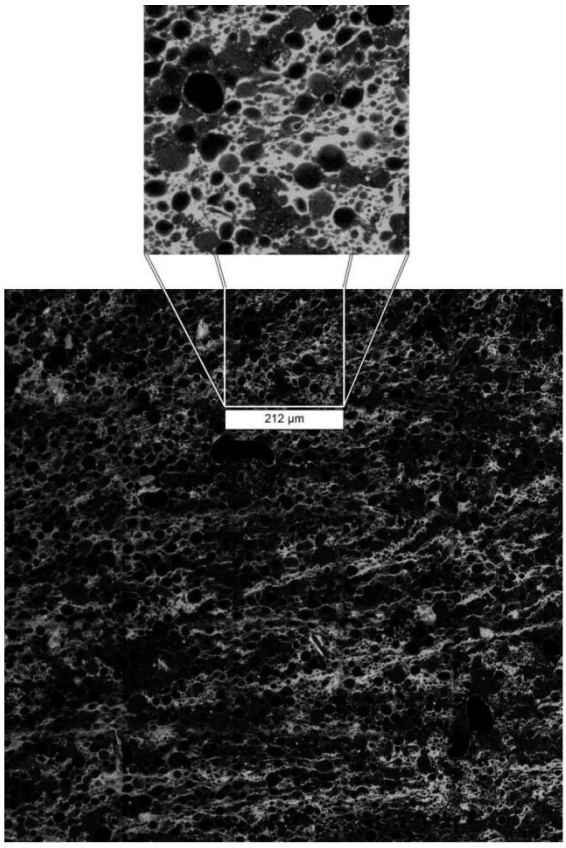

Figure 5: Wheat dough mixed with 75 rpm in a Microfarinograph. Protein was stained with Rhodamine B (gray). The large micrograph has 1060x1060 µm, the zoomed, upper micrograph has 212x212 µm (with increased saturation).

molecular weight glutenin polymers leads to the characteristic wheat dough extensibility and gas retention properties (Autio and Salmenkallio-Marttila, 2001). However, especially at the beginning, the protein network is not homogenously distributed, but interfused with clustered areas of mainly starch granules. This effect could be detected in undermixed dough in a z-kneader (Calderón-Domínguez et al., 2003) as well as in a model dough system, composed of potato starch, wheat gluten, and water (Parada and Aguilera, 2011). An increased mixing time reveals a more homogeneous distribution of starch and gluten. This is often described as an extension and thus as protein films. Therefore the visible area of the protein surface increases, the depth decreases and a continuous gluten phase occurs. Dough mixed to its optimum (maximum resistance) exposes a network of interconnected gluten which is evenly interfused by starch granules (Peighambardoust et al., 2010). The described protein matrix with spread starch granules could also be confirmed in the model system mentioned (Parada and Aguilera, 2011). This means that the properties of the single components (gluten and starch) are the most important factors that form the characteristic microstructure of wheat dough. However, minor components will affect the proportions and the results. In sum, the gluten forms entanglements. Herein, just small areas interact and some separated regions do not show any interaction. This fact explains the elasticity and the possibility of stretching gluten and dough: At the beginning, coiled chains of glutenin subunits are stretched (till breakage of secondary bonds) and afterwards slippage is caused by the entanglement points (Lindsay and Skerritt, 1999).

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

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

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

5.2 Modification of wheat dough microstructure

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

5.2.1 Ingredients

As described above hydration only starts and the development of dough is only made possible with the addition of water to flour. Observations with CLSM on pretzel dough with three different *water contents* showed that, unlike the control and with high water dough, the protein network in low water dough could not form a continuous structure like in. Thus, dough extensibility tests revealed distinct lower extensible measures for the low water dough which could be a result of suboptimal protein formation. Pretzel dough is typically produced using limited water addition which affects the development of the gluten network (Seetharaman et al., 2004). However, it is not entirely clear how water modifies the dough microstructure. Further studies on the variation of the water content could help to ascertain better insight into the hydration process of flour in relation to the protein microstructure development.

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

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

Further, the effect of the addition of *sodium chloride* on wheat dough has been investigated by two current studies. Lynch et al. (Lynch et al., 2009) published 3D images of wheat dough (stained starch and protein) with relatively low NaCl additions. The protein network was described to show larger diameters with increased uniformity of orientation with increasing NaCl. However, no major structural changes were rated. In the study of Beck et al. (Beck et al., 2012) higher additions of NaCl were used and binarized micrographs of the protein network were described. The network radically changed its structure, from spread, uniform, but more isolated protein particles at 0 g NaCl 100 g^{-1} flour to longer and larger protein strands with some junctions and branches. This effect is explained by neutralized repulsing forces of amino acids and therefore a more compact protein structure, however, with increased water phase in between. At the same time a significant increase of the complex shear modulus with increasing NaCl addition was analyzed. This means that in this case the large proteins strands lead to an increased stiffness, as well as increased elasticity. A further image analysis of the processed micrographs could lead to a better understanding of this behavior. First steps in this directions were already showed in rye dough, however, without structural elements (Beck et al., 2009).

5.2.2 Mixing process

The distinct effect of mixing on the microstructure of dough has already been described in chapter 5.1.4. Different forms of this process represent greatly varied types and intensities of mechanical energy input such as shear, rotational and extensional deformations and therefore modify the microstructure. Micrographs of GMP extracted from mixed wheat dough (in a z-blade mixer) and simple sheared dough revealed no detectable particles in the mixed wheat dough. However, the sheared dough showed an aggregation of glutenin particles. An explanation would be that the shearing in the experimental setting (30 min) led to an continuous protein network at the beginning (as shown in the native dough micrograph after 15 min) and subsequently afterwards induced aggregation of the glutenin particles due to the shear forces (Peighambardoust et al., 2005). The specific mechanical energy (SME) showed a distinctly higher value in the shearing process of Spring flour (277 to 181 kJ kg^{-1} after 45 min). In summary dough shearing affects fewer breaks of particular structures of GMP compared to z-mixing where the particles are disrupted (at the same energy input). Another study (Peighambardoust et al., 2006) analyzed the microstructure of dough by CLSM and compared a z-blade mixer and shear induced dough preparation. The shear induced processing led at the beginning to a heterogeneous protein structure (more than some 100 µm), where protein domains and starch granule rich domains could be detected. The direction of the shear flow led to an orientation of these domains. An increased shearing time (up to 45 min) revealed either a breaking up of the larger proteins into smaller fragments and therefore a more homogeneous protein phase or a similarly sized yet still available large protein domain, depending on the used flour (Soissons/Spring flour). These results are distinctly different to those gained in a z-blade mixer. A further study by Peighambardoust (Peighambardoust et al., 2007) used a special device to apply a specific shear. In particular, the flow profile in a cell affected the gluten structure formation. Furthermore, the breaking up of protein domains into a homogeneous network was defined as a time-independent process in the microscopic domain but determined by the shear and the initial size of the protein domains. The dependency on the shear has been shown in a study using different shear rates (Peressini et al., 2008). The large protein domains gained by dough at low shear rate were not analyzed with increasing shear rates, due to fracturing. It was therefore assumed that these structures are quite weak.

5.3 Numerical interpretation using image analysis

The quantification of the structural elements of images is necessary to indicate structural alterations based on processing or ingredient variation on any resolution scale (Kaláb et al., 1995). Furthermore, the comparison of a large number of different samples is enabled. Only with the gained numerical structure parameters is an objective correlation of the microstructure with rheological and other analysis methods

possible. Due to the very limited studies in the field of wheat dough in combination with CLSM, a wider range of studies is discussed.

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

Schluentz et al. (Schluentz et al., 2000) showed differences in the *protein matrix of developed, partially developed and nondeveloped wheat dough*. The developed dough had the most detectable protein matrix (analyzed with SEM). The disadvantages of SEM (as sample preparation) were avoided by Lee et al. (Lee et al., 2001) who used CLSM for comparable experiments. Additionally, they used partially developed dough, created with shear or extensional deformation in a rheometer. The development of the dough resulted in an increase in the complex shear modulus G*. Partially developed dough shows higher stiffness due to preparation (in a rheometer) with extensional deformation than with simple shear deformation. Furthermore, they analyzed the detectable protein matrix using the percentage of pixels with high gray scale values and revealed significant differences (nondeveloped dough < shear deformation < extensional deformation < developed dough). The protein matrix varied from around 11 to 40% of the total area. It could be deduced that the strength of the dough is directly correlated with the amount of protein matrix in the dough microstructure. The formation of the protein network is related to the type of deformation and on the total energy addition.

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

Rye dough microstructure was studied with the aid of image analysis, whereby rye dough has strongly deviating mechanical behavior and therefore different processing necessities compared to wheat dough (Beck et al., 2011). Parkkonen et al. (Parkkonen et al., 1997) used fluorescent microscopy to dye cell walls in rye dough, analyzed the cell wall area, and compared these values with the falling number which is related to gelatinization properties during heating and α-amylase activity. It could be shown that dough with a high number of cell walls lead to high falling numbers which was related with rigid, stable dough. Another study on rye analyzed the effect of Transglutaminase, which catalyzes the formation of protein cross-links, on rheological and microstructural properties (Beck et al., 2009). They analyzed rye dough treated with different concentrations of the enzyme by the use of the protein size and the number of protein fractions. Standard rye dough showed small fragments of proteins without a coherent structure. However, enzyme addition leads to linkages of rye proteins which elongated the protein structures. Decreasing bread volume upon a specified enzyme concentration was explained by a too strong protein aggregation.

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

6. Conclusions and future trends

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

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

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2.3 Dough microstructure: Novel analysis by quantification using confocal laser scanning microscopy

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Dough microstructure: Novel analysis by quantification using confocal laser scanning microscopy

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ABSTRACT

The effect and the correlation of water addition to flour on the microstructure and viscoelastic properties of wheat flour dough were investigated using confocal laser scanning microscopy and a spectrum of rheological methods. Dough with water addition in the range of 52.5–70.0 g water 100 g^{-1} flour was investigated using a stickiness test, uniaxial elongation test, and fundamental rheology like small amplitude oscillatory shear measurement and a creep recovery test. A method for quantifying the microstructure of dough protein gained by CLSM was established with image processing and analysis. The complex shear modulus decreased rapidly with water addition due to the plasticization effect of water molecules and increased mobility in the continuous phases. Elastic behavior, determined as loss factor tan δ and relative elastic part J_{el} decreased. Rheological tests showed high linear correlations with each other (r of |0.66|–|0.98|). Image analysis measurements (average size, area fraction, perimeter, circularity, and fractal dimension) showed high linear correlations (r of |0.66|–|0.85|) with water addition and rheological attributes like the complex shear modulus (r = 0.85) and J_{el} (r = 0.86). CLSM in combination with image processing and analyzing has proven to be an applicable and powerful tool for examining and quantifying dough protein microstructure. Hence, it was possible to prove the dependency of rheology on the microstructure of dough.

1. Introduction

Bread contributes a high part of the daily food-intake and is an important ready-to-eat processed food. Therefore the understanding of bread's pre-product dough is of high interest for a successful and controlled production. In particular its fundamental mechanical properties are most significant to deduce its behavior during processing and to investigate the interactions among its components (Berland & Launay, 1995). Dough is a complex, non-linear, and time dependent viscoelastic system, and thus the rheological properties of dough are difficult to characterize. Therefore it is a challenging task to obtain reliable and meaningful data (Breuillet, Yildiz, Cuq, & Kokini, 2002).

Nevertheless, or exactly therefore, there is a range of methods available to measure the dough's rheology. The empiric rheological properties of dough can be measured by recording z-blades mixers (e.g. farinograph or mixograph) and with load-extension tests. These methods are widely used for successful quality control in the bakery and milling industries. However, they only sparsely fulfill the requirements of fundamental rheological measurement systems, e.g. a rheometer. Dynamic testing methods basically developed for polymer rheology are beneficial in assessing different viscoelastic properties of dough (Berland & Launay, 1995). Controlled stress and strain conditions in oscillatory measurements with small amplitudes (SAOS) are appropriate because they do not largely affect or destroy structures of dough. Furthermore it is easier to calculate and therefore eliminate the effect of different geometries (like mixing geometries in comparison to plate–plate geometries). Further possibilities are creep recovery tests which show good relations to the baking properties (Van Bockstaele, De Leyn, Eeckhout, & Dewettinck, 2008).

In dough the main structurally relevant elements are starch granules, water-soluble and insoluble proteins and entrapped air. After energy addition during kneading, the hydrated proteins start to become interconnected resulting in a continuous spatial network which is classically described as the continuous gluten phase. The viscoelastic protein network consists of glutenin (elastic properties) and gliadin (viscous properties) as the main water-insoluble proteins (Koehler, Kieffer, & Wieser, 2010). The second continuous phase consists of free water where starch granules and water-soluble components are located (Breuillet et al., 2002) and the third phase consists of dispersed gas.

The three phases result in the microstructure of the dough. The microstructure of food determines, among others things, the appearance,

Abbreviations: A^K, Area under the curve; A^S, Work of adhesion; A_F, Area fraction; ØA, Average size; ΣA, total size; BU, Brabender Units; C, Circularity; CLSM, Confocal laser scanning microscopy; C^S, Cohesiveness; D^S, Stickiness; E^K, Dough extensibility; FD, Fractal dimension; G′, shear storage modulus; G″, shear loss modulus; $|G^*|$, Complex shear modulus; J_{el}, Relative elastic part of J_{max}; $J_{max.}$, Maximum creep compliance; J_r, Recovery compliance; P, perimeter; ΣP, Particle count; R^k_{max}, Dough resistance; SAOS, Small amplitude oscillatory shear measurement; tan δ, Loss factor.

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shelf life, taste perception and rheology. Letang et al. (1999) have already shown that the microstructure is essential to compare the evolution of different doughs. It can be seen as the linkage between the molecular properties of the ingredients and the apparent macroscopic properties of the product. Understanding the microstructure by microscopic techniques in relation to macroscopic properties would enable the improvement of existing products and an effective design of new ones (Blonk & Vanaalst, 1993). With this objective a number of microscopy and imaging techniques have already been used to investigate and explain the microstructure of dough and their effects and changes during the development and processing of dough.

Confocal laser scanning microscopy can be applied to gather this microstructure by a dynamic and nearly non-invasive observation. An important feature of CLSM is the acquisition of a specific focal section of the sample. This enables the visualization of interactions between structural elements without the necessity to prepare the samples by fixing and dehydration. CLSM has been proven to be a helpful instrument in cereal science. Applications include the analysis of gluten-free products (Moore, Heinbockel, Dockery, Ulmer, & Arendt, 2006), the bubble size distribution in baked goods (Deshlahra, Mehra, &Ghosal, 2009), glutenin particles in wheat dough (Peighambardoust, van der Goot, Hamer, & Boom, 2005), and bread crust (Primo-Martin et al., 2006). For determining structure, processing of the images obtained by CLSM is necessary to objectively distinguish image areas containing information from those containing background. As a second step, analysis of the processed data is necessary to obtain structure-related data such as area fraction or circularity of particles or aggregates. Although some CLSM micrograph analyses are known for analyzing the diameter of maize starch granules (Nagano, Tamaki, & Funami, 2008), the amount of protein over gray level content (Lee, Ng, Whallon, & Steffe, 2001) or protein particle size distribution of treated rye proteins (Beck, Jekle, Selmair, Koehler, & Becker, in press), no broader analysis of structural elements in the field of dough is known. Furthermore, statistical correlations with rheological or other physical properties are not yet available up to date.

The microstructure of dough can be modified by varying the amount of water. Water is considered to play the most important role in the viscoelastic properties of dough due to its influence on the development of the gluten protein network (Skendi, Papageorgiou, & Biliaderis, 2010). Water has several important functions in dough, such as serving as a solvent for components or as the medium for enzymatic and redox reactions. In addition, water acts as a mobility enhancer due to its molecular weight (Mani, Tragardh, Eliasson, & Lindahl, 1992). The protein phase could constitute the continuous phase of dough at the microscopic level (Amemiya & Menjivar, 1992) and is said to be the most important factor influencing dough rheology.

However, until now no quantitative analysis of the microstructure of dough has been performed and therefore the dependency between the microstructure and the rheology is no more than a logical assumption or a deduction from other fields in natural science. Therefore, in this study a wide bandwidth of dough rheological properties as a function of water addition to flour was for the first time correlated with structural measurements of micrographs of dough protein gained by CLSM and image analysis.

2. Materials and methods

2.1. Ingredients

For all measurements wheat flour type 550 (standard German wheat flour) harvested 2008 from Rosenmühle, Ergolding, Germany was used. Moisture content, water absorption, falling number and ash content were determined according to methods of the International Association for Cereal Science and Technology (ICC) 110/1, 115/1, 107/1 and 104/1 (ICC, 1994), respectively. Protein content was analyzed using the Kjeldahl Method (EBC) (Anger, 2006). All measurements were done in triplicate. The dye for CLSM was rhodamine B, Sigma-Aldrich Chemie Gmbh, Munich, Germany. Sodium chloride (NaCl) was obtained from Südsalz GmbH, Heilbronn, Germany.

2.2. Dough preparation

The formula comprised 50.0 g of wheat flour (corrected to 14% moisture), 0.9 g NaCl and the amount of distilled water needed to reach dough consistency of 500 BU. Analysis of optimal water absorption, dough development time, degree of softening of dough containing NaCl, together with the preparation of the samples for all other analyses, was performed using a DoughLab equipment with 50 g bowl (Perten Instruments GmbH, Hamburg, Germany). Flour was premixed for 60 s to reach the defined temperature of 30 °C in the bowl, water including dissolved NaCl was added and the dough was mixed at a mixing speed of 63 rpm. The mixing time of all dough was set to obtain optimum dough consistency (with a maximum of 480 s). Water addition to flour was set from the optimum quantity (measured with NaCl) to 0, ±5, ±10, +15, and +20%, respectively. With these levels of moisture it was still possible to create a cohesive and homogeneous mass.

2.3. Stickiness measurement

Dough machinability was determined using a TA.XT2 Texture Analyzer (TPA) (Stable Micro Systems, Godalming, UK) and a dough stickiness rig as described by Chen and Hoseney (Chen, 1995). The Chen and Hoseney cell was used with a cylindrical probe of 25 mm diameter. Pre-test speed and test speed was 0.5 mm s^{-1}, post-test speed 10 mm s^{-1}, applied force 40 g, return distance 4 mm, contact time 0.1 s, trigger force 0.05 N and way (before test) 4 mm. Dough was placed into the cell after kneading and rested for 10 min at 30 °C. Duplicate determinations of each single dough with 12 single measurements were performed. The following parameters were recorded: stickiness (D^S), work of adhesion (A^S) and cohesiveness (C^S).

2.4. Uniaxial dough measurement

The uniaxial measurement of the dough to obtain values for extensibility was executed with the TPA using a SMS/kieffer rig (Stable Micro System, Godalming, Great Britain). The mode was measure force in tension, option return to start, pre-test speed 2.0 mm s^{-1}, test speed 3.3 mm s^{-1}, post-test speed 10.0 mm s^{-1}, distance 75.0 mm and trigger force 0.05 N. After dough preparation 20 g was placed directly in the form and proofed for 10 min at 30 °C before analysis. Output values were R^k_{max} (maximum resistance), E^k (extensibility) and A^k (area under the curve). The results are consistent with those obtained using the Brabender Extensograph (Grausgruber, Schoggl, & Ruckenbauer, 2002). Each dough was produced twice and at least 6 measurements per dough were performed.

2.5. Rheometer settings and procedure

The dough samples were analyzed with an AR-G2 rheometer (TA instruments, New Castle, USA). A smart swap peltier plate temperature system (30 °C) with a 40 mm plate–plate geometry (serrated surface to avoid slippage) and a gap of 2 mm was used. An 8 g sample was cut from the dough to avoid extra stress on the sample. The dough was placed between the plates, the excess of dough sample was trimmed and paraffin was applied on the free surface of dough to prevent sample drying during the test. The sample was allowed to rest for 10 min in order to allow a built-up residual stress due to the clamp of the sample. A thermal cover was used to reduce the thermal gradient of the patterns. First an oscillatory frequency sweep was

carried out and after this a creep recovery step. A triplicate determination for each water level was made.

