Enzyme-assisted separation and hydrolysis of gluten – options for intensification

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–
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Abbreviations

AX	arabinoxylan
BU	Brabender units
DH%	degree of hydrolysis
SWG	self-washed gluten from Ibis wheat flour
VWG	vital wheat gluten
VWG + starch	vital wheat gluten plus added starch
WE-AX	water-extractable arabinoxylan
WFG	gluten present in Ibis wheat flour
WU-AX	water-unextractable arabinoxylan

Symbols

a_w	water activity (–)
E	enzyme concentration
E_0	initial enzyme concentration
G'	elastic modulus (Pa)
G''	viscous modulus (Pa)
$J_{c,max}$	maximum creep compliance (kPa^{-1})
J_{el}	relative elastic part (–)
k_m	substrate concentration at which $V = \frac{V_{max}}{2}$
S	solid concentration
S_m	substrate concentration (Michaelis-Menten eq.)
$tan\ \delta$	loss factor (–)
V	velocity of reaction
V_{max}	maximum velocity of reaction

CHAPTER 1

General introduction

1.1 Introduction

Food production is the largest consumer of water in the world. Approximately 70% of the world water is used for agricultural, 20% for industrial, and 10% for domestic use [30]. In Europe, 48% of the water is used by industry and 8–15% of this water by the food industry [101]. This is of importance because freshwater is a scarce good in many regions of the world. Predictions say that less than 50% of the world's population will have an adequate water supply in 2025 [27].

Among the crops produced, wheat is the third most important one in the world [95] and a very important food source. Besides using wheat as a whole, for instance in breadmaking, wheat is also separated into its individual constituents, primarily gluten and starch. Fig. 1.1 illustrates that the world gluten production is still a fraction of the world wheat production, but increases disproportionately.

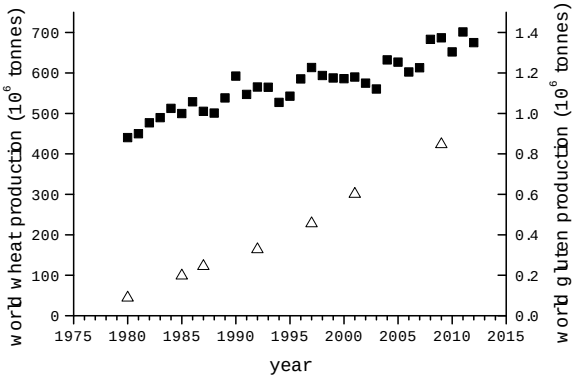

Fig. 1.1: Development of world wheat production (■, primary y-axis, [41]) and wheat gluten production (△, secondary y-axis, [31]) in million tonnes.

After separation, gluten and starch can be utilized in its native form or further processed, for instance by means of hydrolysis. Both, the separation and the hydrolysis of starch and gluten, currently consume copious amounts of water, which is undesirable from an environmental and economic point of view. Therefore, this thesis aims at investigating enzyme-assisted starch–gluten

separation and the subsequent hydrolysis of gluten at low water concentrations, with an emphasis on the gluten hydrolysis.

The following introduction gives a summary of wheat and its constituents and the state of the art in starch–gluten separation and gluten hydrolysis. Afterwards, potentials and drawbacks of intensified wheat processing are shown, followed by an outline of this thesis.

1.2 Wheat

Wheat benefits from its tolerance to diverse climates: it can be grown from Scandinavia and Russia in the North, to Argentina in the South [121]. Wheat is generally cultivated as common wheat (*Triticum aestivum*) for use in bread, Asian noodles, cakes or pastries, and as durum wheat (*Triticum durum*) for use in pasta or couscous [40].

Wheat is commonly distributed in its milled form as wheat flour, which contains about 70% starch, 12% protein, 2% lipids, 2–3% non-starch carbohydrates (arabinoxylan, etc.), and 14% water depending on the type of flour and the season [129]. In the following, the properties of these fractions will be summarized.

1.2.1 Wheat starch

Wheat starch consists of the two glucans amylose and amylopectin, with 20–30% amylose and 70–80% amylopectin in most wheat starches [121]. Amylose has a linear polymer structure with a polymerization degree of several hundred up to 10^4 [141]. Amylopectin has a branched polymer structure with a polymerization degree of 10^5–10^7. Starch is semi-crystalline with 30% of the mass being crystalline and 70% amorphous [8]. Wheat starch is present in form of granules that can be divided into a bigger A-type and a smaller B-type with diameters of 15–35 μm and 2–10 μm [124]. When wheat is hydrated and mixed at temperatures below 50 °C, starch maintains its granular form and is physically entrapped in the gluten network [118].

1.2.2 Wheat gluten

Wheat Gluten, the major protein of wheat, has been defined as the cohesive, viscoelastic, proteinaceous material prepared as a by-product of the isolation of starch from wheat flour [32]. Gluten consists of gliadins and glutenins, which both contribute to the unique viscoelastic properties of wheat dough. Gliadins are sticky when hydrated and mainly contribute to the viscosity and extensibility of dough [148]. They are present in gluten as α-, β-, γ-, and ω-gliadin with molecular masses of 30–80 kDa [129]. Gliadins are mainly present as monomeric proteins due to their low charge densities and low number of disulphide bridges [11]. In contrast, hydrated glutenins are both cohesive and elastic and contribute to the dough strength and elasticity [148]. When reduced with a reducing agent, glutenins can be divided into two subunits: high molecular weight (HMW) and low molecular weight (LMW) glutenins with molecular masses of 65–90 and 30–60 kDa [129]. In wheat gluten and flour, glutenins are present as polymeric proteins linked via disulphide bridges; the resulting clusters reach molecular masses larger than 10,000 kDa [148]. Wheat gluten is rich in the amino acids glutamine and proline. Furthermore, except for lysine, it contains all essential amino acids necessary for humans in the required amounts [31].

1.2.3 Wheat arabinoxylan

Arabinoxylan (AX) is a cell wall component of wheat. The structure of AX is a linear backbone of β-1,4 linked xylan with arabinose side-chains [137], with typical A/X-ratios of 0.5 to 0.83 [61]. AX can be divided into water-extractable (WE-AX) and water-unextractable (WU-AX) arabinoxylan in a ratio of one WE-AX to three WU-AX [129]. WE-AX and WU-AX have similar structures [10]; the possible reason for the water-extractable nature of WE-AX are incomplete crosslinking with other cell wall components, and small structural differences compared to WU-AX. Moreover, initial, incomplete enzymatic degradation in the kernel may play a role for the water-extractable nature [28].

1.2.4 Other ingredients

Most of the non-starch polysaccharides in wheat flour are arabinoxylans. The remaining 15% consist of β-glucan, cellulose and arabinogalactan-peptides [52].

Wheat lipids are present as 50% polar and 50% non-polar lipids. Furthermore, they are divided into free, bound, and starch lipids. The free lipids show a strong tendency to 'bind' to gluten during mixing [129].

Wheat gluten represents 78% of the proteins present in wheat. Besides, wheat also contains 15% water-soluble albumins and 7% globulins that are soluble in dilute salt solutions [8].

