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Interrelation between arabinoxylan and protein during network formation over enzymatic modification in rye based food systems

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Meinen Eltern

Publications

Peer reviewed publications

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

1. **Döring, C., Hussein, M. A., Jekle, M. Becker, T.: On the Assessments of Arabinoxylan Localization and Enzymatic Modifications for Enhanced Protein Networking and its Structural Impact on Rye Dough and Bread. Food Chemistry; 2017. DOI 10.1016/j.foodchem.2017.02.053**

2. Grossmann, I., Doering, C., Jekle, M., Becker, T., Koehler, P.: Compositional Changes and Baking Performance of Rye Dough as Affected by Microbial Transglutaminase and Xylanase. Journal of Agricultural and Food Chemistry. 2016. DOI 10.1021/acs.jafc.6b01545

3. **Döring, C., Grossmann, I., Roth, M., Jekle, M., Koehler, P., Becker, T.: Effect of rye bran particles on structure formation properties of rye dough and bread. Journal of Food Processing and Preservation; 2016. DOI 10.1111/jfpp.12998**

4. Roth, M., Döring, C., Jekle, M., Becker, T.: Mechanisms Behind Distiller's Grains Impact on Wheat Dough and Bread Quality. Food and Bioprocess Technology; 2015. 9:2, 274-284. DOI 10.1007/s11947-015-1620-y

5. **Döring, C., Nuber, C., Stukenborg, F., Jekle, M., Becker, T.: Impact of arabinoxylan addition on protein microstructure formation in wheat and rye dough. Journal of Food Engineering; 2015. 154, 10-16. DOI 10.1016/j.jfoodeng.2014.12.019**

6. **Döring, C., Jekle, M., Becker, T.: Technological and Analytical Methods for Arabinoxylan Quantification from Cereals. Critical Reviews in Food Science and Nutrition; 2016. 56:6, 999-1011. DOI:10.1080/10408398.2012.749207**

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Contents

Abbreviations

AAn	/	amino acid
a_w	-	water activity
AX	/	arabinoxylan
c	g mL^{-1}	concentration
CLSM	/	confocal laser scanning microscopy
CO_2	/	carbon dioxide
EC	/	enzyme commission
GHF	/	glycoside hydrolase family
Gln	/	glutamine
Glx	/	glutamate or glutamine
$\|G^*\|$	Pa	complex shear modulus
HMW	/	high molecular weight
H_2O	/	dihydrogen monoxide
H_2SO_4	/	sulfuric acid
K	-	power law constant
Lys	/	lysine
LMW	/	low molecular weight
M_r	-	relative molecular mass
N	/	nitrogen
n	-	strain-hardening index
NaCl	/	sodium chloride
Pro	/	proline
R	m	bubble radius
S	/	sulphur
SCXI	/	secale cereal xylanase family
TG	/	transglutaminase
t	sec or min	time
tan δ	-	loss factor
U	µmol min^{-1}	unit
WEAX	/	water-extractable Arabinoxylan
WUAX	/	water-unextractable Arabinoxylan

XIP	/	xylanase inhibiting protein
Xyl	/	xylanase
σ	Pa	stress
ε	s^{-1}	strain rate
η	Pa s	viscosity

Summary

The processing of food raw materials into their respective products has a clear objective: The creation of high-quality end-products with specific properties according to the corresponding requirements and expectations of the consumers. The relationship between the structure and function of products during processing plays a key role and is a fundamental base in fulfilling expectations. In the case of rye dough and bread, the structure-function relationship is, in addition to starch, mainly determined and modified by the non-starch carbohydrate arabinoxylan (AX). The structure-function relationship by AX is attributed to the missing ability of rye proteins to form a network with adequate structure, providing characteristics such as elasticity, gas-holding properties and volume. Although many investigations have aimed to improve rye dough and bread characteristics by modifying AX, they remain of minor quality in comparison with wheat. Previous investigations have demonstrated the necessity and the essential role of a functional protein network to realise the aforementioned characteristics. In this current thesis, the role of AX in a possible protein network formation was investigated in detail. Based on the results, a new approach for the creation of a protein network, using the structure-forming enzymes endoxylanase (Xyl) and transglutaminase (TG), with the required characteristics, such as increased gas-retention capacity and elasticity, was investigated. This study found that, in addition to being the decisive structure-forming component, AX also determine physical distances between rye proteins, as demonstrated by microscopic analyses. By building a covering layer around proteins, AX prevent the accessibility of rye proteins to each other. Based on this knowledge, the covering of rye proteins by AX was successfully modified using Xyl treatments. This approach facilitated increased crosslinking of rye proteins using TG. The potential of a protein network development was proven by image analysis. The resulting changes of the dough structure and its characteristics, compared with the untreated dough, were analysed via rheological and baking investigations. The combination of both enzymes increased the elasticity significantly, by 28.0 % in the case of the untreated dough; viscosity decreased by 19.3 %; and crumb hardness increased significantly, by 22.3 %, whereas density decreased by 10.1 %. The decreased density indicates an increased structure formation using both enzymes in combination. In summary, the combined addition of the structure-forming enzymes Xyl and TG led to a coherent protein network with improved dough and bread

characteristics when compared with separate or no enzyme addition. The present investigations provide novel insights into the structure-function relationship of rye dough and bread processing and offer new methods of further modification.

Zusammenfassung

Die Verarbeitung von Nahrungsmittelrohstoffen zu qualitativ hochwertigen Endprodukte mit den jeweiligen geforderten optischen, sensorischen und texturellen Eigenschaften stellt die Lebensmittelbranche täglich vor neue Herausforderungen. Zentrale Rolle zur Erreichung einer gleichbleibenden Qualität spielt dabei die Kenntnis über die Inhaltstoffe sowie deren Mitwirken auf die Struktur-Funktions-Beziehung der jeweiligen Lebensmittelmatrix. Im Falle von Roggenbackwaren, wie durch eigene Untersuchungen bestätigt, ist die Struktur und ihre Funktionen überwiegend durch das Nicht-Stärke-Polysaccharid Arabinoxylan (AX) bestimmt. Dies ist bedingt durch die fehlende Fähigkeit von Roggenproteinen ein Proteinnetzwerk auszubilden, welches Strukturbildungseigenschaften wie Elastizität und Gasrückhaltevermögen bestimmt. Trotz zahlreicher Untersuchungen hinsichtlich der Modifizierung von AX zur Verbesserung der Teig- und Broteigenschaften von Roggen sind diese im Vergleich zu Weizen immer noch von geringer Qualität. Dies wiederum demonstriert die Notwendigkeit eines funktionalen Proteinnetzwerks zur Erreichung der genannten Eigenschaften. Auf Grund dieser Tatsache wurde in der hier vorliegenden Arbeit mittels kombinierten Einsatzes der strukturmodifizierenden Enzyme Endoxylanase und Transglutaminase ein neuer Ansatz zur Schaffung eines funktionalen Proteinnetzwerks erarbeitet.

Mittels Modellteigen konnte gezeigt werden, dass AX in hohen Konzentrationen (5 – 8 %), wie in Roggen vorherrschend, die Ausbildung eines kohärenten Proteinnetzwerks verhindern. Diese Ergebnisse wurde durch nicht-invasive mikroskopische Aufnahmen mittels konfokalen Laser Scanning Mikroskop anhand farbstoffgebundenen Antikörpern gegen AX bestätigt, welche erstmals die Lage der AX im System Teig visuell darstellen und diese als physikalische Barriere zwischen den Proteinen identifizieren. Auf Basis dieser Erkenntnisse wurde die Struktur der AX und Proteine durch den Einsatz der Enzyme zielgerichtet modifiziert. Deren Einfluss hinsichtlich des Aufbaus eines Proteinnetzwerkes konnte mittels rheologischen, visuellen und technologischen Methoden analysiert und statistisch signifikant bestätigt werden. Korrelationsanalysen zwischen den Ergebnissen der Untersuchungsmethoden bestätigen die erzielten Ergebnisse. Durch den kombinierten und zielgerichteten Einsatz strukturmodifizierender Enzyme konnte somit beim Roggen eine Netzwerkstruktur aus rohstoffeigenen Proteinen mit den gewünschten Funktionen reproduzierbar aufgebaut werden.

1 Introduction

Rye dough and bread can be produced from wholemeal flour containing bran, germ and endosperm particles or from refined flour without bran and germ particles (Parkkonen et al., 1994). The final appearance (texture and colour) of bread is, following the recipe and the process of bread production (mixing, fermenting, baking), mainly attributed to the amount of ingredients located in the different fractions (bran, endosperm, germ, etc.) of the grain kernel. Rye flour itself is structured by a complex assembly of various ingredients, such as starch, non-starch **polysaccharides (mainly AX), proteins**, lipids, vitamins and minerals (Arun, 2005). The processing of these ingredients with water and energy input (e.g. mixing) leads to the formation of new or advanced and complex structures between the ingredients, which, in turn, leads to the final dough. Dough can be described as a water-flour system characterised by viscoelastic behaviour (Sivam et al., 2010). During heat treatment, the dough transforms into a spongy crumb covered by a crispy crust (Ali Al-hebeil, 2013). However, rye dough and bread are a challenging matrix regarding structure processing, which is mainly attributed to the missing ability of native rye proteins to form an adequate protein network (as described for wheat proteins) caused by the molecular structure of the proteins and, probably, by the high concentrations of AX present in rye flour.

Food scientists are attempting to find new methods of modifying existing, and designing novel structures, because dough structure formation is an important criterion for final bread quality. Structure formation is more than the consolidating and processing of ingredients. On the scientific level, structure formation somewhat resembles engineering skills. The present or missing interactions of the ingredients, or the modification of the ingredients itself by mechanical, chemical and biological treatments, significantly influence the **structure** and, thus, the **function**, appearance and quality of the end product. In other words, the structure modification of the molecules can realise the intended function. This insight processing of **structures** with specific **functions**. Rye dough and bread structure and their functionality can be mainly described and characterised by the following 10 evaluation characteristics in Figure 1: compressibility, extensibility, elasticity, viscosity, stability, kneadability, connectivity, porosity, expandability and processability.

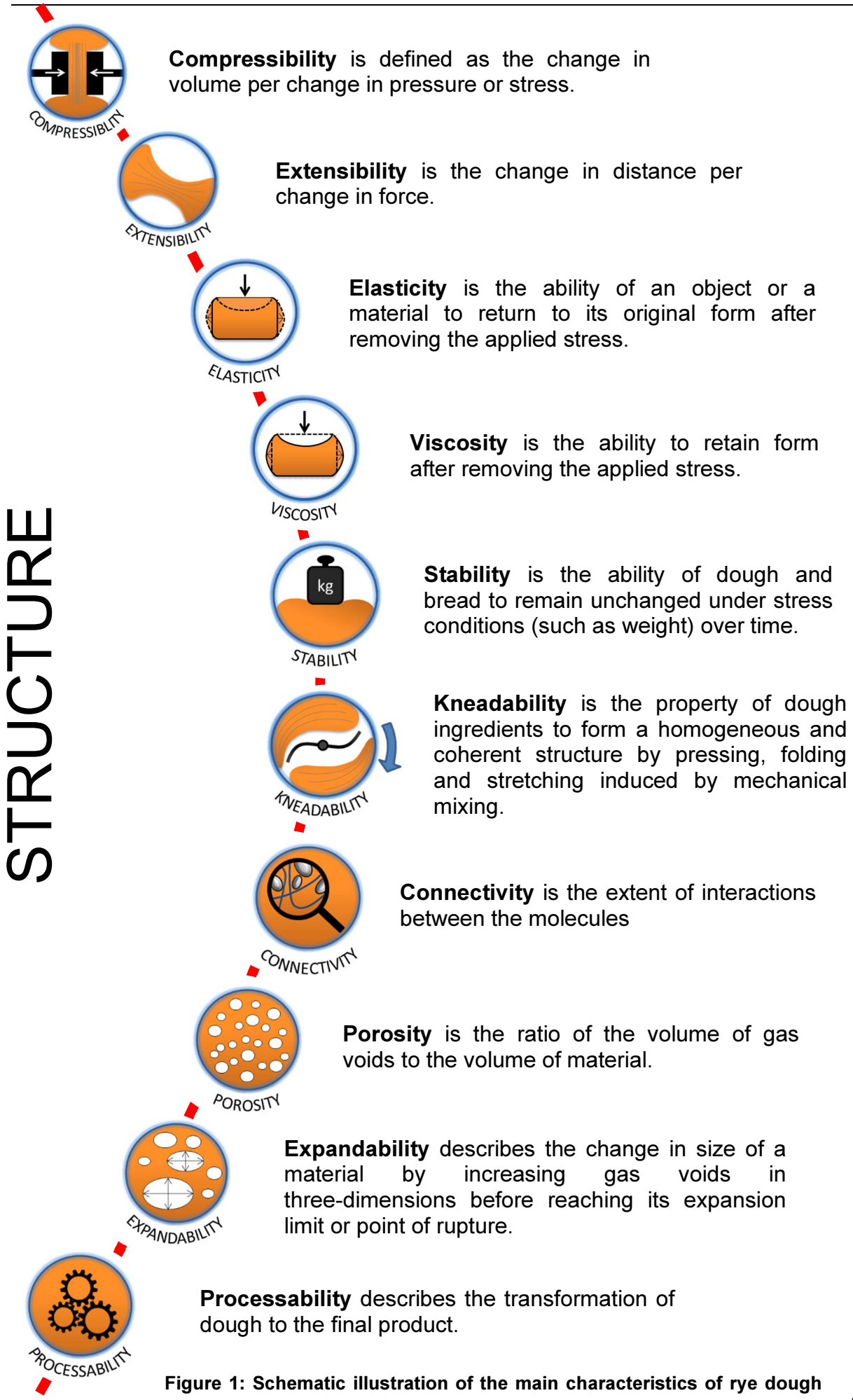

Figure 1: Schematic illustration of the main characteristics of rye dough and bread structure.

A change in structure leads to a change of functionality. The characterisation of the dough structure is performed at different levels of resolution: molecular, nanoscopic, microscopic and macroscopic (Autio and Laurikainen, 1997; Jekle, 2012). Each level of resolution provides different and further insights into the structure-function relationship of dough and bread. A conceptual illustration of the structure at different scales of size and its evaluation characteristics is in Figure 2.

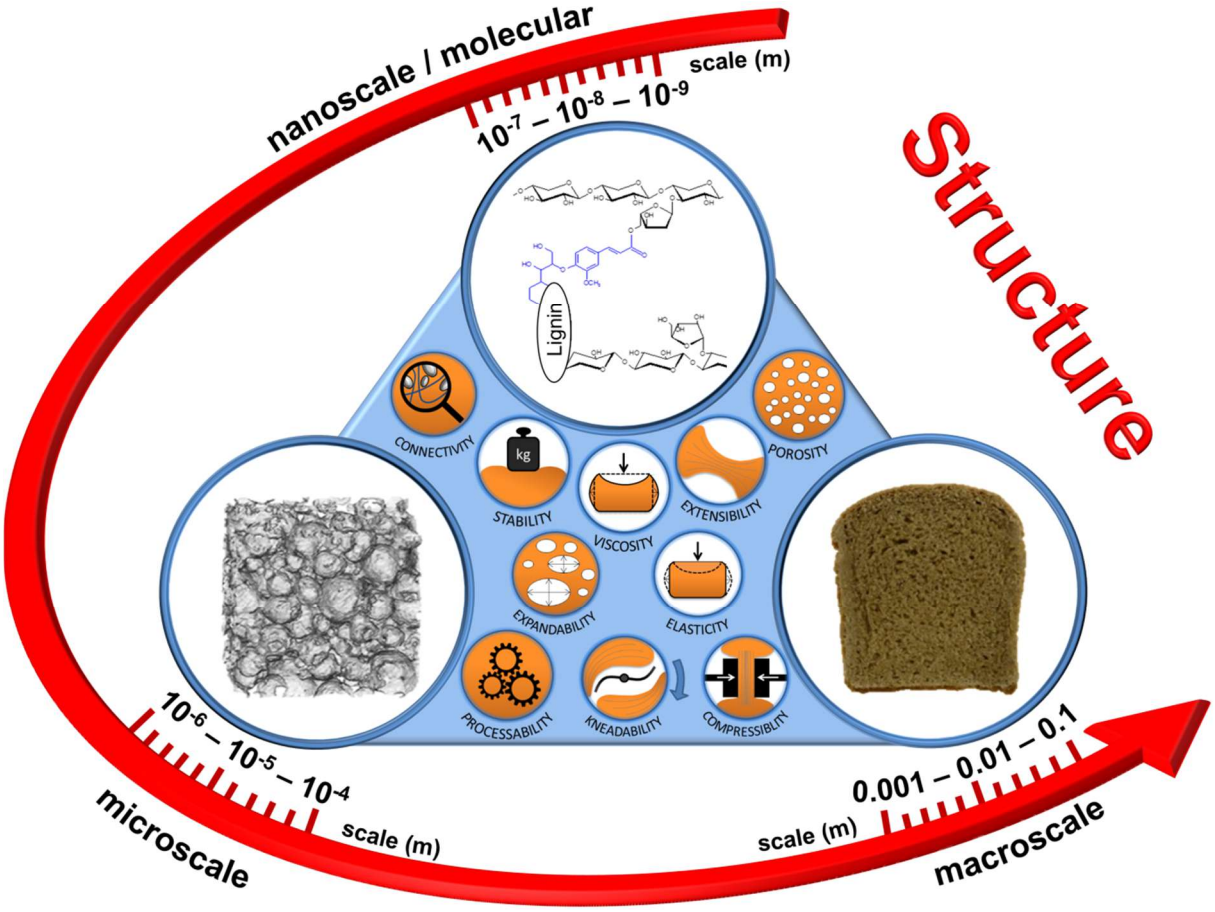

Figure 2: Schematic illustration of different scales of rye dough and bread structure size and structure evaluation characteristics.

A fundamental understanding of the ingredients, such as molecular structure or physicochemical characteristics, is a basic requirement for creating new or modified structures. The following sections provide a comprehensive description of rye dough and bread processing's most-discussed ingredients: rye AX and protein.

1.1 Arabinoxylan and its potential for enzymatic restructuration

Arabinoxylan play a key role in rye dough and bread structure formation. This role is mainly related to the missing ability of rye proteins to form a gluten network (Gellrich et al., 2003, 2004; Kipp et al., 1996). For wheat dough and bread preparation, AX play a minor role in structure formation; but for rye dough and bread, AX are, after starch, the dominant structure-forming component. For rye dough and bread structure formation, the positive effects of AX are linked to physicochemical characteristics (see Section 1.3).

Arabinoxylan are composed of the two monosaccharides: arabinose and xylose. The backbone of the AX molecule consists of β-1,4-D-xylopyranosyl units, which can be substituted for α-L-arabinofuranosyl units at position 2 and/or 3 of the xylose-chain. The substitution results in four different molecular structures: unsubstituted xylopyranosyl, monosubstituted xylopyranosyl at O-2, monosubstituted xylopyranosyl at O-3 and disubstituted xylopyranosyl at O-2.3. The α-L-arabinofuranosyl substituent can be linked to ferulic acid by an ester bond. Ferulic acid, in turn, can form interactions with other flour ingredients, such as lignin via diferulic bonds, or can be connected to tyrosine residues of proteins by oxidative enzymes (tyrosinase and laccase) (Mattinen et al., 2005). This substitution pattern and the interactions yield a three-dimensional (3D) network that significantly influences the physicochemical properties of AX (Hoffmann et al., 1991; Izydorczyk and Biliaderis, 1992; Vinkx et al., 1993). The following physicochemical properties of AX are mainly influenced by the different substitution patterns and molecule sizes: **extractability, viscosity** and **water-binding capacity**.

One of the most-discussed physicochemical attributes of AX is **extractability** by water. Arabinoxylan can be divided into water-extractable AX (WEAX) and water-unextractable AX (WUAX). This characteristic feature depends on several factors, as discussed in Section 2.2. Both WEAX and WUAX have significant yet different influences on final dough and bread structure. First, WUAX decrease dough foam stability by forming intrusions in dough gas cells, which lead to destabilisation and increased coalescence of the dough gas cells (Courtin and Delcour, 2002). For wheat dough, especially high concentrations of WUAX decrease the gluten network yield. In particular, WUAX interact with protein particles via the formation of diferulic acid bonds. This action leads to lower gluten extensibilities and to lower contents of glutenin macropolymers (Wang et al., 2003).

For rye dough preparation, high concentrations, especially of HMW (high molecular weight) WEAX are desired. Izydorczyk and Biliaderis (2007) reported that the positive effect of WEAX occurs especially at higher pressure in the gas cells during the initial baking process. The theory behind that statement is the ability of WEAX to increase the **viscosity** of the liquid film around the gas bubbles. Intrinsic viscosities for WEAX of 2.75 to 8.5 dl/g have been reported (Courtin and Delcour, 2002; Izydorczyk and Biliaderis, 1995). The higher viscosity of the liquid film leads to a stabilisation of the air bubbles, especially in the early baking phase, which, in turn, increases loaf volume and decreases crumb firmness (Courtin and Delcour, 2002). With increasing concentration of low molecular weight (LMW), both of WEAX and AX-oligosaccharides, the positive effect of gas cell stabilisation decreases. A change in viscosity of aqueous solutions affected by AX depends on the molecular structure of AX. Arabinoxylan with a low substitution pattern form a three-fold, left-handed helix, which is relatively flexible compared with the low number of hydrogen bonds (H-bonds) between two contiguous xylosyl residues. On the other hand, an increase in the substitution pattern leads to a stiffer molecule (WUAX). The molecule becomes more extended, which indicates an increased viscosity of aqueous solutions (see Figure 3). Despite the increased viscosity of WUAX compared with WEAX, the stiffer and more extended molecule leads to intrusions in gas cells, which destabilise the gas bubbles, as described above. The degree of substitution also influences the **water-binding capacity** of AX. For polysaccharides such as AX, there are three types of hydration: intramolecular between the hydroxyl groups of the polysaccharide; by the formation of two hydrogen bonds between one water molecule and one single polysaccharide chain; and through hydrogen bonds between several water molecules. The latter primarily occur during the formation of gels (Chaplin, 2003). The differences in water-binding capacity between WUAX and WEAX are discussed in detail in Section 2.2. Consequently, the water-binding capacity and the viscosity and solubility depend on the molecular size, the ratio of arabinose to xylose, the distribution pattern of substituents and the amount of ferulic acid. Figure 3 provides an overview of the dependency between viscosity and extractability.

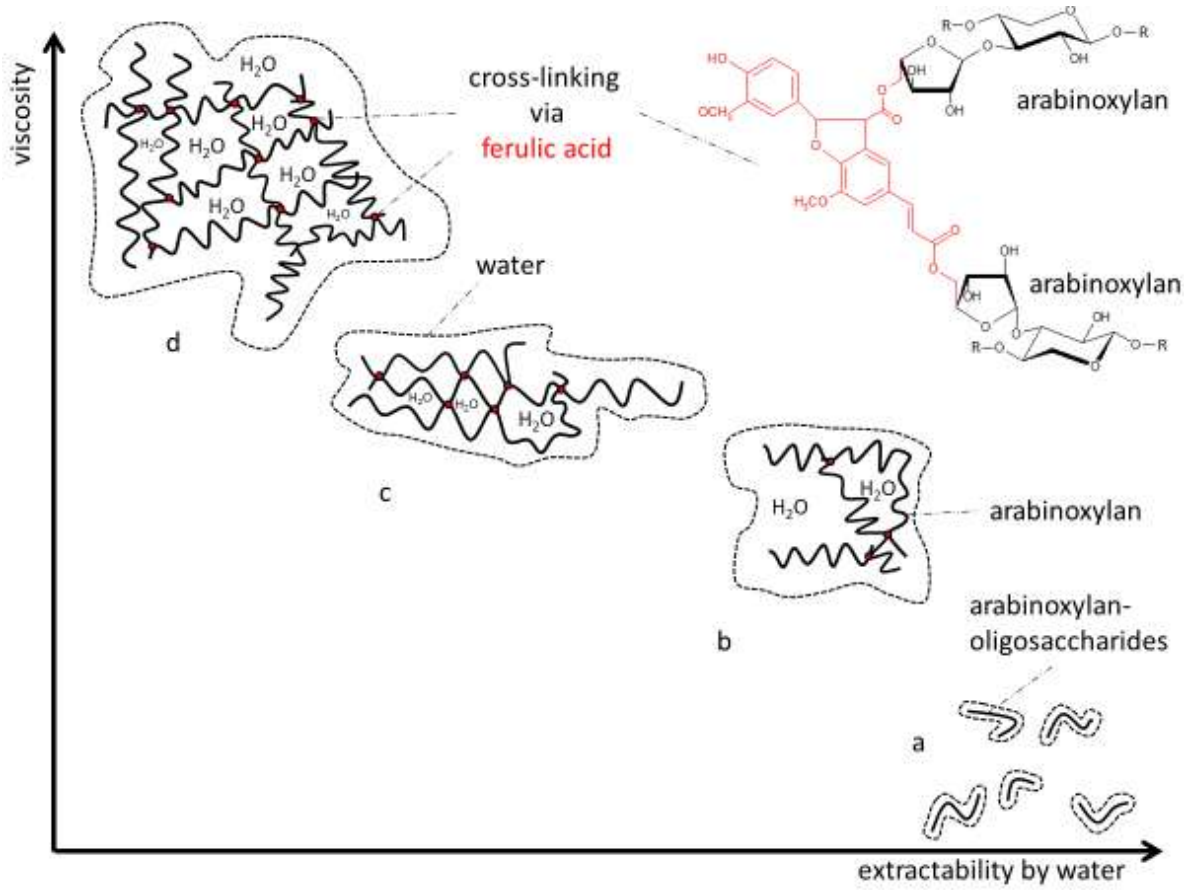

Figure 3: Schematic illustration of dependency between viscosity and extractability of AX as a function of structure and molecular size. a) AX-oligosaccharides; b) WEAX (LMW, low degree of substitution); c) WUAX (HMW, high degree of substitution); d) oxidative crosslinked AX.

Enzymes are globular protein complexes that catalyse chemical reactions with considerable efficiency and specificity (Baldwin, 2000). For rye dough and bread production, several enzymes are known and well characterised to improve final product quality, such as rheological properties, gas retention, crumb firmness and bread volume (Courtin and Delcour, 2002; Martínez-Anaya and Jiménez, 1997; Polizeli et al., 2005). For rye dough formation, Xyl are, in addition to α-L-arabinofuranosidases, feruloyl esterases and exo-1,4-β-xylosidases, the best studied and most common enzymes for AX modification. Figure 4 provides an overview of the different cleavage sites of the mentioned AX-modifying enzymes.

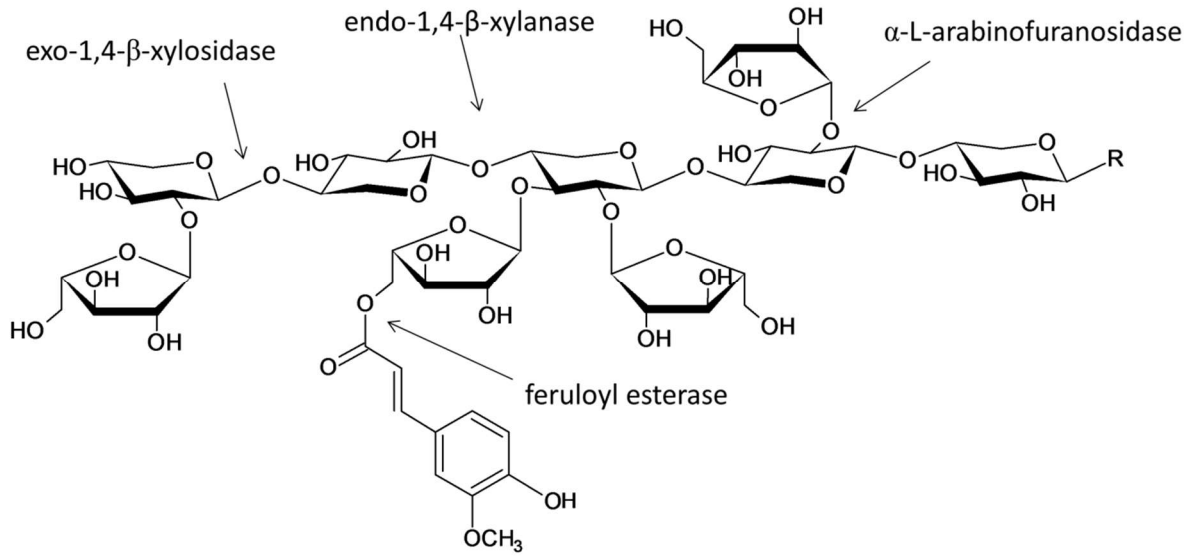

Figure 4: Schematic representation of the hydrolytic action of AX-modifying enzymes.

Endoxylanase (endo-1,4-β-xylanase; EC 3.2.1.8), hereinafter referred to as xylanase (Xyl), is listed as EC 3 in the EC number classification of enzymes and is further classified regarding its molecular mass, molecular structure and catalytic properties into the glycoside hydrolase families 10 and 11 (later referred to as GHF 10 and GHF 11). Xyl cleave the (1,4)-bond of the β-1,4-D-xylopyranosyl backbone. The hydrolysis leads to a degradation of the polysaccharide to xylan-oligosaccharides and xylobiose (Courtin and Delcour, 2001).

The hydrolysis itself mainly depends on two enzyme characteristics: **substrate selectivity** and **substrate specificity**.

Substrate selectivity describes the relative activity of Xyl towards WUAX and WEAX substrates (Moers et al., 2005). For example, a high enzymatic activity for WUAX was detected for a Xyl isolated from *Bacillus subtilis* (GHF 11); whereas, WEAX were minimally affected (Courtin and Delcour, 2001; Courtin et al., 2001). On the other hand, a Xyl isolated from *Aspergillus aculeatus* (GHF 10) displayed high activity against WEAX and low activity against WUAX (Frederix et al., 2003). These results indicate that the activity of Xyl from *Bacillus subtilis* is more effective against larger AX molecules than the Xyl from *Aspergillus aculeatus* (Courtin and Delcour, 2001). Thus, substrate selectivity significantly influences the final physicochemical properties of the AX molecule. Furthermore, a target structure modification of AX can be performed using knowledge of the enzymes substrate selectivity.

A further characteristic that influences the enzymatic action is **substrate specificity**. Depending on the structural features of the active site of Xyl, differences in the

catalytic actions concerning the degree of xylose substitution are reported. Xylanase from the GHF 11 are more specific for cleaving the xylan backbone at unsubstituted regions because their activity is lowered by the presence of arabinose substituents. In contrast, Xyl belonging to GHF10 have a higher cleavage rate of the xylan linkages at substituted regions of the xylan backbone (Berrin and Juge, 2008; Biely et al., 1997; Collins et al., 2005; Maslen et al., 2007; Motta et al., 2013). Concerning the classification of the Xyl, different substitution patterns of the AX molecule are evident, and these have different physicochemical properties. In addition to substrate selectivity, knowledge of substrate specificity is necessary for targeted structure formation of AX and, thus, the final dough and bread characteristics.

For completeness, the presence and influence of **inhibitors** are also important for Xyl activity. Inhibitors are substances that influence and minimise the catalytic reaction of enzymes. Recently, for rye, two inhibitors against Xyl activity have been discovered. The Secale cereale L. xylanase inhibitor (SCXI) represents a group of inhibitors (SCXI I – IV) with similar specificity and structure (Goesaert et al., 2002). The second inhibitor is the XIP-type (Xyl inhibiting protein) endoxylanase inhibitor family. The SCXI displays inhibitory activity only against the GHF 11, but the XIP-type Xyl inhibitor family inhibits both the GHF 10 and 11 (Elliott et al., 2003; Goesaert et al., 2003). In addition to these inhibitors, further non-proteinaceous Xyl inhibitors are known, such as metal ions (Hg^{2+}, Ag^{+}, Cu^{2+}, Fe^{2+} and Mn^{2+}), glycerol, ethanediol and several sulphhydryl reagents, as well as sugars such as xylose, arabinose and xylotriose (Dekker and Richards, 1976).

1.2 Proteins and their potential for enzymatic restructuration

Rye proteins are classified according to their solubility by the fractionation procedure of Osborn in glutelins (extracted using dilute acid or alkali), albumins (extracted using water), prolamins (extracted using alcohol) and globulins (extracted using dilute salt solutions). The following ranges are mentioned in the literature: albumine/globuline: 27 – 45 %, prolamine: 19 – 66 % and, for gluteline, concentrations of 8 – 10 % (Chen and Bushuk, 1970; Gellrich et al., 2003). Another way to classify rye proteins is based on their physiological behaviour. Two groups are mentioned: **storage proteins** and **non-storage proteins** (Verwimp et al., 2012). The latter include, in addition to the Osborn fractions **albumin** and **globulin,** mainly rye enzymes and their inhibitors.

The Osborn fraction **prolamin**, in relation to rye called **secalin**, forms the major part of the rye storage proteins (Gellrich et al., 2003). Due to their amino acid composition, the prolamins can be subdivided into S-rich, S-poor and HMW secalins. For the S-rich or γ-secalins, there are two groups of polypeptides, depending on their molecular weight: **γ-40K-secalins** and **γ-75K-secalins**. There are two structural domains for the γ-secalins: N-terminal and C-terminal (Kreis et al., 1985; Shewry et al., 1982) with different conformations (Shewry and Tatham, 1990; Tatham and Shewry, 1991). In addition to their structural classification, the γ-secalins also differ in their aggregation behaviour.

The **γ-40k-secalins**, which consist of, among others, 18 % of prolamin (Pro) and approximately 35 % of glutamine (Glx), and account for 26 % of the total secalin fraction, appear as monomers containing intramolecular disulphide bonds (Field et al., 1983; Gellrich et al., 2003; Shewry et al., 1983). The relative molecular mass (M_r), as determined by electrospray-ionisation quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS), ranges from 32,141 to 32,446 (Schalk et al., 2017).

The **γ-75k-secalins,** which consist of 23 % Pro and 40 % Glx and account for 45 % of the total secalin fraction, are mainly present as disulphide-linked aggregates (Gellrich et al. 2003, Shewry et al. 1982). The determined M_r of the γ-75k-secalins ranges from 52,313 to 60,476 (Schalk et al., 2017). The differences in the aggregation behaviour between γ-75k-secalins and the γ-40k-secalins are, among other reasons, caused by the presence or absence and the position of the cysteine residues. Regarding stereochemistry, only cysteine residues in favourable positions can form disulphide bonds (Kipp et al., 1996).

The S-poor or **ω-secalines,** which mainly consist of Pro (39 – 43 %) and Glx (28 – 31 %), account for approximately 19 % of the total secalin fraction (Gellrich et al., 2003). The M_r ranges from 39,004 to 39,457 (Schalk et al., 2017). ω-secaline are further characterised by a high amount of phenylalanine and the absence of sulphurous amino acids. ω-secalines mainly appear as monomers (Field et al., 1983; Gellrich et al., 2003; Tatham and Shewry, 1991).

The **HMW secalins** consist of 13 % Pro and about 33 % Glx and display high concentrations of glycin, in contrast to the secalin fractions described above (Field et al., 1982; Gellrich et al., 2003; Shewry et al., 1988). The M_r ranges from 78,173 to 85,154 (Schalk et al., 2017). HMW secalins are detected as aggregates and represent, with a share of 5 % of the total secalin fractions, the lowest amount (Field

et al., 1983; Gellrich et al., 2003; Shewry et al., 1983). The N-terminal amino acid sequences of the HMW secalins are homologous to those of the HMW subunit of wheat (Field et al., 1982; Shewry et al., 1988).

The fourth and final Osborn fraction, which is not soluble in alcohol, is referred to as **glutelin** or, in relation to rye, as **secalinin**. These polymers are stabilised by disulphide bonds. However, the treatment of glutelins with a reducing agent leads to subunits, which are soluble in alcohol. These subunits are rich in prolin and glutamin (Field et al., 1983; Field et al., 1982; Gellrich et al., 2003; Kipp et al., 1996; Shewry et al., 1983). Secalinins mainly comprise the following proteins: HMW secalins, γ-75k-secalins and γ-40k-secalins, with shares of 26 %, 52 % and 12 %, respectively (Gellrich et al., 2003).

Despite having partially homologous regions, rye proteins do not form a protein network as wheat proteins do. In comparison with wheat, rye has fewer storage proteins and a high ratio of alcohol-soluble proteins to insoluble proteins (Chen and Bushuk, 1970; Gellrich et al., 2003; Preston and Woodbury, 1975). Furthermore, rye storage proteins differ regarding disulphide structure and, thus, in their aggregation behaviour. The aggregation behaviour of γ-75k-secalins is restricted due to the absence of additional cysteine residues in the C-terminal domain that forms intermolecular disulphide bonds (Gellrich et al., 2004). Therefore, the formation of a large protein network as described for wheat is limited.

The enzyme TG performs protein crosslinking. This enzyme (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) catalyses acyl-transfer reactions between the acyl group of Glx and the amine group of lysine (Lys), as well further primary amines (Gerrard et al., 2001; Motoki and Seguro, 1998).

In this case, the non-storage rye proteins are important because they contain high proportions of Lys compared with the storage proteins. In addition to the non-storage proteins, storage proteins are also important due to the high proportions of Glx (Dexter and Dronzek, 1975; Gellrich et al., 2003; Preston and Woodbury, 1975).