2.5.1. Oscillatory frequency sweep

During the oscillatory frequency sweep the frequency was varied from 0.1 to 100.0 Hz at constant strain amplitude (0.1% strain). Preliminary tests indicated that the deformation was well within the linear viscoelastic region of the sample. Dynamic rheological properties of samples were recorded by the shear storage modulus G′ and the shear loss modulus G″. The loss factor tan δ was calculated with the equation

$$\tan\delta = G''\,(G')^{-1}. \quad (1)$$

2.5.2. Creep recovery test

A constant shear stress τ_0 of 250 Pa at 30 °C was applied to the dough for 180 s and afterwards removed ($\tau_0 = 0$ Pa). Van Bockstaele et al. (2008) found the best correlation of creep recovery measurements to wheat bread baking quality at this stress. Dough relaxation was recorded for 360 s, providing that the recovery time was long enough for the steady state to be reached. Strain values were collected as a function of time. Final data were expressed in terms of compliance:

$$J(t) = \gamma(t)\,\tau_0^{-1}, \quad (2)$$

where J is the compliance, γ the strain, and τ_0 the constant stress applied during creep. Creep measurement parameters included the time and stress dependent recoverable shear deformation, the creep compliance J_{max} (at t = 180 s of the creep phase). The creep recovery compliance J_r (at t = 360 s of the recovery phase) is a measure of the material's elasticity and describes the mechanical energy stored in the sample during the creep phase. The relative elastic part J_{el} [−] was calculated by:

$$J_{el} = J_r\,(J_{max})^{-1}. \quad (3)$$

2.6. Dough preparation for CLSM

Dough for CLSM was prepared as described above in a 50 g DoughLab bowl at a mixing speed of 63 rpm. Fluorescent dye rhodamine B was added in a concentration of 0.001 g 100 mL^{-1} water. The dye was solubilized in distilled water before mixing to ensure homogenous distribution in the dough. After mixing 2.3 g dough was cut from the inner part of the dough with scissors and was transferred to a specimen shape (diameter 18 mm, height 0.8 mm) before the application of a glass cover slip. Afterwards the dough was proofed for 10 min at 30 °C. A Ti–U inverted research microscope with an e-C1plus confocal system (Nikon, Düsseldorf, Germany) and a 20× objective was used. Proteins were monitored as fluorescence images (λ_{exc} = 543 nm, λ_{em} = 590/50 nm) with 1024 x 1024 pixel resolution (212 x 212 μm) in a constant z-position. Seven independent positions on the x–y-axis were recorded for each dough sample. Each variable was prepared twice. Rheological analysis of dough with added rhodamine B showed no significant differences from dough prepared without dye.

2.7. Digital image processing and analysis

For each dough type 14 digital image analyses were performed using the image processing and analysis open source Java software ImageJ (version 1.42q, National Institutes of Health, Bethesda, Md, USA). The images were pre-processed by changing to grayscale (8 bit). To apply the segmentation of protein to background a fuzzy thresholding algorithm of Huang et al. (1995) was used. The function was applied to formalize the characteristic relationship between a pixel and its related region (the object or the background). The binary images obtained were analyzed for protein features: particle count (ΣP), total area (ΣA), average size (ØA), area fraction (A_F), perimeter (P), circularity (C), and fractal dimension of the image (FD). Computation of the circularity was based on the equation

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

where A is the area of the specific particle and P is the perimeter of the particle.

The fractal dimension FD is a measure to characterize the roughness in an image and can be associated with the complexity of forms (Sarkar & Chaudhuri, 1992). Basically the fractal dimension measures the rate of added structural details with an increased resolution of magnification (Smith Jr, Marks, Lange, Sheriff Jr, & Neale, 1989). To measure FD a set of square boxes with a defined box size was used to cover the border of the particles. An algorithm for the "box counting" method was used to calculate the capacity fractal dimension. The algorithm counts the number of boxes with a length of 2 to 64 pixel size needed to cover a one pixel binary border (protein to background). The log of the number of the boxes which are necessary to cover the border is noted as a function of the log box size. The fractal object gives a straight line with a negative slope. The equation for the calculation of the FD is:

$$FD = \frac{\log(N)}{\log\left(\frac{1}{r}\right)}, \quad (5)$$

where N is the number of boxes and r is the length of the side of the box. Complex or rougher gray level images lead to a high value of FD while smoother or simple images mean a low value.

2.8. Statistical analysis

The statistical significance of results was assessed using single-factor analysis of variance (ANOVA) with the software Statgraphics Centurion (version 15.1.02 Statpoint Technologies Inc. Warrenton, Va, USA) at the 5% significance level. To explore relationships between variables a simple linear regression analysis was calculated. The correlation coefficient r was used to illustrate the degree of correlation.

3. Results and discussion

3.1. Physicochemical characteristics of wheat flour

A falling number of 382 ± 10 s (n = 5) showed a low α-amylase activity. The protein content of the wheat flour used was 11.56 ± 0.18 g 100 g^{-1} flour d.m. (n = 2), ash content 0.67 ± 0.01 g kg^{-1} flour (n = 3) and moisture content 13.73 ± 0.12% (n = 5). Flour analysis following ICC 115/1 (based on dough without NaCl) showed a water absorption of 60.2 ± 0.2%, dough development time 2.9 ± 0.5 min, dough stability of 17.1 ± 1.4 min, and degree of softening of 24.8 ± 6.8 BU. Analysis of the dough containing 1.8% NaCl (based on flour weight) as used in all experiments revealed 58.3 ± 0.1%, 3.9 ± 2.2 min, 18.8 ± 0.1 min, and 5.3 ± 2.6 BU, respectively. The alteration of the results due to addition of NaCl was based on an improved gluten network formation owing to the charge shielding by sodium and chloride ions allowing the protein side chains to converge.

3.2. Stickiness measurement

Stickiness of dough is an important quality parameter in bakery production because it can be used as a processability parameter for

dough. Dough can adhere to proofing baskets and conveyor belts and therefore create problems in automated bakeries (Beck, Jekle, Hofmann, & Becker, 2009). Therefore, the effect of different water additions to wheat flour on stickiness D^S, work of adhesion A^S and cohesiveness C^S was determined (Table 1). Water addition from 52.5 to 70.0 g 100 g^{-1} flour showed a significant increase of stickiness by 131%. The adaption to a second order polynomial curve exhibited a highly significant correlation ($r^2 = 0.909$, $p \leq 0.01$). Furthermore, cohesiveness ($r^2 = 0.962$, $p \leq 0.01$) and A^S ($r^2 = 0.976$, $p \leq 0.01$) increased exponentially. Previous studies stated that dough stickiness was influenced by water content (Beck et al., 2009). As dough stickiness is the force of adhesion between the dough and the plunger of the TPA, it can be concluded that due to the water excess the contact area between dough and plunger increases and strengthens the adhesion. van Velzen (van Velzen, van Duynhoven, Pudney, Weegels, & van der Maas, 2003) suggested that dough stickiness is mainly a consequence of the magnitude of hydration of gluten molecules. This will promote the gluten molecules' migration to the upper dough layers due to a higher mobility. Furthermore, the high increase of C^S is related to the plasticizing effect of water. The more water there is in the dough, the more intermolecular space is exhibited between the structural elements (mainly protein phase, starch granules). Therefore the cohesiveness and the extensibility increases until the adhesion forces of the plunger are too weak or the dough itself disrupts. In summary, the measurement of dough stickiness can be considered as a variation of dough rheology measurements.

3.3. Uniaxial dough measurement

Uniaxial extension tests are commonly used to investigate the behavior of dough. In this study the Kieffer dough and gluten extensibility rig test was performed on wheat dough with increasing amounts of water as shown in Table 1. Standard dough with water addition of 58.3 g 100 g^{-1} flour had an extensibility E^K of 24.54 ± 2.29 mm, a resistance R^K_{max} of 0.397 ± 0.076 N, and an area under the curve A^K of 3.13 ± 0.72 N mm, respectively. All three values were significantly affected by increasing water addition. Dough extensibility significantly increased by 40% whereas R^K_{max} declined by 69% and A^K by 29%.

In addition to a high viscosity to prevent gas cells rising, dough should be extensible to prevent a disruption of gas cell membranes in order to achieve a good bread volume (Sliwinski et al., 2004). Zaidel et al. (2009) examined an increased extensibility of weak and strong flour dough with increasing water contents which is in accordance with the current study. The increase can be explained by the enhancement of mobility in dough due to the low molecular weight of water. Therefore the soft continuous phase of dough increased and enabled a higher dough elongation. Furthermore, owing to the increased water content, fewer noncovalent bonds were developed due to the dilution effect. The effect of altered energy input due to softer dough was minimized in this study by adapting the dough kneading time.

3.4. Fundamental rheological measurements

Small amplitude oscillatory shear (SAOS) measurements enable the acquisition of dynamic rheological functions, without altering the microstructure of dough which consists of an internal network. Thus in Table 2 oscillatory frequency sweep results are shown. To determine the effect of different frequencies, 1 and 10 Hz are illustrated as examples. All samples possessed higher G′ than G″, indicating that all doughs were a highly structured material and had a firm, elastic-like behavior. Increased water addition from 52.5 to 70.0 g 100 g^{-1} flour decreased |G*| by 85/83% (1/10 Hz), G′ by 85/84%, and G″ by 84/82%. The loss factor tan δ increased by 7% at 1 and 10 Hz. In addition to SAOS, creep recovery tests were performed to gain a wide range of rheological data from low to high strains (shown in Table 2). Creep compliance rose by 7013% as an exponential function of water addition ($r = 0.985$, $p \leq 0.01$) and J_{el} decreased by 78%.

Increased water addition led to a decrease of G′ and G″. The current study confirmed other works (Berland & Launay, 1995; Navickis, Anderson, Bagley, & Jasberg, 1982) by providing evidence of the significance of these characteristics over a high bandwidth of water levels. The results of SAOS indicate a high relative uniformity of G′, G″ and |G*| as a function of the two frequencies analyzed. The elastic properties of dough need an optimal hydration of the flour ingredients. If there is too little water the gluten molecules do not fully hydrate (Navickis et al., 1982). However, the results of the current work demonstrate that the proteins are already hydrated at the minimum water addition (−10% from the optimum water addition): the decrease of the relative elastic part ($r = -0.910$, $p \leq 0.01$) and the increase of the loss factor ($r = 0.800$, $p \leq 0.01$) as a linear function of water addition indicate that gluten molecules have a high affinity to free water. If, in addition to the plasticizing effect of the water molecules, other effects such as an increased hydration from minimal to optimal water addition would take place, a non-linear regression would be expected. Another supporting negative consequence on the viscoelastic properties of dough is the strong plasticizing effect of water. The independency of tan δ from the water content observed in other studies (Berland & Launay, 1995; Letang et al., 1999) cannot be confirmed, even if tan δ increased by just 7%.

The plasticizing effect of water was proven by SAOS with |G*| and by the creep-recovery test with J_{max}. The complex shear modulus (at 1 and 10 Hz) as a measurement of stiffness decreased as an exponential function of water addition ($r = -0.987$, $p \leq 0.01$) and the creep compliance as an inverse measurement of the dough's stiffness exponentially increased ($r = 0.985$, $p \leq 0.01$). The observed softening effect of water can be associated with the enhanced mobility effect of water. In polymers a shifting from the rubbery state (presence of entanglement network) to a flow state (disappearance of network due to slippage) can be observed with the addition of water. Water serves as a low molecular weight diluent or plasticizer to dough (a high polymer system) (Heddleson, Hamann, Lineback, & Slade, 1994). In summary, increased moisture content softens dough and decreases the elastic properties in small amplitude and high shear stress tests.

Table 1
Dough stickiness D^S, cohesiveness D^C and work of adhesion A^S measurements (n = 24) as well as uniaxial-extension measurements (resistance R^k_{max}, extensibility E^k) (n = 12) of wheat dough with different water addition. Results are shown as mean±standard deviation. Values in the same column that are significantly different from the lowest water addition (52.5 g water 100 g^{-1} flour) are marked (*$p \leq 0.05$).

Water addition [%]	Stickiness			Uniaxial-extension		
	D^S [N]	C^S [mm]	A^S [N mm]	A^K [N mm]	R^K_{max}[N]	E^K [mm]
52.5	0.295 ± 0.039	0.008 ± 0.001	0.451 ± 0.105	3.888 ± 0.631	0.706 ± 0.102	19.87 ± 1.42
55.4	0.431* ± 0.039	0.015* ± 0.002	0.668* ± 0.088	3.293* ± 0.586	0.433* ± 0.077	23.45* ± 2.34
58.3	0.549* ± 0.035	0.025* ± 0.003	0.839* ± 0.078	3.127* ± 0.723	0.397* ± 0.076	24.54* ± 2.29
61.2	0.592* ± 0.034	0.036* ± 0.003	1.228* ± 0.086	3.040* ± 0.324	0.369* ± 0.063	27.12* ± 2.24
64.1	0.617* ± 0.039	0.058* ± 0.008	2.196* ± 0.195	2.570* ± 0.460	0.258* ± 0.032	31.13* ± 5.13
67.1	0.662* ± 0.031	0.086* ± 0.007	2.923* ± 0.261	3.089* ± 0.404	0.246* ± 0.027	33.61* ± 2.56
70.0	0.680* ± 0.038	0.114* ± 0.013	3.912* ± 0.413	2.743* ± 0.368	0.216* ± 0.021	27.88* ± 3.14

Table 2
Oscillatory frequency sweep measurements shown as complex shear modulus |G*|, shear storage modulus G′, shear loss modulus G″ and loss factor tan δ at 1 and 10 Hz. Results are shown as mean ± standard deviation (n = 3). Values in the same column that are significantly different from the lowest water addition (52.5 g water 100 g^{-1} flour) are marked (*p ≤ 0.05).

Water addition [%]	Frequency oszillation test								Creep-recovery test	
	1 Hz				10 Hz					
	G′ [10^3Pa]	G″ [10^3Pa]	|G*| 10^3 [Pa]	tan δ [−]	G′ [10^3Pa]	G″ [10^3Pa]	|G*| [10^3Pa]	tan δ [−]	J_{max} [10^{-3} Pa-1]	J_{el} [−]
52.5	33.5 ± 3.7	11.3 ± 1.2	35.4 ± 3.9	0.34 ± 0.00	51.3 ± 5.1	20.3 ± 2.0	55.1 ± 5.5	0.40 ± 0.00	0.24 ± 0.03	0.66 ± 0.01
55.4	22.2* ± 1.3	7.7* ± 0.3	23.5* ± 1.3	0.35 ± 0.01	34.3* ± 1.8	14.0* ± 0.4	37.0* ± 1.8	0.41 ± 0.01	0.38* ± 0.04	0.64* ± 0.03
58.3	13.9* ± 0.1	4.7* ± 0.1	14.7* ± 0.1	0.34 ± 0.01	21.8* ± 0.3	9.0* ± 0.3	23.6* ± 0.2	0.40 ± 0.02	0.78* ± 0.03	0.63* ± 0.01
61.2	11.5* ± 0.7	4.0* ± 0.2	12.2* ± 0.7	0.35* ± 0.00	18.9* ± 1.9	7.6* ± 0.6	20.4* ± 2.0	0.40 ± 0.01	1.00* ± 0.24	0.61* ± 0.01
64.1	89.3* ± 0.5	3.2* ± 0.2	9.5* ± 0.5	0.36* ± 0.01	14.6* ± 0.9	6.0* ± 0.4	15.8* ± 1.1	0.41 ± 0.01	2.71* ± 0.11	0.48* ± 0.01
67.1	64.2* ± 0.6	2.4* ± 0.3	6.8* ± 0.7	0.36* ± 0.01	10.4* ± 0.9	4.5* ± 0.5	11.3* ± 1.0	0.43* ± 0.02	6.56* ± 0.42	0.28* ± 0.02
70	51.3* ± 0.1	1.8* ± 0.0	5.5* ± 0.1	0.36* ± 0.00	8.4* ± 0.2	3.6* ± 0.1	9.1* ± 0.1	0.42* ± 0.01	17.29* ± 1.08	0.15* ± 0.01

3.5. Correlation of dough rheological results

Several rheological methods were used to evaluate the effect of water addition to flour. Thus one of the main questions was how these methods correlate with the water addition and with each other. Therefore in Table 3 the coefficients of correlation (r ≥ |0.60|) of most of the rheological measurements of dough and water addition are shown. In addition, the complex shear modulus, the shear loss and storage modulus at 1 and 10 Hz had a high negative correlation (r ≥ −0.91|) with water addition to flour. The relatively elastic part J_{el} of the creep-recovery test had a higher linear correlation (r = −0.910) with water addition than the loss factors of the SAOS, and both measurements were linearly connected, with a correlation coefficient of −0.763 (1 Hz) and −0.691 (10 Hz, data not shown).

The results of the uniaxial elongation dough test showed a significant linear correlation with other rheological measurements. In particular R^K_{max} showed a high dependency on |G*|. This fact emphasizes the importance of the elongation test. Furthermore the analysis of variance exhibited a highly significant correlation of the results of the stickiness measurement with the water addition (r ≥ 0.935). Wang et al. (1996) have already mentioned a strong correlation between the viscoelastic properties of dough with sensory stickiness. This assumption could be proven in this study with a linear correlation between D^S and |G*|. This validates the consideration that dough stickiness measurement is firstly dependent on the rheological behavior of dough and is secondly a type of rheological measurement itself. At the beginning of this method a bulk modulus is applied to the sample and in the recovery step the cohesive forces are influenced by the modulus of elasticity. The study shows high correlations of the applied rheological measurements. Therefore it can be recommended to minimize the amount of different methods in the future without losing important rheological data of the analyzed sample.

3.6. CLSM micrographs

One advantage of CLSM is the z-sectioning which inhibits damage to the structure of the sample. Fig. 1 shows optical sections of wheat dough with increasing water addition. All doughs were mixed to their optimum. Protein (black) was stained by rhodamine B. Gluten is a complex protein composed of protein submits linked by covalent and non-covalent bonds. The molecular properties result in a three-dimensional (micro) structure. Fig. 1 illustrates a considerable variation of the protein network with varying water content. Image analysis of the micrographs revealed the area fraction and the perimeter for A: 40.0%, 67.9 μm, B: 36.8%, 57.7 μm, C: 32.4%, 48.4 μm, and D: 24.3%, 33.3 μm, respectively. Micrograph A with the lowest water content exhibits a slightly clustered, though interconnected network of protein filaments. Between the protein strands some still empty areas can be detected but on the other hand the proteins are more compact compared with micrograph B. This means that the protein network is not evenly distributed. Due to the stiffness of the dough (|G*| of 35377 ± 3906 Pa), the developed protein network was stretched which is indicated by the orientation of the fibrils in the micrograph. This could be due to the high but short energy input during kneading (adjusted kneading time).

Micrograph B (optimum water addition with 1.8 g NaCl 100 g^{-1} flour) shows a uniformly distributed and widely aligned domain of proteins. Chemical bonds like hydrogen, ionic, hydrophobic and covalent bonds are responsible for the integrity of the dough's protein film. This leads to an entangled and physically cross-linked network. The white holes in the continuous network indicate an embedding of starch granules whereas a black ring around a white hole signals an inclusion of air bubbles.

A stronger clustering was obtained in micrograph C (64.1 g water addition 100 g^{-1} flour). Proteins seem to be more aggregated with more empty background which consists of e.g. water, starch granules,

Table 3
Correlation coefficient (r ≥ |0.60|) of significantly linear correlations between dough rheological measurements and water addition (*p ≤ 0.05, **p ≤ 0.01).