1.3 From wheat flour to gluten hydrolysate

Refining of wheat into its constituents generates value since the separated ingredients have many more applications than the wheat itself. If we consider a typical wheat process starting with the wheat and having the hydrolysate as the end product, then the process starts with the milling of the wheat kernel, followed by the separation of the starch and the gluten. The separated starch fraction is then either used in its native form, predominantly in bakery products [85], or the starch is often hydrolyzed and used for syrups, an important ingredient for beverages, or as washing detergent [58]. The separated gluten fraction can also be either used in its native form or further be processed among others by means of hydrolysis. We will now discuss starch–gluten separation and gluten hydrolysis in more detail.

1.3.1 Starch–gluten separation

Wheat starch and gluten are separated industrially by processes such as the Martin or the batter process [129]. The Martin process is the traditional process to separate starch and gluten and still in use today. Here, wheat flour is wetted until a dough forms. In a second step additional water is added to the dough, washing away the starch and other components from the gluten. In a next step, centrifugation separates the starch and the water-soluble components.

In the batter process, approximately equal amounts of water and flour are mixed. A batter forms, which is mechanically broken up by adding more water. The gluten aggregates formed coalesce to gluten clusters, which are low in starch, and form a curd-like structure. The gluten curds are then removed using gyrating sieves [54, 129].

Starch–gluten separation can be enhanced by using xylanases. Xylanases (endo-1,4- β-xylanases, EC 3.2.1.8) are hydrolases preferably degrading the xylan backbone of arabinoxylan (AX), causing a decrease in degree of polymerization. Xylanases differ in their ability to hydrolyze WU-AX and WE-AX. They are commonly used in the baking industry to improve the dough manageability and the bread volume [36].

AX have a relatively high water holding capacity. In total, AX have been reported to hold up to ten times their weight in water [28] and been suggested to hold up to a quarter of the water present in dough [5, 15]. When xylanases are added to hydrated wheat, they reduce the water-holding capacity of AX and thus the dough or batter viscosity. The reduced viscosity then results in an improved agglomeration of the gluten. This, however, has only been reported for WE-AX hydrolysis [24, 45]. Mild hydrolysis of WU-AX, on the other hand, influences the separation negatively because WU-AX solubilizes upon hydrolysis, which increases the viscosity of the dough or batter. Only when WU-AX is severely hydrolyzed to a low molecular mass, the viscosity decreases again and hydrolysis of WU-AX also results in improved gluten agglomeration [47].

1.3.2 Wheat gluten hydrolysis

Wheat gluten is considered to be a byproduct of the production of wheat starch and therefore available in large quantities at moderate prices, cheaper than milk or soy proteins [31]. In industry, native wheat gluten is for instance used as a bread improver, as a binder in meat products and pet food, and to fortify flours e.g. in breakfast cereals [32]. However, its further application in the food industry is limited because native wheat gluten is scarcely water-soluble close to its isoelectric point at pH 6–7. In addition, it has low emulsifying activity

and does not produce steady foams [111]. One possibility to overcome these limitations is to hydrolyze wheat gluten.

Enzymatic hydrolysis of wheat gluten improves the water solubility at neutral pH [23, 69]. Mild enzymatic hydrolysis is mainly performed to alter functional properties such as foaming and emulsifying properties [80, 92], and in bakery to soften hard wheat gluten [37]. Extensive enzymatic hydrolysis is performed to use gluten for instance as a savory flavoring agent [51], in sports nutrition [13], and for the production of hypoallergenic foods [25].

Wheat gluten can be hydrolyzed using acids or enzymes (i.e. proteases). The usage of acids is accompanied by several disadvantages, such as the formation of undesirable tastes and toxic components [25]. Furthermore, the use of acids leads to large amounts of salt after neutralization of the product. Enzymes, on the other hand, possess several advantages over acids like milder process conditions and no undesirable side reactions, while the functional properties of the final product can be better controlled by choosing specific enzymes [68]. Furthermore, enzymes are biodegradable and can remain in the final product when inactivated. On the downside, enzymatic hydrolysis usually results in incomplete hydrolysis and may lead to products with a bitter (off-)taste [22]. In most cases, the bitterness problem can be resolved using specific proteases. However, these proteases are costly and determine the overall costs of the final product to a large extent [38].

Proteases (EC 3.4) are classified according to the nature of their catalytic site (aspartic, glutamic, metallo, cysteine, serine, and threonine proteases) and their origin (microbial, animal, plant) [81, 98]. Furthermore, it is divided between proteases hydrolyzing internal peptide bonds (endopeptidase or proteinase) and proteases cleaving terminal peptide bonds or di- or tripeptides (exopeptidase or peptidase) [48]. Proteases are very diverse regarding their specificity: Some proteases are exclusively specific towards a unique peptide bond, such as the angiotensin-converting enzyme [81]. Other proteases are unselective and randomly cleave many different peptide bonds, which allows the hydrolysis to higher degrees of hydrolysis (DH%). Commercial enzyme mixtures often contain proteases with different catalytic sites and exo and endo

activities [98]. In this thesis, only commercial enzyme mixtures are used with the aim to hydrolyze to high DH%.

1.4 Intensification of the process

As mentioned, the separation and the hydrolysis of gluten currently consume copious amounts of water: Conventional starch–gluten separation often requires 10 to 15 L of water per kg of dry matter [119]. Typical wheat gluten concentrations for enzymatic hydrolysis at neutral pH are around 20% [22, 84, 152] equivalent to 4 L of water per kg dry matter.

Alternatively, starch and gluten can be separated by applying a curvilinear shear flow in an in-house building shearing device (Wageningen University, The Netherlands) allowing more concentrated conditions. This has been extensively studied at water contents down to 42% (i.e. 0.7 L water per kg dry matter) [103, 104, 133, 134, 135]. However, little is known about combining the addition of xylanases with shear-induced starch–gluten separation. Furthermore, little is known about the rheological behavior of dough in general at water contents below 40% and the addition of xylanases.

Likewise, wheat gluten hydrolysis may be performed at higher solid loadings. To the best of our knowledge, this has never been studied. Also the hydrolysis of other proteins at high solid contents has rarely been investigated [16, 71, 113]. A little more is known about high-solid hydrolysis of polysaccharides, for instance for the hydrolysis of cellulose and lignocellulose for biofuel production [43, 59, 72, 110] or wheat starch [6, 130, 131].

Generally, aiming at lower water concentrations provides several benefits: (1) System capacity: A higher solid concentration results in a larger system capacity either by a higher throughput in existing equipment or by smaller reactors, which reduces the capital costs. (2) Energy usage: At higher solid concentrations, less energy is required for heating and cooling during the process and the consequent dehydration of the product, which reduces the operating costs. Furthermore, wheat gluten is often dried employing ring driers at 20% moisture, which involves mixing of wet gluten with dry gluten [31]. Hence, ring drying requires less addition of dry gluten when the preceding process is performed

at higher solid concentrations. (3) Wastewater: Higher solid concentrations produce less wastewater with inherently lower treatment and disposal costs while more of the raw material will end up in the product, instead of in the wastewater. (4) Downstream processing: Higher product concentrations can facilitate the downstream processing and lower the downstream processing costs [59, 94].

