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**A novel approach for structural analysis of high viscose starch
based products during heating**

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„Lernen ist wie Rudern gegen den Strom. Wer aufhört treibt zurück“

Laotse, chin. Philosoph, 4 - 3 Jh. v.Chr.

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Preface and peer reviewed publications

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

- 1. Schirmer, M., Jekle, M., Becker, T.: Starch gelatinization and its complexity for analysis, Starch/Stärke, 66 (2014), 1-12, doi:10.1002/star.201300071**
- 2. Schirmer, M., Zeller, J., Krause, D., Jekle, M., Becker, T.: In situ monitoring of starch gelatinization with limited water content using confocal laser scanning microscopy, European Food Research and Technology, 239 (2014), 247-257, doi:10.1007/s00217-014-2213-0**
- 3. Schirmer, M., Jekle, M., Arendt, E., Becker, T.: Physicochemical and morphological characterization of different starches with variable amylose/amylopectin ratio, Food Hydrocolloids, 32 (2013), 52–63, doi:10.1016/j.foodhyd.2012.11.032**
4. Schirmer, M., Jekle, M., Arendt, E., Becker, T.: Physicochemical interactions of polydextrose for sucrose replacement in pound cake. Food Research International, 48 (2012), 291–298, doi:10.1016/j.foodres.2012.05.003
5. Paquet-Durand, O., Solle, D., Schirmer, M., Becker, T., Hitzmann, B.: Monitoring baking processes of bread rolls by digital image analysis. Journal of Food Engineering, 111 (2012), 425-431, doi: 10.1016/j.jfoodeng.2012.01.024
- 6. Schirmer, M., Jekle, M., Becker, T.: Quantification in Starch microstructure as a function of baking time. Procedia-Food Science, 1 (2011), 145-152, doi:10.1016/j.profoo.2011.09.023**
7. Schirmer, M., Hussein, W.B., Jekle, M., Hussein, M.A., Becker, T.: Impact of air humidity in industrial heating processes on selected quality attributes of bread rolls. Journal of Food Engineering, 105 (2011), 647-655, doi: 10.1016/j.jfoodeng.2011.03.020

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Notation

Latin Letters

Symbol	Unit	Meaning/Definition
c_p	JK^{-1}	heat capacity (constant pressure)
D_F	m	ferret diameter
D_{rot}	$\text{radians}^2 \cdot \text{s}^{-1}$	rotational diffusion coefficient
E_a	$\text{kJ} \cdot \text{mol}^{-1}$	activation energy
G^*	$\text{Pa} \cdot \text{s}$	complex shear modulus
G'	$\text{Pa} \cdot \text{s}$	storage modulus
G''	$\text{Pa} \cdot \text{s}$	shear loss modulus
k	$\text{s}^{-1} \cdot \text{mol}^{-1}$	reaction rate constant
K_0	s^{-1}	reaction frequency factor
p-value	-	probability value
P	m	perimeter
P_s	m	starch perimeter
r	-	coefficient of correlation
R^2	-	coefficient of determination
R	$\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$	gas constant
sd	-	standard deviation
t	h	time
t_p	h	peak time
T	$^{\circ}\text{C}$	absolute temperature
T_C	$^{\circ}\text{C}$	conclusion temperature (DSC)
T_{end}	$^{\circ}\text{C}$	gelatinization end temperature (DSC)
T_G or T_{max}	$^{\circ}\text{C}$	gelatinization temperature (CLSM)
T_o	$^{\circ}\text{C}$	onset temperature (DSC)
T_P	$^{\circ}\text{C}$	pasting temperature
\bar{x}	-	average
w/w	%	weight per weight
$\bar{\phi}A$ or $\bar{\phi}AC$	m^2	average granule size

Greek Letters

Symbol	Unit	Meaning
α	-	gelatinized starch fraction
$\dot{\gamma}$	s^{-1}	shear rate
ΔH	J	enthalpy
Δt	h	time interval
η	$\text{Pa} \cdot \text{s}$	viscosity
λ	nm^{-1}	wavelength
λ_{em}	nm^{-1}	emission wavelength
λ_{ex}	nm^{-1}	excitation wavelength
λ_{rel}	nm^{-1}	relaxation time
ρ	$\text{kg l}^{-1} / \text{kg m}^{-3}$	density (mass per volume)
τ_0	MPa	yield stress

Indics

Symbol	Meaning/Definition
a	activation
c	conclusion
em	emission
end	end/final
ex	exication
F	ferret
max	maximal
n	number/sample amount
o	onset
p	peak
P	pasting
rel	relaxation
s	starch

Abbreviations

Symbol	Meaning/Definition
A	ungelatinized starch
AACC	american association of cereal chemistry
AM	amylose
AP	amylopectin
B	breakdown
BEPT	birefringence end point temperature
C	circularity
CFD	computational fluid dynamic
CLSM	confocal laser scanning microscopy
CO ₂	carbon dioxide
Con A	concanavalin A
db	dry base
DP	degree of polymerization
DSC	differential scanning calorimetry
EA	enzymatic analysis
EM	electron microscopy
EtOH	ethanol
FFA	free fatty acids
FV	final viscosity
G	solubilized/gelatinized starch
H ₂ O	water
HPV	hot paste viscosity
ICC	international association for cereal science and technology
LM	light microscope
LPL	lysophospholipids
n.d.	not detectable
NaCl	sodium chloride
NMR	nuclear magnetic resonance
PLM	polarized light microscope

Continued on next page

Symbol	Meaning/Definition
PLS	least squares regression
PV	peak viscosity
rpm	rotations per minute
RVA	rapid visco analyzer
S	setback
TEG	terminal extent of starch gelatinization
TPA	texture profile analysis
UN/DESA	department of economic and social affairs
WRC	water retention capacity

Summary

Analytical instruments and applications are important tools to characterize and improve raw materials used in food production. This knowledge is fundamental for the process optimization and serves as a useful instrument for product development. For cereal based foods many standardized analytical systems are known. Those systems are well described in many publications especially concerning the raw materials, however without correlation to end product characteristics. The main aim of this thesis is to obtain a global view of the desired components with connections to production parameters applied in food industry. In order to achieve this objective allowing to observe complex food systems exemplary on bread, new innovative methods were combined with well-known microscopic techniques.

As starch is the main component of wheat based product such as bread it was chosen for further investigation. The study of analytical methods to investigate structural changes of starch during heating revealed that they are all working with water in excess. Considering that wheat dough is a complex food system with limited water content no in situ analysis of starch gelatinization under actual condition is possible until now. The newly developed method enabling micrographic analyses for numerous structural features is based on confocal laser scanning microscopy (CLSM) combined with image analyzing techniques. Structural and morphological changes can be quantified and discussed in detail. The relationship between heat treatment and structural features was first proven with different starch suspensions by common thermo physical analytical techniques such as differential scanning calorimetry (DSC).

The new method was used to investigate the onset of starch gelatinization by using threshold values, which are based on the first derivatives, where values of CLSM and DSC showed the highest correlation. The gelatinization temperature that is in micrographs obtained through the shape and size analysis of starch granules is highly depending on the water content.

In summary, a visual online detection system to investigate changes in starch granules on a microstructural scale during heating was developed. This in situ system monitors the structural changes of starch granules such as starch gelatinization with the advantage of being unaffected by secondary factors.

Zusammenfassung

Zur Beurteilung von Rohstoffcharakteren bei der Lebensmittelherstellung sind analytische Untersuchungsmethoden zwingend notwendig. Das daraus resultierende Wissen dient als Grundlage zur Prozessoptimierung, Qualitätsverbesserung und stellt einen nützlichen Bestandteil der Produktentwicklung dar. Speziell im Bereich der Getreideanalytik existiert bereits eine große Vielfalt unterschiedlichster Analysenmethoden, deren Standardisierung und Betrachtungsweise primär auf spezifische Rohstoffeigenschaften ohne einen direkten Produktbezug beruhen. Im Bereich der Stärkeanalytik finden typische Verfahren zur Analyse der strukturellen Veränderungen von Stärke während der Erhitzung primär unter Wasserüberschuss statt.

Ziel dieser Arbeit ist die Verknüpfung aus analytischem Hintergrundwissen mit der Evaluierung einer neuartigen Analysenmethode zur Beurteilung komplexer Lebensmittelsysteme mit limitierendem Wassergehalt am Beispiel Brot. Dabei liegt der Fokus dieser Arbeit auf der globalen Betrachtungsweise zwischen Rohstoffeigenschaften und Endproduktparametern.

Die Konfokale Laser Scanning Mikroskopie (CLSM), kombiniert mit Bildanalysetechnik, ist eine nützliche Methode, um komplexe Lebensmittelsysteme und ihre Transformationsprozesse während der thermischen Behandlung besser zu verstehen. Dieses neue Verfahren ermöglicht es numerische Strukturmerkmale aus mikroskopischen Aufnahmen zu generieren. Zur Analyse dieser Strukturveränderungen und deren Einfluss auf das fertige Produkt wurde ein mikroskopisches Online-System mit digitaler Auswertung entwickelt und mit etablierten Analysenmethoden wie der Differential Scanning Kalorimetrie (DSC) evaluiert. Unter Verwendung von Schwellenwerten, basierend auf den ersten Ableitungen mit der höchsten Korrelation zwischen Werten der CLSM und DSC, konnten strukturelle und morphologische Veränderungen quantifiziert und zur Ermittlung der Verkleisterungstemperatur von Stärke genutzt werden.

Zusammengefasst dient die visuelle bzw. mikroskopie-basierte Online-Methode zur Beurteilung der Verkleisterungstemperatur am Beispiel Stärke. Dieses „in situ“-System ermöglicht die Analyse von Stärkeverkleisterungseigenschaften unabhängig von Sekundärfaktoren und Wassergehalt zur Verbesserung von Produktqualität und Prozessoptimierung.

1 Introduction

For almost all cultures across the world bakery products like bread are the most important staple product marketed. Bread is one of the earliest “processed” foods made and consumed by mankind. This is due to the unproblematic shelf-life, the balanced nutrient composition, multiple ways of preparation and use, food safety as well as the high energy density.

The minimum formula for bread production consists of flour compounds, water, salt and CO₂. The flour compounds used in bakery products are especially from cereal, non-cereal (e.g. tuber) and pseudo-cereal (e.g. amaranth). Whereby, the cereal group especially with wheat, which is one of the major grains in the diet of vast number of the world’s population and, therefore, plays an important role in the usage for bakery products, particularly for bread.

The most wheat breads contain flour separated from endosperm with a reduced mineral content. Typically wheat flour (characteristic for baked goods - like German flour Type 550) consists of approximately 70-80 % starch and 12-14 % protein. This means around 48 % starch and 8 % protein per baked bread. Thus it is obvious that starch is the major bread ingredient. For a better understanding how flour components influence the end product, a separated consideration of these main constituents is typical. Many publications discuss the gluten network regarding the influence on the end product quality (Veraverbeke and Delcour 2002; Falcão-Rodrigues, Moldão-Martins et al. 2005; Primo-Martín, Pijpekamp et al. 2006; Sroan, Bean et al. 2009). The impact of protein microstructure on rheology and processing performance as a structure-function relationship in wheat dough is discussed in detail by Jekle (Jekle 2012). Additionally there exist many publications about the characteristics of cereal starch and their structural changes during modification, but mostly without a correlation to the end product quality (Lund and Lorenz 1984; Singh, Kaur et al. 2007; BeMiller 2011; Le Thanh-Blicharz, Lewandowicz et al. 2012; Maeda, Kokawa et al. 2013; Li, Xie et al. 2014). However, the role of starch is revised. The use of new biological, chemical and physical techniques increase the interest in the relationship between starch structure and functionality. The statement that starch granules sometimes are called in rheological effects only as filler (like “glass beads”) in bread production is changed (Englyst, Hudson et al. 2006). There is more than the particle shape, sizes and size distribution. It is well known that the

presence of starch granules is important for dough and bread quality, but the analytical analyzing spectrum is limited.

1.1 Structure composition of cereal based starch

Like other cereal grains, the wheat kernel contains three main anatomical parts: the bran, the endosperm and a germ (figure 1). The germ is located at the bottom side of the kernel and mainly consists of lipids and additional vitamins. It is linked to the surrounding starchy endosperm, which provides starch and protein in case of germination. To protect these valuable resources from the influence of the environment several layers called bran surround the endosperm as well as the germ. The external bran layers are fiber. The fiber layers are containing a high proportion of vitamins and minerals. In table 1 a typical range of chemical composition of wheat grain is indicated.

Table 1: Chemical constituent distributions as percentage in kernel fractions of wheat ^{a, c} (MacMasters, Hinton et al. 1971), ^b (Belitz, Grosch et al. 2001), ^d (Morrison 1988).

Anatomical Part Fraction	Kernel ratio (%) ^a	Distribution of chemical constituents in wheat (%)						
		Carbohydrates ^b				Protein ^c	Minerals/ Vitamins ^b	Fat ^c
		Pentosans / Hemi- cellulose	Cellu- lose	Starch	Sugar			
Bran	3.8-4.2	43.1	35.2	14.1	7.6		7/15	5.1-5.8
Pericarp	5.0-8.9					2.5		0.7-1.0
Testa	0.2-1.1					1.5		0.2-0.5
Aleuron	4.6-8.9					14.2	61	6.0-9.9
Endosperm	74.9-86.5	2.4	0.3	95.8	1.5	74.5	20/83	0.8-2.2
Germ	2.0-3.9	15.3	16.8	31.5	36.4	3.0	12/25	11.3
Embryonic axis	1.0-1.6							10.0-16.0
Scutellum	1.1-2.0				4.5			12.6-32.0

The starch granules are primarily produced in annual plants like wheat for energy storage over a long growing period. In wheat and other plants, starch granules are embedded in superordinate amyloplasts mainly into the endosperm (figure 1). Wheat has two types and sizes of starch granules. The large lenticular (lens-shaped) A-type granules are >15 µm (in long dimension) and the small, spherical B-type granules are 5-15 µm (in diameter). A-type starch granules contribute more than 70 % (max. 90 %) of the total starch weight, but only a percentage of around 3 to 5 % (max. 10 %) of the total starch granule number. Thereby, B-type starch granules

account more than 90 % of the total granule number but with less 30 % (min. 10 %) of the total weight of wheat starch (Eliasson and Larsson 1993; Raeker, Gaines et al. 1998; Peng, Gao et al. 1999; Chiotelli and Le Meste 2002). These starch granules contain numerous components, which can be divided into two groups: the first comprises the major components amylose (AM) and amylopectin (AP). The second group assembles the minor components like proteins, lipids and minerals (Tester, Karkalas et al. 2004; Copeland, Blazek et al. 2009). The components build up due to their distribution and configuration a typical granule structure depending on crystalline, semi-crystalline and amorphous shells. Gallant, Bouchet et al. (1997) introduced the blocklet concept, which is an additional level of granule structural organization between the growth rings and the level of lamellae. According to this concept, starch granules are composed of alternating crystalline hard shells and semi-crystalline soft shells. These shells are made up of more or less spherical blocklets, which are stacked on top of each other and contain a number of amorphous and crystalline layers. The diameter of the blocklets differs between the two different shells. The thickness of the shells decreases towards the granule exterior. However, variations in AM and AP substantially affect the overall granule geometrical packing arrangement (Jane 2006; Jane 2007). AM is a linear and slightly branched homopolysaccharide buildup of α -(1 \rightarrow 4)-linked D-glucose units with less than 1 % of α -(1 \rightarrow 6) branch points. It displays a molecular weight in the range of 10^5 - 10^6 Da (Ball, Guan et al. 1996) and an average degree of polymerization by number (DP_n) ranging from 0.6×10^3 to 5.2×10^3 (Takeda, Maruta et al. 1992; Hanashiro, Tagawa et al. 2002) depending on its botanical origin. The much larger polymer AP is highly branched and contains α -(1 \rightarrow 4)-glucosidic linkages and about 5 % of α -(1 \rightarrow 6) branch points. It has a molecular weight of 10^7 - 10^9 Da (Ball, Guan et al. 1996) and a DP_n within the range of 0.7 - 26.5×10^3 (Takeda, Shibahara et al. 2003). In comparison to AM molecules, the AP branch chains are relatively irregular and have a broad length distribution with an average length of about 19-31 units (Hizukuri 1985; Tester, Karkalas et al. 2004). The molecular architecture of AP, i.e. the length of the branch chains and the placement of branch linkages, varies considerably between starches from different cultivars. The basic structure for all kinds of AM and AP distribution is out of glucose units (figure 1).

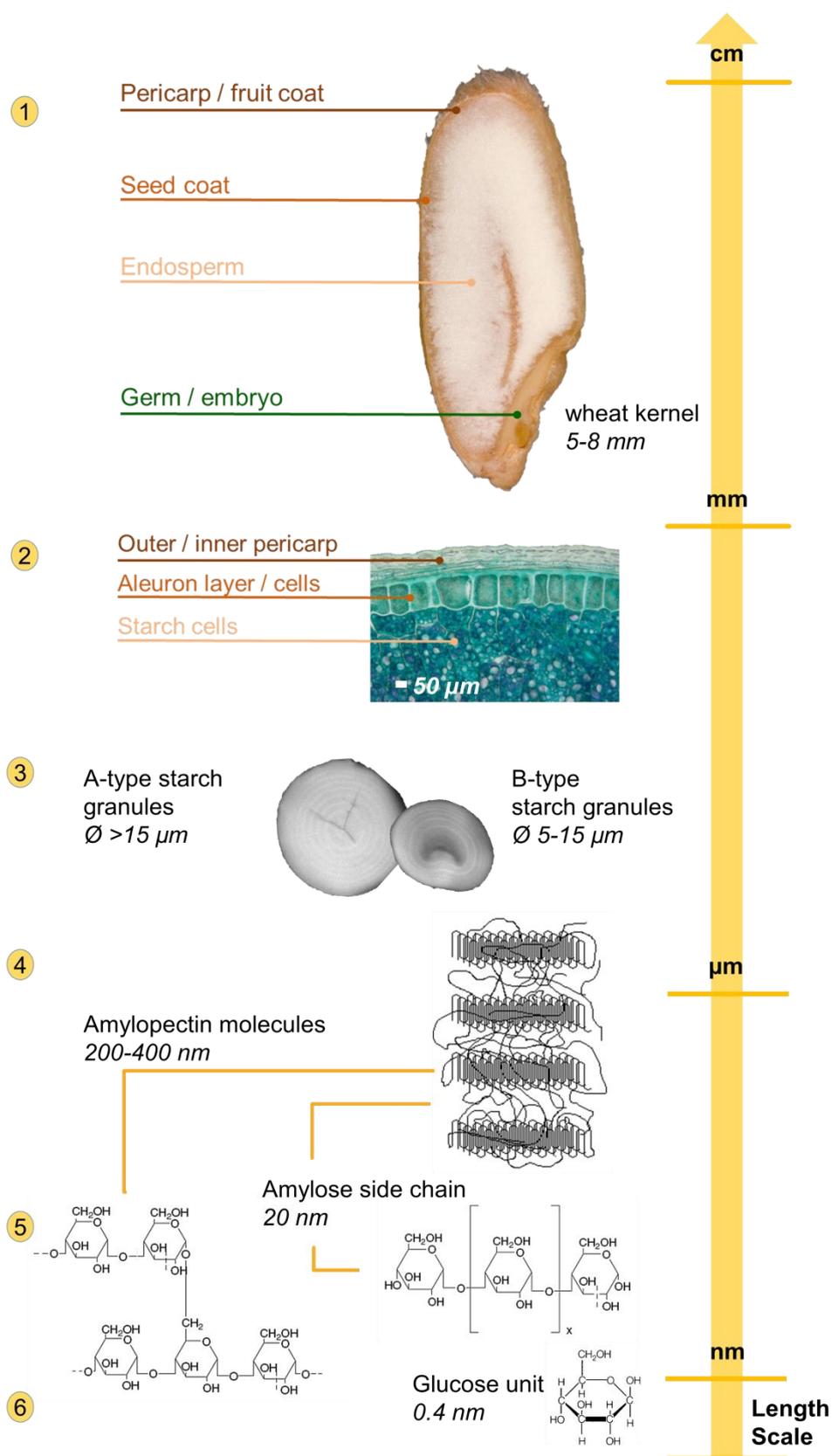


Figure 1: Length scales with the different levels of structural organization of wheat grain. Spanning six orders of magnitude: 1, wheat grain; 2, amyloplasts with embedded starch granules; 3, A/B-types of starch granules; 4, schemata of semicrystalline structure; 5, Amylose/Amylopectin chain; 6, glucose unit.

1.2 Starch structure and their classification in different levels

There are different possibilities to analyze starch structure and their influence on the end product texture combined with -quality. The term structure in combination with an order of magnitude (micro, macro) is often used to describe the structural properties of materials such as food (Jekle and Becker). Thereby, it is clustered in four scales which were based on the configuration dimensions: molecular-, nanoscopic-, microscopic-, and macroscopic-scale. In all scales starch structure could be analyzed in different ways and analytical systems.

For food production different modifications of starch sources are necessary. These treatments of starch can be classified into physical, chemical and physicochemical ones. Thereby, the thermal treatment, which is part of physicochemical changes is the most common one. It is applied on starch based products and is very important for structure fixation of bakery products. The most important benefit to end-product quality is a relationship between internal starch structure and the macroscopic properties of starch dependent changes. For example the amount of starch gelatinization, which is influencing the retrogradation properties is indirectly analyzed by the softness of bread crumb structure. The greatest challenge is to link physicochemical starch properties with information on different structure levels. Thereby, a classification of these typical scales (molecular, nano, micro, and macro) to control the end product quality is required. For a better understanding of this purpose to any starch based food an allocation into two clusters is applied in this thesis: the **analytical level** and the **product level**. The analytical level includes all analyzes which are not measuring directly the product structure. The product structure is depending on the production process and analyzed in the product level. Typically, the analysis in the analytical level is depending on specific process conditions like starch to water content and heating rate. Mainly the research is focused on the performance of one ingredient, like starch or protein. Contrary, in the product level all ingredients independent from production condition are determined. By the usage of typical analyzing systems both levels should represent the product texture combined with -quality. The objective of the combined execution of both levels is to achieve a significant product analyzes (see figure 2).

For an explanation, both systems are described in the following chapter, together with a detailed possible relation between starch structure and bread quality.

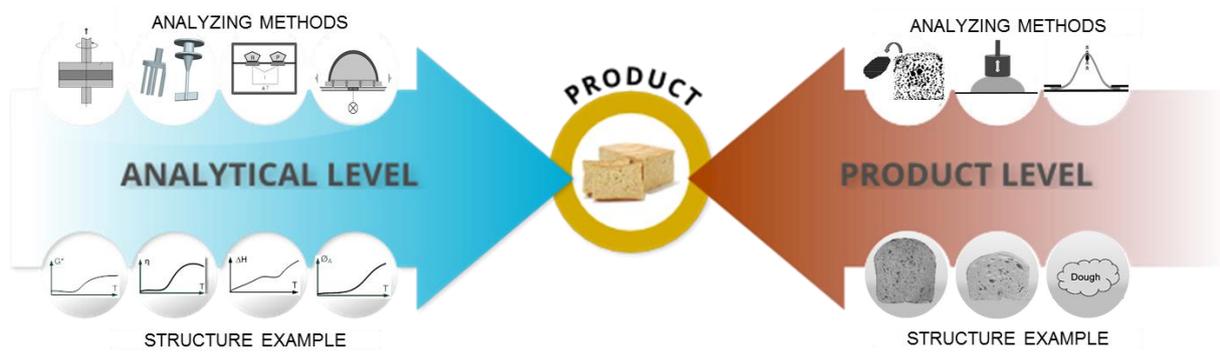


Figure 2: Clustering into analytical and product level. This example presents a wheat flour-water system, like wheat bread to point out corresponding analyzing methods connected to their structure results.



1.2.1 Consideration of analytical level

The quality assurance of raw materials is a central part of the production processes to allow constant food values. Thereby, the structural behavior analyzed before the production process is mandatory for a controlled product development.

In connection to the starch heating process a better knowledge about chemical composition of AM and AP, the starch formation (size, shape), -cluster (A- and B-type), and -structure (surface, starch damage) are necessary to relate these results to the end product.

In research, a huge amount of publications present relation between **AM/AP** distribution and heating parameters. For example, the molecular properties such as chain-length distribution, branch structure, molecular weight and gyration ratio were described (Yoo and Jane 2002). Additionally pasting properties, swelling power, solubility, and dispersed volume fraction measurement and gel stability are well known (Liu, Li et al. 2010; Sánchez, Dufour et al. 2010). Analyzes with a polarized light microscope on starch granules from beaked crumb samples results, that AM rich zones were found in center of starch granules, whereas the outer zones are rich in amylopectin (Hug-Iten, Handschin et al. 1999). For a detailed view to research, it was found out that increased AP results in a higher swelling power, a lower pasting temperature, a higher peak viscosity and poorer consistency of the cold paste (Abdel-Aal, Hucl et al. 2002), together with an increased gelatinization temperature and enthalpy (measured by DSC) (Hayakawa, Tanaka et al. 1997; Fredriksson, Silverio et al. 1998; Sasaki, Yasui et al. 2000; Abdel-Aal, Hucl et al. 2002). A general connection between analytical methods and the end product quality with a texture and sensory evaluation is difficult. In most cases there are only indirect statements to the end product. For instance an AP content increase offers a softer and stickier crumb with open and irregular pore structure and excessively volume shrink. Whereby, increased AM is responsible for setting of the finer crumb structure (Ghiasi, Hosney et al. 1984; Lee, Swanson et al. 2001; Bhattacharya, Erazo-Castrejón et al. 2002).

In addition to the chemical composition, starch granules of various botanical origin also differ in **formation** like (ellipsoidal, oval, spherical, polygonal, elongated, irregular, lenticular and disk) size (diameters ranging from about 0.1 to 200 μm), size distribution (uni-, bi- or polymodal) and occurrence in the amyloplasts (individual or compound) (Jane, Kasemsuwan et al. 1994; Pérez and Bertoft 2010) (see table 2). In dependence on starch gelatinization it is important to know about the specific character of starch granules, because of their differences in shape and size. Both are influencing the physicochemical characteristics of starch (Lindeboom, Chang et al. 2004). In general, in a starch mixture the small granule starch had a higher pasting temperature than large granule starch (Myllrinen, Autio et al. 1998; Pancha-arnon, Pathipanawat et al. 2008).

Table 2: Specific characteristics of starch granules from selected botanical origin (Lorenz 1990; Zheng 1997; Qian and Kuhn 1999; Tester and Karkalas 2002; Alvarez-Jubete, Auty et al. 2010).

Starch	Type	Distribution	Shape	Size (diameter) (μm)
Barley	Cereal	Bimodal	Lenticular (A-type) Spherical (B-type)	15-25 2-5
Maize	Cereal	Unimodal	Spherical/ Polyhedral	2-30
Millet	Cereal	Unimodal	Polyhedral	4-12
Rice	Cereal	Unimodal	Polyhedral	3-8 (Single) 150 (Compound)
Rye	Cereal	Bimodal	Lenticular (A-type) Spherical (B-type)	10-40 5-10
Oat	Cereal	Unimodal	Polyhedral	3-10 (Single) 80 (Compound)
Wheat	Cereal	Bimodal	Lenticular (A-type) Spherical (B-type)	> 15 5-15
Pea	Legume	Unimodal	Reniform (single)	5-10
Amaranth	Pseudo cereal	Unimodal	Polygonale	1-2
Buckwheat	Pseudo cereal	Bimodal	Polygonale	2-14
Quinoa	Pseudo cereal	Bimodal	Polygonale	0.5-3
Tapioca	Root	Unimodal	Spherical/Lenticular	5-35
Potato	Tuber	Unimodal	Lenticular	5-100

There exists further important knowledge about **A- and B-type** granules. It is well known that the large A-type granules are more crystalline than B-types (Chiotelli and Le Meste 2002) and B-types absorb a higher rate of water depending on higher surface-to-volume ratio (D'Appolonia and Gilles 1971; Petrofsky and Hosenev 1995; Stoddard 1999; Chiotelli and Le Meste 2002). The start of gelatinization and first peak enthalpy was in all cases behind the A-type granules (Eliasson and Karlsson

1983; Peng, Gao et al. 1999; Chiotelli and Le Meste 2002; Sahlström, Bævre et al. 2003). This lower enthalpy (measured by DSC) for the gelatinization of B-type granules suggests a lower percentage of organized arrangements or a lower stability of the crystals than in A-type (Chiotelli and Le Meste 2002). Higher lipid content for B-type granules is supported with an increased enthalpy of the transition of amylose-lipids complex. Which results in a greater enthalpy of the second endothermic transition for the B-type granules (Raeker, Gaines et al. 1998; Chiotelli and Le Meste 2002). The better stability of amylopectin observed in B-type granules may be related to the amylose-lipid complex formation during heating, which would obstruct the gelatinization process (Buléon, Colonna et al. 1998). Moreover, the endotherm of starch gelatinization represent essentially the difference between the endothermic energy, associated with granule swelling, melting of crystallites and the exothermic energy associated with hydration of starch and formation of amylose-lipid complexes (Kugimiya and Donovan 1981). Thereby, the greater amount of internal lipids in B-type granules (as well as better hydration) may generate a lower endothermic energy (enthalpy underestimation).

Soulaka and Morrison (1985) proposed the optimum range of 25-35 % B-granules for baking performance. Which corresponds with the statement that large starch granules (A-type) are related to gas cell stability, open pore structure of the crumb and causes pore cells coalescence (Hayman, Hosenev et al. 1998).

The **surface** of wheat starch including their protein film has been suggested to be necessary for the granule structure of starch. The proteins of starch granule surface are composed by storage proteins, starch biosynthetic enzymes, friabilin and puroindolines, 30 kDa proteins and 60 kDa starch granule bound starch synthase (Baldwin 2001). Proteins, especially in the surface of starch ghosts (gelatinized starch) and its importance for maintaining the integrity of the structures after gelatinization (“defined as gelatinized starch granule envelopes after the majority of internal starch polymers have been released”) was high concentrated in the shell of gelatinized starch ghosts (Han and Hamaker 2002). Changes of the composition in starch granule surface shows the possibility for influencing the rheological behavior and functional properties of starch systems (Larsson and Eliasson 1997; Barrera, Bustos et al. 2013). Thereby, the **starch damage** together with the enzyme activity especially α -amylase activity are two starch-surface-related values which are

influencing starch properties, gelatinization and thereby bread quality. Typically starch damage relates to mechanical damage of starch, for example obtained during milling (Liu, Ma et al. 2011). The level of the granular integrity depends on wheat hardness and milling technique. Damaged starch granules hydrate rapidly and are susceptible to enzymatic hydrolysis (Ranhotra, Gelroth et al. 1993). Increased starch damage shifts to lower gelatinization values like onset temperature and enthalpy measured by DSC (Morrison, Tester et al. 1994; Yoo and Jane 2002). Rheological analyzes of starch gelatinization results in reductions of peak viscosity, final paste viscosity, breakdown and setback by increased starch damage ratio (Barrera, Bustos et al. 2013). Dependent on the end product quality starch damage could be effects both - positive and negative. As mentioned, damage starch has much greater water retention capacity, which could be positive for storage properties of bread as well as negative in dependence on dough stickiness and proofing stability. Additionally, by a rise of hydration speed the gelatinization process is also increasing and therefore three main phenomena: The crust coloration will be more intense depending on caramelization and maillard-reaction. The texture of dough and crumb increase in stickiness due to excessive starch hydration and the volume of bread can be improved providing that the retention of fermentation gas is controlled.