	Water addition	|G*| 1 Hz	tan δ 1 Hz	J_{max}	J_{el}	R^k_{max}	E^k	A^k	D^S	A^S
|G*| 1 Hz	−0.919**									
tan δ 1 Hz	0.800**	−0.744**								
tan δ 10 Hz	0.660**	−0.622**	0.731**							
J_{max}	0.813**									
J_{el}	−0.910**	0.710**	−0.763**	−0.936**						
R^k_{max}	−0.901**	0.949**	−0.734**		0.708**					
E^k	0.868**	−0.888**	0.687**		−0.664**	−0.891**				
A^k	−0.728**	0.791**				0.842**	−0.673**			
D^S	0.935**	−0.981**	0.636*	0.635*	−0.740**	−0.939**	0.887**	−0.757**		
A^S	0.952**	−0.811**	0.750**	0.926**	−0.970**	−0.794*	0.713**	−0.609**	0.837**	
C^S	0.955**	−0.808**	0.757**	0.908**	−0.974**	−0.797**	0.745**		0.826**	0.994**

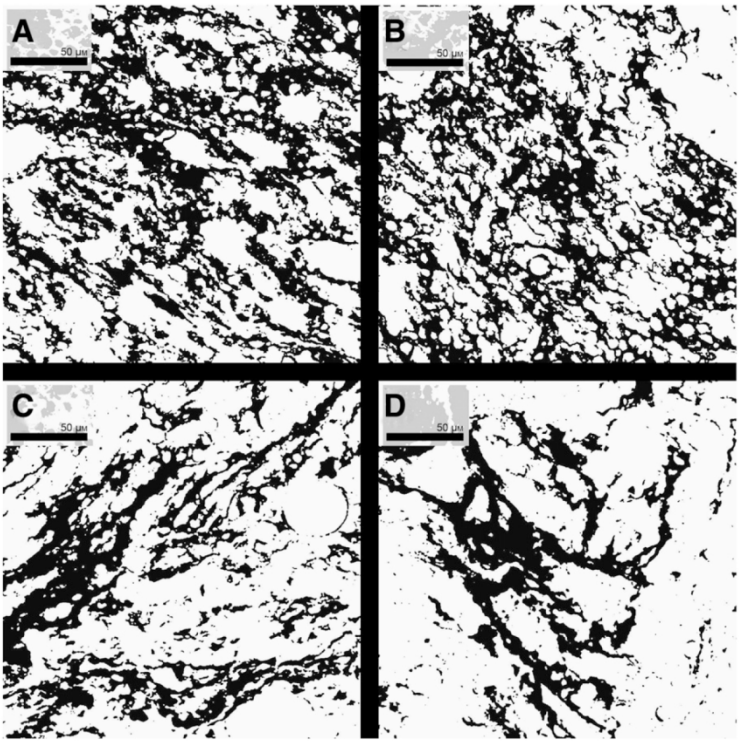

Fig. 1. Binary CLSM micrographs of wheat dough with 52.5 g (A), 58.3 g (B), 64.1 g (C) and 70.0 g (D) water addition 100 g^{-1} flour. Proteins are displayed in black.

and air bubbles. The extensibility of the dough nearly reached its optimum (Table 1) due to the still developed interconnected network. Nevertheless the more clustered domains of protein already resulted in a decrease of the dough resistance by 63%.

The highest water addition of 70.0 g 100 g^{-1} flour in micrograph D entailed a highly aggregated protein phase which is scattered, clustered and less interconnected. The signal revealed less incorporated starch granules which is in accordance with Letang et al. (1999), who stated that starch granules are less visible in highly hydrated dough than in hydrated dough due to a covering of continuous film. The reduced dough extensibility shown in Table 1 is based on this clustered structure.

3.7. Image analysis of the microstructure

Fig. 1 enables to explain the microstructural properties of the protein phase in the different doughs and a first subjective linkage to rheological behavior. However, micrographs taken from CLSM do not have a high visually detectable homogeneity. For a consequent quantitative extraction of the structural features of the proteins processing of the images obtained by CLSM is necessary to objectively distinguish image areas containing information from those containing background. Therefore images from different sections of the dough were processed and analyzed with the image analyzing tool imageJ (Table 4). Particle count ΣP and circularity C of the protein particles showed a fluctuation and therefore a low significance compared with the values obtained, whereas total area ΣA, average size ØA, perimeter P, fractal dimension FD and area fraction A_F (Fig. 2) showed more or less significant differences when water addition was increased.

The total area of detectable proteins was decreased by 28%, in accordance with the area fraction. Due to the increased water addition the real protein content was diluted by 10.1%. The difference between real and visually detectable dilution could be deduced to the alteration of the hydration properties of the flour components. One reason could be an increased hydration, expansion or congregation of starch granules due to excess of free water. Therefore the development of the protein network could be hindered and a lower area fraction is detected.

Table 4
Parameters related to protein properties obtained after image processing of CLSM micrographs as affected by the addition of water during dough preparation. Results are shown as mean±standard deviation (n = 14). Values in the same column that are significantly different from the lowest water addition (52.5 g water 100 g^{-1} flour) are marked (*$p \leq 0.05$).

Water addition [g water 100 g^{-1} flour]	Particle count ΣP [−]	Total area ΣA [10^4 µm^2]	Average size ØA [µm^2]	Perimeter P [µm]	Circularity C [−]	Fractal dimension FD [−]
52.5	500 ± 42	15.29 ± 0.79	308 ± 32	62.3 ± 5.7	0.767 ± 0.017	1.871 ± 0.008
55.4	535 ± 67	13.50* ± 1.16	258* ± 53	52.8* ± 5.8	0.780 ± 0.019	1.880* ± 0.012
58.3	580* ± 62	14.50 ± 0.97	252* ± 26	55.2* ± 3.7	0.776 ± 0.013	1.877 ± 0.010
61.2	572* ± 51	12.71* ± 0.82	225* ± 33	52.5* ± 7.1	0.761 ± 0.012	1.884 ± 0.010
64.1	527 ± 55	12.85* ± 0.95	247* ± 36	53.9* ± 6.7	0.772 ± 0.018	1.894* ± 0.010
67.1	508 ± 85	11.76* ± 1.57	240* ± 62	44.1* ± 8.2	0.797* ± 0.024	1.903* ± 0.016
70.0	557 ± 116	11.01* ± 1.09	184* ± 39	36.3* ± 6.3	0.828* ± 0.019	1.910* ± 0.010

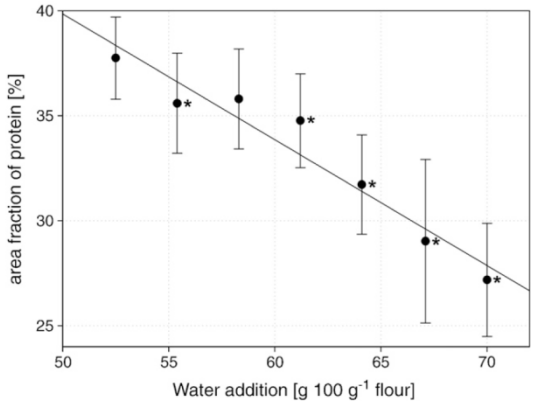

Fig. 2. Area fraction A_F [%] of proteins as affected by the addition of water during dough preparation. Results are shown as mean ± standard deviation (n = 14). Values marked with an asterisk are significantly different (*$p \leq 0.05$) from 52.5 g water addition 100 g^{-1} flour.

Nevertheless the measurements of the image analysis do not include the density of the components which influences the results. Therefore a less interconnected and spread amorphous fibril suprastructure of the gluten molecules and thus a higher density could pretend a relative loss in proteins.

Changes in rheological properties can be distinctly explained by the decrease of ØA by 40% and P by 42%. Due to smaller protein films and fibrils, there is a lower cohesion and elasticity. Fewer interconnected molecules created a less resilient network of proteins in dough. The small water molecules could interact with the network by hydrogen bonds between polypeptide chains and water molecules and therefore reduce the bonding forces between the chains (Entwistle & Rowe, 1979). This fact could also be an explanation for the increased extensibility of the dough. The fractal dimension exhibited a small but significant increase of 2%. This means that the surface of the proteins gets rougher. A more irregular surface structure of the highly hydrated proteins can be deduced. Furthermore, the results of the microstructure and the rheological properties of dough are not in accordance with some other studies (Berland & Launay, 1995; Letang et al., 1999). These studies reported that the loss factor tan δ is independent of the water content of dough and is therefore a parameter of the structure level of the dough's protein network. One study (Letang et al., 1999) deduced that there are no fundamental changes in the structure of dough between 45 and 60 g water 100 g^{-1} flour. However, due to the new investigations in this study, it can be assumed that water addition has an evident quantitative impact on the microstructure of dough.

3.8. Correlation of dough microstructure measurements with rheological results

The rheological behavior of foods and in particular of dough is often linked to the microstructure of the samples. Nevertheless, real relations are rarely proven, since discussion is mostly based on illustrative example. Although these investigations have improved the understanding of the behavior of dough, a quantitative analysis of the correlation of the microstructure of dough to its rheological properties has not yet been possible. In this study, in addition to the rheological evaluation of various dough samples, the microstructure of dough, namely its protein structure, was investigated and the results correlated (Table 5). All parameters, other than the particle count ΣP, showed highly significant linear relations to the water addition to flour.

In particular, the area fraction A_F exhibited a high negative correlation ($r = -0.850$) to water addition. The complex shear modulus revealed correlation coefficients up to 0.786 with A_F. Thus a wider dispersed protein network, which is evident in more and wider fibrils and especially films, leads to a higher stiffness of dough. The loss factor tan δ showed significant but low correlation coefficients from |0.500| to |0.599| with A_F, FD, and C. From this fact and mainly from correlations between the rheological properties of dough, it can be concluded that tan δ cannot be taken as a proper parameter to describe the structural level of the protein network. Furthermore, the results indicate that a variation of water addition has a distinct effect on the dough's microstructure, as mentioned above.

Additionally, A_F showed high correlations with the rheological parameters. The smaller the visually detectable phase of protein became, the more the extensibility E^K in the uniaxial elongation test increased. At the same time the resistance to elongation R^K_{max} decreased. This means that small protein particles enable a specific mobility between phases in dough which weakens the dough and decreases the resistance. Lee et al. (2001) deduced from CLSM micrographs that weak dough has the lowest content of protein matrix and strong dough the highest, which could be quantitatively proven in this study. Furthermore, it is reported that starch–starch interactions are an important source of elasticity in dough (Amemiya & Menjivar, 1992). The current results show the strong relation of the relative elastic part J_{el} of a creep recovery test ($r = 0.799$) with the visual area fraction of proteins. Therefore it can be assumed that starch–starch interactions play a less important role for the elastic properties of dough. In summary, for the first time it was possible to prove quantitatively the assumption of the relationship between the microstructure of dough with its rheological properties.

4. Conclusion

Dough's microstructure is assumed to be the basis of its viscoelastic behavior. Until now, it was hardly possible to prove this assumption mathematically. Therefore it was necessary to develop a new method to obtain values from microscopic image data to enable correlation analysis. The combination of novel techniques such as CLSM with processing and analysis of the micrographs obtained proved to be a powerful method to determine quantitatively the microstructure of dough proteins. The significant relations of the image data such as the area fraction or perimeter with the varied water amount in dough demonstrated the suitability of the established method.

Table 5
Correlation coefficient ($r \geq |0.60|$) of significantly linear correlations between dough rheological measurements and parameters from image analysis (*$p \leq 0.05$, **$p \leq 0.01$).

	Water addition	Fundamental rheology			Stickiness and uniaxial elongation					Image analysis				
		|G*| 1 Hz	J_{max}	J_{el}	R^k_{max}	E^k	D^S	A^S	C^S	ΣP	ØA	A_F	FD	C
ØA	−0.656**	0.657*	−0.642*		0.6232*		−0.722**	−0.650*	−0.611*	−0.668**					
A_F	−0.850**	0.786**	−0.688**	0.799**	0.764**	−0.760**	−0.810**	−0.792**	−0.810**		0.691**				
FD	0.803**	−0.746**	0.650*	−0.746**	−0.720**	0.720**	0.766**	0.746**	0.763**		−0.729**	−0.986**			
C	0.668**		0.848**	−0.856**				0.756**	0.771**			−0.724**	0.674**		
P	−0.740**	0.658*	−0.735**	0.735**	0.613*		−0.729**	−0.750**	−0.733**		0.902**	0.862**	−0.873**	−0.738**	

Moreover, rheological analysis of dough with varied water addition in the range of 52.5–70.0 g water 100 g^{-1} flour showed a rapid decrease of stiffness as well as elastic characteristics of the dough. These results could be confirmed in SAOS as well as in creep recovery tests.

In detail, it was proven that A) most of the rheological methods had highly significant correlations with each other, due at least to the variation of water addition to wheat flour, B) the established method is successfully applicable to investigate the dough's microstructure, in particular its protein microstructure and C) there are high linear correlations between the protein microstructure of dough and its rheological properties. Therefore the methodology enables new investigations into the complex composition of the chemical and physical interactions of dough in future. In summary, CLSM has proven to be a powerful tool for examining the dough protein microstructure.

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2.4 Effects of acidification, sodium chloride, and moisture levels on wheat dough: I. Modeling of rheological and microstructural properties

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ORIGINAL ARTICLE

Effects of Acidification, Sodium Chloride, and Moisture Levels on Wheat Dough: I. Modeling of Rheological and Microstructural Properties

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Abstract The rheological attributes of polymers as wheat dough are strongly related to its microstructure. To quantify dough protein microstructure confocal laser scanning microscopy combined with image analysis was used. The effect of three experimental factors pH (addition of lactic acid and sodium hydroxide), water addition, and sodium chloride (NaCl) addition on empirical and fundamental rheological properties as well as microstructural protein properties were studied and modeled by applying a response surface methodology. The obtained models revealed high correlations between the experimental factors and the complex shear modulus (R^2=0.97), dough resistance (R_{max}^k; R^2=0.91) and stickiness (R^2=0.93). Furthermore it was possible to determine microstructural attributes as the area fraction (R^2=0.88) and Feret's diameter (R^2=0.86) as a function of pH, water and NaCl addition. Especially measures of R^k_{max} revealed highly significant correlations with the protein microstructure as the branching index (r=0.79).

Keywords RSM · CLSM · Confocal · Sodium chloride · Image analysis · Gluten · Microstructure

Abbreviations

A^S	Work of adhesion
A_F	Area fraction
ØA	Average size
BI	Branching index
BU	Brabender units
C	Circularity
CLSM	Confocal laser scanning microscopy
C^S	Dough cohesiveness
D^S	Stickiness
D_F	Feret's diameter
E^K	Dough extensibility
FD	Fractal dimension
G'	Shear storage modulus
G"	Shear loss modulus
|G*|	Complex shear modulus
J_{el}	Relative elastic part of J_{max}
J_{max}	Maximum creep compliance
J_r	Creep recovery compliance
NaCl	Sodium chloride
P	Perimeter
ΣP	Particle count
PH	pH
R^k_{max}	Dough resistance
RSM	Response surface methodology
R^2	Adjusted square correlation coefficient of the fitting model
S	Sodium chloride
tan δ	Loss factor
W	Water

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Introduction

Cereal dough is commonly based on a combination of flour, water, salt, additives, and other ingredients. The distribution of water during the first part of mixing causes the components to absorb water and swell, and the mechanical energy input from the mixers results in an organized continuous protein matrix. The dough matrix can be seen as a bicontinuous system where the described protein matrix forms one

phase and the liquid phase, containing soluble substances and insoluble starch, the other.[1] Another more detailed view of dough is a three phase system, also with a protein network, with a continuous phase of free water where the starch granules and water-soluble components are located and the gas phase which is entrapped during the mixing process and an optional proofing time, forms the third phase.

The behavior of these phases during mechanical energy input specifies the dough's rheological properties.[2] The term rheology is defined as the study of flow and deformation of materials.[3] In wheat dough the properties of a non-Newtonian viscous liquid and a Hookean elastic solid must be analyzed simultaneously. Therefore, this dough can be classified as a viscoelastic material in which gluten proteins have a dominant rheological impact.[4] The characterization of these properties illustrates an effective prediction, for example of process behavior, and can finally lead to the control of food product quality.[5–7] Hence, rheological analysis is commonly used to assess the properties of dough, and to gain knowledge of the functions of dough ingredients and structure.[8]

Rheological assessment is quantitatively linked with the molecular structure of polymers.[3] Because of this, rheology has been used to describe the molecular structure of starch and gluten.[9] The quantity of the connections (based on the molecular level) between the polymers constitutes the microstructure of materials. This microstructure determines the appearance, shelf life and taste perception of the end product. A number of microscopic and imaging techniques have already been used to investigate and understand the microstructure of dough in particular.[10] In cereal science, scanning electron microscopy,[11–13] light (fluorescence) microscopy [14–16] and confocal laser scanning microscopy (CLSM), as a further development of the light microscopy,[7,17–20] have been applied to gain insight into the microstructure of grain, dough, and their components such as starch and proteins.

The CLSM enables the acquisition of a specific focal section of a sample without the necessity of a complex preparation such as fixing or dehydration. This nearly non-invasive observation facilitates the visualization of cross-links between structural elements or the kinetics of visual changes over time. However, the micrographs only deliver a section of the microstructure. For the extraction of structural features it is therefore necessary to process and analyze several images to obtain structural measures.[10] Some studies have already applied the image processing of CLSM features to cereal science,[11,14,21–25] however, this work barley covers structural features such as the circularity or the perimeter of a particle or object in correlation with analyzed properties of the dough.

A method for quantifying the protein microstructure of wheat dough was applied in a work of Jekle and Becker [10] and correlations with rheological properties of dough could be proofed. The correlation analysis revealed high significant linear correlations between image analysis measurements and fundamental as well as empirical rheological attributes. In the current study this methodology is evaluated by the use of response surface methodology (RSM). The possibilities of RSM in the application in cereal chemistry and science has been proven by different studies.[9,26–28] RSM can be used to evaluate the relative significance of several factors. It is an empirical statistical modeling technique which even works in the presence of complex interactions. Quantitative data obtained from designed and randomized experiments are used for multiple regression analysis to develop multivariate equations simultaneously.[29] In the current work, the experimental factors are the pH of dough (3.2–6.8) and the addition of water (47.9–68.7 g 100 g^{-1} flour) and NaCl (0.0–5.0 g 100 g^{-1} flour). These, previously examined, variables cause distinct changes of rheological and structural properties in wheat dough.[7,19,30–36] However, neither a complete observation of their combined effect on empirical and fundamental rheological attributes or a microstructural analysis has been performed on these variables.

In summary, the aims of the present study are a) to determine the influence of the experimental factors pH, water and NaCl addition on rheological and microstructural properties in wheat dough, b) to validate the methodology of the quantification of the protein microstructure using a RSM and c) to calculate the correlations between these attributes. Therefore, rheological relations in the protein microstructure can be evaluated.

Materials and Methods

Basic Ingredients and Analysis

For the dough preparation wheat flour type 550 harvested 2008 from Rosenmühle, Ergolding, Germany was used. Moisture content, water absorption, α-amylase activity and ash content were determined according to the International Association for Cereal Science and Technology's (ICC) principles 110/1, 115/1, 107/1 and 104/1,[37] respectively. The protein content was analyzed using the Kjeldahl Method (EBC).[38] For CLSM micrographs rhodamine B from Sigma-Aldrich Chemie Gmbh, Munich, Germany was used. Sodium chloride (NaCl), lactic acid, and sodium hydroxide solution were obtained from Südsalz GmbH, Heilbronn, Germany; VWR International GmbH, Darmstadt, Germany; and Merck, Darmstadt, Germany, respectively.

Experimental Design

A central composite in a three block design was used with three variables for a total of 60 experiments (Table 1). The

design was created out of a factorial screening design in two blocks 2^3 with two centerpoints per block, two replications and the addition of starpoints. The design was randomized and arranged in 9 blocks. The three variables were pH (*PH*), sodium chloride addition (*S*, g 100 g^{-1} flour) and water addition (*W*, g 100 g^{-1} flour). The experimental conditions of the center point were *PH*=5.0, *S*=1.8 g 100 g^{-1} flour and *W*=58.3 g 100 g^{-1} flour.

Dough Preparation

Dough was prepared in a DoughLab in a 50 g bowl (Perten Instruments GmbH, Hamburg, Germany). Wheat flour (50.0 g, corrected to 14 % moisture) was premixed for 60 s to reach the defined temperature of 30 °C in the bowl. Water including NaCl, lactic acid, and/or sodium hydroxide solution was added in agreement with the experimental design (see Table 1) and dough was mixed at a mixing speed of 63 rpm. The amount of lactic acid or sodium hydroxide solution needed to reach the required pH was analyzed by three nonlinear regression equations of 0.0, 1.8 and 3.6 g 100 g^{-1} flour and lactic acid concentrations of 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 g 100 g^{-1} flour. As mixing conditions have great influence on rheology,[39] each dough was mixed until optimal development (maximum 480 s). The optimal mixing time of each dough was based on pre-examinations.