On the downside, aiming at lower water concentrations also creates new challenges: (1) Mixing problems: A higher solid concentration results in an increased viscosity. This increased viscosity may lead to insufficient mixing and can be highly energy consuming in conventional stirring tanks [72]. Furthermore, the control of reaction parameters, such as pH and temperature is hampered at high solid concentrations. Besides, water acts as the solvent for the enzyme and as the transport medium for the substrate and the product. Thus, higher solid concentrations can lead to diffusional limitations. (2) Water availability: The water activity decreases at low water contents, which influences the enzyme activity and possibly the thermodynamic equilibrium of the hydrolysis reaction. (3) Enzyme inhibition and inactivation: Higher amounts of substrate and product can lead to enhanced substrate and product inhibition, influencing the enzyme kinetics. Furthermore, proteases can be substrate to other proteases, which can result in self-inactivation. At higher solid concentrations, often higher enzyme contents are needed. Therefore, higher enzyme contents can also lead to higher rates of self-inactivation.

1.5 Thesis Outline

The objective of this research is to investigate the effect of increased dry matter concentrations and enzyme usage on a full wheat process chain from wheat flour to a wheat gluten hydrolysate. A schematic overview of this thesis is given in Fig. 1.2. This research study is divided into two parts: First, the role of xylanases in high dry matter dough is studied with the aim to further reduce the water content in the shear-induced starch–gluten separation. It is hypothesized that the water held by AX is released during the enzymatic degradation of AX, giving the opportunity to further increase the dry matter content. Second, the role of

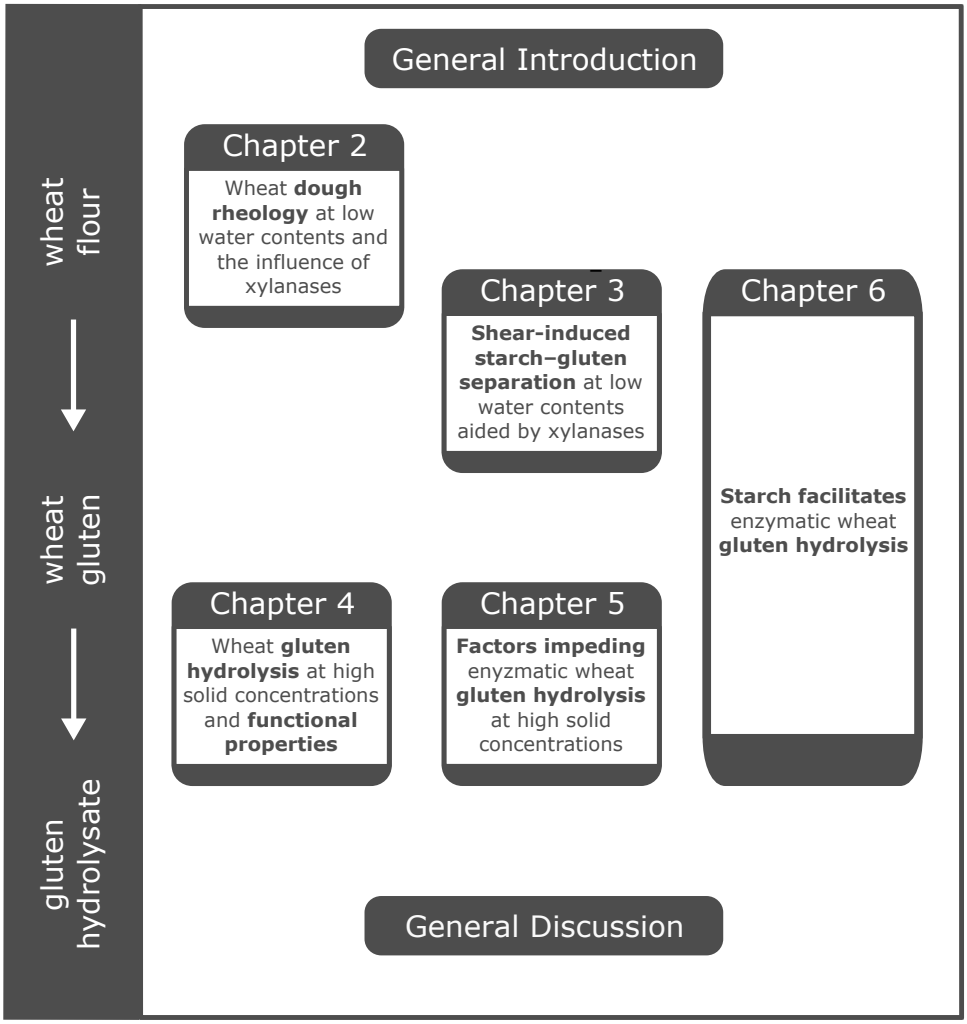

Fig. 1.2: Schematic overview of the thesis.

water in wheat gluten hydrolysis is studied with the aim to perform wheat gluten hydrolysis at concentrated conditions.

Chapter 2 describes the effect of low water contents on wheat dough rheology and how xylanases influence dough rheology. Farinograph, frequency sweep, and creep-relaxation measurements are performed at dough water contents down to 34%.

Chapter 3 describes the influence of xylanases on the shear-induced starch–gluten separation by releasing water associated with arabinoxylan. It is studied how low water contents and xylanases influence the separation mechanism and the protein content of the gluten-rich clusters.

Chapter 4 introduces enzymatic wheat gluten hydrolysis at high solid concentrations and studies how the solid concentration during hydrolysis affects the functional properties and the molecular mass distribution of the resulting hydrolysates. Furthermore, the industrial potential of high-solid wheat gluten hydrolysis is shown. Despite the advantages of high-solid gluten hydrolysis, a lower degrees of hydrolysis at increased wheat gluten concentrations and constant enzyme-to-substrate ratios is observed. In **chapter 5** we study possible causes of this concentration effect, namely mass transfer limitations, enzyme inhibition, enzyme inactivation, water activity, and the plastein reaction.

Chapter 6 studies wheat gluten hydrolysis in wheat flour at high solid contents before starch and gluten are separated. Wheat flour and vital wheat gluten are hydrolyzed at different solid but the same protein contents. Furthermore, the influence of starch on wheat gluten hydrolysis is evaluated by hydrolyzing vital wheat gluten with starch addition and by investigating the influence of the wheat flour concentration from 20% to 70% (w/w) on the hydrolysis.

The general discussion in **chapter 7** summarizes and generalizes the main findings of this thesis and compares the current status in starch–gluten separation and gluten hydrolysis with the concentrated separation and hydrolysis processes developed in this study. In the end, future prospects in high-solid wheat gluten hydrolysis are briefly discussed.