Beside these structural behavior analyzed before the production process, connected to evaluation systems like the DSC, mathematical models for a quantitative evaluation of the starch gelatinization process exist. These models should support information about physicochemical changes in bread during baking process.

For modelling starch gelatinization most authors refer to follow principle kinetic model:



where **A** represents **ungelatinized starch**, **S** **swollen granules** and **G** **solubilized/gelatinized starch**. The **reaction rate constants** k_1 and k_2 according to Arrhenius equation depending on temperature. Both phase changes are irreversible and follow the first-order kinetics:

$$\frac{dC_A}{dt} = -k_1 C_A \quad (2)$$

$$\frac{dC_G}{dt} = -k_2 C_S \quad (3)$$

If the initial **concentration of ungelatinized starch** is C_{A0} the kinetic model is:

$$\alpha = \frac{C_G}{C_{A0}} = 1 + \frac{k_2}{k_1 - k_2} e^{-k_1 t} + \frac{k_1}{k_2 - k_1} e^{-k_2 t} \quad (4)$$

where α is the **gelatinized starch fraction**.

According to recognized publications, modelling of starch gelatinization kinetics in starch to water systems follows additionally a first-order kinetics:

$$(1 - \alpha) = e^{-Kt} \quad (5)$$

where K is the **reaction rate constant** and t is the **time**. The reaction rate constant (K) depends on temperature, according to the Arrhenius equation:

$$K = K_0 e^{-\frac{E_a}{RT}} \quad (6)$$

where K_0 is the **reaction frequency factor**, E_a is the **activation energy**, R is the **gas constant** and T is the **absolute temperature**. Whereby, the activation energy (E_a) is in a temperature range of 50-100 °C between 59 and 306 kJ mol⁻¹. Zanoni, Schiraldi et al. (1995) calculated from calorimetric data (DSC) according to the Arrhenius equation a $K_0 = 2.8 \cdot 10^{18} \text{ s}^{-1}$ and $E_a = 138 \text{ kJ mol}^{-1}$.

Thereby, the extent of **gelatinized starch fraction** is depending on a specific time interval:

$$\alpha_t = 1 - (1 - \alpha_{t-\Delta t}) e^{-K_T \Delta t} \quad (7)$$

This model can be applied both to experimental temperature profiles and to temperature profiles calculated at each site within the product according to the model for heat and mass transfer described above. In the former, Δt is equal to the time interval between two temperature measurements; in the latter, Δt is equal to the time interval applied to solve equations of Zanonis` model (Zanoni, Peri et al. 1995).

Based on these initial studies new work was done to correlate these studies with models used to predict heat and water transfer during bread baking (Zanoni, Pierucci et al. 1994; Zanoni, Peri et al. 1995; Zanoni, Schiraldi et al. 1995). Additionally to the analytical level all these models need a relation to the product level. Because of the dependency on specified analyzing system the molecular mechanism is uncertain.

Typically, these structural changes as well as mathematical models are performed and described in specific analyzing system and medium. As already mentioned the effect of gelatinization on a medium depends on several physicochemical values. Additionally all analyzing methods typically run without recipe additives. Thereby, the major impact is from process parameters and specific starch water medium. The overlapping as well as a mixture of these two groups results in typical physicochemical interaction of structural starch changes. Typically the **heating rate**

depending on heating time and temperature, has a major effect on gelatinization. Faster heating of a starch suspension results in a rise of granule swelling by reaching their maximum swelling and contribution to viscosity before their disruption begins to disturb the viscosity (Huberlant 2003). Besides the granule swelling other pasting properties like rise of gelatinization temperature as well as a declined enthalpy are affected by higher heating rates (Karapantsios, Sakonidou et al. 2000). Those results in a smaller amount of leached amylose into the extragranular matrix together with a weaker amylose network formation (Palav and Seetharaman 2007). Both could be influence the heat transfer into the system as well as contrasts with evaluation respectively calculation of main values. Next to time/thermal impacts, the pasting properties are connected on shear characteristics with the **shear rate**. Native, unswollen starch granules are usually not cracked in the suspension before cooking and can be safely dispersed with high speed mixing or homogenization. In this way, so called “shear thickening” or “dilatants flow” of starch water systems happens. Shear thickening behavior of non-Newtonian flow like starch suspension is described by particle hydro clustering. These hydro clusters are created by domination of hydrodynamic forces over interparticle forces at large shear rates and stresses (Ptaszek 2010; Crawford, Popp et al. 2013). Experiments on “flash” gelatinization showed a strong influence of initial mixing conditions on pasting. Different mixing rates results in typically textural changes of the analyzed material (Karapantsios, Sakonidou et al. 2000). An increase of mixing rate during heating accelerates the heat transfer and shear itself can enhance swelling of highly cross-linked granules (Karapantsios, Sakonidou et al. 2000). This results in an increased paste viscosity after shearing (Kuhn and Schlauch 1994) and may be due to a progressively better fluid agitation together with a better heat transport from the heater to the suspension (Karapantsios, Sakonidou et al. 2000). Contrary to shear thickening at ambient temperatures, an increase of granules disintegration by physical interaction like shear rate is caused, if starch granules already start to swell by temperature rise. Thereby, swollen partly gelatinized starch granules can be disrupted by shearing, resulting in a loss of viscosity and textural stability. If the shear rate is too high, it can reduce the final viscosity (shear thinning) by fragmenting granules (Doublier 1981). These effects are rising by lower water content, high temperature and lower pH value.

In compliance to these knowledge the relation between starch properties and bread-baking performance are not fully developed. Typical mathematical models are calculated in dependence to molar mass and a specified starch or flour system, which is not transferable to changing cereal quality and process parameters. Additionally there exist no parameter depending on recipe components. However, for bread production recipe components with their influences and impact on starch gelatinization are important. To give an overview following chapter summarizes the influence on starch gelatinization of most relevant recipe component of the bread production considered on analytical level.

1.2.2 Recipe/dough components and their influences and impact on starch gelatinization

For the production of wheat bread the understanding of complex interaction between the main recipe/dough components is important. Small amounts of added ingredients are to enhance dough performance during food processing and improve the quality (texture, sensory and shelf life) of backed bread. To analyze the rheological and technological effect it is important to have a huge fundamental knowledge about wheat flour ingredients and the processing of starch-based foods. Without a consideration of interaction between recipe components the relations between additives and starch is reviewed. In case of wheat bread there exist a huge amount of different components like proteins, lipids and salts, which interact with starch during heating. Their main influences on starch gelatinization and the end product quality is specified in the following chapter.

Proteins are an important compound in a lot of baked goods. Basically they are separated in external- and cereal based proteins. In wheat bread processing the cereal based proteins are more important which have a high range in wheat flour between 7 to 15 % (Belitz, Grosch et al. 2008). Cereal based proteins could also be distinguished based on their different functions. On one hand there are the non-gluten proteins (15-20 % of total wheat protein) playing a minor role in bread making and on the other hand the gluten proteins (80-85 % of total wheat protein), which are indispensable for the structure in all baked goods. For further characterizing the wheat proteins and their quality differences, they are splitted in four groups through

Osborne fractionation: albumins (soluble in water), globulin (soluble in salt solution), gliadin (soluble in 70 % ethanol), and glutelin (partially diluted in acid or base). The unique property of gluten-wheat proteins is its ability to porous achievement and the essential volume increase while production of wheat bread (Auger, Morel et al. 2008; Jiang, Kontogiorgos et al. 2008). The wheat gluten proteins are most responsible for the formation of a continuous visco-elastic gluten network structure while bread making. Gluten-network is formed by energy input, it typically emerges during the mixing process, between gliadin, glutenin and lipids (Murray 2011). During baking process a combination of changes in protein surface hydrophobicity, disulphide interchanges and formation of new disulphide cross-links proceed (Weegels, de Groot et al. 1994; Lavelli, Guerrieri et al. 1996).

The interaction between starch and proteins is very specific and profound. A correlation between swelling behavior, shear sensitivity, and protein content of starch was found in literature, but is not specified (Eliasson and Tjerneld 1990). The proteins inhibit the swelling of starch in a water system and thus retard the gelatinization of starch. A possible solution is the competition of proteins and starch for the available water. The proteins delay the pasting process by increasing the gelatinization temperature and decrease the gelatinization intensity with a non-specified correlation to the baked bread (Micard and Guilbert 2000; Stathopoulos, Tsiami et al. 2006).

Lipids could be classified mainly in two parts of origin: non-starch lipids (from membranes, organelles and spherosomes) and starch lipids. Both, starch and non-starch lipids have important functional interactions in food systems. Mainly non-starch lipids are used at the dough mixing process and enhance the bread making manufacturing. While processing two main effects are observable. First, the non-starch lipids 'bind' on gluten or the starch granule surface. Whereby, the unbranched $\alpha(1\rightarrow4)$ -glucan chains from helices with a hydrophobic interior interact with small non-polar molecules and hydrophobic domains of amphiphilic molecules such as fatty acids, monoglycerides and surfactants (BeMiller and Huber 2000). Secondly, polyunsaturated fatty acids are oxidized by wheat lipoxygenase, yielding hydroxyperoxides and free radicals. Besides that they affect dough rheological properties and crumb color (Hoseney 1994). In fact of heating process, lipids can disturb granule swelling, presumably by occluding the starch and by this prevent

hydration. They accelerate pasting properties and lower the temperature at which the starch develops its maximum viscosity (Deffenbaugh and Walker 1990).

Lysophospholipids, in particular lysophosphatidylcholine or lysolecithin, are the major constituents of the starch lipids. The minor components described by amylose-lipid complexes are positively correlated to amylose content (0.8-1.2 % for wheat starch) (Morrison and Gadan 1987; Villwock, Eliasson et al. 1999). Generally, amylose-lipid complexes can be naturally present in starch or can be formed during pasting of starch in presence of lipids (Evans 1986; Morrison, Tester et al. 1993). The chain length of amylose and lipid influences the hydrolysis of starch and thereby the gelatinization (Copeland, Blazek et al. 2009; Alsaffar 2011). Granule swelling and solubilization is delayed, if the complexation takes place with amylose and increase the gelatinization temperature (Ghiasi, Hosney et al. 1982; Tang and Copeland 2007). The amylose-lipid complexation and the amylose crystallization are responsible from the amount of lipids (Eliasson and Wahlgren 2000; Tufvesson, Skrabanja et al. 2001). The reduction of amylose leaching upon the formation of amylose-lipid complexes can reduce inter-granule cohesion and hardness of granules which lead to a softer crumb and increase the shelf life in bread (Chinachoti and Vodovotz 2001).

Focused on salt addition, there is a competition of **salts** (like sodium- and calcium chloride) and starch for the free available water in the system. However, the effect of salts on starch gelatinization is not solely due to the decreased availability of water. At excess and limited water conditions higher sodium chloride (NaCl) concentration (up to 7-9 % w/w total) leads to a decreased gelatinization enthalpy and a higher gelatinization temperature (Wootton and Bamunuarachchi 1979; Chungcharoen and Lund 1987; Lii and Lee 1993; Chiotelli, Pilosio et al. 2002; Day, Fayet et al. 2013). Wootton and Bamunuarachchi (1980) explained the decrease in enthalpy could arise from the influence of sodium and chloride ions in water on starch and their interactions. The rate of starch retrogradation correlates with the moisture content (Beck, Jekle et al. 2011). Therefore, by decreasing water mobility, salt reduces the rate of water migration from the crumb to the crust. This results in a more hydrated system, which stales at a lower rate when compared to a system where no salt is present (He and Hosney 1990). In case of salt reduction the effects of other salts are of interest as well, even if not commonly encountered in food. For example

sodium sulfate increases the gelatinization temperature of potato starch from 62 to 80 °C, while sodium bromide reduces it to 44 °C (Sudhakar, Singhal et al. 1992). DSC measurements from Beck, Jekle et al. (2011) showed that gelatinization values are influenced primary by the characteristics of cations. Whereby the ions have interplay to starch and could be explained by the hydration effect. The energy of hydration depends on the diameter and the charge of cations (Ahmad and Williams 1999; Maauf, Che Man et al. 2001; Viturawong, Achayuthakan et al. 2008). An increase of solvation is depending on increase charge and decrease ions (Marcus 1991; Beck, Jekle et al. 2011). Differences in hydration shell formation by various solutes is suggested to be one of the most important factors of starch gelatinization (Jane 1993).

Only few research articles reported about the influence of NaCl on insufficient water systems like extruded snacks or breakfast cereals. Day, Fayet et al. (2013) analyzed the effect of NaCl concentrations (1, 2, 3 and 4 % w/w total) on different starch to water ratios (s:w from 1:0.45 to 1:0.25). Small reduction of water content typically has strong influence on the gelatinization temperature of starch. A reduction of water below 1:0.35 does not influence the gelatinization process with a salt concentration > 2 % (w/w total). With no regard on the water content, the interaction of Na⁺ with the hydroxyl groups of starch reduces the initial swelling of amorphous regions. Summarized, salts - especially cations - regulate the thermal transitions of starch resulting in a rise of gelatinization temperature with decrease of gelatinization intensity.

In summary, when recipe components such as proteins, lipids, and salts are added to the aqueous phase of starch-based food, the pasting properties change. The intensity of this influence correlates with the starch to water content. However, there is a huge research residue of process understanding for limited and insufficient water contents, in dependence to typical analyzing methods. As already mentioned all typical systems are depending mostly on water excess.

PRODUCT

PRODUCT LEVEL

1.2.3 Consideration of product level

The structure and phase transitions of starch are important aspects which have a profound influence on macroscopic attributes such as texture, sensory, appearance, water-holding capacity (retrogradation) and process stability (Biliaderis 1991). The consumers' acceptance of wheat products like bread is very strong related to sensory attributes depending on the bread texture and therefore on the product level (Alina Surmacka 2002).

Wheat bread production is a complex process because of the interaction of single components. The typical bread production process can be divided into four main process parameters (see figure 3): dough processing (mixing), molding (piece dough processing), proofing and baking. Dough processing, molding and proofing are a part of reversible one whereby the baking process is known as an irreversible process step.

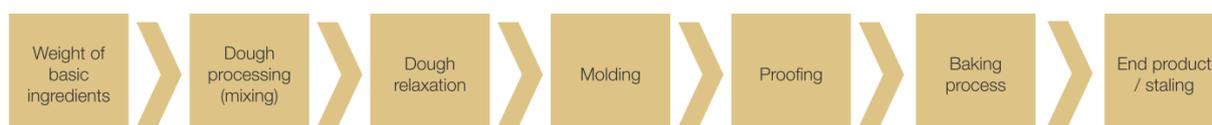


Figure 3: Main steps of typical wheat bread production

While all these production steps, analyzed on the product level are possible. Whereby, baking is the crucial part determining the transformation from dough/paste to the final product. Baking is characterized to stabilization of a porous structure by altering the molecular configuration of the polymeric components in the cell walls through the application of heat. In addition it is the final and mostly irreversible production process, which includes protein denaturation and starch gelatinization. With a rheological approach it converts viscoelastic dough into elastic bread which is depending on mentioned process parameters and recipe components. The main product transformations are the increase of volume by gas expansion, color

formation and the porous structure by crust and crumb fixation initiated by protein denaturation and starch gelatinization (Sablani, Marcotte et al. 1998). Bread and thereby all processes around baking can be clustered as a system containing three different regions (Purlis and Salvadori 2009):

- (1) Crumb: inner zone (wet), where only a slow dehydration occur and temperature is $< 100\text{ }^{\circ}\text{C}$
- (2) Evaporation front: between inner and outer (crumb and crust), where water evaporates and temperature is $\leq 100\text{ }^{\circ}\text{C}$
- (3) Crust: outer zone (dry), where dehydration takes place and temperature is $> 100\text{ }^{\circ}\text{C}$

A common approach is to consider parameters of the end product to conclude starch gelatinization values. But all analyzes which are done after the baking process could be only used as an indirect measurement of starch gelatinization.

For analyzing the macrostructure of baked bread a lot of different methods are already established. Typical systems are specified for measuring volume, crust and crumb features which all depend on starch gelatinization but without a significant correlation. Former publications showed the possibility of crust analyzing systems and established already a system for characterizing crumb thickness, crispness, volume and color. Especially the volume and crust color are typical for a non-destructive characterization of the product quality (Zanoni, Peri et al. 1995; Schirmer, Hussein et al. 2011). For structure analyzes on mouth feeling or storage values, mainly the crumb texture and crumb porosity are important. Thereby, two systems are useful tools: A typical texture analyzing system for structural features like firmness, stiffness and stickiness as well as a digital pore analyzing system for features like pore size, -distribution and -count. The starch gelatinization is apparently necessary for the change in gas retention from the dough during heating. Based on dynamic rheological properties from the dough during baking and the microscopic structure of baked bread these changes were analyzed especially at typical gelatinization temperatures (He and Hosney 1991). As an example figure 4 shows macro structure analysis of crumb firmness depending on baking time with different water addition and baking temperature. These results of crumb texture analysis present a linear increase of the rate of firmness during baking, which depends on baking temperature and starch to water ratio, which are obtained by the starch gelatinization process.

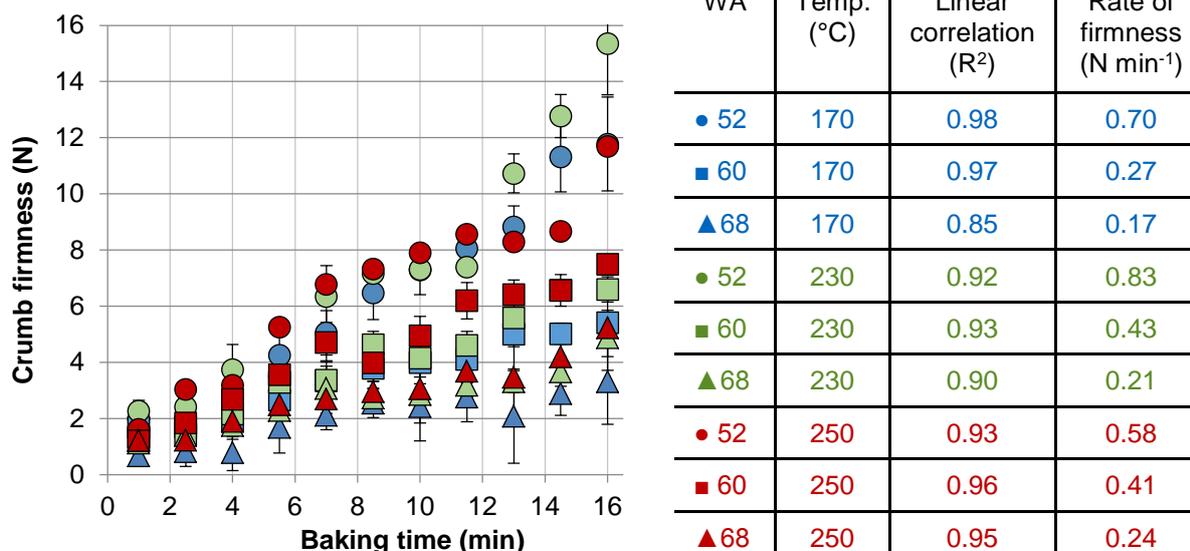


Figure 4: Examples of a significant linear correlation ($p < 0.005$) of crumb firmness depending on baking time with different water addition (WA depending on 100 g_{flour}) and baking temperature ($n=5$) (Schirmer, Jekle et al. 2012).

All these macroscopic features are indirect methods for the evaluation of process parameters and changes of raw material properties, which are influencing the end product. As already mentioned there exists no adequate possibility for analyzing starch changes while baking. There exist only statements without an analytical background, like the description of the oven rise by the start of gelatinization temperature (onset) (Eliasson and Larsson 1993).

There exist already 'models' which distinguished how important the consideration of starch gelatinization is. By a three-dimensional computational fluid dynamic (CFD) model of starch, a gelatinization model for bread baking was analyzed (Therdthai, Zhou et al. 2004). Thereby, the degree of gelatinization was only used to assure the completion of baking under various oven operating conditions. A partial least squares regression (PLS) model using results from grain and flour analysis to evaluate bread characteristics, results with the best variance including flour pasting properties (Sahlström, Bævre et al. 2003).

Mathematical models typically depend on experimental and theoretical approaches to describe the simultaneous heat and mass transfer in a porous medium. Therefore, some efforts have been made to understand the baking process. One of the most important theory is the evaporation and condensation inside the pore structure (Sablani, Marcotte et al. 1998; Purlis and Salvadori 2009; Schirmer, Jekle et al.

2012). Which was theoretically modeled by Mack, Hussein et al. (2013). The process of this model can be divided into four steps:

- (1) Water evaporates at warmer side of foam bubbles, absorbing latent heat of the vaporization
- (2) Vapor moves through the gas phase
- (3) Vapor condenses at the colder side of the foam bubble, setting free its latent heat
- (4) Heat and water are transported by conduction and diffusion, respectively, through a dough membrane to the warmer side of the next foam bubble, where the series of processes can start all over again

Thermodynamically considerations on product level focus on heat convection and conduction, and moisture migration due to diffusion and convection to describe physical, chemical and structural properties of products. Thereby, several studies have been done to find correlations between product quality and baking settings from an experimental design. As shown in the schematically description of figure 5 the inner crumb structure for example is build up by an asymptotically temperature from 90 to 100 °C while the surface tends to the oven temperature (Zanoni, Pierucci et al. 1994; Purlis and Salvadori 2009; Schirmer, Jekle et al. 2012; Mack, Hussein et al. 2013). The heat and water transfer in the product depends on the gas expansion of the dough system (volume increase) (Hadiyanto, Asselmann et al. 2005). In most models the importance of physicochemical components changes like the starch gelatinization is underestimated. These chemical modified components influence the physical parameters primary by changes of the water content, heat flux and density. In dependence there exist already models for simultaneous heat and mass transfer in bakery products to describe the complex mass and heat phenomena inside the product during baking (Hadiyanto, Asselmann et al. 2005). Additionally Therdthai, Zhou et al. (2002) described by mathematical models the effect of the baking temperature profile and time on the weight loss, crust color and internal temperature. All studies are with a promising model to predict the product quality but without the relationship to the microscopic scale (like starch component). The problem is that typically all these models are applied with the assumption that all parameters are constant during baking (Hadiyanto, Asselmann et al. 2005). There is a possibility for further work to increase the variable dependent parameters like baking temperature

and water content etc., but without a possibility to validate the huge amount of different raw material and their composition.

The effect of different gelatinization profiles on final bread quality is still unknown and very difficult to analyze. In summary, much of the research has focused on experimental and/or mathematical modeling of thermal and water aspects in the baking process as well as into the product. Only limited amount of published papers exist on quantitative evaluation of physicochemical changes in bread during the baking process.

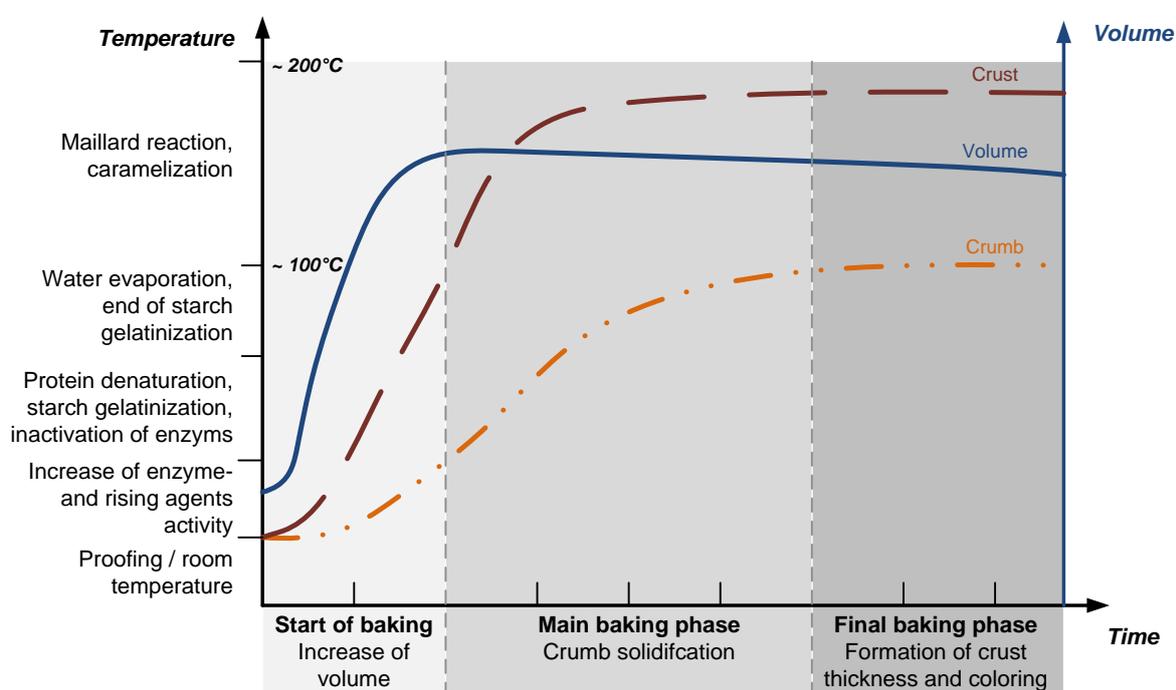


Figure 5: Schematically description of the transition from dough/paste to the baking product differentiated temporally in three main phases.

1.3 Thesis outline

The previous chapters pointed out that starch granules - next to protein - are the main component of wheat flour and their gelatinization induces major structural changes during bread baking. These changes are however highly depending on physicochemical properties. Exogenous and endogenous factors have a great influence on the individual constituents of wheat flour including starch and therefore also on the baking values and the end product quality. Wheat bread seen from physicochemical point of view has a defined structure exhibiting a low-viscosity. It is characterized as a complex system due to interaction of single components during thermal treatment. Whereby, the process of gelatinization in limited water systems like dough/bread influences, considering parameters such as recipe constituents, preparation and baking conditions, of the final product attributes. In the production process, baking is the crucial step determining the transformation from dough to the baked product. The application of heat alters the molecular configuration of the polymeric components in the cell walls resulting in the stabilization of the porous structure. To consider these uncontrollable factors different approaches applied on raw material including analytical and product based measurements were discussed in the introduction section. In case of the molecular level huge information about structural changes of starch while heating (in excess of water) can be gained. Typically, these information`s do not exist for a complex systems including bread, in which starch is surrounded by other components. These components as demonstrated in the previous chapter have a huge influence on the gelatinization of starch. These problems can only be overcome if the gelatinization of starch is investigated on the macroscopic level where starch is incorporated in a matrix of other components. However, all known methods can only measure the gelatinization in a complex product without the application of a model function. Due to a lack in knowledge about the actual causes and extend of changes in the product evoked through changes in starch structure measurements on the microscopic level are required. A connection between both levels and their analyzing systems makes correlations between structural changes of the starch and the end product quality almost impossible. In order to find a possibility for analyzing these modifications, an accurate definition of the starch gelatinization process is prerequisite.

For this purpose new methodologies for visual and microscopic detection of starch changes could achieve the target. Typical visual analyzing methods are not able to

determine in situ starch properties like the gelatinization temperature. They are usable for analyzing main components of complex food structure such as starch and protein however they are not leading to quantitative factors. By employing special image analyzing tools, established for medical technology, more information can be obtained than from conventional probing techniques (Jekle and Becker 2015). Out of this, the confocal laser scanning microscope (CLSM) enables a noninvasive way to obtain these information. Additionally, the CLSM presents a powerful tool to achieve increased sharpness and therefore well-resolved images from selected levels as well as a three dimensional outlook of the samples. The CLSM is already a frequently applied method to visualize starch gelatinization 'offline' (Moore, Juga et al. 2007; Primo-Martín, van Nieuwenhuijzen et al. 2007; Alvarez-Jubete, Auty et al. 2010; Jekle and Becker 2011). Depending on these research knowledge the following study questions were formulated:

- *Is the morphological characterization of starch by CLSM a useful tool for digital micrograph analyzes?*
- *Which differences of starch properties exist between starch systems with excess water and limited water (dough/bread products)?*
- *Can thermally induced changes of starch be analyzed during thermal treatment?*
- *Could an analyzing system, depending on 'real' recipe parameters and composition give information about the starch gelatinization?*

These fundamental research questions are strongly linked to baking problems. Concerning the global growth of the baking industry, two main characteristics are of special interest. This is on the one hand side the standardized product quality and on the other hand the energy consumption. In both cases the baking process designates the most important step during the production. The in-depth understanding of the product alteration while heating enables a better control of the process. A relevant practical example (depending on energy and product quality) should underpin these statements.

A typical problem in toast-bread production is the so called "waist-formation" of the bread where the sides shrink to the middle. To solve this problem, which is assumed to be caused by an insufficient gelatinization of starch, empirically the baking time and temperature are increased. However, until now no prove of this assumption is

existent. In order to verify this the analysis and the understanding of the starch gelatinization under real conditions is required. This could allow a better controllability and suggestibility of the gelatinization process by the adaptation of the baking conditions. Further also leading to an increased knowledge about the influence of recipe components on phenomena such as the waist-formation. With the obtained knowledge the baking time and temperature could be adjusted precisely in order to save costs as well as to ensure a constant product quality and decrease the food waste depending on production.

Consequently is the main objective of this thesis to develop an in situ analyzing system allowing to understand and evaluate the structure of complex food systems and especially their transformation processes during heat treatment. To develop this target an online system combined with a digital analyzing tool would be necessary.

To achieve this main objective the following research steps, further discussed in the next chapters of this thesis, were required:

- (I) The definition of thermal induced structural changes of starch and their transmissibility to analytical methods
- (II) Visual and morphological characterization by micrograph analyzes using CLSM of variable amylose / amylopectin ratio
- (III) Development of a new analyzing system suitable for highly viscose products, for a better understanding of micro-structural and thermal aspects
- (IV) Further development of the new in situ analyzing system allowing to determine the gelatinization temperature in a variety of products with limited water content

2 Results

2.1 Summary of results

The publications are summarized in this chapter, followed by the copies of the individual publications.

Part I <i>Chapter 2.2</i> <i>Page 31-42</i>	Starch gelatinization and its complexity for analysis
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The term “gelatinization” of starch generally describes an irreversible structural change observable on all product design scales ranging from micro to macro level. These structural transformations of starch during thermal impact and in following production steps are highly depending on several different aspects, which are however not specified in a sufficient manner.

In order to achieve a better understanding of these heat induced changes it is necessary to cluster the influencing aspects into the following two categories, namely raw material properties and process parameters. The development of physical analytical methods with their corresponding gelatinization aspects in relation to their process parameters is illustrated in the following chapters. Based on the current knowledge it becomes apparent that no analytical system is present allowing to investigate starch gelatinization and the resulting structural changes on different length scales in food products. Therefore, the application of a specified non-invasive online analyzing system is recommended to follow the starch gelatinization within a complex food matrix.

Part II <i>Chapter 2.3</i> <i>Page 43-54</i>	Physicochemical and morphological characterization of starch with different amylose / amylopectin ratio
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Starch-rich raw materials are widely used in the food industry. Their functionality and end-use applications are markedly influenced by starch characteristics. Starches with varying amylose (AM) and amylopectin (AP) content are of particular interest due to their ability to influence and modify the texture, quality and stability of starch-based food products. The present study shows the influence of the AM/AP content on physicochemical and morphological properties of a range of starches (Maize = 3 %, 23 %, 71 %; Potato = 2 %, 21 %; Wheat = 28 %; Barley = 3 %, 25 % AM content w/w of starch).