Functional Dough Properties

Stickiness Measurement Dough machinablity or handling was assessed by stickiness measurement using a TA-XT 2 texture profile analyzer (TPA) (Stable Micro Systems, Godalming, UK) and a dough stickiness rig. Both stickiness and uniaxial dough measurement settings followed the method described by Jekle and Becker.[10] Stickiness (D^S), work of adhesion (A^S), and dough cohesiveness (C^S) were recorded and 12 separate measurements were performed per experiment.

Uniaxial Dough Measurement Dough extensibility tests were performed by a TPA using a SMS/kieffer rig (Stable Micro System, Godalming, Great Britain). The following measurements were recorded: R^k_{max} (maximum resistance) and E^k (extensibility), which are consistent with those obtained using the Brabender Extensograph.[40] In each experiment at least 6 measurements were performed.

Rheometer Settings and Procedures The dough was analyzed with an AR-G2 rheometer (TA instruments, New Castle, USA). A 40 mm plate-plate geometry (serrated surface on both plates to avoid slippage) and a gap of 2 mm was used at a fixed temperature of 30 °C (with a thermal cover). A sample of 8 g was cut from the dough and placed between the plates. The excess dough was trimmed and paraffin was applied to the free surface of the dough to prevent sample drying during the test. The dough was allowed to rest for 10 min (to dissipate a built-up residual stress due to the clamp of the sample). First, an oscillatory frequency sweep was performed with a frequency sweep from 0.1 to 100.0 Hz at constant strain amplitude (0.001). Preliminary tests indicated that the deformation was well within the linear viscoelastic region of the sample. Dynamic rheological properties were recorded by the complex shear modulus |G*| and the loss factor tan δ, which was calculated as the ratio of the shear loss modulus G" to the shear storage modulus G'. Afterwards, a creep recovery test was performed with the following settings: A constant shear stress τ_0 of 250 Pa at 30 °C for 180 s. After the constant stress, the sample was recorded for 360 s, providing the recovery time was long enough for the steady state to be reached. Final data were expressed in terms of the stress dependent recoverable shear deformation, the creep compliance J_{max} (at t=180 s of the creep phase), and the relative elastic part of the creep-recovery test J_{el}, calculated by $J_{el}=J_r\ (J_{max})^{-1}$, with J_r as the creep recovery compliance (at t=360 s of the recovery phase).

CLSM Micrographs and Digital Image Processing and Analysis

For the acquisition of CLSM micrographs a Ti-U inverted research microscope with an e-C1plus confocal system (Nikon, Düsseldorf; Germany) and a 20 x (N.A.= 0.75) objective was used. Dough was prepared as described above with an addition of rhodamine B at a concentration of 0.001 g 100 mL^{-1} water. The protein phase was monitored as fluorescence image (λ_{exc}= 543 nm, λ_{em}=590/50 nm) with 1024×1024 pixel resolution (212×212 μm) in a constant z-position. From every experiment seven independent positions on the x-y-axis were recorded and analyzed. The addition of rhodamine B did not significantly influence ($p>0.05$) the rheological properties of the dough.

The images were processed using the image processing and analysis open source Java software ImageJ (version 1.42q, National Institutes Health, Bethesda, Md, USA). After pre-processing by changing to grey-level, proteins were isolated from the background using the fuzzy thresholding algorithm of Huang et al..[41] With this algorithm the characteristic relationship between an object and background expressed in a pixel and its related region could be illustrated. The binary micrographs were analyzed by the features of proteins: particle count (ΣP,

Table 1 Central composite design in 3 blocks with the experimental factors pH (*PH*), sodium chloride (*S*) and water addition (*W*)

Run	pH (*PH*) [-]	NaCl (*S*) [g 100 g^{-1} flour]	Water addition (*W*) [g 100 g^{-1} flour]
1	4.00	0.00	52.5
2	5.00	1.80	58.3
3	5.00	1.80	58.3
4	6.00	0.00	64.1
5	6.00	3.60	52.5
6	4.00	3.60	64.1
7	6.00	3.60	64.1
8	5.00	1.80	58.3
9	4.00	0.00	64.1
10	6.00	0.00	52.5
11	4.00	3.60	52.5
12	5.00	1.80	58.3
13	4.00	0.00	52.5
14	5.00	1.80	58.3
15	5.00	1.80	58.3
16	6.00	0.00	64.1
17	6.00	3.60	52.5
18	4.00	3.60	64.1
19	6.00	3.60	64.1
20	5.00	1.80	58.3
21	4.00	0.00	64.1
22	6.00	0.00	52.5
23	4.00	3.60	52.5
24	5.00	1.80	58.3
25	4.00	0.00	52.5
26	5.00	1.80	58.3
27	5.00	1.80	58.3
28	6.00	0.00	64.1
29	6.00	3.60	52.5
30	4.00	3.60	64.1
31	6.00	3.60	64.1
32	5.00	1.80	58.3
33	4.00	0.00	64.1
34	6.00	0.00	52.5
35	4.00	3.60	52.5
36	5.00	1.80	58.3
37	5.00	1.80	68.7
38	5.00	1.80	58.3
39	5.00	1.80	47.9
40	3.21	1.80	58.3
41	5.00	0.00	58.3
42	6.79	1.80	58.3
43	5.00	5.02	58.3
44	5.00	1.80	58.3
45	5.00	1.80	68.7
46	5.00	1.80	58.3
47	5.00	1.80	47.9
48	3.21	1.80	58.3
49	5.00	0.00	58.3
50	6.79	1.80	58.3
51	5.00	5.02	58.3
52	5.00	1.80	58.3
53	5.00	1.80	68.7
54	5.00	1.80	58.3
55	5.00	1.80	47.9
56	3.21	1.80	58.3
57	5.00	0.00	58.3
58	6.79	1.80	58.3
59	5.00	5.02	58.3
60	5.00	1.80	58.3

[-]), average size (ØA, [μm^2]), area fraction (A_F, [%]), perimeter (P, [μm]), circularity (C, [-], calculated by $4\ \pi\ A\ P^{-2}$, where A is the area of the specific particle and P is the perimeter of this particle), Feret's diameter (D_F, [μm]) and the fractal dimension of the image (FD, [-]).[10] The fractal dimension is a characterization of the roughness of an image.[42] It can be associated with the complexity of forms where complex or rougher grey level images lead to a high value of FD while smoother or simple images mean a low value. Further, the "branching index" BI was established, where $BI = P\ F^{-1}$.

Statistical Analysis

Multivariate analysis (stepwise regressions) and response surface plots (main effects plots) were performed using Statgraphics Centurion (version 15.1.02 Statpoint Technologies Inc. Warrenton, Virginia). The design enabled approximations of the measured data (*y*) using RSM. The second-order polynomial model (Eq. 1) includes all interaction terms:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}{x_1}^2 + b_{22}{x_2}^2 + b_{33}{x_3}^2 \quad (1)$$

Where y is the response data, x_1-x_3 are the experimental factors, b_0 the constant, b_1-b_3 the linear coefficient, b_{11}, b_{22}, b_{33} the quadratic coefficient, and b_{12}, b_{13}, b_{23} the interaction coefficient. The significant interactions of the three experimental factors *PH*, *S* and *W* to the 14 dependent variables $|G^*|$, tan δ, J_{el}, $R^K{}_{max}$, E^K, D^S, A_F, FD, ΣP, D_F, P, ØA, C and BI, respectively, were estimated using this equation. Furthermore a linear regression analysis was performed to explore the relationships between variables.

Results and Discussion

Physicochemical Characteristics of the Wheat Flour

The flour had the following values: protein content 11.56±0.18 g 100 g^{-1} flour d.m. (n=2), ash content 0.67±0.01 g kg^{-1} flour (n=3), moisture content 13.73±0.12 g 100 g^{-1} flour (n=5), falling number 382±10 s (n=5). Results obtained by ICC method 115/1 showed: water absorption 60.2±0.2 % (n=3), dough development time 2.9±0.5 min (n=3), dough stability 17.1±1.4 min (n=3), degree of softening 24.8±6.8 BU (n=3). The method was repeated with a dough containing 1.8 % NaCl (dissolved in water): 58.3±0.1 %, 3.9±2.2 min, 18.8±0.1 min, and 5.3±2.6 BU, respectively. The observed changes owing to the addition of NaCl have already been described in the literature.[7,19] The reasons for this are a strengthened gluten network formation due to changes in the overall excess charge of the protein forming amino acids and thus less water absorption capability of the dough.

Effects of the Experimental Factors pH, Sodium Chloride and Water Addition on Dough Properties

Analytical data from the experimental design (Table 1) were fitted to multiple regressions to estimate response surfaces on dependent quality variables (Table 2). Significant coefficients (p<0.05) of the stepwise regression fitting model are shown. The achieved mathematical model can be expressed in equations of unscaled variables. Equation 2 exemplarily shows this for |G*|,

$$|G*| = 868695 - 75184PH - 821S - 19449W + 4735PH^2 + 130W^2 + 352WPH. \quad (2)$$

Empirical and Fundamental Rheological Properties

The rheological attributes (shown in Table 2) revealed a high dependency on the design variables water addition, pH, and NaCl addition. All measures were significantly affected by the variables except for the loss factor tan δ and the relative elastic part of the creep recovery test J_{el} as a function of NaCl addition.

The variation of the water addition also revealed quadratic effects on |G*|, J_{el} and $R_{max}{}^{K}$. The decrease of |G*| (Figure 1) as a function of the water addition confirms other studies in which the water addition was varied in a linear experimental design.[10] This serves as an affirmation for the validity of the experiment design used. The main effect plots show the dependency of the respective measures of the experimental factors on all other factors held constant at a value midway between their highs and lows. The observed

Table 2 Coefficients (95 % confidence interval) of the experimental factors of the stepwise regression fitting model[a]

Factor[b]	|G*|	tanδ	J_{el}	R^{K}_{max}	E^{K}	D^{S}	A_F	FD	ΣP	D_F	P	ØA	C	Bl
Constant	868695	0.464	-2.728	11.93	-9.274	-3.956	167.6	1.162	242.3	19.17	88.86	6666	0.680	25.35
PH	-75184	0.030	-0.244	0.129	0.786	0.863	12.49	-0.050	32.18	-6.369	-2.158	-48.58	0.065	0.169
S	-820.9	ns.	ns.	-0.148	-7.632	-0.184	-13.17	0.055	-151.0	7.678	7.842	ns.	-0.054	-0.926
W	-19449	-0.009	0.153	-0.367	0.395	0.052	-4.940	0.025	ns.	0.180	-0.202	-195.9	-0.001	-0.659
S^2	ns.	ns.	ns.	-0.021	ns.	ns.	ns.	ns.	ns.	0.175	0.685	ns.	ns.	ns.
W^2	130.0	ns.	-0.001	0.003	ns.	ns.	0.038	0.000	ns.	ns.	ns.	1.582	ns.	0.005
PH^2	4735	-0.012	0.215	ns.	ns.	-0.049	-1.259	0.005	ns.	0.579	ns.	ns.	-0.007	ns.
SW	ns.	ns.	ns.	ns.	ns.	0.003	ns.	ns.	ns.	-0.070	-0.202	ns.	0.001	ns.
SPH	ns.	ns.	ns.	0.047	1.529	ns.	2.106	-0.009	26.785	-0.734	ns.	ns.	0.003	0.135
WPH	351.9	0.002	ns.	-0.003	ns.	-0.006	ns.	ns.	ns.	ns.	ns.	ns.	ns.	ns.
R^2	0.967	0.682	0.827	0.911	0.518	0.927	0.884	0.855	0.670	0.863	0.536	0.465	0.378	0.820

[a] The experimental factors were pH (*PH*), sodium chloride (*S*) and water addition (*W*)

[b] |G*|, complex shear modulus, tan δ, loss factor, J_{el}, relative elastic part of creep recovery test, R^{K}_{max}, resistance, E^{K}, extensibility, D^{S}, stickiness, A_F, area fraction, FD, fractal dimension, ΣP, particle count, D_F, Feret's diameter, P, perimeter, ØA, average size, C, circularity, BI, branching index, ns, no significant effect, R^2, adjusted square correlation coefficient of the fitting model

softening effect of the water is based on the plasticizing effect of the water molecules. Dough or gluten can be considered as a polymer.[43] The addition of water to polymers causes a shift from the rubbery state to a flow state. Meanwhile the entanglement network disappears due to slippage. The loss factor as the ratio of the loss modulus to the storage modulus reveals a linear increase with higher water addition. This means that the rate of elastic behavior decreases. In addition to the fundamental rheological measures uniaxial load-extension measurements were also performed. The resistance R^K_{max} shows a decrease with increased water addition. The soft continuous phase of the dough increases and fewer noncovalent bonds can be developed due to the dilution with water molecules. This leads to less resistance of the dough against elongations in the extensibility rig used. The stickiness is also positively influenced by the water addition, which supports the results of other studies.[10,44,45] The change of D^S can be ascribed to the increased excess of water on the contact area between the dough and the plunger which results in increase of adhesion. Van Velzen et al. [45] suggested a second reason: the migration of hydrated gluten molecules to the upper dough layers due to the increased mobility of higher water content.

The chemically adjusted decrease of the pH results in an overall increase in stiffness, reported as |G*| with a local minimum at pH 5.6. Shiau and Yeh [46] reported a decreasing apparent viscosity of noodle dough at 50 °C. However, the observed range of pH was distinctly different and the results could indicate an inflection point at a pH of around 5 to an increasing apparent viscosity with decreasing pH. One reason could be the change from a negative charge over pH 6 to a positive net charge of wheat flour under pH 6.[47] Further, other authors also reported an increase in stiffness (viscosity) with decreasing pH [30,33] in an pH range of about 6 to 4. Therefore, a slight change of the conformation of the proteins could occur and more water is bound to the proteins, wherefrom less water is available in the dough system. An increased swelling of gluten proteins with decreased pH as already been investigated by Schober et al..[35] The mentioned inflection point of the behavior of |G*| in the study of Shiau et al. [46] could be also recognized in the slope of tan δ. Additionally, the present results also reveal a decreasing loss modulus under pH 5.6. Since tan δ is the ration of G" to G', this indicates more elastic-like behavior. The modification of rheological measures with pH could be based on the pH-dependent SH/SS interchange reaction in the protein phase.[33] Another theory is the effect from oxidative gelation reactions involving polysaccharide-polysaccharide or polysaccharide-protein covalent cross-linkings (pentosans cross-linked by ferulic acids).[48] The resistance R^K_{max} does not show a quadratic effect from the pH (Table 2 and Figure 1). However, R^K_{max} positively linearly correlates with the pH, which also indicates a change in the conformation of the proteins. The behavior is in accordance with results from cracker sponges.[49] The analysis of the stickiness D^S exposes a decreased value below pH 5.2. The discussed rheological properties could to some extent be a result of the change in the overall net charge due to its pH dependency. A neutral charge causes less repulsion forces and less space for water molecules between the proteins. These repulsion forces increase with increasing charge, and more water molecules can be attach to the protein strands whereby less mobile water is available in the system. Therefore the

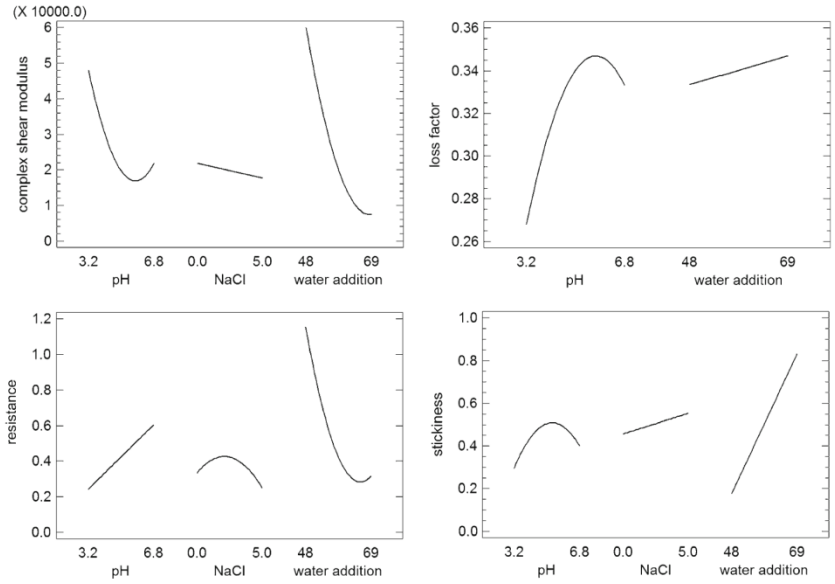

Fig. 1 Main effect plots of the complex shear modulus |G*|, the loss factor tan δ, the resistance R^K_{max}, and the stickiness D^S as a function of significant ($p<0.05$) experimental parameters

stickiness decreases. Further, |G*|, tan δ, R^K_{max} and D^S had interaction effects between water addition and pH.

The results revealed a slight decline of |G*| with increased NaCl addition to the dough. Other studies showed different variant results: small increase of |G*| (only significant from 0 to 2.5 and 3.00 g NaCl 100 g^{-1} flour,[7,19] and no significant different values of |G*|,[33,34] whereby dough in these studies was produced without an adjusted development time for every amount of NaCl addition. However, the addition of NaCl has a distinct impact on dough development time: at pH 4 and a water addition of 52.5 g 100 g^{-1} flour or 64.1 g 100 g^{-1} flour the development time changes with an addition of NaCl from 0 to 3.6 g 100 g^{-1} flour from 3.9±0.6 to 1.9±0.2 g 100 g^{-1} flour or 2.2±0.1 to 1.6±0.0 g 100 g^{-1} flour, respectively (n= 2). With an adaptation of the development time the pure effect of the changes on the rheological properties can be shown. The charge of the gluten molecules in dough normally leads to a small repulse of each other. This charge could be slightly neutralized with the addition of sodium chloride. Therefore, its hydration could be reduced and more water would be available in the system, leading to a softening of the dough. The increase of D^S with increased NaCl addition is in accordance with the study of Beck et al. [7,19] in which the sodium chloride concentration was linearly increased from 0 to 40 g 100 g^{-1} flour.

Additionally, the adjusted square correlation coefficient of the stepwise regression fitting model proves high accuracy in predicting the rheological data as a function of the experimental factors pH, sodium chloride and water addition. Especially |G*|, R^K_{max}, D^S and J_{el} reveals high coefficients of determination of 0.97, 0.91, 0.93 and 0.83, respectively.

Image Analysis Measures

Proteins are a major determinant of the rheological properties of wheat dough.[50] The usage of CLSM enables the visualization of the developed protein network. The laser light of the CLSM can permeate the surface and therefore scans deeper layers without a modification of the visualized section. In combination with an image analyzing and processing tool, the effects of process and recipe variables on the protein microstructure of wheat dough can be detected directly. The variation of the experimental factors pH, water, and NaCl addition reveals significant effects on the measures of the protein phase in the micrographs by image processing (expect water addition to ΣP and NaCl addition to ØA, shown in Table 2). This underlines the possibilities of this methodology in obtaining direct insights into the structural composition of dough. Furthermore, Table 2 shows that the numerical models of A_F, FD, D_F, BI can well predict the measures with adjusted square correlation coefficient of 0.88, 0.85, 0.86 and 0.82, respectively.