CHAPTER 2

Wheat dough rheology at low water content and the influence of xylanases

Abstract

We report the effect of low water content and xylanases on wheat dough rheology. Farinograph, dynamic oscillation, and creep-recovery measurements were performed using water concentrations from 34–44.8% (total basis). A water reduction from 43.5–44.8% to 34% increased G' and G''-values, increased resistance upon mixing as evidenced by higher Farinograph Brabender Unit values, and decreased the maximum creep compliance by 1–2 orders of magnitude. Furthermore, it was investigated whether the influence of the water content on G' and creep-relaxation tests was similar at higher water contents previously reported with this study at low water content. The results obtained at these low water conditions were in line with extrapolated data from literature. Addition of an endoxylanase with a higher selectivity for water-unextractable arabinoxylans and an endoxylanase with a higher selectivity for water-extractable arabinoxylans both resulted in lower G' and Farinograph dough consistencies and an increase in maximum creep compliance. The major influence of both xylanases was the release of water with possible water reductions of 2–5% (on water basis). However, no distinct differences between the two xylanases were observed.

This chapter has been submitted as:
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2.1 Introduction

Mixing water and wheat flour at the right proportion results in a heterogeneous dough system mainly consisting of a hydrated gluten network with embedded starch granules. It is the contribution of the gluten proteins that allows wheat to form a viscoelastic and strain-hardening dough. However, a dough is only obtained in a relatively small concentration range of about 30–55% (total basis) water. Lower water contents result in a cohesive and wetted powder; higher water contents result in a batter.

Since the biopolymers in wheat are immiscible, water is present in each respective phase and at the interphases [44]. As a result, water influences the rheological behavior of dough. It has been reported that increasing water contents reduce Farinograph Brabender Units (BU) [79] and G' and G'' in dynamic oscillation measurements [49, 63]. In creep-relaxation tests, the creep compliance increases with increasing water contents at constant stress, whereas the elastic contribution decreases [63, 88].

Wheat dough rheology is mainly studied in literature with the goal to relate it to the breadmaking quality at water concentrations representing optimal water absorption and higher. To the best of our knowledge, wheat dough rheology has only been described to water concentrations as low as 41–42% [79, 83, 122]. Studying dough rheology at lower water concentrations would extend our understanding of the role of water in dough. Moreover, aiming at lower water concentrations in processes in general is advantageous from an environmental and economic point of view. First, more concentrated conditions result in larger system capacities and in less drying energy and time in case the final product needs to be dried [57]. Second, more concentrated conditions require less water and produce less wastewater with inherently lower treatment and disposal costs while more of the raw material will end up in the product instead of in the wastewater. In practice, wheat doughs with water contents below optimal water absorption can for instance be used in pasta production using durum wheat and extrusion processes [74], or in shear-induced starch–gluten separation [55].

The amount of water added to dough is determined by the water needed to fully hydrate the gluten. However, wheat contains water absorbing components

such as arabinoxylan (AX) that compete with gluten for water [144, 145]. AX is present in wheat flour as water-extractable arabinoxylan (WE-AX) at 0.5% (w/w) and as water-unextractable arabinoxylan (WU-AX) at 1.5% (w/w) [129] with water-holding capacities up to 9.9 g water/g WU-AX and 6.3 g water/g WE-AX [28]. Hydrolyzing AX by means of xylanases could release water and make it available for gluten hydration, which would reduce the overall water addition to dough. The approach of using reduced water contents with the help of xylanases has been used previously for pasta production [14]. However, to the best of our knowledge, low water content in combination with xylanase addition have never been studied regarding wheat dough rheology.

2.2 Materials & Methods

2.2.1 Materials

Soissons wheat flour from a single, common wheat cultivar was obtained from Meneba (Rotterdam, The Netherlands). The water and protein content were 14.4±0.4% and 10.7±0.4% (N×5.7), respectively (all by weight). Farinograph optimal water absorption was 55.8% on 14% moisture basis (AACC-method 54-21). Shearzyme 500L was kindly provided by Novozymes A/S (Bagsværd, Denmark). Shearzyme is a monocomponent endoxylanase from *Aspergillus aculeatus* with a greater selectivity for WE-AX [45]. Grindamyl POWERBake 950 was kindly supplied by Danisco A/S (Brabrand, Denmark). Grindamyl POWERBake 950 is an endoxylanase from *Bacillus subtilis* with a greater selectivity for WU-AX [109]. The xylanases will be referred to as Shearzyme and Grindamyl in the following. Tap water was used in all experiments.

2.2.2 Water and enzyme contents

Water contents of 34%, 36%, 38%, 39.5%, 41.5%, 43.5%, and 44.8% (based on total weight) were used in this study similar to Hardt et al. [55]. This corresponds to Farinograph water absorptions of 29.7%, 33.8%, 38.1%, 41.5%, 46.3%, 51.5%, and 55.1% (all on 14.4% flour moisture basis). It was assumed that the wheat flour consisted of 0.5% WE-AX and 1.5% WU-AX [129]. Enzyme-to-substrate

ratios of 1:6 (0.17 g enzyme/g WE-AX) in case of Shearzyme and 1:9 (0.11 g enzyme/g WU-AX) in case of Grindamyl were used to ensure a high enzyme activity and to limit inhibition.

2.2.3 Farinograph

A 50-g mixer connected to a Do-corder E330 equipped with a PL2100 computer interface and controller unit (Brabender, Duisburg, Germany) for torque measurement was used to evaluate the Farinograph dough characteristics according to AACC-method 54-21 at 30 °C. Farinograph dough consistencies are typically expressed in Brabender Units (BU), with 1 BU = 0.00196 Nm in a 50-g mixer.

2.2.4 Dynamic oscillation and creep-recovery measurements

Tap water at 35 °C was added to wheat flour in a 10-g Farinograph mixer and mixed for 5 min at 40 °C. The temperature of 40 °C was chosen to have a balance between a practical temperature for dough mixing and the optimum temperature of Grindamyl and Shearzyme of 50 °C [109] and 70 °C [89], respectively. The mixing time was kept constant to allow constant times of the xylanases to be active. Nevertheless, all samples with water contents higher than 34% had peak times of 6 min or below (Fig. 2.1). After mixing, the samples were sealed in a plastic bag and rested at room temperature for 15 min before application in the rheometer.

Dynamic oscillation and creep-recovery measurements were performed employing a Physica MCR 301 rheometer (Anton Paar, Germany). A plate–plate geometry was used with profiled plates and a plate diameter of 25 mm, operated at 20 °C. A piece from the inside of the dough from the plastic bag was placed on the rheometer. The sample was gently trimmed after compressing and fixed at a 2 mm gap. At 34% water content, the compression to the 2 mm gap could take up to 15 min. Drying was prevented by placing a wetted tissue next to the plates combined with a solvent trap.

Dynamic oscillation measurements. G', G'', and consequently $tan\ \delta = G''/G'$ were determined over a frequency of 0.1 Hz to 10 Hz. The strain was

0.01% and measured to be within the linear viscoelastic region. The samples were allowed to rest an additional 5 min on the rheometer before the measurement, i.e. the samples rested a total of 20 min, allowing a careful balance between relaxation and a possible drying of the sample. Moreover, a resting time of 20 min is common in literature, e.g. [49, 63]. Resting times were kept constant because of the xylanase addition. Experiments were performed in triplicate.