Starches have been analyzed in terms of their chemical composition, water retention capacity, morphological characteristics, and pasting/thermal properties. The changes in starch granule morphology during gelatinization were monitored by Confocal-Laser-Scanning-Microscope (CLSM). The different analysis revealed that waxy-starches (AP > 90 %) had a high water retention capacity (1.2-1.5 times higher) and developed higher paste viscosities (up to 40 % for maize; 43 % for barley). The swollen granules were highly susceptible to mechanical breakdown and solubilized faster. Higher AM contents showed inhibition of an extensive granule swelling and lowered the paste viscosity. The exceptional integrity of the high-AM starch even prevented its gelatinization at atmospheric pressure. Significant differences in physicochemical and morphological properties between the starches from regular, high-AM and waxy strains have become evident, no direct relationship between the AM/AP contents and the internal growth ring structures of the starch granules could be identified by CLSM. The waxy starches had a higher gelatinization temperature (up to 2 °C) and enthalpy (up to 20 %), which indicates a higher crystalline and molecular order.

Part III <i>Chapter 2.4</i> <i>Page 55-62</i>	Quantification in starch microstructure as a function of baking time
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The purpose of this study was the characterization of micro structural and thermal aspects of starch gelatinization in wheat dough/crumb during bread baking. The microstructure of starch granules was examined by confocal laser scanning microscopy (CLSM) and evaluated by an image analyzing tool. Supporting crystallinity changes in wheat dough/crumb were analyzed by differential scanning calorimetry (DSC) and calculated by the content of terminal extent of starch gelatinization (TEG).

The micrograph of processed CLSM data showed starch structure changes during baking time. After gelatinization the starch fraction itself was inhomogeneous and consisted of swollen and interconnected starch granules. Image processing analyses showed an increment of mean granule area and perimeter of the starch granules. The results of DSC were examined to present an equation which provides a mean of predicting TEG values as a function of baking time. CLSM and DSC measurements present high significant linear correlation between mean starch granule area and TEG ($r = 0.85$).

The possibility to combine CLSM with thermal physical analytical techniques like DSC in the same experiments is useful to obtain detailed structural information of complex food systems like wheat bread. Finally, it offers the option to enlarge the knowledge of microstructural starch changes during baking in combination with physicochemical transformation of starch components.

Part IV*Chapter 2.5**Page 63-72*

In situ monitoring of starch gelatinization with limited water content using confocal laser scanning microscopy

The gelatinization of starch is crucial for the production of bakery products. Therefore, the examination of the characteristics and the extent of this process are of fundamental importance for research and product development. Typical analysis methods for study structural starch changes during heating are performed in excess of water. Considering that wheat dough is a complex system with reduced water content an option to analyze starch gelatinization under actual product conditions is missing. Therefore, an in situ method in a confocal laser scanning microscope (CLSM) equipped with a heating system was developed to monitor the starch gelatinization in samples with different flour to water ratios ((m/v) 1:0.39, 0.49, 0.51, 0.53, 0.55, 0.57, 0.59, 0.98, 4.91, 5.63 and 7.14). The new method was used to investigate the start of starch gelatinization temperature (T_G) by using thresholds-based, on first derivatives with highest correlation between T_G of CLSM and onset temperature (T_o) of differential scanning calorimetry (DSC). A highly significant linear correlation between T_G of A-type granules and T_o was observed ($R^2 = 0.903$). The size and shape of granules with sample 1:0.98 shows a clear impairment against samples with water content above. The newly modified in situ method allows the measurement of starch gelatinization in different flour to water ratio independent of secondary factors. Consequently, it can be used for complex starch-based-system with reduced water content to investigate the actual starch granule structure disintegration.

2.2 Starch gelatinization and its complexity for analysis

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REVIEW

Starch gelatinization and its complexity for analysis

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The term “gelatinization” of starch generally describes an irreversible structural change observable on all product design scales ranging from micro to macro level. These structural transformations of starch during thermal impact and in following production steps are highly dependent on several different aspects, which are, however, not sufficiently specified. In order to achieve a better understanding of these heat-induced changes it is necessary to cluster the influencing aspects into the following two categories, raw material properties and process parameters. The development of physical analytical methods with their corresponding gelatinization aspects, in relation to their process parameters, is illustrated in this review. Based on the current knowledge it becomes apparent that no analytical system is present which would allow the investigation of starch gelatinization and the resulting structural changes on different length scales in food products. Therefore, the application of a specified non-invasive online analyzing system to follow the starch gelatinization within a complex food matrix is recommended.

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Confocal laser scanning microscopy / Differential scanning calorimetry / Physical methods / Physicochemical changes / Rapid visco analyzer / Thermal methods

1 Introduction

As functional ingredient, carbohydrates take a special position in many types of food [1]. However, particularly complex carbohydrates such as native starch are poorly digestible without thermal treatment during production inducing an irreversible structural change of the desired final product [2]. Thus the gelatinization of starch is provoked by many food processing operations including, amongst others, the hot-extrusion of cereal based products, baking process of bread, and pastries as well as cooking of sauces and fillings.

Until now a lot of research has focused on thermally induced physicochemical changes of the starch structure

referred to as gelatinization [3–6]. The progression and the extent of gelatinization is primarily determined by raw material properties as well as the applied product parameters (see Fig. 1). From the side of the raw material properties the gelatinization process is influenced by characteristics of the starch granules: composition, morphology, molecular architecture, and molecular weight [7–9]. Beside these, for the production of the final product additional ingredients are added incorporating different components majorly influencing the gelatinization process [10, 11]. Furthermore, the addition of water is of particular interest, because the starch-to-water ratio determines the extent of gelatinization [12, 13]. Most studies focused on systems with an excess of water, although in food systems the water content is limited or insufficient [14]. Only few studies considered the effect of different water contents on starch gelatinization [12, 15–17]. In addition to the material properties, the methodology itself and thus the manner in which the structural changes of starch are studied has a major influence on the gelatinization due to the different process parameters.

A wide range of analytical systems offers the possibility to analyze structural changes of starch, all differing in mode of application and construction. Changes of the process

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Abbreviations: BEPT, birefringence end-point temperature; CLSM, confocal laser scanning microscopy; DSC, differential scanning calorimetry; LM, light microscopy; PLM, Polarized light microscope; RVA, rapid visco analyzer

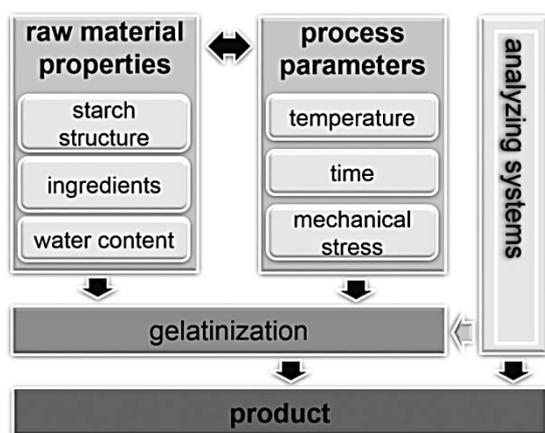


Figure 1. Influence of material properties and process parameters on the progression and extent of gelatinization. Physical analytical systems are used to investigate the gelatinization process and product conditions without correlation to process.

parameters such as heating and/or shear rate of the system influence the gelatinization characteristics and thereby also impede an independent interpretation of the individual effects [18–20]. All of these factors influencing the gelatinization characteristics are of further importance as they affect food production resulting in changes of the product conditions such as the product texture, saccharification as well as other quality values.

Therefore this review aims to evaluate the state of knowledge in the field of starch gelatinization characteristics. Furthermore, it describes the physical analytical methods with their gelatinization aspects in relation to the process parameters.

2 Molecular, granular, and physicochemical structure of starch

The stability, transformation, and physical properties of starch containing materials are largely dependent on internal and external factors. Internal factors are determined by the botanical sources, including the properties and nature of the amorphous and crystalline structure of native starch granules. Starch types from different botanical sources vary in size (diameters ranging from about 0.1 to 200 μm), morphology (ellipsoidal, oval, spherical, polygonal, elongated, irregular, lenticular, and disk), size distribution (uni-, bi-, or polymodal), and occurrence in the amyloplasts (individually or as compounds) [21, 22]. In addition, external factors like the cultivation area and the climate also influence starch properties [3]. Usually, starches consist of semicrystalline parts consisting of microcrystalline regions bound to amorphous regions of flexible chain segments [23]. Starch granules contain numerous components, which can be

Table 1. Physicochemical properties of amylose (AM) and amylopectin (AP) [75, 76]

Property	Amylose	Amylopectin
Molecular structure/branches	Mainly linear/ primarily α -1-4	Highly branched/ α -1-4; α -1-6
Molecular weight	10^5 – 10^6 Da	10^7 – 10^9 Da
Iodine bonds/color	20%/blue–black	<1%/red–purple
Digestibility by β -amylase	100%	Approx. 60%
Dilatation in aqueous solutions	Unstable	Stable
Solubility	Low/barely soluble	High
Gelatinization temperature	Low	High
Melting temperature	Low	High
Amylose–lipid complex	Very high amount	No
Gel formation	Firm, irreversible	Soft, reversible
Films	Coherent	Not readily formed
Viscosity	Low	High
Thickener	Poor	Good
Shear stability	Relative stable	Instable
Adhesive forces	Weak	Strong
Freezing–thawing stability	Unstable	Stable
Retrogradation rate	High	Low

divided into major and minor components. The major components in starch include amylose (AM) and amylopectin (AP), which make up for 98–99% of the dry weight of the starch granule [24–26]. Both (AM and AP) are composed of anhydroglucose units and their chains can thus be represented as $(\text{C}_6\text{H}_{12}\text{O}_6)_n$, whereby n is variable (in length) depending on the distribution of the different polymers. The ratio of the two starch molecules (in starch granules) as well as their molecular configuration strongly influences the product properties [27]. The most important physicochemical properties of AM and AP are summarized in Table 1. AM is located in the amorphous and semi-crystalline regions, whereas the majority of AP chains are found within the crystalline regions [28]. Starch granules show different degrees of crystallinity ranging from 15 to 45% [29]. The second group contains of minor components such as proteins, lipids, pentosans, and minerals (*e.g.*, phosphorus and silica) [6, 26]. Proteins (<0.6% of friabilin) and integral lipids (depending on AM content, up to 2% LPLs, and FFA) are out of these the most abundant and technologically important ones. The botanical origin of the starch and its purification during extraction in the course of manufacturing influences the quantity of the protein and lipids. The water content of native cereal starch is about 10–12%. Exceptions are some tuber and roots with water contents of about 14–15% [6, 26].

3 Physicochemical properties of starch in water while heating

When starch is heated in water physicochemical interactions between AM and AP are changing the starch granule

structure. As a result, different structural changes of starch granules can be detected: glass transition, gelatinization/melting and the melting of the amylose–lipid complex (for schematic description, see Fig. 2).

The glass transition (described) in suspensions is a temperature-induced transition of an amorphous “glassy” state to a progressively more rubbery state [30]. This “absorption of water” into amorphous regions is certainly possible before the irreversible steps within the gelatinization process are completed. Depending on the semicrystalline starch structure, the exact thermal event for glass transition can be difficult to detect [31]. The concentration and temperature dependency of the glass transition is affected by factors such as the gas barrier properties of amorphous starch (permeability and porosity), density, free volume and the cohesive energy density of starch suspensions [30]. The structural changes resulting from the glass transition facilitate the hydration and dissociation of double helices in crystallites. The dissociation of the crystallites is initiated after the glass transition temperature of amorphous regions, and is called gelatinization.

Gelatinization accounts for the majority of the structural changes of starch granule that take place during heating of a system with a specific starch-to-water ratio. These changes occur over a defined temperature range characteristic for different sources of starch due to the specific properties [32]. The gelatinization is initiated by the absorption of water into the granules leading to the hydration of the amorphous shell and the simultaneously occurring disruption of hydrogen bonds [33]. At a certain degree of granule expansion caused through hydration, the destabilizing and disruptive stresses induced by the swelling of the amorphous rings is transmitted further to the lamellar crystallites. This process is mediated by long AP chains that interconnect the side chain clusters within the semi-crystalline regions as well as within the two alternating semi-crystalline and amorphous regions. Continuing heat transfer results in irreversible changes ascertained with starch granule gelatinization. This includes the melting of starch crystallites, starch

solubilization, and leaching out of starched granules, which can be seen in a loss of birefringence and increase in suspension viscosity [33–36].

In summary, the starch gelatinization process is defined as the destruction of molecular order within the starch granule including all concomitant and irreversible changes resulting in alteration of its properties. The term gelatinization can be applied for all starch containing samples (showing different starch-to-water ratios) [12, 15, 16].

Amylose–lipid complexes can be naturally present in cereal starches [37, 38] or formed subsequently to gelatinization of starch in the presence of lipids. Amylose complexes can be divided into two distinguishable states: the less ordered type I and semicrystalline type II, which are so called amylose–inclusion complexes. Type I complexes typically dissociate at temperatures between 95 and 105°C [39, 40], while type II complexes are formed by heating the amylose–ligand mixture to temperatures exceeding 90°C [39]. The endothermic enthalpies of both amylose complexes have been described as being very similar and fairly independent of the chain length of the lipid. Therefore, in some publication a description of only one type of amylose complex can be found [41–43]. On the basis of the high dissociation temperature typically no melting of amylose–inclusion complexes is observed in many food products.

In Fig. 2 the state and occurring phase transitions of starch while heating are summarized. Heating a starch–water mixture firstly undergoes the reversible glass transition, which can be analyzed by sensitive DSC. If the temperature is increased further, the irreversible gelatinization process can be detected. Analytical methods to investigate these structural changes in dependence on the starch-to-water ratio are discussed in the following chapter. Depending on AM and lipid content and if temperatures of 90°C are exceeded the formation of amylose–lipid complexes can be detected using DSC. During cooling the amorphous structure in AM starts to recrystallize followed by the recrystallization of amorphous regions in AP during storage.

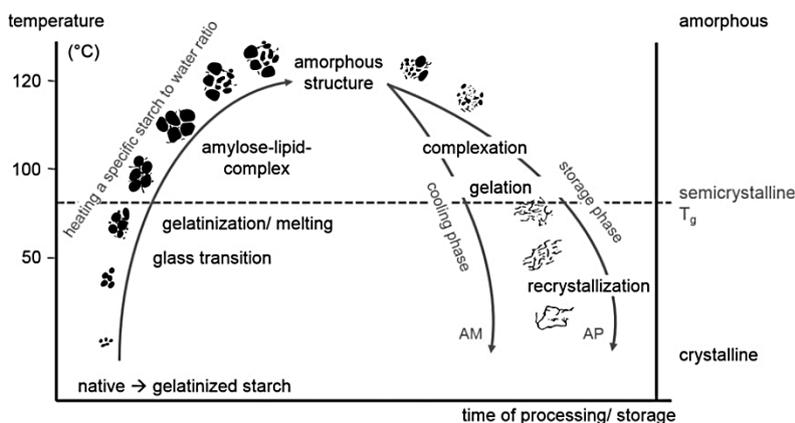


Figure 2. Phase diagram showing the state and phase transition of starch when applying a temperature profile. Starch undergoes a transition from a crystalline to an amorphous structure when heated and a subsequent recrystallization when cooled and during storage (T_g , gelatinization temperature; AM, amylose; AP, amylopectin).

4 Fundamental approach for analyzing starch gelatinization

As starch is typically contained within a structural and chemical complex food matrix its molecular and physico-chemical structure cannot be determined directly. Therefore, chemical, enzymatical, and physical methods have to be applied to investigate structural changes of starch. In order to study starch modification during heating, physical analytical methods are generally preferred. As the methods on the milling and baking industry are standardized applications they were selected for further investigation. These methods are typically based on empirical rheological systems provided to analyze systems with an excess amount of water including the rapid visco analyzer (RVA) or amylograph as well as viscograph. Changes in viscosity as a result of granule swelling and the solubilization (leaching) of macromolecules allow characterizing important steps in the gelatinization process. An advantage of these methods is the adaptability of shear, heating and/or cooling rate based on the analyzed sample. Typically, the viscosity analysis begins at temperatures between 30 and 50°C lying below the gelatinization temperature of starch. In Fig. 3 a description of a viscosity measurement is given showing a heating and cooling temperature profile [44]. When the applied temperature exceeds the starch granule gelatinization temperature swelling and partial rupture of the granules is initiated shown by an increase in viscosity. Thus the pasting temperature (1; T_P) and the peak viscosity (3; PV) represent the most meaningful values for an unspecific description of starch gelatinization (depending on viscosity). Whereas T_P represents the beginning of gelatinization and PV the intensity of gelatinization, both viscosity-based. To ensure comparability of these rotating viscometer systems, homoge-

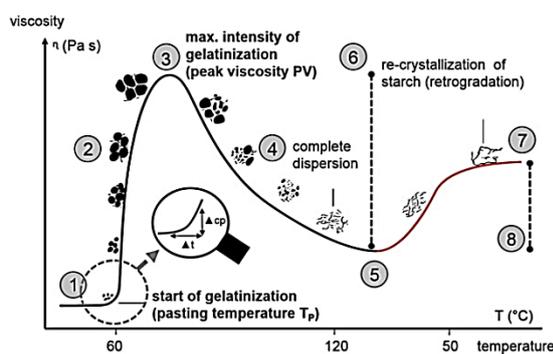


Figure 3. Viscosity measurement of starch in an excess amount of water, applying a temperature profile including a heating and cooling step. (1) Gelatinization onset (T_P , pasting temperature), (2) hydration of starch granules, (3) max. intensity of gelatinization (PV, peak viscosity), (4) enzymatic and shear destruction of starch granules, (5) minimum viscosity (HPV, hot paste viscosity), (6) viscosity loss (B, breakdown), (7) final viscosity (FV), and (8) paste hardening (S, setback).

neity is essential for calculating the pasting temperature and peak viscosity. Additionally, an adaptation of the method considering temperature determination and different heating rates are prerequisites for a precise evaluation of gelatinization values. All described viscometers are constructed differently using different stirrer geometries and filling volumes based on different standardization regulations of ICC or AACC. Besides these device variables variations in the flour-to-water ratio and heating rates for analyzing starch properties are found. Furthermore, there exist differences in how to measure the temperature of the sample. In the amylograph the temperature sensor is placed directly in the suspension compared to the RVA where the temperature is measured externally. To clarify this contrast between the described systems, all relevant values are presented in Table 2 [44]. It is to be noted that there is only a description about method implementation given without any information about process or sample values. In addition, also the evaluation and calculation of main values (pasting temperature (T_P) and the peak viscosity (PV)) can differ leading to different gelatinization values.

As already mentioned in the introduction, many food systems are heated in industrial applications exhibit limited or insufficient water content. Such systems cannot be analyzed by the presented viscometric systems. For a better understanding of the importance of the sample water content, the effect of different starch-to-water ratios on the main starch gelatinization values (T_G = start of gelatinization, I_G = gelatinization intensity) is illustrated in the following phase diagram (Fig. 4).

The water content in most food systems limits the degree of starch gelatinization. Considering the two most important gelatinization values for the production of food (T_G and I_G) only very few publications focus on the influence of the starch-to-water ratio [12, 15, 45]. Increasing the water content in food leads to an increase in gelatinization enthalpy and decrease in gelatinization temperature [12, 45–49]. The rise in gelatinization temperature in foods with limited water content can be explained by the large plasticizing effect of water on biopolymers: when the water content is shifted to very low values even small changes in water content affect the gelatinization values majorly [50]. The main factor causing an increase in gelatinization temperature is the decrease in available (free) water due to a reduction of water content. Thus the amount of energy to crack the crystalline structure of starch is greatly increased [51].

In summary, reducing the water content in food systems changes the gelatinization properties. Owing to this phenomenon (as also shown in Fig. 4) it is important to point out the need for systems able to analyze starch gelatinization in samples with limited and insufficient water content. The following chapter will give an overview of all existing systems to analyze starch gelatinization with limited water content.

Table 2. Summary of the discussed physical analytical methods with the interpretation of gelatinization values in relation to product applications. The data presented are based on values obtained from literature.^A [77], ^B [78]

Type/function	Method	Concentration (s:w ratio)	Heat-rate ($^{\circ}\text{C min}^{-1}$)	Rheol. geometry	Gelatinization values	Interpretation	Product application
Rheometric	Empirical	Rapid visco analyzer (RVA)	12 ^A		Excess starch-to-water ratios between 1:7.14 and 1:5.56 ^A (starch to water)	Start of gelatinization → Intensity of gelatinization → Speed of gelatinization → Viscosity, enzymatic activity → Heat, acid, shear stability → gel formation → Retrogradation, freezing–thawing stability →	Malt, mash Sauce Pasta, rice Malt Pudding Pudding Dough, bread
	Empirical	Amylograph	1.5 ^B		Excess 1:5.63 ^B (starch-to-water)	Starting temperature (T_p) Gelatinization maximum (AU) Gelatinization temperature (T_g) [79]	Malt, mash Sauce
	Fundamental	Rheology (oscillation measurement)	Unlimited		Stiffness (G^*) Inflection point (P) [52]	Intensity of gelatinization → Start of gelatinization →	Bakery products Malt
Calorimetric	DSC	Insufficient, limited, excess	Indefinite	–	Onset temperature (T_0) Peak temperature (T_p) End temperature (T_{End})	Start of gelatinization →	Malt
Microscopic	Light microscopy (LM), polarizing light microscopy (PLM)	Insufficient, limited, excess	Offline unlimited	–	Enthalpy of gelatinization (ΔH) [15] Start of granule increase (ΔA) Average size (\varnothing_A) Circularity (C) Ferret diameter (L_F) Perimeter (P) BEPT (98% of the granules lost birefringence) [80]	Intensity of gelatinization Start of gelatinization → Intensity of gelatinization →	Bakery products Malt Bakery products
	Confocal laser scanning microscopy (CLSM)	Insufficient, limited, excess	Mostly Offline Indefinite	–	Start of granule increase (ΔA) Average size (\varnothing_A) Circularity (C) Ferret diameter (L_F) Perimeter (P) [73, 81]	Intensity of gelatinization Start of gelatinization → Intensity of gelatinization →	Bakery products Malt Bakery products

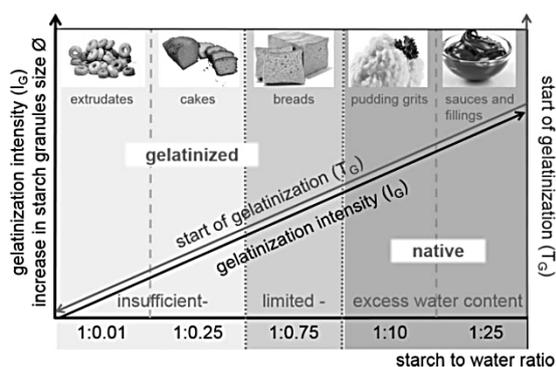


Figure 4. Phase diagram illustrating selected product samples with different starch-to-water ratios and the influence on the main gelatinization values (T_G , start of gelatinization; I_G , gelatinization intensity). Insufficient water content leads to limited gelatinization, whereby an excess of water results in total gelatinization. With an increase in water content T_G decreases whereas I_G increases.

5 Systems for analyzing starch gelatinization with limited water content

As mentioned the physical analytical methods established in the milling and baking industry do not allow measuring starch gelatinization under real conditions when the amount of water is limited. Following results of innovative methods based on fundamental rheological, calorimetric, and micro-

scopic principles have been implemented offering possibilities of analyzing starch gelatinization even in systems with limited water content. Table 2 summarizes all currently known and applied physical analytical methods and their interpretation of gelatinization.

5.1 Fundamental rheological systems for analyzing starch gelatinization with limited water content

With a rheometer starch gelatinization can be analyzed with oscillation tests (strain or stress controlled) by applying a specific temperature profile comparable to those of the viscograph. Measurements with rheometers are independent of the sample amount and construction, generally equipped with a plate–plate or plate–cone geometry. By using a temperature–ramp test (equivalent to the micro-baking test), starch systems with reduced water content can be measured [52–54]. The main advantage of oscillation measurements is the non-destructive product analysis.

The complex shear modulus G^* (stiffness of samples illustrated in Fig. 5) obtained with an oscillation test characterizes the structural changes during pasting. The temperature at the point where stiffness starts to increase is defined as the inflection point (P_i). P_i is a typically used value for analyzing the onset of gelatinization. G^* , which is calculated after the performed experiment represent the gelatinization intensity. The change in stiffness (P_i), as well as the gelatinization intensity measures the gelatinization

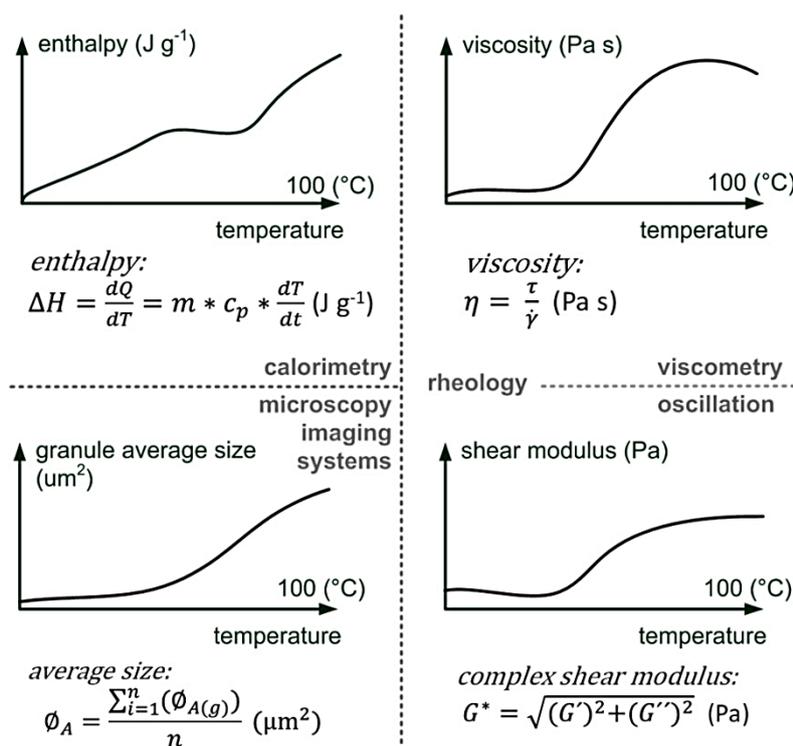


Figure 5. The three main groups of physical analytical methods to characterize starch granules in food systems during thermal processes. (top left) calorimetry uses enthalpy (ΔH), (bottom left) microscopy imaging systems uses average granule size (ϕ_A), (top right) viscometry (which belongs to rheology) uses viscosity (η) and (bottom right) oscillation (also part of rheology) uses the complex shear modulus (G^*) to characterize the gelatinization process.

progress indirectly through changes in the rheological properties of the sample. These values are highly influenced by secondary components such as salt, sucrose and proteins, which are added during production. These components influence the viscosity, rheological properties and consequently bias the measurement, as the changes cannot clearly be attributed to changes induced by the gelatinization process. However, rheological systems offer compared to viscometers the possibility to analyze the gelatinization process of samples with reduced water content in a non-destructive way [52, 55, 56].

5.2 Calorimetric system to analyze gelatinization with limited water content

The DSC as a thermoanalytical technique is a valuable tool for identifying structural changes by recording energy flow. The loss in crystallinity occurring during starch gelatinization is measured as a first-order thermal transition [57]. When analyzing gelatinization, four parameters are determined:

onset temperature (T_O), peak temperature (T_P), end temperature (T_C), and the enthalpy of gelatinization (ΔH) (see Fig. 5) [58]. The enthalpy, the integrated area under the thermal transition peak can be referred to the intensity of gelatinization.

For characterizing the start of gelatinization, T_O serves as useful value. In contrast to a viscometer system, DSC enables to measure suspension as well as systems with limited water content [59]. To demonstrate the spectrum of application possibilities of DSC Table 3 list the (obtained) gelatinization values of various starch sources with different starch-to-water ratios.

5.3 Microscopic systems to analyze starch gelatinization with limited water content

5.3.1 Classic microscopic systems

The following section presents some well-known microscopic analyses used in combination with imaging techniques that

Table 3. Gelatinization of different starch sources measured by DSC

Starch source	Temp. profile (°C)	Gelatinization values			Start to end (°C)	Heating rate (°C min ⁻¹)	Ratio (starch:water)	Reference
		Start (°C)	Maximum (°C)	Enthalpy (mJ mg ⁻¹)				
Potato	20–120	54.0	58.8	9.7	10.5	10	1:3	[82]
Potato	5–150	58.6	63.0	19.6	13.6	4	1:2	[83]
Potato	5–150	58.0	62.5	18.5	12.5	4	1:2 (db)	[84]
Potato	20–140	60.4	64.4	16.9	9.3	10	1:5 (db)	[85]
Wheat	0–150	51.0	—	13.4 (db)	24	10	1:8 (db)	[45]
Wheat	20–120	56.2	61.1	9.7	10.4	10	1:3	[82]
Wheat	5–150	51.5	56.2	10.6	10.1	4	1:2	[83]
Wheat	5–150	54.4	58.7	11.8	9.2	4	1:2 (db)	[84]
Wheat	5–150	52.5	58.7	10.6	19.0	10	1:4.3 (db)	[86]
Wheat	27–127	58.4	63.2	10.0	10.6	10	1:3	[87]
Wheat	30–100	54.6	62.5	11.5	18.8	10	1:2.3	[88]
Wheat	40–120	48.1	53.6	14.8 (db)	9.9	1	1:2.3	[76]
Wheat	40–120	49.3	54.4	14.8 (db)	9.5	1	1:2.3	[76]
Wheat	25–150	57.6	62.4	6.1	17.1	5	1:4	[89]
Wheat	25–165	44.62	62.2	14.3 (db)	50.2	10	1:1.5	[90]
w-A-type		45.93	60.15	11.1 (db)	41.3			
w-B-type		50.33	64.61	8.12	38.7			
Wheat waxy	30–100	55.6	66	11.8	24	10	1:2.3	[88]
Wheat waxy	40–120	48.1	54.7	17.1	15.4	1	1:2.3	[76]
Wheat waxy		51.6	57	17.9	13.7			
Wheat waxy		51.1	56.8	17.5	15			
Barley	25–150	61.3	65.5	11.4	11.4	10	1:3 (db)	[91]
Barley waxy	25–150	59.3	64.9	13.0	22.5	10	1:3 (db)	[91]
Barley high-AM	25–150	61.0	67.9	6.0	15.6	10	1:3 (db)	[91]
Maize	33–123	63.9	70.3	11.0 (db)	—	10	1:3.3	[92]
		67.1	71.5	8.0 (db)				
		64.7	71.1	11.0 (db)				
Maize waxy	33–123	66.7	71.5	13.0 (db)	—	10	1:3.3	[92]
Maize high-AM	33–123	70.6	79.8	4.0 (db)	—	10	1:3.3	[92]

have been proven suitable for analyzing surface and internal structures of starch granules.