The image analysis used reveals a decrease of the area fraction A_F and the perimeter as a function of water addition to flour. This confirms the result of an earlier study where water addition to flour was linearly varied with a high resolution.[10] The reasons for this are the dilution effect of the added water and a disturbed structuring of the protein network as well as influences on the density of the detectable proteins. The established measure, the branching index BI, stands for the spreading of the proteins. A BI of 3.14 implies a round shape or a circle. As BI gets higher, the more the shape of the proteins spreads. A higher water addition leads to protein particles with a rounder shape

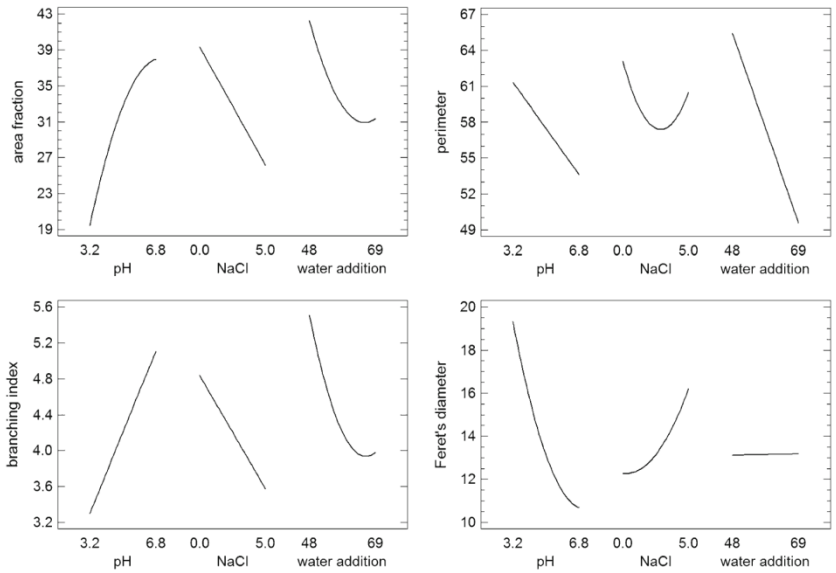

Fig. 2 Main effect plots of the image analysis measures area fraction A_F, perimeter P, branching index BI, and Feret's diameter D_F as a function of significant (p<0.05) experimental parameters

Table 3 Coefficient of significant linear correlations between the rheological properties and measurements of the image analysis (*$p \leq 0.05$, **$p \leq 0.01$)

	fundamental rheology				stickiness and uniaxial elongation				image analysis						
	J_{max}	J_{el}	$\lvert G^* \rvert$	tan δ	E^k	R^k_{max}	D^S	A^S	C^S	ΣP	ØA	A_F	P	C	D_F	FD
J_{el}	-0.952**															
$\lvert G^* \rvert$	-0.469**	0.600**														
tan δ	0.483**	-0.564**	-0.353*													
E^k	-0.450**	-0.450**	-0.458**	0.536**												
R^k_{max}		0.316*	0.651**													
D^S	0.558**	-0.676**	-0.900**		0.332*	-0.653**										
A^S	0.845**	-0.854**	-0.672**		0.370**	-0.500**	0.765**									
C^S	0.737**	-0.756**	-0.679**		0.413**	-0.488**	0.706**	0.948**								
ΣP		-0.297*	0.377**	0.550**	0.597**											
ØA		0.286*	0.762**	-0.294*	-0.322*	0.454**	-0.407**			-0.561**						
A^F						0.585**				0.497**	0.307*					
P	-0.565**	0.606**	0.593**	-0.517**	-0.445	0.277*	-0.529**	-0.434**	-0.363**	-0.443**	0.560**					
C	0.316*	-0.359**		0.396**							0.300*		-0.411**			
D_F				-0.557**	-0.428**	-0.709**				-0.773		-0.774**	0.405**	-0.470**		
FD						-0.615**	0.313*			-0.415**	-0.394**	-0.993**		-0.289*	0.732**	
BI			0.303*			0.793**	-0.394**			0.458**		0.919**	0.306*		-0.739**	-0.912**

due to less interconnected strains that results from the plasticizing effect of the water molecules.

Schober et al. [35] analyzed gluten with a CLSM after 2 h of swelling in solutions of deionized water and the addition of a lactate buffer and NaCl. A pH of 3.9 caused an increased swelling and a filmlike structure. However, the current results, that analyze of a wheat dough 10 min after kneading, achieve a decline of A_F and BI as well as increasing P, D_F and FD. There are two explanations for this effect: on the one hand the proteins reveal a denser structure with decreasing pH values. The decreased number of proteins still appear as strands, with increased width and fewer interconnections. Furthermore, the Feret's diameter increases more than the perimeter and the branching index decreases due to fewer connected strands. Another evidence for this behavior is the reduced A_F with reduced pH. More compact proteins strains could result in a loss of detectable protein area by the image processing tool. The second possibility which would also underline the results of Schober et al. [35] is the theory of more dissolved proteins due to the reduced pH. Proteins strands unfold due to the increased intramolecular electrostatic repulsion at lower pH and a partial dissolution of strands occurs.[51] Therefore the detectable area of a cohesive protein structure is reduced.

The variation of the NaCl addition reveals significant effects ($p<0.05$) on the measures of the visual protein quantification except for the average size of the proteins. The main effects plots in Figure 2 illustrate a decrease of A_F and BI as well as an increase of D_F. The proteins strands are enlarged but also show a more scattered or isolated structure whereby the BI decreases. The protein structure appears more elongated, and this is reflected in the increased Feret's diameter. The dense microstructure could be based on the shielding effect of the salt anions (Cl^-) at the positive net charge of the gluten molecules which leads to decreased intra- and intermolecular electrostatic repulsion of the molecules.

Correlation of Dough Rheology with Image Analysis Measures

In polymer and food analysis microstructure and rheology are often interrelated; however, hardly any work has been done in the correlation of these two aspects in cereal science. Jekle and Becker [10] have established a method to quantify dough's microstructure, thereby enabling correlation with rheological results. Table 3 shows the multivariate data handling of the measured analytical rheological and microstructural variables of the current work, providing information on the significant correlations within the parameters. The correlation coefficients were obtained by Pearson correlation analysis. Firstly the rheological attributes show high significant correlation. The maximum creep compliance J_{max} correlates linearly with J_{el} (r=-0.952) and the empirical rheological measures A^S (r=0.845) and C^S (r=0.737). The high shear stress of 250 Pa seems to describe the adhesion and cohesion forces described by the stickiness measurement adequately. Furthermore, |G*| has high significant correlation with D^S (r=-0.900). This means that stickiness linearly decreases with increased dough stiffness even during the variation of three experimental factors. Stiffer dough has less molecular mobility, whereby, the contact area between dough and corresponding surface decreases due to decreased viscosity of the dough as a function of time. In summary, besides tan δ, rheological attributes consistently correlate linearly.

The analyzed microstructural values of the dough's protein phase also revealed significant correlations with rheological attributes ($r=\left|0.277\right| - \left|0.793\right|$). In general, the correlations do not indicate high dependencies between the dependent variables. However, some values indicate a relationship between the rheological and microstructural variables: the perimeter of the proteins correlates with the relative elastic part of J_{max} with r=0.606. An increased perimeter means that the protein network is more widely spread which could lead to more connection points as well as a more interlocked network and therefore a higher elasticity. Further, the dough resistance R^k_{max} linearly correlates with the Feret's diameter (r=-0.709) and the branching index (r=0.793). The negative dependency of the dough resistance on the diameter of the proteins can be explained by elongated protein structures. The longer (and thinner) the proteins, the less resistance to an uniaxial extensibility appears. Therefore the polymers can easily slide past one another. If elongation occurs broadly, rather than in just one direction (represented by a high branching index), the resistance increases as the network becomes more interlocked. This is presented by the linear correlation of the branching index with the dough resistance (r=0.793). The current experimental design with its broad range of variation implies that next to the protein microstructure the major component of wheat flour, the starch granules, also have a considerable effect. This can be indicated by studies of Chiou et al. [52] who showed changes in starch granule morphology depending on salt concentrations as well as Lee et al. [53] who revealed differed molecular conformations (molar sizes and radius of gyration) of starches depending on the pH.

Conclusion

The experimental design and response surface methodology enabled the determination of significant effects on empiric and fundamental as well as structural attributes. Almost all experimental factors (pH, water addition and NaCl addition) significantly affect the properties of dough (except for sodium chloride on the fundamental elastic properties tan δ and J_{el} and on ØA as well as the water addition on ΣP). For

instance minimum stickiness is attained by the addition of 49.0 g water 100 g^{-1} flour, 3.6 g NaCl 100 g^{-1} flour and a pH of 3.2, which represent the cornerstones of the experimental design and therefore the extreme values. An addition of 68.1 g water 100 g^{-1} flour, 3.8 g NaCl 100 g^{-1} flour and a pH of 6.8 leads to an increased extensibility which could be a sign for good bread making performance.[54] Furthermore the gained data facilitate the modelling of quantified protein microstructure with good correlations (R^2 up to 0.86) and demonstrate the validity of the used methodology. In summary, the study confirmed the influence of the protein microstructure on rheological attributes of wheat dough and the dependency of this microstructure from the pH, sodium chloride, and water addition. However, influences of the experimental factors on the starch morphology and behavior are assumed to play another important role.

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2.5 Effects of acidification, sodium chloride, and moisture levels on wheat dough: II. Modeling of bread texture and staling kinetics

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ORIGINAL ARTICLE

Effects of Acidification, Sodium Chloride, and Moisture Levels on Wheat Dough: II. Modeling of Bread Texture and Staling Kinetics

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Abstract The effect of three experimental factors pH (addition of lactic acid and sodium hydroxide), water, and sodium chloride (NaCl) addition on wheat bread making performance (volume, baking loss, crumb firmness, crumb grain features) and the crumb staling during storage was studied. The staling behavior was modeled with the Avrami equation and with linear regressions. All bread quality parameters were reliably modeled using response surface methodology (up to R^2=0.97). The crumb staling behavior was better described by a linear regression than by the rate constant k of the Avrami equation (R^2=0.87 / R^2=0.36). The highest volume can be achieved with the experimental values pH 5.39, 0.41 g NaCl 100 g^{-1} flour and 68.7 g water 100 g^{-1} flour. Correlation analysis revealed significant linear dependency of dough rheology (complex shear modulus) on the firmness of the bread crumb (r=0.73) and staling attributes ($r \geq 0.73$). Dough microstructural properties showed significant but low correlation with bread making performance attributes.

Keywords Crumb · Avrami · Response surface methodology · Image analysis · Staling · Microstructure

Abbreviations

A_F^P	Pore void fraction
$ØA^P$	Average pore size
BL	Baking loss
C^P	Pore circularity
C^S	Dough cohesiveness
D_F	Dough protein Feret's diameter
D_F^P	Pore diameter
D^S	Dough stickiness
F_t	Crumb firmness
F_{96h}-F_{2h}	Extent of firming
F_{lin}	Firming rate
$\lvert G^* \rvert$	Complex shear modulus
H^P	Crumb holes
J_{el}	Relative elastic part of J_{max}
J_{max}	Maximum creep compliance
k	Rate constant
n	Avrami exponent
NaCl	Sodium chloride
ns	No significant effect
PH	pH
ΣP^P	Pore count
ΣP	Particle count
ρ	Density
RSM	Response surface methodology
R^2	Adjusted square correlation coefficient of the fitting model
S	Sodium chloride
t	Storage time
Θ	Uncrystallized material at time t
V	Volume
W	Water

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Introduction

The consumption of wheat bread constitutes a large portion of the daily food-intake in many countries. The bread production process starts with the mixing of the pre-product dough with the main components flour, water, yeast, sodium

chloride, and other quality enhancing ingredients. After water binding, the gluten proteins form a viscoelastic continuous phase. This interconnected spatial network has a dominant rheological impact on the dough.[1] Therefore, dough rheology is often analyzed to explain and understand the mechanical behavior of dough and its structure depending on the addition of different ingredients.[2] The microstructure and rheological behavior of dough with respect to the variables pH, water, and sodium chloride addition has already been comprehensively investigated in part I of the present study.[25] These properties are often related to the quality of the end product.[3,4] As the dough proofs, the rheological attributes influence gas bubble growth and gas retention properties. In the final baking process, these dough characteristics determine the crumb appearance and the final product quality.[5] With the temperature increase the viscosity of the dough rapidly changes due to starch gelatinization and protein denaturation. Thus, the bread crumb is characterized as a composite foam material.[6] However, the formed bread crumb and its firmness undergo a continuous change during storage. Although it is clear that staling behavior is responsible for huge economic loss in the baking industry and for consumers, the overall mechanisms behind staling are still being discussed. Staling involves changes in flavor as well as in texture. These changes are reflected in the mouth feeling due to a loss of crumb softness and crumbliness. The phenomenon is often attributed to the starch retrogradation or more precisely amylopectin retrogradation.[7,8] Nevertheless, several researchers claimed that bread firming is more complex and not synonymous with starch retrogradation.[9,10] Crumb firming might be a result of starch-gluten interactions or rather cross-linking between gluten and gelatinized starch;[11,12] however, other studies have revealed that the influence of gluten on firming is not essential.[13] The addition of other ingredients such as lipids,[14] hydrocolloids,[8,15] enzymes,[16,17] sodium chloride,[10] and water[6] may alter the staling kinetics of breads. The analysis and modeling of the crumb texture alteration during storage enables a better understanding of staling mechanisms. Thereby non-linear regression models, like a modified Avrami equation, are reasonable tools for crumb firming studies. The Avrami equation is generally used for the alteration of crystallization properties in a restricted solid matrix.[18] The fraction of crystallization which will still occur is described as an inverse exponential function of some power of time.[19] Additionally, the model is used to describe the staling of bread crumb parameters[18,20–22] over time. The non-linear equation can be utilized as a description of the transformation from one phase (state of matter) to another, and consequently to describe the increase of firmness during storage. However, linear regressions of the firming of bread crumbs have also been reported during the first 3 days of storage[23] for both white and whole bread ($R^2 \geq 0.92$).[24] The objective of this study was a) to determine the influence of the experimental factors pH, water, and sodium chloride addition on the bread making performance of standard wheat breads, b) to examine the influence on the staling behavior as well as the comparison of nonlinear and linear regressions and c) to correlate the results of the rheological and microstructural properties of the dough[25] to these features.

Materials and Methods

Ingredients

For all experiments wheat flour type 550 (standard German flour) from Rosenmühle, Ergolding, Germany (harvested 2008) was used. Its ingredients have been previously analyzed:[25] moisture content of 13.7±0.1 g 100 g^{-1} flour (n=5), ash content of 0.7±0.0 g kg^{-1} flour (n=3), and protein content of 11.6±0.2 g 100 g^{-1} flour d.m. (n=2). The baking quality relevant properties were: falling number, 382±10 s (n=5); water absorption, 60.2±0.2 % (n=3); dough development time, 2.9±0.5 min (n=3); dough stability, 17.1±1.4 min (n=3); and degree of softening, 24.8±6.8 BU (n=3). Dough containing 1.8 g sodium chloride 100 g^{-1} flour revealed a water absorption of 58.3±0.1 %. Sodium chloride was obtained from Südsalz GmbH, Germany; dry yeast (fermipan red, *Saccharomyces cerevisiae*) from Casteggio Lievitii srl, Casteggio, Italy; lactic acid from VWR International GmbH, Darmstadt, Germany; and sodium hydroxide from Merck, Darmstadt, Germany.

Experimental Design

A central composite in three block design with three variables was used as described in part I of the present study.[25] The 60 experiments were randomized. Variations of the pH (*PH;* 3.2–6.8), the sodium chloride addition (*S;* 0.0–5.0 g 100 g^{-1} flour), and the water addition (*W;* 47.9–68.7 g 100 g^{-1} flour) were used as experimental factors. The center point had the following parameters: PH=5.0, S=1.8 g 100 g^{-1} flour and W=58.3 g 100 g^{-1} flour.

Bread Preparation

Dough containing 1.0 g dry yeast 100 g^{-1} flour and the amount of water, sodium chloride, lactic acid and sodium hydroxide following the experimental design was prepared in a spiral mixer type SP 12 A-3 (Diosna Dierks & Söhne GmbH, Osnabrück, Germany). After blending the ingredients at 100 rpm for 1 min the dough was mixed at 200 rpm. The mixing time was adjusted for every experiment to the maximum of the power consumption curve of

the software of the mixer (version 2.0), with a maximum of 10 min. The dough end temperature was set to 28 °C due to an adjustment of the water temperature. Afterwards the dough was proofed at 30 °C and 80 % relative humidity for 15 min, weighed at 700±0.5 g pieces, molded, placed in pans, and proofed again for 60 min. Then the dough was baked for 30 min at 230 °C in a multideck oven (Matador Store 12.8, Werner & Pfleiderer Lebensmitteltechnik Sachsen GmbH, Sohland, Germany) with a 2325 mL initial steam injection m^{-3} baking room. After the baking process, the volume (V) [ml] was measured using the laser based volumeter BVM (TexVol Instruments AB, Sweden). The baking loss (BL) was calculated by the ratio of the difference between dough piece and bread weight to dough weight [%] and the density (ρ) by the ratio of bread weight to bread volume [g ml^{-1}]. The breads for the staling tests (up to 96 h) were stored in hermetically sealed plastic bags with an exposure to gas (80 % N_2, 20 % CO_2) at 20±1 °C.

Instrumental Texture Profile Analysis and Avrami Model

For the texture profile analysis (TPA), the bread was cut to 25 mm slices. A compression test with two repeating cycles on the Texture Analyzer TVT-300 XP (TexVol Instruments AB, Sweden) was performed. The bread slices were 40 % compressed with a plunger of 36 mm diameter and an applied force of 0.098 N, at a test speed of 1.7 mm s^{-1}, a post-test speed of 10 mm s^{-1}, a time between cycles of 15 s, and a trigger force of 0.049 N. The firmness was recorded following AACC method 74–09. The results were analyzed using the Avrami equation:

$$\Theta = (F_\infty - F_t)(F_\infty - F_0)^{-1} = \exp(-kt^n), \quad (1)$$

where Θ is the fraction of the recrystallization still to occur or of the uncrystallized material at time t. F_o, F_t and F_∞ are experimental values (crumb firmness) at time zero (F_o), at time t (t=4, 24, 48 and 72 h), and infinity (or limiting value defined as F_∞=96 h), k is the rate constant, and n is the Avrami exponent. Further, the firming rate (F_{lin}) [N h^{-1}] was calculated by a linear regression of the measured firmness values as a function of storage time. The firming rate is defined as the slope of the linear function. Another value for the description of staling was the extent of firming (F_{96h}-F_{2h}) [N] which is the difference between the firmness values after 96 h and 2 h.

Bread Crumb Image Analysis

The breads were cut to 25 mm slices and digital images were obtained with a Canon EOS 40 D digital camera with standardized manual settings and standardized light in a black box. The images were processed and analyzed with the open source software ImageJ (version 1.42q, National Institutes Health, Bethesda, Md, USA) which runs in java. The images were set to greyscale (8 bit), and for the segmentation of pore to crumb a Fuzzy thresholding algorithm of Huang et al.[26] was used. Gonzales-Barron et al.[27] compared seven thresholding techniques with the k-means clustering algorithm and gained good results using this threshold algorithm to analyze bread crumbs. A special advantage was that it isn't biased by large pores in bread crumb images. The binary images collected were analyzed by the features of the crumb grain: pore area fraction (or void fraction, A_F^P) [%], average pore size ($ØA^P$) [mm^2], pore count (ΣP^P) [−], pore diameter (D_F^P; calculated as Feret's diameter) [mm], pore circularity (C^P) [−] and crumb holes (H^P) [−]. Crumb holes were defined as pores with D_F^P >5 mm. From every experiment six images were recorded and analyzed.

Statistical Analysis

The software Statgraphics Centurion (version 15.1.02 Statpoint Technologies Inc. Warrenton, Virginia) was used for a multivariate analysis (stepwise regressions) and for response surface plots (main effects plots). The experimental design used enables the approximation of the measured data (y) using a response surface methodology (RSM). Additionally, the results were analyzed using a linear regression analysis to explore the relationships between the variables.

Results and Discussion

Effects of the Experimental Factors pH, Sodium Chloride, and Water Addition on Bread, Texture, and Firming Properties

According to the experimental design,[25] the data from the bread analysis were fitted to multiple regressions to estimate the response surfaces. The resulting equations are listed in Table 1. The significant coefficients (p<0.05) of the stepwise regression fitting model are expressed in equations of unscaled variables of the form:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (2)$$

where y is the response data, x_1–x_3 are the experimental factors, b_0 the constant, b_1–b_3 the linear coefficient, b_{11}, b_{22}, b_{33} the quadratic coefficient, and b_{12}, b_{13}, b_{23} the interaction coefficient.