Creep-recovery measurements were performed by applying 1000 Pa for 1 min and recording the relaxation for 5 min at 0 Pa. The shear stress of 1000 Pa was chosen to allow strains above 0.5% at 34% water content. Samples were allowed to rest an additional 15 min on the rheometer before the measurement. The maximum creep compliance $J_{c,max}$ (kPa^{-1}) after 1 min and the creep recovery compliance J_r (kPa^{-1}) after another 5 min were recorded. Subsequently, the relative elastic part $J_{el} = J_r/J_{c,max}$ was derived. Experiments were performed in triplicate.

2.2.5 Statistical analysis

A two-factor analysis of variance was performed to test significant differences ($\alpha = 0.05$) in water content and enzyme addition using SPSS version 19.0. We chose an ANOVA because it allowed the comparison of samples with and without xylanase addition in the whole water content range used, using more data points for comparison. The results of all two-factor ANOVAs can be found in appendix A.1. Data were normally distributed based on the Shapiro–Wilk test. Logarithmic values were used to test significant differences in dough consistency, G' and $J_{c,max}$ for different water contents. Experiments at 34% water content were single experiments, since the applied forces at 34% water were close to the rheometer's limit. Therefore, they were excluded in the ANOVA. Significance tests for $J_{c,max}$ were only performed up to water contents of 39.5%. In the following text, the word *significant* will only be used to refer to statistically significant results of the ANOVA.

A simple power law fit: $a \times x^n$ was used to fit the dough consistency and G' as a function of the water content similar to Lefebvre and Mahmoudi [78]. We would like to stress that the primary goal was expressing the non-linear

relationship between the respective rheological parameter and the water content and not to obtain the most suitable fit.

2.2.6 Digitizing data from literature

Data from literature was extracted using the software Engauge Digitizer 4.1. To reduce the number of displayed publications for a better clarity of the graphs, only studies with at least three different tested water contents were taken into consideration. For frequency sweeps G'-values had to be measured at 1 Hz and strains and temperature did not exceed 0.2% and 30 °C. In several studies, the water content was given as water absorption instead of water content on total basis; we corrected for this. A water content of 14% was assumed for those wheat flours for which no water content was specified.

2.3 Results

2.3.1 Farinograph

Fig. 2.1 shows the influence of low water content and xylanase addition on the Farinograph peak time and consistency after 30 min. Decreasing the water

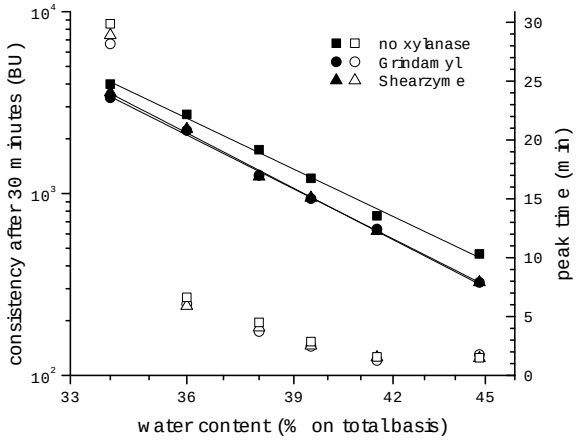

Fig. 2.1: Farinograph dough consistency after 30 min (closed symbols) and peak time (open symbols). $R^2 > 0.99$ for all power law fits. Please note the log–log plot. The entire mixing profiles are shown in appendix A.2.

content resulted in longer peak times for water contents below 40%. The addition of both xylanases showed no statistically significant influence on the peak time. At 34% water content, the peak time increased noticeably and it might be doubtful whether it was actually reached after 30 min, when the experiment was ended. This was the only water concentration at which the gluten was not sufficiently hydrated to form a coherent dough within 5 min.

The dough consistency in Farinograph experiments is generally expressed in Brabender Units (BU). We plotted the averaged values recorded of the last 30 s of every experiment (i.e. after 30 min of running) to allow enough time for AX hydrolysis to occur. Without enzyme the dough consistency increased upon water reduction. This water effect could be described by a power law ($R^2 > 0.99$). The addition of Shearzyme and Grindamyl significantly decreased the dough consistency at every water content, with the decrease being more pronounced at higher water contents. No significant differences were found between Shearzyme and Grindamyl regarding dough consistency and peak time. The entire mixing profiles of the different water contents can be found in appendix A.2.

2.3.2 Dynamic oscillation measurements

Fig. 2.2 displays the influence of low water content and xylanase addition on G', G'', and $tan\ \delta$. Decreasing the water content from 43.5% to 34% led to a fortyfold non-linear increase in G' and G'' (Fig. 2.2a). Increasing the frequency from 0.3 to 10 Hz showed the typical increase in G' and G'' following power law, but this frequency-caused increase was rather independent of the water content; the power law index n remained between 0.18 to 0.22 for G' and 0.24 to 0.28 for G'' in the frequency range of 1 to 10 Hz.

The addition of Shearzyme and Grindamyl significantly decreased G' at all water contents compared to dough without xylanase (Fig. 2.2b). However, no significant difference in G' was found between Shearzyme and Grindamyl. Furthermore, the addition of both enzymes increased $tan\ \delta$ at water contents below 43.5% (Fig. 2.2c). At 43.5% water content, $tan\ \delta$ also slightly increased for no xylanase addition from 0.310 to 0.349. Nevertheless, overall, the increase in $tan\ \delta$ was statistically significant for Grindamyl compared to no xylanase

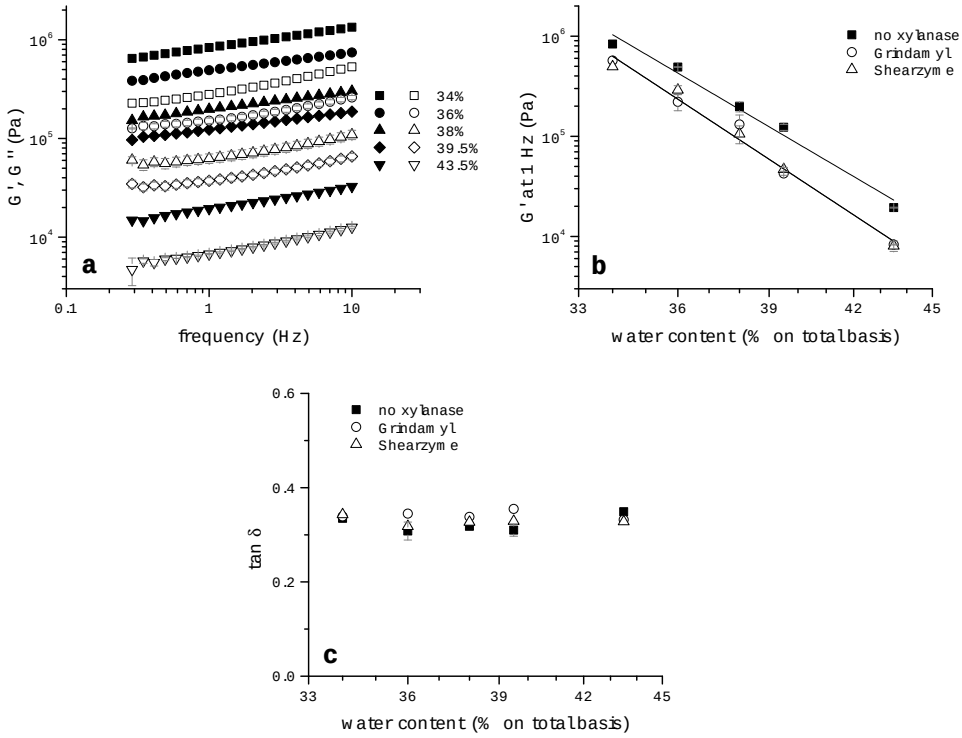

Fig. 2.2: (a) Influence of the water content on G' and G'' at different frequencies without enzyme addition. The legend indicates the water content. Closed and open symbols represent G' and G'' respectively. Error bars represent the standard deviation and are only shown for G'' but were similar for G'. (b) Influence of the water content on G' at 1 Hz with and without xylanase addition. $R^2 > 0.98$ for all power law fits. (c) *tan* δ at 1 Hz at different water contents. Standard deviation is only shown if > 0.03. Please note the log–log plot.