Microscopic systems present a useful tool for studying head induced microstructural changes in starch. The earliest microscopic system is based on light microscopy (LM; max. resolution 200 nm) and is still a useful tool for conventional applications [60]. Using LM as an off-line technique, thermally dependent structural changes in starch granules can be detected visually [61, 62]. By staining starch granules and analyzing them with light microscopy Collison and Chilton [63] were able to analyze the cracked granules with regard to the gelatinization process. They found out that starch samples containing a starch-to-water ratio up to 1:0.3 (db) showed no visually observable changes such as cracks. Any changes that occurred with lower water content were not reflected by this staining technique. At starch-to-water ratio of 1:0.55 (db) or greater cracks in starch granules were seen.

Polarized light microscope (PLM) additionally illuminates the samples with polarized light. This allows researchers to visualize the disruption of the semi-crystalline structure of starch granules during gelatinization seen by the loss of their birefringence and disappearance of the “maltese” cross [45, 64–66]. The typical swelling pattern is an indicator of the onset of gelatinization corresponding to T_O (DSC) [67]. The birefringence end-point temperature (BEPT, *i.e.*, the temperature at which 98% of the granules have lost their birefringence) is defined as the intensity of gelatinization. Generally a linear correlation between T_P of DSC and visually detected BEPT exists [67, 68]. Analyses of different starch-to-water ratios up to 1:0.6 (db) showed only few observable structural changes (>90% birefringence). At a ratio greater than 1:0.5 (db), most granules showed a swelling with around 2% birefringence [69]. In both presented microscopic systems (LM, PLM) the induced changes during gelatinization can only be observed at starch-to-water ratios greater than 1:0.3 (db). The microscopic techniques are further also limited by the lack in quantitative evaluation possibilities of the results. The use of image analysis tools in combination with microscopic techniques allows to gain more information from the visual inspection [70].

5.3.2 Advanced microscopic systems

Confocal laser scanning microscopy (CLSM; max. resolution 150 nm) is already an established offline method used for visualizing starch gelatinization and applied in many studies [70]. CLSM represents a major improvement compared to conventional LM by using point illumination and a photo detector focusing on the same spot, crucially increasing the image quality [71]. Additionally, CLSM enables to examine samples under actual conditions or during a dynamic process such as heating. Furthermore, by the use of specific fluorescence stains desired areas or components can be stained selectively without interfering with other parts or

components [72, 73]. The offline detection of structural changes of starch granules with subsequent image analyses serves as useful tool in order to analyze the starch gelatinization process. Using CLSM, the average granule size (\varnothing_A) (Fig. 5), the perimeter (P), the feret diameter (D_F = (perpendicular) maximum distance between two parallel lines restricting the starch granule) and circularity ($C = P/2\sqrt{\pi A}$) are the most important parameters to describe the gelatinization process [70, 74]. Assuming a defined gelatinization process, the temperature-dependent increase in average granule size (\varnothing_A) can be considered as the start of gelatinization. With the total increase in average size a significant value for the gelatinization intensity is obtained.

All presented methods have helped to gain knowledge about starch gelatinization and the process leading to it. However, until now no method has been found/exists able to analyze starch gelatinization online during an industrial process in combination with an image analysis.

6 Conclusions

The detectability of the starch gelatinization process in different starch–water systems was reviewed focusing on the dependence of several investigation methods on raw material properties and process parameters. Raw material properties and process parameters were distinguished in order to point out the importance of measuring systems to analyse the gelatinization independently from the applied method parameters. These systems are indispensable for universal use in order to describe the gelatinization process in complex matrices and for various applications.

Based on the measuring method, the described systems can be divided into rheological, calorimetric, and microscopic systems (see Fig. 5). The reviewed rheological methods can be separated into empirical viscometers (viscometry, with viscosity (η) to determine gelatinization) and fundamental rheometer systems (oscillation, with complex shear modulus (G^*) to determine gelatinization). In both cases changes in viscosity of the sample, caused by starch granule swelling and solubilization (leaching out) of macromolecules, are used to characterize important steps of the gelatinization process. The major drawback of these empirical viscometers such as RVA and amylographs as well as viscographs is that they cannot determine the gelatinization properties of food with limited or insufficient water content. They further show a lack in comparability as different devices and ways of calculating and evaluating the starch-dependent viscosity changes are used. However, due to the standardized measuring procedure and the easy handling of these methods they are still used for a wide range of applications. The fundamental rheometers additionally allow measurements in systems with limited water content.

In microscopic systems the starch gelatinization (determined by using average granule size ($\bar{\varnothing}_A$)) can be analyzed in a non-destructive way. CLSM further allows offline detection of visual changes of the starch granules during the gelatinization more independently from other recipe components due to differential staining. The DSC (with enthalpy (ΔH) to determine the viscosity) on the other hand is the only system to measure the gelatinization online and in a non-destructive way, following the changes in starch granules during the heating process. Therefore, a system combining a visual offline system with a calorimetric system, could overcome the lack of a specific non-invasive online analyzing system able to investigate the starch gelatinization within a complex food matrix in a precise manner. In conclusion, to compare the gelatinization properties of starch in complex food systems it is essential to analyze it under real conditions using the same process parameters and product formula. Depending on the physicochemical properties (and process parameters), the method to investigate the starch gelatinization needs to be selected wisely in order to obtain product-dependent gelatinization values. The use of specific analytical methods helped to gain comprehensive knowledge about the gelatinization process. In contrast to the statement in Fig. 1 a connection exists between the methodology, the process parameters and physical analytical systems, influencing the progress and extent of gelatinization in food products shown in Fig. 6.

However, to further increase the understanding of thermally induced structural changes during food production, novel approaches are needed to study starch gelatinization online. Based on the analysis of all established techniques, CLSM combined with image analyses techniques, offers the greatest potential in understanding complex food systems. To follow the transformation of food

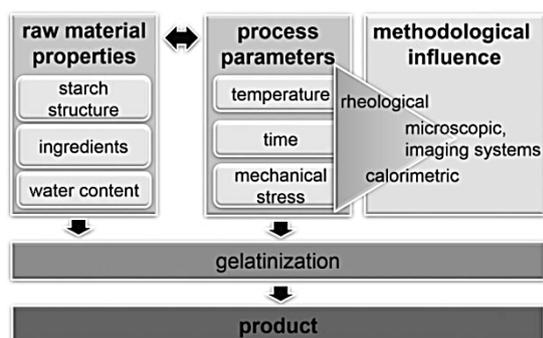


Figure 6. Dependence of the gelatinization on the methodology and the influence of process parameters and raw material properties. The methods are listed starting with the method showing the greatest dependence on product parameters. In contrast to the statement in Fig. 1, a connection between methodology, process parameters and physical analytical systems exists, which influences influencing the progress and extent of gelatinization in food products.

components during thermal processes, the combination of an online system with a digital analytical tool would be necessary to evaluate the structural changes.

7 Nomenclature

η	viscosity (Pa s)
$\dot{\gamma}$	shear rate (s^{-1})
τ_0	yield stress (Pa)
t	time (s)
G^*	complex shear modulus (stiffness) (Pa)
G'	storage modulus (Pa)
G''	loss modulus (Pa)
m	mass (kg)
c_p	heat capacity (constant pressure) ($J K^{-1}$)
T	temperature ($^{\circ}C$)
$\bar{\varnothing}_A$	average granule size (μm^2)
P	perimeter (μm)
D_F	feret diameter (μm)
C	circularity (–)
ΔH	enthalpy ($J g^{-1}$)
T_O	onset temperature (DSC) ($^{\circ}C$)
T_C	conclusion temperature (DSC) ($^{\circ}C$)
T_P	pasting temperature (RVA) ($^{\circ}C$)
	Peak temperature (DSC) ($^{\circ}C$)
PV	peak viscosity (Pa s)
t_p	peak time (min)
HPV	hot paste viscosity (Pa s)
B	breakdown (Pa s)
FV	final viscosity (Pa s)
S	setback (Pa s)
D_{rot}	rotational diffusion coefficient (s^{-1})

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2.3 Physicochemical and morphological characterization of starch with different amylose/amylopectin ratio

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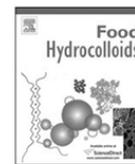
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Physicochemical and morphological characterization of different starches with variable amylose/amylopectin ratio

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ABSTRACT

Starch-rich raw materials are widely used in the food industry. Their functionality and end-use applications are markedly influenced by starch characteristics. Starches with varying amylose (AM) and amylopectin (AP) content are of particular interest due to their ability to influence and modify the texture, quality and stability of starch-based food products. The present study shows the influence of the AM/AP content on physicochemical and morphological properties of a range of starches (Maize = 3%, 23%, 71%; Potato = 2%, 21%; Wheat = 28; Barley = 3%, 25% AM content w/w of starch).

Starches have been analyzed in terms of their chemical composition, water retention capacity, morphological characteristics, and pasting/thermal properties. The changes in starch granule morphology during gelatinization were monitored by confocal laser scanning microscopy (CLSM). The different analysis revealed that waxy-starches (AP>90%) had a high water retention capacity (1.2–1.5 times higher) and developed higher paste viscosities (up to 40% for maize; 43% for barley). The swollen granules were highly susceptible to mechanical breakdown and solubilized faster. Higher AM contents showed inhibition of an extensive granule swelling and lowered the paste viscosity. The exceptional integrity of the high-AM starch even prevented its gelatinization at atmospheric pressure. Significant differences in physicochemical and morphological properties between the starches from regular, high-AM and waxy strains have become evident, no direct relationship between the AM/AP contents and the internal growth ring structures of the starch granules could be identified by CLSM. The waxy starches had a higher gelatinization temperature (up to 2 °C) and enthalpy (up to 20%), which indicates a higher crystalline and molecular order.

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1. Introduction

Starch is the major energy storage reserve carbohydrate synthesized in many parts of plants and represents the second most abundant biopolymer on earth, next to the organic compound cellulose (Eliasson, 2004). It plays an important role as functional material in the food and non-food industries and serves as an essential source of energy for human and animal nutrition. Starch granules contain numerous components, which can be divided into two groups: the first comprises of the major components amylose (AM) and amylopectin (AP) and the second is made up of the minor components of starch (proteins, lipids and minerals) (Copeland, Blazek, & Salman, 2009; Tester, Karkalas, & Qi, 2004). The ratio of the two α -glucans in starch granules as well as their molecular

structure influence e.g. the solubility, gelatinization temperature, viscosity, gelation and retrogradation properties of starch and therefore represent major parameters for the quality, texture and stability of starch- or flour-based products (Blazek & Copeland, 2008).

In general, the ratio of AM to AP and their structural variability strongly depend on the botanical origin. Regular starches contain approximately 70–80% AP and 20–30% AM, waxy starches less than 10% AM and high-AM starches more than 40% AM (Tester et al., 2004). In order to obtain starches with specific pasting properties and other technologically relevant characteristics, several approaches have been made which aimed to increase the granule AM or AP content. Such breeding programs are based either on non-genetic modification in terms of traditional breeding and selection of agronomically well-adapted varieties or on genetic modification or rather manipulation of the expression of genes involved in starch-biosynthetic pathway (Blazek & Copeland, 2008; Morell & Myers 2005).

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Various analytical methods have been used to estimate the AM and AP contents of starch and to determine the molecular structures of these two starch polysaccharides (Herrero-Martínez, Schoenmakers, & Kok, 2004; Kugimiya & Donovan, 1981; Mestres, Matencio, Pons, Yajid, & Fliedel, 1996; Moorthy, Andersson, Eliasson, Santacruz, & Ruales, 2006; Wang, Li, et al., 2010; Wang, Yu, et al., 2011), (Grant, Ostenson, et al., 2002; Kobayashi, Schwartz, & Lineback, 1985; Scott, Jane, & Soundararajan, 1999; Stawski, 2008). In comparison, different imaging techniques which have proven to be suitable for analyzing starch granule surface or internal structure are light microscopy including polarizing, bright-field (Atkin, Cheng, Abeysekera, & Robards, 1999), and fluorescence microscopy (Goossens, Derez, & Bhar, 1988), atomic force microscopy (Baldwin, Adler, Davies, & Melia, 1998) and electron microscopy including scanning (Jane, Kasemsuwan, Leas, Zobel, & Robyt, 1994) and transmission electron microscopy (Yamaguchi, Kainuma, & French, 1979). But in all cases there were not any morphological characterization as well as quantification of different AM/AP content by microscopic analysis as well as correlations between analytical and microscopic analysis. As a basis for the determination of the AM/AP content of total starch by confocal laser scanning microscopy (CLSM), an enzymatic method based on the specific formation and precipitation of AP-Concanavalin A (Con A) complexes, after a pre-treatment of the sample to solubilize resistant starch and to remove lipids and free D-glucose was used. In this study, CLSM, which uses laser technology in combination with fluorescence imaging, was used to represent first the visualization of starch granule morphology with the objective for the determination of the AM/AP content.

Different starches are used in many applications in the food industry and therefore in almost all applications, starches were heated before use. When starch granules are heated in the presence of water, several changes at molecular and structural level occur. These alterations of starch structure are related to the granule-specific gelatinization behavior, which is strongly affected by a number of inherent characteristics: granule composition (in particular the ratio of AM to AP), granule size, granule molecular architecture (especially the AP branch chain lengths and distribution) and molecular weight of AM and AP molecules (Jane, Chen, et al., 1999; Lindeboom, Chang, & Tyler, 2004). The progress and extent of the thermal gelatinization of starch are affected by a wide range of external factors, including applied heating temperature, moisture content, heating time, heating rate, presence of further ingredients in food or non-food products, processing conditions, starch preparation and storage conditions (Beck, Jekle, & Becker, 2011; Spigno, & De Faveri, 2004). AP is suggested to be responsible for granule swelling while the fraction of AM–lipid complexes may retard the swelling and induce an increase in gelatinization temperature (Jane et al., 1999). Former publications are dealing with the visualization of gelatinized starches as well as analysis of pasting properties and thermal changes, but without combined consideration.

In this study, morphological, rheological and thermal characteristics of different starches were studied. Thereby, CLSM has been used to investigate the granule characteristics as well as the gelatinization behavior of starch granules visualized and analyzed by a micrograph processing tool. These values were combined with pasting properties analyzed by rapid visco analyzer (RVA) and thermal changes measured by differential scanning calorimetry (DSC). The main objectives of this study are to characterize and compare the morphological and physicochemical properties of selected starches of cereal and tuber origin. The samples have either been currently used in the food industry or were less established therein and significantly ($p \leq 0.05$) differ in AM/AP content. On the basis of the morphological characteristics and physicochemical properties, an effect of the AM/AP content on the sample properties is going to be evaluated.

2. Materials and methods

2.1. Starch samples

The starch samples used and their respective sources were maize starch (National Starch & Chemical GmbH, Hamburg, Germany), high-AM maize starch (HYLON® VII, National Starch), waxy maize starch (AMIOCA™ National Starch), potato starch (AVEBE FOOD, Veendam, The Netherlands), waxy potato starch (ELIANE™ 100, AVEBE FOOD), wheat starch (Merck KGaA, Darmstadt, Germany), barley starch (Grain Processing Corporation, Iowa, USA) and native waxy barley starch (Lyckey PU 91 000, Kampffmeyer Nachf. GmbH Ratzeburg, Germany) which are a selection of important starches for the food industry. The selected starches evinced following AM contents (w/w of starch) Maize = 3%, 23%, 71%; Potato = 2%, 21%; Barley = 3%, 25%.

2.2. Analysis of chemical and physicochemical properties of samples

The sample moisture content (%) was determined thermogravimetrically using the moisture analyzer MLB-50-3 (Kern & Sohn GmbH, Balingen-Frommern, Germany) by weight loss from the initial weight. The crude protein content of the starches and flours was determined on the basis of the Kjeldahl method using a Kjeltec™ 2400 Auto Analyzer Unit. For the conversion of the percentage nitrogen to crude protein content, the factor 6.25 was used. The crude lipid content (i.e. free lipids) was quantified using the AACC Method No. 30-25.01 (2010). The ash content was determined according to the ICC Standard Method No. 104/1 (1990) and related to the dry matter of the sample substance. The water retention capacity (WRC) was determined by measuring the water uptake of the samples (at approx. 20 °C) according to the standard AACC Method No. 56-11.02. It is expressed as percent weight of solvent retained by the sample in a gel pellet after centrifugation and decantation related to the sample weight on a 14% moisture basis. For the determination of the AM/AP content of total starch, the AM/AP assay procedure, utilizing the commercially available kit (Megazyme International Ireland Ltd.), was followed according to the recommendation of the manufacturer. This enzymatic method is based on the specific formation and precipitation of AP–Concanavalin A (Con A) complexes, after a pre-treatment of the sample to solubilize resistant starch and to remove lipids and free D-glucose. The test kit includes relative standard deviations of <5% for pure starches. The total starch content of the samples was measured enzymatically using an assay kit according to the standard AACC Method No. 76.13. The starch content measurement includes the major components amylose and amylopectin, the minor components of starch (protein, minerals, and lipids) are not detected. The damaged starch content of the samples was determined using an assay kit in accordance with the AACC Method No. 76-31.01 (2010). The method is based on the enzymatic susceptibility of damaged starch granules. Each measurement was performed in duplicate.

2.3. Visual characterization by confocal laser scanning microscopy (CLSM) and micrograph analysis

Aqueous sample suspensions (10 g kg⁻¹) were prepared by dispersing 50 mg of the sample in an appropriate volume of distilled water while gently stirring for 2 min. Aliquots (500 µL) of the suspension were transferred into 1.5 mL microtubes and 40 µL of aqueous Nile Blue solution (0.1 g 100 mL⁻¹) were added. After mixing thoroughly by repeated pipetting up and down, the stained solutions were incubated at 20 °C for 3 h. The swollen and gelatinized starch samples were prepared by heating the starch–water

suspensions (10 g kg^{-1}) for 0.5, 1.0, 2.0 and 4.0 min in a boiling water bath. 500 μL aliquots of the samples were immediately transferred into 1.5 mL microtubes, cooled on ice and further prepared for microscopy as described above. For CLSM observation the stained suspensions were transferred to specimen shapes and each well was covered with a glass cover slip. The glass cover slips were sealed with nail polish and additionally fixed with adhesive tapes. The confocal micrographs of the samples were obtained by means of an inverted CLSM (ECLIPSE Ti-U, equipped with 3 lasers (448 nm, 543 nm, 635 nm), 3 detectors (510/30 nm, 590/50 nm, 650 LP nm), EZ-C1 Software (V3.80) from NIKON Instruments Inc., New York, USA) in the fluorescence mode. A $60\times$ oil immersion objective was used. The wavelength used to generate fluorescence of starch granules was 635 nm and the emission filter 650 LP was selected. All the micrographs were recorded at a 1024×1024 pixel resolution in different z-positions. A pixel dwell time of 59.28 μs was chosen to improve the signal to noise ratio and to obtain a higher micrograph quality. The gain was adjusted for each sample to avoid excessive background fluorescence and micrograph saturation. Different magnifications ($\leq 212^\circ \times 212^\circ \mu\text{m}$) were used to reveal structural details of the samples. Each of the samples was prepared twice and about 10 independent positions on the x–y axis were recorded per sample. In compliance to Schirmer, Jekle, and Becker (2011) and Jekle and Becker (2011a, 2011b) a number of granule size and shape characteristics of the starch samples were analyzed using the open source ImageJ (version 1.46e, National Institutes Health, Bethesda, Md, USA). The images were pre-processed by changing to grayscale (8 bit). To apply the segmentation of starch granules as well as starch “ghosts” a fuzzy threshold algorithm of Huang and Wang (1995) was used. The function was applied to formalize the characteristic relationship between a pixel and its related region (the granule or the background). After thresholding a special algorithm (watershed) was used for cell counting. Furthermore the segmentation of the starch grain used by watershed algorithm was an important part for the implementation of starch grain analysis. By the usage of binary images, the following parameters of the starch granules were measured: average size ($\bar{\theta A}$) (μm^2), perimeter (P) (μm) and circularity (C) (–) which is a unit of the roundness (unit circle = 1). Calculation of the average size was based on the equation:

$$\bar{\theta A}_c = \frac{\sum_{i=1}^n (\theta A_c)_i}{n}$$

2.4. Pasting properties by rapid visco analyzer (RVA)

The pasting properties of the starch samples evaluated using the rapid visco analyzer (Newport Scientific Pty Limited, Warriewood, Australia) with thermocline control and data collecting software. The method used was the RVA™ General Pasting Method with a standard temperature/shear profile. The samples were mixed at 50 °C for 10 s by 960 rpm and 90 s by 160 rpm, heated up by a heating rate of $0.2 \text{ }^\circ\text{C s}^{-1}$, stopped at 95 °C, kept for 162 s, shut down by a heating rate of $0.2 \text{ }^\circ\text{C s}^{-1}$ and hold 120 s at 50 °C before test ended. For each starch sample, 3.0 g of material (on a basis of 14% moisture) were weighed into an aluminum RVA sample canister. 25.0 g of distilled water were added to create a 9.2% (w/w) dispersion of the sample. The sample–water mixture in the aluminum canister was subsequently stirred with a mixing paddle for a few seconds to prevent the formation of lumps. Then, the canister, together with the stirring paddle, was inserted into the RVA unit and the test was started. Average values for peak viscosity (PV) (mPa s), hot paste viscosity (HPV) (mPa s), final viscosity (FV) (mPa s), pasting temperature (T_p) (°C), peak time (t_p) (min), breakdown ($P = PV - HPV$) (mPa s) and setback ($S = FV - HPV$)

(mPa s) were obtained for each sample from quadruplicate RVA viscosity measurements.

2.5. Thermal properties by differential scanning calorimetry (DSC)

The thermal characteristics of the starch samples were analyzed using a Diamond DSC (with Pyris Series Software (V10.1), Perkin Elmer Inc., Connecticut, USA) equipped with a thermal analysis data station. The sample (2 mg) was directly weighted into a standard non-hermetic aluminum pan and 6 mg of distilled water were added. Thus, a sample:water ratio of 1:3 was achieved. The suspension was gently homogenized by stirring it up with a needle in order to obtain the wetting of the sample at the bottom of the pan. The pan was immediately sealed with a crimper and allowed to equilibrate for 10 min at 20 °C before being loaded to the DSC unit. The analysis was carried out by heating the pan from 40 to 100 °C using a heating rate of $10 \text{ }^\circ\text{C min}^{-1}$. A sealed empty aluminum pan was used as inert reference. The melting point and the enthalpies of indium were used for temperature and heat capacity calibration of the instrument. The applied nitrogen steam had a flow rate of 20.0 mL min^{-1} . The sample specific gelatinization parameters, namely onset temperature (T_o) (°C), peak temperature (T_p) (°C), conclusion temperature (T_c) (°C) and temperature range ($\Delta T = T_c - T_o$) (°C), were determined from the DSC thermograms by means of the Pyris Series software. In addition, the enthalpy of gelatinization (ΔH) (J g^{-1}), which is associated with the loss of crystalline and molecular order during starch gelatinization, was obtained by numerical integration of the area under the thermal transition peak above the extrapolation line. All measurements were conducted in quintuplicate and the average values of the thermal parameters were calculated.

3. Results and discussion

3.1. Chemical and physicochemical compositions

With regard to the chemical composition of total starch, considerable differences in the total AM/AP contents between the cultivars were detected (Table 1). The regular starches displayed species-specific AM contents with a maximum average AM content occurring in wheat starch (27.8%) and a minimum average AM content occurring in potato starch (20.9%). The AM contents of the waxy starches, however, showed not significantly huge different for the cereal and tuber cultivars (2.2–3.4%). The high-AM maize starch displayed, as expected, the highest AM content among all analyzed starches (71%).

The WRC values of the starches are shown in Table 1 respectively. The waxy starches were characterized by substantially higher WRC compared to their regular counterparts (90.2% vs. 58.6% for waxy maize starch, 79% vs. 65.4% for waxy potato starch and 87.1% vs. 70.6% for waxy barley starch). Higher WRC for waxy starches could be explained by the molecular structure of the major, extensively branched starch component AP, which is considerably more complex than that of AM. The molecular size and unit chain distribution of AP effects the formation of hydrogen bonds (Sasaki & Matsuki, 1998). Regular maize and wheat starch were very similar in WRC and exhibited the lowest WRC among all analyzed starches. Regular potato starch showed a higher WRC when compared to regular maize and wheat but a lower WRC when compared to regular barley starch. The WRC of the starches was assumed to be rather species-specific and not dependent on granule size. The high WRC of the starch samples with anomalous starch composition might be strongly influenced by their respective granule architecture. On the other hand, the purity of the samples has to be taken into account, as well.

Table 1Chemical composition and physicochemical properties of starch samples with an accuracy value of the test method, $\bar{X} \pm \text{sd.}$, ($n = 2$).

Starch type	Protein (% w/w db)	Lipid (% w/w db)	Ash (% w/w db)	Starch (% w/w db)	AM (% w/w of starch)	Starch damage (% of starch)	Moisture (% w/w)	WRC (% w/w)
Maize								
Regular	0.37 ± 0.02 ^a	0.21 ± 0.05	0.07 ± 0.01	96.3 ± 1.9	22.7 ± 0.6	1.30 ± 0.01 ^a	12.6 ± 0.06 ^a	58.6 ± 0.1 ^a
hAM	0.56 ± 0.01 ^b	0.21 ± 0.07	0.13 ± 0.01	92.2 ± 0.1	71.0 ± 2.3	1.74 ± 0.09 ^b	12.8 ± 0.03 ^b	109.9 ± 0.7 ^b
Waxy	0.20 ± 0.01 ^c	0.12 ± 0.03	0.07 ± 0.01	94.3 ± 0.8	2.5 ± 0.4	1.91 ± 0.04 ^c	12.9 ± 0.02 ^b	90.2 ± 3.4 ^c
Potato								
Regular	0.08 ± 0.01	0.19 ± 0.02	0.33 ± 0.01	93.4 ± 0.6	20.9 ± 3.7	0.46 ± 0.05 ^a	14.6 ± 0.06 ^a	65.4 ± 1.6 ^a
Waxy	0.08 ± 0.05	0.13 ± 0.04	0.28 ± 0.01	91.7 ± 1.2	2.2 ± 0.6	0.73 ± 0.06 ^b	16.9 ± 0.10 ^b	79.0 ± 2.5 ^b
Wheat								
Regular	0.19 ± 0.02	0.14 ± 0.04	0.16 ± 0.01	92.8 ± 0.1	27.8 ± 0.5	1.97 ± 0.11	13.0 ± 0.09	58.9 ± 0.9
Barley								
Regular	0.32 ± 0.01 ^a	0.18 ± 0.05	0.18 ± 0.01	94.8 ± 0.2 ^a	24.7 ± 0.8	1.14 ± 0.02 ^a	11.6 ± 0.08 ^a	70.6 ± 0.3 ^a
Waxy	0.38 ± 0.01 ^b	0.27 ± 0.06	0.21 ± 0.01	92.3 ± 0.6 ^b	3.4 ± 0.7	1.91 ± 0.04 ^b	13.9 ± 0.14 ^b	87.1 ± 0.6 ^b

Means in the same row with different letters are significantly different ($\geq 3 = \text{One-Way ANOVA}$; $\geq 2 = t\text{-Test}$, $p < 0.05$).

The tuber starches contained a lower level of protein and a higher level of ash compared to the cereal starches. Higher moisture contents of the tuber starches were also evident. These chemical traits of potato starch are in agreement with previously reported chemical characteristics of tuber and root starches (Hoover, 2001; Tester et al., 2004). The high ash content might have been associated with the presence of higher phosphorus contents and the moisture content might have been related to the B-type polymorphic form of potato starch. In contrast, the maize starches exhibited the lowest ash contents and the high-AM maize cultivar contained the highest amount of protein. The lipid contents of the starch samples could not be exactly quantified due to inherent inaccuracies of the Soxhlet method related to low-lipid content determination. In addition, a low efficiency of the Soxhlet method for the extraction of polar and bound lipids with apolar solvents was previously reported (Xiao, Mjøs, & Haugsgjerd, 2012). In agreement with data reported in the literature (Vasanthan & Hoover, 1992), the Soxhlet data showed that the levels of lipids in the different starches were low. The cereal starches contained higher quantities of lipids compared to the potato starches. The highest and lowest amounts of lipids were present in waxy barley starch and waxy maize starch, respectively. This variation in percentage of minor components depends on plant origin, polysaccharide composition, endosperm physical structure and texture and starch isolation method (Lindeboom et al., 2004; South & Morrison, 1990). All starches were characterized by high total starch contents ($>90\%$ w/w db). Regular maize starch showed the highest average purity (96.29%) and the lowest average purity was found in the waxy potato cultivar (91.65%). Due to the fact that the analyzed starches had been commercially acquired from various suppliers, minor differences in starch composition are likely attributable to qualitative differences in extraction and purification method. Regular starches were characterized by lower starch damage levels than their waxy counterparts. These higher levels of starch damage in non-regular starches indicated a lower resistance of their granules to mechanical stress. Among all analyzed starches, the tuber starches possessed the lowest starch damage levels. This characteristic feature might be the result of a more gentle extraction and refining process in comparison to the isolation of cereal starches.

3.2. Visual characterization by confocal laser scanning microscopy (CLSM) and micrograph analysis

In a preliminary experiment six fluorescent dyes, FITC, Nile blue (hydrogen sulfate), Safranin O, Acridine Orange, Rhodamine B and APTS, were used and their staining efficiency was evaluated.

In line with previous studies, the preliminary experiment revealed that the dyes were different in affinity or selectivity (Dürrenberger, Handschin, Conde-Petit, & Escher, 2001; Jekle & Becker, 2011a, 2011b; Schirmer et al., 2011). As a result, different intragranular dye concentrations and overall micrograph contrasts were obtained. There is only little or no information available on the way in which extent the dyes interact with starch granules. In the present study, CLSM micrographs revealed that the water-soluble basic oxazine dye Nile blue was the most suitable fluorescent staining agent for the observation of native starch granules (Schirmer et al., 2011; Tajalli, Gilani, Zakerhamidi, & Tajalli, 2008). The other fluorescent reagents stained starch granules as well, but the less intense or insufficient labeling resulted in heterogeneous staining and reduced optical contrast within granule fine structures.