All modeled equations of the baking performance, firmness, and firming values as well as the crumb image analysis

Table 1 Coefficients (95 % confidence interval) of the experimental factors of the stepwise regression fitting model[a]

Baking performance				Firmness					Avrami		Staling		crumb grain					
Factor[b]	V	ρ	BL	F_{2h}	F_{4h}	F_{24h}	F_{48h}	F_{72h}	F_{96h}	k	n	F_{96h}-F_{2h}	F_{lin}	A_F^P	$ØA^P$	$ΣP^P$	H^P	D_F^P	C^P
Constant	−8416	3.015	−8.157	525.2	297.2	648.5	826.4	880.1	881.8	−0.08346	0.64492	675.2	7.111	−23.85	−5.305	−127.1	−129.5	−0.1444	0.8455
PH	2099	−0.7417	5.820	−61.70	−68.10	−129.8	−163.4	−178.2	−172.3	0.0015	ns.	−102.85	−1.160	4.032	1.169	172.0	19.42	0.3414	0.0148
S	184.8	0.0263	0.1307	15.44	13.69	17.97	30.59	39.31	29.21	0.0025	0.12765	17.83	0.2450	18.59	0.5649	−3.959	4.991	0.3303	−0.0461
W	135.8	−0.0195	−0.06475	−12.83	−3.342	−8.698	−11.49	−12.02	−12.07	0.0052	0.00352	−11.838	−0.1194	0.3322	0.0942	ns.	2.537	0.0109	0.0040
S^2	−45.82	0.0151	−0.0338	−0.19531	1.237	1.594	1.842	2.076	3.564	−0.00111	ns.	2.500	0.02256	−0.7109	−0.0069	−10.33	−0.5609	−0.0136	0.0031
W^2	−0.7553	0.0000	0.0035	0.1117	0.0088	0.0357	0.044899	0.0463	0.0493	0.0000	ns.	0.05768	0.00056	ns.	−0.0002	−0.0537	−0.0178	0.0002	0.0000
PH^2	−164.0	0.0518	−0.2772	6.068	4.518	8.617	10.16	11.09	11.15	ns.	ns.	5.51797	0.0643	−0.3470	−0.0612	ns.	−1670	−0.0154	−0.0010
SW	−1.788	−0.00038	−0.0030	−0.14877	−0.1613	−0.2208	−0.3495	−0.4112	−0.3767	0.0000	−0.0024	−0.2275	−0.0028	−0.2215	−0.0083	−2.077	−0.0480	−0.0041	0.0005
SPH	−4.469	−0.00505	−0.0391	−0.71173	−1.057	−1.289	−2.119	−3.064	−2.311	−0.00028	ns.	−1.5971	−0.0205	−0.8435	−0.0430	ns.	−0.1542	−0.0205	0.0020
WPH	−4.804	0.0030	−0.04409	ns.	0.3468	0.6363	0.9380	1.023	0.8945	0.0000	ns.	0.73791	0.0080	0.0696	−0.0056	2.414	−0.0204	−0.0011	−0.0004
R^2	0.974	0.950	0.921	0.603	0.939	0.927	0.936	0.938	0.904	0.358	0.480	0.870	0.888	0.848	0.952	0.680	0.962	0.950	0.836

[a] The experimental factors were pH (*PH*), sodium chloride (*S*) and water addition (*W*)

[b] V, volume; ρ, density; BL, baking loss; F_t, firmness after time t; k, rate constant; n, Avrami exponent; F_{96h}-F_{2h}, extent of firming; F_{lin}, firming rate; A_F^P, pore void fraction; $ØA^P$, average pore size; $ΣP^P$, pore count; H^P, crumb holes; D_F^P, pore diameter; C^P, pore circularity; ns, no significant effect; R^2, adjusted square correlation coefficient of the fitting model

show highly adjusted square correlation coefficients. Excluding F_{2h}, the Avrami constants n and k as well as the pore count of the crumb, the adjusted R^2 exhibit a range of 0.836 to 0.974. This means that the equations can very precisely predict the quality values as a function of the experimental factors.

The effects of the pH (*PH;* 3.2–6.8), sodium chloride (*S;* 0.0–5.0 g 100 g^{-1} flour), and water addition (*W;* 47.9–68.7 g 100 g^{-1} flour) on the baking volume are shown in Table 1 and Figure 1. The calculated prediction equation shows an adjusted R^2 of 0.974. Therefore, it is possible to predict the volume of the end product with a high accuracy directly by the values of the *PH*, *S* and *W*. The three experimental factors show linear, quadratic as well as interaction effects on the baking volume. The real volume of the products was used in the current study, as the dough pieces were weighed (700±0.5 g) before proofing and the volume and the specific volume [ml g^{-1}] correlated highly significant (R^2=0.999, $p \leq 0.01$). The addition of water from 47.9 to 68.7 g 100 g^{-1} flour leads to an overall increase of the bread volume. This could be a result of an increased dough extensibility by water addition, which is shown by the experimental design in the previous publication.[25] Water molecules act as a plasticizer to the dough components and structure, whereby the cohesion of the components decreases and the extensibility increases. This prevents the disruption of gas cell membranes and leads to an increased bread volume. Skeggs et al.[28] also showed an increased bread volume with increasing water addition over the level of optimum water absorption, however, with a maximum and therefore as a parabolic function. The deviations may occur due to the different mixers, the used strong flour (dough stability of 17.1±1.4 min) and the optimized kneading time in the current study.

The addition of sodium hydroxide and lactic acid significantly alters the volume (and density) of the breads. Figure 1 shows the decrease of the volume with decreasing pH at a water addition of 58.3 g 100 g^{-1} flour and a sodium chloride addition of 1.8 g 100 g^{-1} flour. However, a decrease of the pH from the original pH of the dough firstly exhibits an increase of the volume, with a local maximum, followed by the described negative effects on the volume. Earlier studies describe either no significant effect on the specific volume at a pH of 5.4 and 5.1 compared to a standard dough without acidification[29] as well as at a pH of 5.3[30] or a decrease of 12 % by reducing the pH from 5.95 to 5.60.[31] However, the current study investigates a wider range of pH (from pH 3.2 to 6.8) whereby effects on the volume can be better detected. A local maximum of the volume due to the decrease of the pH by gaseous acetic acid has been published by Segushi et al.[32] Wheat dough is very sensitive to changes of the pH and the ionic strength which directly affect the components gluten, starch, and hemicellulose, and therefore

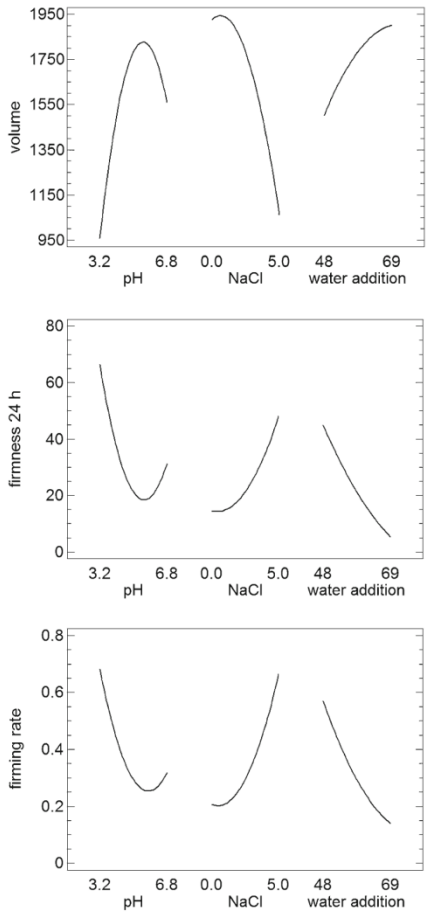

Fig. 1 Main effect plots of the bread quality values volume (V) [mL], firmness after 24 h storage (F_{24h}) [N], and firming rate (F_{lin}) [N h^{-1}] as a function of significant ($p<0.05$) experimental parameters

the structure and the mechanical behavior of dough up to the end product. Decreased pH values result in a change of the conformation of the proteins, more water can be bound and less water is available for the matrix. Due to the overall positive net charge of the proteins at lower pH the intramolecular electrostatic repulsion is increased, the proteins start to unfold, expose more reactive groups and a dissolution of the strands starts.[33]

The modeled equation of the rheological properties of dough in this range of pH exhibited a reciprocal behavior of the complex shear modulus as a measure of dough firmness and extensibility.[25] This means, the stiffer and less extensible the dough, the less it can withstand the increased pressure in the gas bubbles by yeast fermentation, resulting less end volume. The addition of NaCl leads first to an initial short increase of volume which is followed by a rapid decrease. At low concentrations NaCl has a positive effect on yeast's leavening ability.[34] The modeled maximum volume at around 0.5 to 1.0 g NaCl 100 g^{-1} flour is in accordance with earlier studies.[10] It seems that at this NaCl concentration an optimum occurs between the gluten strengthening effect (described in the first part of the study), the gas holding capacity and the osmotic stress on the yeast cells. Due to the osmotic stress and altered permeability of the cell wall, the metabolism of the yeast is influenced and less CO_2 is produced. Furthermore there is an effect of NaCl concentration on the lag phase (prolonged) and thus on the growth of yeast cells.[35] Therefore the gas bubbles in the dough matrix reveal less pressure. In combination with an increased protein strength due to the Na^+ and Cl^- ions the dough the extension of the volume is lowered. An optimized end-product volume can be achieved with the experimental values pH 5.39, 0.41 g NaCl 100 g^{-1} flour and 68.68 g water 100 g^{-1} flour. Figure 2 shows the response surface plot of the end-product volume as a function of the experimental factors pH and water addition at a constant NaCl addition of 1.8 g 100 g^{-1} flour. The mentioned effects of the water addition and the change of the pH on the bread volume can be visually detected. The variation of the pH leads to a stronger alteration of the volume as the effect of the water addition (Figure 2). The decrease of the pH exhibits first an increase of the volume followed by a strong reduction. This effect is not affected by the water content. The water addition slightly increases the bread volume. The combination of low water addition and low pH exhibits a stronger negative effect on the bread volume.

The firmness values after 4 h to 96 h of storage (at 20 °C) can be modeled with a high correlation coefficient ($R^2 \geq 0.927$). The firmness after 24 h of storage (F_{24h}) (Figure 1) shows a reciprocal behavior compared to the end-product volume. It seems that the firmness of the bread crumb mainly depends on the volume and therefore on the void fraction of the crumb pores. The void fraction is mainly based on the CO_2 production of the yeast cells and on the viscoelastic properties of the dough which affects in combination the expansion rate of the gas bubbles. This is the basis for the gas holding properties of the dough and the later fixed void fraction in the crumb. The higher the void

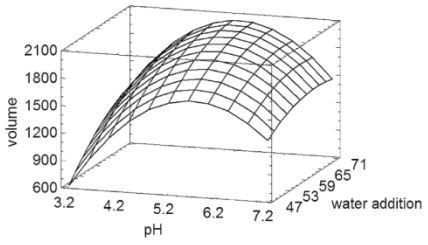

Fig. 2 Response surface plot of end product volume (V) [mL] as a function of the variables pH [−] and water addition [g 100 g^{-1} flour] (at a standardized NaCl addition of 1.8 g 100 g^{-1} flour)

fraction, the higher the volume of the bread and the lower the content of crumb material which resists deformation when measuring the firmness. A linear correlation between the (specific) volume and the firmness was already mentioned in different studies.[24,36]

The change of the firmness values over time were described by the difference of the firmness value of the last and the first measurement, the extent of firming F_{96h}-F_{2h} and by a linear model using the slope of the equation as the firming rate F_{lin}. Furthermore, the values were fitted to the nonlinear Avrami equation. This equation generally describes the kinetics of crystallization. This can be used to characterize the recrystallization of starch molecules during storage after a thermal treatment such as the baking process.[10] Further, the function has already been used to describe the firming properties of bread crumbs.[22] The rate constant k of the equation describes the development of the firming over time. The higher k, the higher the firming process.[37] The Avrami exponent n indicates the nucleation and growth mode of the crystals.[38] Although the firmness values of the individual experiments could be consistently fitted with the Avrami equation with a high correlation ($R^2 \geq 0.94$), it was not possible to describe the resulting exponents as a function of the experimental values ($R^2 \leq 0.480$). However, the predicted equation of the linear parameter firming rate F_{lin} had a high square correlation coefficient of 0.888. In summary, the model of the linear firming rate can be better predicted than the more complex Avrami model as a function of pH, NaCl and water addition. All three variables show significant effects on the firming rate. The firming rate increases with increased NaCl concentration and pH reduction and decreases with the addition of water to the dough. However, it is not possible to detect the causal dependency of this effect. It can be assumed that the change of the bread's volume has a greater effect on the firming rate than the possible direct chemical effect of the independent variables does since the specific volume of breads correlates with its firming rate.[21,39] Furthermore, Armero et al.[18] showed the dependency of bread crumb firming on the volume and initial crumb firmness. On the other hand, a study of Skeggs et al.[28] described the effect of breads of different dough weights in which the higher dough weight (300 g) resulted in a softer bread crumb. These different initial crumb firmness values resulted in the same firming rate. In summary it seems to be most suitable to assume that the experimental variables affect the volume as well as the initial firmness and these factors affect the firming rate.

Next to the volume of baked goods, the macroscopic structure of the crumb is an important quality parameter for the costumer. The surface, a characteristic of the crumb, is affected by the more or less connected bubbles, or rather pores of different sizes, in the bread and results in the sliceability of the bread. For the evaluation of the crumb, an image analysis tool was used to gain features of the crumb grain. The size and shape of the pores was often used to classify the quality of crumbs.[40,41] In the current study many crumb grain features such as the pore void fraction (or area fraction, A_F^P), the average pore size ($\varnothing A^P$), the pore count (ΣP^P), the pore diameter (D_F^P), the pore circularity (C^P) and the crumb holes (H^P) were investigated (see Figure 3). The pore void fraction shows a behavior that relates to the volume of the products. The higher the void fraction of both the dough after proofing and the bread after thermal heating increases, the higher the volume.

The water addition shows, however, no quadratic effect on the void fraction in contrast to volume development. In the observed range, an increase of water in the dough results in a linear increase of the void fraction of the end-product. Furthermore, the pore void fraction shows no maximum as a

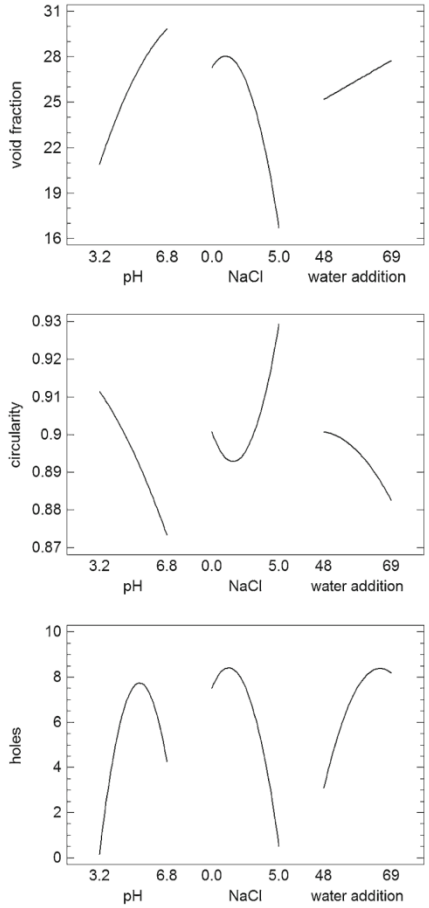

Fig. 3 Main effect plots of the crumb image analysis values pore void fraction (or pore area fraction, A_F^P) [%], pore circularity (C^P) [−], and crumb holes (H^P) [−] as a function of significant ($p<0.05$) experimental parameters

function of the pH variation whereas a clear volume maximum can be detected. The experimental factors affect the number of crumb holes (D_F^P >5 mm) and the pore diameter (Table 1) to the same extent as the area fraction. However, a maximum of crumb holes (8 holes per slice of bread) can be detected at pH 5 (at optimum water addition of 58.3 g 100 g^{-1} flour and sodium chloride addition of 1.8 g 100 g^{-1} flour). These results show that the formation of crumb holes (or the fracture of the crumb) is not necessarily combined with an increased area fraction of the crumb pores.

The shape descriptor circularity (C^P) reveals that with higher area fraction the evenness of the pores decreases. This can be explained firstly by the methodology (limitation of pore detection) and secondly by the creation of the holes. In some cases crumb holes (or large crumb pores) occur through the coalescence of smaller pores in the dough or during the transition between dough and crumb during baking. Therefore the evenness of the shape and the circularity is decreased in comparison to smaller bubbles in the dough.

Correlation Analysis

Gluten proteins are a crucial factor for the rheological behavior of wheat dough and the texture of wheat breads.[42] The rheological and microstructural behavior of wheat dough over a wide range of experimental factors were investigated in part I of the present study.[25] The obtained features are correlated with the bread quality properties obtained on the basis of the same experimental design and the significant correlations ($r \geq 0.5$) within these values are shown in Table 2. The baking performance properties reveal high linear correlations among each other. In particular the bread volume is linearly connected with the values of the firmness and the firming. The assumed negative correlation between the volume and the firmness (F_{24h}) could be proven ($r=-0.898$). This fact has already been described,[36] however the current study shows its general validity due to the wide range of variations. The density exhibits a positive and a slightly higher (in comparison to the volume) correlation coefficient with F_{24h} ($r=0.919$). The values describing the firming F_{96h}-F_{2h} and F_{lin} are significantly linear correlated with the volume ($r=-0.879$; $r=-0.872$), the density ($r=0.892$; $r=0.886$) and the firmness after 24 h of storage ($r=0.968$; $r=0.953$). The high dependency between the volume, the firmness, and the firming behavior of wheat breads could be clearly demonstrated. Furthermore, the crumb grain features reveal high linear correlations with the baking performance properties. However, as already assumed in the discussion of the main effect plots, the pore void fraction is not properly dependent from the end product volume ($r=0.688$). By contrast the average size of the pores shows a higher correlation coefficient ($r=0.869$) with the bread volume. This means that the end volume is not only based on the extension of the gas bubbles but might also be based on a change of the density of the matrix itself (crumb cell walls). Additionally, the pore count does not show any significant effect on the volume. This fact could also explain the lower correlation of the pore void fraction with the volume (in comparison to the average pore size). The amount of pores alters the void fraction with a low dependency to the volume. Nevertheless, the average size and diameter of the pores are highly positively correlated with the volume and negatively with the density of the wheat breads. The

Table 2 Coefficients of significant linear correlations (≥0.5) between the end product quality properties as well as dough rheological and microstructural properties ($p \leq 0.05$)

	Baking performance properties				Dough						
					Rheology					Microstructure	
	V	ρ	BL	F_{24h}	$\lvert G^* \rvert$	J_{max}	J_{el}	D^s	C^s	ΣP	D_F
V		−0.984	0.814	−0.898	−0.520				0.571		−0.597
p	−0.984		−0.809	0.919					−0.556	−0.520	0.635
BL	0.814	−0.809		−0.828	−0.598		−0.547		0.776		
F_{24h}	−0.898	0.919	−0.828		0.730			−0.597	−0.694		
F_{96h}-F_{2h}	−0.879	0.892	−0.760	0.968	0.733			−0.577	−0.634		0.511
F_{lin}	−0.872	0.886	−0.738	0.953	0.738			−0.585	−0.619		0.518
A_F^P	0.688	−0.628	0.677						0.561		
$ØA^P$	0.869	−0.834	0.865	−0.789		−0.516	0.504		0.748		
$ΣP^P$											
H^P	0.904	−0.843	0.776	−0.812	−0.643	0.538	−0.575		0.676		
D_F^P	0.884	−0.851	0.869		−0.537				0.754		
C^P	−0.699	0.652	−0.649	0.573		−0.576	0.591		−0.550		

higher the bread volume, the more holes the crumbs exhibit, since the average size of the pores increases and more pores can be defined as holes (D_F^P >5 mm) (r=0.904). Furthermore, the firmness of the crumb after storage reveals high correlation coefficients ($r \geq 0.96$, data not shown), with the exception of F_{2h}. It seems that 2 h after baking the cooling of the product and perhaps the retrogradation of amylose is too uneven. This is also confirmed by the low R^2 (0.603) of the stepwise regression fitting model of F_{2h} (Table 1).