Transglutaminase is isolated from several species, including animals, plant tissues and microorganisms. The pH range for TG activity from microorganisms is 4 – 9, whereas the optimum is pH 7. The optimum temperature is 50 °C. Temperatures lower than 40 °C and higher than 60 °C reduce the enzyme activity by approximately 50 % (Katsuya et al., 1996). For proteins, the addition of TG leads to the formation of a protein network. For wheat dough, a weak gluten network can be transformed into

a strong network by adding TG (Larré et al., 2000). For dough structure characteristics, TG can increase dough extensibility, elasticity and water-holding capacity (Basman et al., 2002; Bauer et al., 2003; Caballero et al., 2007). Köksel et al. (2001) reported that a wheat dough treated with TG displayed, in comparison with untreated dough, an increased complex shear modulus (G*), which is related to an increase of the molecular average weight of the proteins. In addition, TG improves wheat bread loaf volume and modifies crumb hardness (Autio et al., 2005; Gerrard et al., 2001). The mentioned positive effects on dough and bread characteristics were also reported for dough and bread made from gluten-free and rye flours (Dłużewska et al., 2015). For the latter, the addition of TG up to 1000 U TG kg^{-1} leads to a more structured and elastic protein network, as well as a higher loaf volume and crumb springiness. On the other hand, the positive effect of TG addition is limited (Beck et al., 2011). In addition to the number of free glutamine and lysine residues, AX are mainly mentioned as hindering protein interaction (Cauvain, 2015).

1.3 Rye dough structure processing

Based on the chemical composition of rye flour, the production of rye dough differs to that of wheat dough (Weipert, 1997). The consistency of rye dough has minor elasticity and gas retention, which leads to a reduced bread volume and a compact crumb (Weipert, 1997). This consistency is mainly attributed to the dough ingredients mentioned in Sections 1.2 and 1.3. The influence of these ingredients on rye dough and bread structure is discussed in detail regarding the main steps of rye bread production, which can be divided into the following three phases: **Dough mixing**, **fermentation** and **baking** (Shah et al., 1999).

Rye dough structure is a complex, semi-solid hydrated system mainly composed of starch, proteins, lipids, non-starch polysaccharides (mainly AX), water and other nutrients and additives (Gan et al., 1999). For rye **dough mixing**, all the ingredients (mostly rye flour, salt, yeast, sourdough and water) are merged into a kneading bowl and mixed. The aim of the mixing process is two-fold: first, to form a homogenous dough. During the initial mixing phase, the flour particles, water and the other mentioned components are brought into close contact by mechanical work. In detail, the dough develops a coherent, 3D viscoelastic structure as a result of the formation of the protein-AX-starch matrix, in which starch granules are consistently detached. Arabinoxylan and proteins can also occur detached or linked to each other via ferulic

acid, as described above. The formation of a protein network compared to that of wheat dough is prevented by two factors: the chemical structure of the proteins and the high level of AX. The AX inhibit the ability of rye proteins to connect and, thus, to form a protein network (Cauvain, 2015). Consequently, for the traditional method of rye bread production, proteins play only a minor role in the structure-forming process of rye dough and bread (Cauvain, 2015). The gluten-starch matrix in wheat dough determines the rheological parameters of extensibility and strength; but for rye dough, the protein-AX-starch matrix determines viscosity and stability (Sroan et al., 2009).

Arabinoxylan form a sticky gel that holds the dough together. In addition, a high content of AX raises water absorption, which leads to increased dough viscosity. The viscosity depends on several factors, such as the relationship of WEAX to WUAX, interactions of AX to flour components (ferulic acid) and the swelling and solubility properties described above. The solubility and swelling properties of AX increase at low pH values (4.1 – 4.9), a level traditionally reduced in rye dough by the use of sourdough (Hammes and Gänzle, 1998). The acidification leads to a partial hydrolyse of WUAX to WEAX. The increased content of WEAX and the reduction of WUAX significantly influence dough viscosity and gas bubble stability during fermentation, as described in Section 2.2. In addition to the chemical structure and interaction of AX with other flour ingredients, processing parameters also influence the final dough and bread appearance. For wheat dough it is usual to characterize dough preparation also by their dough development time. In the case of rye dough, this would not be meaningful, because time-dependent protein networking does not occur. Rye dough must be mixed gently to keep the mechanical stress short, especially towards the end of the mixing time. The usual mixing time and energy input for wheat dough preparation would destabilise the structure of rye dough due to the high native content of AX in the raw material: bound water is released and the dough softens, becomes stickier and loses its dimensional stability. This effect results in flat bread with compact crumbs. The optimal mixing time and energy input depends on other processing conditions, such as rye flour composition and enzymatic activity (Seibel and Weipert, 2001). The second aim of the mixing process is the incorporation of gas cell nuclei by trapping air through the action of surface renewal in the dough matrix (Campbell and Mougeot, 1999). The gas cell nuclei are thought to be the start of the gas bubble growth. In addition to the incorporation of air via overlapping dough sheets caused by the mixing process, the choice of mixer

geometry and energy input strongly influence the number and size of the entrained gas bubbles (Cauvain and Young, 2006).

At the end of the mixing process, the dough is divided and moulded into the desired forms. As required for wheat dough, a resting period after mixing for protein relaxation is unnecessary due to the absence of a protein network. However, for over-mixed or intensively mixed dough, a short rest induces the rebinding of released water during mixing.

The next step, which significantly influences the rye dough structure, is the **fermentation** process. The common temperature for the fermentation process is set between 28 °C and 34 °C. At this temperature range, the production of carbon dioxide (CO_2) by *saccharomyces cerevisiae* is optimal. The relative humidity is set between 75 % and 85 %. The high relative humidity prevents the dough surface drying, which, in turn, prevents deficit in appearance of the final bread. During fermentation, the metabolism of yeasts chemically transforms carbohydrates into CO_2 and ethyl alcohol as the principal finished compounds. Yeast performance determines the CO_2 and ethanol production, whereas gas retention determines the final dough structure, appearance and volume. Gas retention mainly depends on three structural factors: the surface tension of gas bubbles, the internal pressure of the gas bubble and the rheology of the protein-AX-starch matrix. From the rheological point of view, the protein-AX-starch matrix determines viscosity and stability. During the fermentation process, the gas bubbles expand, with strain rates of 10^{-3}/sec - 10^{-4}/sec (Dobraszczyk, 1997).

Gas cell growth results in an expansion of the protein-AX-starch matrix, causes a thinning of the dough matrix surrounding the expanding gas cells and, if the expansion does not stop, dough matrix rupture (Kokelaar et al., 1996). However, the thinner dough film can resist further extension by increasing stress. This phenomenon is called strain hardening and occurs at stress rates proportionally higher to the applied strain rate (Dobraszczyk and Roberts, 1994; Van Vliet et al., 1992). Equation 1 describes the power law relation between stress and strain:

$$\sigma = K\varepsilon^{n} \qquad (1)$$

where σ is the stress, K is the power law constant, ε is the strain and n is the strain-hardening index (Dobraszczyk et al., 2003; Sroan et al., 2009). Good dough stability

occurs at strain-hardening indices (n) ≥ 1, or is expressed by an exponential relationship for n ≥ 2 (Dobraszczyk, 1997; Dobraszczyk and Roberts, 1994; Dobraszczyk and Salmanowicz, 2008). For rye dough, a strain-hardening index of 1.3, present in an exponential relationship, was observed. This result indicates that strain-hardening occurs less in rye dough and is more pronounced in wheat dough. This finding is attributed to the formation of a gluten network, which provides extensibility and strength to wheat dough. Wheat flours with poor gluten quality have low strain-hardening indices (1.7 – 1.8) (Kokelaar et al., 1996). Low strain-hardening indexes are directly linked to decreased bread volumes and irregular crumb structures due to early gas bubble coalescence and dough rupture. This link further explains the poor bread-making performance of rye dough. These phenomena reveal the importance of the formation of a protein network with specific contextual requirements.

In addition to the rheology of the protein-AX-starch matrix, gas retention is further determined by gas bubble stability, their surface tension and their internal pressure.

The number and distribution of gas bubbles mainly depend on the mixing process and on dough-processing steps such as portioning and moulding (Gan et al., 1995). The growth and, thus, the stability of the gas bubbles depend on dough composition, cell size, yeast cell metabolism and availability of fermentable sugars (mono- and disaccharides). Bubble growth further depends on the permeability, coalescence, surface tension and internal pressure of the gas cell (Bloksma, 1990; Chiotellis and Campbell, 2003; Turbin-Orger et al., 2012). The rate of gas bubble growth (dR/dt), is described by Equation 2:

$$\frac{dR}{dt} = \frac{R(\Delta P - 2\sigma)}{4\eta} \qquad (2)$$

where R is the bubble radius, t is the time, η is the viscosity, σ is the surface tension and ΔP is the difference of bubble pressure (interior) and atmospheric pressure (Mitchell et al., 1999). With increasing bubble size, the gas bubbles expand until they rupture.

To describe the effect of structure on gas bubble formation, it is necessary to consider the role of the lamella (Figure 5 a and 5 b).

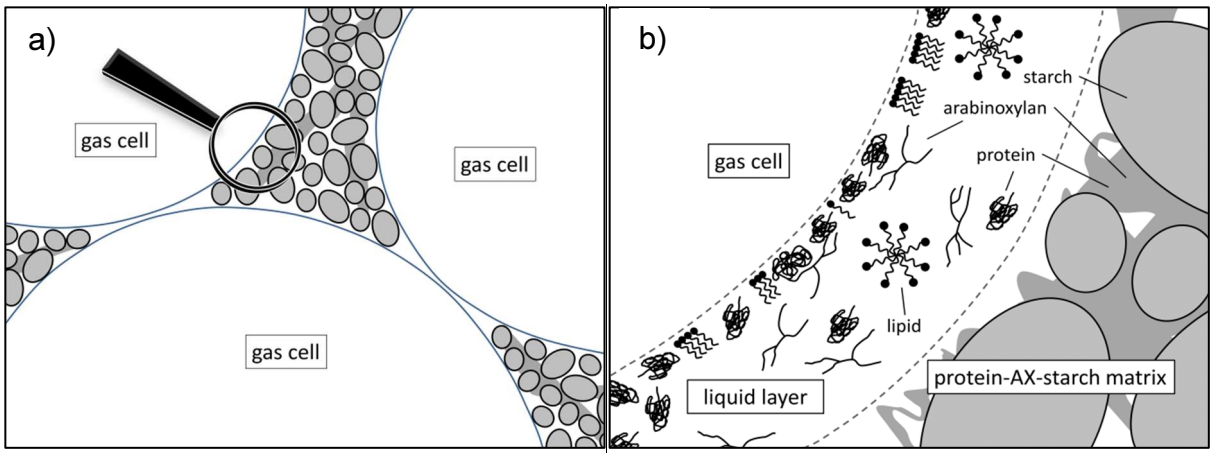

Figure 5: a) Section of rye dough showing the gas cells and the protein-AX-starch matrix. b) Three main parts of dough presented in detail: gas cell, lamella (liquid layer) and protein-AX-starch matrix.

The lamella acts as an enclosure of the gaseous bubbles and, thus, influences the growth and the coalescence of gas cells produced by fermentation. The stability of these films depends on the viscosity and the surface tension, as well as the elastic force caused by gas compressibility (Wilde, 2012). Competition between these forces results in the equilibrium state.

From the chemical point of view, as seen in Figure 5 b, the lamella of rye dough gas bubbles mainly consists of proteins, lipids and AX (i.a. WEAX). The proteins allow the stabilisation of the lamella by adhering to the surface of the gas cell. The lipids destabilise the protein layer by decreasing the surface tension of the film, but lipids in high quantity take the place of proteins and stabilise the film (Wilde, 2012). Arabinoxylan, especially WEAX, assume the main function for film stabilisation by increasing its viscosity and by stabilising the interfacial layer between the protein-starch matrix and the gas cell. However, WUAX decrease gas cell stabilisation by forming intrusions in the gas cells during fermentation (Courtin and Delcour, 2002). To minimise the influence of WUAX, Xyl can be added. The addition of Xyl to the dough leads to a decrease in the ratio of WUAX to WEAX in favour of WEAX. Further degrading of HMW WEAX to LMW WEAX reduces the positive effect of film stabilisation. A further degrading by overdosage leads to the formation of oligosaccharides or to monomers xylose and arabinose and decreases the aforementioned advantages of WEAX on the dough and bread characteristics.

The third phase in rye dough and bread structure formation is the **baking** process. This process occurs directly after the fermentation process. Common baking temperatures for rye bread are between 200 °C and 240 °C initially, then falling to

180 – 220 °C, depending on the desired final bread appearance. During the baking process, both heat and mass transfer occur (Sakin et al., 2009). During the initial phase, steam (approx. 0.5 L) is used to enhance the development of bread volume. The steam accumulates on the dough surface, blocking the vapour transfer from the dough and delaying the drying of the surface until the dough irreversibly merges into crust and crumb (Schirmer et al., 2011). Additionally, the condensed steam accumulated on the initially cold dough enhances heat conduction to the inner dough matrix.

During baking, the dough undergoes two main endothermic transitions:

- The gelatinisation of starch. This process occurs at a temperature of approximately 60 °C. The combination of water and heat breaks down the intermolecular bonds of starch molecules, allowing greater water retention and, thus, dissolving the starch granules in a plasticised form, so viscosity increases. This process highly depends on the ratio of water to starch and is influenced by the amount of AX. Flours with high AX concentrations, mainly WEAX, perform much better in bread making than those with a low AX content. This effect is linked to the water holding capacity of AX, whereby starch gelatinisation delay (Weipert, 1993, 1997). It could be also conceivable that AX cover the starch granules; resulting in limited starch gelatinisation and therefore in poor bread appearance (Heinz et al., 2015; Kaiser et al., 2015). Clarification of this hypothesis is needed and should be part future experimental work. However, it can be concluded, that the degrees of starch gelatinisation depend on the amount of water available in the dough systems.

- The denaturation of proteins. This process occurs at temperatures between 50 °C and 80 °C. During this transition, the protein extensibility and cohesivity decrease and the stability increases.

The baking stage is further characterised by the rapid expansion of gas cells (strain rates of 10^{-3} - 10^{-4}/sec) due to the increased pressure initiated by thermal treatment during baking (Dobraszczyk, 1997). Notably, gas pressure rises proportionally with temperature, following the Gay-Lussac Law. Therefore, once the gas cells rupture due to the increased pressure, the gas diffuses throughout the structure. From the baking point of view, this action is known as ‘end of oven spring’. The heat treatment further transforms the foamy dough into a spongy crumb, as is characteristic of the

final product bread. The volume increase in dough is in addition to a thermal gas expansion caused by the release of carbon dioxide and water vapour. Volume increase by carbon dioxide occurs at low temperatures (50 – 60 °C). Gas bubbles, created and trapped by mixing, can receive carbon dioxide from yeast fermentation. Carbon dioxide production is related to the yeast activity and stops at temperatures (approx. 45 °C and more) that deactivate the yeast. Volume increase by water vapour occurs at high temperature. The water held by the matrix turns into vapour and expands the matrix. Dough expansion by vapour depends, in part, on the water level of the dough, where and how it is held in the matrix and the rate at which heat is input (Bloksma, 1990).

Despite all these positive effects of AX on rye dough and bread production, the final structure and its function are not comparable to those of wheat products. This issue illustrates the need for a well-developed and aggregated protein network.

1.4 Thesis outline

For rye dough and bread processing, AX play a key role in structure formation (structure-function relationship) and, thus, the final appearance. Therefore, much work has been carried out to understand the role of AX in rye dough structure formation (Buksa, 2016; Cyran and Cygankiewicz, 2004; Parkkonen et al., 1994; Weipert and Zwingelberg, 1980). Analyses of the AX structure, of interactions between AX molecules (via oxidative crosslinks of ferulic acid), of AX molecules of other flour components (proteins, lignin) and of physicochemical properties such as viscosity, water-holding capacity and extractability have been performed (Buksa et al., 2016; Kipp et al., 1996; Kühn and Grosch, 1988; Piber and Koehler, 2005). Moreover, several theories exist that explain how optimal concentrations and substitution patterns of AX stabilise and improve rye dough structures, whereas variations can finally weaken the structure (Courtin and Delcour, 2002; Izydorczyk and Biliaderis, 2007).

Furthermore, with the exception of the work by Beck et al. (2011), the role of rye proteins in rye dough structure formation has not been the focus of food scientists. This lack, in addition to the assumption that AX hinder rye proteins from aggregating, is attributed to the widespread assumption that rye proteins do not aggregate according to their chemical structure. Although rye proteins are not directly involved in rye dough structure formation, isolated rye proteins do have the ability to

aggregate (Gellrich et al., 2003). Furthermore, it has been assumed that crosslinked rye proteins can take over a part of structure formation in rye dough and, hence, increase the final structure and appearance of rye dough and bread.
Therefore, the following hypothesis is postulated: *Crosslinked rye proteins can take over the main part of structure formation in rye dough and bread and, thus, improve the existing structure properties, such as elasticity, stability and expandability.*

This hypothesis is based on the following theory. It is assumed that excessive degradation of AX by Xyl (endo-1,4-Xylanase derived from *Bacillus subtilis*) will increase the accessibility of the proteins. The excessive degradation will be reached by overdosing Xyl. The increased accessibility by AX degradation, in turn, could induce the formation of a protein network according to protein crosslinks during mixing. Figure 6 is a schematic drawing of the influence of AX on protein accessibility and the role of Xyl.

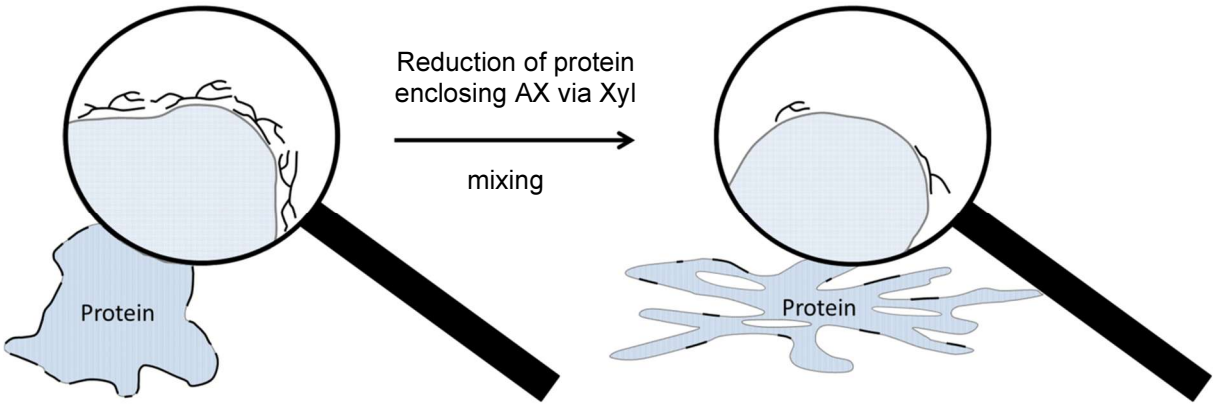

Figure 6: Schematic drawing of the protein behaviour during mixing, depending on the AX content. The reduced AX content leads to an increased protein access and stretching.

Although an increased interaction of rye proteins and, thus, a more elastic structure is expected, the protein network cannot sustain the high forces during mixing, proofing or oven rises due to the low ability of rye proteins to connect according to their chemical structure. To enhance the protein interaction and, thus, the formation of a coherent and functional protein network as described for wheat dough, the use of the protein-crosslinking enzyme TG (transglutaminase derived from *Streptoverticillium* sp.) is envisaged. Rye protein crosslinking by TG seems to be optimal due to the high Glx and Lys content of 23.6 mol/100 mol and 3.1 mol/100 mol amino acid, respectively (Belitz et al., 2004). It is further expected that the improvement will lead to increased bread volume and more elastic crumb. To

investigate the influence of TG on protein network formation, different enzyme concentrations, including overdosing, were applied. The mechanism of protein crosslinking, illustrated by the enzyme TG, is displayed in Figure 7.

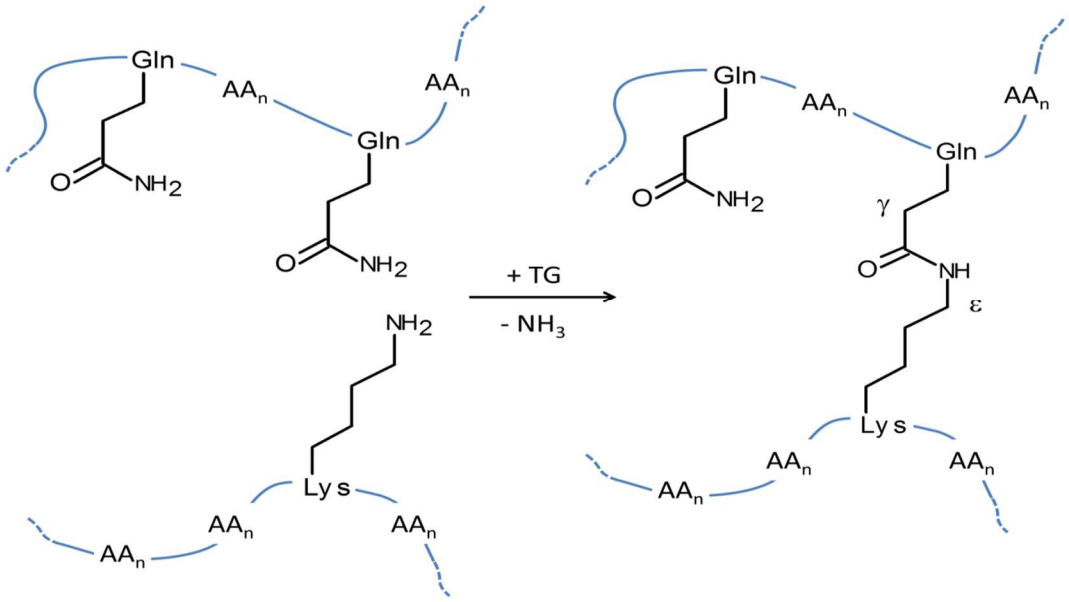

Figure 7: Transglutaminase catalysed reaction between glutamine (Gln) and lysine (Lys) residues of proteins (AAn: amino acid). The catalytic reaction leads to ε-(γ-glutamyl)lysine bonds.

The improvement of structure formations as described above has not been investigated for rye dough and bread in general. Furthermore, the formation of structures by enzymatic action is far from clear and deserves detailed study. Therefore, the main research topics of this thesis focus on the mechanism of rye AX and rye protein on rye dough and bread structure formation. Further focus is placed on the enzymatic modification of rye AX and proteins for structure optimisation.
To clarify, the thesis covers the following points:

1. A critical summary of the effect of different AX patterns (WUAX, WEAX, xylooligosaccharide) on dough and bread structures, as well as the detection and quantification of AX in cereal-based products.
2. Analysis of the structure-function relationships of rye dough and bread.
3. Effect of AX on structure formation in rye dough and bread.
4. Localisation of AX in rye dough via innovative microscopy and image analysis.
5. Rye dough and bread structure formation and its functional optimisation via rye proteins via the usage of TG and Xyl.

To realise the formation of improved structures by enzymatic treatment, the focus is on the dough. Compared with flour or bread, dough appears the most appropriate phase according to its surrounding conditions, such as temperature and water content. The investigations reveal the role of AX and protein on the structure-function relationship and further increase our understanding of the role of enzymes on the structure formation of (rye) dough and bread.

1.5 Methods

This chapter describes the methods for dough and bread analysis.

1.5.1 Analytical investigations

The methods used for analytical investigations are summarised in Table 1:

Table 1: Applied methods for analytical investigations

Analysis	Method	Source	For detailed description see following Section:
Moisture content	ICC Method 201	ICC – International Association for Cereal Science and Technology	Sections 2.3, 2.4, 2.5
Protein content	Kjeldahl Method	EBC – European Brewery Convention	Sections 2.3, 2.4, 2.5
Ash content	AACCI Method 08-12.01	AACC International. Approved Methods of Analysis 1999a,	Sections 2.3, 2.4, 2.5
Solvent-retention capacity	AACCI Method 56-11.02	AACC International. Approved Methods of Analysis	Section 2.3
β-glucan content	AACCI Method 32-23.01	AACC International. Approved Methods of Analysis	Section 2.3
Total starch	AACCI Method 76-13.01	AACC International. Approved Methods of Analysis	Sections 2.4, 2.5
Starch damage	AACC Method 76-33.01	AACC International. Approved Methods of Analysis	Section 2.5
	ICC Method 172	ICC – International Association for Cereal Science and Technology	Section 2.4

1.5.2 The role of water in measuring viscoelastic dough properties

For typical rye dough production, all the ingredients (mostly rye flour, salt, yeast, sourdough and water) are merged into a kneading bowl and mixed. The final product properties, such as bread volume and crumb hardness, vary according the dough firmness (in the present study expressed as complex shear modulus [|G*|]: too firm and the bread appearance will be poor (low bread volume, compact crumb); too soft and the dough will flow and result in poor bread appearance (Cauvain, 2003). The desired dough firmness depends on the amount of added water. Varying the water content or modifying the ingredients changes the amount of non-bound water and, thus, the viscoelastic properties of the dough. Depending on the parameters (e.g. elasticity, viscosity) that determine the influence of the modifications, misinterpretation of the results is possible. To minimise the influence of different dough firmness on the viscoelastic properties of the dough, each dough was set to the same dough firmness by varying the water addition. The procedure of dough firmness adjustment by water addition to a fixed value was performed using the trial-and-error method. Further settings and a detailed description of the procedure are in Section 2.4.

1.5.3 Rheological investigations

To determine the viscoelastic properties of enzyme-treated dough, oscillation tests and creep-recovery tests were performed using an AR G2 Rheometer (TA Instruments-Waters LLC, New Castle) with a plate-plate geometry (d = 40 mm).
Oscillation tests were performed to analyse the influence of enzymes in a non-destructive manner. Creep-recovery tests were performed to determine the viscoelastic behaviour at higher (structure-destructive) shear rates. The settings and the procedure of the methods are described in detail in Sections 2.3, 2.4 and 2.5.

1.5.4 Dough fermentation monitoring

Maximum dough height, total volume of gaseous release and gas retention were determined as described in the manual of the manufacturer of the Rheofermentometer F3 (Chopin-Groupe, 2000). A quantity of 315 g of dough was placed in the chamber and fermented for 3 h at 30 °C.
A detailed description of the procedure is explained in Section 2.3.

1.5.5 Protein and arabinoxylan visualisation via Confocal Laser Scanning Microscopy

Visual detection of AX was performed using Confocal Laser Scanning Microscopy (CLSM) from Nikon (Düsseldorf, Germany). The visualisation of dough ingredients has traditionally been performed by staining techniques such as Rhodamine B (protein), Nile Blue (starch) and Calcoflour (β-glucan).

For protein detection, fluorescent dye Rhodamine B (c = 0.001 g 100 mL^{-1}) from Sigma-Aldrich Chemie GmbH (Munich, Germany) was added on top of the sample dough. After one minute, the specimen shape was covered by a cover slip. Protein structure was analysed using a 60 x objective.

Micrograph analysis was performed using image processing open source Java software ImageJ (Version 1.42q, National Institutes of Health, Bethesda, Md, USA) with the DoMiQ (dough microstructure quantification) method described by Jekle and Becker (2011). The following protein features were analysed: perimeter (the length of the outside boundary of the selection) and feret diameter (the longest distance between any two points along the selection boundary).

Further settings and a detailed description of the procedure are in Sections 2.4 and 2.5.

No dye is known for the direct visualisation of AX. The known AX-staining techniques are based on the immunolabeling of AX using dye-labelled antibodies or an inactive fluorescent-labelled Xyl binding to AX. For all techniques, good results were achieved concerning AX detection, and the applied techniques were then used to visualise AX in grain cell walls. No information exists regarding whether these techniques work for AX detection in dough.

For AX detection in dough, a polyclonal antibody-FITC solution, acquired from Coring System Diagnostix GmbH (Gernsheim, Germany), was diluted with bovine serum albumin (BSA) buffer in a ratio of 1:10. Then, 20 mL of the AX antibody suspension was added to the top of the sample dough and stored at 30 °C with 80 % RH for 30 min. Then, non-bound antibodies were removed by washing the dough surface carefully with the BSA buffer. The AX structure was analysed using a 60 x objective.

The method and procedure developed to visualise AX in dough are described in detail in Section 2.5.

1.5.6 Quantitation of arabinoxylan

The content of total AX and WEAX of the bran fractions and rye flour was determined by the hydrolysation of the AX with 4.0 M H_2SO_4. Monosaccharides after hydrolysation were analysed by anion exchange chromatography with pulsed amperometric detection. The method was performed according to Houben et al. (1997), with small modifications. The modifications are described in detail in Section 2.3.

The quantification of WEAX in rye bread was performed using gel permeation high-performance liquid chromatography (GP-HPLC). Analysis of the data was conducted using Agilent Galaxie software. The method is described in detail in Section 2.3.

1.5.7 Bread characterisation

For bread characterisation, bread volume was analysed using a laser-based volumeter BVM-L370 (TexVol Instruments AB, Viken, Sweden) according to AACCI Method 10-05.01. Crumb hardness was analysed using a TVT-300 XP Texture Analyser (TexVol Instruments AB, Viken, Sweden) following AACC Method 74-10.02. Bread weight was determined using a laboratory balance (Kern QKE 8K005, Kern & Sohn, Balingen-Frommern, Germany). The density was calculated using the ratio of bread weight and bread volume.

Further settings and a detailed description of the procedure are in Sections 2.3 and 2.5.

2 Results (thesis publications)

2.1 Summary of main results

This chapter summarises the thesis publications, followed by the original paper.

PART 1 TECHNOLOGICAL AND ANALYTICAL METHODS FOR ARABINOXYLAN QUANTIFICATION FROM CEREALS

PAGES 32 – 44

One of the most-discussed rye grain ingredients with substantial influence on dough structure is the non-starch polysaccharide AX, which consists of a backbone of β-1.4-D-xylopyranosyl residues with randomly linked α-L-arabinofuranosyl units. Isolated and added as dough additive, AX influence dough structure significantly, including both desirable and undesirable effects. In addition to the concentration in flour, the effects of AX depend on several structural and physicochemical characteristics, such as the arabinose-to-xylose ratio, molecular weight and linkages to further dough ingredients. These characteristics also depend on the applied extraction method. Therefore, the first thesis publication provides an extensive overview of common extraction methods (enzymatical, chemical and physical) for AX isolation from different grain sources. Additionally, analytical methods for AX quantification are summarised and compared with each other. The review summarises and discusses the effect of AX on dough and bread structure. The conclusion is that not only are the structure (oligosaccharides, water-extractable and water-unextractable AX) and, thus, the physicochemical characteristics significantly influenced by the extraction method, but also the yield of the AX. The appropriate selection of the extraction method results in an accumulation of AX with selected physicochemical characteristics. From the technical point of view, AX, depending on their physicochemical characteristics, greatly impact the dough structure and, thus, the final dough and bread characteristics. Despite all the positive and negative effects of AX on dough and bread structure, there is insufficient information on the role of AX on protein network formation and, thus, on the final dough and bread structure.

Authorship contribution: Döring, C. Study design, literature search and interpretation of results, manuscript writing; Jekle, M. Critical review of study design and manuscript draft; Becker, T. Supervision and critical review of manuscript.

PART 2 EFFECT OF RYE BRAN PARTICLES ON STRUCTURE FORMATION PROPERTIES OF RYE DOUGH AND BREAD

PAGES 45 – 55

The elucidation of the structure formation and the characterisation of rye dough and bread matrices remain challenging. The aim of the present study is to gain a deeper insight into the role of AX in rye dough and bread structure formation and how it can be influenced. Therefore, the influence of different rye bran concentrations (0.0 – 20.0 %), with varying particle sizes (125 – 250 µm and 355 – 500 µm) and different processing methods, including the addition of endo-1,4-β-xylanase, were investigated in rye dough and bread. The influence of different treatments on the structure and the associated changes in dough function were evaluated using rheological methods, fermentation and baking tests. The results reveal no interruptions in the rye dough structure through the addition of bran particles (i.e. no structure failures were detected). However, the addition of bran particles increased water absorption, which consequently increased dough firmness, expressed as complex shear modulus (|G*|), and decreased the bread volume. In contrast, the addition of Xyl in combination with bran particles up to 15.0 % displayed similar rheological (tan δ and |G*|) and baking behaviour (spec. bread volume and crumb firmness) to rye dough samples without bran or the Xyl addition. This finding is attributed to the release of water from AX caused by enzymatic hydrolysis of the xylan backbone. Thus, rye dough and bread structure are mainly dominated by the concentration and water absorption of AX. Furthermore, these investigations indicate that rye proteins do not play a significant role in structure formation in a traditional production process. For further measurements, the adjustment of the dough firmness by water calibration should be examined to analyse the influence of water absorption caused by AX on dough and bread structure.

Authorship contribution: Döring, C. Study design, literature search, analytical method development, data analysis and interpretation, manuscript writing; Grossmann, I. Study design, literature search, analytical method development, data analysis and interpretation, manuscript writing data creation and analysis; Roth, M. Analytical

method development and interpretation of data of flour components; Jekle, M. Critical review of study design and manuscript draft; Koehler, P. Supervision and critical review of manuscript; Becker, T. Supervision and critical review of manuscript.
In the ‘Results and Discussion’ (page 7, column 1) of the present publication ‘Effect of rye bran particles on structure formation properties of rye dough and bread’ the phrase ‘On the contrary, in rye dough a gluten-starch matrix as described for wheat dough does not exist, thus there is no bran related decrease of Hm and R’ is misunderstandable and should be deleted.

PART 3 IMPACT OF ARABINOXYLAN ADDITION ON PROTEIN MICROSTRUCTURE FORMATION IN WHEAT AND RYE DOUGH

PAGES 56 – 62

To gain further insights into the role of AX in the protein microstructure formation of wheat and rye model dough, the impact of different AX concentrations (wheat and rye flour replaced by 0.0 - 10.0 g AX 100 g^{-1} flour) were investigated. This study assumed that AX, especially in high concentrations, could act as a physical barrier during the network formation of wheat and rye proteins. The final dough firmness, expressed as complex shear modulus (|G*|), of each wheat and rye model dough was adjusted using water calibration to a fixed value of 21,500 Pa and 12,100 Pa, respectively. This action was necessary because AX absorbs high amounts of water, which affects the final dough firmness and, thus, the rheological behaviour of the final dough. The influence of AX on protein network formation and dough performance was studied using fundamental rheological tests and microscopic imaging via CLSM. With increasing AX concentration, an increase in loss factor (tan δ) was observed (rye: 285 %; wheat: 148 %), which proves the negative influence of AX on the rheological behaviour of dough, especially in high concentrations as it occurs in rye flour (5 – 8 % AX). Image analysis of the micrographs confirms the negative influence of AX on the protein network formation of wheat and rye model doughs for AX concentrations higher than 5 %. Correlation analysis, especially between the perimeter of the protein network and the loss factor (tan δ), found significant values for both wheat (r = -0.76) and rye (r = -0.88) model doughs. In summary, the results

confirm the hypothesis that AX plays an important role in network formation in wheat and rye dough.

Authorship contribution: Döring, C. Study design, literature search, analytical method development, data analysis and interpretation, manuscript writing; Nuber, C. Data creation and analysis; Stukenborg, F. Data creation and analysis; Jekle M. Critical review of study design and manuscript draft; Becker, T. Supervision and critical review of manuscript.

In the conclusion of the present publication ‘Impact of arabinoxylan addition on protein microstructure formation in wheat and rye dough’ the term ‘protein formation’ is misunderstandable and should be replaced by ‘protein network formation’. The tenth sentence of the conclusion should be also corrected as follows: With increasing concentration of AX up to 10.0% AX (wheat model flour replaced by AX), an increase in tan δ of the wheat model dough of 148% was reported.

PART 4 ON THE ASSESSMENT OF ARABINOXYLAN LOCALIZATION AND ENZYMATIC MODIFICATION FOR ENHANCED PROTEIN NETWORKING AND ITS STRUCTURAL IMPACT ON RYE DOUGH AND BREAD

PAGES 63 – 72

From the results obtained in the previous study, it is evident that the protein network formation in rye dough is mainly dominated by the presence of AX. To confirm this finding and to understand the effect of AX on the formation of a possible rye protein network, two main objectives were examined. First, to identify whether AX inhibits rye protein interaction by protein covering, fluorescent-stained antibodies that bind specifically to the AX molecules were used. By means of image analysis, AX was identified as the main component enclosing the proteins. These findings confirm the hypothesis that AX encloses the proteins and thereby possibly prevents protein interaction. Therefore, the second aim was as follows: to identify whether protein network formation can be enhanced using AX-degrading (endo-1,4-β-xylanase) and protein-crosslinking enzymes (transglutaminase, TG). Using Xyl, it was assumed that the reciprocal protein accessibility increases, whereas the use of TG increases the network formation by protein crosslinking. In this case, it was of further interest to know whether the enzyme addition positively impacts rye dough and bread

appearance. The effect of three enzyme concentrations alone and in combination of Xyl (0.0, 0.1, 0.3, 0.5 g/kg flour) and TG (0.0, 1.0, 3.0, 5.0 g/kg flour) on rye dough micro- and macrostructure (CLSM, Rheometer) and baking performance (crumb hardness, density) were investigated. In these experiments, dough firmness, expressed as complex shear modulus (|G*|), was adjusted using water calibration to a fixed value of 21,500 Pa. Sensitive analysis found multiple effects of enzyme addition on dough structure. Image analysis revealed that the protein network percentage area could be increased significantly up to 38 %. Furthermore, baking performances indicated significantly increased bread volumes (approx. 11 %) in contrast to the results achieved for untreated dough and bread. These results also reveal that higher enzyme concentrations (Xyl > 0.3 g/kg flour; TG > 3.0 g/kg flour) reverse the detected positive effects. The present investigations offer further insight into the mechanism of rye protein network formation and how it can be modified.

Authorship contribution: Döring, C. Study design, literature search, analytical method development, data analysis and interpretation, manuscript writing; Hussein, M. Mathematical and statistical analysis; Jekle M. Critical review of study design and manuscript draft; Becker, T. Supervision and critical review of manuscript.

2.2 TECHNOLOGICAL AND ANALYTICAL METHODS FOR ARABINOXYLAN QUANTIFICATION FROM CEREALS

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Technological and Analytical Methods for Arabinoxylan Quantification from Cereals

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Arabinoxylan (AX) is the major nonstarch polysaccharide contained in various types of grains. AX consists of a backbone of β1.4D-xylopyranosyl residues with randomly linked αʟarabinofuranosyl units. Once isolated and included as food additive, AX affects foodstuff attributes and has positive effects on human health. AX can be classified into waterextractable and waterunextractable AX. For isolating AX out of their natural matrix, a range of methods was developed, adapted, and improved. This review presents a survey of the commonly used extraction methods for AX by the influence of different techniques. It also provides a brief overview of the structural and technological impact of AX as a dough additive. A concluding section summarizes different detection methods for analyzing and quantification AX.