addition. The increase in *tan* δ for Shearzyme addition was not statistically significant.

2.3.3 Creep-relaxation measurements

Fig. 2.3 depicts the influence of the water content and xylanase addition on the maximum creep compliance $J_{c,max}$ and the relative elastic part J_{el}. Increasing the water content from 34% to 43.5% resulted in a non-linear increase in $J_{c,max}$ of more than two orders of magnitude with and without xylanase addition (Fig. 2.3a). Furthermore, an increase in slope was observed above 39.5%. Xylanase addition significantly increased $J_{c,max}$.

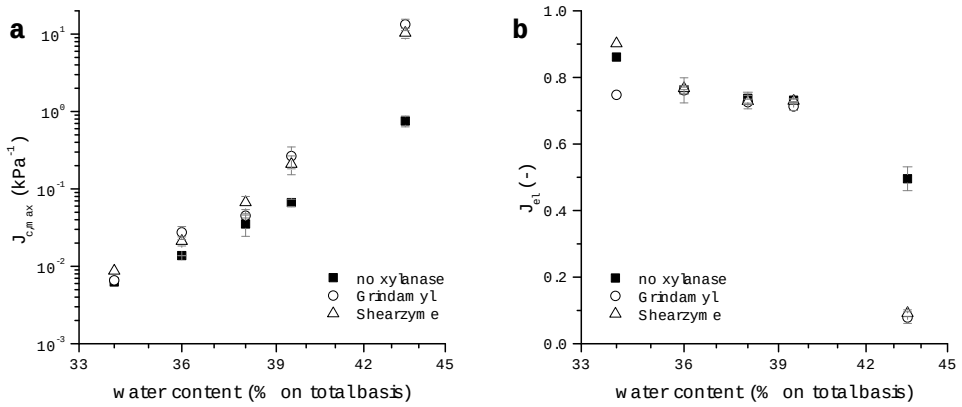

Fig. 2.3: Creep-recovery tests. (a) $J_{c,max}$ after 1 min at an applied shear stress of 1000 Pa. (b) J_{el} after another 5 min at 0 Pa. Error bars represent the standard deviation. Please note the log–log plot.

At water contents below 40%, xylanase addition had no significant influence on J_{el} (Fig. 2.3b). However, higher water contents overall resulted in significantly lower J_{el} at all water contents, although at water contents below 40% the effect was rather small. For instance, J_{el} remained between 0.71 and 0.77 between 36% and 39.5% water content. The scatter in J_{el} at 34% can be traced back to the fact that experiments were only performed once. At 43.5% water content, J_{el} decreased considerably with and without xylanase addition compared to water contents $< 40\%$. With xylanase addition this decrease was even more distinct with $J_{el} < 0.1$. No statistically significant differences between Shearzyme and Grindamyl were found in $J_{c,max}$ and J_{el} at all water contents.

J_{el} and $tan\ \delta$ both characterize the elastic properties of the dough. The reason why J_{el} (Fig. 2.3b) showed a greater dependency on the water content than $tan\ \delta$ (Fig. 2.2c) can be related to the applied forces. While $tan\ \delta$ measurements were performed in the linear viscoelastic region of the dough, J_{el} measurements were performed outside the linear viscoelastic region. Consequently, the applied torque values in the rheometer were 12 to 1100 times higher for J_{el} than for $tan\ \delta$ at 1 Hz depending on the water content and xylanase used.

2.4 Discussion

Decreasing the water content from 43.5% to 34% resulted in an increase in dough consistency (Fig. 2.1), G' and G'' (Fig. 2.2), and a decrease in $J_{c,max}$ (Fig. 2.3) of 1–2 orders of magnitude. The addition of both xylanases showed the same effect as the addition of water. Below, we will evaluate the role of water in wheat dough rheology at very low water content and compare our results to wheat doughs at higher water contents. Furthermore, we will discuss the effect of xylanase addition on wheat dough rheology.

2.4.1 Farinograph

The observed increase in dough consistency for decreasing water contents without xylanase addition (Fig. 2.1) is in agreement with the trend obtained by Létang et al. [79], obtaining similar BU-values in a water content range of 41.5% to 45%. However, opposed to our study, water content and dough consistency were presented to be linearly correlated at water contents from 41.5% to 50.5% [42, 79]. Daniels [29], though, also showed a non-linear relationship between the water content and the torque down to 34% water content, albeit in a different mixing device.

2.4.2 Dynamic oscillation measurements

Small deformation rheological tests, such as dynamic oscillation measurements, indicate the linear viscoelastic behavior of wheat dough. While it is agreed that the presence of starch narrows the linear viscoelastic range of wheat dough [66, 126], the influence of starch–starch, starch–protein, and protein–protein interactions on G' (and G'') has remained controversial. Different authors suggested that starch–starch and starch–protein interactions determine G' to a large extent at small deformations, since weak wheat flours with consequent higher starch contents and starch–gluten blends with higher starch contents showed higher G' than strong wheat flours and starch–gluten blends with lower starch contents [66, 67, 107]. In contrast, Lefebvre [76] pointed out that starch granules are unable to form networks. Instead, they act as large particles with hydrodynamic forces dominating over particle-particle interactions. This supports

that the protein determines the linear viscoelastic behavior in small deformation tests.

Investigating the influence of a distinct wheat component on G' by changing the component's content inevitably changes the contents of all other components and their ratios to one another. Therefore, also the role of water must be considered to understand the influence of starch and gluten on G'. For instance, Uthayakumaran et al. [126] showed that replacing wheat gluten by starch decreased G' at constant water contents, which seems contradictory to the findings mentioned regarding flours high in starch. However, the authors further showed that starch addition increased G' when performing the experiments at optimal water absorption instead of at constant water contents. Fig. 2.2b illustrates that decreasing water contents increased G' at 1 Hz in a non-linear fashion. Obviously, the total water content, the amount of free water, and the water distribution among the wheat components need to be considered when understanding wheat dough rheology.