3.2.1. Morphological characterization by micrographs of different starches

Fig. 1 shows selected confocal laser scanning micrographs of the native regular, high-AM and waxy starches. The confocal laser scanning micrographs of the regular, waxy and high-AM starches revealed significant differences in granule size and shape between plant species. A number of characteristic size and shape parameters of the starch granules obtained using ImageJ analysis are summarized in Table 2. The three maize starches exhibited detectable morphological variation among the different maize cultivars. High-AM maize starch consisted of significantly smaller granules than its regular and waxy counterparts analyzed by the perimeter ($P = 38.4 \mu\text{m}$, $39.4 \mu\text{m}$ and $29.3 \mu\text{m}$ for regular, waxy and high-AM maize starch, respectively) and the granules average size ($\text{OA} = 106.4 \mu\text{m}^2$, $107.4 \mu\text{m}^2$ and $62.3 \mu\text{m}^2$ for regular, waxy and high-AM maize starch, respectively). A small number of granules was also observed to be elongated rod-like, triangular or filamentous in shape. This observation is in agreement with previous findings reported by Glaring, Koch, and Blennow (2006) and Jiang, Horner, et al. (2010), Jiang, Jane, et al., 2010. The latter proposed a model of formation of these starch granules in which fusion of two starch granules in the same amyloplast occurs at an early stage of kernel development. Regular and waxy maize starch closely resembled one another in terms of granule shape and size. In comparison to maize starch, granules from wheat and barley starch showed a clear bimodal size distribution. The large A-type granules of wheat starch exhibited non-uniform, lenticular or disk shapes. The shape of the smaller B-type wheat starch granules was predominantly spherical or ellipsoidal. In contrast, barley starch

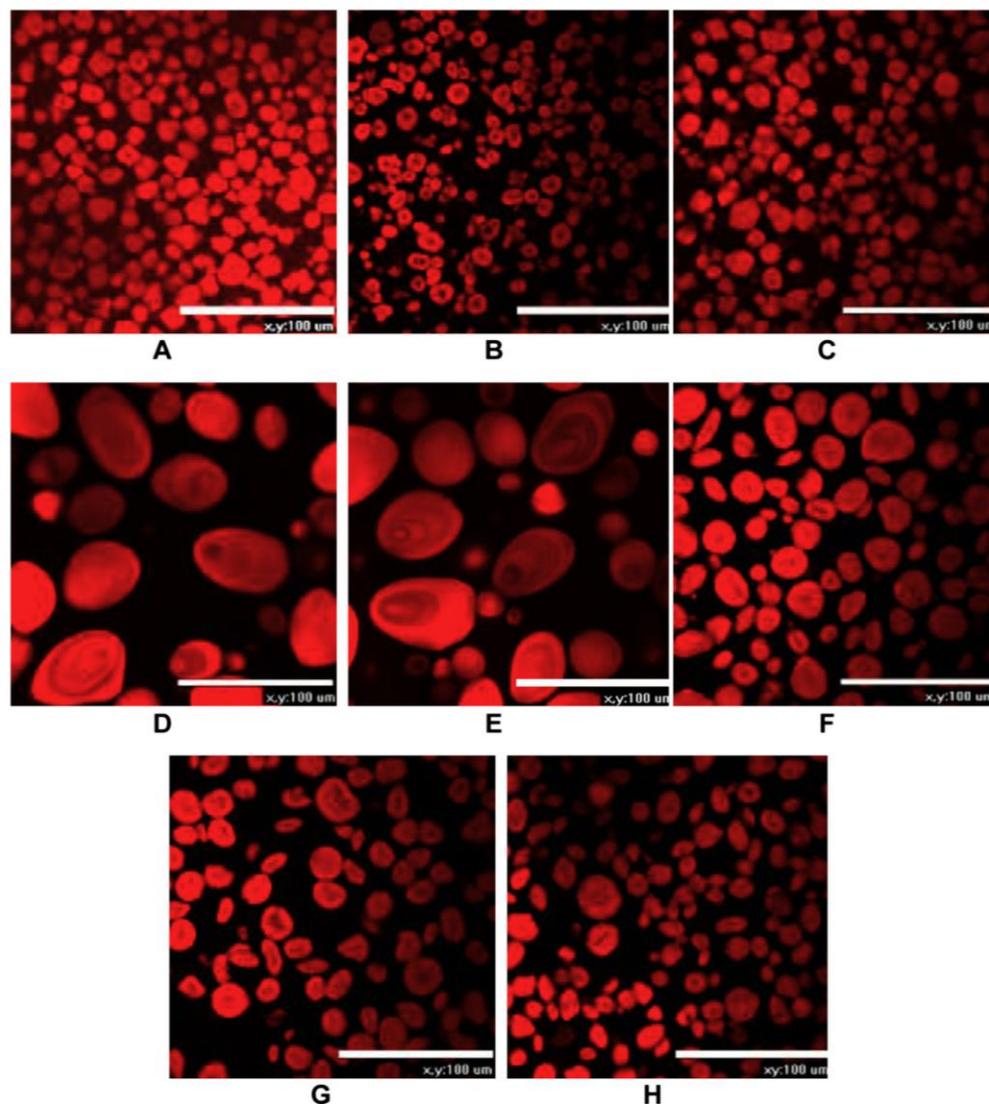


Fig. 1. Confocal laser scanning micrographs of starches from (A) regular maize, (B) High-AM maize, (C) Waxy maize, (D) Regular potato, (E) Waxy potato, (F) Regular wheat, (G) Regular barley and (H) waxy barley. Scale bar: 100 μm .

displayed large lenticular A-type granules and small irregular-shaped, elongated B-type granules. The B-type granules of waxy barley starch seemed to be smaller and more spherical in shape than those of regular barley, whereas the larger A-type granules apparently displayed similar size and shape. This different in granules type is a reason of high standard deviation of micrograph analysis. The granules average size of the regular wheat and barley starch granules were very similar analyzed by the perimeter ($P = 53.8 \mu\text{m}$ and $52.7 \mu\text{m}$ for regular wheat and barley starch, respectively) and granule average size ($\text{OA} = 223.9 \mu\text{m}^2$ and $195.2 \mu\text{m}^2$ for regular wheat and barley starch, respectively). The waxy barley starch granules, in contrast, had a smaller average size ($\text{OA} = 141.7 \mu\text{m}^2$). The potato starches displayed exceptionally large granules compared to the cereal starches. Both regular and waxy potato starch granules were also distributed in a typical

bimodal fashion and round to oval in shape. Similar to the waxy barley starch granules, the waxy potato starch granules were of smaller average size than their regular counterparts ($\text{OA} = 738.2 \mu\text{m}^2$ and $601.9 \mu\text{m}^2$ for regular and waxy potato starch, respectively). The larger granule fraction was exclusively ellipsoid in shape and the smaller granule fraction was basically spherical in shape analyzed by the circularity ($C = 0.71, 0.61$) against maize, wheat and barley ($C > 0.80$). Previous studies have described the microscopic analysis of granule size and shape characteristics using scanning electron microscopy or light microscopy in combination with micrograph analysis (Jiang, Jane, et al., 2010; Torres, Troncoso, Díaz, & Amaya, 2011). In the present study, the characterization of starch granule morphology using CLSM and micrograph analysis has proven to be a reliable and promising technique.

3.2.2. Visual and image processing analysis of starch gelatinization characteristics of confocal laser scanning micrographs

CLSM imaging has also been used to investigate the internal structures as well as the gelatinization characteristics of starch granules. Fig. 2 represents aqueous suspensions of different starch samples heated for 0–3 min. After a special heating time the granules (ghosts) were no more detectable. The starch granules showed markedly different resistance to swelling and disintegration which is shown by micrograph analyzes in Table 2. During heating expansions of all the starches seems to proceed in all directions. Cereal starches granules expand equally in length and width, whereas the oval-shaped potato starch (D, E) granules more in length than in width expand which is analyzed by the circularity (heating time of 3 min, $C = 0.71, 0.65, 0.64$ and 0.52 for regular maize (A), wheat (F), barley (G) and potato (D), respectively).

Table 2

Size characterization and structural features of confocal laser scanning micrographs of the starch samples determined by image processing, $\bar{X} \pm \text{sd.}$, ($n = 3$). After a heating time of 3 min the most starch granules (ghosts) were no more detectable by the digital analyzing system.

Starch type	Heating time (min)	$\bar{\text{O}}\text{A}$ (average size) (μm^2)	P (perimeter) (μm)	C (circularity) (–)
Maize				
Regular	0.0	106.4 ± 4.9	38.4 ± 0.9	0.83 ± 0.02
	0.5	336.1 ± 37.5 *	68.1 ± 4.9 ***	0.84 ± 0.02
	1.0	809.3 ± 104.1 ***	111.7 ± 7.7 ***	0.76 ± 0.02 **
	2.0	882.4 ± 18.2 ***	117.9 ± 1.1 ***	0.78 ± 0.00 *
	3.0	1144.9 ± 328.4 ***	152.9 ± 23.7 ***	0.71 ± 0.03 ***
High-AM	0.0	62.3 ± 3.3	29.3 ± 0.9	0.83 ± 0.01
	0.5	78.1 ± 17.7	26.5 ± 4.0	0.62 ± 0.02 ***
	1.0	142.1 ± 39.3 ***	36.0 ± 5.8 ***	0.61 ± 0.01 ***
	2.0	206.4 ± 33.2 ***	42.3 ± 4.7 ***	0.56 ± 0.01 **
	3.0	213.9 ± 16.1 ***	43.4 ± 2.6 ***	0.54 ± 0.01
Waxy	0.0	107.4 ± 7.2	39.4 ± 1.7	0.78 ± 0.02
	0.5	666.4 ± 214.3 ***	108.5 ± 22.5 ***	0.71 ± 0.05 **
	1.0	1071.1 ± 210.1 ***	139.8 ± 10.9 ***	0.67 ± 0.04 **
	2.0	1235.8 ± 191.6 ***	152.3 ± 21.0 ***	0.63 ± 0.04 ***
	3.0	1376.481 ± 240.8 ***	149.1 ± 16.8 ***	0.63 ± 0.04 ***
Potato				
Regular	0.0	738.2 ± 165.0	100.5 ± 12.0	0.71 ± 0.05
	0.5	4592.9 ± 1895.1	302.6 ± 45.6 **	0.59 ± 0.07
	1.0	4486.2 ± 1514.9	306.8 ± 45.2 **	0.57 ± 0.04 *
	2.0	6682.6 ± 2091.0 **	318.6 ± 39.0 ***	0.57 ± 0.09 *
	3.0	6983.8 ± 4383.2 **	381.6 ± 125 ***	0.52 ± 0.05 **
Waxy	0.0	601.9 ± 50.2	96.4 ± 3.2	0.61 ± 0.02
	0.5	947.7 ± 214.3	124.6 ± 13.2	0.68 ± 0.01
	1.0	1057.3 ± 568.7	151.1 ± 61.5	0.62 ± 0.15
	2.0	2210.6 ± 236.3 *	232.6 ± 38.9 *	0.54 ± 0.13
	3.0	2639.8 ± 1113.2 *	275.5 ± 75.3 **	0.38 ± 0.01
Wheat				
Regular	0.0	223.9 ± 16.6	0.0 ± 0.0	0.82 ± 0.03
	0.5	348.0 ± 43.6	74.7 ± 7.3 *	0.72 ± 0.06 **
	1.0	397.3 ± 129.3 **	88.4 ± 19.8 ***	0.61 ± 0.06 ***
	2.0	421.5 ± 91.7 **	88.7 ± 11.0 ***	0.64 ± 0.04 ***
	3.0	533.8 ± 103.2 ***	99.5 ± 10.1 ***	0.65 ± 0.02 ***
Barley				
Regular	0.0	194.7 ± 9.2	52.6 ± 1.8	0.81 ± 0.01
	0.5	612.2 ± 28.4 ***	101.3 ± 2.2 ***	0.71 ± 0.03 **
	1.0	750.4 ± 105.3 ***	121.1 ± 9.4 ***	0.62 ± 0.01 ***
	2.0	954.7 ± 90.7 ***	136.5 ± 10.8 ***	0.65 ± 0.04 ***
	3.0	1282.7 ± 183.1 ***	159.6 ± 16.1 ***	0.64 ± 0.05 ***
Waxy	0.0	141.7 ± 16.6	0.0 ± 0.0	0.00 ± 0.00
	0.5	1396.2 ± 321.1 ***	159.6 ± 26.7 ***	0.69 ± 0.04 ***
	1.0	1057.0 ± 164.9 ***	130.7 ± 12.2 ***	0.75 ± 0.02
	2.0	1268.3 ± 239.5 ***	153.6 ± 19.0 ***	0.65 ± 0.03 ***
	3.0	1292.8 ± 270.6 ***	156.7 ± 21.5 ***	0.64 ± 0.03 ***

Results are shown as mean $\bar{X} \pm 95\%$ confidence interval, $n = 3$. From 0.0 min heating time significant different values are marked (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

During heating, the large regular potato starch (D) granules presented a faster structural resolution compared to the cereal starch granules (heating time 0.5 min = more than 6 times for potato- and around 1.5 times for wheat starch (F)). Especially the initial expansion showed huge differences between regular and irregular starches analyzed by structural features. Waxy starches showed an increase of the rate and degree of granule swelling, whereby a decrease was analyzed for high-AM maize (B). At prolonged heating, the gelatinization process of waxy starch ends earlier, resulting in complete disruption of the granules after 2 min.

The high-AM maize (B) starch exhibited an increased resistance to granule swelling and disintegration. Its granules only swelled to a very limited extent (heating time 3 min, $\bar{\text{O}}\text{A} = 213.9 \mu\text{m}^2$ against $1376.5 \mu\text{m}^2$ for regular maize (A)) and together with low increase of perimeter. This phenomenon of restricted granule expansion is based on the physical characteristics of AM molecules in high-AM maize starch (B). It is suggested that the facilitated interaction of linear AM chains induces higher granule integrity (Colonna & Mercier, 1985). A complete gelatinization and granule disruption after heating for 3 min was observed for the waxy cereal starches. They rapidly and uniformly expanded in size and disintegrated more quickly than their regular counterparts (refer to Table 2). In the first stage of heating, CLSM imaging revealed the appearance of a large central cavity at hilum level which expanded as the heating time increased. This observation confirmed that starch granule gelatinization starts in the hilum size and proceeds toward the periphery (Singh, Singh, Kaur, Singh Sodhi, & Singh Gill, 2003). During gelatinization, mobilized AM molecules diffused out of the granule interior into the surrounding aqueous medium. As a consequence of this progressive dissolution of starch polymers with increasing heating time, Nile blue molecules mainly redistributed from the granule interior to the leached AM and AP chains in aqueous solution. This resulted in an apparent increase in background fluorescence signal.

However, it should be noted that the micrographs were recorded at various gain settings. For each sample the instrument gain setting was adjusted to optimize signal intensity of the stained gelatinized granule remnants and to minimize background fluorescence. Thus, the micrographs did not allow an analysis of the fluorescence intensities.

3.2.3. Visualization of starch granule internal structure

An additional aim of the present study was to detect a possible relationship between the Nile blue-staining patterns of the granules and their respective AM/AP contents. CLSM micrographs of the individual starch granules at different magnifications are presented in Fig. 3. Growth rings were clearly visualized in all native starch samples except for the high-AM maize starch (B). The sharpest and most intensive growth ring structures were revealed in the large regular (D) and waxy potato starch (E) granules. The growth rings were ellipsoid-shaped and deposited around the eccentric hilum. In contrast to the potato starches, the cereal starches displayed a central hilum. Exemplary the high-AM maize (B) and waxy barley starch (H) granules displayed severe internal cracks, which might be related to the altered starch metabolism of starches or the higher susceptibility to mechanical damage (Bettge & Morris, 2000; Blennow, Hansen, et al., 2003). Defects in starch granule morphology might also have been caused by differing isolation, drying and milling conditions (Grant, 1998). The Nile blue staining of the regular and waxy cereal starches revealed a pattern of alternating concentric structures with different intensities of fluorescence. In comparison to the potato starch (D) granules, fluorescence contrast between the stained and unstained or weakly stained growth rings was less pronounced. Due to the characteristic layered structure of the detected growth rings, the rings were

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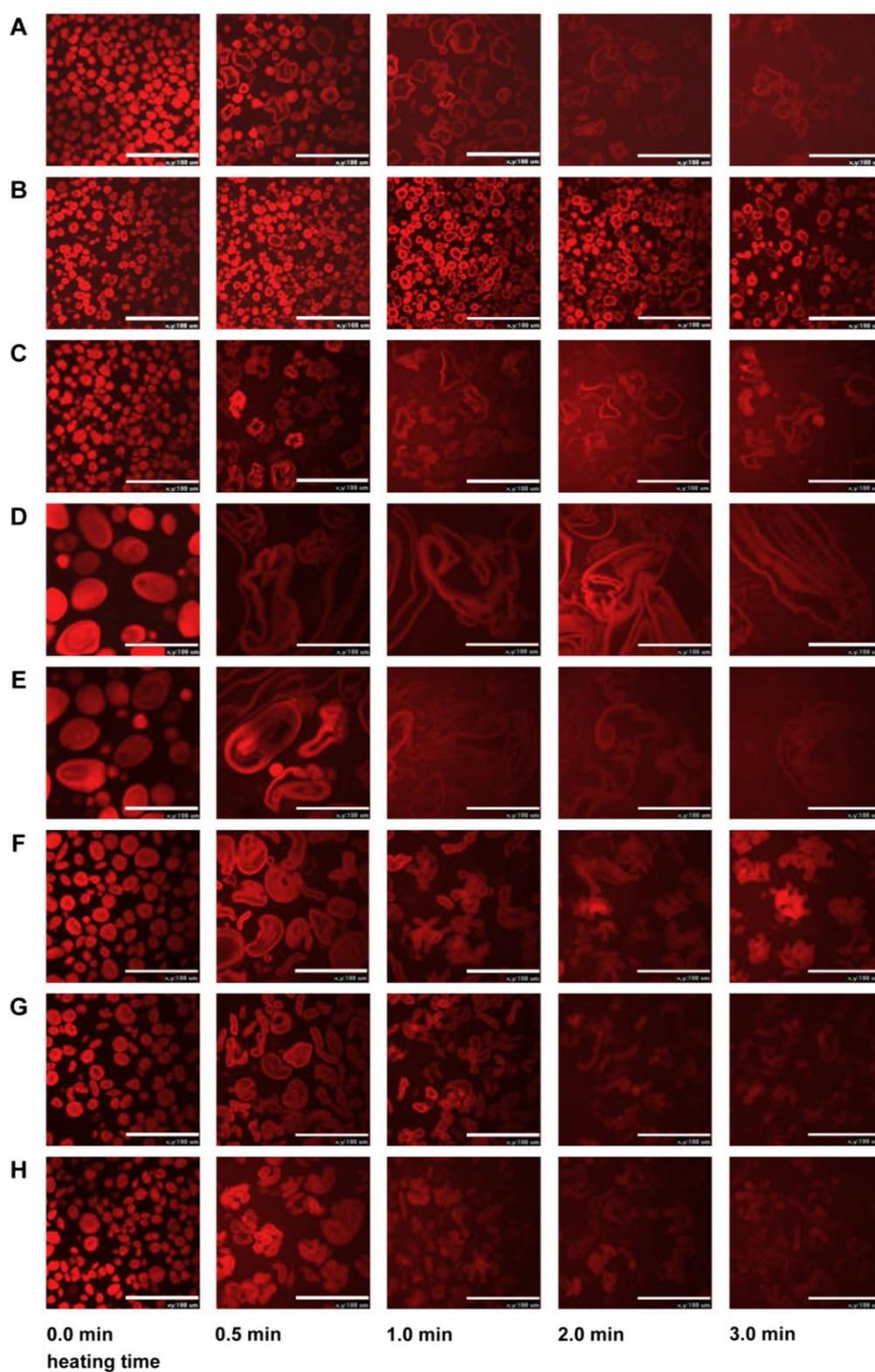


Fig. 2. Confocal laser scanning micrographs of starches from (A) regular maize, (B) High-AM maize, (C) Waxy maize, (D) Regular potato, (E) Waxy potato, (F) Regular wheat, (G) Regular barley and (H) waxy barley during gelatinization. Scale bar: 100 μm.

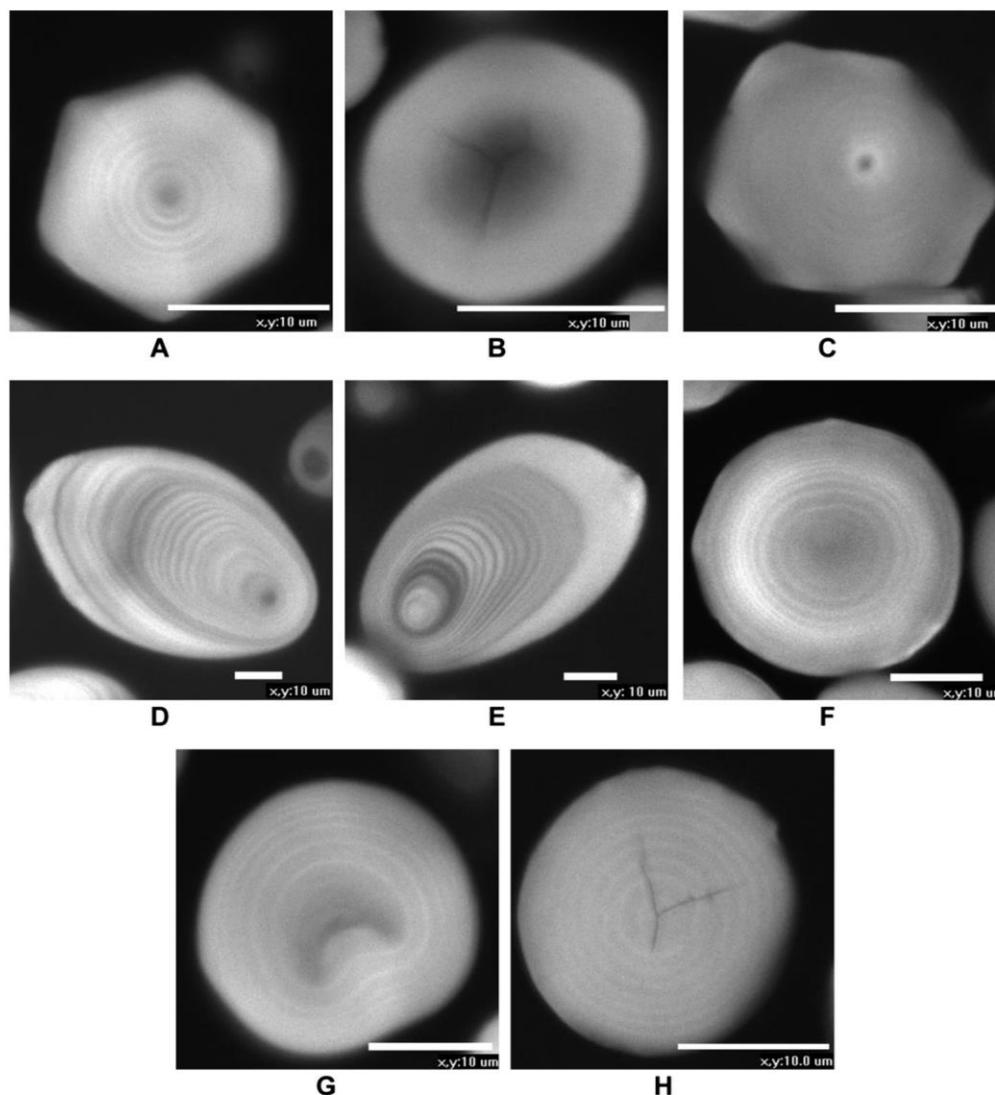


Fig. 3. Confocal laser scanning micrographs of the internal structure of starches from (A) regular maize, (B) High-AM maize, (C) Waxy maize, (D) Regular potato, (E) Waxy potato, (F) Regular wheat, (G) Regular barley and (H) waxy barley. Scale bar: 10 μm .

assumed to correspond to the aforementioned alternating semi-crystalline and amorphous domains. Thus, the use of Nile blue did not allow a direct differentiation between AM and AP molecules in the granule. Both molecules are composed of linear chains of α -(1 \rightarrow 4)-D-glucans and differ with respect to the frequency and length of α -(1 \rightarrow 6) branches but with a chemical similarity of AM and AP molecules. The highly branched AP molecules are predominantly deposited in the semi-crystalline growth rings with the majority of the branch chains being ordered within crystalline cluster structures. In contrast, AM has been suggested to be mainly located within the amorphous zones of the two distinct granule domains (Pérez & Bertoft, 2010).

The high-AM maize starch granules showed no existence of growth rings. The granules were uniformly stained and growth rings were obscured. This observation suggests that the fluorescent

dye accumulated in the AM-rich domains of the granule. Nile blue, which might predominantly exist in its protonated form in aqueous solution, is proposed to possess an enhanced affinity to the amorphous background region of starch granule structure. It might preferentially penetrate into the less ordered and therefore, more accessible regions of the granule, which are also known to be highly susceptible to enzymatic attack (Blazek & Gilbert, 2010). It is further the amorphous growth rings that first undergo molecular disordering by water-absorption and swelling during the early stages of starch gelatinization (Jenkins & Donald, 1998). In line with the importance of granule porosity for enzymatic degradability of native starch, peripheral granular pores and channels were considered to facilitate the access of Nile blue to the granule interior (Blazek & Gilbert, 2010; Glaring et al., 2006). The fluorescence contrast obtained with Nile blue could be explained by nonspecific

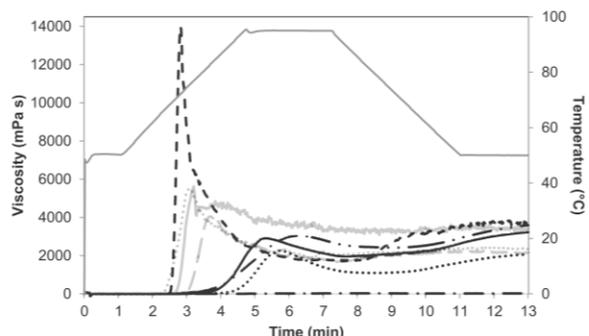


Fig. 4. Pasting profiles of regular, waxy and high-AM starches measured by RVA with special temperature profile (—); maize (•), waxy maize (---), high AM-Maize (- - -), potato (• - •), waxy potato (—), wheat (---), barley (· · · ·), waxy barley (· · · ·).

hydrophobic interactions between the dye and the amorphous zones of the granules. It resulted in the visualization of the AM-enriched and less ordered parts.

In the regular and waxy potato starch granules, the thickness of the dark growth rings, which were supposed to represent the semi-crystalline domains, was smaller than that of the bright colored amorphous rings. In the cereal starch granules, ring width and periodicity differed from that in potato starch granules. The width of the dark rings appeared to be greater than that of the bright rings. There was also no detectable difference between the growth ring structures in granules from regular and waxy varieties of the same plant species. Similar to the potato starch granules, a decrease in ring width from the granule center to the periphery could be observed. Earlier published scanning and transmission electron microscopy studies have revealed that the average thickness and number of growth rings varied depending on the botanical origin and AM content of the starch (Li, Vasanthan, Hoover, & Rosnagel, 2003; Yuryev, Krivandin, et al., 2004). However, the same studies have also brought up controversial results for the average thickness of the semi-crystalline growth rings with increasing AM content. Reports on the thickness of the amorphous growth rings, on the other hand, are rare. The amorphous rings might be similar in size to the semi-crystalline rings. A small-angle X-ray scattering study showed further that the average AP cluster size, i.e. the combined size of one crystalline and one amorphous lamellae, remained unaffected by the AM and AP content (Jenkins & Donald, 1995). All these data suggest that it

would be difficult to find a relationship between the thickness of the growth rings and the AM/AP content of the starch samples. The obtained Nile blue staining pattern might also have been influenced by the excessive hydration of the granules and the preferential swelling within their less dense amorphous domains. A penetration of Nile blue into the amorphous or crystalline lamellae cannot be further excluded. In conclusion, the different fluorescence staining patterns are assumed to be caused by inhomogeneities within the starch granule matrix. There could not be identified any difference in staining between AM and AP. Details on the nature of the interaction between Nile blue and starch granule structural components were not known.

3.3. Pasting properties

Fig. 4 illustrates representative RVA pasting patterns of the different starches. In addition, characteristic pasting parameters of the samples generated by RVA are summarized in Table 3. Considerable differences in RVA pasting characteristics were observed among the starch samples depending on plant species and chemical composition of total starch. The starches from waxy maize and barley displayed a higher average peak viscosity (PV) and breakdown (P) and a lower total setback (S) and pasting temperature (T_p) compared to their regular counterparts. During the initial heating process, paste viscosities of waxy maize and barley starch suspension rapidly increased and reached the first maximum after a short heating time. A further increase in heating temperature caused the quick disintegration of swollen starch granules. As a result large breakdown viscosities could be recorded. Upon cooling, the paste viscosities only increased slightly which indicated the lack of AM and resulted in reduced setback values. These observations suggest that waxy cereal starch granules in aqueous suspension show reduced thermal stability and thus start to gelatinize at a lower temperature. They swell very quickly and increase the paste viscosity when heated above a critical temperature. The high susceptibility of swollen waxy starch granules to thermal and mechanical breakdown facilitates granule rupture and induces loss of paste viscosity. The reduced AM content in waxy starches results in limited AM leaching and in the formation of a low-viscous matrix after cooling (Gunaratne, Ranaweera, & Corke, 2007). In contrast to AM molecules, which exhibit a pronounced tendency to retrograde during the cooling process of the cooked starch paste, the AP molecules are known to recrystallize over an extended period of time. Thus, the viscosity of waxy starch pastes only increases gradually upon cooling and ageing. Similar pasting

Table 3
Rheological and pasting properties of starches as measured with the RVA, $\bar{X} \pm \text{sd}$, ($n = 4$).

Starch type	T_p (°C)	PV (mPa s)	t_p (min)	HPV (mPa s)	FV (mPa s)	P (mPa s)	S (mPa s)
Maize							
Regular	74.2 ± 0.4 ^a	2910 ± 42 ^a	5.30 ± 0.03 ^a	1965 ± 39 ^a	3227 ± 31 ^a	945 ± 16 ^a	1262 ± 28 ^a
High-AM	n.d.	23 ± 2 ^b	6.81 ± 0.10 ^b	18 ± 2 ^b	22 ± 1 ^b	6 ± 1 ^b	4 ± 2 ^b
Waxy	71.9 ± 0.1 ^b	4085 ± 53 ^c	3.68 ± 0.02 ^c	1592 ± 55 ^c	2155 ± 54 ^c	2493 ± 18 ^c	563 ± 49 ^c
Potato							
Regular	66.9 ± 0.2 ^a	14090 ± 64 ^a	2.83 ± 0.02 ^a	1666 ± 79 ^a	3778 ± 124 ^a	12424 ± 106 ^a	2039 ± 254 ^a
Waxy	67.9 ± 0.3 ^b	5687 ± 81 ^b	3.21 ± 0.02 ^b	2884 ± 164 ^b	3402 ± 104 ^b	2803 ± 115 ^b	518 ± 113 ^b
Wheat							
Regular	65.4 ± 0.2	3046 ± 27	6.27 ± 0.10	2417 ± 82	3590 ± 52	629 ± 65	1173 ± 84
Barley							
Regular	68.0 ± 1.4 ^a	2279 ± 39 ^a	5.7 ± 0.10 ^a	1089 ± 57 ^a	2070 ± 23 ^a	1190 ± 50 ^a	981 ± 56 ^a
Waxy	64.0 ± 0.1 ^b	5544 ± 55 ^b	3.1 ± 0.03 ^b	1794 ± 44 ^b	2334 ± 32 ^b	3750 ± 59 ^b	540 ± 61 ^b

Means in the same row with different letters are significantly different ($\geq 3 = \text{One-Way ANOVA}$; $\geq 2 = t\text{-Test}$, $p < 0.05$).

characteristics of waxy starches have been previously reported for various botanical sources e.g. waxy wheat (Blazek & Copeland, 2008; Hung, Maeda, & Morita, 2007; Yoo & Jane, 2002), waxy barley (Song & Jane, 2000) and waxy maize (Jane et al., 1999; Yoo & Jane, 2002). All these waxy starches also revealed a lower T_p , higher PV, lower FV, larger breakdown as well as little setback compared to their regular counterparts measured using RVA or viscoamylograph. Among all the starches that have been investigated in this study, maize starches displayed the highest T_p and regular wheat starch displayed the lowest T_p . The magnitude of the parameters peak viscosity (PV), hot paste viscosity (HPV) and final viscosity (FV) for regular cereal starches was also observed to follow the order of wheat starch > maize starch > barley starch. In contrast to their cereal counterparts, the tuber starches showed different paste viscosity patterns. The regular and waxy potato starches were both characterized by a higher PV, which, however, was positively related to the AM content. In addition, the RVA parameters of regular potato starch except for T_p and HPV were found to be higher than those of the other starches. Its pasting profile displayed a very sharp peak in combination with a major thinning during cooking. The pasting pattern of waxy potato, on the other hand, showed a lower PV and a reduced shear thinning during cooking. The pasting curve even exhibited an extremely irregular shape. These remarkable differences in PV between regular and waxy potato starch are consistent with recent findings of Varatharajan, Hoover, Liu, and Seetharaman (2010). Higher AM content of regular potato starch enabled greater intermolecular interaction between potato AM and AP molecules, maintaining granule integrity. Thus, the regular granules were enabled to extensively enlarge in size and to reach a higher PV. On the other hand, the fragile waxy potato starch granules swelled and solubilized rapidly, leading to lower PV, rapid P and lower S.