Keywords Dietary fiber, enzymatic treatments, extraction, instrumental analysis, structure, technologic properties

INTRODUCTION

In recent years, pentosans which can be divided into arabinogalactan (AG) and arabinoxylan (AX) were in focus of many researches, in which especially AX was of main interest. A lot of research was done to extract, analyze, and identify AX as well as to analyze its influences on human health and its functional properties in foodstuff (Glitsø et al., 2000; Izydorczyk and Biliaderis, 2007; Izydorczyk and Dexter, 2008; Qiang et al., 2009). Especially, the chosen way of extraction determines different AX attributes such as molecular weight, water absorption, and AX content, which were so far been underestimated.

For human diet, AX is described as a helpful ingredient to prevent a lot of diseases like diabetes type 2, intestine cancer, and cardiovascular disease. In the intestine, AX stimulates the growth of probiotic bacteria which, in addition, has a positive effect on human health (Izydorczyk and Biliaderis, 2007). Furthermore, AX absorbs water and increases fecal bulk which decreases the concentration of potential carcinogens in the intestine (Izydorczyk and Biliaderis, 2007). Next to degradation of AX, a slight increase of acetic, butyric, and propionic acid concentration in the colon was also observed, which may result in reducing colon cancer (Aura et al., 2005). For diabetes type 2, it is assumed that AXOS lower the postprandial glucose level and insulin response.

There exists several publications about the positive effects of dietary fibers and how they influence the glycemic index (Torsdottir et al., 1991).

Extracted, purified, and added as food additive, AX can also impart technological properties to food (e.g. bakery products) such as an increased water binding capacity, foam stability as well as modified textural properties, and improved shelf life (Vinkx and Delcour, 1996).

Nevertheless, the detected positive properties of AX, especially in dough, are mainly determined by the AX structures and its compounds to other grain ingredients. In addition to the purification and concentration steps, but more dominantly the methodologies of AX extraction influence the structure of the achieved AX molecules. Therefore, different laboratory extraction methods of grain cultivars are being developed and optimized as in wheat, rye, and barley as well as for AX in food matrices such as dough and bread. For AX fortification and technological use (in foods), further researches have been carried out for different extraction techniques and solvents. Furthermore, the knowledge about easy, efficient and cheap extraction methods can be helpful for later improvement of

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functional and technological properties of food systems with AX (e.g. bakery goods) but also to facilitate research about positive effects of AX for human health.

A lot of time and effort has been spent to develop possible ways of extraction, isolation, fractionation, and detection of AX, due to the high content of AX in different cereal grains, the proven positive effects on human health and food properties such as bakery products as well as for analyzing and characterizing the AX molecule. The objective of this manuscript is to review present laboratory scale methods for extraction of AX. It also summarizes the impact of the different extraction techniques in respect to the achieved AX structure and its application as dough additive.

Structural Characteristics of AX

For all plant tissues and cereals, the general molecular structure of AX is similar (Vinkx et al., 1993). The main structure is described as a linear molecule that consists of a β1.4D-xylopyranosyl backbone with side chains of αLarabinofuranosyl linked at position 2 and/or 3 of the xylose-chain. This results in four different molecular structures of AX: unsubstituted xyl*p* (xylopyranosyl), monosubstituted xyl*p* at O2, monosubstituted xyl*p* at O3, and disubstituted xyl*p* at O2.3 (see Fig. 1) (Izydorczyk and Biliaderis, 1992; Vinkx et al., 1993; Izydorczyk and Biliaderis, 2007). The content of AX depends on its cereal source and its location in the grain. Concentration of AX increases from the inner (endosperm) to the outer layers (bran) (Lempereur et al., 1997). Table 1 summarizes AX contents of different cereal sources out of different cereal locations.

For AX characterization, the ratio of arabinose to xylose (ara/xyl) is described and investigated for different types of grains. Generally, the ratio of ara/xyl in AX from wheat endosperm (0.50–0.60) (Hoffmann et al., 1991; Dervilly-Pinel et al., 2001) and rye endosperm (0.48–0.55) (Bengtsson and Åman, 1990) is lower than in wheat bran (0.57–1.07) (Shiiba et al., 1993) and rye bran (0.48–0.78) (Ebringerová et al., 1990; Nilsson et al., 1996), respectively. The ratio of ara/xyl plays an important role for the solubility of AX. Unsubstituted regions of AX show an increased tendency of aggregation and thus become insoluble. The smooth unbranched regions without arabinose residues form aggregates due to stabilization by hydrogen bonds (Andrewartha et al., 1979; McCleary and Prosky, 2001; Courtin and Delcour, 2002; Kóhnke et al., 2011). Especially, Andrewartha et al. (Andrewartha et al., 1979) demonstrated that an ara/xyl ratio lower than ~0.43 causes a drastic drop in water solubility. For a more intensive investigation of the AX structure, it is necessary to be extracted. Extraction procedure depends on the type of AX since there are waterextractable arabinoxylan (WEAX) and waterunextractable arabinoxylan (WUAX) present in grain.

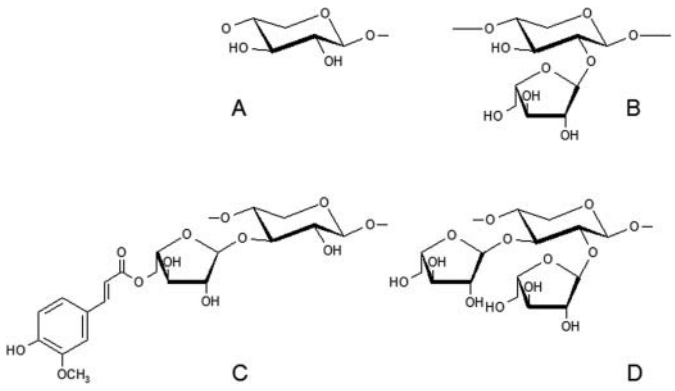

Figure 1 Identified AX molecules: (A) unsubstituted xylp; (B) monosubstituted xylp at O-2; (C) monosubstituted xylp at O-3 connected with ferulic acid; (D) disubstituted xylp at O-2,3.

Functionality and effects concerning the structural and functional properties of AX are not only dependent on its concentration in the plant but also on the constitution of the side chains. Differences in the AX properties are based on the aforementioned kind of substituent. Izydorczyk and Biliaderis (Izydorczyk and Biliaderis, 1995) found out that most of the arabinofuranosyl molecules of AX are present as monosubstituted residues. Only a small percentage of these side chains consist of more than one arabinofuranosyl substituent. These oligomeric side chains are attached to each other via 1→2, 1→3, and 1→5 linkages. Moreover, the relative amount of monosubstituted xylose at O-2 is low but especially these kinds of molecules were found in significant amounts in isolated barley and rye fractions (Ebringerová et al., 1990; Vinkx et al., 1995a). In addition to αLarabinofuranosyl and β-D-xylopyranosyl residues, small contents of other sugar residues such as galactopyranose and 4-O-methyl-α-D-glucuronic acid were detected (Höije et al., 2006).

In addition, ferulic acid (FA) and related derivate such as *p*-coumaric acid and sinapic acid play an important role as side chain constituents. In grains, FA is connected to lignin in the cell wall matrix. AX is linked to the cell walls by an ester bond between the FA carboxyl group and the hydroxyl group at C-5 position of the αLarabinofuranosyl side chain (Fig. 2; red structure) (Nilsson et al., 1996; Lempereur, Rouau et al., 1997; Bataillon, Mathaly et al., 1998; Courtin and Delcour, 1998; Nino-Medina et al., 2010). The mechanism of binding to the cell wall was described in detail by Ishii (Ishii, 1997). Concerning this attachment, FA can act as cross-link between AX as well as AX and Lignin (Tan, Hoson et al., 1991). For cross-linking AX side chains, two distinct mechanisms have been described: the photochemical induces dimerization as the predominant mechanism and on the other hand the oxidative coupling of dehydrodimers via peroxidases (Fig. 3) (Fry, 1979; Ford and Hartley, 1990; Hartley and Morrison, 1991). FA which is not bound to dehydrodimers can also connect to other cell wall constituents such as proteins, lignin, or cellulose by covalent and noncovalent interactions (Iiyama et al., 1990; Ralph et al., 1998; Piber and Koehler, 2005). These cross-links of cell wall components influence the wall

Table 1 AX levels of different cereals

Source	Total AX content [%]	WEAX content [%]	Analyzing method	Reference
Wheat				
Whole meal	4.07–6.02[ab]	0.37 – 0.56[ab]	Auto analyzer (spectrophotometer)	(Lempereur et al., 1997)
Whole meal	4.0–9.0[b]	0.3 – 0.9[b]	—	(Fincher and Stone, 2004)
Whole meal	n.d.	1.0[b]	gas liquid chromatography	(Ragaee et al., 2001)
Whole meal	5.5[b]	0.8[b]	HPAEC	(Krahl et al., 2009)
Whole meal	2.15–2.77[a]	0.50–0.51[a]	gas chromatography	(Dornez et al., 2008)
Flour	n.d.	0.35–0.54[ab]	gas liquid chromatography	(Loosveld et al., 1997)
Flour	n.d.	0.42[b]	gas chromatography	(Cleemput et al., 1995)
Flour	n.d.	0.31 – 0.44[a]	gel permeation chromatography	(Courtin and Delcour, 1998)
Flour	n.d.	0.44[ab]–0.71[ab]	gas liquid chromatography	(Dervilly-Pinel et al., 2001)
Flour	1.77–2.59[ab]	0.41–0.50[ab]	gas liquid chromatography	(Rouau et al., 1994)
Bran	24.1	0.9	gas liquid chromatography	(Maes and Delcour, 2001)
Bran	n.d.	1.5[c]	HPSEC	(Schooneveld-Bergmans et al., 1998)
Bran	19[b]	n.d.	HPLC	(Bataillon et al., 1998)
Bran	22.6[b]	1.8[b]	gas liquid chromatography	(Maes and Delcour, 2002)
Rye				
Whole meal	7.6	n.d.	gas liquid chromatography	(Bengtsson and Åman, 1990)
Whole meal	5.3–8.4[a]	2.6–4.0[a]	gas liquid chromatography	(Hansen et al., 2003)
Whole meal	n.d.	0.07–0.35[ab]	size exclusion chromatography	(Nilsson et al., 2000)
Whole meal	7.1–12.2[b]	0.6–2.4[b]	—	(Fincher and Stone, 2004)
Whole meal	n.d.	1.8[b]	gas liquid chromatography	(Ragaee et al., 2001)
Whole meal	n.d.	0.66	gas chromatography	(Delcour et al., 1999)
Whole meal	7.0[b]	1.4[b]	HPAEC	(Krahl et al., 2009)
Whole meal	7.3[b]	n.d.	gas chromatography	(Vinkx et al., 1995)
Flour	n.d.	2.10[b]	gas liquid chromatography	(Dervilly-Pinel et al., 2001)
Bran	n.d.	1.7–7.7	gas liquid chromatography	(Figueroa-Espinoza et al., 2004)
Other cereals				
Oat whole meal	2.2–4.1[b]	0.2[b]	—	(Fincher and Stone, 2004)
Barley whole meal	4–8[b]	0.4[b]	—	(Fincher and Stone, 2004)
Barley whole meal	4.38–7.79[ab]	n.d.	gas chromatography	(Henry, 1986)
Barley flour	n.d.	0.29–0.46[ab]	gas liquid chromatography	(Dervilly-Pinel et al., 2001)
Triticale flour	n.d.	0.55[b]	gas liquid chromatography	(Dervilly-Pinel et al., 2001)
Malt (wheat)	5.5[b]	1.5[b]	HPAEC	(Krahl et al., 2009)
Malt (rye)	7.0[b]	2.9[b]	HPAEC	(Krahl et al., 2009)
Different wheat milling fractions	1.44–30.66[b]	0.48–1.71[b]	gas chromatography	(Delcour et al., 1999)

[a]Different varieties
[b]Dry matter
[c]Glucuronoarabinoxylan
HPAEC, High Performance Anion Exchange Chromatography; HPLC, High Performance Liquid Chromatography; HPSEC, High Performance Size Exclusion Chromatography; n.d., not determined

characteristics in the original plant tissue such as flexibility, adherence, extensibility, and plasticity (Fry Stephen and Miller Janice, 1989). Apart from that, the mentioned cross links also influence the solubility of AX. It was shown that waterunsoluble AX are 8 to 39 times highly cross-linked than water-soluble AX (Bunzel et al., 2001). Therefore, researchers concluded that the solubility of AX does not only depend on the kind of arabinose substituent but also on the degree of cross-linking by diferulic acid (Michniewicz et al., 1990; McCleary and Prosky, 2001) and on the substitution pattern of AX (Köhnke et al., 2011). Up to now, there are only few indications for a possible classification scheme of AX into WEAX and WUAX. In a certain range of branching, the xylan backbone is water soluble; above and below it is not soluble, but this value can be shifted by the amount of linked arabinose units and interconnections for example FA. Especially, the ratio of WEAX to WUAX has an important influence on dough and bread characteristics as described below.

Technological Properties of AX

An addition of isolated AX to dough revealed a competition of AX with other flour components concerning the hydration of particles and influenced dough and bread characteristics (Michniewicz et al., 1991). For measuring water absorption, many analyzes were done by the farinograph. Originally, the farinograph is used for rating wheat flour characteristics such

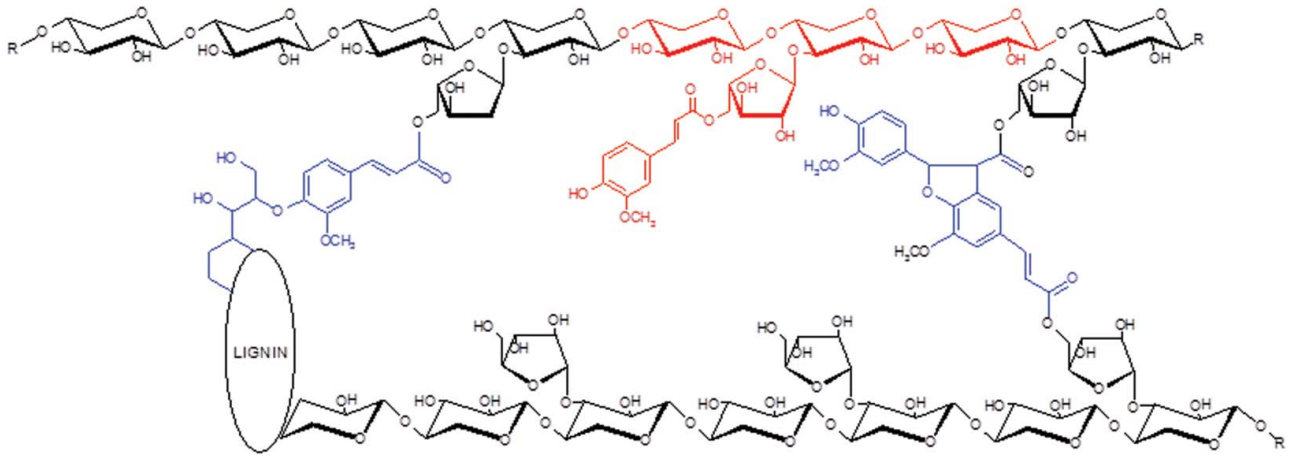

Figure 2 Identified AX structure according to Bunzel and Steinhart (Bunzel and Steinhart, 2003): Red structure: Isolated and identified ferulic acid AX fragments. Blue structure: Ferulic acid association, which can act as cross links between AX and Lignin.

as water absorption or dough development time (DDT) by a consistency measurement. For water soluble pentosans and water insoluble pentosans, consisting of approximately 70% AX, water holding capacities of 4.4 and 9.9 times their weight

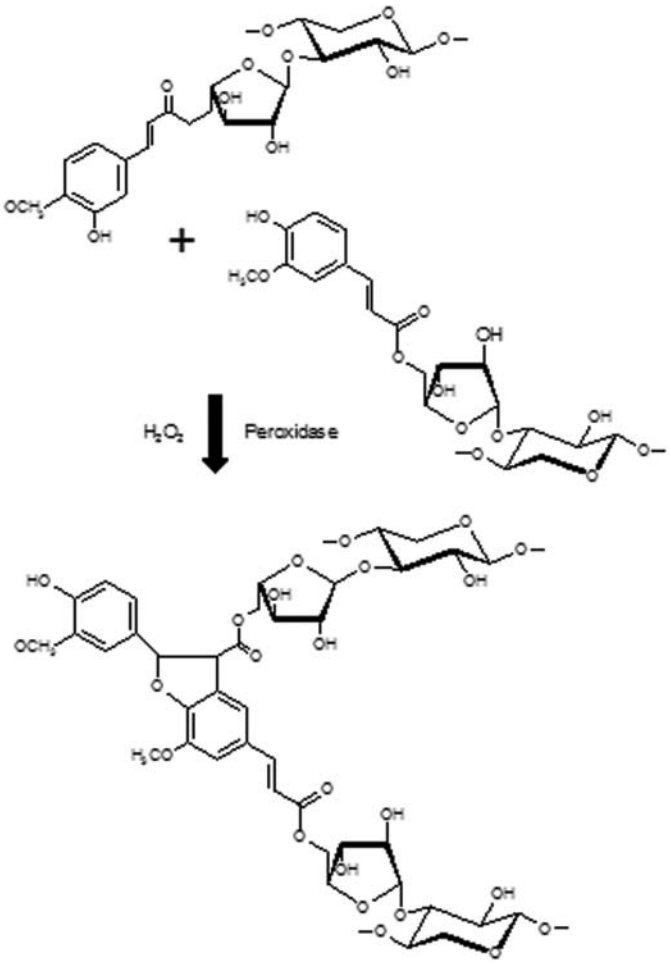

Figure 3 Oxidative coupling of ferulic acid.

was reported for wheat flour, respectively (Kim and D'Appolonia, 1977). Girhammar and Nair (Girhammar and Nair, 1992) reported wheat water soluble and insoluble pentosans water holding capacities of 11 and 10 times of their weight, respectively. Jelaca and Hlynka (Jelaca and Hlynka, 1971) showed that water soluble pentosans absorb 9.2 times their weight of water and water insoluble pentosans 8.0 times their weight. Worth to mention, the uncertainty in the water absorption measured by the farinograph is caused by the AX itself or by the interactions of AX with other flour components such as FA linkages which could result in a gel network and therefore in a higher water absorption as described later. Several scientists proposed different mechanisms how water is generally bound in polysaccharides. Water is associated with solutes in a number of ways such as water absorption on hydrophilic sites of the molecule by hydrogen bonding or water held by a network (Labuza and Busk, 1979; Chen et al., 1984; Chaplin, 2003). Thus, the determination of water absorption is basically dependent on the kind of water binding. It is assumed that these attributes are affected by the molecule structures themselves as well as by the pattern of intramolecular and intermolecular bonds. In addition, it is also assumed that these abilities apply equally for water-extractable and water-unextractable AX. WUAX is not able to dissolve in water due to its pattern of structure as described before. Nevertheless, it is assumed that WUAX are able to bind water by capillarity action and by hydrogen bonding (Chaplin, 2003). Further, investigations prove that the content of potential reactants with AX such as FA also have a strong influence on the water holding capacity of AX (Pentoans) (Izydorczyk and Biliaderis, 1992). Consequently, for measuring the water absorption capacity of AX, it is necessary to determine the content and the degree of connectivity of possible AX reactant. At this point, more research work is needed to analyze the process of water binding capacity.

A significant increase in DDT was shown by Microfarinograph investigations of flour, fortified with 2% AX. The most

effective increase was gained for water soluble pentosans from rye. The DDT increased from 5.5 (control) to 10 minutes. Determined DDTs with addition of waterextractable and waterunextractable pentosans from wheat were eight and 7.5 minutes, respectively (Michniewicz et al., 1991). It was further analyzed that the amount and the molecular weight of AX (HMW and LMW) as well as the AX source like wheat or rye and the layer (bran, aleurone layer or endosperm) have an important influence on the extent of these effects (Izydorczyk and Biliaderis, 1992; Biliaderis et al., 1995). The fractionation of the HMW and LMW AX took place after extraction via gel permeation chromatography. In another study, it was shown that the water-holding capacity can be increased by oxidative cross-linking (by H_2O_2/peroxidase, laccase/O_2 or chemical systems) of ferulic acid polymers (see Fig. 3) which results in water-holding capacities up to 100 g of water per gram of polymer (Nino-Medina et al., 2010). In addition, it was further investigated, that waterextractable pentosans and also AX, treated with oxidants, formed a gel network (Izydorczyk et al., 1990). The gelling potential is influenced by the molecular weight, the degree of branching, which can be separated during the extraction via ethanol or ammonium sulfate in AX with low and high ara/xyl ratios as described later, and phenolic acid content as well as the AX source (Rattan et al., 1994). DervillyPinel et al. (Dervilly-Pinel et al., 2001) have shown that gels from rye and barley result in stronger gels than those from wheat and triticale, this effect has also an important influence on dough rheology. Rattan et al. (Rattan et al., 1994) demonstrated in frequency sweep tests that AX, treated with peroxidase and H_2O_2, show an initial increase of storage modulus (G') followed by a plateau region, but generally G' dominates over G''. For untreated AX solutions, the loss modulus (G'') predominates over the G' in the frequency test range. It is also mentioned that the rheological behavior of this AX solution changed from a viscous solution to a solid-like material. For wheat, rye, and triticale AX, similar rheological behaviors have been reported (Dervilly-Pinel et al., 2001). In addition, Köhnke et al. (Köhnke et al., 2011) changed the AX pattern by the use of the specific enzyme arabinofuranosidase and demonstrated that the solubility of AX depends on its substitution pattern. With this finding, Köhnke et al. also demonstrated that the functional properties of AX can be changed by the use of Enzymes. For dough making, it is suggested that WEAX have a positive effect on the dough structure and dough stability, especially when standing higher pressure in the gas cells in the beginning of the baking process (Izydorczyk and Biliaderis, 2007). For WUAX, a decrease in dough stability, loaf volume, and other bread characteristics was analyzed (Courtin and Delcour, 2002), it was also demonstrated that the waterunextractable part can form physical barriers and interfere directly or delay the process of gluten formation. As a conclusion, the gluten has a lower extensibility as well as a lower rate of aggregation (Wang et al., 2003). Dornez et al. (Dornez et al., 2009) demonstrated that AX can be weakened by the use of xylanases. Beck et al. (Beck et al., 2011) analyzed the effect of transglutaminase on rye proteins cross-linking and assumed that the interaction is limited by the presence of pentosans.

In general, it can be summarized that the effect of AX on dough and bread characteristics depends on the pattern of AX structure, the ara/xyl ratio, and the molecular size of AX (Biliaderis et al., 1995). Due to the different features of WEAX and WUAX, a separation of these molecules is beneficial for analyzing the effect of AX on food processing and enables therefore a quality optimization.

Methods of Extraction and Isolation of AX

AX is identified as a useful ingredient to impart some functional properties into food beside its positive metabolic effect on human health. For the isolation of AX, especially from various types of grains, different extraction methods are known. Recently, the (most) common methods for isolating AX in labor scale involve aqueous and alkaline extraction but also enzymatic and ultrasound treatment (Elbegzaya et al., 2010). WEAX are described to be bound weakly in the plant tissue cell wall and thus can easily be extracted with the help of water. The extractability of WUAX depends on the degree of arabinose substituents, on the substitution pattern of AX as well as on the degree of cross links between FA and other cell wall components as described above. In this case, for cleaving these bonds, more sophisticated extraction methods have to be applied. For the isolation of WUAX from the cell wall, chemicals with a higher dissolving effect like NaOH, $Ba(OH)_2$, or enzymes like xylanase and esterase as well as ultrasound are needed. Moreover, for several methods, researchers implemented different intermediate steps to isolate AX such as: stirring, heating, or enzyme treatments to increase extraction yield of AX along with the degree of purity. The knowledge about functionality and applicability of different extraction methods is essential to gain the highest AX yield possible. Moreover, the chosen extraction methods also influence the functional properties of AX.

Pre-purification of AX Sources

Cereals consist of starch, nonstarch polysaccharides, different proteins, lipids, and other minor components. For AX recovery, these components are obstructive and thus need to be removed step by step from the whole matrix.

First, the grain samples are milled, then heat treatment is applied to inactivate sample immanent endogenous enzymes (Cleemput et al., 1993). Hartmann et al. (Hartmann et al., 2005) also used this pre-purification step successfully for flour and bread. For dough, however, enzymes were inactivated under reflux with 90 % (v/v) ethanol for 30 minutes.

For the removal of proteins, Fincher and Stone (Fincher and Stone, 2004) treated wheat flour with 80% ethanol before isolating WEAX. Such treatments result in water-soluble pentosans with about 2% protein content. In their work, Courtin and Delcour (Courtin and Delcour, 1998) analyzed a wheat pentosan

concentrate, a byproduct of the industrial wheat starch–gluten separation process. Before analyzing the contained AX, proteins were removed by the addition of clay as flocculation agent. To obtain the maximal protein absorption, the wheat pentosan concentrate was suspended in water, pH was adjusted to 3.5, and afterwards the clay–protein precipitate was separated by centrifugation and discarded. Vansteenkiste et al. (Vansteenkiste et al., 2004) used the same purification method for solely crude wheat WEAX. Before clay treatment, the samples were thermally purified at 100°C for 20 min for enzyme inactivation. In detail, Dervilly-Pinel et al. (Dervilly-Pinel et al., 2001) analyzed different flours and the effect of cooking in ethanol (80%) prior to extraction and confirmed that ethanol-treated flours were less contaminated by proteins than untreated flours.

In a next step, after centrifugation and separation of proteins, αamylases and glucosidases were added to degrade starch and saccharides (Nilsson et al., 1996). Hansen et al. (Hansen et al., 2003) processed a sample with thermostable αamylase at 100°C for one hour. Afterwards, the remaining starch fragments were completely degraded by amyloglucosidase (Hansen et al., 2003). For maize bran, analogous proceedings are published (Carvajal-Millan et al., 2007).

The mentioned pre-purification steps were instrumental in achieving more purified AX and facilitate the later extraction step. Nevertheless, these purification steps go along with exposition of AX. The subsequent process steps deal with the virtual extraction of WEAX and WUAX, respectively.

Extraction of WEAX

As already mentioned, most of the water soluble AX are only weakly bound in the cell wall tissue of the plant and can easily be isolated by moderate extraction methods. Here, water is the main reagent for isolating AX. The described methods slightly differ in its extraction parameters as temperature, time, and amount of purification steps.

After sample extraction from different types of grains by stirring with water, most methods involve centrifugation of the aqueous suspensions, leading to aqueous supernatant which contains the WEAX and an insoluble residue. In some studies, the supernatant was directly taken for analyzing WEAX (Hashimoto et al., 1987; Lempereur et al., 1997; Delcour et al., 1999). In other studies, the WEAX containing supernatant was additionally purified prior to final WEAX quantification. The supernatant was heated (app. 90°C) and the precipitating soluble proteins were removed by filtration, adsorbed with ammonium sulphate, by clay or by centrifugation (Cleemput et al., 1993; Rattan et al., 1994; Loosveld et al., 1997). For rye whole meal and wheat flour, Vinkx et al. (Vinkx et al., 1993) and Rattan et al. (Rattan et al., 1994) hydrolyzed residual starches and other polysaccharides by addition of αamylase and/or amyloglucosidase. After incubation, the solution was centrifuged and the supernatant was filtered with Celite or the enzymes were inactivated by heating the solution for 30–60 minutes at 85–95°C. Furthermore, Rattan et al. (Rattan et al., 1994) dialyzed the supernatant with water instead until no sugar was detectable in the dialysate. Some other researchers skip this step and centrifuged the supernatants directly after cooling for yielding AX solutions (Loosveld et al., 1997; Ragaee et al., 2001).

To achieve a concentration of WEAX, it was precipitated with ammonium sulfate solution or with ethanol (80–90 %) and dried in an oven for 24 hours at 45°C, 7 hours at 40°C, respectively (Cleemput et al., 1993; Cleemput et al., 1995; Nilsson et al., 2000). These two chemicals can also be used for fractional AX precipitation by stepwise addition. Nevertheless, with an increasing concentration of ethanol and ammonium sulfate solution, there was a detectable increase in the ratio of ara/xyl in the achieved fractions of the wheat flour samples, respectively (Izydorczyk and Biliaderis, 1992; Cleemput et al., 1995). These results could not be confirmed by Courtin and Delcour (Courtin and Delcour, 1998) who investigated a wheat pentosan concentrate in which the ratio of ara/xyl did not increase by an ethanol concentration.

In addition to the separation via ethanol, Cleemput et al. (Cleemput et al., 1993) reported, that a concentration of 65% (v/v) yielded the best separation between the two pentosan polysaccharides AX and AG. On the other hand, Loosveld et al. (Loosveld et al., 1997) reported no separation of WEAX and water extractable AG-peptides by the mentioned ethanol concentration.

Another technique of AX separation involves adsorption of water-soluble hemicelluloses with tris-HCl buffer on a diethylaminoethyl (DEAE)-Sepharose (CL-6B) column. The AX concentrates from the DEAE column were loaded onto a Sephacryl (S-200) column and the achieved AX fractions were pooled and dialyzed against dest. water. For further experiments, the solution was frozen immediately and freeze-dried (Shiiba et al., 1993). Nilsson et al. (Nilsson et al., 2000) used the same technique for fractionation of a crude AX extract. For this, a DEAE-cellulose column was activated with sodium borate, the AX extract was applied on the column and eluted by dest. water. The achieved fractions were pooled, dialyzed against dest. water and freeze-dried.

For the isolation of WEAX from dough or bread, the samples were freeze-dried, milled, and analyzed as described above for flour (Cleemput et al., 1997; Hartmann et al., 2005).

The extraction by water seems to be an easy way for an isolation of WEAX. The complexity of extraction is less and no special tools or chemicals are needed which implies lower costs. In addition, due to the effect of easy extractability of WEAX, this fraction shows different functional properties to WUAX such as water absorption, dough development or gel formation as described above. For dough and bread preparation, the WEAX are described as more beneficial as the WUAX. The content of WEAX depends on the source and is far less than the content of WUAX. Although regarded as water soluble, the AX bound to the cell tissue are not or only in small quantities isolated by these methods.

Extraction of WUAX

For the extraction of WUAX, more complex and more sophisticated methods are necessary than for WEAX. Beside longer stirring times, higher temperatures and more complex purification steps, the extraction solvent have an essential effect on AX yield and purity. The extraction chemicals can be differentiated into alkaline and acidic solvents.

For WU-AX determination, Gruppen et al. (Gruppen et al., 1991) treated wheat flour and wheat bran samples with a series of solvents: Dimethylsulfoxide (DMSO), urea, hydroxylamine hydrochloride (NH_2OH-HCl) in phosphate buffer and sodium hydroxide (NaOH). After continuous stirring for 16 hours at 20°C and centrifugation, the residues were reextracted with the respective solvent, diluted with water and centrifuged again. The supernatants were combined; the pH value adjusted to 7.0 and finally dialyzed with distilled water. When using hydroxylamine hydrochloride, the samples were dissolved in sodium carbonate buffer and adjusted to pH 5.0 and 7.2.

A further extraction solvent was a sodium carbonate (Na_2CO_3) solution. For extraction with this solvent, the samples were extracted for 16 hours at 4°C, centrifuged, neutralized, dialyzed with deionized water, and analyzed. The achieved residues were re-suspended in sodium carbonate and sodium borhydride, extracted for three hours at 20°C, centrifuged, re-extracted with solvent, water, and analyzed.

The next solvent described for extraction was a saturated barium hydroxide ($Ba(OH)_2$) solution. The samples were extracted with this solvent for 16 hours at 20°C. After centrifugation, the residues were re-extracted with solvent and centrifuged again. Both supernatants were combined, neutralized, and dialyzed against sodium acetate buffer and water. The achieved residues were treated with acetic acid and extracted with water. After centrifugation, the supernatants were combined, dialyzed with water, and analyzed. With $Ba(OH)_2$, it was possible to extract approximately about 80% of pure (less glucose) AX present in wheat flour and wheat bran. This was the highest concentration of AX achieved by the different extraction solvents.

Equal amounts of AX were obtained using NaOH, on the contrary fewer amounts were extracted using DMSO and urea. For NH_2OH–HCL solution, different yields were obtained depending on the pH such that at pH 7.2 more AX were extracted than at pH 5.0. Moreover, different FA concentrations for both pH values were observed. This indicates that more ester linkages are broken at pH 7.2, which results in higher AX contents (Gruppen et al., 1991). In addition, the structure of AX and therefore their functional properties can be changed by different pH values during extraction.

Bergmans et al. (Bergmans et al., 1996) applied the method of Gruppen et al. using $Ba(OH)_2$ as described before for selective extraction of WUAX from wheat bran. Using this methodology, Nilsson et al. (Nilsson et al., 1999) achieved an extraction of 44.3% WUAX in rye bran. In matters of NaOH extraction, Bataillon et al. (Bataillon et al., 1998) extracted a wheat bran sample with NaOH at different concentrations (10, 20, 40 or 80% corresponding of NaOH/starting bran) and temperatures (20, 40, 60, and 80°C) for six hours after removing lignin with sodium chlorite. After centrifugation, the supernatants were cooled, the pH was adjusted to 4.8, cooled down, and centrifuged. The acidic supernatants were purified by microfiltration and spray-dried. The effective concentration for the extraction of AX was 80%, independent of extraction temperature. For lower concentrations, the temperature had a slight influence on the extraction efficiency.

For the extraction of WUAX, chemicals with a high dissolving effect are necessary. WUAX make up to 95% of total AX in the different kinds of bran. In dependence on the source of plant tissue, it is possible to extract up to 80% of total WUAX. Among the solvents described above, $Ba(OH)_2$ seems to be the most effective solvent for extraction WUAX out of different cell wall matrices. Conversely, the high-dissolving effect of those chemicals changes the ara/xyl ratios and therefore the physiochemical properties of AX. It is reported that the ara/xyl ratio increases with ethanol, ammonium sulphate as well as barium hydroxide concentration for WEAX and WUAX (Izydorczyk and Biliaderis, 1992; Schooneveld-Bergmans et al., 1999). However, these treatments are more complex and associated with higher chemicals costs.

Enzymatic Extraction of AX

Enzymatic treatment of plant tissue can have several aims: AX with linkage to the cell wall of plant tissue can be disconnected from that, WUAX can be split in that way to convert them into WEAX or WEAX can be degraded into its molecular components, namely xylose and arabinose. For that aim, the commonly applied enzymes are (1→4)-β-endo-xylanase (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), αL-arabinofuranosidase (EC 3.2.1.55), or feruloyl-esterase (EC 3.1.1.2) (Benamrouche et al., 2002).

Especially, endoxylanases attack the AX backbone and change the functionality of the polymers (Dornez et al., 2009). Endoxylanases convert WUAX into WEAX and these will be further degraded into their single components: arabinose and xylose (Petit-Benvegnen et al., 1998; Courtin and Delcour, 2002). Benamrouch et al. (Benamrouche et al., 2002) investigated the influence of a (1→4)-β-endo-xylanase treatment on wheat bran. After pre-purifying a soft wheat cultivar, the resulting liquid was considered as freed from starch. Xylanase action was initiated and supported by constant stirring for 24 hours at 60°C. Afterwards, enzymes were deactivated by heating at 100°C for 10 minutes and the achieved solution was centrifuged. The supernatant was assayed for sugar content. Analysis of carbohydrates accounts for 63% of total dry matter, in which 40% were arabinose and xylose. Furthermore, 50% of the wheat bran AX was solubilized by enzymatic treatments. This result was confirmed by Beaugrand et al. (Beaugrand et al., 2004), who used the same hydrolyze conditions as Benamrouch et al.

Another method for extracting pentosans (AX and AG) was described by Hong et al. (Hong et al., 1989) who used a multicomponent enzyme system, declared as Meicellase. This enzyme system includes cellulases, β-glucosidase, xylanase, β-xylosidase and α-L-arabinosidase. Extraction was done for 18 hours at 30°C in a shaking water bath. Carbohydrate measurements were done for water-soluble, enzyme-extractable, and total pentosans (Hashimoto et al., 1987; Hong et al., 1989). In this case, the pentosan content instead of the AX content was determined and by further degrading it would be possible to determine the actual AX content.

A general way for isolating AX by enzymatic treatments is given in the following clause. To make AX available for further processing, first it has to be split from lignin that constitutes the cell wall. Since ferulic acid acts as bridge between lignin and AX, feruloyl-esterase can be used to split the ester linkage between arabinose and ferulic acid. This reaction takes place in water at a moderate temperature. In this specific reaction, the content of accessible WEAX and WUAX in the matrix is increased. This pool of AX is further used for the determination of the whole AX content in the grain. For the transformation of WUAX into WEAX, highly specified enzymes such as endoxylanases, xylanases, and arabinofuranosidases are used. The transformation of WUAX into WEAX depends on the reaction time and concentration of the chosen enzymes. To decrease the degree of intermolecular linkages, the bridge former, ferulic acid, has to be cleaved. This can be performed by the use of feruloyl-esterase. Therefore, all accessible AX should be present as WEAX and WUAX without intermolecular linkages. Now, WEAX and WUAX can be isolated and be used for either fortification of other food systems or can further be degraded for a quantification of AX. For further degradation, the next step comprises the removal of side chains from the xylose backbone of both WEAX and WUAX. For this purpose, arabinofuranosidase is commonly used. In the last step, the xylose backbone is exposed to xylanases. This results in a total liquefaction of former AX into separate arabinose and xylose molecules which can further be determined quantitatively and represent the total amount of AX.