The wheat flour used in this study had a dry matter-to-water ratio of 1.23:1 at optimal water absorption. This ratio reached almost 2:1 at 34% water content. Consequently, the competition for water among the wheat ingredients increased at these low water content. The question is, whether water depletion below 40% water content favored one of the phases, e.g. the gluten phase, regarding the water distribution. The results presented indicated that the slopes of the graphs of the dough consistency (Fig. 2.1) and G' (Fig. 2.2b) as function of the moisture content remained constant, which suggests that decreasing water contents down to 34% water content decreased the amount of free water rather than water binding to starch, gluten, etc. As a result, the dough consistency and G' increased at lower water contents, since water acts as a plasticizer in dough [35], but not because the mechanism of hydration changed. This argumentation is supported by literature. As mentioned, water is present in dough in each respective phase (i.e. gluten, starch, AX, etc.) and at the interphases [44]. Here, dough is hydrated by the first and second monolayers, by capillary condensation, and by free water [114]. Using NMR-T_2 relaxation times, Ruan et al. [114] observed the formation of a liquid "free" water phase sufficient to mobilize the flour constituents at water contents above 35%, which relates to the lowest water

content used in this study. Furthermore, it was shown that at water contents above 33% the amount of free water increases for increasing water contents whereas the amount of tighter-bound water remains constant [35].

To obtain a better view on the impact of the water content on the slope of the non-linear decrease in G', Fig. 2.4 compares the influence of the water content on G' at 1 Hz at low water content (Fig. 2.2b) with higher water contents up to 51% reported in literature.

Our results from 34% to 43.5% water content fall in line with the values in literature at higher water contents. G' reduces in a steady, non-linear fashion over the whole water content range in which a dough is formed. This is displayed by the similar trends of the power law exponent n, with $-10 < n < -19$ in literature and $n = -15.4$ in this study and corresponding high R^2. The differences in G' at constant water contents can be explained by differences in the wheat cultivar, with consequent differences in protein content, protein composition, and water absorption. Furthermore, the behavior of $tan\ \delta$ also supports that water acts as

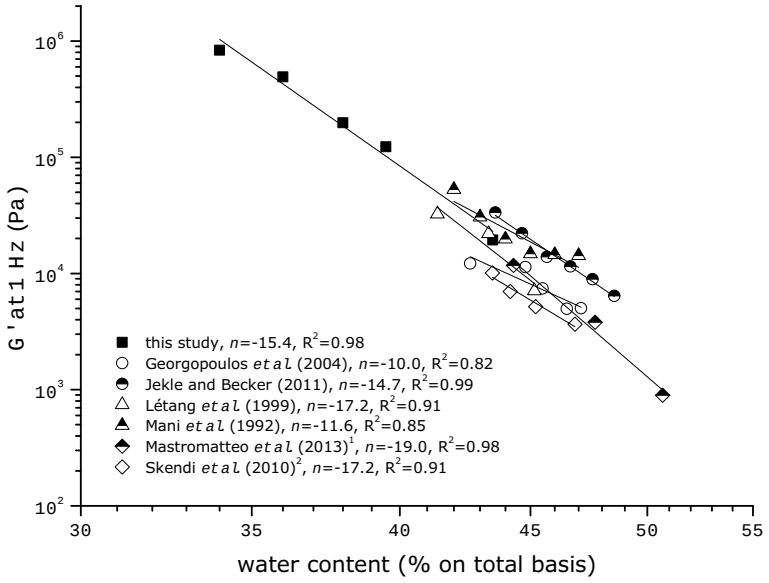

Fig. 2.4: Influence of the water content on G' at 1 Hz. Comparison with literature at a water content range from 34% to 51%. Trend lines are power law fits. Power law exponent n and R^2 are given in the legend derived from a simple power law fit: $a \times x^n$. Please note the log–log plot. [1] durum wheat. [2] wheat cultivar Yekora. Sources: Georgopoulos et al. [49], Jekle and Becker [63], Létang et al. [79], Mani et al. [83], Mastromatteo et al. [88], Skendi et al. [122].

a plasticizer in dough that changes the values of the rheological parameters, but not the hydration of dough as such. Fig. 2.2c shows *tan* δ to be independent of the water content at water contents below 40%. Even a further water content increase to 43.5% affected *tan* δ only slightly, with at most a 13% increase. This is in line with most studies found in literature, revealing either no influence of the water content on *tan* δ [9, 79], or only a slight increase in *tan* δ for increasing water contents [63].

2.4.3 Creep-relaxation

Since water acts as a plasticizer in dough, applying a constant stress will increase $J_{c,max}$ when adding water, as observed in Fig. 2.3a. However, a change in slope for the non-linear increase was observed for water contents above 40% because high strains generally lead to disorientation of the dough structure. Hence, $J_{c,max}$ was also a function of the elastic and viscous deformation. At higher water contents the influence of the viscous part increased as seen in Fig. 2.3b, which explains the change in slope observed in Fig. 2.3a. Fig. 2.5 compares our results for $J_{c,max}$ at low water content with higher water contents up to 51% reported in literature.

Similar to our observations, the slope of the non-linear increase in $J_{c,max}$ is generally larger at higher than at lower water contents. Also, a change in slope can be seen in some studies for increasing water contents [63]. The observed differences in $J_{c,max}$ in the different studies can be explained by differences in the wheat cultivar and the applied stresses.

While in the linear viscoelastic region $J_{c,max}$ is independent of the force applied, $J_{c,max}$ increases with increasing force in the non-linear viscoelastic region. The transition from linear to non-linear viscoelastic region has been shown to occur above 10 Pa for a common wheat cultivar at optimal water absorption [128], which is considerably lower than the forces used and depicted in Fig. 2.5. The question is whether the higher increase in $J_{c,max}$ at higher water contents was only caused by disorientation and damage of the dough structure [127, 147], or whether the water content also explains the higher increase in $J_{c,max}$ and the consequent decline in J_{el}. To test this, Fig. 2.6 shows the effect of the total strain on the recoverable, elastic strain. The approach to compare the

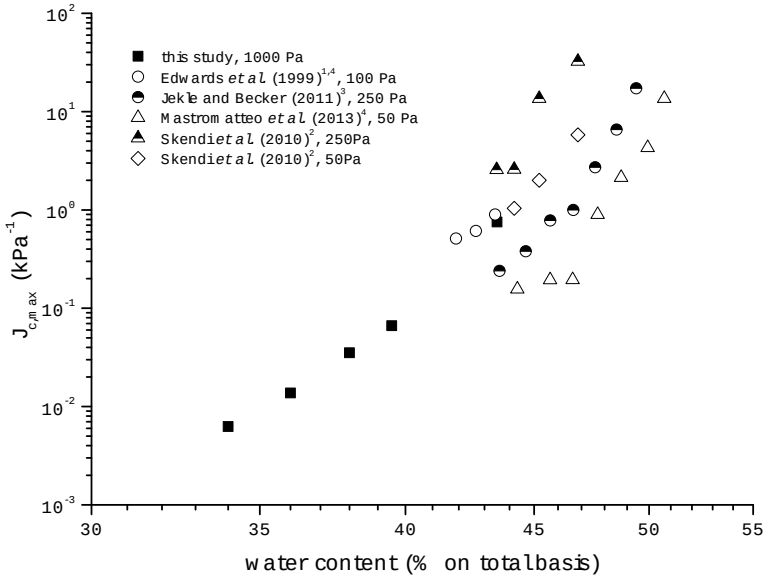

Fig. 2.5: $J_{c,max}$ recorded after 1 min. Comparison with literature at a water content range from 34% to 51%. The legend indicates the literature source and the applied stress. Please note the log–log plot. [1] wheat cultivar Durex. [2] wheat cultivar Yekora. [3] $J_{c,max}$ recorded after 3 min. [4] durum wheat. Sources: Edwards et al. [39], Jekle and Becker [63], Mastromatteo et al. [88], Skendi et al. [122].

total strain with the elastic strain has been used previously by Lefebvre [77] for creep times ≥3 h at constant water contents. Here, we compare the total strain to the elastic strain of our study with other studies at short creep times. In all studies, higher strains were achieved with higher water contents, except for van Bockstaele et al. [128] who used higher stresses.