Hence, the different pasting patterns of the analyzed cereal and potato starches imply that intrinsic factors other than AM/AP content affect starch functional properties. Potato starches contain high levels of phosphate monoesters, which are covalently bound to the AM and AP fraction. The negatively charged phosphate groups induce a more rapid and greater extent of granule swelling, low T_p , high PV and a retarded retrogradation of the potato starch. The absence of starch lipids and phospholipids in tuber starches intensifies the various effects of phosphate monoesters on thermal pasting behavior (Hoover, 2001; Keetels, Visser, van Vliet, Jurgens, & Walstra, 1996). In contrast, regular cereal starches and high-AM maize starch are characterized by high phospholipid levels: These lipids are complexed with AM single helices or long branch-chains of AP, retard granule swelling and prevent the AM from leaching during starch gelatinization (Jane et al., 1999). Thus they reduce PV and increase T_p . Waxy cereal starches, which contain negligible amounts of phosphorus and lipids, quickly develop viscosity and reach high PV at lower T_p (Jane et al., 1999; Singh, Singh, et al., 2003; Tester & Morrison, 1990).

Both the pasting behavior of starch and the degree of granule swelling are further strongly dependent on starch granule size (Fortuna, Januszewska, Juszczyk, Kielski, & Palasiński, 2000; Zaidul, Yamauchi, et al., 2007). Native starches with a high proportion of large granules, e.g. potato starch, display a unique swelling capability and form highly viscous pastes (Sanchez, Dufour, Moreno, & Ceballos, 2010; Singh, Singh, et al., 2003). However, an effect of granule size on T_p was not evident. The high PV of regular potato starch correlates with an extensive AM leaching, negating the effect of granular swelling when compared to waxy potato starch (Varatharajan et al., 2010). The study revealed no significant differences in chemical composition (protein, lipid and ash content) and AP branch chain-length distribution between regular and waxy potato starch.

The high-AM maize starch displayed the lowest RVA pasting parameter (PV, HPV, FV, breakdown and setback) among the set of starches analyzed in this study. These results indicated, that the granules of high-AM maize starch were less susceptible to thermal treatment and mechanical shearing during measurement in the RVA. Hence, their restricted swelling and resistance to disintegration led to a minor increase in paste viscosity, a slight breakdown and a negligible setback. The high thermal stability of the high-AM starch compared to the insufficient thermal stability of the regular and waxy starches thus does not allow the use of the common RVA technology. Concerning the use of a High Pressure RVA, which applies high temperature, elevated pressure and shear, the viscous behavior of the high-AM maize starch could be assessed equally (Case, Capitani, et al., 1998). The high granule integrity of the high-AM maize starch is reported to be caused by strong molecular bonding forces within the granule interior.

3.4. Thermal properties

Table 4 summarizes the endothermic transition temperatures (T_0 , T_p and T_c), gelatinization temperature ranges (ΔT) and enthalpy changes (ΔH) of the native starch samples on a dry weight basis. The thermal analysis showed that the chosen starch samples were different in T_p and ΔH with respect to their AM content. This observation is in agreement with a previous study of Cooke and Gidley (1992) who stated that thermal transition characteristics of native regular, waxy and AM-rich starches would be highly dependent on the AM/AP content and the respective crystalline and molecular order. In contrast to the regular and waxy starches, the high-AM maize starch showed one irregular-shaped endothermic peak at the upper limit of the measured temperature range.

In comparison to their regular counterparts, the waxy starches were characterized by higher average ΔH which suggests that they had a more compact physical structure. This result agreed well with previous findings for maize (Jane et al., 1999), potato (Varatharajan et al., 2010), wheat (Hung et al., 2007) and barley starch (Matsuki, Yasui, Sasaki, Fujita, & Kitamura, 2008). Previous X-ray diffraction studies of regular, waxy and high-AM starch granules have shown that starch granules markedly differ in crystallinity. Waxy starches displayed a dense, highly ordered structure whereas the regular starches were less ordered and more loosely packed. Thus, it can be assumed that a decrease in starch crystallinity correlates with an increase in AM content (Hung et al., 2007). As a result of the higher percentage of AP in waxy starches, more thermal energy is needed

Table 4
The endothermic transition temperatures and enthalpy changes observed for starch gelatinization in water measured by DSC, $\bar{X} \pm \text{sd.}$, ($n = 5$).

Starch type	T_0 (°C)	T_p (°C)	T_c (°C)	ΔT (°C)	ΔH (J g ⁻¹)
Maize					
Regular	66.7 ± 0.1 ^a	71.1 ± 0.1 ^a	74.9 ± 0.2 ^a	8.2 ± 0.2 ^a	10.7 ± 0.4 ^a
High-AM	n.d.	n.d.	n.d.	n.d.	n.d.
Waxy	66.6 ± 1.9 ^a	71.8 ± 0.1 ^b	76.6 ± 0.4 ^b	10.0 ± 2.0 ^b	12.9 ± 0.6 ^b
Potato					
Regular	61.8 ± 0.1 ^a	65.5 ± 0.1 ^a	70.0 ± 0.2 ^a	8.2 ± 0.1 ^a	14.9 ± 0.2 ^a
Waxy	63.6 ± 0.1 ^b	69.2 ± 0.2 ^b	75.0 ± 0.3 ^b	11.3 ± 0.2 ^b	15.8 ± 0.8 ^b
Wheat					
Regular	53.3 ± 1.8	60.6 ± 0.2	64.9 ± 0.5	11.6 ± 2.1	8.5 ± 0.5
Barley					
Regular	60.1 ± 0.2 ^a	63.5 ± 0.1 ^a	66.7 ± 0.3 ^a	6.6 ± 0.1 ^a	9.4 ± 0.4 ^a
Waxy	57.9 ± 0.2 ^b	63.2 ± 0.1 ^b	68.8 ± 0.2 ^b	10.9 ± 0.3 ^b	10.8 ± 0.2 ^b

Means in the same row with different letters are significantly different ($\geq 3 = \text{One-Way ANOVA}$; $\geq 2 = t\text{-Test}$, $p < 0.05$).

to disrupt intermolecular bonds, e.g. hydrogen-bonds linking adjacent chains or helices, within starch structure (Colonna & Mercier, 1985). In the current study, the waxy starches of maize and potato had a higher T_m than their regular counterparts. The waxy barley starch, however, showed a slightly lower T_p compared to its regular counterpart. The highest T_o and T_p values were obtained for the maize starch cultivars followed by potato, barley and wheat starch cultivars. On the other hand, the highest ΔH values were calculated for the potato starches followed by the maize, barley and wheat starches. These differences in T_p and ΔH between the starches of different botanical origin were suggested to be mainly influenced by the crystallite perfection together with a range of other factors such as lipid and phosphorus content, AP branch chain lengths and distribution, starch polymorphism and length of AP chains involved in crystalline arrangements. A study with regular wheat and barley starch has revealed their defective crystalline structure and suggested a contribution of this defect to the low T_p (Jane et al., 1999). The study also suggested that the high concentration of phosphate monoesters in potato starch together with the B-type polymorphism of this starch, contributed to a lower T_p . It has further been reported that the ΔH of potato starches was positively related to the high percentage of long AP branch chains. This is usually indicated by larger enthalpy changes. The difference in ΔH between the regular cereal and potato starches might also be explained by the formation of helical inclusion complexes between AM molecules and free lipids during gelatinization. The formation of AM–lipid complexes within the regular cereal starches is an exothermic process and might result in a decrease of ΔH (Eliasson, 1986).

The gelatinization temperature range of the different starches also revealed a considerable variation. The barley starch exhibited the lowest range whereas the highest range had been assumed for the high-AM starch. These results were consistent with previous data reported in the literature (Jane et al., 1999). In general, the phase transition of one single starch granule might take place over a temperature range of 1–2 °C in excess water. The gelatinization of the whole population of starch granules, however, has been suggested to occur within a broader temperature interval (Fredriksson, Silverio, Andersson, Eliasson, & Åman, 1998). The different temperature ranges and gelatinization temperatures indicated that there were considerable differences in internal granular organization. A comparison of the results with previously published data needs the same fundamental applied DSC methods. Substantial variation was found in terms of sample preparation, sample/water ratio, heating rate and other measurement conditions (Yu & Christie, 2001).

4. Conclusion

The aim of the study was to evaluate relationships between the AM/AP content and the chemical, visual, pasting and thermal characteristics for the different samples.

All waxy starch samples were characterized by higher starch damage contents, which suggest a lower resistance to mechanical damage. The WRC of the starch samples was strikingly influenced by the AM/AP content, whereby the waxy starches retained more water. This finding might be explained by their altered granule architecture.

The CLSM analysis revealed that the starch granules significantly varied in size and shape. Micrograph analysis of CLSM pictures showed that the highest average size was measured for the regular potato starches ($\bar{\phi}A = 738.2 \mu\text{m}^2$), whereas the smallest average size was found for the high-AM-maize starches ($\bar{\phi}A = 62.3 \mu\text{m}^2$). An influence of the anomalous starch composition on the average size of the waxy and high-AM starch granules was evident. These

starches were found to possess a smaller average size, indicating an influence of their altered starch metabolism on the granule size distribution pattern. However, no obvious relationship between the specific staining patterns and the AM/AP contents of the starches could be identified. Depending on gelatinization characteristics the waxy starch granules were extremely fragile, swelled rapidly and disintegrated and solubilized easily.

The AM/AP ratio significantly affected the pasting properties of the analyzed samples. The RVA viscosity profiles revealed that high AP contents were associated with extensive granule swelling upon heating and high shear thinning of the aqueous pastes. However, upon cooling, the waxy starch pastes only became viscous very slow due to the lack of AM. Thus, the pastes displayed a higher PV and breakdown but a lower setback and T_p than their regular counterparts. During the heating phase, the RVA viscosity patterns of the starches reflected the temperature induced changes of the granules studied under CLSM. The differences in pasting behavior among the cereal and potato starches could be attributed to species specific variations in e.g. AM/AP ratio, phosphorus and lipid contents, AP branch chain-length distribution and granule size.

By DSC analysis all waxy starches showed a higher enthalpy of gelatinization (ΔH) and a lower gelatinization temperature (T_o) which is consistent to previous measurements like the peak viscosity (PV) analyzed by RVA. This observation suggested that waxy starch granules possess a more compact physical structure and a higher crystalline and molecular order. Similar to the RVA analysis, differences in thermal properties between the samples of different botanical origin were suggested to be dependent on lipid and phosphorus content, AP branch chain-lengths and distribution, but also on crystallite perfection, starch polymorphism and length of AP chains involved in crystalline arrangements.

In conclusion, this study shows significant differences in the morphological and physicochemical characteristics of starches with varying AM/AP contents. Compared to their regular counterparts, the waxy starches had a higher gelatinization temperature and enthalpy, which indicates a higher crystalline and molecular order. The primary advantage of micrograph analysis is the huge detectable amount of micrographs together with the micrograph conversion to a number format. These features offer the possibility for analyzing the starch granule changes in excess as well as in limited water systems during thermal processing.

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2.4 Quantification in starch microstructure as a function of baking time



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Quantification in starch microstructure as a function of baking time

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Abstract

The purpose of this study was the characterization of micro structural and thermal aspects of starch gelatinization in wheat dough/crumb during bread baking. The microstructure of starch granules was examined by *confocal-laser-scanning-microscopy (CLSM)* and evaluated by an image analyzing tool. Supporting crystallinity changes in wheat dough/crumb were analyzed by *differential-scanning-calorimetry (DSC)* and calculated by the content of *terminal extent of starch gelatinization (TEG)*. The micrograph of processed *CLSM* data showed starch structure changes during baking time. After gelatinization the starch fraction itself was inhomogeneous and consisted of swollen and interconnected starch granules. Image processing analyses showed an increment of mean granule area and perimeter of the starch granules. The results of *DSC* were examined to present an equation which provides a mean of predicting *TEG* values as a function of baking time. *CLSM* and *DSC* measurements present high significant linear correlation between mean starch granule area and *TEG* ($r = 0.85$). The possibility to combine *CLSM* with thermal physical analytical techniques like *DSC* in the same experiments is useful to obtain detailed structural information of complex food systems like wheat bread. Finally, it offers the option to enlarge the knowledge of microstructural starch changes during baking in combination with physicochemical transformation of starch components.

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Keywords: bread crumb; starch gelatinization; CLSM; DSC.

1. Introduction

The change from dough to bread during thermal heating process entails important structural modifications which depend on specified process conditions [1-5]. The resting time - used for dough rise and structure relaxing - is followed by the baking process which is an irreversible process causing physical and chemical changes of the product components with the objective of a specified volume and stabilized crumb structure. The volume depends on the oven-rise which is driven by the gas expansion.

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These gases, mainly - air, EtOH, CO₂ and water vapour, contribute to the bubble inflation during baking [6]. After the oven-rise, whilst thermal heating, protein denaturizes, starch gelatinizes and the crust formation creates a stabilized product.

Starch is the main component of wheat bread and its gelatinization induces major structural changes during baking. The knowledge about the degree of gelatinization is an important factor for the control and optimization of thermal heating processes. This gelatinization process involves partly irreversible structural changes of the starch granules with concurrent loss of its molecular order or crystallinity [7]. The swollen granules and partially solubilized starch act as essential structural elements of bread.

Various analytical methods have been used to describe starch gelatinization, e.g. ultrasonic, viscometers, *enzymatic analysis (EA)*, *nuclear magnetic resonance (NMR)*, X-ray crystallography, thermal- (*DSC*) and microscopic-analysis like *electron microscopy (EM)* and *light microscopy (LM)* [8-10]. Especially *LM* presents a valuable method for the study of the microstructure changes in starch [11-14]. Several authors [12, 15] have pointed out the difficulty involved in preparing dough and bread samples for microscopy. The disruption of the protein network and a distorted image of the bread crumb due to hydration during fixation [12] and staining are procedural methods. The shrinkage of starch and protein as a consequence of dehydration is also described. To validate these problems the *confocal laser scanning microscopy (CLSM)*, which is a technique for obtaining high resolution optical images with depth selectivity, was used by some authors [16-17]. The main advantage of *CLSM* is the ability to acquire in-focus images from selected depths without sample destruction. *CLSM* is already an approved method to visualize starch gelatinization. Primo-Martin et al. [18] e.g. used the confocal laser technique compared with *LM* to visualize the starch crystallinity in bread crust.

Besides these microscopic methods, the *differential scanning calorimetry (DSC)* - in which the thermal energy is required for maintaining a given rate of temperature changes - was used as one of the main tools for the investigation of thermally induced starch gelatinization during the course of the baking process [19]. Several parameters can be defined by *DSC*: gelatinization temperature (T_{max} , corresponding to that where half of the granules have lost their birefringence), initial or onset temperature (T_{on} , where birefringence loss starts) and final or end temperature (T_{end} , where 90 % of the granules have lost their birefringence) [20]. These temperatures, especially the gelatinization temperature, are characteristic of the biological origin of starch and a reflection of its internal structure.

Up to now all microscopic methods are restricted methods because they only focus on a group of objective of the structural changes. The usage of an image analyzing system is a crucial factor for the quantification of the results. The microstructure of starch granules was examined by *confocal laser scanning microscope (CLSM)* and analyzed by an image processing tool. Supporting crystallinity changes in wheat dough/bread were analyzed by *differential scanning calorimetry (DSC)* and calculated by the *terminal extent of starch gelatinization (TEG)*. The aim of this study was to characterize the micro and thermal structure of starch gelatinization in wheat dough/crumb during baking and to combine both analyzing systems to enhance their explanatory power.

2. Materials and Methods

2.1. Ingredients

All ingredients were weighed and mixed first under slow for 1 min at 53 rpm followed by faster mixing for 6 min at 106 rpm (Diosna laboratory kneaders with group controller, Multimixing S.A. GmbH, Osnabrück). An optimum of the dough temperature of 28 °C was maintained by tempering with the used distilled water. After mixing the dough was rested for 20 min at 30 °C and a relative moisture of 80 % (KOMA Koeltechnische Industrie, Roermond, Niederlande). Subsequently breads of 150 g were weighted, formed and placed in a tin (conical 110x70x80 mm, bottom 100x60 mm) (BICO GmbH,

Pfaffenhofen, Germany). Proofing was performed at 30 °C and relative moisture of 80 % for 60 min. For all baking tests a deck oven (Matador Store 128, Werner & Pfleiderer GmbH, Sohland, Germany) was used with different baking temperatures and a steam amount of 0.930 L(H₂O) m⁻³. The experiments were carried out by an independent linear experimental design shown in Table 1. The maximum of baking time was measured by means of the temperature profile of the experimental design. By measurements it was shown that a maximum time of 16 min was necessary to reach a crumb temperature of 98 °C (at the coldest point of crumb). After baking the breads were immediately cooled in liquid nitrogen for further analysis.

Table 1. Experimental design with different baking time, temperature setting (°C) and water addition (g (100 g flour)⁻¹); n = 2.

Baking time (t) (min)	Temperature setting (°C)	Water addition (g (100 g flour) ⁻¹)
1 – 16	170/190/210/	52/60/68
steps of 3 min	230/250	

2.2. Microstructure and image analysis

Dough/crumb slices were cut from the middle of the breads. Analysing samples were cut from the inner part of the slices using a scissor and were transferred to a specimen shape (diameter 18 mm, height 0.8 mm). A 2 % agar solution was added to the samples. After the agar solution got solidified, the specimen shape was abraded by a razor blade. To stain the starch, around 50 µl of a 0.1 g (100 ml (distil H₂O))⁻¹ Nile Blue solution (AppliChem Biochemica GmbH, Darmstadt, Germany) was added to the sample and kept for 20 min. When staining procedure was completed a glass-cover slip was placed and fixed on the specimen shape.

A confocal laser-scanning system (Nikon, Düsseldorf, Germany) with a 60 x oil immersion objective and an Ar/Kr laser was used. Starch was monitored as fluorescence images ($\lambda_{exc} = 638$ nm, $\lambda_{em} = 650$ nm) at pixel resolution of 1024 x 1024 (212 x 212 µm) in a constant z-position. Five independent positions on the x-y-axis were recorded of each dough sample done by duplicate.

For each experiment these ten digital images were analyzed using the image processing and analysis freeware Mac Biophotonics ImageJ (version 1.42q, National Institutes Health, Bethesda, Md, USA). The images were pre-processed by changing to grey-level (8 bit). An image thresholding method which is based on minimizing the measures of fuzziness on an input image was used [21]. This procedure is used to denote the characteristic relationship between a pixel and its belonging region. After thresholding a special algorithm (watershed) was used for cell counting. Furthermore the segmentation of the starch grain used by watershed algorithm was an important part for the implementation of starch grain analysis.

The gained segmented binary images were analyzed by the features of starch granules: *mean starch granules area* ($\emptyset A_c$) and *starch perimeter* (P_s). Computation of the *mean starch granules area* was based on the equation:

$$\emptyset A_c = \frac{\sum_{i=1}^n (\emptyset A_c)_i}{n} \quad (1)$$

2.3. Thermal analysis of starch gelatinization

Usually two endothermic peaks are observed in the *differential-scanning-calorimetry* (DSC) curve when a starch/water mixture is heated up to 150 °C with excess amount of water, while three endothermic

peaks are observable when the sample is achieved with limited amount of water. The first peak is attributed to moisture-dependent disorganization of starch crystallites, and the second reflects the “melting” of the remaining crystallites [22]. The third peak is thought to be related to order–disorder transition of amylose–lipid complexes [23]. Therefore it will be reasonable that the first peak (at around 65 °C) and the second (>110 °C) are responsible for starch gelatinization (order–disorder transition). In this work, however, only the first peak in the DSC-thermogram was targeted to be integrated to give the enthalpy responsible for starch gelatinization, as a first-order estimation. The reason for this choice was that the crumb temperature during baking is not more than 100 °C [24-25]. With a decrease in moisture content the second peak tends to shift to a higher temperature; even to a temperature above 100 °C when the moisture content decreases under $0.67 \text{ g}_{\text{water}} \text{ g}^{-1}_{\text{starch}}$ [26]. In contrary, the first peak remains at about 60 °C.

Breads for DSC analysis were cooled for 1 hour. After cooling, breads were cut in the centre to get a slice. Samples of 30 - 40 mg crumb were put in an empty stainless steel pan (capacity of 40 μl). The pan was closed with a cover (pans and cover, TA Instruments Ind., Germany).

DSC measurements were performed with a Perkin-Elmer Diamond DSC calorimeter (Perkin-Elmer Corp., Germany). Indium was used to calibrate the system. The samples were heated from a temperature of 30 up to 100 °C with a heating rate of $10 \text{ }^\circ\text{C min}^{-1}$ [8]. During DSC measurement an empty stainless steel pan (capacity of 40 μl) was used as a reference. The enthalpy of the sample was expressed in J g^{-1} (db). All experiments were measured by triplicate. The enthalpy of dry mass m_{dry} at specified time (5) and the gelatinization rate were calculated as

$$\Delta H_t = \frac{\Delta h_t * 100 [\%]}{m_{\text{dry}} [\%]} \quad (2)$$

$$TEG (\%) = \frac{\Delta H_0 - \Delta H_t}{\Delta H_0} \quad (3)$$

Whereby TEG is the percentage of gelatinization, ΔH_0 is the enthalpy at initial baking time t_0 and ΔH_t is the enthalpy at sample baking time.

3. Results and Discussion

3.1. Microscopic changes of starch structure during baking

CLSM was applied in order to visualize the starch gelatinization of bread crumbs during baking process. Some authors have already described the microstructure of dough and bread by the use of CLSM [16, 27-29] but not as a function of baking time including an image-analysing-system. Figure 1 shows an example of starch gelatinization during baking from 1 to 16 min (temperature: 230°C; water addition $60 \text{ g (100 g}_{\text{flour}})^{-1}$):

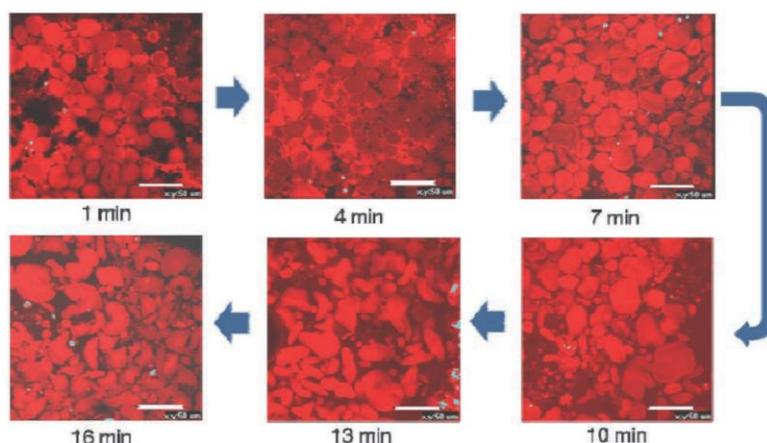


Fig. 1. Examples of CLSM pictures, required during the baking process (1 – 16 min)

Figure 1 visualizes that after a baking time of 7 min the first starch granules began to gelatinize. Starch granules got larger, swollen and lost the oval or round shape. The images recorded at 16 min baking time show a maximum of starch gelatinization. The figure shows that the starch granules appear to be inhomogeneous and irregular. The black bodies contained no protein or starch granules and probably there are residual components of the crumb (air bubbles and/or water) which are not fluorescent. During baking time the volume of starch granules increase. Further baking time >7 min led to an increase of broken starch granule structures depending on water accumulation.

For characterizing of the starch gelatinization the mean granule area, the -perimeter and the -roundness were analyzed. Figure 2 (A) shows main effects between mean granule area and baking time as well as baking temperature. With current baking time as well as with increasing temperature a significant ($p < 0.05$) linear increase of mean granule area was found ($r = 0.79$). Additionally, the perimeter (Figure 2 (B)) significantly increased by an increment of baking time and temperature ($R^2 = 0.81$) as well as the granule roundness correlated significant negative to baking time. All al cases there was no significant correlation based on the different water addition.

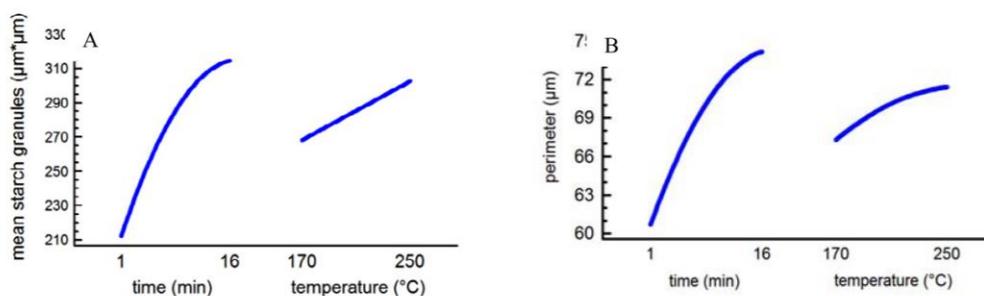


Fig. 2. (A) mean starch granule area (μm^2) and (B) perimeter (μm) as a function on baking time (min) [n=60] as well as baking temperature ($^{\circ}\text{C}$) [n=50]

3.2. Thermo-analytical analysis of dough/crumb during baking

DSC thermograms were gained from samples separated from parts of dough/crumb selected at different baking times (before baking (t_0) and from 1 min to 16 min at an interval of 3 min). At t_0 the thermogram shows a gelatinization endothermic peak with a maximum between 67 to 69 °C, which demonstrates the minimum of gelatinization rate (= 0 %) in specified experiments. Contrary the highest value of the enthalpy integrated between T_O (peak onset) and T_E (peak end) is analyzed (2.9 to 3.0 J g⁻¹ dry mass). Those values are very close to those found in the literature for wheat flour gelatinization in a limited water system [30]. The area of the endothermic peak continuously decreased with an increase of baking time and finally disappeared for crumb after 10 min of baking. In all experiments gelatinization has already been started after 1 min of thermal heating, but with a low value of $TEG \leq 5$ %. The significant values of non-gelatinized starch amount found in crumb samples ($TEG \leq 81$ %) after 7 min were explained by the crumb temperature lower than T_{max} . For baking time at 4 min, a higher TEG has been revealed by an increase of water addition. For example there is a duplication of TEG between water additions of 52 % to 68 %. These results are depending on two different aspects. On the one hand with an increasing water addition more water for starch gelatinization is available. On the other hand with higher water addition the thermal transfer into the crumb increase based on the fact that the heat transmission coefficient of water and water vapour is higher than the heat transmission coefficient of air. These aspects could be supported by the literature which were analyzed by Fukuoka et al. [30] who found that the gelatinization rate is a function of temperature and moisture content. At longer baking times ($t \geq 10$ min) no significant thermo-analytical changes were detected in these samples.

Table 2. Temperatures (°C), enthalpies (J g⁻¹) and gelatinization rate (%) of dough/crumb at different baking times (min) and water addition (w_a) (g (100g flour)⁻¹)

w_a	Δt (min)	ΔH_i (J g ⁻¹)	TEG (%)
52	0	2.9±0.2	0
	1	2.9±0.1	0.7±2.5
	4	2.2±0.1	25.0±4.6
	7	0.6±0.1	80.9±1.9
	10	0.4±0.0	86.9±1.9
60	0	2.9±0.1	0
	1	2.9±0.1	1.1±1.5
	4	1.9±0.1	34.4±4.3
	7	0.5±0.1	81.4±1.8
	10	0.1±0.05	100
68	0	3.0±0.3	0
	1	2.8±0.2	5.4±6.0
	4	1.3±0.1	53.3±3.1
	7	0.6±0.0	80.0±1.1
	10	0	100

4. Conclusion

During bread baking starch granules swell and gelatinize. The conditions, in which these phenomena occur, determine the quality of bread. This study presents the combining of multiple analytical techniques

for a better understanding of the starch gelatinization. *CLSM* was used to explain some of the phenomena which occur during gelatinization caused by thermal heating. Significant ($p < 0.05$) linear correlation between the *TEG* and the starch perimeter ($r = 0.76$) as well as the mean starch granules area ($r = 0.85$) until a total gelatinization rate of 100 % were found. Summarized the dependence between micro- and thermal-structural changes of starch could be shown. The possibility of combining *CLSM* with thermal-analytical techniques in the same experiments using specially designed stages offers the possibility to receive detailed structural information of complex food systems like wheat bread.

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2.5 In situ monitoring of starch gelatinization with limited water content using confocal laser scanning microscopy

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ORIGINAL PAPER

In situ monitoring of starch gelatinization with limited water content using confocal laser scanning microscopy

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Abstract The gelatinization of starch is crucial for the production of bakery products. Therefore, the examination of the characteristics and the extent of this process are of fundamental importance for research and product development. Typical analysis methods for studying structural starch changes during heating are performed in excess of water. Considering that wheat dough is a complex system with reduced water content, an option to analyze starch gelatinization under actual product conditions is missing. Therefore, an in situ method in a confocal laser scanning microscope (CLSM) equipped with a heating system was developed to monitor the starch gelatinization in samples with different flour to water ratios [(m:v) 1:0.39, 0.49, 0.51, 0.53, 0.55, 0.57, 0.59, 0.98, 4.91, 5.63 and 7.14]. The new method was used to investigate the start of starch gelatinization temperature (T_G) by using thresholds based on first derivatives with highest correlation between T_G of CLSM and the onset temperature (T_o) of differential scanning calorimetry. A highly significant linear correlation between T_G of A-type granules and T_o was observed ($R^2 = 0.903$). The size and shape of granules with a ratio of 1:0.98 show a clear impairment against samples with higher water content. The newly modified in situ method enables the measurement of starch gelatinization in different flour to water ratios independent of secondary factors. Consequently, it can be used for complex starch-based systems with reduced water content to investigate the actual starch granule structure disintegration.

Keywords Fluorescent staining · Image processing · Differential scanning calorimetry

Introduction

Several analysis methods have been used to study different aspects of starch gelatinization during heating. They can be divided in two categories: Direct methods and indirect methods. Indirect methods mainly include rotation viscosimeters such as the rapid visco-analyzer (RVA), the amylograph/viscograph and the rheometer. Indirectly, a change in viscosity characterizes important sections of the gelatinization process. However, for analyzing starch gelatinization with reduced water content, due to high viscosity ranges, these typical rotation viscosimeter systems are inapplicable. As an exception, rheological analyses with oscillation tests (equipped with a plate–plate or plate–cone geometry) offer the possibility to determine starch gelatinization characteristics with reduced water [27, 32]. Nevertheless, it is important to consider the effect of secondary factors on the measured viscosity. Such secondary factors can be flour constituents such as proteins, lipids and fiber in the raw material or the composed food matrix, water and recipe additives (such as NaCl and sugar) [13, 18, 35]. But they also comprise process parameters such as the temperature or the shear rate [39]. The complexity of food ingredients and processing parameters influences the viscosity and further rheological values. Consequently, the significance of indirect analysis methods for the investigation of starch gelatinization characteristics is reduced [1].