Enzymatic treatments seem to be a good alternative for isolating AX from different plant tissues. Certainly, the relevant enzymes are presently not economically available on the market for isolating AX in large scale (Elbegzaya et al., 2010). Furthermore, enzymatic treatments are problematic when treating AX with high ratios of ara/xyl as well as highly branched AX since the enzymes are hindered by the side chains (Lequart et al., 1999; Dervilly-Pinel et al., 2004). In addition, the degree in hydrolyzing the AX structure depends on the specific enzyme (Courtin and Delcour, 2002). Another disadvantage of the use of enzymes arises from the steric structure of the plant tissue. Other components such as lignin are known to hinder the accessibility of enzymes in the tissue. Due to that, the extraction yields of AX are less compared to methods that were mentioned in the chapters before (Lequart et al., 1999; Benamrouche et al., 2002). On the other hand, investigations mentioned by Köhnke et al. (Köhnke et al., 2011) demonstrate, that specific functional characteristics of AX can be adjusted by the use of specified enzymes. Enzymes can be used for target-orientated, specific modification of biopolymers. The findings lead to the conclusion that the currently known enzymes are able to change specific AX structures by selective hydrolysis which leads to AX with defined functional properties. Especially in the case of structural and functional changing of AX (as well as biopolymers) using enzymes, yet more research is needed.

Extraction of AX via Ultrasound

Ultrasound (US) has been used for a gentle extraction of low-molecular materials and polymers for more than 20 years (Sun et al., 2002; Sun and Tomkinson, 2002). Thus for the extraction of AX, ultrasound has successfully been applied. Elbegzaya et al. (Elbegzaya et al., 2010) analyzed the influence of US on extraction performance of nonwater extractable Glucurono-AX from rye bran. The rye sample was milled, purified, and the WEAX removed. Afterwards, the achieved rye sample was treated with US in combination with alkaline hydrogen peroxide ($NaOHH_2O_2$) and aqueous NaOH (aNaOH), respectively. For comparison, extraction of WUAX without US was done, as well. For aNaOH determinations, 20 g purified WUAX were suspended, 1, 2, and 3% aNaOH was added, heated up to 50°C and treated with US for 10 minutes at 120 W and 24 W cm^{-2}. The highest yield in AX was measured with a use of 3% aNaOH. The results also show that the additional US treatment yields a lower ara/xyl ratio which means more cleavage of arabinose from the xylose backbone instead of breaking it. The content of extracted AX with US (131 g kg^{-1} raw bran) was slightly higher than without US (128 g kg^{-1} raw bran). NaOH-H_2O_2 treatment with and without US resulted in 175 and 170 g kg^{-1} raw bran, respectively. Especially, the reduced extraction time of 10 min with US compared to former 240 minutes without US was mentioned positively. It was assumed that US accelerates saponification of esters between phenolic acids and AX due to alkaline treatment. This mechanism was also mentioned by Hromádková et al. (Hromádková et al., 1999) and Sun et al. (Sun et al., 2004). Another explanation was given by Hollmann and Lindhauer (Hollmann and Lindhauer, 2005), who describe that the extractability of AX will be influenced positively by removing lignin by H_2O_2 treatments. It was described, that the relative molecular weight is negatively influenced by US. Due to US exposure, the relative molecular weight was reduced from 770 to 480 kDa. The reducing of molecular weight was effected by the high energy input of US to remove AX from the cell wall.

Ultrasound as a single means of extraction does not perform better in terms of extraction quantities but has enormously positive effects on reaction times.

Quantification of AX

After extraction, different analyzing methods for the detection and quantification of the AX content are commonly used. These measurements provide information about the structure and the composition and thereby deliver knowledge about the characteristics of solubility, molecule size, etc.

One method was based on the hydrolysis of AX into arabinose and xylose combined with high performance anion exchange chromatography (HPAEC). For the determination of AX content, the sample was dispersed in buffer (prepared from sodium acetate and acetic acid or tris(hydroxymethyl)-amino-methane and hydrochlorid acid (HCl)), treated with HCl and thus hydrolyzed. After cooling, the sample was neutralized by addition of NaOH. Samples which contain starch were treated with glucose oxidase/catalase to convert glucose into gluconic acid, since a peak overlay between glucose and pentosan monosaccharides can occur. The outcome of this was a longer dwell time of gluconic acid by the HPAEC and thereby better chromatographic separation of arabinose and xylose peaks (Houben et al., 1997).

Another way for the hydrolysis of AX was described by Shiiba et al. (Shiiba et al., 1993) with trifluoroacetic acid. Trifluoroacetic acid was added to the AX isolates and the solution was aerated with nitrogen for one minute. After heating in an oven at 105 °C for two hours for hydrolyzing AX, the solution was evaporated at 50°C to remove trifluoroacetic acid from the sample. Before injection into the HPLC, the sample was dissolved in dest. water and filtered. Compared to the method of Houben et al. (Houben et al., 1997) and Krahl et al. (Krahl et al., 2009), the monosaccharides were analyzed by HPLC at 80°C. Other researchers used sulphuric acid for the hydrolysis of WEAX with small variations in time and temperature (Izydorczyk et al., 1991; Rattan et al., 1994; Beaugrand et al., 2004; Hartmann et al., 2005; Carvajal-Millan et al., 2007).

In the study of Rantanen et al. (Rantanen et al., 2007) different sample preparations and analyzing methods were arranged and compared. For HPLC sample preparation, a rye flour sample was hydrolyzed with 2 M HCl for four hours at 100°C and neutralized with 4 M NaOH (2 mL). The second sample was hydrolyzed with 1 M sulfuric acid for 30 minutes at 120 °C and hydrolyzed with 10 M NaOH. As the results show, the treatment with sulfuric acid showed the highest yield in xylose (65%). As described by the researchers, the hydrolysis with sulfuric acid gave the best recovery of the carbohydrate composition provided by the manufacturer.

Bataillon et al. (Bataillon et al., 1998) adopted a hydrolyzation method for measuring AX by HPLC from Hoebler et al. (Hoebler et al., 1989) and Gruppen et al. (Gruppen et al., 1992) with small modifications: Before hydrolyzation of the monosaccharides, the samples were pretreated with 72% (w/w) sulfuric acid for a solubilization of semi-crystalline structures, followed by hydrolysis with high concentrated sulfuric acid for two hours at 100°C. The monomeric sugars were analyzed by HPLC fitted with a Biorad Aminex column HPX87H at 40°C. Gruppen et al. (Gruppen et al., 1991; Gruppen et al., 1992) developed the instruction above for analyzing AX by gas chromatography, then the AX content was calculated by equation 1 (Houben et al., 1997; Krahl et al., 2009)

$$\mathrm{AX} = 0.88 * (\%\mathrm{D-xylose} + \%\mathrm{L-arabinose}) \quad (1)$$

Especially for calculating the AX content, Courtin and Delcour (Courtin and Delcour, 1998) used the following equation (equation 2):

$$\mathrm{AX} = 0.88 * [(\%\ \text{arabinose} - 0.7 * \%\text{galactose}) + \%\ \text{xylose}] \quad (2)$$

The factor 0.88 was applied for the conversion of free sugar residues to anhydro sugars as present in polysaccharides (Hashimoto et al., 1987).

Another method for analyzing AX was realized by gas chromatography (GC) measurement. The GC can only be used for samples which are gaseous or, although undecomposed, vaporizable. However, the monosaccharides in their native form are not directly vaporizable, for that specific reason, the sample hydrolysats were treated with different chemicals for converting the monosaccharides into alditol acetates (highly volatile). These alditol acetates were used for GC injection as described by Englyst and Cummings (Englyst and Cummings, 1984) as well as by Cleemput et al. (Cleemput et al., 1993). This method was also used by many other researchers with only small modifications in sample preparations (Vinkx et al., 1993; Rouau et al., 1994; Figueroa-Espinoza et al., 2004).

Some researchers also used a colorimetric phloroglucinol method for analyzing AX. The samples were treated with a special extraction solution and placed in a boiling water bath for 25 minutes. After a rapid cooling in cold water, the absorbance at 552 and 510 nm was measured. Calculation of the percentage of pentosans can be achieved by equation 3:

$$\mathrm{P}\ (\%) = (\mathrm{A}_{550\ \mathrm{nm}-510\ \mathrm{nm}} * \mathrm{S} * \mathrm{D} * \mathrm{V} * 0.88 * 100) * \mathrm{F}^{-1} \quad (3)$$

Where $\mathrm{A}_{550\ \mathrm{nm}\text{-}510\ \mathrm{nm}}$ is the difference of absorbance between 550 and 510 nm; *S* is the slope of the xylose calibration plots; *D* is the dilution factor (50); *V* is the volume [mL] of extract; 0.88 is the polymerization factor; and *F* is the mass [g] of the flour (dry matter). The contents of AX, determined by different analyzing methods are shown in Table 1.

The most-used method of AX characterization is the detection of its molecular weight. This determination provides information about different AX sizes from the different plant tissues as well as the extent of the extraction treatments on the molecular size. Different methods were applied by the scientists such as sedimentation test, gel filtration chromatography

and size exclusion chromatography (SEC) with different detectors such as laser light scattering analyzer or refractometer. Molecular weights of two different purified fractions from wheat bran, analyzed by SEC and determined with a refractometer detector, ranged from 300.000 to 350.000 Da (Shiiba et al., 1993). Hartmann et al. (Hartmann et al., 2005) define molecular weights of wheat and rye flour between 5.000 and 110.000 Da, as estimated by SEC with RI (refractive index) and UV detection. Molecular weight measurements of rye samples, analyzed by SEC and detected with a multi angel light scattering detector, resulted for AX at 124.000 (Nilsson et al., 2000). For alkali-extractable wheat flour AX, an average molecular weight of 850.000, analyzed by laser light scattering detector, was reported (Gruppen et al., 1992). In the same study, for the sample subfractions, the molecular weights varied between 260.000 and 650.000 Da.

The research about impact of endogenous non-starch polysaccharide hydrolyzing enzymes on molecular weight of wheat flour AX measured molecular weights between 5.500 and 853.000 for three different cultivars. The eluate was monitored by refractive index detection (Cleemput et al., 1997).

Rattan et al. (Rattan et al., 1994) reported a molecular weight of eight different wheat flours, measured by limiting viscosity between 134.000 and 204.000.

The results show that many suitable methods are available for AX quantification. It can be assumed that none of the methods mentioned is better suited for AX quantification than another. This statement is confirmed by comparing the achieved results of the different analyzing methods shown in Table 1. In order to evaluate the comparability of the different methods, test series with the same analyzing probe (grain, harvest year, growing area, etc.) are necessary.

CONCLUSION

AX represents a considerable part in cereal grain plant tissues. They protect and provide stability as well as flexibility to the cell wall tissue. The highest deposits in the grain are placed in the outer layer. They are bound by covalent and non-covalent cross-links to other plant tissue polymers such as lignin, proteins, and cellulose. The ability of AX to improve food systems (e.g. bakery goods) and to affect human health positively stimulated many researchers to find effective ways of extracting AX and making it appropriate for technological application such as bread improvement. The methods mentioned are used for extraction, characterization, and quantification of AX in laboratory scale. For AX extraction, the chosen method, the solvents, and the concentration of the solvents determines the purity, yield, ara/xyl ratio, and the solubility of the AX. Nevertheless, not all of the available AX can be isolated by the methods presented. From the process point of view, it was not yet successful to isolate AX without fragmentation of isolates and consequently changes of physical properties and molecular weights. Therefore, more research is recommended to delimit extraction to AX without other impurities. In addition, especially for commercial AX extraction, more efficient (higher AX contents) and cheaper techniques are necessary to fortify and improve foodstuffs by AX such as beverages and bakery goods.

Another possibility for enhancing different kinds of especially flour-based products by AX could be the addition of AX-rich matrices such as finely ground bran (without extraction) to rye dough. However, up to now no information exists about the influence of the milling process on the ground bran and the final ratio of WUAX and WEAX. Currently there is less research done about the influence of wheat bran particle size on wheat dough structure and even no results are available about finely ground rye particles and their impact on the rheological behavior of rye dough. Especially, the necessity for these results was pointed out in this review. For the future development of natural and nutrition-rich rye products, the knowledge about the rheological influence of rye bran is essential.

A possible way to reach technological benefits could be the modification of polysaccharides structures (e.g. AX) by use of specific enzymes. For rye products, enzymatic treatment seems to be a good alternative in terms of AX modification and therefore to improve dough and bread characteristics.

For understanding and controlling the effect of enzymatic treatment, more knowledge and usage of existing analyzing methods of these structures are necessary.

ABBREVIATIONS

ara	=	arabinose
ara/xyl	=	ratio arabinose to xylose
AG	=	arabinogalactans
AX	=	arabinoxylan
AXOS	=	arabinoxylan-oligosaccharide
DDT	=	dough development time
FA	=	ferulic acid
G′	=	storage modulus
G″	=	loss modulus
lG*l	=	complex shear modulus
HMW	=	high molecular weight
LMW	=	low molecular weight
WEAX	=	water-extractable arabinoxylan
WUAX	=	water-unextractable arabinoxylan
xyl	=	xylose
xyl*p*	=	xylopyranosyl
US	=	ultrasound.

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2.3 EFFECT OF RYE BRAN PARTICLES ON STRUCTURE FORMATION PROPERTIES OF RYE DOUGH AND BREAD

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EFFECT OF RYE BRAN PARTICLES ON STRUCTURE FORMATION PROPERTIES OF RYE DOUGH AND BREAD

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ABSTRACT

Rye possesses higher nutritional value compared to wheat; and has recently gained increasing attention. However, rye is still a challenging matrix regarding structure characterization and processing. The effects of different concentrations (0.0–20.0%) and sizes (125–250 and 355–500 μm) of added rye bran particles, as well as processing methods on dough formation and bread structure were investigated. Rheological studies on dough showed decrease of loss factor by 11.0% whereas the complex shear modulus increased by 114.0%. Baking tests showed volume reduction as well as increased crumb hardness by bran addition. Xylanase addition and bran particles up to 15.0% showed similar rheological and baking performance like rye without bran and xylanase. These findings highlight the importance of arabinoxylans as main structure-forming component of rye dough and their influence on mechanical properties.

PRACTICAL APPLICATIONS

With increasing demand worldwide to nutritious and alternative bread tastes, rye bran represents a natural ingredient with high content in fiber. In contrast to traditional rye bread, the enhancement of rye flour with bran increases water absorption which influences shelf life positively in addition to nutritional enrichments. However, due to poor structure formation based on the molecular structure of the rye proteins and the increased amount of the polysaccharide arabinoxylan, rye dough is still a challenging matrix regarding structure characterization and processing. The addition of further ingredients such as rye bran makes the way of processing even more difficult. In this paper, the way of rye bread production enriched with bran is investigated. The paper demonstrates impressively the role of mechanical treatment of bran as well the role of enzymes to minimize the influence of bran on final dough and bread structure.

INTRODUCTION

The quality of baked-products in general is defined by three factors: optical appearance, sensory attributes and textural properties. Among these, texture exhibits a special challenge because special requirements such as uniform pore size distribution and soft but elastic crumb have to be achieved. It is well known that beside dough processing parameters (type of energy input and mixing time) and oven settings (time, steam and temperature), in particular, dough ingredients (starch, protein, arabinoxylan [AX]) and their chemical composition are responsible for the final dough and bread characteristics. The main difference between wheat and rye dough during processing is the weaker ability of the latter to form a viscoelastic structure. Unlike wheat proteins, rye proteins do not form a viscoelastic protein network upon mixing with water. This is attributed to differences in the aggregation behavior of 75k γ-secalins and high-molecular-weight (HMW-) secalins compared to the low-molecular-weight (LMW-) and HMW-glutenin subunits of wheat (Wieser and Kieffer 2001; Verwimp *et al.* 2007; Beck *et al.*

2011). In regard to rye dough, the pentosans, mainly consisting of AX, are crucial for the bread processing performance of rye flour (Verwimp *et al.* 2007). AX consist of a linear (1,4)-β-D-xylopyranosyl backbone which can be substituted at the O-2 and/or O-3-positions with α-L-arabinofuranose. They have been classified into water-extractable arabinoxylan (WEAX) and water-unextractable arabinoxylan (WUAX). To modify the AX structure as well the WEAX to WUAX ratio, xylanases have commonly been used. Xylanases hydrolyze the glyosidic linkage between xylose moieties in the xylan backbone. Both WEAX and WUAX are able to absorb many times their own weight of water, and this affects the dough properties and bread characteristics (Izydorczyk and Biliaderis 1992; Courtin and Delcour 2002; Gonçalves *et al.* 2012; Döring *et al.* 2015). The beneficial effect of AX in wheat dough has been explained by an increase of the viscosity of the dough aqueous-phase due to hydration of WEAX, which positively influences the gas retention capacity. Furthermore, an increase of the WEAX content results in a higher bread volume and in a softer and more homogenous bread crumb. It has been suggested that WEAX form a secondary network that amplifies the gluten network (He and Hoseney 1991; Vinkx and Delcour 1996; Courtin and Delcour 2002; Döring *et al.* 2015). Unfortunately, only little information is available on the role of AX in the structure formation properties of rye dough, and rye based bread is also not considered in detail.

Bran or bran fractions are often added in breadmaking to increase the dietary fiber content and, thus, the nutritional value of the products (Wang *et al.* 2002; Gélinas 2013; Gélinas *et al.* 2015). In wheat dough and wheat-based bread, it is well known that the addition of bran negatively affects the dough processing properties and bread characteristics such as bread volume and crumb hardness. The most evident effect of bran addition is the reduction of bread volume. In wheat bread enriched with wheat bran, Schmiele *et al.* (2012) found a decrease of the specific bread volume by up to 58.0% compared to the control sample without bran addition. Zhang and Moore (1999) and de Kock *et al.* (1999) showed not only a decrease in bread volume with increasing bran content but also a variation of the specific bread volume depending on the wheat bran particle size. Further effects of bran supplementation are changes in crumb hardness (Salmenkallio-Marttila *et al.* 2001; Wang *et al.* 2002), increased water absorption and altered pasting properties (Schmiele *et al.* 2012; Banu and Aprodu 2015). The effects mentioned before have mainly been attributed to an interference of the gluten network and a change in the hydration properties of the dough constituents. Wang *et al.* (2002) reported that the gluten formation was caused by interactions between bran particles and gluten proteins. All in all, some work has been done on the effects of bran addition in wheat bread-making, but information is scarce on how bran addition affects the properties of rye dough and bread. On one hand, bran particles have been reported to disturb dough formation, on the other hand, AX as an important constituent of bran could be expected to positively affect the dough due to their high water-binding capacity.

Therefore, the aim of the work presented here was to study the effect of rye bran addition on the properties and structure of rye dough and the quality of rye bread. Furthermore, bran addition should be combined with enzymatic modification of AX by a xylanase to get insight into the significance of AX for the functional properties of rye dough. For this purpose, in this work, rye dough and rye bread were analyzed, to get further information about their forming properties. To realize this aim, rye bran was added in order to investigate its impact on the dough structure during preparation and to analyze the effect of bran particles on the structural formability such as rheological behavior, CO_2 retention and bread volume. On one hand, bran particles are mentioned to disturb the dough formation. On the other hand, bran consists mainly of AX which is expected to influence the dough properties positively by enzymatic hydrolysis. To fulfill this aim, different rye bran particle sizes (125–250 and 355–500 μm), different concentrations (0.0, 5.0, 10.0, 15.0 and 20.0%) as well as different times (direct and indirect bran additions) of bran additions were considered, and the effect of a xylanase in combination with bran addition was analyzed. Predicting the final dough and bread characteristics were achieved by means of chemical analysis, rheology, dough fermentation and baking measurements. The relevance of this work is driven by the missing evidence in the literature; concerning the influences on the rye dough structural formability.

MATERIAL AND METHODS

Raw Materials

A commercial rye flour (Type 1150, ash content 1.15% dwb; Rosenmühle, Ergolding, Germany) was used. Rye bran was purchased from a commercial mill (SchapfenMühle, Ulm, Germany). The bran was sieved in a sieve tower to obtain two fractions with different particle size distribution (small size: 125–250 μm; large size: 355–500 μm). Further ingredients for final dough preparations was instant dried yeast (Uniferm GmbH & Co. KG, Werne, Germany), distilled water, sodium chloride (Südsalz GmbH, Bad Friedrichshall, Germany), lactic acid (purity: ∼90%; AppliChem GmbH, Darmstadt, Germany) and the commercial xylanase Grindamyl® H 361 (Danisco AS, Bagsvaerd, Denmark). Ash content, β-glucan content and water absorption of the rye flour and the bran particles were determined according to AACCI

methods 08-12, 32-23 and 56-11.02 (AACC International. Approved Methods of Analysis 1999a, 1999b, 1999c). Protein content (N x 6.25) was determined using the Kjeldahl Method (EBC) (Anger 2006).

Solvent Retention Capacity

SRC was analyzed according to AACCI method 56-11.02 (AACC International. Approved Methods of Analysis 2000). For determination, 5 ± 0.050 g flour/bran were weight into 50 mL test tubes and diluted in 30 mL distilled Water. After mixing for 5 min and resting for 30 min, the suspensions were centrifuged for 10 min at 6,000 × g at room temperature (RT). The supernatants were decanted and the test tubes drained at 90° angle for 10 min on a paper towel. Afterward, the test tubes were weighted and the SRC was calculated according to

$$\%\mathrm{SRC}=\left[\frac{\text{gel weight}}{\text{sample weight}}\times\left(\frac{86}{100-\text{sample moisture}}\right)-1\right]\times 100 \quad (1)$$

Quantitation of Total AX and WEAX in Rye Bran and Flour

The content of total AX and WEAX of the bran fractions and rye flour was determined according to the method of Houben *et al.* (1997) with small alterations: To determine the total AX content, 0.1 g of the sample was dispersed in 4.0 mL of distilled water and 4.0 mL of 4.0 M H_2SO_4 and subsequently hydrolyzed for 60 min in boiling water in a normal laboratory screw-cap test tube. After cooling to RT, 8.0 mL of 4.0 M NaOH was added to neutralize the solution. The solution was diluted 1:10 and filtered through a 0.45 µm Chromafil PET 45/25 membrane filter (Macherey Nagel, Düren, Germany). A 10 µL of the final extract was injected to a Dionex ICS5000 high performance anion-exchange chromatography. A CarboPack PA 10 Analytical column (Dionex, Sunnyvale, California) was used as stationary phase with an isocratic eluent of 50 mM NaOH used as mobile phase for final detection of monosaccharides by pulsed amperometric detection. The total AX content was calculated from the concentrations of xylose and arabinose residues according to Courtin and Delcour (1998).

To quantitate WEAX, 0.1 g of the sample was stirred in 15 mL of distilled water for 60 min at 30C in a normal laboratory screw-cap test tube. After centrifugation (15 min, 4,000 × g, RT), 1 mL of the supernatant was transferred into a test tube, dispersed with 1 mL of 4.0 M H_2SO_4 and hydrolyzed for 60 min in boiling water. After cooling to RT, 2.0 mL of 4.0 M NaOH was added to neutralize the solution. Due to the inferior separation of glucose and xylose on the CarboPack PA 10 column (Dionex, Sunnyvale, California), a treatment with glucose oxidase was necessary. In all the experiments, WEAX was considered of relevance in contrary to WUAX.

Dough Preparation

For dough preparation, 2.0% sodium chloride, 1.8% dry yeast, 76.0% water and 0.8% lactic acid based on 100.0% flour or flour-bran mixture (corrected to 14% moisture content) was used. Lactic acid was used to minimize the activity of endogenous α-amylase. To determine the effect of bran on the dough quality, variations in bran content (0.0, 5.0, 10.0, 15.0 and 20.0% flour replaced by bran) and time of addition (direct: bran was directly added to the flour before kneading; indirect: bran was added to the final dough in an extra kneading step after dough preparation) of bran were investigated. Therefore, three different procedures were chosen:

1. All ingredients were directly added into a z-kneader bowl (50 and 300 g) (Brabender, Duisburg, Germany). Mixing parameters were 63 rpm for 5 min at 20C. Later on, this procedure will be referred to as "direct bran addition."
2. Dough without addition of bran was prepared according to procedure 1 and mixed for 4 min. Then, hydrated bran was added and mixing was continued for another 90 s. Mixing speed was reduced from 64 to 32 rpm to minimize damage to the dough structure by the bran. During the first 4 min of dough mixing (without bran), the bran was hydrated by soaking in water in a separate vessel. The amount of water was determined by the SRC method. Later on, this procedure will be referred to as "indirect bran addition."
3. Xylanase powder (0.075% based on 100.0% flour or flour-bran mixture; 100 U/g Xylanase at pH 4.2) was used as additional ingredient and a dough was prepared according to procedure 1. Later on, this procedure will be referred to as "xylanase addition."

Dough Analysis

An AR G2 Rheometer (TA Instruments-Waters LLC, New Castle) with a plate-plate geometry ($d = 40$ mm) was used to conduct the rheological measurements. After dough preparation (direct and indirect bran additions, xylanase addition), 4 g of the dough were gently placed between the plate-plate geometry. The chosen gap for measurements was 2,000 µm and the temperature of the geometry was 30C simulating the temperature in a proofing chamber. After a relaxation time of 10 min, oscillation tests were performed according to the procedure of Houben *et al.* (2010) with small modifications: Deformation 0.075% (value was determined via stress sweep and is in accordance with the finding

of Gräber (1999)). Three experiments were repeated threefold and averaged.

Dough fermentation was monitored using a Chopin Rheofermentometer F3 (Chopin S.A., Villeneuve-La-Garenne, France). A quantity of 315 g dough was placed in the chamber for 3 h fermentation at 30C. The maximum dough height (Hm) in mm, total volume of released gas (mL) and the gas retention (%) were determined (Chopin-Groupe 2000).

Baking Test and Bread Analysis

For bread preparation, dough was prepared as described before with small modifications: For procedure one and three, all ingredients were mixed in a spiral kneader (Diosna Dierks & Söhne GmbH, Osnabrück, Germany) for 4 min at 100 rpm and followed by 1 min at 200 rpm. For procedure two, the soaked bran was added to the final dough and mixed together with the dough for 60 s at 100 rpm.

After kneading, the dough was directly scaled into 300 g pieces, hand-molded, placed into backing-tins and proofed at 30C for 45 min at 80.0% relative humidity. The bread was baked with initial steam subjection of 0.5 L at 230C (top heat) and 200C (bottom heat), falling to 200C (top and bottom heat) in a rack oven (Matador MD 120, Werner & Pfleiderer, Dinkelsbühl, Germany) for 40 min. After baking the final bread was cooled to RT for 2 h before weight and volume was determined using a BVM-L370 volumeter (TexVol Instruments, Viken, Sweden).

Crumb firmness (hardness) was analyzed using a TVT-300 XP Texture Analyser (TexVol Instruments AB, Viken, Sweden). The procedure was performed according to AACCI method 74-10.02 (AACC International. Approved Methods of Analysis 1999c). Four replicates from two different sets of baking tests were analyzed and averaged.

Quantitation of WEAX in Rye Bread

A slice with a thickness of 1 cm was cut from the center of the bread. The slice was cut into cubes of 1 cm^3 and the cubes were frozen, freeze-dried and grinded on a Retsch mill (ZM 200, Retsch, Haan, Germany). WEAX was extracted from the freeze-dried bread powder following a method described by Ragaee *et al.* (2001) with some modifications. A 1.5 g of dough was extracted with 15 mL of distilled water for 90 min under continuous stirring. The slurry was centrifuged (3,500 × g, 30 min, RT) and 300 µL of phosphate buffer (50 mmol/L, pH 6.9) were added to the supernatant. To remove the remaining starch, the supernatant was incubated with 0.4 mg thermostable α-Amylase (Sigma A 4551) at 95C for 60 min. The mixture was cooled to RT, centrifuged and incubated with 0.1 mg of amyloglucosidase (Sigma 10115) at 60C overnight after addition of 300 µL of acetate buffer (1 mol/L, pH 5.0). The mixture was again centrifuged and the pH of the supernatant was brought to a value 3.0 with hydrochloric acid (1 mol/L). The supernatant was stirred with 600 mg of montmorillonite (Sigma 69904) for 30 min at RT to remove the proteins. Before centrifugation, the pH of the slurry was adjusted to pH 7 with sodium hydroxide (2 mol/L). The supernatant (WEAX) was filtered through a 0.45 µm membrane and used for gel permeation high-performance liquid chromatography (GP-HPLC) analysis.

GP-HPLC with refractive index detection of the WEAX was conducted on a Yarra 3u SEC-2000 column (300 × 7.8 mm, 3 µm, separation range 1,000–300,000) (Phenomenex, Aschaffenburg, Germany) with a security guard precolumn (GFC-2000, 4 × 3.0 mm ID, Phenomenex, Aschaffenburg, Germany). A Jasco HPLC (PU-1580; Gross-Umstadt, Germany) was used in combination with a Jasco autosampler (AS-2057 Plus), a Jasco HPLC Pump (PU-1580), a Jasco degaser (DG-980-50) and a Jasco RI-2031 Intelligent Refractive Index Detector. The eluent was 0.1 mol/L sodium chloride in distilled water at a flow rate of 1 mL/min and the injection volume was 100 µL. Analysis of the data was conducted with Agilent Galaxie software (version 1.10.0.5590). The area under the curve of the GP-HPLC curves of the WEAX from bread were determined and given as arbitrary units (a.u.).

Statistical Analyses

One-way analysis of variance (ANOVA) followed by Turkey's test was performed to evaluate the significance ($P < 0.05$) of the results using the Software GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, California).

RESULTS AND DISCUSSION

Physicochemical Characteristics of Raw Materials

The chemical composition of the raw materials used is shown in Table 1. For further discussion, the SRC and the AX content are of great interest. The SRC provides information about the water absorbed by the raw material: The higher the SRC value, the more water is absorbed, and this has an effect on the final dough and bread characteristics (mainly dough and bread firmness). AX is an important component for rye dough structure formation, especially WEAX have been reported to have a positive influence on the dough characteristics. All results are shown as mean values ± SD, $n = 3$ (threefold repetition).

TABLE 1. CHEMICAL COMPOSITION* OF RAW MATERIALS

Parameter†	Rye flour	Small bran size (125–250 μm)	Large bran size (355–500 μm)
SRC [mL/g]	1.50 ± 0.04	2.10 ± 0.11	2.65 ± 0.00
Protein content (dwb) [%]‡	9.40	12.70	13.30
Ash content [%]	1.18 ± 0.01	6.45 ± 0.04	5.46 ± 0.47
β-Glucan (dwb) [%]	1.57 ± 0.02	6.27 ± 0.11	5.03 ± 0.12
WEAX [%]	1.98 ± 0.22	1.91 ± 0.05	1.55 ± 0.15
Total AX [%]	2.56 ± 0.18	12.00 ± 0.44	10.81 ± 0.72

* Mean values ± standard deviation of triplicate determinations ($n = 3$).

† SRC, solvent retention capacity; dwb, dry weight basis; WE, water-extractable; AX, arabinoxylan.

‡ Standard deviation ≤ 5 %.

Fundamental Rheological Analysis

Oscillation measurements were performed in a nondestructive way to investigate the influence of rye bran particles (rye flour replaced by rye bran up to 20.0%) on the rheological properties of rye dough. The effect of different procedures (direct and indirect bran additions, xylanase addition) and the bran particle size (125–250 μm) on the complex shear modulus ($|G^*|$) and the loss factor ($\tan \delta$) are shown in Fig. 1. For $|G^*|$, no significant differences were obtained between the direct and indirect bran additions. Replacement of flour by rye bran (up to 20.0%) increased $|G^*|$ by 100.2% and 106.6% for the direct and indirect methods of bran addition, respectively. Xylanase addition led to significant decrease of $|G^*|$ for all bran concentrations (0.0–20.0%) in comparison to the direct and indirect bran additions. This can be explained by the release of water by AX due to modification by xylanase. Replacement of flour by rye bran (up to 20.0%) and xylanase addition increased $|G^*|$ by 113.6%. For all bran concentrations and methods of addition, the increase of $|G^*|$ with increasing bran concentration can be explained by the increased water-binding capacity of the bran particles. However, the curve trends were nearly the same for each measurement. This leads to the conclusion that indirect bran addition and xylanase addition had no dominant influence on the viscoelastic behavior of rye dough; thus, the dough structure changes could not be correlated to the addition of xylanase or bran. This assumption is confirmed by the values of the loss factor $\tan \delta$ (Fig. 1). For the direct and indirect bran additions (up to 20.0%), $\tan \delta$ decreased by 6.4%, and for the xylanase addition it decreased by 11.5%. However, this only reflects the influence of water because the differences between the direct and indirect bran additions as well as the xylanase addition were not significant ($P > 0.05$). Rye dough supplemented with large bran particle sizes (355–500 μm) showed rheological behavior similar to the dough treated with small bran particle sizes for both direct and indirect bran additions (Fig. 2). Values for $|G^*|$ were not significantly different between direct and indirect bran additions. This lead to the conclusion that the structure of rye dough is different from wheat dough because it cannot be destroyed or enhanced by bran addition. Addition of rye bran (up to 20.0%) increased $|G^*|$ by 80.6% and 94.7% with direct and indirect bran additions, respectively. However, in the case of xylanase addition, a 20.0% replacement of flour by rye bran affected an increase of $|G^*|$ by only 46.6%. It has to be emphasized that the small bran particles had a stronger influence on $|G^*|$ than the larger ones. This could be related to slower water binding of large versus small bran particles due to lower surface-to-mass ratio. Although the SRC results (Table 1) showed higher water retention capacity for large bran particles; nevertheless, the lower surface-to-mass ratio in comparison to small bran particles appears to be the main reason for the reduced water absorption and, therefore, the lower value of $|G^*|$. Obviously, the SRC values cannot be

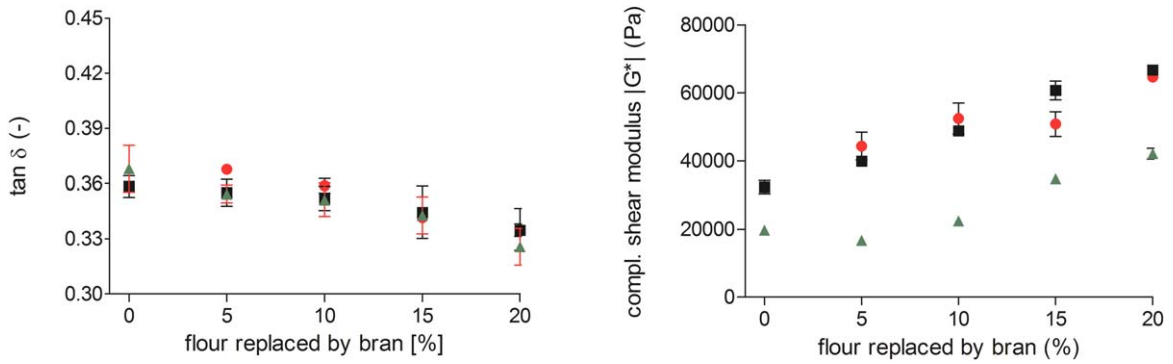

FIG. 1. EFFECT OF RYE BRAN ADDITION (PARTICLE SIZE: 125–250 μm) ON THE LOSS FACTOR TAN Δ AND THE COMPLEX SHEAR MODULUS $|G^*|$OF RYE DOUGH AS AFFECTED BY THE METHOD OF DOUGH PREPARATION. (•), DIRECT BRAN ADDITION; (■), INDIRECT BRAN ADDITION; (△) XYLANASE ADDITION ($n = 3 \pm$ SD)

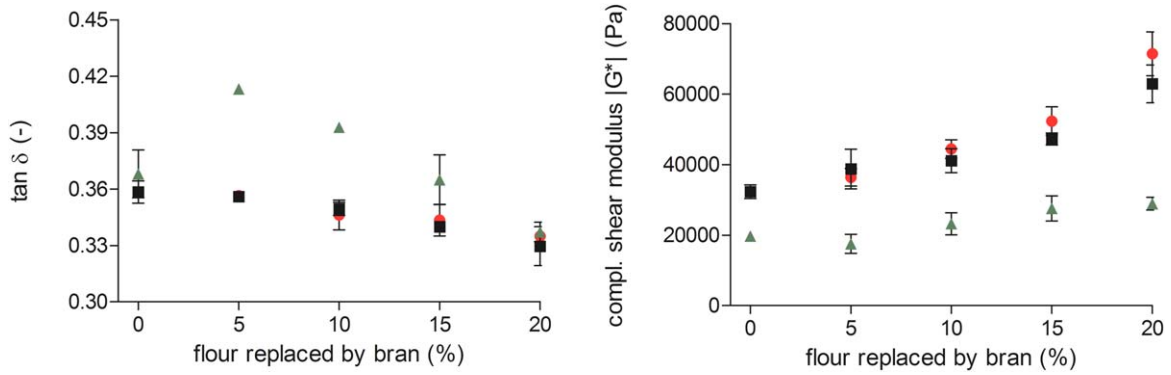

FIG. 2. EFFECT OF RYE BRAN ADDITION (PARTICLE SIZE: 355–500 μm) ON THE LOSS FACTOR TAN Δ AND THE COMPLEX SHEAR MODULUS |*G**|OF RYE DOUGH AS AFFECTED BY THE METHOD OF DOUGH PREPARATION. (•), DIRECT BRAN ADDITION; (■), INDIRECT BRAN ADDITION; (△) XYLANASE ADDITION ($n = 3 \pm$ SD)

directly related to |*G**| because of the applied force during centrifugation in the SRC method, which affects the results; the higher the force, the lower the SRC becomes. Another possible explanation is that water absorption described by dough firmness (|*G**|) is the result of the entire dough system and is dominated by AX present in flour rather than by bran particles as described by Girhammar and Nair (1992). The combination of xylanase and larger particles influenced the dough microstructure expressed by tan δ (Fig. 2). In comparison to the dough with combined application of xylanase and bran with small particle size, larger bran particles increased tan δ, especially for bran concentrations of 5.0–10.0%. tan δ of dough with direct and indirect bran additions matched each other for increasing bran concentrations. It can be assumed that both the reduced water absorption of the large bran particles and the replacement of flour by bran led to reduced water binding and, therefore, less elasticity of the dough. However, increasing the bran concentration (15.0–20.0%) compensated for the released water and the dough became more elastic, expressed by a decline in the tan δ. Furthermore, it is worth to mention that the final dough structure appeared not to be influenced by the bran particles as in wheat dough. From the rheological measurements, it can be concluded that the addition of rye bran particles of different sizes (125–250 and 355–500 µm) up to 20.0% have an indirect influence on the microstructure of rye dough. These results are in accordance to the findings of Parkkonen *et al.* (1994); they found an influence of different particle sizes obtained from rye wholemeal on the rheological behavior of rye dough. In contrast to rye dough, the addition of bran particles to wheat dough directly shows a negative influence on the rheological properties (Hartikainen *et al.* 2013). For wheat, Gan *et al.* (1992) described a dilution of the structure forming component gluten by addition of fiber fractions to wheat dough. As demonstrated by Wang *et al.* (2002), water unextractable solids directly decrease the gluten yield and influence the rheological properties of wheat dough. The changes of the rheological behavior of rye dough seem to be only attributed to a change of the available water content and not to the destabilization of the structure-forming components as described for wheat. The available water content and its distribution in rye dough are mainly determined by the content of AX and other water-absorbing additives. Therefore, particles such as bran interfering with the structure of wheat dough do not appear to have a significant effect on the structure and functional properties of rye dough in contrast to wheat dough.