The consideration of the rheological dough properties reveal that the complex shear modulus $|G^*|$, as a measure of the dough firmness, linearly correlates with the firmness of wheat breads after 24 h of storage (r=0.730). Additionally, F_{96h}-F_{2h} and F_{lin} are linearly dependent on $|G^*|$ (r=0.733; r=0.738). The dough stiffness influences the initial crumb firmness and therefore the staling behavior of the breads. This means that small amplitude oscillatory shear measurements are a helpful tool in predicting crumb firmness and firming behavior in wheat breads with the variation of pH, water, and sodium chloride addition. The dough cohesiveness C^S demonstrates similar (but negative) correlation coefficients such as $|G^*|$. The dough cohesiveness or the so called dough strength, was analyzed by a stickiness measurement with a Chen and Hoseney Rig in a Texture Analyzer and can be considered as an empirical rheological value.[43] Further, a linear dependency between C^S and the diameter of the crumb pores (D_F^P) and the average pore size ($ØA^P$) can be calculated (r=0.754 and r=0.748, respectively). This means that the rheological properties influence the appearance of the crumb grain features as a measure of dough cohesiveness. The higher the measured dough cohesiveness, the higher the expansion potential of the dough and the larger the diameter of the crumb pores of the end-product. Other rheological values as the compliance J and the relative elastic part of J_{max} J_{el} exhibit low correlations with the end product quality values.

The values of the protein network, assessed by dough microstructure quantification (DoMiQ), do not reveal high correlations with the end-product quality features. However, the diameter of the wheat dough proteins (D_F) show a linear correlation (r=0.635) with the density of the breads. The wider the protein network expands, the higher the density of the breads. This could be based on a higher resistance of the dough towards the expansion of the gas bubbles during proofing and thermal treatment. Although some correlations between the protein network and rheological properties of the dough were shown (see part I of the publication), during the baking process the behavior of the starch presents an important factor concerning the end-product quality. Starch is more or less inert while mixing and proofing. However, starch gelatinization takes place due to the thermal impact; the starch granules hydrate, enlarge, and induce together with the denaturized protein the bread crumb. These alterations during baking were not monitored with the current methodology; whereby, this cannot be taken into account.

Conclusion

This research demonstrates that RSM can properly be applied to model the bread making performance of wheat bread as a function of the pH (3.2–6.8), water (47.9–68.7 g 100 g^{-1} flour) and sodium chloride addition (0.0–5.0 g 100 g^{-1} flour). All experimental values showed significant effects on bread volume, density, baking loss, firmness values, staling attributes (linear and Avrami model), and crumb grain features void fraction, average pore size, pore count, pore diameter, pore circularity, and crumb holes (except the water addition on the pore count).

An optimized end-product volume can be reached with the experimental values pH 5.39, 0.41 g NaCl 100 g^{-1} flour and 68.7 g water 100 g^{-1} flour, and a minimized staling rate with a pH 4.92, 1.11 g NaCl 100 g^{-1} flour and 68.7 g water 100 g^{-1} flour. This means that a maximum addition of water, a slightly decrease pH (around pH 5), and an approximately halved standard NaCl addition (based on 1.75 g NaCl 100 g^{-1} flour) achieves the best bread making performance of wheat flour. However, sensory disadvantages are not taken into account which will mainly occur due to the reduced NaCl content. The results revealed that during a short storage period, which will be mainly applied for oven-to-sales products, linear regressions of the staling behavior can be better predicted as attributes of the Avrami equation although the firming of the individual batches can be well described by the Avrami equation ($R^2 \geq 0.94$).

Furthermore, it could be shown that the dough rheology linearly correlates with the firmness of the bread crumbs and can be used to study bread making potential. Especially small amplitude oscillatory shear measurements ($|G^*|$) show significant correlations with the bread crumb firmness after 24 h of storage (r=0.73). Because of the dependency on the initial crumb firmness values $|G^*|$, can be also correlated with the linear firming behavior of bread crumbs.

The dough protein microstructure (gained by CLSM in combination with image processing), which was previously associated with dough rheology, reveals significant but low correlations with the bread making performance. To predict these attributes a microstructural investigation of both protein structure and starch granules and its alteration of the morphology from dough to the end product has to be proposed.

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3 DISCUSSION

Wheat protein is the dominant factor influencing dough rheology (Goesaert et al., 2005; Thiele et al., 2004; Wieser, 2007). Rheological characteristics such as extensibility and gas retention capability are based on physical interactions on the microscopic scale (0.1-100 µm) (Autio et al., 2001; Lefebvre et al., 2000; Peressini et al., 2008), which covers the resolution of light and laser based microscopy. In the current thesis, the microstructures were acquired by confocal laser scanning microscopy (CLSM) and were quantified with a developed image processing and analyzing method (Dough Microstructure Quantification, DoMiQ) in order to create a wheat dough structure-function relationship on a micro- to macroscale.

The protein microstructure was modified in an initial experimental design (chapter 2.3) by a variation of water addition to flour because water is considered to have an outstanding role in the viscoelastic properties of dough due to its influence on the microstructure (Skendi et al., 2010). Further, the blending or mixing of water with biomolecules enables the creation of structure in foods (Rao, 2007). On the one hand, the results confirmed the advantages of CLSM, such as the dynamic and nearly non-invasive observation of a specific focal section for evaluating dough protein microstructure. On the other hand, the variation of the ratio water to wheat flour lead to a distinct change in the protein microstructure after mixing, as shown in Figure 3-1.

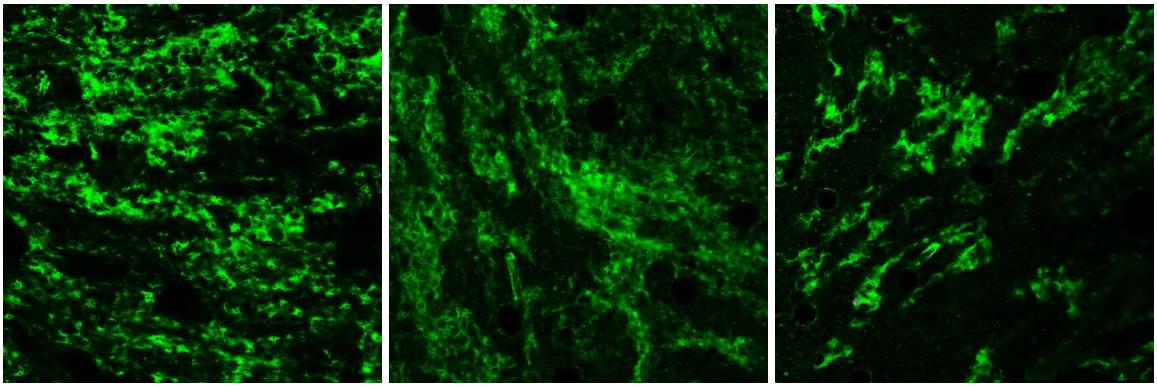

Figure 3-1: Wheat dough protein microstructure with varied water content, in 637 x 637 µm^2. Left: 52.5 g water 100 g^{-1} flour; middle: 58.3 g water 100 g^{-1} flour; right: 64.1 g water 100 g^{-1} flour. Rhodamine B was dissolved in the water to stain the proteins (green).

The visual microstructure was developed from a slightly orientated network, with some empty areas between the strands and clusters (low water content), to a homogenously distributed network (standard water addition). A further increase in the water addition lead to more strongly clustered and less interconnected aggregates. The rheological influence of the water addition on dough can be extrapolated from these micrographs (Figure 3-1): Elasticity decreased with increased water addition and the system softened due to the weakened protein network. Further, the plasticization effect of water was based on a strong non-reactive filling function (Masi et al., 1998) and the enhanced mobility of the liquid phase due to its low molecular weight (Mani et al., 1992). The fundamental and empirical rheological analysis of wheat dough in chapter 2.3 characterized the plasticizing effect of water in detail. All micrographs revealed basic structural protein elements from aggregates, coarse protein domains, to homogenously distributed strands as described in earlier studies (Dürrenberger et al., 2001; Peighambardoust et al., 2006). However, the microstructural element glutenin macro polymer GMP, which was described in several publications (see chapter 1.1) as having a spherical particle shape, could not be visualized, regardless of water addition. The GMP is described as a round protein particle extractable from wheat flour and dough (by sodium dodecyl sulphate, in the insoluble gel-layer), and is disintegrated due to mechanical rupture during the mixing process (Don et al., 2003a; Don et al., 2003b). At the optimal dough development time, GMP should be still extractable: however, microscopic detection after extraction decreases with increased mixing time (Campos et al., 1997; Don et al., 2003b). It can be assumed that larger and more widely spread protein morphologies are formed out of the GMP particles in the dough matrix itself. Every dough presented in this thesis was mixed to its optimal dough development time (if detectable), and regarding the GMP theory, the particles should still be available and thus detectable. However, CLSM is unable to distinguish any particles in the dough matrix, even if the proteins are more aggregated. As such, a critical analysis of whether GMP particle morphology is just an artifact which appears due to the extraction methodology and its high shear impact (centrifugation) should be undertaken even though the existence of GMP after extraction is not doubted, and well established in cereal science.

In summary, CLSM is well suited to visualizing wheat dough protein microstructure and its alterations. However, the aim of the current thesis was to find a structure-function

relationship based on a mathematical approach. Therefore a method for dough microstructure quantification (DoMiQ) was established, as shown in Figure 3-2. For this, the pre-processing of the original micrograph comprises a change to gray level, binarization, and noise reduction. Following noise reduction, regions smaller than 2 pixels are deleted, and after segmentation, structural features are analyzed using the open source Java software imageJ (version 1.42q, National Institutes of Health, Bethesda, Md, USA) (Collins, 2007; Rajwa et al., 2004).

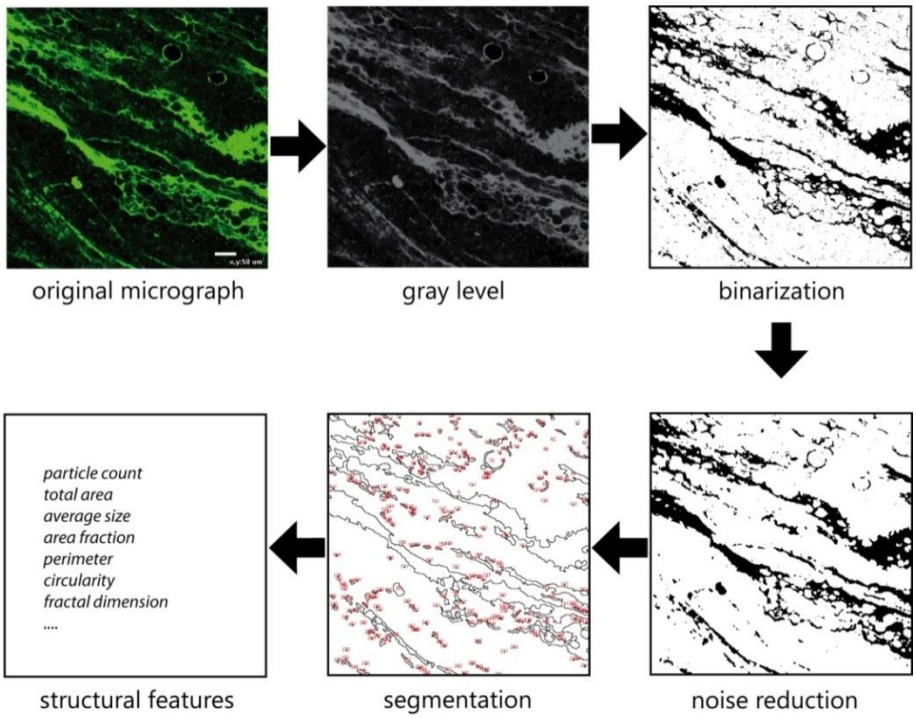

Figure 3-2: Operational sequence of the established method for the quantification of a wheat dough protein microstructure "DoMiQ". A scale bar (50 µm) is included in "original micrograph".

The DoMiQ method provided quantitative structural details of the morphology of wheat dough protein in a non-invasive matter for the first time. The analyzed measures were particle count (ΣP, [-]), total area (ΣA, [µm^2]), average size (ØA, [µm^2]), and area fraction (A_F, [%]) as well as the shape descriptors perimeter (P, [µm]), circularity (C, [-]), Feret's diameter (D_F, [µm]), and the fractal dimension of the image (FD, [-]). Further, the effect of the specific dependent variables on the dough's macrostructure was evaluated. Since it was expected that the description of the branching of protein aggregates would be a meaningful value for the morphology of the network, the measure "branching index" (BI, [-]) was introduced. It characterizes the surface of the region of interest (expressed by the perimeter P) in a

dimensionless relation to its extension (expressed by the (Feret's) diameter D_F) and is calculated by

$$BI = \frac{P}{D_F}. \quad (3\text{-}1)$$

The minimal reachable BI value is theoretically 2.0 with an object as a straight line (where $P=2D_F$). A perfect circle has a BI of 3.14. Figure 3-3 shows three different particle morphologies with related BI (calculated with imageJ). The significance pertaining to dough proteins is discussed after the following paragraph.

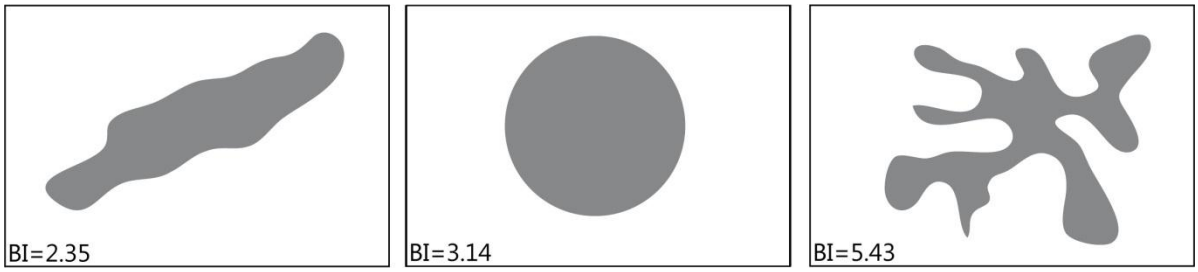

Figure 3-3: Visualization of three particle morphologies with different branching indexes. Left: a long and thin morphology with a BI of 2.35; middle: a circular morphology with a BI of 3.14; right: a branched morphology with a BI of 5.43.

The analysis of the micrographs in chapter 2.3 revealed a repeatable acquisition of measures such as the area fraction A_F of the standard (58.2 g water 100 g^{-1} flour) with 35.8 ± 2.38% (n=14 micrographs). Also, structural features such as the perimeter P showed a reasonable standard deviation of 3.7 µm with a mean of 55.2 µm. A comparison of the water addition levels revealed significant differences from the lowest water addition to all other variations (52.5 up to 70.0 g water 100 g^{-1} flour). This fact statistically replaces earlier views on the microstructure by environmental scanning electron microscopy (Létang et al., 1999) which assumed no fundamental changes in the structure of dough following water addition of 45 to 60 g water 100 g^{-1} wheat flour. Other authors described a rheological alteration of wheat dough due to the filler function of water and due to water's function as a lubricant which increases the relaxation of dough (Masi et al., 1998). In contrast to these invasive previous works, the current micrographs, structural features, and rheological characterization confirmed tremendous changes from an interconnected network, resulting in stiff and less extensible dough at a low water addition, to a clustered, less entangled network with more plastic and less elastic properties at high water additions. In dough with a water addition above 64.1 g 100 g^{-1} flour, the alteration of the microstructure was based

on a low energy input during mixing as an optimum mixing time was no longer detectable (over 20 min). Due to the plasticization effect of water in the system, the protein aggregates cannot be distributed to a homogenous network and are revealed in the analyzed clustered status.

The value BI, postulated in this thesis as a measure of the ratio of protein perimeter to (Feret's) diameter, was applied to a related experimental set-up (not included in the thesis publications in chapter 2) in which the addition of water to another wheat flour batch was varied in steps comparable to chapter 2.3 (Jekle and Becker, 2011). Figure 3-4 shows two micrographs of a standard water addition (A) and an increased water addition (B). The proteins in micrograph B are considerably less spread out and reveal stronger and less branched strands or aggregates.

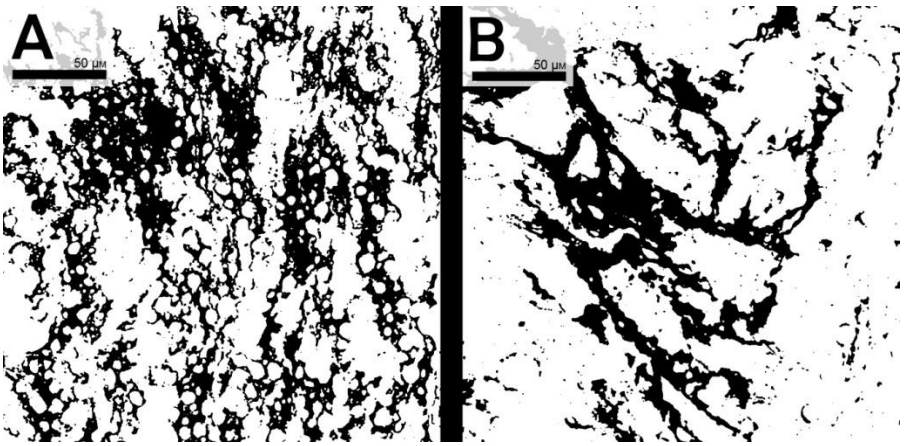

Figure 3-4: Binary CLSM micrographs of wheat dough protein with 58.3 g (A) and 70.0 g (B) water addition 100 g^{-1} flour. A water addition of 58.3 g water is the optimal water addition for this flour; 70.0 g is equivalent with a 20% increased water addition. Proteins are displayed black.

The DoMiQ method exhibited highly significant dependencies between the water addition and the branching index BI ($r = -0.92$) (Figure 3-5). The graph illustrates a more spread out protein structure with low water addition; whereas, a simpler or less branched structure exhibits with increased water addition due to less interconnected aggregates that result from the plasticizing effect of the water.

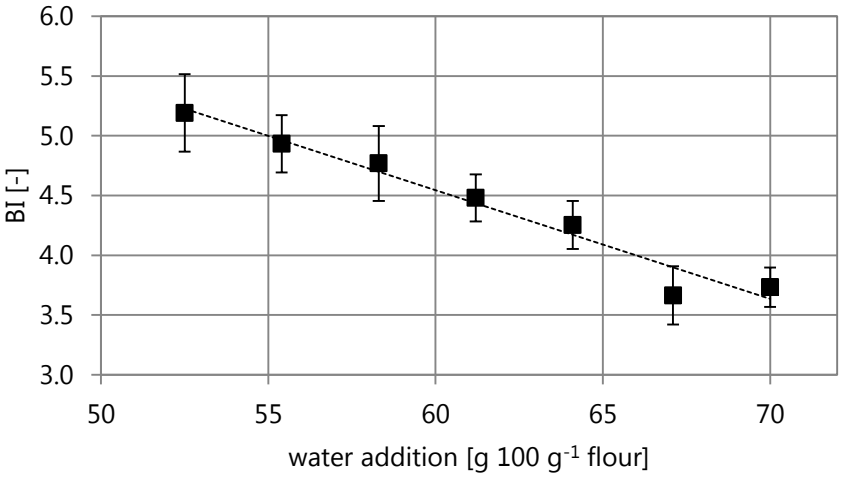

Figure 3-5: Branching index BI of the protein microstructure as a function of the water addition to flour (based on (Jekle and Becker, 2011)).

To validate the results in a wider range of experiments, a multivariate analysis of an experimental design with three variables (water, sodium chloride addition, and pH value by acidification with lactic acid) was performed using response surface methodology (RSM). The selection of the three parameters was reasonable due to their high impact on wheat protein microstructure (Beck et al., 2012a; Jekle and Becker, 2011; Schober et al., 2003). The variables showed a significant influence on all microstructural measures such as A_F, FD, ΣP, D_F, P, ØA, C, and BI (expect water addition on ΣP). This means that a) the protein microstructure can be modified by sodium chloride addition and pH value and b) it can be quantified by DoMiQ. A second-order polynomial model of the protein microstructure partly revealed a highly adjusted R^2. In particular, the fitted model of A_F explained 88.4% of the variability of the protein area fraction as a function of water addition, sodium chloride addition, and the pH value. The mean absolute error (MAE), as the average value of the residuals, was 1.83. The BI had an adjusted R^2 of 82.4% with a MAE of 0.25. This confirms the universal applicability of the method for quantifying dough protein microstructure.

That the rheology of food comprises macroscopic responses determined by changes and characteristics on a microscopic scale (José Miguel, 2005; Rao, 2007) is an accepted fact in cereal science (Beck et al., 2012a; Bloksma, 1990b; Lefebvre et al., 2000). However, it had yet to be mathematically proven. The current thesis facilitated the proof due to the variation of water addition and a correlation of the results of the successfully established method to extract structural protein features with fundamental and empirical rheological properties.