Direct methods comprise all microscopic and calorimetric systems. They offer the possibility to investigate thermal-based starch changes independent from the viscosity and the influence of secondary factors. Furthermore, direct methods make it possible to separately observe the effect of secondary factors on the gelatinization properties of starch. This ensures that all constituents which may affect the gelatinization are considered in the final

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result. In a differential scanning calorimeter (DSC), starch gelatinization can be monitored directly, since changes in the amount of heat between the sample and the reference are measured as a function of energy [23]. The DSC is an established method to analyze gelatinization at different starch to water ratios and it can be used to compare varying starch sources [6, 12, 30, 33]. During the last years, a novel analysis method for the visual detection of a microstructure is gaining importance in food science. In this case, the understanding of the material behavior is based on visual characterizations. The confocal laser scanning microscopy (CLSM) enables an off-line view on the microstructural level of complex food systems [2, 20, 21, 28, 31, 33]. The usage of CLSM for analyzing structure components of dough and bread is already an established method. Since many important processes in food production depend on thermal treatments, the CLSM has been used to analyze the effect of heating on the food matrix after each heating step. Unfortunately, a continuous measurement has never been applied. Additionally, analyses were typically performed without a digital analyzing tool to evaluate the structural product changes during a thermal treatment [15, 16, 36, 40].

The main constituents of wheat bread are wheat flour, salt and water. The two main constituents of the flour, namely gluten and starch, clearly have a huge influence on the properties of the final product. In former publications, typically the structural changes of proteins were discussed [20, 24]. The primary objective of the current study is to provide a novel approach for an in situ monitoring of starch gelatinization by modification of the CLSM. The established method was used to evaluate the start of starch gelatinization temperature for samples with different flour to water ratios with detection based on thresholds. For the validation, a comparison with an established direct method was necessary. For this purpose, the non-invasive DSC method was used. The goal of this in situ monitoring was to allow a quantitative determination of the start gelatinization temperature through the alternation of the granule's morphology by heating of a dough-like system.

Materials and methods

Sample preparation

For imitating wheat dough, samples were prepared with wheat flour (Type 550, Rosenmühle, Germany), different amounts of distilled water and 2 g sodium chloride (Südsalz GmbH, Heilbronn, Germany) per 100 g flour. The ions of sodium chloride are known to influence the gelatinization [7]. For dough preparation, the following flour to water ratios were used (m:v): 1:0.39, 0.49, 0.51, 0.53, 0.55, 0.57,

0.59, 0.98, 4.91, 5.63 and 7.14 (based on 14 % flour moisture content). In accordance with AACC method 54-21.02, a torque measuring z-kneader (doughLAB; Perten Instruments, Germany) was used to determine the optimum water absorption. The ratio of 1:0.59 refers to the sample of a standard wheat dough. The two ratios with the highest water amount correspond to the standard protocol of a rapid visco-analyzer (1:5.63) and an amylograph (1:7.14). They were used to allow a comparison among of these methods for further studies.

Analysis of chemical properties of wheat flour

Relating to the used wheat flour, the total starch content (80.5 ± 0.75 % (db); $n = 3$) of the sample was measured enzymatically using an assay kit (Megazyme International, Ireland Ltd.) according to the standard AACC Method No. 76.13. The flour moisture content (12.1 ± 0.3 %; $n = 3$) was determined thermogravimetrically using the moisture analyzer MLB-50-3 (Kern & Sohn GmbH, Balingen-Frommern, Germany).

Analysis of pasting properties via differential scanning microscopy

A Diamond differential scanning calorimeter (DSC) equipped with a thermal analysis data station (Pyris Series Software (V10.1), Perkin Elmer Inc., Connecticut, USA) was used to measure gelatinization temperatures and enthalpy. The produced samples were equilibrated for 30 min before analysis. Approximately 30 mg of probe material was weighed into pans and immediately sealed with a crimper. An empty sample pan was used as reference. The samples were heated from 40 to 100 °C using a heating rate of 10 °C min⁻¹. By using the melting point and the enthalpies of indium, the temperature and heat capacity of the instrument were calibrated. DSC determines pasting parameters such as onset temperature (T_o) (°C), peak temperature (T_p) (°C) and end temperature (T_{End}) (°C). Thermograms from DSC were analyzed via Pyris Series software. In order to obtain the enthalpy of gelatinization (ΔH) (J g⁻¹), the area under the thermal transition peak above the extrapolation line was numerical integrated. All measurements were done in triplicates.

Analysis of chemical, physicochemical and visual pasting properties by CLSM

A confocal laser scanning microscope (CLSM, ECLIPSE Ti-U) with three lasers (448, 543, 635 nm), three detectors (510/30, 590/50, 650 LP nm), EZ-C1 Software (V3.80) from NIKON Instruments Inc. (New York, USA) and equipped with a heating plate (Instec, Software Win

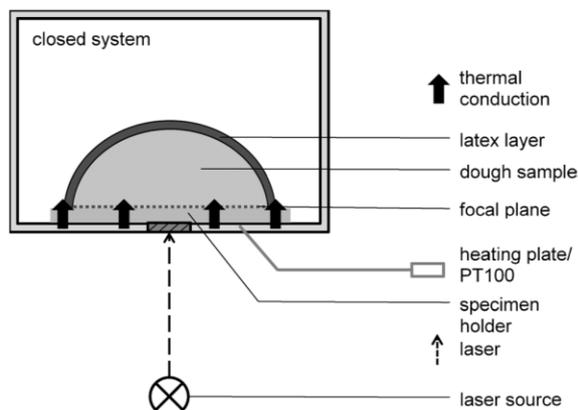


Fig. 1 Model of the experimental arrangement for non-liquid/dough similar systems. Samples are heated by thermal conduction from *bottom* (heating plate). The laser probes the sample from below. The small sample amount, a heating rate of $1\text{ }^{\circ}\text{C min}^{-1}$ and the short distance between the source of heat and the focal plane of the CLSM ensure that the detected temperature is nearly equal to the actual sample temperature. The latex layer prevents a loss of sample humidity

Temp for MK100 (V1.0.110311), Colorado, USA) was used to observe the changes in dough while heating up to $100\text{ }^{\circ}\text{C}$. The samples were prepared by manually dispersing wheat flour, sodium chloride and water with the respective stain concentration. The dye solutions Nile Blue (AppliChem GmbH, Darmstadt, Germany), Safranin O (Sigma-Aldrich Chemie GmbH (Steinheim, Germany), Rodamine B (Merk KGaA, Darmstadt, Germany), Periodic acid–Schiff (PAS) (Carl Roth, Steinheim, Germany) were obtained by solubilizing a specific amount (varied from 0.001, 0.01, 0.2, 0.3, 0.5 $\text{g } 100\text{ ml}^{-1}$ for all dyes) of dye with distilled water. All dyes were added to the dough as an aqueous solution to the mixing water. For the liquid flour–sodium chloride–water systems (1:7.14, 5.63, 4.91 and 0.98), a specimen holder was required. Therefore, small aluminum caps with a central borehole were fixed with a small glass cover slip underneath. Furthermore, special heat-resistant glue was used to get the equipment sealed at temperatures up to $100\text{ }^{\circ}\text{C}$. Non-liquid samples (1:0.39, 0.49, 0.51, 0.53, 0.55, 0.57 and 0.59) were pressed onto the glass cover slip and sealed with latex which prevents drying out. Afterward, confocal micrographs of the samples were taken by modified CLSM in the fluorescent mode. This novel measurement system is described in Fig. 1. Depending on the used dye, (see evaluation of different dyes) an Ar ion (488 nm) and a He/Ne laser (543 nm) with a 590/50 nm and a 650 LP detector, respectively, were applied and the sample was observed through a $40\times$ immersion objective. All micrographs were recorded with the Nikon NIS-Elements ND2 software. A scan size of 512×512 pixel resolution was set.

In order to amplify the signal, the dwell time was reduced to $21.6\text{ }\mu\text{s}$. The samples were heated directly in focus by the usage of the heating plate. Thereby, baking was simulated by heating from $40\text{ }^{\circ}\text{C}$, holding the temperature due to acclimatization reasons for 10 min, continued with a permanent rate of $1\text{ }^{\circ}\text{C min}^{-1}$ up to $80\text{ }^{\circ}\text{C}$. In preliminary tests, this heating rate was found out as the best. Below a temperature of $50\text{ }^{\circ}\text{C}$, swelling of the starch granules is usually minimal [14]. Micrographs were recorded every minute (in accordance to a heating rate of $1\text{ }^{\circ}\text{C min}^{-1}$) and analyzed from two separated samples.

Micrograph analysis via image evaluation

Randomly, 10 A-type granules and 10 B-type granules were irregularly selected. Thereby, imaging offers the ability to record structural components for each individual particle and to distinguish among individual granules. The micrographs were changed to grayscale (8-bit), and a threshold (Huang dark) was used to generate a binary form, analyzed according to Schirmer et al. [33]. Thereby, following parameters were determined:

Average size (\varnothing_A) (μm^2), perimeter (P) (μm), feret diameter (D_F = maximum distance between parallel lines tangent to the starch granule profile) (μm) and circularity ($C = P/2\sqrt{\pi A}$) (–).

Determination of starch start gelatinization temperature based on CLSM

A-type ($>15\text{ }\mu\text{m}$) granules offer a higher growing potential and are stronger affected by heating influences compared to B-type ($5\text{--}15\text{ }\mu\text{m}$) granules [38]. The large A-type granules have slightly faster start of gelatinization temperature than the small B-type granules [11]. For these reasons, A-type granules were chosen for the development of a sensitive method that allows capturing gelatinization-based alterations. With a transformation according to Eq. 1, all values of granule average size at $40\text{ }^{\circ}\text{C}$ were set to 1.00 to allow a comparison among the samples. This normalization enables to observe and compare different size gaining rates within the different water contents. Thereby, $\varnothing_A(x)$ is the normalized average granule size and $\varphi_i(x)$ the average size of the i th granule under the temperature $x \in [40, 100]$, $i \in \{1, 2, \dots, n\}$ where n is the number of granules. The normalized average size $\varnothing_A(x)$ is defined by Eq. 1:

$$\varnothing_A(x) := \frac{1}{n} \sum_{i=1}^{n=10} \frac{\varphi_i(x)}{\varphi_i(40)} \quad x \in [40, 100] \quad (1)$$

The determination of starch gelatinization temperature based on CLSM (T_G) was carried out using thresholds based on two different approaches: by standard deviation

and first derivatives. Due to fluctuations in the measurements, these thresholds were investigated iteratively to find the most reliable gelatinization temperature. The output was compared to the reference values of DSC measurement. The threshold with highest correlation between CLSM and DSC was taken as final result.

Threshold defined by standard deviation

The first approach is based on the standard deviation of all measurement points out of one TA investigation. This threshold was summed up to the normalized start point of one, with $i = 0:0.05:1$ (see Eq. 2):

$$\text{threshold} = 1 + i * \bar{\sigma} \quad (2)$$

The intersection point of the threshold with the analyzed data points was determined by linear interpolation of the first point above and the last one below the defined threshold.

Threshold defined by first derivative

In the second approach, the first derivative of the measurements over temperature is calculated. For each point, the derivative is approximated via the Taylor polynomial and the surrounding points. A detailed description for calculating the derivative can be found at Hoffmann and Chiang [19]. Iteration was performed on derivative values between 0 and 0.03 in steps of 0.001 predicting the most probable temperature value compared to the DSC standard measurement.

Results and discussion

Evaluation of different dyes

In the present study, changes in starch gelatinization under heating were observed. Therefore, an appropriate

heat-resistant dye which is highly affine for wheat starch was required. As the sample contains not only pure starch and water but also proteins in a dough similar matrix, it is important to select a dye which provides these specific demands. In literature, many studies can be found to analyze starch components using different dyes. Safranin O, Nile Blue [2, 17, 33], Rhodamine B [5, 8, 10, 24, 28] and Periodic acid–Schiff (PAS) [4] are the chemicals mostly used and established in the starch-based food research. All these typical dyes were screened to find the best dye quality for the problem of interest. Single CLSM micrographs with varying quality due to the different selected dyes are illustrated in Fig. 2. The mentioned dyes were observed under same conditions and added to the sample in different concentrations according to values found in the literature (see above). To compare the results, the flour to water ratio was set constant to 1:0.59. This represents a standard dough formula based on the results of the water absorption determined through Farinograph analysis. All dyes were added to the dough as an aqueous solution to the mixing water. The used dyes and the corresponding concentrations varied from 0.001, 0.01, 0.2, 0.3, 0.5 g 100 ml⁻¹ for all dyes except for PAS, which was stained by drop technique (app. 50 µl for the used drop). PAS was added to the dough and washed out after 10 min with distilled water. PAS offered a good micrograph quality but lost its fluorescence at proceeding temperatures from 65 °C and higher. Usage of Rhodamine B resulted in micrographs of good resolution but also with restrictions of strong contrasts. Both Nile Blue and Safranin O provided good micrograph quality with high contrasts and a homogenous labeling of the granules. A condition for the required dye was a specific staining of the starch granules and Safranin O turned out to deliver the most satisfying results analyzed by image evaluation. On the one hand, a concentration had to be determined, which reveals micrographs of a high brightness caused by the fluorescence dye. On the other hand, an overdosed use, which influences the natural behavior of the

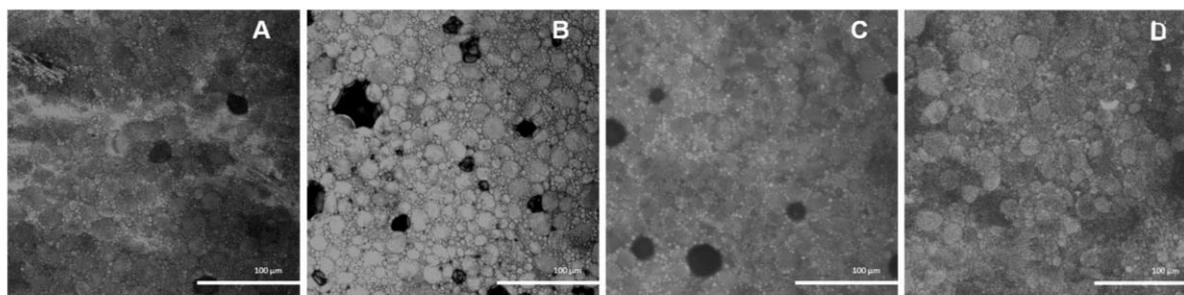


Fig. 2 Differently stained samples at a flour to water ratio 1:0.59 (m/v) (containing at 50 °C) with exemplary micrographs. Used dyes and concentration: Nile Blue with 0.5 g 100 ml⁻¹ (a), Safranin O

with 0.3 g 100 ml⁻¹ (b), Rhodamine B with 0.5 g 100 ml⁻¹ (c) and PAS applied with drop technique (d). Scale bar 100 µm

sample, had to be avoided. At low concentrations, the dye showed a stronger affinity to proteins but shifted to starch granules at higher amounts. To ensure a complete labeling of the granules and prevent adulteration effects, a concentration of 0.3 g Safranin O per 100 ml of distilled water was set constant for the following studies.

Visual characterization of starch structure by CLSM and image analysis

In the present study, the morphological alterations resulting through heating of flour–water–sodium chloride mixtures were studied by CLSM. It is known that particle geometry affects the characteristics and behavior of particulate

materials [3]. During the heating process, the CLSM micrographs revealed significant differences in granule size and shape. Thereby, up to a temperature of 70 °C, structural analysis was practicable. At higher temperatures, the granules were not separately detectable (see Fig. 3).

Figure 3 represents an example of confocal laser scanning micrographs of 1:0.59 heated up to 80 °C. The observation that bigger A-type granules seem to be spherical under increased water amounts and B-type granules were considered oblate spheroid in shape [38] could not be repeated due to the low water contents. In all cases, samples showed a spherical shape. With decreasing water amount, samples turned more cavernous and showed black air bubble which are typical in a dough-like system. The starch granules showed markedly

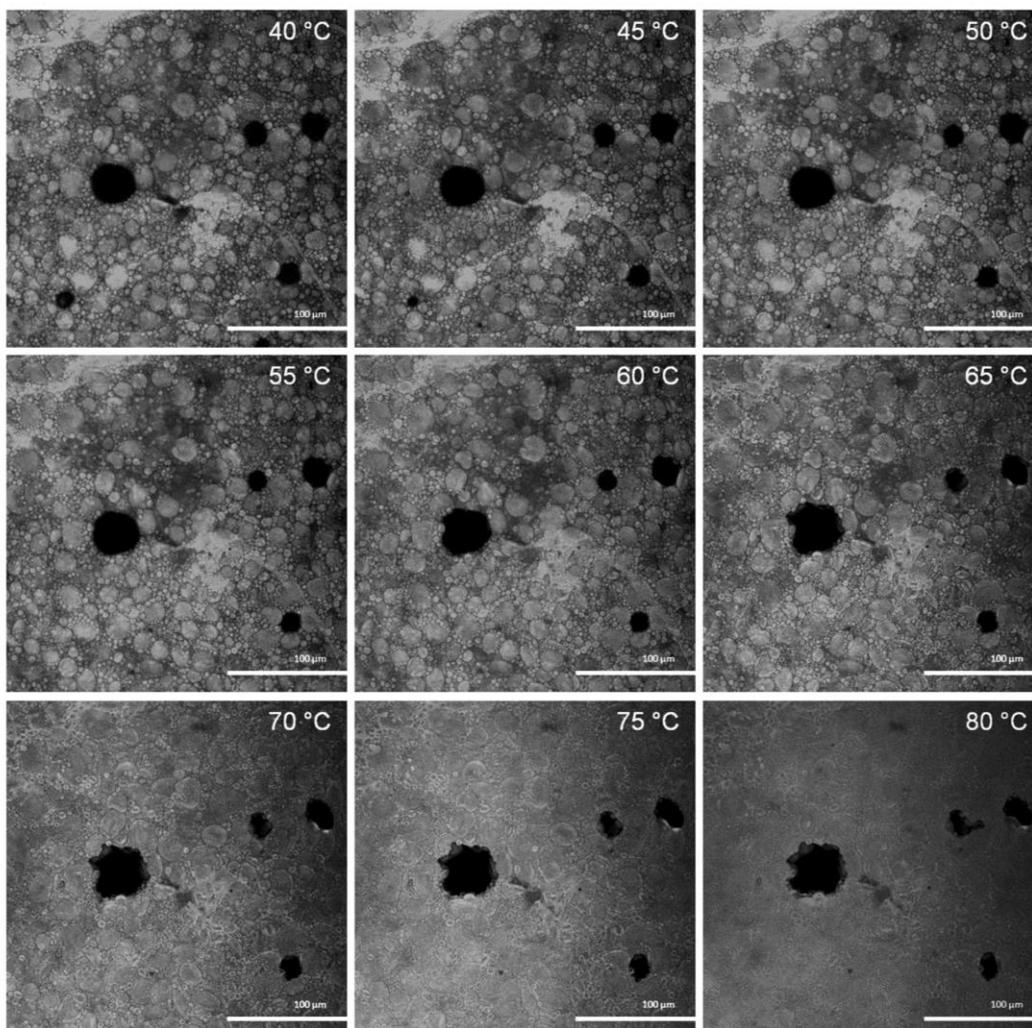


Fig. 3 Example of confocal laser scanning micrographs at a flour to water ratio 1:0.59 (m/v) under heating. Micrographs show the proceeding starch alterations every 5 °C. Scale bar 100 µm

different resistance to swelling and disintegration compared to the aqueous suspension. Not only the total size at exceeding temperatures is reduced but also the average granule size increase is differing along the different water amounts. In general, the lower water content reduced swelling and results in a reduced rise of average size during heating. For instance, 1:7.14 raised their size up to $\Delta\phi_A = +176\%$ of initial size while the reduced water content samples of 0.39 limited swelling only to $\Delta\phi_A = +8\%$. The determined initial size (40 °C) varies within the different samples and seems not to follow a specific rule but more to be influenced by the selection of the chosen granules. However, referring to the aqueous suspension, most of the dough similar samples reveal smaller size values which may result from the increased dry base and the accompanied loss of available space. A- and B-type granules show a similar behavior under thermal treatment. But it has to be mentioned that micrograph processing is hindered and induces an increased error rate due to smaller shape of B-type. In general, B-type granule size starts at around $30\ \mu\text{m}^2$ at 40 °C and rises up to $40\ \mu\text{m}^2$ at 70 °C (depending on water content). The perimeter and feret diameter of each sample are correlating with the measured size and increase with similar rates. This observation is in line with a low circularity decrease which reveals that expanding occurs uniformly in same length and width. Particularly, the initial size influences the circularity. The smaller B-type granules offered markedly increased circularity with values around 0.98 and a small decrease under heating. Bigger A-type granules showed lower circularity (around 0.91) and remain with a reduced loss along the heating process compared to the aqueous suspensions. These effects are also seen by the black air bubbles, which are getting more irregular in shape. All size and shape data of A-type granules are summarized in Table 1. The varying size of the selected granules is naturally occurring whereby a higher standard deviation indicates an increased size range and a low standard deviation indicates a more homogenous size distribution of the granules. At high temperatures, micrograph processing remains hindered and thus not applicable. The granular shape of the starch starts to disappear, and only remnants of granules are recognized [28].

The normalized granule average size depending on heating profiles of A- and B-type granules with varying water content is shown in Figs. 4 and 5. The smaller B-type granules were assumed to be spherical while bigger A-type granules were considered oblate spheroid in shape [26, 38]. Under heating, the granules increase and show high size gains according to their water composition. A-type granules of 1:7.14 content almost tripled their size, 1:5.63 increased up to 1.7 and 1:0.59 up to 1.3 times of initial. Size values of B-type granule of different starch to water ratios show nearly similar profiles but are constantly shifted to decreased levels. For example, B-type granules of 1:7.14 content swelled up to 2.4 times, 1:5.63 increased

to up to 1.4 times and 1:0.59 up to 1.3 of initial size. It was revealed by comparing size gains of A-type and B-type granules that growing potential is increased for the bigger A-type granules [11, 25, 34]. The onset of size gaining is similar among all samples but reveals different gradients. Additionally, it depends on increased amylopectin content of B-type against A-type granules. Which is also increasing the gelatinization temperature [22].

Samples with higher water amounts presented an increased growth of its feret diameter compared to the granule average size. The granules expanded more in length than in width. Circularity of sample 1:7.14 decreased from 0.90 to 0.85 ($\Delta C = 0.05$) while 1:0.98 barely lost their shape from 0.91 to 0.90 ($\Delta C = 0.01$). In the compact microstructural configuration of systems with lower water contents, granules are not able to follow their natural affection to expand ellipsoidal and seem to be disabled by surrounding particles.

The particularly missing shape at high temperatures (>70 °C) inhibits image processing. This is caused by the fact that swelling of starch granules ends in a final disruption. The shapes disappear slightly with raising temperature and complicate image analysis. Based on the certain range of 10 randomly chosen A- and B-type granules, the determination of distribution is restricted, which hinders comparison with previous studies using mean values of all granules [31, 37]. However, possible result variations can be explained by different granule densities, in particular, parts of the focal size. An increased appearance causes a reduced availability of water per starch granule in that region. The varying size of the selected granules is naturally occurring whereby a higher standard deviation indicates an increased size range. In contrast, a low standard deviation indicates a more homogenous size distribution of the granules. In summary, the available water of the system is influencing two facts: the time-dependent granule increase as well as the granule average size after heating. Both cases are a fact of granule disintegration decrease correlation with reduction in water content. Furthermore, these effects are more pronounced for A-type granules than for B-type, which could be explained by a higher lipid content of the small wheat granules [29]. It seems that there is a less ordered arrangement of the polysaccharide chains in the smaller granules when compared with the larger ones. These differences in functional properties of small and large granules seemingly show that the granule size distribution is influencing the differences in gelatinization.

Detection of starch gelatinization (onset-) temperature (T_0) based on DSC

The analyzed gelatinization and enthalpy properties of different samples measured with the differential

Table 1 Extract of temperature profiles for characteristic size determined through micrograph processing. Means of A-type granules at different flour to water ratios (m/v), ($n = 3$, mean value \pm SD)

Temperature (°C)	40	50	60	65	70
1:7.14					
Average size (μm^2)	450.5 \pm 56.0 ^a	452.6 \pm 36.2 ^a	513.7 \pm 94.9 ^a	832.1 \pm 287.7 ^b	1,244.1 \pm 385.2 ^c
Perimeter (μm)	78.1 \pm 5.9	78.0 \pm 3.9	83.2 \pm 80.9	106.9 \pm 20.1	132.6 \pm 20.9
Feret diameter (μm)	27.2 \pm 2.2	27.1 \pm 1.3	29.0 \pm 2.6	37.4 \pm 6.3	46.8 \pm 6.2
Circularity (–)	0.90 \pm 0.0	0.90 \pm 0.01	0.89 \pm 0.01	0.88 \pm 0.0	0.85 \pm 0.0
1:5.63					
Average size (μm^2)	476.1 \pm 31.2 ^a	478.2 \pm 122.7 ^a	510.7 \pm 58.1 ^a	619.7 \pm 104.3 ^a	808.8 \pm 154.0 ^b
Perimeter (μm)	79.4 \pm 2.8	79.5 \pm 3.2	82.1 \pm 4.3	90.2 \pm 7.2	104.4 \pm 10.7
Feret diameter (μm)	26.6 \pm 0.9	26.5 \pm 1.0	27.3 \pm 1.4	30.2 \pm 2.7	35.3 \pm 4.0
Circularity (–)	0.91 \pm 0.01	0.91 \pm 0.0	0.91 \pm 0.0	0.91 \pm 0.0	0.90 \pm 0.0
1:4.91					
Average size (μm^2)	408.2 \pm 47.8 ^a	411.0 \pm 54.1 ^a	469.2 \pm 31.8 ^a	721.1 \pm 182.3 ^a	998.5 \pm 261.3 ^b
Perimeter (μm)	73.3 \pm 4.6	73.5 \pm 5.2	78.7 \pm 3.0	93.4 \pm 6.4	113.2 \pm 4.1
Feret diameter (μm)	24.6 \pm 1.4	24.7 \pm 1.7	26.6 \pm 1.0	31.6 \pm 1.9	39.5 \pm 4.5
Circularity (–)	0.92 \pm 0.0	0.92 \pm 0.0	0.92 \pm 0.01	0.91 \pm 0.0	0.89 \pm 0.01
1:0.98					
Average size (μm^2)	318.0 \pm 12.5 ^a	310.3 \pm 16.9 ^a	316.7 \pm 33.2 ^a	371.0 \pm 61.0 ^a	472.8 \pm 5.2 ^b
Perimeter (μm)	66.3 \pm 0.3	64.6 \pm 1.3	65.5 \pm 3.2	70.3 \pm 5.2	79.6 \pm 0.6
Feret diameter (μm)	22.8 \pm 0.6	22.1 \pm 0.1	22.6 \pm 1.2	24.2 \pm 1.6	27.6 \pm 0.2
Circularity (–)	0.91 \pm 0.01	0.91 \pm 0.02	0.90 \pm 0.0	0.90 \pm 0.01	0.90 \pm 0.00
1:0.59					
Average size (μm^2)	377.6 \pm 45.0 ^a	379.4 \pm 50.5 ^a	396.5 \pm 32.1 ^{a,b}	439.5 \pm 38.8 ^{a,b}	457.5 \pm 48.6 ^b
Perimeter (μm)	71.9 \pm 3.9	71.8 \pm 4.4	73.2 \pm 2.5	75.2 \pm 3.5	78.7 \pm 3.9
Feret diameter (μm)	24.3 \pm 1.0	24.3 \pm 1.3	25.0 \pm 0.4	25.6 \pm 1.0	26.6 \pm 1.2
Circularity (–)	0.90 \pm 0.01	0.91 \pm 0.0	0.91 \pm 0.01	0.91 \pm 0.01	0.91 \pm 0.01
1:0.57					
Average size (μm^2)	326.0 \pm 167.4	326.4 \pm 170.3	360.9 \pm 221.4	370.0 \pm 178.3	377.0 \pm 222.3
Perimeter (μm)	64.9 \pm 18.2	64.9 \pm 18.7	67.3 \pm 22.5	68.4 \pm 19.6	69.1 \pm 22.6
Feret diameter (μm)	22.0 \pm 5.6	22.2 \pm 6.1	22.8 \pm 7.0	23.01 \pm 6.5	23.69 \pm 7.5
Circularity (–)	0.91 \pm 0.01	0.91 \pm 0.01	0.92 \pm 0.01	0.92 \pm 0.01	0.91 \pm 0.02
1:0.55					
Average size (μm^2)	323.9 \pm 216.6	327.8 \pm 227.8	344.8 \pm 249.0	368.5 \pm 194.8	378.1 \pm 254.8
Perimeter (μm)	63.4 \pm 22.0	63.3 \pm 22.7	65.0 \pm 24.2	69.9 \pm 23.2	68.8 \pm 24.4
Feret diameter (μm)	21.7 \pm 6.9	21.8 \pm 7.3	22.2 \pm 7.8	22.7 \pm 8.0	23.7 \pm 7.9
Circularity (–)	0.92 \pm 0.01	0.92 \pm 0.0	0.91 \pm 0.01	0.91 \pm 0.01	0.91 \pm 0.01
1:0.53					
Average size (μm^2)	346.5 \pm 24.2	349.6 \pm 24.8	368.5 \pm 19.2	398.5 \pm 14.5	417.5 \pm 16.1
Perimeter (μm)	66.7 \pm 4.2	68.0 \pm 2.5	70.0 \pm 2.2	73.2 \pm 1.7	74.9 \pm 1.4
Feret diameter (μm)	22.7 \pm 1.8	23.1 \pm 1.1	23.7 \pm 1.3	24.3 \pm 0.5	25.3 \pm 0.1
Circularity (–)	0.92 \pm 0.01	0.92 \pm 0.0	0.92 \pm 0.01	0.91 \pm 0.0	0.91 \pm 0.0
1:0.51					
Average size (μm^2)	358.8 \pm 47.0	352.7 \pm 29.8	357.8 \pm 23.9	389.6 \pm 23.2	402.6 \pm 24.8
Perimeter (μm)	69.1 \pm 4.9	68.5 \pm 3.4	68.7 \pm 3.0	69.1 \pm 3.0	73.1 \pm 3.0
Feret Diameter (μm)	23.1 \pm 1.7	23.2 \pm 1.1	23.4 \pm 1.2	24.3 \pm 1.1	24.8 \pm 1.1
Circularity (–)	0.92 \pm 0.0	0.92 \pm 0.0	0.92 \pm 0.01	0.92 \pm 0.01	0.91 \pm 0.01
1:0.49					
Average size (μm^2)	289.5 \pm 18.3	289.6 \pm 28.5	296.7 \pm 22.4	314.4 \pm 31.5	324.7 \pm 41.3
Perimeter (μm)	61.9 \pm 1.7	61.6 \pm 2.8	62.5 \pm 2.3	63.5 \pm 2.3	65.3 \pm 3.4