Dough Development of Rye Dough Enriched With Rye Bran

To obtain more information on the formation and stability of the structure of rye dough, fermentation studies with dough differing in preparation and bran particle size were performed (Table 2). In general, different bran concentrations significantly ($P < 0.05$) affected the maximum dough development (Hm) height during a 3 h fermentation. Similar results were reported for wheat dough enriched with fibers (Ktenioudaki *et al.* 2012; Roth *et al.* 2015). For the small bran size particles, the direct and indirect bran additions decreased Hm with increasing bran level (between 0.0 and 20.0%) by approximately 31.3 and 33.1%, respectively. For xylanase, the corresponding decrease of Hm was approximately 35.9%. However, no significant differences ($P > 0.05$) were analyzed for the direct and indirect methods between 0.0% and 10.0% bran additions. For the method of xylanase additions, significant differences ($P < 0.05$) were analyzed for each bran addition. Furthermore, the use of xylanase gave a significant increase ($P < 0.05$) of dough height (up to 37.2%) in comparison to the direct and indirect bran additions. The addition of large bran particles led

TABLE 2. INFLUENCE OF DIFFERENT DOUGH PREPARATIONS (DIRECT AND INDIRECT BRAN ADDITION, XYLANASE ADDITION) AND BRAN PARTICLE SIZES (SMALL, LARGE) ON DOUGH DEVELOPMENT AND GASEOUS RELEASE OF RYE DOUGH. RESULTS ARE SHOWN AS MEAN VALUES ± STANDARD DEVIATION ($n = 2$)

	Small bran size (125–250 μm)			Large bran size (355–500 μm)		
Ratio rye flour : rye bran	Dough development Hm [mm]	Gas release Total volume [mL]	*R* [%]	Dough development Hm [mm]	Gas release Total volume [mL]	*R* [%]
Direct bran addition						
100:00	$25.70 \pm 2.83^{a/\alpha}$	$1103.00 \pm 145.66^{a/\alpha}$	$94.70 \pm 0.28^{a/\alpha}$	$25.70 \pm 2.83^{a/\alpha}$	$1103.00 \pm 145.66^{a/\alpha}$	$94.70 \pm 0.28^{a/\alpha}$
90:10	$23.65 \pm 1.63^{a/\alpha}$	$1034.00 \pm 171.12^{a/\alpha}$	$93.30 \pm 1.41^{a/\alpha\beta}$	$22.50 \pm 0.28^{a/\alpha}$	$959.50 \pm 157.68^{a/\alpha}$	$96.00 \pm 2.40^{a/\alpha}$
80:20	$18.15 \pm 0.35^{b/\alpha}$	$976.00 \pm 36.77^{a/\alpha}$	$96.45 \pm 0.35^{a/\alpha}$	$17.15 \pm 1.20^{b/\alpha}$	$1013.50 \pm 3.54^{a/\alpha}$	$95.75 \pm 1.49^{a/\alpha}$
Indirect bran addition						
100:00	$25.70 \pm 2.83^{a/\alpha}$	$1103.00 \pm 145.66^{a/\alpha}$	$94.70 \pm 0.28^{a/\alpha}$	$25.70 \pm 2.83^{a/\alpha}$	$1103.00 \pm 145.66^{a/\alpha}$	$94.70 \pm 0.28^{a/\alpha}$
90:10	$21.95 \pm 1.63^{a/\alpha}$	$1164.50 \pm 57.28^{a/\alpha}$	$89.60 \pm 1.27^{a/\alpha}$	$23.20 \pm 0.42^{a/\alpha}$	$934.50 \pm 13.44^{a/\alpha}$	$96.15 \pm 0.21^{a/\alpha}$
80:20	$17.30 \pm 1.27^{b/\alpha}$	$994.50 \pm 166.17^{a/\alpha}$	$92.55 \pm 2.33^{a/\alpha}$	$17.10 \pm 0.71^{b/\alpha}$	$810.00 \pm 83.44^{a/\alpha}$	$97.30 \pm 0.42^{a/\alpha}$
Xylanase addition						
100:00	$35.25 \pm 0.35^{a/\beta}$	$1244.50 \pm 17.68^{a/\alpha}$	$93.50 \pm 0.14^{a/\alpha}$	$35.25 \pm 0.35^{a/\beta}$	$1244.50 \pm 17.68^{a/\alpha}$	$93.50 \pm 0.14^{a/\alpha}$
90:10	$29.25 \pm 0.21^{b/\beta}$	$1270.00 \pm 48.08^{a/\alpha}$	$97.30 \pm 1.27^{a/\beta}$	$30.50 \pm 0.14^{b/\beta}$	$1267.00 \pm 69.30^{a/\beta}$	$86.50 \pm 0.42^{b/\beta}$
80:20	$21.30 \pm 0.28^{c/\beta}$	$1408.50 \pm 2.12^{b/\beta}$	$97.55 \pm 1.63^{a/\alpha}$	$23.90 \pm 0.14^{c/\beta}$	$1208.00 \pm 140.01^{a/\beta}$	$85.35 \pm 2.05^{b/\beta}$

Hm: height of maximum dough development expressed in millimeter [mm]; total volume: Total volume of gas release [mL]; *R*: retention coefficient in percent [%], retention volume divided by the total gas release.

Significant differences were determined by one-way ANOVA followed by Tukey's test ($P < 0.05$). Same letters indicates means which are not significantly different from one another. Two types of comparisons were made: 1. Different Latin letters indicate significant differences between means in the same column for each dough preparation. 2. Different Greek letters indicate significant differences between means in the same column for equal ratios of rye flour to rye bran of different dough preparations.

to the same trend of dough development as described for the small particles. The decrease of Hm with increasing bran concentration is linked to an increasing dough firmness as expressed by $|G^*|$ in Figs. 1 and 2, which was likely to impact the ability of the dough to rise. This leads to the conclusion that in rye dough the bound water is the limiting factor for dough development. This assumption is confirmed by the values of the retention coefficient (R). For the small bran size, no significant differences for R were obtained between the bran concentrations for each method of addition (except indirect method 10.0% bran addition and xylanase method 10.0% bran addition; $P < 0.05$). The addition of large bran size particles on R also showed no significant differences between the direct and indirect methods. Significant differences were obtained between xylanase addition and both bran addition methods (direct and indirect) at 10.0% and 20.0% of bran addition. The continuous high R, of min. 85.4%, indicated that the structure was still intact. For wheat dough, several authors described that decreasing Hm and R are related to an interruption of the gluten-starch matrix (Parkkonen *et al.* 1994; Le Bleis *et al.* 2015). On the contrary, in rye dough a gluten-starch matrix as described for wheat dough does not exist, thus there is no bran-related decrease of Hm and R. For dough supplemented with xylanase, an increase of the dough stability was reported. This was due to a xylanase-induced increase of the WEAX content, thus, boosting the viscosity of the dough lamellae (liquid films surrounding the gas voids), which has a positive effect on the stability of the gas cells during fermentation (Courtin and Delcour 2002).

Quality Characteristics of Rye Bread

The influence of different bran concentrations, bran particle size ranges and dough preparation methods on the specific volume, crumb hardness and WEAX levels of rye breads were also determined. The two particle size ranges of added bran had similar effects on the bread volume (Figs. 3 and 4). The addition of small-sized bran particles (up to 20.0% with direct bran addition) reduced the specific bread volume by 8.5%, and in the case of larger bran particles the specific volume also dropped by 7.3%. Thus, a reduction of the particle size of the bran had only a weak effect on the specific bread volume of rye bread in contrast to wheat bread (Noort *et al.* 2010). For wheat dough, crosslinking of cell wall AX to gluten proteins via ferulic acid has been postulated to minimize gluten network formation, thus leading to decreased bread volume (Wang *et al.* 2003, 2004). In rye dough, (gluten) proteins are of minor importance for network formation because of their different protein conformation compared to gluten proteins of wheat (Wieser and Kieffer 2001; Verwimp *et al.* 2007). Pentosans, mainly AX, are thought to contribute most to the structure and functional properties of rye dough and, thus, also on bread structure formation (Cauvain and Young 2007). Concerning the dough preparation methods, no significant ($P < 0.05$) differences were

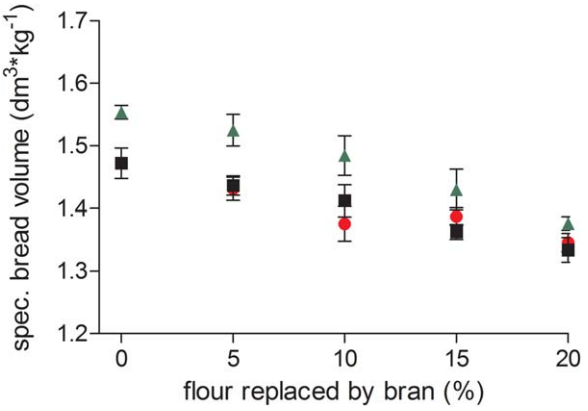

FIG. 3. EFFECT OF RYE BRAN ADDITION (PARTICLE SIZE: 125–250 μm) ON THE SPECIFIC VOLUME OF RYE BREAD AS AFFECTED BY THE METHOD OF DOUGH PREPARATION. (•), DIRECT BRAN ADDITION; (■), INDIRECT BRAN ADDITION; (△) XYLANASE ADDITION ($n = 3 \pm$ SD)

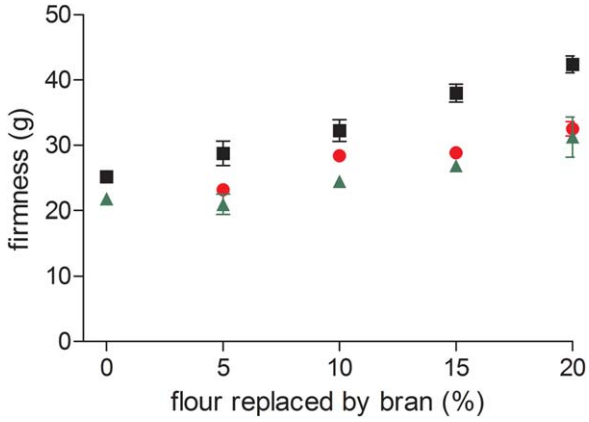

FIG. 5. EFFECT OF RYE BRAN ADDITION (PARTICLE SIZE: 125–250 μm) ON THE CRUMB HARDNESS OF RYE BREAD AS AFFECTED BY THE METHOD OF DOUGH PREPARATION. (•), DIRECT BRAN ADDITION; (■), INDIRECT BRAN ADDITION; (△) XYLANASE ADDITION ($n = 3 \pm$ SD)

achieved between the direct and indirect bran additions. For the indirect addition, it was assumed that the specific volume increases because the particles are not expected to disturb the rye dough structure during formation and therefore no lack of structure would occur. However, no significant differences were achieved. For the different particle sizes of the bran, in comparison to direct and indirect bran additions, the combination with xylanase addition caused a significant ($P < 0.05$) increase of the specific volume up to a bran concentration of 15.0%. This can be explained by an increased amount of WEAX, which are thought to stabilize the gas bubbles during fermentation, thus leading to an increased volume (Courtin and Delcour 2002). This is also shown later. It is worth to mention that the addition of xylanase and rye bran (up to 15.0% for both particle ranges) led to the same specific volume as in the case without xylanase and bran addition. The effects of bran and/or xylanase addition on the firmness of rye bread crumb (2 h after baking) are shown in Figs. 5 and 6. Addition of small bran particles up to 20.0% (direct bran addition) increased the crumb firmness by 25.5% and for the large bran particles by 24.6%. For the small-sized bran, no significant ($P > 0.05$) differences of the crumb firmness were obtained for both the direct bran addition and the xylanase addition (except a bran addition of 10.0%). However, indirect bran addition led to a significant ($P < 0.05$) increase of the crumb firmness at all bran concentrations, which was not the case for the addition of larger particles (355–500 µm) by indirect bran addition. A possible reason is the lower specific surface area and water-binding capacity of large versus small bran particles leading

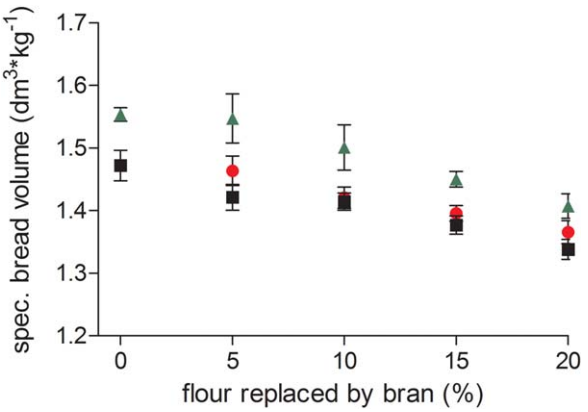

FIG. 4. EFFECT OF RYE BRAN ADDITION (PARTICLE SIZE: 355–500 μm) ON THE SPECIFIC VOLUME OF RYE BREAD AS AFFECTED BY THE METHOD OF DOUGH PREPARATION. (•), DIRECT BRAN ADDITION; (■), INDIRECT BRAN ADDITION; (△) XYLANASE ADDITION ($n = 3 \pm$ SD)

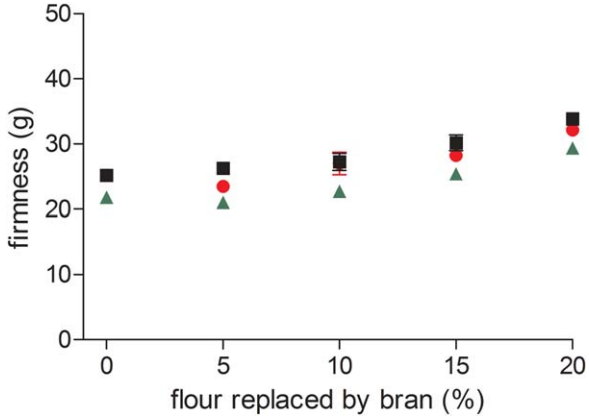

FIG. 6. EFFECT OF RYE BRAN ADDITION (PARTICLE SIZE: 355 – 500 μm) ON CRUMB HARDNESS OF RYE BREAD AS AFFECTED BY THE METHOD OF DOUGH PREPARATION. (•), DIRECT BRAN ADDITION; (■), INDIRECT BRAN ADDITION; (△) XYLANASE ADDITION ($n = 3 \pm$ SD)

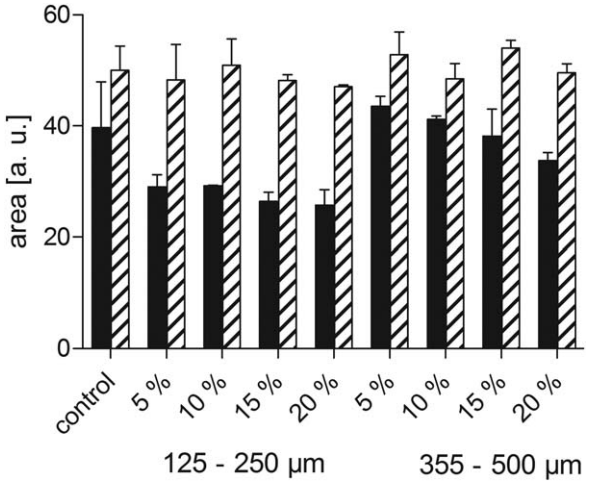

FIG. 7. TOTAL AREA UNDER THE CURVE OF GEL PERMEATION CHROMATOGRAMS OF WEAX. BLACK COLUMNS, BREAD FROM DOUGH WITH DIRECT BRAN ADDITION; SHADED COLUMNS, BREAD FROM DOUGH WITH XYLANASE ADDITION; a.u., ARBITRARY UNITS ($n = 3 \pm$ SD)

to reduced firmness. Firmness measurements of bread with and without xylanase and without bran addition showed a significantly ($P < 0.05$) lower crumb firmness compared to bread supplemented with bran and/or xylanase, this is due to increased bread volume. Furthermore, xylanase addition up to 15.0% led to the same crumb firmness as rye bread with bran addition only. It can be summarized, that the application of xylanase in rye bread-making increased the final bread quality. Furthermore, the addition of xylanase plus bran (all concentrations) enhanced the final bread characteristics such as specific volume and crumb hardness. As mentioned before, xylanase is known to increases the content of WEAX, which has a positive effect on gas cells' stabilization during fermentation and on the early stage of oven rise. To confirm this theory for bran-containing rye bread, the content of WEAX (absorbance areas) as affected by the method of bran and/or xylanase addition was determined by GP-HPLC-RI analysis. Breads obtained from dough with direct bran addition and xylanase addition (different bran concentrations and both particle size ranges) were analyzed. Bread prepared by indirect bran addition was not shown because of insignificant ($P > 0.05$) differences in the absorbance area of the permeation chromatograms compared with the rye bread prepared by direct bran addition. The results are shown in Fig. 7. Except of the standard bread, the application of xylanase led to a significant increase ($P < 0.05$) increase in the obtained absorbance area of WEAX compared to the bread without xylanase. Furthermore, this experiment also showed that after addition of bran with smaller particle size, the xylanase-induced increase of the WEAX content was higher than in the bread containing large bran particles. This was due to the higher specific surface of the small particles leading to more efficient formation of WEAX compared to the large particles. Altogether, this leads to the conclusion that WEAX positively influence the dough and bread structure confirming the hypothesis of Courtin and Delcour (2002).

CONCLUSION

The present study showed how the structural properties of rye dough are affected by rye bran particles with and without addition of xylanase. The type of bran addition (direct/indirect), as well as the particle size (small/large) provide comparable effects on dough properties and bread quality. Thus, it can be concluded that functionally relevant components, such as AX, are not affected by bran particles during mixing. It appears that bran particles only weakly interfere with the structure of rye dough because of its quite robust structural nature. However, the addition of xylanase affects the structural properties of rye dough and leads to improved bread quality. The study also demonstrated that the rye dough and bread structures are mainly dominated and influenced by AX. However, it is still not clear how AX influences the dough and bread characteristics in detail. More work is necessary to unravel how AX influences dough formation, dough properties and bread quality. It will be also interesting to know if rye protein can form a kind of network after AX have been degraded by xylanase. All in all, this work successfully highlights the impact of different additives and constituents on rye dough structure from a chemical, rheological and end-product structural point of view. The work contributes to closing the gap in the literature addressing this crucial issue for rye dough, which is considered a healthy alternative to wheat with good nutritional value.

ACKNOWLEDGMENTS

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2.4 IMPACT OF ARABINOXYLAN ADDITION ON PROTEIN MICROSTRUCTURE FORMATION IN WHEAT AND RYE DOUGH

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Impact of arabinoxylan addition on protein microstructure formation in wheat and rye dough

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ABSTRACT

The aim of this study was the identification and characterization of the impact of different Arabinoxylan (AX) concentrations (0.0%; 2.5%; 5.0%; 7.5%; 10.0%) on the protein microstructure formation of wheat and rye model dough which were adjusted to the same dough firmness by water calibration. The microstructural characterization was achieved by means of confocal-laser-scanning-microscopy micrographs that were evaluated by an image processing tool. The rheological behavior of the AX treated dough was characterized by oscillation and creep recovery tests. Wheat and rye dough micrograph analyses exhibited a decrease in perimeter and feret diameter of the protein network with increasing AX concentration of approx. 63% and 54%, respectively. Rheological investigations showed that the viscoelastic part of the wheat and rye model dough determined loss factor ($\tan\delta$) increased with increasing AX concentration by 148% and 285%, respectively. The results prove the impact of AX on protein microstructure formation in dough.

1. Introduction

The rheological behavior of cereal dough such as viscosity and elasticity is commonly based on the formation of a continuous protein network during mixing (Dobraszczyk, 2004; Wieser, 2007).The formation of the protein network not only depends on the mixing parameters such as energy input, time, dough ingredients (water, sodium chloride) but also on the protein characteristics (Kuktaite et al., 2007; Wang et al., 2003a). In wheat dough, gliadin and glutenin are the known protein fractions that form a continuous protein matrix by disulphide bonds and intermolecular noncovalent interactions (Verwimp et al., 2007). Especially the low molecular weight (LMW) and the high molecular weight (HMW) glutenin subunits form protein networks with molecular weights between 500,000 to more than 10 million Da. (Wieser, 2007; Wieser et al., 2006). For rye dough, no such continuous protein network is mentioned in literature. One reason could be the difference in the molecular structure of rye proteins (secalins and glutelins) in contrast to wheat proteins (gliadin and glutenin) (Verwimp et al., 2007). For rye proteins, differences in the aggregation behavior of 75 k γ-secalins and HMW secalins related to the aggregation behavior of the LMW and HMW glutenin subunits of wheat are mentioned for a limited protein network formation (Verwimp et al., 2007; Wieser and Kieffer, 2001).

Another reason could be the presence of the predominant non-starch polysaccharide Arabinoxylan (AX). AX consists of a β-1.4-D-xylopyranosyl backbone with side chains of α-L-arabinofuranosyl linked at position 2 and/or 3 of the xylose-chain and plays a key role for final bread and dough quality (Döring et al., in press; Izydorczyk and Biliaderis, 1992). Especially in high concentrations, AX seems to influence dough and bread quality negatively. It is mentioned that AX in higher concentrations disturbs the protein network formation during dough development and could affect the dough properties. This applies particularly for wheat dough where the dough extensibility and gluten yield are extensively influenced by the presence of AX (Michniewicz et al., 1991; Wang et al., 2003b). Wang et al. presumes that AX is affecting the protein network formation of wheat protein (gluten) by competing for water. It was further presumed, that AX can form physical barriers and interfere directly with the process of gluten network formation which could have negative effects on protein network formation and gluten yield (Wang et al., 2003a). Wang et al. (2004) also presume that AX interacts by covalent cross-linking with the proteins which influences the rheological

Abbreviations: AX, Arabinoxylan; *c*, concentration; $|G^*|$, complex shear modulus; G', storage modulus; G'', loss modulus; tan δ, loss factor; CLSM, Confocal Laser Scanning Microscopy; *d*, diameter; Da, Dalton; λ_{exc}, excitation; λ_{em}, emission; J_{max}, maximum creep compliance; J_r, creep recovery compliance.

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properties of the gluten network. In contrast, Hilhorst et al. (2002) found no evidence for a cross-linking between AX and proteins in wheat dough.

The results and interpretation of the mentioned literature leads to the hypothesis that AX hinders, especially in high concentrations, the proteins to develop a structured network. To prove this hypothesis and to interpret the role of AX on dough characteristics such as dough rheology, the impact of different AX concentrations on wheat and rye protein network formation was investigated. This was performed using model dough, which allows the analysis of the influence of AX on the protein network without the influence of further ingredients such as β-glucan or lipids. Additionally, to interpret and compare the achieved results, all dough were adjusted to the same dough firmness by water calibration. The adjustment of water allows predicting the rheological behavior of dough with different AX concentrations excluding the influence of different dough firmness, which could distort the final results significantly. Investigations were carried out to examine the influence of different AX concentrations on protein network formation. Furthermore, predicting the final dough and bread characteristics was achieved by means of rheological investigations as well as micrographs by Confocal Laser Scanning Microscopy (CLSM) of the final protein network. The results of the rheological and visual properties of the dough were correlated to the AX concentrations. The presented investigation provides detailed information of the AX impact on protein formation in wheat and rye dough. Thus, describing its functionality and elaborating the detailed process of protein formation.

2. Materials and methods

2.1. Raw Materials and chemical composition

For dough preparation, standard wheat flour (type 550) and rye flour (type 1150), from Rosenmühle (Ergolding, Germany), was acquired. For the model dough, wheat starch was acquired from Sigma–Aldrich Chemie GmbH (Munich, Germany) and wheat protein from Kröner-Stärke (Ibbenbüren, Germany). Rye starch and protein was acquired from Lyckeby Culinar AB (Kristianstad, Sweden). Rye AX was obtained from Megazymes (Wicklow, Ireland). The rye AX has a purity of ~90% (dwb). Further components of the AX sample are Starch (0.2%), β-Glucan (0.1%), protein (2.5%) and ash (4.0%). The ratio of arabinose to xylose is 38–62. The molecular weight of the AX is 440 kDalton. Further ingredients for final dough preparations were distilled water and sodium chloride (NaCl) (Südsalz GmbH, Bad Friedrichshall, Germany). Moisture contents were analyzed according ICC Method 201 (ICC – International Association for Cereal Science and Technology). Total starch and starch damage of the samples were analyzed by AACCI Method 76-13.01 (AACC International. Approved Methods of Analysis) and ICC Method 172 (ICC – International Association for Cereal Science and Technology, 2011), respectively. Protein contents were determined by using the Kjeldahl Method (EBC) (Anger, 2006). Water retention (only with distilled water) of the wheat and rye flour as well for the wheat and rye starch was determined by Solvent Retention Capacity (SRC) (AACC International. Approved Methods of Analysis). The analyses on the raw materials were performed in duplicates.

2.2. (Model) Dough preparation

For standard wheat dough preparation, wheat flour type 550 (50.0 g; corrected to 14% moisture), NaCl (0.9 g) and distilled water, depending on finale dough firmness of 4900 mN m, were mixed together in a Z-Blade mixer (50 g bowl). The settings of the trials were according to AACCI Method 54-21.02 (AACC International. Approved Methods of Analysis). After reaching the final dough firmness of 4900 mN m (9.8 mN m = 1 FU), the procedure was stopped and the dough firmness was re-measured by a Rheometer as described later (see Section 3.3). For the rye dough (rye flour type 1150), the same procedure was performed with one modification: Dough firmness was adjusted to 2940 mN m as described by Beck et al. (2012). After reaching 2940 mN m, the final dough firmness was also re-measured by a Rheometer. For wheat dough a firmness expressed as complex shear modulus ($|G^*|$) of 12,100 Pa was measured. In the case of rye dough a $|G^*|$ of 21,500 Pa was measured.

After analyzing the dough firmness of the standard dough, prepared with Z-Blade mixer in the Rheometer, the standard dough was produced once more using a Glutomatic system 2000 (Perten, England). Dough mixing by Glutomatic was developed to produce smaller amounts of dough compared to the Z-Blade mixer. Dough mixing in the Glutomatic required some necessary modifications: 1. The Glutomatic bowl was covered with a plastic foil (Fig. 1). 2. The water pump was switched off. 3. The distance between the glutomatic mixing hook and the bowl was increased after 20 s of mixing to 80 mm. This was necessary to reduce the energy intake and was performed by a metal spacer (Fig. 1). Mixing time to reach the prescribed dough firmness was 4 min 50 s.

For preparing the model dough, the dry components starch, Gluten protein, AX and NaCl were weighed into the Glutomatic bowl and homogenized by a spatula before adding water. For the wheat model dough preparations; the protein to starch ratio was adjusted to 1:9 and for the rye model dough the protein to starch ratio was adjusted to 1:12 (Chevallier and Colonna, 1999). To determine the effect of AX on the protein microstructure, different AX contents (0.0%, 2.5%, 5.0%, 7.5%, and 10.0% flour was replaced by AX) were added. The water addition was adjusted for each model dough to reach the same dough firmness ($|G^*|$) which was related to the dough firmness ($|G^*|$) determined in the Rheometer of the standard wheat and rye dough. The adjustment of dough firmness was performed to eliminate the influence of different dough firmness on the rheological properties of the dough samples as discussed later. Table 1 displays the determined water contents for

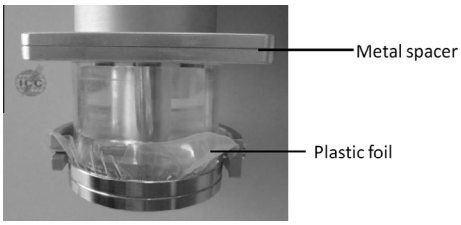


Fig. 1. Modified Glutomatic mixing system for (model) dough preparation. Metal spacer increases distance between Glutomatic mixing tool and bowl to reduce energy intake during mixing. Plastic foil seals the original Glutomatic bowl.

Table 1
The determined water contents for each AX concentration to reach the analyzed dough firmness of standard wheat and rye dough: standard wheat dough firmness $|G^*|$: 12,100 Pa; standard rye dough firmness $|G^*|$: 21,500 Pa.

AX conc. (%)	Wheat model dough Water content based on 100 unit mass flour	Rye model dough Water content based on 100 unit mass flour
0.0	57.8	55.6
2.5	64.5	62.5
5.0	72.0	69.0
7.5	80.0	75.5
10.0	94.0	85.0

each AX concentrations depending on dough firmness of the wheat and rye standard dough. Afterwards, the model dough was mixed in the Glutomatic as described above for the standard dough.

2.3. Rheological settings and analyzes

For the rheological measurements, an AR G2 Rheometer (TA Instruments-Waters LLC, New Castle, USA) with a plate–plate geometry (d = 40 mm) was used. After dough preparation, 3 g of the dough were gently placed between the plate–plate geometry. The chosen gap for measurements was 2000 μm and the temperature of the geometry was 30 °C for simulating the proofing room temperature. Excess dough between the plate–plate geometry was softly removed by a razor blade. To prevent a drying-out of the sample, the margin was sealed with paraffin oil. After a relaxing time of 10 min, an oscillation test was performed following the procedure of Houben et al. with small modifications (Houben et al., 2010): The applied deformation of 0.075% was determined by a deformation test and is well within the linear viscoelastic region of the samples. The chosen frequency was 1.0 Hz. Final data were expressed in terms of the loss factor $\tan\delta$. $\tan\delta$ describes the ratio of G'' to G', where G'' describes the loss modulus and G' denotes the storage modulus. Consequently, a lower $\tan\delta$ reflects higher network elasticity. $\tan\delta$ was calculated using the following equation:

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

To gain further data from high strains, a creep recovery test was performed. The added shear stress during the creep test was 50 Pa for 300 s. For the recovery test, no shear stress was applied for 900 s. Final data were expressed as creep recovery compliance J_r (at t = 900 s of the recovery phase). The measurements were performed in threefold.

2.4. Microscopic settings and analyzes

For a visual analysis of the protein microstructure, dough was gently cut from the main dough with a scissor and was transferred to a specimen shape (diameter 18.0 mm, height 0.8 mm). Staining the protein microstructure, 40 μl of fluorescent dye rhodamine B (c = 0.001 g 100 ml^{-1}) from Sigma–Aldrich Chemie GmbH (Munich, Germany) was added on top of the sample dough. After one minute, the specimen shape was covered by a cover slip. A Confocal Laser Scanning Microscope from Nikon (Düsseldorf, Germany) and a 60 × objective was used to analyze the protein microstructure. Thirteen independent micrographs on the xy-axis were captured for each dough sample. The measurements were performed in three different dough samples. Proteins were monitored in fluorescence micrographs (λ_{exc} = 543 nm, λ_{em} = 590/50 nm) with 1024 × 1024 pixel resolution (212 × 212 μm) in a constant z-position. Micrograph analyses were performed using image processing open source Java software ImageJ (Version 1.42q, National Institutes of Health, Bethesda, Md, USA) with the DoMiQ (Dough Microstructure Quantification) Method described by Jekle and Becker (2011). For further discussion, following protein features were analyzed: Perimeter (the length of the outside boundary of the selection) and feret diameter (the longest distance between any two points along the selection boundary).

2.5. Statistical analysis

All data were statistically evaluated using the software GraphPad Prism (GraphPad Software, Inc., La Jolla, CA 92037 USA). One-way analysis of variance (ANOVA) was used to evaluate statistical significance of the results, where the significance of difference is defined at $P < 0.05$. Correlation coefficient r was used to describe linear correlations between the measurements.

3. Results and discussion

3.1. Physicochemical characteristics of raw materials

Starch damage of wheat flour, rye flour, wheat starch and rye starch are 4.71 ± 0.04%, 4.61 ± 0.01%, 4.55 ± 0.48% and 4.17 ± 0.02%, respectively. Total starch amount are 58.42 ± 9.14% (dwb), 53.16 ± 0.90% (dwb), 92.33 ± 1.96% (dwb) and 92.63 ± 3.00% (dwb), respectively. The protein contents of wheat flour, rye flour, wheat protein, rye protein, wheat starch and rye starch are 11.93 ± 0.12%, 9.04 ± 0.22%, 65.45 ± 2.18%, 61.20 ± 0.29%, 0.19 ± 0.03% and 0.10 ± 0.00%, respectively. Solvent retention capacity of wheat flour, rye flour, wheat starch and rye starch are 63.52 ± 0.03%, 137.79 ± 0.60%, 61.69 ± 0.31% and 68.65 ± 0.46%, respectively.

3.2. Model dough

The aim of this study was the rheological and visual characterization of the impact of different AX concentrations on the protein network formation in wheat and rye dough. In order to realize this aim, there exist two possibilities: One could be the addition of AX to commercial flour. However, it will not be possible to reduce the AX contents in commercial flour to a repeatable fixed content. Additionally, the usage of AX degrading enzymes does not guarantee a constant AX concentration in the final flour. The second possibility is the usage of native flour ingredients (e.g. starch and protein) to define a model flour/dough. In this case it will be possible to guarantee a constant concentration of the ingredients. Therefore, the usage of the latter (model flour/dough) was chosen to fulfill the aim.

3.3. CLSM analysis of the protein microstructure

Visual analysis using CLSM provide information of the dough microstructure and the sample is analyzed in a nondestructive manner (Jekle and Becker, 2015). To minimize the influence of different dough firmness ($|G^*|$) on the investigations, all wheat and rye model dough were adjusted to the same dough firmness by the variation of the water addition in relation to the dough firmness determined in the standard dough (standard wheat dough firmness: 12,100 Pa; standard rye dough firmness: 21,500 Pa). The adjustment of dough firmness was performed to eliminate the influence of different dough firmness on the rheological properties of the dough samples as discussed later. Table 1 displays the determined water contents for each AX concentrations depending on dough firmness of the wheat and rye standard dough. Two binary micrographs for each AX concentrations are exemplarily shown (Figs. 2 and 3). The proteins are shaded white.

Fig. 2 shows the impact of different AX concentrations on the protein microstructure after mixing of the wheat model dough. CLSM micrograph A without AX displays a stretched and well-formed protein network and its fibrils are orientated in the same direction. The protein fibrils of CLSM micrograph B are still orientated and the protein network is still visible but starts to be lightly distributed and spread (black areas imbedded in the protein microstructure) by the addition of 2.5% AX. The feret diameter seems to be more decreased which could be explained by the interruption of the protein network formation by AX. From micrograph B, it could be assumed that an AX concentration of around 2.5% is optimal for the final protein network formation. On one hand, the protein network is a bit weakened which should have a positive effect on the

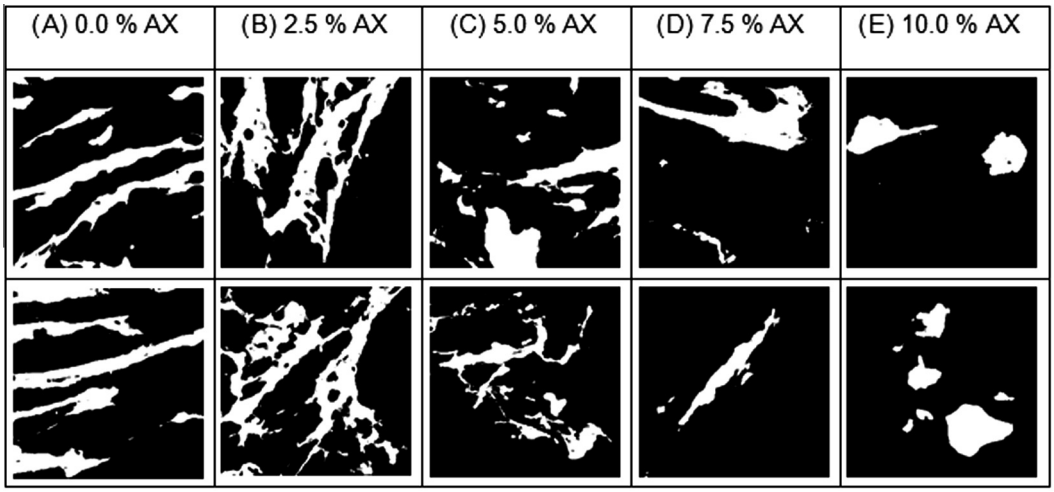


Fig. 2. Binarized CLSM micrograph of wheat model dough with different AX contents (flour replaced by AX): (A) 0.0% AX, (B) 2.5% AX, (C) 5.0% AX, (D) 7.5% AX and (E) 10.0% AX. Protein network is shaded white. Size: 212 × 212 μm (1024 × 1024 px).