Variation of water addition was chosen as water is a key factor in the creation of edible structures of foods (Rao, 2007). Highly significant linear correlations (r) were found among others between A_F and the rheological measures |G*| (0.79), R^k_{max} (0.76), E_k (−0.76), and D_s (−0.81). This provides a general statement: The more protein is detectable with DoMiQ (A_F), the stiffer, less sticky, and less extensible the dough is. This proves the visual evaluation of SEM micrographs by Lee et al. (Lee et al., 2001) and a further interpretation of the results of Schober et al. (Schober et al., 2003) (also see chapter 2.2) that a low content of detectable protein microstructure weakens wheat dough. This was also described as low protein density (Schober et al., 2003), meaning low gray intensity. Considering the structural features, the following correlations were found: circularity C with J_{max} (0.85), J_{el} (−0.86), C_S (0.77); FD with |G*| (−0.75), J_{el} (−0.75), R^k_{max} (−0.72), D_s (0.77); and perimeter P with |G*| (0.66), J_{max} (−0.74), J_{el} (0.74), R^k_{max} (0.61), D_s (−0.73). The variation of one parameter reveals the interrelation between rounder (>C) and therefore less branched (smaller surface or perimeter, <P) and a softer and less elastic dough. In reverse, the dough becomes stronger (stiff, elastic) with more surface contact between the protein aggregates and the surrounding matrix. Furthermore, the results of the variation of three ingredients in the RSM design showed some high significant linear correlation between P and |G*| (0.59), J_{max} (−0.57), and J_{el} (0.61), between BI and R^k_{max} (0.79), and between D_F and R^k_{max} (−0.71). The rheological impact of a branched and therefore enlarged contact area (>P, >BI) due to spacious and connected protein strands is also evident in this experimental design. This morphological detail also explains the interpretation of gluten protein micrographs that a less fragmented and more organized protein network (described by branched and interconnected areas) leads to a higher viscoelastic behavior. However, the variation of water, sodium chloride addition and pH value in 2.4 resulted overall in lower correlations than the single ingredient variation. Therefore, the mode of action of sodium chloride and acidification is critically discussed in the next paragraph.

Next to the influence on the microstructure, the experimental variables influenced as well the molecular and nanoscopic scales. A decreasing pH value results in a slight change of the protein configuration due to an increase of net positive charge of the proteins (Galal et al., 1978). Furthermore, the reaction rate of SH/SS interchanges is pH-dependent (Song and Zheng, 2007). This means that mechanisms in the molecular and nanoscopic scale could

occur, directly influencing the macroscopic behavior without having any relation to the microstructure. Sodium and chloride ions change the protein fraction on a nanoscale level as well due to neutralized repulsion forces in the proteins (Kirkwood, 1943), this considerably effects the microstructure (see chapter 2.4 and own works (Beck et al., 2012a)). The change of the microstructure determines in turn the macrostructure; however, there could be also direct impacts from the nano- to the macrostructure. To explain these effects, a possible simple experimental set-up would address the addition of ascorbic acid to wheat flour-water systems. Ascorbic acid influences the molecular and nanoscopic scale of wheat gluten (Every et al., 2008; Koehler, 2003a, b) and thus dough macroscopic behavior, such as increased dough strength (Aamodt et al., 2003). The addition quantity is low so that the amount of molecules will only affect crosslinking abilities and not the structure itself. A comprehensive investigation of its effects on the molecular/nanoscopic, microstructural, and macrostructural level could reveal and/or close causality breaks in the argumentation.

The often declared structure-function relationships of gluten proteins are mostly based on the molecular and nanoscopic levels (Belitz et al., 1986; Hamer and van Vliet, 2000; MacRitchie, 1999; Mejia et al., 2007; Southan and MacRitchie, 1999). The current thesis enables an enlarged structure-function relationship on a microstructural level which is strongly determined by physical effects. A further improvement of the method would be to enlarge the current micrographs (212x212 µm) to a millimeter scale without losing details so that a larger microstructural area could be acquired and analyzed. For this purpose, both an automation of the imaging with a construction for a movable sample and a compiling of the micrographs have to be applied.

The schematic protein network models presented in chapter 1.1 (Belton, 2005; Bloksma, 1990a; Létang et al., 1999; Singh and MacRitchie, 2001) are, on the one hand, the basis for the protein microstructure and therefore the material's mechanical behavior; however, they do not represent the status of the microstructure itself. Therefore a dough protein microstructure model with different properties is illustrated in Figure 3-6. The results of the current thesis are implemented in this protein model. It is a physical approach that describes the network with strands and aggregates which become joined through physical entanglements. The mechanical behavior of scheme A is stiffer and more elastic than scheme B. This can be detected by the smaller circularity and a higher perimeter of the

protein aggregates and strands, respectively. The rounder and less branched (<BI) protein aggregates in B reveal more deformation due to stress during elongation or mixing, resulting in a decreased dough resistance value. The higher perimeter in A induces a higher contact area between the protein and the surrounding matrix (starch, liquid phase) as well as to adjacent protein aggregates. Therefore higher friction energies have be to overcome, resulting in a strengthening of the material (A2). Converting the nanoscopic model of loops and trains to the microscale, a higher branching of the protein structure leads to an increased elasticity of the system.

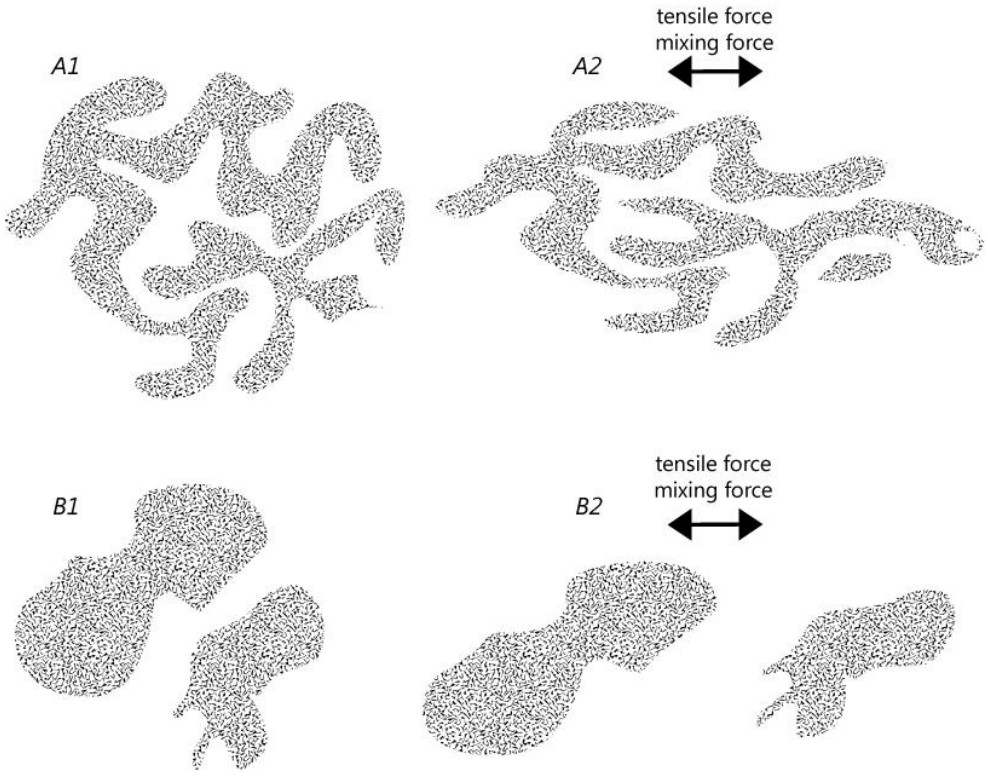

Figure 3-6: Illustration of wheat dough protein model in the microscale level (<100 µm) under relaxed (1) and stressed (2) conditions. The schemes in A represent a stiffer mechanical behavior and B a softer and less elastic material.

The prediction of the end product qualities based on a dough analysis would enable better control of the production of baked goods as well as a defined creation of specific properties. However, the micro- and macrostructure undergo tremendous changes during the heating process of the dough matrix. Nevertheless, the analysis of the results of the experimental design of chapter 2.5 revealed that the dough macrostructure, more precisely the complex shear modulus |G*|, determined the bread crumb firmness as well as the firming rate during storage. The higher the firmness of the dough, expressed in a high |G*|

value, the firmer the bread crumb and the higher the firming rate. During the fermentation step (in the following named proofing) carbohydrates are metabolized to carbon dioxide (CO_2) which diffuses from the dough matrix into the cells leading to an expansion by more than an order of magnitude (Martin, 2004). The complex shear modulus is a measure of the viscous forces $P_v(t)$, which together with the hydrostatic pressure $P_h(t)$ and the surface tension $P_s(t)$, defines the expansion of the gas cells during proofing (Grenier et al., 2010). The lower the viscous forces, the higher the expansion rates (with an local maximum (Jekle, 2011)), the higher the volume of the bread and therefore the lower the firmness of the crumb. The detected effect on the firming rate can in general be explained in two ways: a) by affecting the components of the matrix during firming (during storage), which could be accomplished by an alteration of starch with ions as theoretically shown in an own work (Beck et al., 2011a); or b) by influencing the matrix components before firming (during dough preparation), in particular by an increased water adsorption of pentosans and damaged starch, respectively, due to higher water addition (Goesaert et al., 2009). The second aspect would affect processing and baking performance, such as the volume of the end product. The volume, in turn, determines the firming rate of the crumb, which was shown by an own special experimental set-up (Jekle and Becker, 2010b), as well as in the practical application in the current thesis (inverse relation between volume and firming rate). Despite these findings, the analysis method of the crumb firmness should be discussed and improved. Even though all studies concerning crumb texture firming are based on texture profile analysis (TPA) (Beck et al., 2012b; Cornford et al., 1964; Hug-Iten et al., 2003; Martin et al., 1991; Rasmussen and Hansen, 2001), the application of this method should be critically evaluated. The higher the product volume, the higher the void fraction and the lower the ratio of crumb touching the plunger of the TPA. This system represents practically the thumb method used in sensory evaluation, but does not deliver information about the firming of the pure crumb matrix itself. The specific porosity (via image processing and analyzing of the crumb) of the crumb texture should be taken into account to normalize the crumb firmness.

A relationship between the dough protein microstructure and the baking performance could not be properly established. Further approaches could improve the capabilities of the microstructure in determining processing and baking performance: Comprehensive

multivariate statistical methods such as the principal component analysis could support revealing relations between the measures, if existent. However, additional information has to be considered in this context and is discussed in the following.

The relation of the protein microstructure with dough macrostructure in ambient temperature has been shown, but during heating this relationship could be disordered. During heating the baking functionality of gluten is reduced by a decrease of extractability and solubility (Schofield and Booth, 1983). This is based on the formation of cross-links and the polymerization of gluten molecules. Furthermore, hydrophobicity increases due to unfolding processes and the exposure of hydrophobic groups. The unfolding increases as well as the elasticity and permanent cross-links are formed with further heating (Lefebvre et al., 1994). It is not clear if these processes mainly take place in the molecular and nanoscopic level or if they also occur in the microscopic level, too. An investigation of temperature dependent changes of the protein microstructure would be necessary to determine this. In addition to protein changes during the heating process, a further point is the consideration of the gas phase in the system. The foam system dough with a porosity of around 7% (Tanner et al., 2011) tremendously increases its porosity and merges into a sponge system. The expansion of dough during oven rise leads to a high rate of strains in the dough system, since the dough cell walls are elongated parallel to the cell surface and perpendicular to the surface. Strain hardening of gluten proteins occurs which is based on the molecular weight distribution (MWD) of the gluten proteins and leads to an increased resistance to biaxial extension. At its optimum, the slippage of the segments between the entanglements of the protein appears without a rupture of the polymeric, entangled network. With lower MWD less dough strength is exhibited, and slippage is prevented with higher MWD due to more entanglements (Sroan et al., 2009). This supports the baking performance of wheat dough by an increased gas holding ability (Dobraszczyk et al., 2003). An online observation of the microstructural changes together with rheological changes including the strain hardening during the heating process could explain the significance of microstructural phenomena.

Another influence on the relation between protein microstructure and baking performance is the relevance of starch on the dough properties during heating. The tremendous increase of dough viscosity during heating is mainly based on the gelatinization process (Salvador et

al., 2006), which strongly influences gas retaining ability and therefore determines end product qualities (He and Hoseney, 1991). These effects are also a function of changes in the microstructure, namely starch microstructure. Therefore, in an own study, wheat starch granules were analyzed with CLSM during a heating process and combined with the, established in this thesis, DoMiQ method in an offline experimental set-up (Schirmer et al., 2011). The increase of the mean granule area as well as the granule perimeter could be significantly detected as well as its linear positive relation with thermal characteristics (differential scanning calorimetry). Even if the quantitative relation with the macrostructure is still pending, this indicates strong impacts on the behavior of dough. Further investigations promise comprehensive insights into the transition from dough to crumb. Efforts should focus on an online investigation during the heating process of starch as well as on the protein microstructure. Simultaneously, an observation of the gas cell growth and pressure during heating (offline usability already proved (Petford et al., 1999)) would deliver new insights in the transition from dough to crumb.

In addition to these real-time experiments the established DoMiQ method would also facilitate new research fields in cereal science such as an observation of pentosans to determine its impact on rye dough characteristics; this would continue current investigations in this field (Beck et al., 2009; Beck et al., 2011b). A further outstanding research field would be the three-dimensional acquisition of structural elements, although it would require a distinctly enhanced processing, analyzing, and structure modeling methodology. To summarize, all investigations should be focused on the approach to enhance the understanding of micro- and macrostructure in order to create specific structural features as a function of processing parameters. Overall, the successfully developed DoMiQ methodology provided detailed and novel knowledge of the structure-function relationship in wheat dough.

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5 APPENDIX

The numbering of the following non-reviewed publications is continued from the peer reviewed publications.

5.1 Non-reviewed papers

17. Verheyen, C., Jekle, M., Becker, T.: Influences on dough development. Baking+biscuit 3 (2012), 40-43.
18. Beck, M., Jekle, M., Becker, T.: Different sterilisation methods for dough proofing carrier hygiene. Baking+biscuit 2 (2012), 14-18.
19. Schirmer, M., Jekle, M., Arendt, E., Becker, T.: Less calories with stevia and polydextrose. Baking+biscuit 1 (2012), 14-18.
20. Schirmer, M., Jekle, M., Becker, T.: Röschecharakterisierung von Kleingebäcken in Abhängigkeit der Gasfeuchte im Backraum. Cereal technology 4 (2011), 144-154.
21. Beck, M., Jekle, M., Becker, T.: Protein cross-linking - a method for improving the quality of rye baked goods. Baking+biscuit 5 (2009), 78-81.
22. Beck, M., Jekle, M., Becker, T.: Proteinvernetzung - Ein Weg zur Verbesserung von Roggenbackwaren. Brot und Backwaren 6 (2009), 36-41.
23. Beck, M., Hofmann, S., Jekle, M., Becker, T.: Untersuchung unterschiedlicher Materialien auf die Eignung als Gärgutträger. Getreidetechnologie 63 (2009) 2, 58-65.
24. Schirmer, M., Jekle, M., Hussein, M. A., Becker, T.: IBO – Intelligenter Backofen. Der Lebensmittelbrief – Ernährung aktuell 19 (2008), 373-375.

5.2 Book contribution

25. El Fawakhry, H., Beck, M., Jekle, M., Hussein, M., Becker, T.: Dough density determination using ultrasound. In Review: bread. Hamburg, Germany: f2m food multimedia GmbH (2010), 110-115.

5.3 **Oral presentations with first authorship**

26. Jekle, M., Becker, T.: Theoretische Grundlagen des Knetens. Frühjahrstagung des Weihenstephaner Institut für Getreideforschung, Freising, Germany, 2012-03-28.
27. Jekle, M., Becker, T.: Quantification and modeling of dough microstructure by the use of image analysis. 2011 AACC International Annual Meeting, Palm Springs, USA, 2011-10-18.
28. Jekle, M., Becker, T.: Implementation of a novel tool to quantify dough microstructure. iCEF11 International Congress of Engineering and Food, Athens, Greece, 2011-05-29.
29. Jekle, M., Hussein, M., Becker, T.: Moderne Messmethoden zur Prozesskontrolle. 18. Internationale Tagung IGV, Potsdam, Germany, 2011-05-09.
30. Jekle, M., Becker, T.: Textural and rheological properties of wheat dough as a function of its microstructure. ICC Cereals & Europe Spring Meeting, Freising, Germany, 2011-04-12.
31. Jekle, M., Becker, T.: Korrelation von Mikrostruktur und rheologischen Kenndaten von Weizenteigen. Sitzung des Ausschusses für Bäckereitechnologie, Detmold, Germany, 2010-12-25.
32. Jekle, M., Becker, T.: Zusammenhang zwischen mikroskopischer Struktur und rheologischen Kenndaten in Weizenteigen. 61. Tagung für Getreidechemie, Detmold, Germany, 2010-06-23.
33. Jekle, M., Becker, T.: Auswirkungen der Textur und Teigausbeute auf die Frischhaltung von Modellweizenbroten. Detmolder Studientage, Detmold, Germany, 2010-02-24.
34. Jekle, M., Solle, D., Hitzmann, B., Becker, T.: Digital image analysis for the optimal control of bread and rolls baking. 5th International Technical Symposium on Food Processing, Monitoring Technology in Bioprocesses and Food Quality Management, Potsdam, Germany, 2009-08-31.
35. Jekle, M., Becker, T.: Auswirkung der Teigausbeute auf rheologische und texturelle Eigenschaften von Modellweizenbroten. 60. Tagung für Getreidechemie, Detmold, Germany, 2009-06-25.

36. Jekle, M., Zeeb, B., Becker, T.: Die konfokale Laser-Scanning-Mikroskopie als Tool in der Getreidetechnologie. Sitzung Ausschuss der Bäckerei-Technologie, Hohenheim, Germany, 2009-03-24.
37. Jekle, M., Moaty, A. A., Hussein, M. A., Becker, T.: Visuelle Teigbewertung. Detmolder Studientage, Detmold, Germany, 2009-02-18.
38. Jekle, M., Zeeb, B., Becker, T.: Die konfokale Laser-Scanning-Mikroskopie als Tool in der Getreidetechnologie. Sitzung Ausschuss der Getreidechemie, Hohenheim, Germany, 2009-02-18.
39. Jekle, M., Hussein, M. A., Mitzscherling, M., Becker, T.: Visuelle Teigbewertung. 59. Tagung für Bäckerei-Technologie, Detmold, Germany, 2008-11-06.

5.4 **Poster presentations with first authorship**

40. Jekle, M. B., T.: Confocal laser scanning microscopy as a promising tool in cereal science. Sitzung des wissenschaftlichen Ausschusses der FEI, Neustadt an der Weinstrasse, Germany, 2010-09-07.
41. Jekle, M., Becker, T.: Confocal laser scanning microspcopy as a promising tool in cereal science. 15th World Congress of Food Science and Technology, Cape Town, South Africa, 2010-08-22.
42. Jekle, M., Solle, D., Hitzmann, B., Becker, T.: Digital image analysis for the optimal control of bread and rolls baking. ProcessNet-Jahrestagung 2009 / 27. DECHEMA-Jahrestagung der Biotechnologen, Mannheim, Germany, 2009-09-08.
43. Jekle, M., Houben, A., Mitzscherling, M., Becker, T.: Amaranthsauerteige – Nutzen und Potenzial. 66. FEI Jahrestagung, Hohenheim, Germany, 2008-09-03.

5.5 **Curriculum Vitae**

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Work Experience

Since 07.2009	**Research assistant, Technische Universität München, Institute of Brewing and Beverage Technology, Workgroup Cereal Process Engineering, Freising, Germany**
11.2007 – 06.2009	**Research assistant, Universität Hohenheim, Department of Process Analysis and Cereal Technology, Stuttgart, Germany**

Education

10.2001 – 10.2007 Universität Hohenheim, Stuttgart, Germany

- Study of Food Technology
- Degree: Diploma, Dipl.-LM-Ing.

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