Table 1 continued

Temperature (°C)	40	50	60	65	70
Feret diameter (μm)	21.0 ± 0.7	21.0 ± 1.0	21.2 ± 0.7	21.4 ± 1.0	22.3 ± 1.0
Circularity (–)	0.92 ± 0.01	0.92 ± 0.01	0.92 ± 0.01	0.92 ± 0.0	0.92 ± 0.0
1:0.39					
Average size (μm^2)	344.6 ± 21.7	341.6 ± 26.0	344.9 ± 27.9	352.0 ± 46.0	372.0 ± 54.0
Perimeter (μm)	67.5 ± 2.9	67.3 ± 3.7	67.7 ± 3.9	69.1 ± 5.2	70.2 ± 6.2
Feret diameter (μm)	22.8 ± 0.8	23.0 ± 1.3	23.3 ± 1.2	23.5 ± 2.0	24.0 ± 2.2
Circularity (–)	0.92 ± 0.0	0.91 ± 0.01	0.91 ± 0.01	0.91 ± 0.0	0.91 ± 0.0

Means in the same row with different letters are significantly different (one-way ANOVA); $p \leq 0.05$

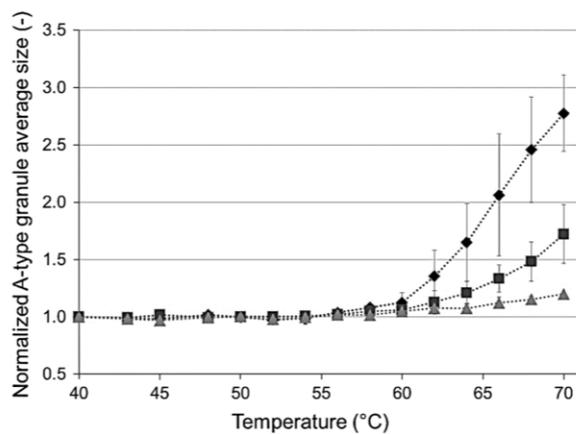


Fig. 4 Heating profiles of A-type granules with varied flour to water ratios [diamond = 1:7.14; square = 1:5.63; triangle = 1:0.59 (m/v)]. Granule average size was normalized to 1.0 for enhanced comparisons ($n = 3$, mean value \pm SD)

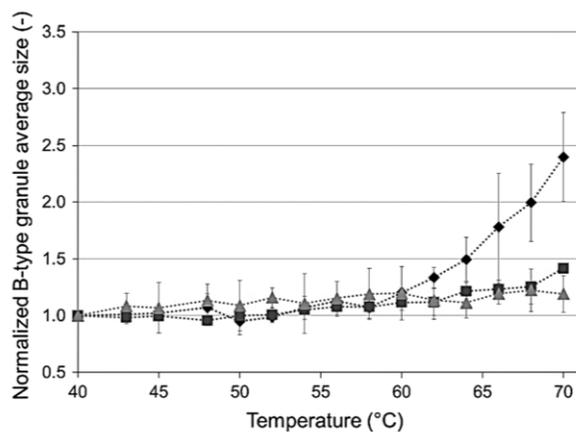


Fig. 5 Heating profiles of B-type granules with varied flour to water ratios [diamond = 1:7.14; square = 1:5.63; triangle = 1:0.59 (m/v)]. Granule average size was normalized to 1.0 for enhanced comparisons ($n = 3$, mean value \pm SD)

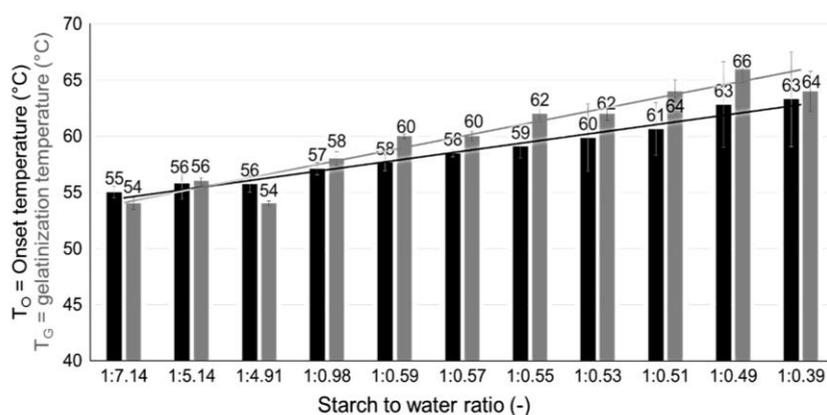
scanning calorimetry (DSC) are summarized in Table 2. With decreased water content, the DSC results show a high significant ($p \leq 0.001$) linear increase of the onset temperature (T_o) ($R^2 = 0.961$). Thereby, 1:7.14–1:4.91 showed nearly the same ranges of T_o (55.7 ± 0.5 °C) which represents a water excess system and water addition above this amount would not significantly influence T_o . While reducing water content, a constant decline of T_o was observed. The highest T_o (63.3 °C) was detected at the sample with the lowest water content (1:0.39). The T_o observations are associated with a decrease in gelatinization enthalpy (ΔH) whereby additionally, sample (1:0.39) showed the lowest $\Delta H = 0.3$ J g $^{-1}$. T_p and T_{end} were determined as well but could not indicate a correlation within the different samples. The remaining parameter of interest is T_o of DSC (thermal), which offers reliable data to compare with the CLSM (visual) method to determine starch gelatinization by granule disintegration.

Evaluation of starch gelatinization temperature (T_G) based on CLSM determined in comparison to DSC

The determination of starch gelatinization temperature based on CLSM (T_G) (visual granule disintegration) was carried out using thresholds based on two different approaches: Threshold was defined by standard deviation and by first derivative. In order to validate the determined gelatinization temperatures based on DSC (T_o), measurements with the CLSM micrograph processing (T_G) were carried out. As already determined by Chiotelli and Le Meste [11], B-type granules have a higher affinity for water at room temperature which could be seen on data fluctuations in Fig. 5. For analyzing T_G , only A-type granules were used, which is depending on earlier start of granule gelatinization [12]. The threshold with highest correlation between DSC and CLSM was taken as final result. Thereby, the usage of the first derivative with a threshold of 0.006 offers the best correlation ($R^2 = 0.903$; Fig. 7). Due to the heterogeneous nature of crystallites present within granules, starch thermal transitions occur over a

Table 2 Gelatinization and enthalpy properties of different samples measured with the DSC ($n \geq 3$ mean value \pm SD)

Flour/water ratio (m/v)	T_o (°C)	T_p (°C)	T_{End} (°C)	Area (mJ)	ΔH (J g ⁻¹)
1: 7.14	55.0 \pm 0.5	62.1 \pm 1.6	70.8 \pm 7.2	40.5 \pm 14.4	0.9 \pm 0.2
1: 5.63	56.4 \pm 0.4	60.9 \pm 1.1	66.3 \pm 1.1	36.3 \pm 5.0	1.0 \pm 0.1
1: 4.91	55.7 \pm 0.7	60.7 \pm 0.5	66.3 \pm 0.6	31.2 \pm 3.2	0.9 \pm 0.1
1: 0.98	57.1 \pm 0.5	63.3 \pm 0.5	72.9 \pm 1.1	44.0 \pm 9.6	1.8 \pm 0.4
1: 0.59	57.5 \pm 0.7	64.9 \pm 0.4	73.6 \pm 1.4	32.5 \pm 7.6	0.8 \pm 0.1
1: 0.57	58.5 \pm 0.3	66.0 \pm 0.4	73.2 \pm 1.2	18.6 \pm 6.4	0.5 \pm 0.1
1: 0.55	59.1 \pm 1.2	66.1 \pm 0.5	75.4 \pm 0.8	26.1 \pm 8.2	0.7 \pm 0.1
1: 0.53	53.9 \pm 3.0	67.1 \pm 2.1	75.6 \pm 2.4	27.3 \pm 6.6	0.7 \pm 0.1
1: 0.51	60.6 \pm 2.4	67.6 \pm 2.3	77.5 \pm 2.1	23.8 \pm 8.7	0.7 \pm 0.3
1: 0.49	62.8 \pm 3.8	67.5 \pm 1.7	75.4 \pm 2.5	22.0 \pm 3.8	0.6 \pm 0.1
1: 0.39	63.3 \pm 4.2	71.3 \pm 3.6	78.1 \pm 3.2	9.1 \pm 2.5	0.3 \pm 0.1

**Fig. 6** Determination of the onset temperature (T_o) with DSC (black) and gelatinization temperatures T_g with CLSM (gray) from samples with varying flour to water ratios (m:v) ($n \geq 3$, mean value \pm SD). For calculation of T_g , the first derivative and a threshold of 0.006

were used. Both measurements show a high significant ($p < 0.001$) increase in temperature showing start of gelatinization by visual (CLSM) and thermal (DSC) granule disintegration ($R^2-T_o = 0.961$, $R^2-T_g = 0.884$)

temperature range rather than at a defined temperature [9]. This smooth gelatinization transition entails hindered accuracy but does not inhibit the determination of a specific temperature using CLSM. Figure 6 gives an overview about the detected gelatinization temperatures (T_g) analyzed by CLSM. The gelatinization temperatures of the aqueous suspensions are markedly reduced compared to the dough similar samples. The detected increase in the gelatinization temperature is in accordance with the literature. BeMiller and Huber [9] wrote that thermal transitions occurring under limited moisture conditions are generally shifted to increased temperatures, depending on the amount of water present for plasticization. Thus, water enters the amorphous parts in decreased rates and reduces swelling. Thereby, irreversible transition from the ordered state to a disordered state is occurring at higher temperatures.

Conclusion

In the present study, starch alterations in model systems with varying flour to water ratios have been observed during thermal treatments. A confocal laser scanning microscope (CLSM) equipped with a heating plate enabled the in situ monitoring of starch gelatinization. Generated CLSM micrographs were analyzed by image processing in order to determine the gelatinization temperatures of dough similar samples.

In the case of starch, gelatinization can be defined as the disintegration of the native granule structure. This process is difficult to describe through measurable values with indirect methods. A-type granules disintegrate faster and they are affected by heating more rapidly than B-types granules. Therefore, A-types were

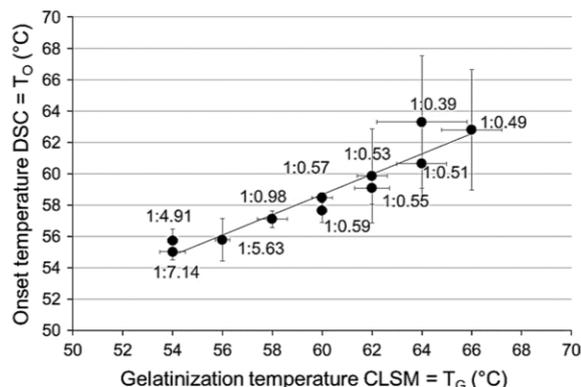


Fig. 7 Highly significant linear correlation ($p \leq 0.001$) between the onset temperature T_o (DSC) and the gelatinization temperature T_g (CLSM) for different flour to water ratios. Coefficient of determination $R^2 = 0.903$

chosen to develop a method to capture a specific temperature describing the start of gelatinization (T_g) by visual detected granule disintegration in a CLSM. To validate the obtained data, onset temperatures (T_o) of the same samples were determined through differential scanning calorimetry (DSC). The threshold used to detect T_g resulted in the highest correlation between CLSM and DSC. The calculation of the first derivative of the measurements over temperature reached a derivative value of 0.006 to predict T_g . These results were in accordance with the CLSM examinations and offered a highly significant linear correlation between T_g and T_o ($R^2 = 0.903$). Consequently, the new in situ monitoring can be used to determine the lowest possible water content for starch gelatinization. At a ratio of 1:0.98, granules remained smaller and more uniform, which indicates less gelatinization in comparison to samples with a higher water content.

In summary, this study presents the possibility to determine the starch gelatinization temperature with different water ratios especially for dough similar models using CLSM. Visual “online” detection of starch granules during heating at a microstructure scale was developed. Additional advantages of this method are the real-time monitoring of the sample temperature and the independence against secondary factors. Compared to liquid systems (max. water content 1:7.41), a flour to water ratio of 1:0.59 (equal to dough systems) achieved only around 40 % of the granule average size at 70 °C. In this study, the granule size increase by disintegration is decelerated as a factor of starch gelatinization. As a result, more than a half of the starch granules were not gelatinized during heating of wheat dough. Considering

that wheat dough is a complex system with limited water content ($\leq 1:0.98$), this in situ monitoring provides the requirements for analyzing starch gelatinization under real product conditions.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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3 Discussion

Wheat starch has an important contribution to food structure and hence its quality. However, for human digestibility a partial gelatinization of starch is necessary. This irreversible production process depends on a large variety of different product as well as process factors. Due to the complexity of wheat breads and their limited water content, the influence of the thermal impact on starch gelatinization was not accurately evaluated until now.

Bread baking is complex, which involves many simultaneous physical, chemical and biochemical changes of the product. The importance of energy efficiency of industrial processes is fundamental for environmental and economic considerations. The process optimum depends substantially on the type of baked good and the desired product characteristics. Baked products are engineered with recipes containing significant quantities of wheat or other cereal flours mainly consisting of starch. As dough transforms into bread during baking, important structural changes that highly depend on the processing conditions as well as on the ingredients are studied (Rouillé, Chiron et al. 2010). The starch granules - next to the gluten - are the main components of bread and their gelatinization during baking induces major structural changes (Köhler 2009). On the macroscopic product level, baking induces the solidification of dough and the change from a foam-like system with incorporated gas bubbles to an open pore system, i.e. a sponge. On the molecular level the swollen granules with partially solubilized molecules act as essential structural elements of dough/bread (Keetels, Visser et al. 1996). To broaden the knowledge of structural changes during heating, the microscopic understanding of starch gelatinization was established. For this purpose starch water suspensions were initially investigated before moving on to complex food such as bread. The evaluation of typical analytical methods was carried out through a literature review concerning starch gelatinization and its analytical complexity. A fundamental approach to examine starch gelatinization was reviewed with emphasis on special systems allowing to analyze dough with limited water content. Confocal laser scanning microscopy (CLSM) in combination with different image analyzing techniques was investigated in order to gain understanding of complex food and their transformation processes during heating. To provide an overview on the applicability of products with limited water content table 3 summarizes the usage of CLSM in complex products like dough and

bread. This summary illustrates the wide spectrum of typical dyes and their concentration dependence on sample preparation. In general, the usage of dyes is in an unique range for specific chemical components. In practical applications a variability depending on the dye concentration can be observed. Rhodamin for example interacts with gluten if present in concentrations around 0.01 g l^{-1} and with starch granules in concentrations around 0.1 g l^{-1} (Baier-Schenk, Handschin et al. 2005). Additionally, no standardized applications including regulations about sample preparation and staining time are available. In order to compare results from different research project a standardization are required. These broad research spectrum summarized in table 3 demonstrates on the one hand the CLSM technique as an already known method and on the other hand points out the limitations of the system imposed by the offline detection. Following aspects support the aim of this work to modify and evaluate a new in situ analyzing system using CLSM.

Depending on differences in flour quality the prediction of the end product properties are very challenging. The structural transformation that may undergo during processing can be influenced in various ways. Whereby, the progression and extent of the thermal gelatinization of starch are affected by internal and external factors (see figure 6).



Figure 6: Overview of internal and external factors influencing the flour, dough and end product quality.

The internal factors are generally dependent on culturing and genetic aspects. Therefore, the botanical origin such as the content and ratio of amylose (AM) and amylopectin (AP), are primary depending on culturing and genetic aspects. To evaluate the suitability of CLSM as a tool for morphological characterization of starch by micrograph analyzes different ratios of AM and AP were analyzed, serves as a selection for internal factors. Heat induced internal structure and morphological changes of different samples were characterized visually. AM and AP dependent changes of gelatinization analyzed with a micrograph and evaluated with an image processing tool correlated with standardized measurements such as differential scanning calorimetry (DSC) and viscometry (RVA).

Besides these internal factors, the flour components and the processing parameters applied to produce bread are considered as external factors. It needs to be considered that the internal factors cannot be influenced and the external factors are majorly determined by the applied process. Therefore, further steps in this research are investigating the analyzability of the influence of external factors such as technological aspects in order to combine them with recipe components. The incorporation of many of these factors as possible allows a better understanding of the starch granules gelatinization. Therefore, the changes in starch granules were analyzed during heating in products with excess as well as with limited water.

As a result the micrographs of CLSM data showed starch structure changes of wheat dough and bread during baking. After gelatinization the starch fraction was inhomogeneous and consisted of swollen and interconnected starch granules. Highly viscous systems initially investigated via micrograph analysis revealed a clear separation of different heating steps. Both, CLSM and DSC measurements present a possibility to characterize the progression of gelatinization as a function of baking time. The possibility of combining CLSM with thermal analytical techniques (DSC) in the same experiments using specially designed stages offers the possibility to receive detailed structural information of complex food systems like wheat bread. For the first time significant microstructural changes of starch - as a transformation of dough towards crumb - were demonstrated during the baking process. Thereby, thermally induced phase transitions and associated structural changes of bread dough lead to an inhibited granule swelling compared to the experiments of starch suspensions. During the first two thirds of baking time the starch granules increased.

At further baking granule rise stopped and an increase of broken starch granules structure depending on water accumulation was seen.

Based on these results it was necessary to find a solution for analyzing starch changes without any influences and indirect way of measurements. Therefore the CLSM-micrographs together with thermal analytical techniques was used as basis for further research. The great prospects of these results are the development of a new in situ monitoring of starch gelatinization. The following innovations namely the real-time monitoring of the sample temperature and the robustness against secondary factors are the main advantages of this new technique. Considering that wheat dough is a complex product with limited water content ($\leq 1:0.98$ flour to water), the in situ monitoring provides the missing requirement for analyzing starch gelatinization under industrial product conditions. This new method enables the possibility for analyzing starch gelatinization during heating, which is typically very complicated due to the complexity of the products and their limited water content.

Summarized, the CLSM-micrographs of real baking and model systems with a heating plate indicate the same behavior: the gelatinization process is limited by the water content. With limited water content starch granules show no typical “ghost fraction”, granule swelling finishes after the absorption of the available water. The results pointed out that the measurable gelatinization process is already completed after around 60 % of the total baking time and once the sample reaches 70 °C. This aspect was already closely discussed in the literature part, where in figure 5 it is demonstrated that the maximum internal temperature of about 98 °C is already reached after two-thirds of baking time. Internal structure fixation, including starch gelatinization is not a limiting factor why the full baking time is required. The last third of baking time is primarily responsible for the formation of the crust and bread color. Depending on the desired product quality a reduction of baking time including the associated energy could be realized.

Based on the discussed analytical methods, starch gelatinization and the associated parameters can be described as follows: **“starch gelatinization depends on measurable parameters describing structural (starch) changes.”**

Regarding this statement, visual (CLSM) and thermal (DSC) detection of starch granule disintegration allow a possible definition of the gelatinization process.

It has to be mentioned that micrograph analysis is highly time-consuming and needs to be automated. Further studies are required to increase micrograph quality, so software can be used for auto-capturing of the granule size and simultaneously determining the gelatinization temperature. Furthermore, this new in situ system should be used to understand the impact of heat during bread formation. Thereby, correlations between the end product in dependence on analytical as well as sensorial product evaluations and the gelatinization content are needed. A sensorial evaluation is important to confirm the completeness of the baking process and ensure a high product quality. Additionally separate consideration on crumb and crust structure should be carried out combined with a texture and color evaluation using established systems (Scanlon and Zghal 2001; Schirmer, Hussein et al. 2011). Due to the effect of granule size on water absorption, gelatinization temperature, granule swelling, viscosity development and digestibility is necessary to broaden the knowledge about internal factors such as the A-to-B granule ratio, which are important to a range of end uses.

Table 3: Commonly used stains for detection from dough and bread related systems by confocal laser scanning microscopy (CLSM).

Application / sample Preparation	Sample / measured ingredients	Dye / Concentration (g 100ml ⁻¹)	CLSM model / Lens	Reference
Crust was separated from the crumb using a knife. Any remaining crumb was carefully removed under the crust. Crust and crumb samples were freeze-dried and grounded (0.25 mm sieve) for analysis.	bread crumb and crust/ starch	FITC 0.24 / rhodamine 0.042 (dissolved in water)	Leica TCS SP (Leica Microsystems, Heidelberg, Germany), Ar/Kr laser	(Primo-Martín, van Nieuwenhuijzen et al. 2007)
Discs of 70 mm diameter and 5 mm thickness were transferred to a cryostat held at -25 °C. 15 µm thick sections were then cut and placed on a microscope slide. One drop of the stain was added to the section and a coverslip was placed on top. After 5 min the stained sections were examined.	biscuit dough/ starch, fat and gluten	nile blue 0.1 (drop technique)	Zeiss LSM310 (Cael Zeiss, Welwyn Garden City, UK), Ne/Ne laser	(Gallagher, Kenny et al. 2005)
Crumb with dimensions of app. 5 x 5 x 3 mm were cut with a razor blade, placed on a microscope slide and stained on the surface.	bread crumb/ starch and protein	nile blue 0.1 (drop technique)	Lecia SP 5	(Alvarez-Jubete, Auty et al. 2010)
Optical analysis of a dough section during rise, in which the protein fraction has been stained.	dough/ protein (starch without dye)	rhodamine	BioRad, MRC 600 CSLM (Zeiss inverted microscope, Ar/Kr laser)	(Blonk and van Aalst 1993)

2.3 g sample was cut from the inner part of the dough with scissors and transferred to a specimen shape (diameter 18 mm, height 0.8 mm) before covering with a glass slip.	dough/ protein	rhodamine 0.001 (dissolved in water)	Ti-U inverted microscope with an e- C1plus (Nikon, Düsseldorf, Germany)	(Jekle and Becker 2011)
In situ observation of the freezing process in wheat dough: Formation of ice and changes in the gluten network. Small pieces (approx. 8 mm ³) of dough or gluten were squeezed between two cover slips. A spacer (0.5 mm thickness) was used (between the two cover slips) to standardize sample thickness. The cover slips were sealed with silica gum to avoid drying out.	dough/ starch and protein	rhodamine 0.001 (gluten) (dissolved in water) 0.01 (starch)	Leica TCS SL (Leica Microsystems, Heidelberg, Germany) Ar- and He/Ne- laser	(Baier- Schenk, Handschin et al. 2005)
Bread/dough was cut into sections of 150 µm thickness with a manual microtome equipped with a knifeholder for conventional razorblades. The sections were stained and incubated for 30 min followed by rinsing with deionized water for 30 min.	dough, bread/ starch and protein	safranin 0.04 (drop technique, starch)/ acid fuchsin 0.01 (protein)	Leica TCS SP (Leica Microsystems, Heidelberg, Germany)	(Dürrenberg er, Handschin et al. 2001)
Samples for dough analyzes were placed onto a slide before covering it with glass slip. To examine the breadcrumb the breads were baked. After baking the breads were cooling for 2 h. A sample of each was taken from the center of the crumb and placed onto the slice and covered with a glass cover slip.	dough, sourdough, bread (gluten-free)/ starch and protein	safranin 0.002 (added to water)	MRC-1024 laser-scanning confocal system (Biorad, UK) mounted on an upright microscope (Axioskop, Zeiss, Germany)	(Moore, Juga et al. 2007)
The obtained dough sample (0.5 g) was cut off and rounded. After compressing on a slide to a thickness of 3 mm the dough was covered with a coverslip to prevent it from drying out. The sample was scanned using confocal microscopy system as proofing continued at room temperature.	dough/ protein	Tetraethyl- rhodamin B 0.01 (added to water)	Leica TCS SP (Leica Microsystems, Heidelberg, Germany)	(Li, Dobraszczy k et al. 2004)

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5 Appendix

5.1 Non-reviewed papers

1. Schirmer, M.: Der Backprozess - Physikochemische Strukturveränderung unter thermischer Erhitzung, *Journal Culinaire*, 52 (2012), 36-42.
2. Schirmer, M., Jekle, M., Arendt, E., Becker, T.: Less calories with stevia and polydextrose. *Baking+biscuit 1* (2012), 14-18.
3. Schirmer, M., Jekle, M., Hussein, M.A., Becker, T.: Entwicklung eines intelligenten Backofens zum optimierten Backen in industriellen Backöfen mittels digitaler Bildauswertung und erfahrungsbasierter Fuzzyregelung, [www.fei-bonn /download/projekte/projekt Datenbank.html/fv_15659/](http://www.fei-bonn/download/projekte/projekt Datenbank.html/fv_15659/) (2011).
4. Schirmer, M., Jekle, M., Hussein, M.A., Becker, T.: Röschecharakterisierung von Kleingebäcken in Abhängigkeit der Gasfeuchte im Backraum. *Cereal technology 04* (2011), 172-184.
5. Schirmer, M.; Jekle, M.; Hussein, M.A.; Becker, T.: IBO – Intelligenter Backofen, Bericht, *Der Lebensmittelbrief*, 19. Jahrgang (2008), 373-375.

5.2 Book contribution

Schirmer, M.; Jekle, M.; Becker, T.: Charakterisierung funktioneller Eigenschaften von Weizen- und Roggenmahlerzeugnissen. Aktuelle Untersuchungs-Methoden, praktische Anwendungen. AgriMedia Verlag (Bergen/Dumme), currently in progress.

5.3 Oral presentations

1. Schirmer, M., Jekle, M., Becker, T.: Online monitoring of starch gelatinization with limited water content using CLSM, AACCI Annual Meeting, Albuquerque, New Mexico, USA, 2013-10-02.
2. Schirmer, M., Jekle, M., Becker, T.: Grundlagen über Getreidestärken, VDB-LG Bayern Frühjahrstagung, Freising, Germany, 2013-03-22.
3. Schirmer, M., Jekle, M., Becker, T.: Verkleisterungseigenschaften während des Backprozess, VDB-LG Bayern Frühjahrstagung, Freising, Germany, 2013-03-22.

4. Schirmer, M., Jekle, M., Becker, T.: Krisenbewältigung bei Hygienebeanstandungen, 62. Tagung für Bäckereitechnologie, Detmold, 2012-11-13.
5. Schirmer, M., Jekle, M., Becker, T.: Der Branchenumbbruch: Stellungnahme zum aktuellen Backwarenmarkt, VDB-Erntegespräch 2012, Freising, 2012-08-30.
6. Schirmer, M., Jekle, M., Becker, T.: Physicochemical and morphological characterization of different starches with variable amylose/amylopectin content, AACCI Annual Meeting, Hollywood, Florida, USA, 2012-10-02.
7. Schirmer, M., Jekle, M., Becker, T.: Strukturanalysen von ultraschallgeschnittenen Modellweizenbrotten während der Erhitzungsphase, GDL-Kongress Lebensmitteltechnologie 2012, Dresden, 2012-09-27-29.
8. Schirmer, M., Jekle, M., Becker, T.: Charakterisierung von Stärken und Mehlen mit differenziertem Amylose- und Amylopektingehalt. 1. Frühjahrstagung des WIG, Freising, 2012-03-28.
9. Schirmer, M., Beck, M., Jekle, M., Arendt, E., Becker, T.: Mikro- und Makrostrukturelle Krumenbildung während des Backprozesses, 26. Detmolder Studientage, Detmold, 2012-02-13.
10. Schirmer, M., Jekle, M., Arendt, E., Becker, T.: Saccharoseersatz mittels Polydextrose zum Einsatz von Stevia als Zuckeraustauschstoff in Massensystemen, 61. Tagung für Bäckereitechnologie, Detmold, 2011-11-15-17.
11. Schirmer, M., Jekle, M., Becker, T.: Strukturveränderungen während des Backprozesses, 62. Tagung für Getreidechemie, Detmold, 2011-06-29-30.
12. Schirmer, M., Jekle, M., Becker, T.: Quantification in starch microstructure as a function of baking time, iCEF11 International Congress on Engineering and Food, Athen, Greece, 2011-05-22.
13. Schirmer, M., Jekle, M., Becker, T.: Micro and macro structural changes in dough/crumb during baking, 3rd International Spring Meeting 2011, Freising, 2011-04-11-13.
14. Schirmer, M., Jekle, M., Arendt, E., Becker, T.: Evaluation of the sugar replacer stevia in confectionary products, 40th Annual UCC Food Research Conference - University College, Cork, 2011-04-01.
15. Schirmer, M., Jekle, M., Becker, T.: Makro- und mikrostrukturelle Veränderungen während der thermischen Behandlung von Weizenbrot.

- Jahrestreffen des Fachausschusses Lebensmittelverfahrenstechnik, Vlaardingen, Niederlande, 2011-03-22.
16. Schirmer, M., Jekle, M., Becker, T.: Selbststeuernde Backofensysteme. 25. Detmolder Studententage, Detmold, 2011-02-21-23.
17. Schirmer, M., Beck, M., Jekle, M., Becker, T.: Physikochemische Stärkeveränderungen während der thermischen Behandlung. Ausschusssitzung der Getreidechemie, Ahrensburg, 2010-11-11.
18. Schirmer, M., Jekle, M., Hussein, M.A., Paquet-Durand, O., Solle, D., Becker, T., Hitzmann, B.: Der intelligente Backofen. 61. Tagung für Bäckereitechnologie, Detmold, 2010-10-27.
19. Schirmer, M., Jekle, M., Hussein, M.A., Becker, T.: Krusteneigenschaften in Korrelation zur Gasfeuchte im Backraum, Vortrag, 60. Tagung für Bäckerei-Technologie, Detmold, 2009-11-03-05.

5.4 Poster presentations

1. Schirmer, M., Jekle, M., Becker, T.: Structural analysis of ultrasound sliced bread during thermal process, XVII Latin American Seminar of Food Science and Technology – ALACCTA, Estado de Paraná, Brazil, 2012-08-05.
2. Schirmer, M.; Jekle, M.; Hussein, W.B.; Hussein, M.A.; Becker, T.: Interaction of humidity characteristics and bread roll crust quality in thermal processes, Poster, 15th World Congress of Food Science and Technology, Cape Town, South Africa, 2010-08-22-26.
3. Schirmer, M., Jekle, M., Hussein, M. A., Becker, T.: Interaction of humidity process and roll crust quality in industrial convection ovens, gemeinsame Sitzung des Wissenschaftlichen Beirats und der Kommission für Technik, Umwelt und Wissenschaft im VDM Freising, 2010-06-30.
4. D. Solle, M. Schirmer, T. Becker, B. Hitzmann: Überwachung von Backprozessen mittels digitaler Bildverarbeitung, 67. FEI-Jahrestagung 2009, „Erfolgsmodell Industrielle Gemeinschaftsforschung“, Hannover, 2009-09-08-10.
5. Schirmer, M.; Jekle, M.; Hussein, W.B.; Hussein, M.A.; Becker, T.: Betrachtung von Strukturbildungsreaktionen bei thermischer Behandlung von Getreideprodukten, ProcessNet-Jahrestagung 2009, 27. DECHEMA-Jahrestagung der Biotechnologen, Mannheim, 2009-09-08-10.

5.5 Curriculum vitae

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- 08/09 – 01/14 Phd Student, Technische Universität München, Institute of Brewing
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- 09/08 – 07/09 Research Assistant, University Hohenheim, Department of Process
Analysis and Cereal Technology,
Hohenheim, Stuttgart, Germany
- 09/98 – 09/02 Baker/ Baker Master, Different Bakeries and Confectionaries,
Gunzenhausen, Unterschwaningen, Röckingen, Stadland, Germany

Education

- 04/10 – 04/12 M.Sc., Master of Research in Food Science and Technology,
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- 10/04 – 08/08 Dipl. Ing., Study of Food Technology, University of Appliance
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„Der beste Weg die Zukunft vorauszusagen, ist, sie zu gestalten.“

Willi Brandt, Federal Chancellor of BRD, 1913 - 1992