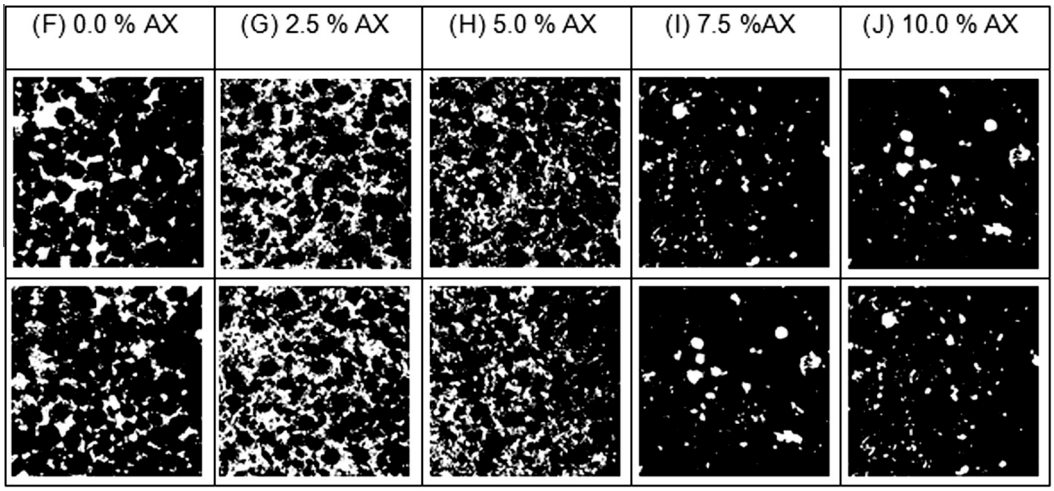


Fig. 3. Binarized CLSM micrographs of rye model dough with different AX contents (flour replaced by AX): (F) 0.0% AX, (G) 2.5% AX, (H) 5.0% AX, (I) 7.5% AX and (J) 10.0% AX. Protein network is shaded white. Size: 212 × 212 μm (1024 × 1024 px).

finale dough and bread volume. On the other hand, the protein network seems to be sufficiently developed to hold back produced gas during fermentation and gives enough stability in the early stage of baking (oven rise). This assumption is confirmed by the analysis of Biliaderis et al. and Buksa et al., who showed that an addition of AX, especially water-soluble AX, up to 1% to wheat flour have a positive effect on the final bread volume (Biliaderis et al., 1995; Buksa et al., 2013). In the work of Delcour et al. (1991) an addition of 2.5% AX-Pentosan to the gluten–starch-flour leads to the highest bread volume. In contrary higher concentrations decrease the finale loaf volume. They suggest that the used protein–pentosan complex does act with the gluten and/or starch. They further explain that such an interaction could be one reason for a decrease of the volume loaf volume above certain concentrations. For commercial wheat flours a concentration of approx. 2.5% AX is naturally occurring content in wheat flours (AX: 2–3%). With increasing AX concentration (5.0% AX, micrograph C), the protein network formation starts to get inhibited. The proteins are irregularly distributed, less stretched and partly agglomerated. With AX concentrations of 7.5% (micrograph D) and 10.0% (micrograph E) no protein network formation is visible. One possibility for decreased protein network formation with increasing AX content could be the dilution of the dough with increasing water content which is necessary to adopt the dough firmness. As described by Jekle and Becker (2011), increased water content leads to more compact and aggregated proteins because of a decreased ability to stretch the protein aggregates with increasing water content. Furthermore, with an increasing AX concentration, no stretching of the proteins was detectable which could also be related to the increasing water content (Heddleson et al., 1994). Both, increased AX concentration and the associated increased water content especially at concentrations higher than 5.0% AX reflect a negative influence on protein development. This could be relevant for wheat flours with low extraction rates and therefore with high AX concentrations. Another theory of a decreasing protein network is that AX and proteins compete for water. However, because of the adjusted water content and the associated elimination of competition for water, this theory will not be pursued further in this work. It is also mentioned that the protein network formation is limited by the AX itself which enclose the proteins (Saulnier et al., 2007). These findings, mentioned previously in the literature is confirmed by the present micrographs (Fig. 2) visualizing for the first time: decreasing protein microstructure formation with increasing AX concentration. It is worth to mention, that the decreasing protein network formation is related to a decrease of total protein amount by increasing AX concentration. However, the change of total

Table 2
Protein microstructure values obtained by image analyses of CLSM micrographs effected by the addition of AX. Results are shown as mean ± standard deviation (n = 13). Values in the same column that are significantly different from the value with the next lower AX concentration are marked ($^{*}p \leqslant 0.05$).

AX conc. (%)		Wheat model dough			Rye model dough	
		Perimeter (μm)	Feret diameter (μm)		Perimeter (μm)	Feret diameter (μm)
0.0	(A)	128.66 ± 56.47	31.85 ± 15.04	(F)	44.96 ± 5.26	12.56 ± 1.44
2.5	(B)	76.66* ± 28.37	17.12* ± 6.58	(G)	45.62 ± 6.05	8.89* ± 1.22
5.0	(C)	59.39* ± 13.72	16.03 ± 3.60	(H)	33.56* ± 5.54	7.87* ± 0.96
7.5	(D)	53.30 ± 21.38	15.87 ± 5.19	(I)	18.06* ± 1.78	5.61* ± 0.42
10.0	(E)	54.61 ± 19.05	15.89 ± 5.26	(J)	14.38* ± 1.25	4.88* ± 0.40

protein content with respect to the AX content is very low (data not shown). Therefore, this influence will not be pursued further in this work.

For the rye model dough (Fig. 3), the same trend of the protein formation with increasing AX concentration was detectable. However, the wheat model dough and the rye model dough cannot be compared directly: First, the final dough firmness of the rye model dough was higher than for the wheat model dough; secondly, rye proteins do not form a protein network (gluten) such as wheat proteins because of the molecular structure of the rye proteins as described by Gellrich et al. (2004), Verwimp et al. (2007).

Fig. 3 shows the influence of an increasing AX concentration on the protein network formation (shaded white) in the rye model dough. In the CLSM micrograph F (0.0% AX), proteins are distributed and apparently connected to each other. Furthermore, the proteins are located around the starch granules (black holes). Although the rye model dough contains no AX, no stretched protein fibrils were detectable as described for the wheat model dough (Fig. 2; Micrograph A). This is due to the fact that rye proteins do not develop a protein network as extensive as wheat proteins (gluten). However, theoretically it should be possible to connect the rye proteins, because no substances such as AX are present that would prevent protein connection (Beck et al., 2011). Looking at graph G (2.5% AX) it is obvious that the proteins are more spread out and distributed. As described for the wheat model dough, an addition of 2.5% AX starts to disturb the protein interaction. Because of the finely distributed proteins, an addition of around 2.5% AX also seems to be optimal for the development of a possible protein network in rye dough. With increasing AX concentration (5.0% AX; graph H), the proteins get further distributed. A big change in the protein microstructure occurred in the concentration range of 5.0% AX (micrograph H) and 7.5% AX (micrograph I). Besides, in CLSM micrograph H the proteins are finely dispersed, no protein network is visible in CLSM micrograph I. The proteins are more agglomerated with more empty background (non-protein area). It is assumed that the dispersion of the proteins is limited by a formation of an AX network which encloses the proteins as described for the wheat dough. Therefore, the proteins are not able to disperse finely as described in micrograph F, leading to images with sparse protein distribution (more empty background). An AX concentration between 5.0% and 7.5% is the common amount existing in commercially available rye flours. Higher AX concentrations seem to have no further effect on the protein distribution on rye model dough.

Despite of the differences in the molecular structure of the wheat and rye proteins, the impact of the AX addition has a similar visual tendency for both protein network formations. For both, an AX addition of 2.5% AX seems to spread the protein network whereas a further addition up to 10.0% decreases the protein formation. In the rye model dough, especially micrographs H (5.0% AX) and I (7.5% AX) explains the low dough extensibility and gas holding characteristics of standard rye dough in contrast to standard wheat dough. As seen in graphs H and I, no protein network formation exists. Next to the hypothesis that AX build a network which disturbs the protein to develop a network, missing protein formation is also attributed to a dilution effect by water as described for the wheat model dough. The higher the AX content, the higher the dilution effect becomes, because of increased water absorption by AX (see Table 1). Especially for rye flours, which have a common AX concentration of approx. 7.5%, the dilution of rye dough by water influences the protein formation negatively and therefore the final dough and bread quality. To counteract this protein interacting barrier as well the dilution effect, the beneficial use of pentosanases, mainly xylanases, would then be exerted for modifying the AX network.

To gain quantitative information concerning the influence of different AX concentrations on the protein network formation of the wheat and rye model dough, the CLSM micrographs were analyzed using the image processing and analysis open source Java software ImageJ with the DoMiQ (Dough Microstructure Quantification) method. Table 2 shows perimeter and feret diameter for the wheat and rye model dough. As seen from the results (Table 2) in the wheat model dough, perimeter and feret diameter are decreasing with increasing AX concentration. Significant differences ($p \leqslant 0.05$) were obtained between 0.0% and 5.0% AX addition for the perimeter. The perimeter of the protein network decreases with increasing AX concentration. Between 7.5% and 10.0% AX no significant differences ($p \geqslant 0.05$) were obtained which leads to the conclusion that no protein network formation occur at AX concentrations higher than 7.5%. Significant differences ($p \leqslant 0.05$) were obtained for feret diameter between 0.0% and 2.5% AX. Between 5.0% and 10.0% AX no significant differences ($p \geqslant 0.05$) were obtained. The feret diameter does not change because of the agglomeration of the proteins. In the rye model, perimeter and feret diameter also decreased significantly; nevertheless, feret diameter values for the rye model dough are lower. This could also be related to the different molecular structure of the rye proteins as described earlier. However, from the results, especially from the values with low AX concentrations, it can be assumed that rye proteins have minor ability to form a network. This assumption is confirmed by the results of Field et al. who detected an aggregation of rye proteins (Field et al., 1983). The presented results, of these investigations, shows for the first time the influence of the AX content on the rheological behavior of wheat dough protein network formation and elaborate the process of protein formation in more detail.

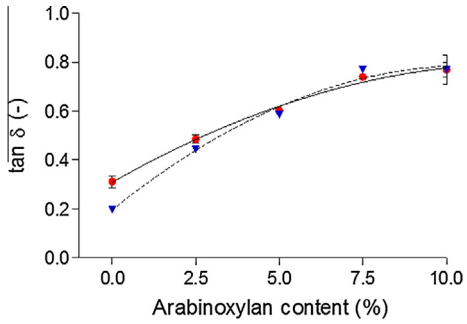
3.4. Fundamental rheological measurements

Oscillation measurements were performed in a nondestructive way to characterize and analyze the influence of AX on the protein microstructure formation in wheat and rye dough. All kind of model dough are adjusted to the same dough firmness by the variation of the water addition in relation to the dough firmness determined in the standard dough (wheat dough firmness $|G^{*}|$: 12,100 Pa; rye dough firmness $|G^{*}|$: 21,500 Pa).

To investigate the effect of different AX concentrations on the protein network, loss factor $\tan\delta$ and creep recovery compliance J_r are monitored. Table 3 summarizes the results of the rheological investigations for the different dough. For wheat model dough, the

Table 3
Values of the rheological analyses of the wheat and rye model dough. Results are shown as mean ± standard deviation ($n = 3$). Values In the same column that are significantly different from the value with the next lower AX concentration are marked ($^{*}p \leqslant 0.05$). Where tan δ is the loss factor and J_r is the creep recovery compliance.

AX conc. (%)	Wheat model dough		Rye model dough	
	Oscillation test tan δ (–)	Creep-recovery test J_r (1/kPa)	Oscillation test tan δ (–)	Creep-recovery test J_r (1/kPa)
0.0	0.31 ± 0.02	0.23 ± 0.11	0.20 ± 0.01	0.13 ± 0.05
2.5	0.49* ± 0.02	0.54* ± 0.08	0.44* ± 0.01	0.34* ± 0.09
5.0	0.60* ± 0.01	1.22* ± 0.26	0.58* ± 0.01	0.65* ± 0.07
7.5	0.74* ± 0.01	4.74* ± 0.28	0.77* ± 0.01	2.48* ± 0.18
10.0	0.77 ± 0.06	5.67 ± 0.77	0.77 ± 0.03	2.67 ± 0.37



addition of AX up to 10.0% increased tan δ by 148% and as well J_r increased ~25 times than the 0.0% AX model dough. In case of rye model dough, the addition of AX increased tan δ by 285% and J_r increased ~21 times than the 0.0% AX model dough.

Fig. 4 displays tan δ for wheat model dough as a function of AX addition. An addition up to 7.5% AX in the wheat model dough differs significantly ($p \leqslant 0.05$). No significant differences for tan δ were noticed between 7.5% and 10.0% AX addition. This leads to the conclusion that at concentrations higher than 7.5% AX no protein network formation occur. This conclusion is confirmed by the CLSM micrographs of the protein network as described above (Fig. 2; Micrograph D and E). Based upon the results: an equation of a second degree polynomial was observed with a goodness of fit of $R^2 = 0.95$. These results are in accordance with the results of the visual analysis as shown earlier. The fitting trend for tan δ as a function of AX concentration was expected from the micrographs' predictions as explained by the influence of AX on the protein network formation. From theory it was assumed that increasing AX concentrations reduces the elasticity of dough. However, a decrease of dough elasticity affected by AX addition occurs only as long as the proteins have the ability to form a network. If the concentration of AX are that high that the proteins cannot form a network, higher AX concentrations should not have a further effect on the dough elasticity. Therefore, an equation of a second order was expected for tan δ as a function of AX content. For the creep recovery test (J_r), also a second order equation was determined. The increasing compliance (J_r) can be explained by an increasing AX addition, two mechanisms seem to be plausible: First, AX can interact with each other by covalent and non-covalent crosslinks which disturb the interaction of the proteins (gluten) with each other whereby the gluten has lower extensibility and a lower rate of aggregation as described by Wang et al. (2003a); Another reason could be the dilution effect by water which is also related to the AX interaction that can absorb a lot of water (Izydorczyk and Biliaderis, 1995). Both theories are supported by the micrograph analysis results as discussed earlier.

For rye model dough, the same trends of rheological behavior with increasing AX concentration were detectable: with increasing AX concentration, the dough becomes less elastic (Fig. 4). An addition up to 7.5% has a significant influence ($p \leqslant 0.05$) on the viscoelastic behavior of the dough. The curve trend can be described by a second degree polynomial ($R^2 = 0.99$), which was expected as described above. The results cannot be directly compared because of the different dough firmness and the different molecular structures of the wheat and rye proteins. Tan δ increases in wheat model dough by 148% and in rye model dough by 285%. For the creep recovery test the same trend of the viscoelastic behavior was detected. In summary, increasing AX concentrations in model flours decreases the elastic properties of the resulting model dough of same firmness levels due to a decrease of the protein microstructure. Further, the protein network formation is decreased because of a dilution effect by water which is also related to the AX network, accordingly absorbs a lot of water. Therefore, in addition to the decreased ability of rye proteins to form a protein network as extensive as wheat proteins, high contents of AX influences the ability of wheat and rye proteins to form a protein network. This also explains the lower ability of standard rye flours to form a protein network because of the natively AX amount of 5–7%.

3.5. Correlation of CLSM micrograph results with rheological results

For wheat model dough (Table 4), strong linear correlations ($r = 0.969$) between AX concentration and the rheological behavior (tan δ) were reached. For the rheological behavior of the creep-recovery test, also a strong correlation coefficient between J_r and AX concentration was obtained for wheat model dough ($r = 0.916$). In other words, the elasticity (J_r) decreases with increasing AX concentration. For J_r and the perimeter of the proteins, a weaker negative correlation coefficient of $r = -0.417$ is shown. Therefore, increasing J_r resembles a decreasing elasticity, consequently protein perimeter values decrease affected by the increasing AX. This leads to the conclusion that an addition of AX to the flour has a direct (reduced protein interaction caused by AX) and an indirect effect (dilution effect caused through water absorption by AX). In this work only the combined effect can be seen, AX showing a negative effect on the elasticity of the dough.

In the case of rye model dough (Table 5), also high linear correlations between tan δ and AX concentration ($r = 0.961$) as well for J_r and AX ($r = 0.925$) were analyzed. For the visual values, strong

Table 4
Results of significant linear correlations between values of rheological analyses and micrograph analyses from wheat model dough ($^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$).

	AX conc.	Wheat model dough			
		Fundamental rheology		Micrograph analyses	
		tan δ	J_r	Diameter	Perimeter
AX conc.	–				
tan δ	0.969***	–			
J_r	0.916***	0.824***	–		
Diameter	−0.458***	−0.581*	−0.502*	–	
Perimeter	−0.575***	−0.761***	−0.417	0.899***	–

Table 5
Results of significant linear correlations between values of rheological analyses and micrograph analyses from rye model dough (*$p \leqslant 0.05$, **$p \leqslant 0.01$, ***$p \leqslant 0.001$).

	AX conc.	Rye model dough			
		Fundamental rheology		Micrograph analyses	
		$\tan\delta$	J_r	Diameter	Perimeter
AX conc.	–				
$\tan\delta$	0.961***	–			
J_r	0.925***	0.888***	–		
Diameter	−0.912***	−0.875***	−0.485	–	
Perimeter	−0.908***	−0.769***	−0.474	0.873***	–

linear correlations between AX concentrations and diameter ($r = -0.912$) as well perimeter ($r = -0.908$) was obtained. Additionally, weaker correlations ($r = -0.769$) were reached for the values of the fundamental rheology and the micrograph analyses.

Because of the high correlation between the AX concentration and the rheological results in addition to the visual values, the AX concentration plays a main role for the formation of the final protein network as discussed earlier.

4. Conclusion

In the present study, the impact of different AX concentrations on the protein microstructure of wheat and rye model dough is visualized. Visual analyses of the model dough with commercial wheat flour common AX concentrations of 2.5% show no negative effect concerning the protein formation. Already concentrations of 2.5% AX affect the protein network resulting in a greater spread and distribution of protein formation. This leads to a decreased elasticity of the dough which should have a positive effect on the final bread characteristics such as volume and crumb hardness (Delcour et al., 1991). Therefore, it is assumed that an addition of around 2.5% to a model flour or the natively existing concentration in commercial wheat flours are optimal for a well-developed protein network formation whereas concentrations higher than 5.0% show negative effects. From the present study, it can be concluded that AX concentrations higher than 5.0% prevent the protein formation. In the case of rye model dough, no protein microstructure was detectable at AX concentrations between 5.0% and 10.0%, which is the natively existing concentration range in commercial rye flour. Nearly the same protein microstructure was detectable for wheat model dough with AX concentrations between 5.0% and 10.0%. The visual analyses were supported by rheological findings. With increasing concentration of AX up to 10.0% AX (wheat model flour replaced by AX), a decrease in $\tan\delta$ of the wheat model dough of 148% was reported. For rye dough, $\tan\delta$ increased by 285%. Furthermore, the addition of AX is strongly correlated to the rheological and visual analyses. In summary, the study confirmed the influence of different AX concentration on rheological and visual attributes of rye and wheat protein microstructure.

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2.5 ON THE ASSESSMENT OF ARABINOXYLAN LOCALISAZATION AND ENZYMATIC MODIFICATION FOR ENHANCED PROTEIN NETWORKING AND ITS STRUCTURAL IMPACT ON RYE DOUGH AND BREAD

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On the assessments of arabinoxylan localization and enzymatic modifications for enhanced protein networking and its structural impact on rye dough and bread

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ABSTRACT

For rye dough structure, it is hypothesised that the presence of arabinoxylan hinders the proteins from forming a coherent network. This hypothesis was investigated using fluorescent-stained antibodies that bind to the arabinoxylan chains. Image analysis proves that the arabinoxylan surrounds the proteins, negatively affecting protein networking. Further, it is hypothesised that the dosing of xylanase and transglutaminase has a positive impact on rye dough and bread characteristics; the findings in this study evidenced that this increases the protein network by up to 38% accompanied by a higher volume rise of 10.67%, compared to standard rye dough. These outcomes combine a product-oriented and physiochemical design of a recipe, targeting structural and functional relationships, and demonstrate a successful methodology for enhancing rye bread quality.

1. Introduction

Rye products have been widely used in human foods due to dietary benefits of different non-starch polysaccharides such as arabinoxylan (AX). Nevertheless, rye-baked products are limited due to the inability of rye flour to form a viscoelastic protein network that can retain gas, such as that found in the gluten network of wheat dough (Courtin & Delcour, 2002). Despite the ability of rye proteins to slightly interact during dough mixing to form a protein network, rye proteins can connect to each other through transglutaminase (TG, EC 2.3.2.13). TG is reported to cross-link high-molecular-weight glutenins into large insoluble protein polymers.

Several studies have shown that TG increases the elasticity, water-holding capability and other functional properties of baked products (Gujral & Rosell, 2004; Tang, Chen, Li, & Yang, 2006; Truong, Clare, Catignani, & Swaisgood, 2004). For rye dough and bread, the addition of TG results in an improved rheological behaviour and increased bread volume. Specifically, the addition of TG (0–1000 U/kg flour) decreased the loss factor $\tan\delta$ ($\tan\delta = G''/G'$) by approx. 19% and increased the bread volume by approx. 6%, technically considered improved behaviour (Beck, Jekle, Selmair, Koehler, & Becker, 2011). Despite the abovementioned positive effects of TG addition on rye dough and bread characteristics, AX limits protein networking (Beck et al., 2011).

Although AX is identified as the major structure-forming component of rye dough, several publications describe its negative influence on the protein network formation of wheat and rye dough (Döring, Nuber, Stukenborg, Jekle, & Becker, 2015; Wang, Rosell, & Benedito de Barber, 2002). AX classified into water-extractable arabinoxylan (WEAX) and water-unextractable arabinoxylan (WUAX) are described to absorb many times their weight of water and influence the properties of the dough and bread characteristics such as viscoelasticity of the dough, bread volume and crumb firmness (Buksa, 2016; Buksa, Nowotna, & Ziobro, 2016; Ma, Xu, Xu, & Guo, 2012). Further, AX in especially high concentrations, such as in the case of rye flour (5–8% AX), has been reported to enclose the proteins, thereby preventing protein interaction (Beck et al., 2011; Döring et al., 2015).

To minimise the limitations of AX on protein networking and bread characteristics, AX-hydrolysing enzymes can be added. The

Abbreviations: AX, arabinoxylan; RH, relative humidity; TG, transglutaminase; Xyl, xylanase; FITC, fluorescein isothiocyanate.

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most commonly used enzyme to improve processing and final characteristics of wheat and rye products is endoxylanase (Xyl). Endoxylanase (1,4-β-xylan xylohydrolase, EC 3.2.1.8) hydrolyses glycosidic bonds within the xylan, resulting in a decrease in the degree of polymerization of the polysaccharides (Sunna & Antranikian, 1997). In general, the addition of Xyl leads to improved dough-processing properties and bread characteristics (increased bread volume, softer crumb) (Courtin, Gelders, & Delcour, 2001). In contrast, an overdose of Xyl leads to reduced dough and bread characteristics because AX degrades into oligosaccharides (Courtin & Delcour, 2002). In summary, based upon literature findings, each enzyme (TG and Xyl), depending on its activity, has a positive effect on wheat dough and bread characteristics (Roccia, Ribotta, Ferrero, Pérez, & León, 2012; Schoenlechner, Szatmari, Bagdi, & Tömösközi, 2013).

The mentioned findings in literature lead to the hypothesis that a synergistic effect should occur, improving rye dough and bread characteristics by combined addition of both enzymes (TG and Xyl) in certain concentrations.

Therefore, the aim of the present study is twofold:

First, to identify whether AX covers rye proteins, thereby possibly preventing rye–protein interaction. In order to achieve this aim, model rye dough with a specific AX concentration is produced and an antibody dye solution stains the AX. Confocal laser scanning microscopy (CLSM) images are captured to trace the location of the AX, and the images are analysed using image processing.

Secondly, to prove that the addition of Xyl increases the accessibility of TG to rye proteins, thus enhancing rye–protein connectivity. As such, rye dough and bread are produced from commercial rye flour, modified by different concentrations of Xyl and TG and examined with respect to protein formation (CLSM), rheological properties (rheometer), bread volume and crumb hardness. All doughs are adjusted to the same dough firmness by water calibration to minimise the influence of different water concentrations on the dough and bread results.

2. Materials and methods

2.1. Raw materials and chemical composition

For model rye dough preparation, rye starch and rye protein were acquired from Lyckeby Culinar AB (Kristianstad, Sweden). Rye starch and protein isolation was performed according to the procedure of Andersson, Andersson, and Åman (2001) with the following modifications: rye flour was suspended in distilled water to 15% dry matter. Afterwards, fiber particles were removed by a centrifugal fiber screener (mesh screen net of 60 μm). To separate the starch from proteins and solubilized pentosans without peptization of the starch granules, the pH of the suspension was adjusted to 11.5 with 0.075 M NaOH. After extraction, undissolved material was settled by running the suspension in a centrifuge (3000*g*) for 6 min. The supernatant was siphoned to a tank and saved for later protein fractionation. The obtained starch was suspended in distilled water and centrifuged two times. Afterwards, the starch was suspended in distilled water, adjusted to pH 6 by addition of HCl (1 M), filtered and gently dried to a moisture content of 13%. Starch content and starch damage were determined according to AACCI methods 76-13.01 and 76-33.01, and found to be 92.63 ± 3.00% (dwb) and 4.17 ± 0.02%, respectively. For rye–protein isolation, the protein fraction obtained during the starch separation process was neutralized to the isoelectric point of pH 4.2–4.3 by addition of HCl (1 M). To initiate protein flocculation, 1% of $CaCl_2$ was added. Afterwards, the flocculated proteins were recovered by centrifugation (3000*g*, 10 min). The proteins were further suspended in distilled water and adjusted to pH 6 with a NaOH solution (1 M). Afterwards, the suspension was carefully spray-dried to avoid heat coagulation of the proteins. Protein contents (Nx5.7) of rye protein and rye starch determined by Kjeldahl method (EBC) (Anger, 2006) were found to be 61.20 ± 0.29% and 0.10 ± 0.00%, respectively. Rye AX was acquired from Megazymes Incorporation (Wicklow, Ireland) with a purity of ~90% (dwb). Additional components of the rye AX sample were starch (0.2%), β-glucan (0.1%), protein (2.5%) and ash (4.0%). The ratio of arabinose to xylose was 38:62. Peak molecular weight (g/mol), weight-averaged molecular weight, number-averaged molecular weight, intrinsic viscosity (dL/g), radius of gyration (nm) and the polydispersity index were 303,000 ± 1370 g/mol, 440,000 ± 5810, 218,000 ± 2920, 4.55 ± 0.03 dL/g, 42.52 ± 2.20 nm and 2.02 ± 0.00, respectively. All of the above data for AX were specified by Megazymes Inc. (Wicklow, Ireland).

Standard rye dough preparation was performed as follows. Rye flour (type 1150) was acquired from Rosenmühle GmbH (Ergolding, Germany). Starch content and starch damage determined according to AACCI methods 76-13.01 and 76-33.01 were 72.14 ± 1.21 g/100 g (dwb) and 4.23 ± 0.27% (dwb), respectively. The protein content (Nx5.7) determined by the Kjeldahl method (EBC) (Anger, 2006) was found to be 8.37 ± 0.27% (dwb). Referring to the measurements of Grossmann, Döring, Jekle, Becker, and Koehler (2016), contents of the protein fractions albumins/globulins, prolamins, glutelins and residual proteins were 2.0 ± 0.0, 3.3 ± 0.0, 1.1 ± 0.2 and 3.1 ± 0.0 g/100 g flour, respectively. WEAX and total AX content were determined according to Kiszonas, Courtin, and Morris (2012) and found to be 1.37 ± 0.42% and 5.73 ± 0.52%, respectively. Further ingredients for the rye dough preparations included distilled water, lactic acid (purity ~90%; AppliChem GmbH, Darmstadt, Germany), NaCl (Südsalz GmbH, Bad Friedrichshall, Germany) and dry yeast (*S. cerevisiae*) (Uniform GmbH & Co. KG, Werne, Germany). Transglutaminase Veron TG (Streptoverticillium sp.) and xylanase Veron RL (endo-1, 4-Xylanase derived from Bacillus subtilis) were provided by AB Enzymes (Darmstadt, Germany). Transglutaminase activity (354.3 ± 6.1 U/g powder; 1 U = 1 μmol of hydroxamate/min; pH 4.2; 30 °C) was determined following the procedure of Folk and Cole (1966). Xylanase activity (12.2 ± 0.2 U/mg; pH 4.2; 30 °C) was determined according to Bailey, Biely, and Poutanen (1992). Referring to the measurements of Grossmann et al. (2016), no further activities were detected for the Xyl-based quantifications of xylosidase, arabinofuranosidase, feruloyl esterase and α-amylase activity.

2.2. Dough preparation

Dough preparation, with respect to all baking, rheometer and CLSM analyses, was adjusted to the same dough firmness to minimise the influence of different water concentrations on the dough and bread results. A commercial rye dough (flour type 1150) was prepared in a Z-Blade mixer and adjusted to a dough consistency of 300 BU according to AACCI method 54-70.01. Afterwards, the dough was measured in the rheometer and the complex shear modulus (G^*) was determined. G^* describes the vectorial sum of G' and G'', thus representing the dough firmness, and was calculated using the following equation:

$$G^* = \sqrt{(G')^2 + (G'')^2} \quad (1)$$

where G'' describes the loss modulus and G' denotes the storage modulus. The determined dough firmness of the commercial rye dough determined by the rheometer and expressed as G^* was 21,500 Pa. All further doughs were adjusted to the same dough firmness of 21,500 Pa by water calibration. The temperature of the geometry of the rheometer was set to 30 °C for the purpose of

simulating proofing room conditions. After placing 3 g of dough between the plates of the rheometer, the gap for measurements was adjusted to 2000 μm. A razor blade carefully removed excess dough between the plate-to-plate geometry. The margin of the dough was sealed with paraffin oil to protect the dough from drying. After a relaxation time of 10 min, an oscillation test was carried out. The applied frequency of the oscillation test was set to 1.0 Hz and the deformation to 0.075%, previously determined by the deformation test. The applied deformation was well within the linear viscoelastic region of the samples; the testing time for determining G^* was 60 s.

For performing CLSM analysis, rye starch (4.08 g), rye protein (0.34 g), rye AX (0.33 g) and NaCl (0.9 g) were weighed into a sealed Glutomatic bowl and homogenised using a spatula. Water (3.61 g) was added to adjust a final dough firmness of 21,500 Pa. After 20 s of mixing, the distance between the bowl and the mixing hook was increased to 80 mm using a metal spacer to reduce the energy intake. The total mixing time was set to 4 min and 50 s.

Rheology measurements were carried out as follows. Rye flour type 1150 (50.0 g; corrected to 14% moisture), NaCl (0.75 g), distilled water (adjusted for dough firmness G^* = 21,500 Pa) and lactic acid (adjusted to a pH = 4.2) were mixed in a Z-Blade mixer (50 g). The mixing time was set to 4 min at 63 rpm at 25 °C. To modify the AX and protein fraction of the rye dough, different concentrations and combinations of TG (0.0, 1.0, 3.0 and 5.0 g enzyme/kg flour) and/or Xyl (0.0, 0.1, 0.3 and 0.5 g enzyme/kg flour) were added to the flour before mixing.

Baking analyses were performed using 720.00 g of rye flour (corrected to 14% moisture), 10.80 g NaCl, 7.20 g dry yeast, distilled water (adjusting to dough firmness of 21,500 Pa), different concentrations and combinations of TG (0.0; 1.0, 3.0 and 5.0 g enzyme/kg flour) and/or Xyl (0.0, 0.1, 0.3 and 0.5 g enzyme/kg flour). The ingredients were mixed at 25 °C for 4 min at 100 rpm and 1 min at 200 rpm in a laboratory mixing machine (DIOSNA, Dierks & Söhne GmbH, Osnabrück, Germany). After kneading, the dough was divided into 300g pieces, hand-molded, placed into backing tins (height/width/depth 11.0 cm/7.0 cm/8.0 cm) and proofed at 30 °C with 80% RH for 50 min. The dough was baked with an initial steam injection of 0.5 L at 230 °C (top heat) and 200 °C (bottom heat), falling to 200 °C (top and bottom heat) in a rack oven (Matador store, Werner & Pfleiderer, Germany) for 55 min.

2.3. Rheological settings and analyses

The rheological measurements were performed on an AR G2 Rheometer (TA Instruments-Waters LLC, New Castle, USA) using a flat plate-to-plate geometry (d = 40 mm). The rheometer settings were the same as above, except that the relaxation time was increased to 10 min and the testing time was set to 50 min. The rheological measurements of enzyme-treated dough, shown in Fig. 2, were considered at the end of the proofing time set after 50 min (10 min relaxation time + 40 min analysis time). The measurements were always performed in triplicate.

2.4. Microscope settings and analyses

For a visual analysis of the AX and protein fractions, model rye dough was prepared with 7.5% AX (0.33 g), gently cut from the main dough and transferred to a specimen shape (diameter 18.0 mm, height 0.8 mm). A polyclonal antibody-FITC solution for specific staining of AX, acquired from Coring System Diagnostix GmbH (Gernsheim, Germany), was diluted with bovine serum albumin (BSA) buffer in a ratio of 1:10. Afterwards, 20 μL of the AX antibody suspension was added to the top of the sample dough and stored at 30 °C with 80% RH for 30 min. Afterwards, non-bound antibodies were removed by washing the dough surface carefully with the BSA buffer. Next, 40 μL of fluorescent dye Rhodamine B (c = 0.001 g/100 mL, from Sigma-Aldrich Chemie GmbH Munich, Germany) was added on top of the sample dough to visualise the protein fractions. After one minute, the specimen shape was covered with a slip, and after a second storage time of 10 min, the dough was analysed. The AX and protein microstructure of the model dough was visualised with a CLSM from Nikon GmbH (Düsseldorf, Germany) with a 60× objective lens. Fluorescence micrographs (λexc = 488 nm, λem = 515/30 nm) with 1024 × 1024 pixel resolution (212 × 212 μm) were used for monitoring the AX in a constant Z-position. Proteins were monitored in fluorescence micrographs (λexc = 543 nm, λem = 590/50 nm) with 1024 × 1024 pixel resolution (212 × 212 μm) in a constant Z-position.

Commercial rye dough was gently cut from the main dough with a scissor and transferred to a specimen shape (diameter 18.0 mm, height 0.8 mm) for the visual analysis of protein fractions. The proteins were stained with Rhodamine B and monitored with a 20× objective lens in fluorescence micrographs (λexc = 543 nm, λem = 590/50 nm) with 1024 × 1024 pixel resolution (686 × 686 μm) in a constant Z-position. Five independent micrographs on the XY-axis were captured for each dough sample. Measurements performed on three dough samples; image analyses were performed using in-house developed software (ImagePro V 0.7) (Hussein, Hussein, & Becker, 2012).

2.5. Baking analyses

After baking, the final bread was cooled to room temperature (2 h) before monitoring weight, volume and crumb hardness. Volume measurements were performed using a laser-based volumeter BVM-L370 (TexVol Instruments AB, Viken, Sweden). For checking the accuracy of the laser-based volume measurements, spot checks of the bread volume were conducted using the rapeseed method according to AACCI Method 10-05.01. The results obtained by the volumeter matched the results of the rapseed method. Bread weight was calculated using a laboratory balance (Kern QKE 8K005, Kern & Sohn, Balingen-Frommern, Germany). The density was calculated using the ratio of bread weight to bread volume (g/mL). Three replicates from three sets of baked samples were analysed and averaged. Crumb hardness was analysed using a TVT-300 XP Texture Analyser (TexVol Instruments AB, Viken, Sweden) following AACC Method 74-10.02. Three replicates from three sets of baked samples were analysed and averaged.

2.6. Sensitivity analysis

The effects of simultaneous enzyme addition (Xyl and TG) were estimated using sensitivity analysis. Sensitivity analysis is a well-known mathematical methodology that expresses the dependency of the outputs (observations) of a system on the inputs (parameter variations). This method is essential for exploring a multidimensional complex space. In this space, several inputs and outputs are present in the absence or minor understanding of the relationships between the input and output (Cacuci, 2003). One of the simple forms of sensitivity analysis is partial derivative-based one-factor-at-a-time (OAT) (Czitrom, 1999). Typically, this is achieved using the sensitivity coefficient (SC), a derivative of an output variable (y) with respect to an input parameter (x), as shown in Eq. (2).

$$SC = \frac{\partial y}{\partial x} \tag{2}$$

The output parameters (y) are the measurements taken for the estimation of the dough and bread properties such as G″ (loss modulus), G′ (storage modulus) for dough and the bread volume (V),

bread density (ρ) and crumb hardness (H). The input parameters are the Xyl (x_{Xyl}) and TG (x_{TG}) concentrations. The coefficients are calculated with partial dependence on one enzyme while keeping the other constant; this way both effects are considered in a simplified manner, as shown in Eq. (3).

$$SC_{Xyl} = \frac{\partial y}{\partial x_{Xyl}}|_{TG=const}, SC_{TG} = \frac{\partial y}{\partial x_{TG}}|_{Xyl=const} \quad (3)$$

Defining an SC > 0.5 and an SC < −0.5 indicates higher proportional and inverse dependence, respectively, of the output parameter with respect to the input parameter, in this case the enzyme concentration. All sensitivity calculations were conducted at the end of the proofing time and normalized to the rye dough at zero enzyme concentrations.

2.7. Image analysis

CLSM opens the possibility of structure-function investigations; several approaches have been developed in the medical sector for neuronal protein investigations (Dinh, Jimenez, Papadakis, Laezza, & Labate, 2015; Ozcan, Negi, Laezza, Papadakis, & Labate, 2015). These methods are based on thresholding the protein branches combined with a de-noising filter (anisotropic), thus not affecting the informative protein-edge-carrying pixels. The exact algorithm implemented in this study, as illustrated in Supplementary Fig. S1 where the protein branch (trace curve C) emerges from a rye protein, is parametrized as a function of the colour intensity distribution. The edges of the protein branches are calculated by the second derivative of the colour distribution perpendicular to the branch direction in a rectangular window aligned with the protein branch; the window scans all angular possibilities emerging from the rye protein until the next branch is found. This algorithm (implemented in ImagePro V 0.7) is used for all CLSM protein network investigations of different doughs with various xylanase and transglutaminase concentrations. The software reports these different protein network properties:

- Network percentage area (NPA): area covered by a protein network as a percentage of the analysed image.
- Average network length (ANL): the average length of the emerging protein branches.

3. Results and discussion

3.1. Evidence of protein enclosure by AX via dye-labelled antibodies

Although the techniques of structural analyses and identification of AX by dye-labelled antibodies or inactivated xylanases are known, no measurements concerning the localization of AX in rye dough exist (Dornez, Cuyvers et al., 2011; Emmie Dornez, Holopainen, Cuyvers, Poutanen, Delcour, Courtin, et al., 2011; Guillon et al., 2004; McCartney, Marcus, & Knox, 2005). Given the advantages of antibodies labelled with fluorescent dyes over commercial fluorescent dyes for structural identification, this technique has been proven to be optimal for determining if AX covers proteins, thereby preventing protein interaction. Accordingly, FITC-labelled antibodies against AX (generated by the Coring System Diagnostix GmbH) were used for localization of AX in rye dough.

Fig. 1 shows an example of a rye dough probe in which localization of proteins (Fig. 1A) and AX (Fig. 1B) by Rhodamine B and FITC-labelled antibodies is indicated, respectively. Indicated by black edges, the AX structure was determined by image processing (Fig. 1C). Differences in the intensity of brightness of the detected AX depends on the concentration and accessibility of antibodies to AX, as described by Dornez et al. (2011). The differences in brightness were also used to differentiate between AX on protein networks (high brightness = high antibody concentration), as seen in Fig. 1B and 1C, and AX dissolved in the dough dispersion (low brightness = low antibody concentration), which were removed by filters. This indicates that AX is located on the proteins and distributed throughout the dough. Differences in the perimeters of the detected AX cycles, as shown in Fig. 1C, depend on the protein size itself or the spatial conformation of the proteins. Therefore, the perimeter varies with the position of the focal plane (Z-axis) where the images are taken. The detected AX that does not form a connected contour is also located next to the proteins. In this case, only a part of the protein is covered by AX. The mechanism of protein covering by AX can be described by the findings of Oudgenoeg et al. (2001), who determined that AX interacts with proteins via ferulic acid. Therefore, the obtained results confirm the hypothesis that AX covers proteins. For further examination, model dough with an excess of Xyl (0.5 g Xyl/kg flour) was prepared and analysed to determine if the addition of Xyl shows a reduction in AX. As seen in Fig. 1D and E, a decrease in protein enclosure by AX occurred from adding excess Xyl. These results are confirmed by processing of the CLSM images (Fig. 1E). In contrast, to Fig. 1B, after Xyl treatment, the dough in Fig. 1E shows more dispersed AX (higher background signal of the AX). This means that the AX has not degraded to its sugar monomers but modification of the protein-bound AX has occured. Further, the proteins are not totally free of AX (Fig. 1E). The addition of excess Xyl alone does not result in increased protein linkage, as seen in Fig. 1D. From these results, it can be concluded that AX encloses the rye proteins. In the scope of this work, only one type of Xyl was used; however, the chemical structure of the raw materials (isolated starch, protein and AX) used in the experiments described in Section 3.1 could be different from those in the commercial rye flour used in the experiments below, due to the extraction procedure, as described by Buksa et al. (2016). Therefore, further work is needed to elaborate if different AX-degrading enzymes (ferulic acid esterase and arabinofuranosidase) can cause further degradation. Additionally, the behaviour of commercial rye flours at different AX concentrations should be analysed to confirm the achieved results and to predict the rheological and technological behaviours of these flours. Based on these findings, the question now is how AX hydrolysis can influence the protein networks.

3.2. Synergistic effect of xylanase and transglutaminase addition on rheological properties

The synergistic effect of Xyl and TG addition was investigated to determine if the combination of AX degrading Xyl and the cross-link promoting TG will enhance the protein networking of rye dough. For that purpose, rheological measurements were performed to examine the response of the storage modulus (G′) and loss modulus (G″) with combined enzyme addition. As described by Caballero, Gómez, and Rosell (2007), it is expected that the addition of TG leads to a significant change in G′ and G″, but no information on the dosing limitations was reported.

In the present work, Xyl (0.0, 0.1, 0.3, 0.5 g/kg flour) was simultaneously tested with the addition of TG (0.0, 1.0, 3.0, 5.0 g/kg). In Fig. 2, G′ is plotted against G″ for both enzyme concentrations. Additionally, regions of low (0.0–1.0 g/kg flour), middle (1.0–3.0 g/kg flour) and high (3.0–5.0 g/kg flour) TG concentrations are clustered with arrowed lines, and constant Xyl concentrations (0.0, 0.1, 0.3, 0.5 g/kg flour) are marked as dotted lines. Following a certain Xyl concentration dotted line through the different regions of TG levels, one can explore the synergistic effect of Xyl and TG on the rheological properties (G′ and G″).

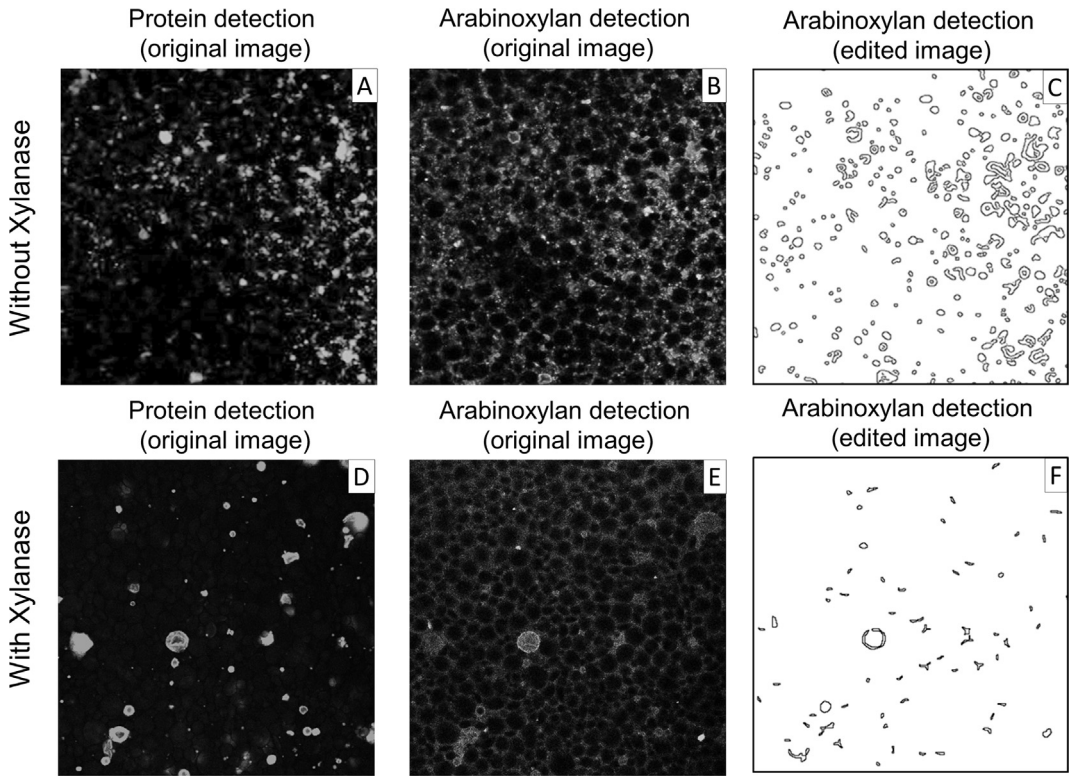

Fig. 1. Location of detected AX fractions in model rye dough (7.5% AX) by fluorescein isothiocyanate (FITC)-labelled AX antibodies. A: Protein visualisation via Rhodamine B. B: AX visualisation via FITC-labelled AX antibodies. C: Detected location of AX structure after image analysis. D: Protein visualisation via Rhodamine B with Xyl treatment. E: AX visualisation via FITC-labelled AX antibodies with Xyl treatment. F: Detected location of AX structure with Xyl treatment (image analysis). Size: 212 × 212 μm (1024 × 1024 pixels).

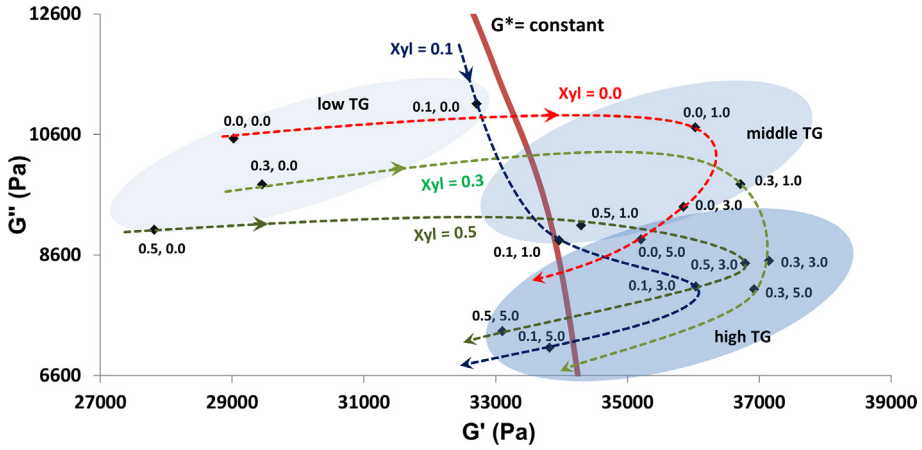

Fig. 2. Effect of different transglutaminase and xylanase concentrations on rye dough rheology shown as storage modulus (G′) vs. loss modulus (G″). Regions of low (0.0 g/kg flour), middle (1.0–3.0 g/kg flour) and high (3.0–5.0 g/kg flour) effects of transglutaminase on rye dough rheology are shown with arrowed dashed lines of constant xylanase concentrations (0.0, 0.1, 0.3, 0.5 g/kg flour). By following a certain xylanase concentration (dotted line) through different regions of transglutaminase levels, one can explore the synergistic effect of xylanase and transglutaminase on the storage modulus (G′) and loss modulus (G″). Different enzymes' concentrations are labelled near the scattered points, with the first digit indicating the xylanase concentration (g/kg flour) and the digit after the comma indicating the transglutaminase concentration (g/kg flour).

The addition of TG led to a significant decrease in the G″ of the dough. The explanation of this decline can be explained by the increased water binding of the protein interacting with increased TG concentration at the same dough consistency (G*). Therefore, the loss modulus (G″) decreases, whereas the storage modulus (G′) is nearly constant. In other words, if the dough consistency (G*) is not fixed to a constant value, G′ would increase due to a stiffer dough caused by increased water binding from improved protein interaction. These results are with the findings of Larré et al. (1998), who analysed an increase in G′ and G″ of gluten gel by 10–50 times and 2–4 times, respectively, by TG addition. The increased cross-linking effect between glutamine and lysine residues of rye proteins is further confirmed in an another report (Autio et al., 2005). The main difference in the data can be seen by following the dotted lines of Xyl = 0.3 g/kg flour or Xyl = 0.5 g/kg flour, and regardless of exceeding the 0.1 g/kg flour Xyl, the dough elasticity (G′) increases with increasing TG concentration up to 3.0 g/kg flour. This is because the water freed by Xyl is rebound by the increased protein interaction caused by TG. Therefore, depending on the final dough consistency (G*) and the

reduced amount of free water from increased protein interaction, the dough becomes more elastic (G′) at the same dough consistency (G*). Nevertheless, the rise in G′ rebounds as soon as the TG level exceeds 3.0 g/kg flour, indicating that this is the maximum dosage of TG to achieve the highest elasticity (G′) and lowest viscosity (G″). The decreased storage modulus (G′) at concentrations greater than 3.0 g/kg flour can be explained by an increased average molecular weight of the protein matrix. As described by Beck et al. (2011), high TG concentrations lead to strongly aggregated protein particles. Strongly aggregated protein particles are assumed to lead to a decrease in G′ because the continuous protein network is reduced.

From another perspective, following the trends shows that when the Xyl levels increase above 0.1 g/kg flour, the viscous term (G″) drops. This can be explained by the synergistic enzymes' activity; increasing the elasticity and more water bound to the network, i.e. enhanced water-holding capability (Gujral & Rosell, 2004; Tang et al., 2006; Truong et al., 2004).

Furthermore, performing a sensitivity analysis using Eq. (3) quantifies the findings. Thus, the SC was calculated for G′ and G″ with variations of xylanase and transglutaminase concentrations following the methodology of OAT, as mentioned in Section 2.6.

Table 1a summarises the calculations of SC, reflecting the effect of enzyme addition on the strength of change in G′ or G″. The first part of Table 1a represents investigation of the Xyl concentration factor while keeping the TG at fixed levels. Stepwise addition of Xyl from 0.0 → 0.1 g/kg is seen to have a strong positive influence (expressed by an SC value of >0.5) on G′ for all concentrations of TG in the range 0.0–5.0 g TG/kg flour. Consequently, the G′ shown in Fig. 2 on the x-axis increased strongly in comparison to the untreated dough, as seen by following the corresponding dotted line (0.1 g Xyl/kg, 0.0 g TG/kg to 0.1 g Xyl/kg, 5.0 g TG/kg).

In the second step, the Xyl concentration is increased (0.0 → 0.3 g/kg flour) and the positive influence on G′ becomes weaker, but is still positive (0.4 < SC < 0.5) and limited to TG concentrations ranging from 1.0 to 5.0 g/kg flour. Further addition of Xyl to a concentration level of 0.5 g/kg restricts the positive effect (negligible effect −0.3 < SC < 0.3) on G′ for all TG concentrations. Fig. 2 is in agreement with this, as seen by following the dotted line 0.5, 0.0 → 0.5, 5.0; the addition of 1.0 g TG/kg flour increases G′ substantially. Further addition (3.0 g TG/kg flour) increases G′, but in comparison to the lower concentration, is less than half the increase; therefore, the addition of Xyl alone has a small increasing effect on G′ (SC = 0.64). The addition of TG leads to a greater effect on G′ (SC = 1.21) than Xyl, but both together leads to the highest increase in G′. This is proven by the SC, showing that G′ is more sensitive to increasing TG (SC = 1.33 at constant Xyl = 0.3 g/kg and rising TG up to 1.0 g/kg) than increasing Xyl (SC = 0.44 at constant TG = 1.0 g/kg and rising Xyl up to 0.3 g/kg). This also shows that TG has a greater effect on G′ than Xyl on its own. Additionally, Xyl and TG decrease G″; however, this effect is also increased by the addition of both enzymes together, as seen in Fig. 2, especially at concentrations of 0.1 g Xyl/kg and 1.0 g TG/kg, which have a strong negative SC of −0.8. On the right side of the table, the effect on G″ is calculated. Xyl addition for all TG concentrations, except at 0.1 g Xyl/kg flour and 0.0 g TG/kg flour, show an opposite effect on G″. The SC is strongly negative in ranges where the addition of Xyl is 0.0 → 0.1 g/kg flour at TG concentrations of 0.0–5.0 g/kg flour. Further addition of Xyl 0.0 → 0.3 g/kg flour shows only a considerable negative SC at a TG concentration of 5.0 g/kg flour. More enzyme addition shows negligible adverse effects (−0.3 < SC < 0.3), therefore leading to the conclusion that Xyl and TG decrease G″. This effect also increases by the addition of both enzymes together, as seen in Fig. 2.

In the second part of Table 1a, the contribution from a gradual addition of TG to changes in G′ and G″ is reported. Regarding G′, the

Table 1a
Sensitivity analysis calculated based on Eq. (3) for the synergetic addition of xylanase and transglutaminase on the final response of storage modulus (G′) and loss modulus (G″) with a maximum of std ± 0.09 for G′ and std ± 0.071 for G″. **1b:** SC calculated on the protein networking properties: Network percentage area (NPA), average network length (ANL) with a maximum of std ± 0.11 for NPA and std ± 0.08 for ANL. **1c:** Sensitivity analysis of the final response of bread measured by means of volume (∀), hardness (H) and density (ρ) with a maximum std of ± 0.03, ± 0.016 and ± 0.035, respectively.

1a	G′-Xyl $\frac{\partial G'}{\partial x_{Xyl}}\|_{TG=Const}$				G″-Xyl $\frac{\partial G''}{\partial x_{Xyl}}\|_{TG=Const}$			
g Xyl /kg flour	g TG /kg flour							
	0.0	**1.0**	**3.0**	**5.0**	**0.0**	**1.0**	**3.0**	**5.0**
0.0-0.1	0.64 ⇧	0.85 ⇧	1.21 ⇧	0.83 ⇧	0.27 ▫	-0.80 ⇩	-1.17 ⇩	-1.65 ⇩
0.0-0.3	0.03 ▫	0.44 ▫	0.47 ▫	0.45 ▫	-0.12 ▫	-0.12 ▫	-0.32 ▫	-0.40 ▫
0.0-0.5	-0.04 ▫	0.18 ▫	0.27 ▫	0.14 ▫	-0.14 ▫	-0.14 ▫	-0.20 ▫	-0.30 ▫
	G′-TG $\frac{\partial G'}{\partial x_{TG}}\|_{Xyl=Const}$			G″-TG $\frac{\partial G''}{\partial x_{TG}}\|_{Xyl=Const}$				
g Xyl /kg flour	g TG /kg flour							
	0.0-1.0	**0.0-3.0**	**0.0-5.0**	**0.0-1.0**	**0.0-3.0**	**0.0-5.0**		
0.0	1.21 ⇧	0.39 ▫	0.21 ▫	0.09 ▫	-0.18 ▫	-0.16 ▫		
0.1	0.85 ⇧	0.40 ▫	0.17 ▫	-0.80 ⇩	-0.39 ▫	-0.33 ▫		
0.3	1.33 ⇧	0.47 ▫	0.27 ▫	-0.36 ▫	-0.32 ▫	-0.24 ▫		
0.5	0.91 ⇧	0.45 ▫	0.14 ▫	-0.68 ⇩	-0.33 ▫	-0.30 ▫		

1b	NPA-Xyl $\frac{\partial NPA}{\partial x_{Xyl}}\|_{TG=Const}$				ANL-Xyl $\frac{\partial ANL}{\partial x_{Xyl}}\|_{TG=Const}$			
g Xyl/kg flour	g TG/kg flour							
	0.0	**1.0**	**3.0**	**5.0**	**0.0**	**1.0**	**3.0**	**5.0**
0.0-0.1	0.62 ⇧	1.04 ⇧	7.45 ⇧	3.2 ⇧	-0.36 ▫	-0.27 ▫	6.37 ⇧	0.73 ⇧
0.0-0.3	0.46 ▫	0.86 ⇧	1.46 ⇧	1.3 ⇧	0.03 ▫	0.48 ▫	0.48 ⇧	0.55 ▫
0.0-0.5	0.24 ▫	0.21 ▫	0.19 ▫	0.0 ▫	0.13 ▫	0.21 ▫	0.18 ▫	0.12 ▫
	NPA-TG $\frac{\partial NPA}{\partial x_{TG}}\|_{Xyl=Const}$			ANL-TG $\frac{\partial ANL}{\partial x_{TG}}\|_{Xyl=Const}$				
g Xyl/kg flour	g TG/kg flour							
	0.0-1.0	**0.0-3.0**	**0.0-5.0**	**0.0-1.0**	**0.0-3.0**	**0.0-5.0**		
0.0	0.70 ⇧	-0.09 ▫	-0.16 ▫	-1.13 ⇩	-0.52 ⇩	-0.34 ▫		
0.1	1.04 ⇧	2.48 ⇧	0.65 ⇧	-0.27 ▫	2.12 ⇧	0.15 ▫		
0.3	2.59 ⇧	1.46 ⇧	0.76 ⇧	1.43 ⇧	0.48 ⇧	0.33 ▫		
0.5	1.04 ⇧	0.32 ▫	0.01 ▫	1.03 ⇧	0.30 ▫	0.12 ▫		

1c	Volume-Xyl $\frac{\partial \forall}{\partial x_{Xyl}}\|_{TG=Const}$				Hardness-Xyl $\frac{\partial H}{\partial x_{Xyl}}\|_{TG=Const}$				Density-Xyl $\frac{\partial \rho}{\partial x_{Xyl}}\|_{TG=Const}$			
g Xyl/ kg flour	g TG/kg flour											
	0.0	**1.0**	**3.0**	**5.0**	**0.0**	**1.0**	**3.0**	**5.0**	**0.0**	**1.0**	**3.0**	**5.0**
0.0-0.1	0.50 ⇧	0.72 ⇧	0.64 ⇧	0.41 ▫	1.30 ⇧	0.66 ⇧	0.86 ⇧	1.39 ⇧	-0.38 ▫	-0.68 ⇩	-0.56 ⇩	-0.36 ▫
0.0-0.3	0.18 ▫	0.20 ▫	0.18 ▫	0.12 ▫	0.28 ▫	0.33 ▫	0.37 ▫	0.48 ▫	-0.15 ▫	-0.18 ▫	-0.16 ▫	-0.10 ▫
0.0-0.5	0.08 ▫	0.07 ▫	0.06 ▫	0.08 ▫	0.23 ▫	0.22 ▫	0.22 ▫	0.23 ▫	-0.07 ▫	-0.05 ▫	-0.07 ▫	-0.07 ▫
	Volume-TG $\frac{\partial \forall}{\partial x_{TG}}\|_{Xyl=Const}$			hardness-TG $\frac{\partial H}{\partial x_{TG}}\|_{Xyl=Const}$			Density-TG $\frac{\partial \rho}{\partial x_{TG}}\|_{Xyl=Const}$					
g Xyl/ kg flour	g TG/kg flour											
	0.0-1.0	**0.0-3.0**	**0.0-5.0**	**0.0-1.0**	**0.0-3.0**	**0.0-5.0**	**0.0-1.0**	**0.0-3.0**	**0.0-5.0**			
0.0	0.79 ⇧	0.27 ▫	0.16 ▫	-0.58 ⇩	-0.38 ▫	-0.26 ▫	-0.67 ⇩	-0.24 ▫	-0.15 ▫			
0.1	0.72 ⇧	0.21 ▫	0.08 ▫	0.66 ⇧	0.29 ▫	0.28 ▫	-0.68 ⇩	-0.19 ▫	-0.07 ▫			
0.3	0.59 ⇧	0.18 ▫	0.07 ▫	0.98 ⇧	0.37 ▫	0.29 ▫	-0.53 ⇩	-0.16 ▫	-0.06 ▫			
0.5	0.34 ▫	0.10 ▫	0.08 ▫	1.11 ⇧	0.37 ▫	0.23 ▫	-0.27 ▫	-0.11 ▫	-0.07 ▫			

step addition of TG (0.0 → 1.0 g/kg flour) shows a strong positive influence on G′ for all Xyl concentrations. A slight decrease seen in the SC once the concentration of Xyl reaches 0.5 g/kg flour is worth mentioning, as indicated earlier and shown in Fig. 2, which is similar to the over-dosage effects. Further addition of TG (0.0 → 3.0 g/kg flour) reflects a weaker positive effect on G′, which strongly decreases to a weak effect (SC < 0.3) at TG stepping from 0.0 → 5.0 g/kg flour.

On the other hand, the response of G″ generally reflects negative sensitivity, as seen in the lower right part of Table 1, except at 0.0 g Xyl/kg flour, where a weak positive sensitivity is observed. This response in G″ matches the dotted line findings in Fig. 2, where the line for constant Xyl (Xyl = 0.0 g/kg flour) shows a slight rise in G″ (10536.7 Pa → 10720.0 Pa) and a strong rise in G′ (29020.0 Pa → 36026.7 Pa). Although there is a slight increase in G″, the SC value (0.09) is small, indicating only a minor change in the structure. Regardless and as mentioned earlier, G″ shows a strong negative sensitivity until TG overdoses above 1.0 g/kg, after which all SCs show weaker negative values.

3.3. Synergistic effect of xylanase and transglutaminase analysed by image visualisation

Visual image analysis provides further insight into the protein network distribution and proves and clarifies the aforementioned rheological results. Visual analyses of the CLSM images after combined enzyme addition were performed (Fig. 3). Supporting the visual evaluation of the images, the routines described in Section 2.7 were implemented on the pictures, removing the noise and tracing the protein network.

After image pre-processing, the NPA and ANL were calculated and used for comparing the effect of different enzyme additions on protein networking.

Fig. 3 summarises the findings from the analysis of the protein network. Accordingly, for each concentration, five images were analysed and the first three doughs with the highest NPA and ANL were ranked. Consistent with the rheological analysis, the NPA and ANL for synergetic enzyme addition of Xyl and TG with concentrations of Xyl = 0.1 g/kg flour and TG = 3.0 g/kg flour, Xyl = 0.3 g/kg flour and TG = 3.0 g/kg flour and Xyl = 0.3 g/kg flour and TG = 5.0 g/kg flour reveal the most branched and spanned protein network. In rheological terms, branched and spanned protein networks for Xyl = 0.1 g/kg flour and TG = 3.0 g/kg flour, Xyl = 0.3 g/kg flour and TG = 3.0 g/kg flour and Xyl = 0.3 g/kg flour and TG = 5.0 g/kg flour represent the highest elasticity, similar to the results in Section 3.2. The sensitivity analysis supports these findings, forecasting the strongest positive impact (SC = 7.45 and 6.37 on the NPA and ANL, respectively) by addition of 0.1 g Xyl/kg flour and 3.0 g TG/kg flour. Furthermore, the stepping of Xyl concentrations from 0.0 → 0.1 and 0.0 → 0.3 g/kg flour for TG concentrations of 1.0, 3.0 and 5.0 g/kg flour showed a strong positive effect on raising the NPA and ANL until overdosage is reached (Xyl concentrations of 0.5 g/kg flour). At the overdosage point, the sensitivity of NPA and ANL are mostly seen to weaken (SC < 0.5), especially for TG concentrations of 1.0, 3.0 and 5.0 g/kg flour. In other words, the protein network projects no further growth, also supported by the findings shown in Fig. 3. On the other hand, overdosage of TG occurs at 5.0 g/kg flour and strongly impacts ANL by weakening its sensitivity and not as strongly influencing NPA (Table 1b).

These results are in accordance with the findings of Jekle and Becker (2011), where G′ increases with increasing total area of proteins, explained by bigger protein films and fibrils. As mentioned above, the increase in elasticity is explained by increasing linkages of the protein network. On one hand, with an increased protein network area, more water can bind, causing the elasticity to

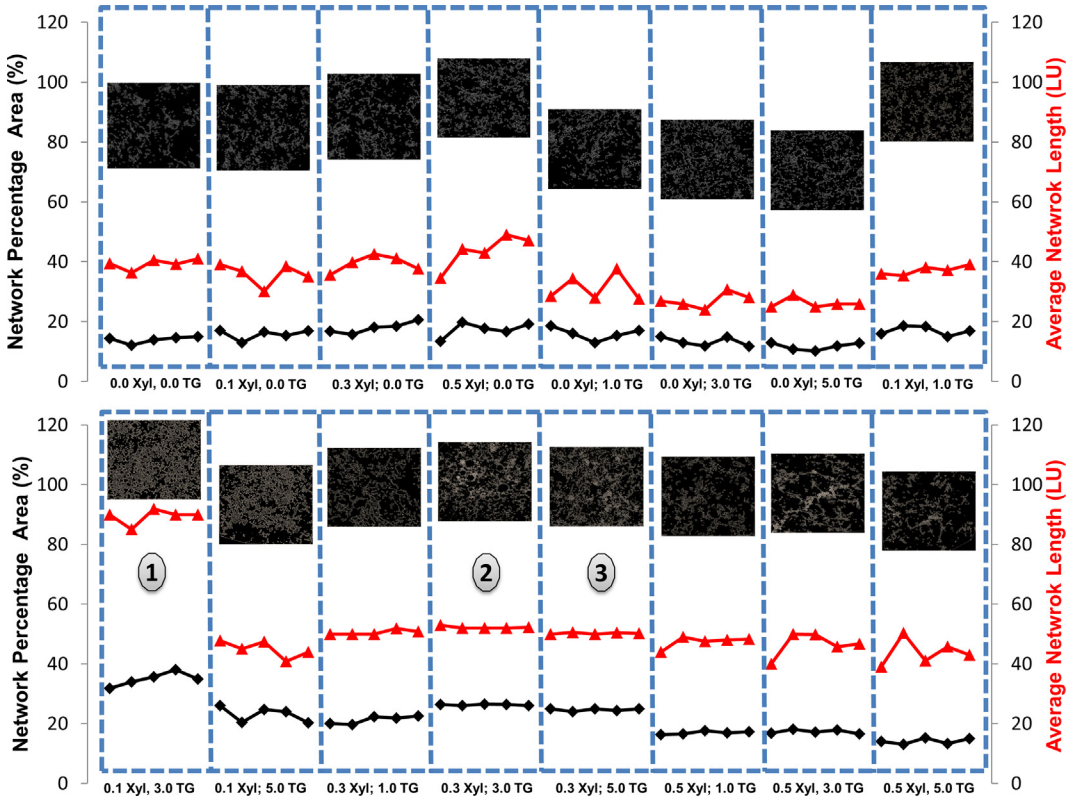

Fig. 3. Protein networking analysis represented by the network percentage area (%) and average network length (LU) for different combinations of xylanase and transglutaminase additions based on analysis of CLSM images with a statistical fivefold repetition. The first three doughs with the highest average network length (LU) and network percentage area (%) are ranked by numbers (1, 2 & 3).

increase at the same dough consistency (G*). On the other hand, increased cross-linking of protein leads to a rise in elasticity, as described by Cornec, Popineau, and Lefebvre (1994). With no enzyme addition, the average network length was 22 LU (length unit; 1 length unit is 0.1 mm) in contrast to the range of 35–93 LU for different cases of enzyme addition. Similarly, the network percentage area with no enzyme addition was 10%, in contrast to the range of 14–38% for different cases of enzyme addition.

3.4. Synergistic effect of xylanase and transglutaminase addition on bread properties

Regardless of the benefits mentioned for simultaneous enzyme activity on the dough, a deeper insight on the end-product for broader and consumer-oriented evaluation is necessary. Thus, the influence on final product measurements of bread volume, crumb hardness and density was quantified. The partitioned domain is plotted in Fig. 4 and divided into low and high hardness, and low, middle and high density. The labelled scattered points in Fig. 4 represent different enzyme concentrations: the first digit represents the Xyl concentration (g/kg flour) and the digit after the comma is the TG concentration (g/kg flour). In Fig. 4, four clusters are seen that enclose the points of the same Xyl concentrations and different TG concentrations. For the cluster for Xyl = 0.0 g/kg flour, all points at TG = 1.0–5.0 g/kg flour displayed low density and low hardness, indicating higher volume. The lowest density occurred at TG = 5.0 g/kg flour (14.79%). In other words, the largest volume rise (16%) compared to standard bread was achieved with no Xyl addition. Nevertheless, Fig. 2 shows that this represents a higher viscosity with bigger voids and crumb structure failures (Fig. 5). On the other hand, at Xyl concentration of 0.3 g/kg flour, a more moderate density reduction is reached (9.66%), which is accompanied by a volume rise of 10.67% compared to standard bread. Moreover, the structural compactness (Fig. 5; Xyl = 0.03 g/kg flour, TG = 3.0 g/kg flour for a visual impression of the crumb comparison) was preserved. With the exception of TG = 5.0 g/kg flour, the edge of the Xyl = 0.3 g/kg flour cluster shows that the higher density is accompanied with increased hardness, reflecting a structural bread failure, as seen in Fig. 5. Obviously, the Xyl concentration shows a more precise classification, but the simultaneous effect of both enzymes is needed to reach the density and hardness levels. From a physico-chemical point of view, Xyl degrades AX, causing more free protein residues that are available to be cross-linked by TG. Additionally, increasing Xyl influences the density that rises to a high limit and yields harder crumbs, definitely reflecting a much stronger network than desired (Fig. 5; Xyl = 0.5 g/kg flour, TG = 0.0 g/kg flour). As such, the gaseous voids encapsulated by the dense network have lower expansion degrees of freedom, causing a drop in volume. Performing sensitivity analysis on the data quantifies the findings and shows if further enzyme addition strongly or weakly contributes to the volume, hardness and density changes. As seen in Table 1c, on the first step addition of Xyl (0.0 → 0.1 g/kg flour) at different TG concentrations, the volume and hardness show a strong positive response and the density displays a strong adverse effect. For example, the addition of 0.1 g Xyl/kg flour decreases the density by an SC value of -0.38. The addition of TG (1.0 and 3.0 g/kg flour) at 0.1 g Xyl/kg flour has a higher influence on density (SC = −0.68 and −0.56), whereas a TG concentration of 5.0 g/kg flour inverts the positive effect of TG addition on density (SC = −0.36). At starting concentrations of TG, the sensitivity of volume and hardness rises gradually. In contrast, the density sensitivity rises at 0.0 TG/kg flour, and then rises again slowly. Once a bigger step in Xyl is used (0.0 → 0.3 g/kg or 0.0 → 0.5 g/kg), all sensitivities fall to weaker contributions.

Further, analysing the stepwise addition of TG from 0.0 → 1.0 g/kg flour at constant Xyl concentrations (0.0, 0.1 and 0.3 g/kg flour) demonstrates a strong positive influence with SC = 0.79, 0.72 and 0.59, respectively. At a TG concentration of 0.3 g/kg flour, the sensitivity starts diminishing until an over-dosage of 0.5 g Xyl/kg flour drops the SC to weak positive effect (SC = 0.34). In other words, the addition of both enzymes declines to a positive contribution for increasing volume. Accordingly, both the hardness and density encounter a similar effect, where hardness shows a stronger

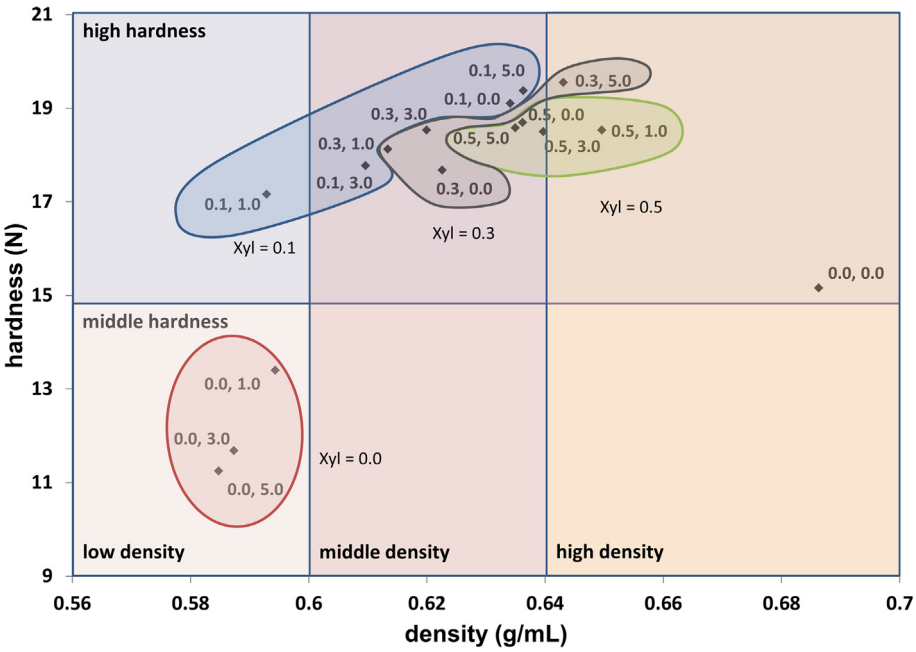

Fig. 4. Scattering of density and hardness levels of different enzyme concentrations labelled, with the first digit indicating the xylanase concentration (g/kg flour) and the second digit indicating the transglutaminase concentration (g/kg flour). The mean value is from four slices of single bread from three different sets of baking.

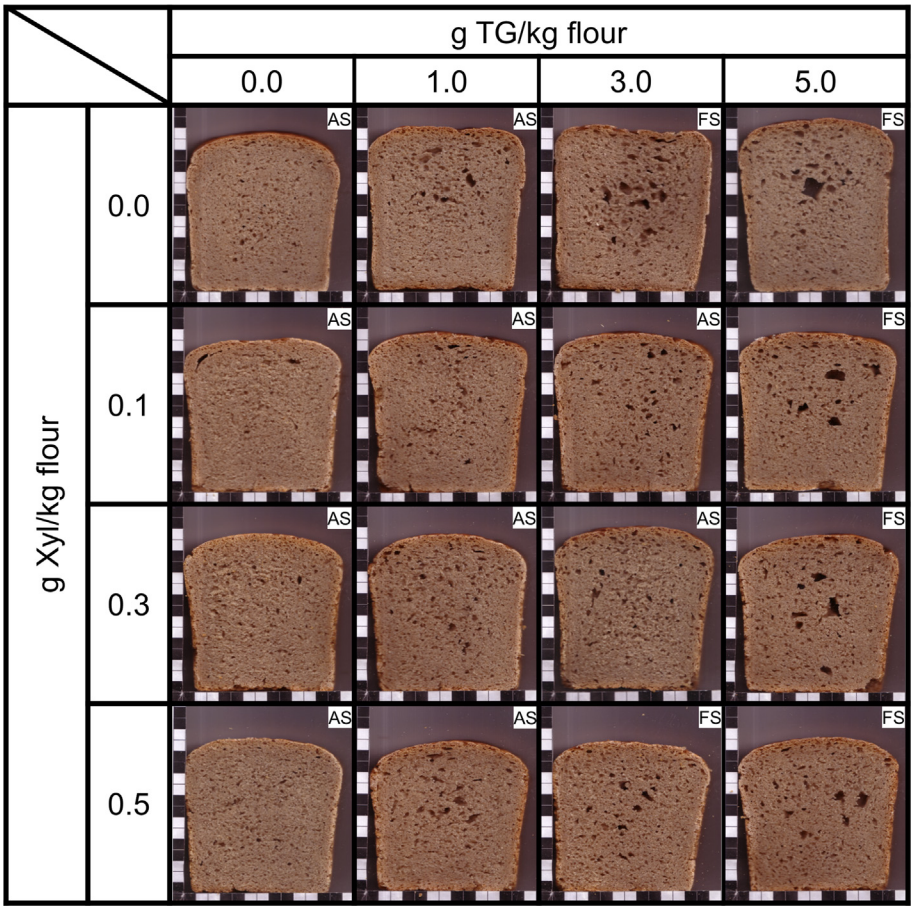

Fig. 5. Images of the crumb structure of bread slices treated with different concentrations of xylanase (Xyl) and transglutaminase (TG). A: Xyl = 0.0, TG = 5.0; B: Xyl = 0.3, TG = 3.0; C: Xyl = 0.5, TG = 0.0 all in g/kg flour. Accepted crumb structures evaluated by optical view are marked as AS (accepted structure). Insufficient crumb structure is marked as FS (failed structure).

sensitivity matching the decline in volume. This also matches the decrease in density that corresponds to the volume rise that strongly diminishes at enzyme concentrations of 0.5 g Xyl/kg flour and 0.0 → 1.0 g TG/kg flour.

4. Conclusions

CLSM images of model rye dough obtained after staining AX with stained AX antibodies clearly prove the hypothesis that proteins are surrounded by AX, especially for high AX concentrations. The encapsulation of proteins by AX was further shown to be reduced by the addition of Xyl. These results give further insight into protein networking and support an understanding of the structure–function relationship of rye dough. Nevertheless, further experiments should be performed with other AX-modifying enzymes and commercial rye flours.

Additionally, the results demonstrate that improved and increased protein interaction from AX degradation via Xyl and cross-linking using TG in rye dough made from commercial rye flour are feasible. These findings show that AX influences rye dough and bread characteristics such as viscoelasticity, bread volume and crumb hardness. The concentration of TG and Xyl can be modified to target the desired structure. The results achieved in the present study include the following:

- When TG $\leq$ 3.0 g/kg flour (and for all Xyl conc. 0.0–0.5 g/kg flour), G′ strongly increases and G″ slightly decreases, meaning the dough becomes more elastic.
- When TG > 3.0 g/kg flour and for all Xyl concentrations (0.0–0.5 g/kg flour), G′ falls strongly and G″ falls slightly. In this case, the dough elasticity decreases.
- When increasing the Xyl for all Xyl concentrations (0.0–0.5 g/kg flour), G′ increases slightly and G′ decreases. That is, with increasing Xyl concentration, the dough becomes more elastic.

Since these findings focused on only one commercial rye flour type (type 1150) and xylanase type, further experiments with different AX-modifying enzymes (ferulic acid esterase, arabinofuranosidase, etc.) should be conducted. Additionally, diverse flour types with different starch concentrations and degrees of damage, as well those with different AX concentrations and molecular structures, should be further investigated. Although the control of firmness by water calibration should have compensated for the free water effect caused by enzymatic degradation of WUAX to WEAX, further research should address this issue and its influence on the observed results. These findings provide a path for product-oriented physiochemical design of recipes that simultaneously targets structure–function relationships, providing a prosperous methodology for enhancing the quality of nutritious rye bread.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.053.

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3 Discussion

Following starch, AX are, at present, the main structure-forming component for rye dough and bread production. In contrast to rye, for wheat dough production, high AX concentrations are undesirable because of the following factors: 1) Depending on the molecule structure, AX absorb high amounts of water. This absorbency leads to competition for water between gluten and AX during dough formation, which influences the final dough and bread structure negatively (Wang et al., 2003). 2) The increased water absorption capacity of AX negatively influences starch gelatinisation (Lineback and Wongsrikasem, 1980). Limited water availability for starch gelatinisation leads to reduced bread quality, as discussed in detail by Schirmer et al. (2014). 3) AX interact with proteins by crosslinking the tyrosine residues of the protein and their aromatic ring of ferulic acid (FA) side chains, thereby affecting protein network formation negatively (Neukom and Markwalder, 1978; Wang et al., 2003; Wang et al., 2004). In traditional rye dough and bread production, the first and third factors are of minor interest due to the reduced capability of rye proteins to form a protein network, as discussed in Section 1.2. The second factor can also be neglected because rye dough is produced with higher amounts of water than for wheat dough. The additional water balances the increased water absorption caused by the naturally higher amount of AX in rye flour. Therefore, compared with wheat, comparable amounts of water are available for starch gelatinisation. In contrast, for rye dough and bread production, high amounts of AX (5 – 8 %) are the dominant structure-forming component. In particular, HMW WEAX improve water distribution and increase molecular interactions between the constituent macromolecules of the dough (Vinkx and Delcour, 1996). This action causes high viscosity in the aqueous phase and positively influences the final bread characteristics, such as crumb structure, crumb firmness and bread volume (Verwimp et al., 2012). The effects of AX, in addition to its concentrations, mainly depend on its molecular weight and structural properties, such as the arabinose-to-xylose ratio, or the interactions with further substituents (e.g. ferulic acid) (Cyran and Cygankiewicz, 2004; Kühn and Grosch, 1988; Weipert, 1997). Accurate knowledge of the molecular structure and the physicochemical characteristics of AX is a basic requirement for creating or modifying structures. Therefore, several technical (enzymatical, physical and chemical) methods for AX isolation and analytical methods for AX quantification from cereals were developed. Furthermore, how the different physicochemical properties

of AX impact dough and bread structure was investigated. The evaluation of current extraction and quantification methods of AX and their effect on structure formation was summarised and discussed in the literature review (Section 2.2).

Great improvements to rye dough and bread characteristics were achieved by using AX modification enzymes (such as endo-1,4-β-xylanase) and oxidative crosslinking via oxidases, as described in Section 2.2 (Autio et al., 1996; Dervilly-Pinel et al., 2001). However, despite the positive effect of enzymatic treatment, rye dough and bread structure remain relatively poor in quality compared with wheat.

To gain deeper insights into the role of AX on the structure-formation properties in rye dough and bread, the effects of different bran particle sizes, bran concentrations and the addition of an endo-1,4-β-xylanase on dough micro- and macrostructure were analysed and discussed (Section 2.3). The results do not reveal any interruptions in the rye dough structure caused by the addition of bran particles (i.e. no structure failures were detected). However, with increasing bran concentrations, the amount of free water decreased, the dough became firmer and the final bread volume decreased. In contrast, the addition of Xyl and the associated release of bound water by AX hydrolysis led to increased gas retention and bread volume. These results occurred due to an increase in WEAX because of the Xyl addition. As described by Courtin and Delcour (2002), WEAX stabilises the gas cells during fermentation and the early stage of oven rise by increasing the viscosity of the liquid film around the gas bubbles. Furthermore, the increased gas retention and bread volume could be linked to increased dough extensibility caused by the released water, which acts as a plasticiser to the structure (Jekle and Becker, 2011). However, these positive effects are limited to the Xyl concentration as described by McCleary (1986). An overdosage of Xyl decreases the dough consistency and leads to sticky dough (McCleary, 1986). The results of this current research demonstrate the dominant role of AX on rye dough structure formation (Section 2.3). In this context, the water absorption of AX especially seems to contribute to structure formation.

Therefore, to control and to minimise the influence of water on the rheological behaviour, and to estimate the role of AX on the structure-function relationships of different dough, further experiments were performed considering final dough firmness, expressed as complex shear modulus (|G*|). For all experiments, wheat and rye dough firmness were set to 12,100 Pa and 21,500 Pa, respectively. These values accord with the target dough firmness for wheat and rye dough of 500 and 300 Brabender Units stated by the approved Methods of the American Association of

Cereal Chemists (according to AACCI Method 54-21.02). This new approach of dough water adjustment for analysing dough structures treated by different techniques provides further insights from a different perspective and helps to understand the relationship between structure and function in detail. In contrast, water adjustment to a fixed value can also be considered critical. Higher water content might cause a dilution effect and, thus, affect the results. Therefore, the decision to adjust the dough firmness to a fixed value via water calibrations must be carefully considered for each experiment. In this context, future investigations should consider the adjustment of dough firmness to a fixed value by water addition for structure elucidation.

All effort was made to improve rye dough and bread structure formation by focusing on AX, but the investigations did not provide any information about the direct effect of AX on rye proteins. This information, however, is essential for the formation of a possible protein network from rye proteins, especially because AX interact with rye proteins (Piber and Koehler, 2005).

Therefore, rye model dough with different AX concentrations (0.0 – 10.0 %) were produced and analysed. Although the model systems did not provide a native system, they did provide a good opportunity to analyse the direct effect of single components (AX) on multi-component systems (as represented by dough in this study). Additionally, undesired side effects caused by components, which are not in focus of the investigations, can easily be eliminated. Therefore, model doughs were composed of rye starch, rye protein, rye AX, NaCl and water. To evaluate the effect of different AX concentrations on a possible protein network formation in rye (model) dough, a model dough of wheat was produced also. These different model doughs were composed of wheat starch, wheat protein, NaCl, water and rye AX.

To eliminate the influence of dough firmness on the rheological parameters of elasticity and viscosity, the samples were adjusted to the same dough firmness using water. This approach is necessary for samples in which the dough firmness changes and, consequently, influences the final dough parameters of viscosity and elasticity. The treatment of samples with varying dough firmness show different values in elasticity and viscosity. In this case, the change of viscoelastic behaviour was not due to a change in elasticity or viscosity but to a change in firmness. Furthermore, the standardisation of dough firmness by water adjustment will improve the evaluation of the results among each other. Another possibility would have been the adjustment of the dough firmness related to the a_w-value of the sample. However,

this approach was not given further consideration because of the results achieved by the firmness adjustment via water calibration. Nevertheless, future investigations should take the feasibility of a_w-value adjustment on the dough and the final bread into account.

The effect on the dough microstructure, especially on the protein network formation, was investigated using CLSM. This technique allows the visual identification of microstructures of different systems in a non-destructive manner. Therefore, CLSM is a very important tool for the analysis of sensitive structures (Jekle and Becker, 2011). An interesting finding was that increasing concentrations of AX lead to a reduction of the protein network for both rye and wheat in nearly in the same manner. At low AX concentrations (0.0 – 2.5 %), the proteins are evenly distributed and form a coherent network (wheat), or are at least located close to each other (rye), whereas at high concentrations (7.5 – 10.0 %) the proteins are widely distributed and less stretched (Figure 8). The results were confirmed by rheological measurements (Section 2.4). The negative effect of AX on the protein network formation is clearly illustrated at concentrations higher than 5.0 % AX. Finally, for refined wheat flour, AX concentrations of 2 – 3 % are common, whereas 5 – 8 % concentrations were analysed for rye flour.

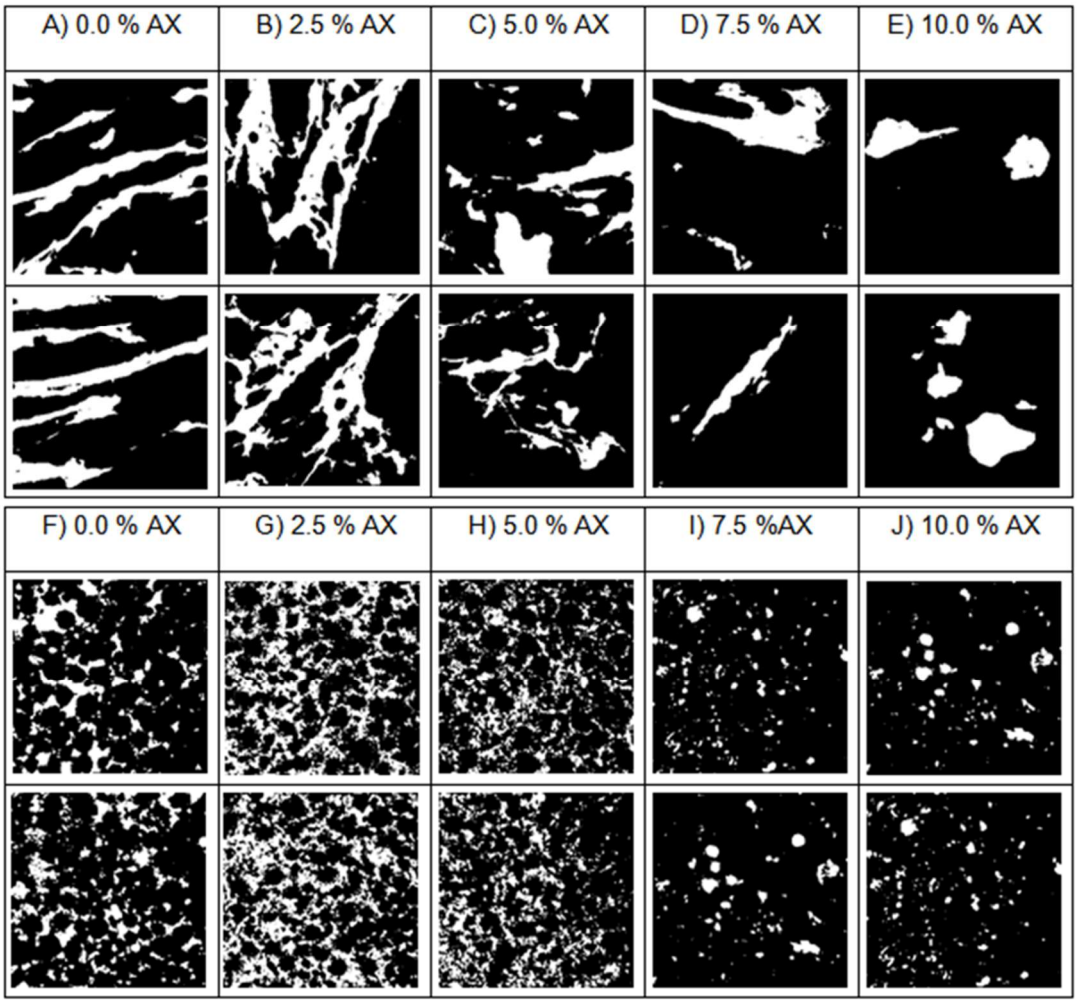

Figure 8: Binarized CLSM micrograph of wheat and rye model doughs with different AX contents (flour replaced by AX): Wheat: A) 0 % AX, B) 2.5 % AX, C) 5.0 % AX, D) 7.5 % AX and E) 10.0 % AX. Rye: F) 0 % AX, G) 2.5 % AX, H) 5.0 % AX, I) 7.5 % AX and J) 10.0 % AX. Protein network is shown in white. Size: 212 x 212 µm (1024 x 1024 px). Fixed firmness for wheat model dough: 12,100 Pa. Set firmness of all rye model doughs: 21,500 Pa.

These results indicate that rye proteins, even if they could connect in the way wheat proteins do, cannot build a protein network due to the required water content for dough preparation and the associated physical distance between proteins.

In the present experiments, the addition of 1 % AX to rye flour (flour replaced by AX) led to an extra water addition of 3 g 100 g^{-1} flour, as seen in Figure 9. For AX gels, water absorptions up to 100 g of water per gram polymer were analysed. In this case, the high hydration capacity was reached via oxidative crosslinking of AX (Izydorczyk et al., 1990).

For the investigations described in Section 2.4, a direct comparison of the water addition required to reach a fixed dough firmness depending on the AX content is not possible due to the different firmness of wheat and rye doughs. The detected water

contents for the model dough are in accordance with the water contents for producing commercial wheat and rye doughs (wheat: AX: 2 – 3 %, water content: 55 – 65 g 100 g^{-1} flour; rye: AX: 5 – 8 %, water content: 70 – 80 g 100 g^{-1} flour).

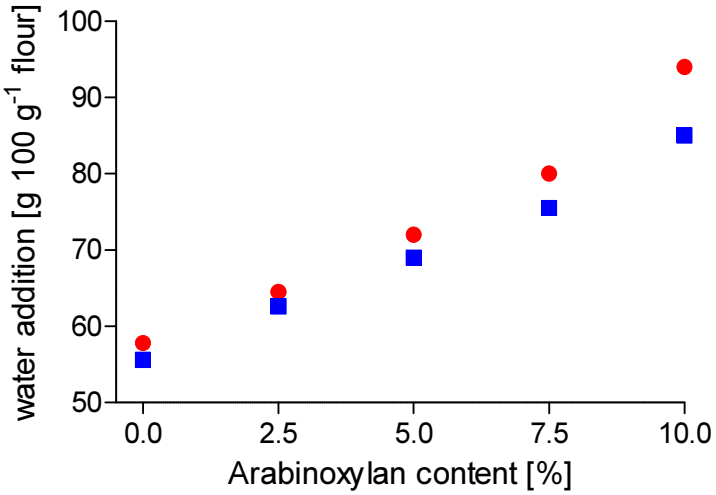

Figure 9: Determined water contents for wheat (●) and rye model doughs (■) to reach the default │G*│ value of 12,100 Pa (wheat model dough) and 21,500 Pa (rye model dough) depending on AX concentration. Linear regression of water addition with increasing AX content is obtained (wheat model dough: y=3.52x+56.08, r=0.98; rye model dough: y=2.87x+55.20, r=0.99). Mean ± Standard deviation; n=3.

Overall, a reduction of AX in native rye flour of up to 2.5 % should reduce the physical distance between the proteins, as seen for the model dough in Fig. 8 for the rye CLSM micrographs for AX concentrations lower than 5 %. This change should improve protein network formation if rye proteins could interact in the same way as wheat proteins. The increased water addition for AX concentrations higher than 5 %, as well as the reduced protein network formation, tends to decrease dough stability, gas-retention capacity and bread volume, as is evident from commercial rye dough and bread. It can be further assumed that wheat flour with AX concentrations higher than 5 % display dough and bread characteristics related to rye flour. These estimations are in accordance with the results of Delcour et al. (1991), who found that an addition of more than 3 % water-soluble pentosan fractions from wheat and rye to a natural flour had a volume-reducing effect. Buksa (2016) examined the highest bread volume by the addition of 3 % AX to the rye model dough, while AX concentrations > 3 % resulted in reduced bread volumes. In comparison with the present work, Delcour et al. (1991) and Buksa (2016) did not consider the role of water as a dilution factor on protein network formation. The investigations performed by Delcour et al. (1991) and Buksa (2016) tend to analyse the influence of AX on

final dough and bread parameters, such as dough yield and bread volume. This present work analysed the reciprocal effect of AX and protein network formation as a function of different water contents. The results suggest that the water content needed for dough preparation influences protein network formation.

To obtain further insights into the role of AX in a possible protein network formation, and to confirm the hypothesis that AX cover proteins, the identification and localisation of AX in rye dough was necessary.

This aim was achieved by visual identification of AX via CLSM. Traditionally, dough structure-forming components are identified by dyes such as Rhodamine B, Nile Blue or Nile Red (Jekle and Becker, 2015). One disadvantage of dyes is the non-specific staining of the sample component, especially in multi-phase systems. For example, Nile Blue stains both starch and proteins. The staining further depends on the accessibility and affinity of the dye to other sample components. Therefore, distinction and interpretation of the structure by image analysis are difficult. For a specific staining of components, fluorescent dyes can be labelled using monoclonal or polyclonal antibodies. Although this technique has been successfully applied to stain and identify AX in grain cell walls (Guillon et al., 2004; McCartney et al., 2005; Ordaz-Ortiz et al., 2004; Willats et al., 2000), it had never been applied to identify AX in dough. For a successful detection of AX using the antibody-labelled technique, the following factors had to be considered. According to Amit et al. (1985), antibodies produced as the immune response to polysaccharides such as AX bind to a specific pattern of the polymer (antigen's epitope), and not only to one molecule. Therefore, to achieve a high visual response to the structure by using dye-stained antibodies, it must be ensured that the antibodies' affinity to the antigen's epitope is not (sterically) hindered by the presence of further molecule residues. Additionally, antibodies bind specifically to the antigen's epitope. If the antigen's epitope differs in structure, the antibodies' affinity to bind decreases (Guillon et al., 2004). This factor is particularly relevant for the detection of AX via antibodies in dough produced from natural flour. Therefore, the AX must first be extracted from the flour to produce antibodies. However, as described in Section 2.2, the extraction techniques influence the final AX structure. Therefore, the structure of the AX for antibody production differs to that in the flour. This difference could result in a limited affinity of the antibodies to bind to the AX in the natural flour, which, in turn, results in a limited visual response of the AX. Both the structure and the accessibility of the antigen´s epitope to AX are important factors for successful visual detection.

A further direct technique for AX visualisation could be using fluorescently labelled Xyl. This technique is based on an inactive fluorescently labelled Xyl binding to AX. As described by the author, the advantage of this technique over the immunolabeling of AX is the faster and easier production of the Xyl probe. Furthermore, the staining protocol is described as fast and easy also (Dornez et al., 2011). However, no information exists about AX visualisation in dough. Based on the mentioned positive effects (easy production, lower costs), future investigations should consider the manageability of the inactive fluorescently labelled Xyl for AX-staining in dough.

For the non-specific visualisation of flour components by adding dye to the dough (staining technique), the choice of dye should be carefully considered since interactions with other sample ingredients can falsify the results of image analysis. In this present work, the use of dye-labelled antibodies to identify the localisation of AX in rye dough was adopted for the first time, as described in Section 2.5. From the images achieved, it is evident that AX are mainly located around the proteins (Figure 10 A, B and C). Moreover, AX are also widely distributed within the dough, as described in Section 2.5. The successful visual detection of AX in rye model dough by dye-labelled antibodies supports the hypothesis that the ability to form a rye protein network is reduced by the presence of AX.

This finding further leads to the hypothesis that increased protein interaction should occur after AX hydrolysis via Xyl.

To increase protein accessibility, Xyl addition was consciously overdosed. Higher concentrations lead to an excessive degradation of AX, which can be recognised by decreased dough firmness, gas-retention capacity and bread volume. This effect is mainly attributed to a reduction of the liquid film viscosity around the gas bubbles.

Note that the enzyme concentrations applied here significantly deviate from those applied and reported by Grossmann et al. (2016). In the present work higher Xyl and TG concentrations were used. This is explained by the planned research objective to form a protein network that take over the main part of structure formation in rye dough and bread. Further experimental clarification is part of planned future work.

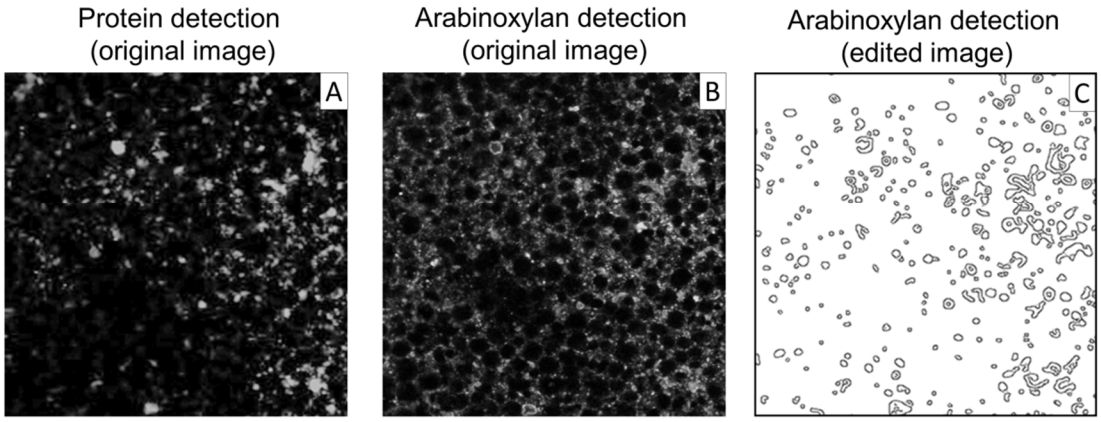

Figure 10: Location of detected AX fractions in model rye dough (7.5 % AX) by fluorescein isothiocyanate (FITC)-labelled AX antibodies. A: Protein visualisation via Rhodamine B. B: AX visualisation via FITC-labelled AX antibodies. C: Detected location of AX structure after image analysis. Size: 212 x 212 µm (1024 x 1024 pixels).

Excessive AX hydrolysation via Xyl results in the release of bound water and, consequently, leads to a dilution effect that influences protein accessibility negatively, as discussed above. Therefore, rye dough firmness, expressed as complex shear modulus (|G*|) of the Xyl-treated dough, was adapted to that of the untreated dough using water adjustment. Using this technique, the results of the rheological measurements (ratio of elasticity to viscosity) were not affected by the increased water content.

To discuss the effect of the enzyme treatments on the dough and bread characteristics, the main results are summarised in the following tables. In Table 2, the results of the Xyl-treated dough and bread are listed.

Table 2: Rheological characteristics of rye dough treated with different endo-1,4-β-xylanase concentrations. G´: storage modulus [Pa]; G´´: loss modulus [Pa]; H: hardness [N]; ρ: density [g/mL]; n=3. The first row in the table represents rye dough without Xyl addition. The columns represent the average storage modulus G' [Pa] of the dough, the percentage deviation of G' (ΔG' %) with respect to the enzyme-untreated dough, the average loss modulus G'' [Pa] of the dough, the percentage deviation of the loss modulus with respect to the enzyme-untreated dough (ΔG'' %), the hardness H [N] of the crumb, the percentage deviation of the hardness (ΔH) with respect to the enzyme-untreated crumb in the first row, the density ρ [g/mL] of the crumb and the percentage deviation in the density with respect to the enzyme-untreated bread (Δρ %).

enzym conc. (Xyl [g/kg flour])	G' [Pa]	Δ G' [%]	G'' [Pa]	Δ G'' [%]	H [N]	Δ H [%]	ρ [g/mL]	Δ ρ [%]
0.0	29020		10537		15.16		0.69	
0.1	32710	12.7	11113	5.5	19.11	26.1	0.63	-8.7
0.3	29456	1.5	9771	-7.3	17.68	16.6	0.62	-10.1
0.5	27816	-4.1	9020	-14.4	18.7	23.4	0.63	-8.7

An instantaneous rise in the storage modulus G' of almost 12.7 %, in comparison with the standard dough, occurred by adding 0.1 g Xyl kg^{-1} flour. For the same Xyl addition, the loss modulus G'' rose to 5.5 %. The crumb hardness increased to 26.1 %, while the crumb density decreased by just 8.7 %. These results can be explained by an increasing concentration of WEAX, stabilising the gas bubbles, which increases the dough elasticity and the gas-retention abilities, reflected in a reduced crumb density. The increased hardness can be traced to evenly distributed and reduced gas bubbles due to the stabilisation effect of WEAX. Therefore, the final crumb structure is more consistent, which increases the crumb hardness. An addition of 0.3 g Xyl kg^{-1} flour, as in the third row of Table 2, increased the elasticity by only 1.5 % and the hardness by 16.6 %, while the density declined by 10.1 %. The decrease in the loss modulus can be explained by a further rise of WEAX. This rise increases the water binding to the network, reflecting the further decrease of viscosity. Xylanase concentrations of 0.5 g Xyl kg^{-1} flour rebound the effect, and the dough became less elastic (-4.1 % relating to 0.3 g Xyl kg^{-1} flour), which is reflected in decreased volume or oven rise. The addition of 0.1 g Xyl kg^{-1} flour had the largest effect on elasticity. In this context, elasticity can be considered the most important impact factor in dough, influencing gas bubble growth and gas retention, and thus, the final bread volume and crumb hardness. Furthermore, increased protein accessibility by TG should occur for Xyl concentrations higher than 0.1 g Xyl kg^{-1} flour. However, before proving this assumption, the influence of TG without Xyl had to be investigated.

Transglutaminase is reported to decrease wheat dough extensibility and to increase dough resistance; thus, changing weak dough into strong dough with increased elasticity and water-holding capability (Gujral and Rosell, 2004; Tang et al., 2006; Truong et al., 2004). In high concentrations, this impact hinders the growth of air bubbles, resulting in a decreased bread volume (Autio et al., 2005; Bauer et al., 2003; Gerrard et al., 2001). The same results were found for TG-treated rye dough. High TG concentrations (900 U TG kg^{-1} flour) resulted in strongly aggregated protein particles, reducing bread volume (Beck et al., 2011). This study's investigations, presented in Table 3, support these findings. The elasticity rose with the addition of TG and slightly rebounded once the concentration exceeded 3.0 g kg^{-1} flour. The hardness seemed to rise continuously with increased TG concentration, and the density maintained an average 14.5 % decrease. The almost continuous decline of the loss modulus can be explained by the rise in the protein networking, which

increases the water-holding capacity and leads to a further decrease in the viscosity. Generally, a TG addition of 3.0 g TG kg^{-1} flour would be the optimal concentration for improving rye dough and bread characteristics. Higher TG concentrations (> 3.0 g TG kg^{-1} flour) lead to structure failures for all Xyl concentrations (0.0, 0.1, 0.3 and 0.5 g Xyl kg^{-1} flour), as described in Section 2.5. Lower TG concentrations (< 3.0 g TG kg^{-1} flour) might have the same storage modulus but a higher loss modulus.

Table 3: Rheological characteristics of rye dough treated with different TG concentrations. G´: storage modulus [Pa]; G´´: loss modulus [Pa]; H: hardness [N]; ρ: density [g/mL]; n=3.

enzym conc. (TG [g/kg flour])	G' [Pa]	Δ G' [%]	G'' [Pa]	Δ G'' [%]	H [N]	Δ H [%]	ρ [g/mL]	Δ ρ [%]
0.0	29020		10537		15.16		0.69	
1.0	36026	24.1	10720	1.7	13.41	-11.5	0.59	-14.5
3.0	35846	23.5	9403	-10.8	11.69	-22.9	0.59	-14.5
5.0	35203	21.3	8860	-15.9	11.25	-25.8	0.59	-14.5

To confirm the hypothesis of possible protein network formation following AX degradation, and to finalise the investigation, the synergetic effect of Xyl and TG addition was investigated. This approach was performed for the first time for rye dough and bread.

Special focus was directed to the TG concentration of 3.0 g kg^{-1} flour while changing the Xyl concentrations (Table 4). The maximum elasticity was observed at a combination of Xyl = 0.3 g kg^{-1} flour and TG = 3.0 g kg^{-1} flour, reaching a 28.0 % higher elasticity than without enzyme addition. A high elasticity reflects the formation of a protein network. The formation of a protein network determines the crumb structure and the crumb density, as described in Section 2.5. The density was compromised to almost 10.1 % lower than the untreated dough. On the other hand, the loss modulus (G'') decreased to almost 19.3 %, which can be attributed to the synergetic activity of enzymes; more water binds to the network and enhances the water-holding capability, which increases the elasticity. As in previous investigations, overdosing rebounds the elasticity and the density. Therefore, the combination of 0.3 g Xyl kg^{-1} flour and 3.0 g TG kg^{-1} flour is the optimal dosing for the maximal improving of dough and bread appearance and the final product quality. These findings are confirmed by the statistical evaluation in Section 2.5.

Table 4: Rheological characteristics of rye dough treated with different endo-1,4-β-Xyl and TG concentrations. G´: storage modulus [Pa]; G´´: loss modulus [Pa]; H: hardness [N]; ρ: density [g/mL]; n=3.

enzym conc. (Xyl/TG [g/kg flour])	G' [Pa]	Δ G' [%]	G'' [Pa]	Δ G'' [%]	H [N]	Δ H [%]	ρ [g/mL]	Δ ρ [%]
0.0/0.0	29020		10537		15.16		0.69	
0.1/3.0	36037	24.2	8079	-23.3	17.78	17.3	0.61	-11.6
0.3/3.0	37150	28.0	8508	-19.3	18.54	22.3	0.62	-10.1
0.5/3.0	36783	26.8	8464	-19.7	18.5	22.0	0.64	-7.2

Notably, for the sole addition of Xyl, the optimal concentration to improve dough characteristics (elasticity) was determined as 0.1 g Xyl kg^{-1} flour (Table 2). The combined addition of Xyl and TG revealed 0.3 g Xyl kg^{-1} flour to be the concentration to increase dough elasticity maximally. The addition of 0.3 g Xyl kg^{-1} flour provided the best effects, whereas higher Xyl concentrations rebounded these effects. In the presence of TG, higher optimum concentrations of Xyl can be explained by an increased accessibility of TG to proteins, resulting in the formation of protein agglomerates and not in evenly distributed protein strands. This effect would also explain the formation of holes in bread crumbs for all Xyl concentrations in combination with TG concentrations of 5.0 g kg^{-1} flour, as well as for the combination of 0.5 g Xyl kg^{-1} flour and 3.0 g TG kg^{-1} flour, as discussed in Section 2.5.

The findings in this present thesis prove the assumption that the final characteristics and appearance of rye dough and bread are significantly influenced by the presence of AX. The addition of enzymes can be used to improve the final dough and bread characteristics, as previously described in Sections 2.2 to 2.5. Despite these results, a more specific experimental design must be developed to investigate further the limits and optimal concentrations of the enzyme treatments, in accordance with the rye dough and bread production process (time, pH). In further investigations, the effect of salt concentration, which influences the water activity, should also be considered.

This information can help to create a more targeted structure, with corresponding characteristics such as improved gas-retention capacity and dough elasticity. In this context, one topic that requires greater study is the influence of further AX-modifying enzymes (α-L-arabinofuranosidases, feruloyl esterases and exo-1,4-β-xylosidases) to enhance protein connectivity with or without protein network-forming enzymes such as TG. In particular, using AX-degrading enzymes α-L-arabinofuranosidases and feruloyl esterases, it would be interesting to evaluate whether protein accessibility increases, and to what extent the ratio of WEAX to WUAX is influenced. As discussed by Courtin and Delcour (2002), WEAX are important for stabilising gas

bubbles. This positive effect was maximal for WEAX concentration around 2.5 to 3.0 % of flour (Delcour et al., 1991). Therefore, an increase of WEAX to the mentioned concentration should be considered when using enzymes. Furthermore, the effect of around 2.5 % AX on protein network structure should be analysed. In low concentrations, AX have positive effects on dough structure formation. Arabinoxylan relax strong protein networks, especially of wheat dough (briefly addressed in Section 2.4), expanding the protein network during fermentation and baking, which influences the mentioned dough and bread characteristics positively. Another interesting topic would be the detection of the new enzyme-created structures in a more analytical manner. From the results achieved in this study (microscopically, rheologically), it could be clearly stated that a coherent protein network was created using the enzymes Xyl and TG.

Furthermore, determining the correlation of the present results on the micro-/macro-level with effects on the molecular level and the proof of molecular crosslinks (i.e. by analysis of protein molecular weight and sizes) needs to be investigated. This information could help to enlighten the mathematical relationship of dough characteristics and structure properties.

Finally, all investigations to improve rye dough and bread structure should be performed considering all the desired characteristics of the final product. To achieve structural features with desired functions, a fundamental understanding of all the involved ingredients is required.

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5 Apendix

5.1 Non-peer reviewed publications

Döring, C., Bellido, G., Jekle, M., Becker, T.: Pound cake quality as a function of time and temperature, baking+biscuit international, 5 (2013), 58-62.

Döring, C., Degen, N., Bellido, G., Jekle, M., Becker, T.: Frischhaltung bei Sandkuchen in Abhängigkeit von Zeit und Temperatur, Brot und Backwaren, 6 (2013), 41-45.

Döring, C., Chen, X., Eichler, R., Voigt, T., Jekle, M., Becker, T.: Weihenstephaner Standard steht für die Backbranche bereit, Brot und Backwaren, 3 (2016), 61-62.

5.2 Conference Contributions

Oral

Döring, C., Houben, A., Jekle, M., Becker, T.: Investigation on the benefit of novel sourdough in wheat bread production, 9th Young European Cereal Scientists and Technologists Workshop, Budapest, Hungary, 2010/04/19.

Döring, C., Jekle, M., Becker, T.: Bedeutung und Modifikation von Arabinoxylan in Roggenteigen, 1. WIG Frühjahrstagung, Germany, 2012/03/29

Döring, C., Langer, A., Jekle, M., Becker, T.: Bedeutung von Roggenkleie in Roggenbackwaren für die Praxis, 2. WIG Frühjahrstagung, Freising, Germany 2013/04/21

Döring, C., Jekle, M., Becker, T.: Qualitätsbeurteilung von Brotgetreide und Mehl III – Neue Einblicke in Backwaren, Getreidefachtagung, Freising, Germany, 2013/06/12

Döring, C., Nuber, C., Stukenborg, F., Jekle, M., Becker, T.: Structural characterization of arabinoxylan addition - Impact on protein microstructure formation of rye and wheat dough, AACCI Annual Meeting, Albuquerque, New Mexico, USA, 2013/10/02.

Döring, C., Nuber, C., Stukenborg, F., Jekle, M., Becker, T.: Proteinentwicklung während des Knetens - alles nur eine Frage des Arabinoxylangehalts?, 3. WIG Frühjahrstagung, Freising, Germany, 2014/04/02

Döring, C., Nuber, C., Stukenborg, F., Jekle, M., Becker, T.: Einfluss von Arabinoxylanen auf die Proteinentwicklung von Weizen- und Roggenteigen, 65. Tagung für Getreidechemie, Detmold, Germany 2014/06/26

Döring, C., Eichler, R., Jekle, M., Voigt, T., Becker, T.: Weihenstephaner Standards für die Backwarenbranche, 65. Tagung für Bäckereitechnologie, Detmold, Germany, 2014/11/12

Döring, C., Jekle, M., Becker, T.: Steuerung der Strukturausbildung von Roggenteigen durch enzymatische Modifikation der Arabinoxylan- und Proteinfraktion, 4. WIG Frühjahrstagung, Freising, Germany, 2015/04/22

Döring, C., Jekle, M., Becker, T.: Bedeutung und Modifikation von Pentosanen für die Teig- und Brotherstellung, Erfa-Tagung, Miltenberg, Germany, 2015/11/10

Poster

Döring, C., Jekle, M., Becker, T.: Investigation of the effect of varying shelf lifeparameter on the pound cake microstructure, ICC Cereals & Europe Spring Meeting, Freising, Germany, 2011/04/12

Döring, C., Jekle, M., Becker, T.: Pound Cake Quality as a Function of Storage Conditions. AACCI Annual Meeting, Savannah, Georgia, USA, 2016/10/23-26