Remarkably, the data from almost all studies form a master curve. At low strains most of the deformation can be recovered. When reaching strains above 10%, the dough deformation is too high and results in disorientation and damage of the dough structure. As a result, the increase in elastic strain levels off and the irreversible strain increases. The irreversible strain is the distance between the line of identity and the data points. Lefebvre [77] showed very similar results for longer creep times by using different wheat cultivars. He further showed that gluten shows exactly the same behavior, meaning that the relaxation behavior of

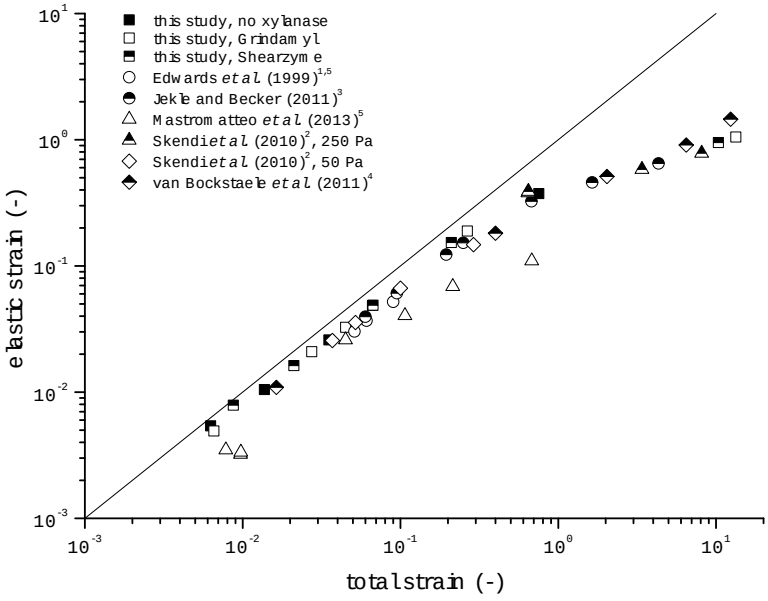

Fig. 2.6: Influence of the total strain on the elastic strain. The solid line shows the line of identity (x = y). By implication, the distance between the line of identity and the data points shows the irreversible strain. The legend indicates the literature source. [1] wheat cultivar Durex. [2] wheat cultivar Yekora. [3] total strain recorded after 3 min. [4] total strain recorded after 5 min. [5] durum wheat. Sources: Edwards et al. [39], Jekle and Becker [63], Mastromatteo et al. [88], Skendi et al. [122], van Bockstaele et al. [128].

wheat dough in the non-linear viscoelastic region is determined by the protein phase.

Fig. 2.6 illustrates that the amount of elastic strain is independent of the water content, the wheat cultivar, and the applied stress; the relaxation behavior of wheat dough is only determined by the total strain. The total strain, of course, can be influenced by the water content and the applied stress. Our results at low water content without xylanase addition only resulted in strains up to 75%. To also see the effect of higher strains, the influence of Shearzyme and Grindamyl on the elastic strain is also depicted in Fig. 2.6. Again, the elastic strain was a function of the total strain.

2.4.4 Xylanase and dough rheology

Hydrolysis of AX decreased Farinograph dough consistency (Fig. 2.1), G' (Fig. 2.2b), and increased $J_{c,max}$ (Fig. 2.3a). This is in agreement with other studies at water contents above 40%, where xylanase addition reduces the dough consistency [60, 64] and G' [146]. The addition of WE-pentosans to gluten, which has the opposite effect than adding xylanase, was shown to decrease $J_{c,max}$ [82].

The impact of Grindamyl and Shearzyme addition on the dough consistency, G', and $J_{c,max}$ decreased at lower water contents. It is known that an increase in substrate concentration results in a lower conversion at constant enzyme-to-substrate ratios. Santala et al. [116] showed reducing hydrolysis rates at water contents below 40% for wheat bran AX hydrolysis, which has also been observed for wheat gluten and other biopolymeric systems [56]. Nevertheless, despite the reduced AX conversion rate at low water content, both xylanases proved to be active at all water contents for all rheological characteristics studied.

AX indirectly impedes gluten network formation by competing for water [144, 145]. In this study, Shearzyme and Grindamyl mainly resulted in the release of water as seen for the reduced BU-values (Fig. 2.1) and G' (Fig. 2.2b). The amount of released water ranged between 2–5% (on water basis, derived from the power law fits) for the dough consistency and G' and increased with increasing water contents; i.e. a dough with xylanase behaved like a regular dough with additional 2–5% water (on water basis). However, we also found a small increase in $tan\ \delta$ at water contents below 40%, which proved to be statistically significant at least for Grindamyl. This shows that Grindamyl addition made the dough slightly more viscous.

Even though Shearzyme and Grindamyl behaved similarly in many aspects, Shearzyme has a greater selectivity for WE-AX and Grindamyl has a greater selectivity for WU-AX [45, 109]. Mild hydrolysis of WU-AX results in the solubilization of WU-AX and increased viscosities [45]. Upon prolonged hydrolysis the molecular weight of this solubilized WU-AX will reduce further, which will reduce the viscosity again. Therefore, higher BU-values for Grindamyl compared to Shearzyme would be theoretically expected during the mixing, which did not occur (see appendix A.2). Also no significant differences

in G', $J_{c,max}$ and J_{el} were found. Only with respect to $tan\ \delta$, a small difference was found between Shearzyme and Grindamyl. One reason why hardly any differences were observed between Grindamyl and Shearzyme could be that hydrolysis was performed at very concentrated conditions. The effect of WU-AX hydrolysis on the water-holding capacity, with a consequent release of water is then much larger than the effect of solubilizing the hydrolyzed WU-AX.

2.5 Conclusions

We studied the influence of low water content on wheat dough rheology down to 34% water content. Decreasing water contents increased Farinograph dough consistency and G', and decreased $J_{c,max}$ in a non-linear fashion of 1–2 orders of magnitude. This non-linear behavior occurred with a steady slope for the dough consistency and G', indicating that simply the amount of free water decreased at low water content with a resulting decline of the plasticizing effect of water.

Furthermore, we studied the possibility of reducing the dough water content while maintaining the rheological behavior of the dough, by addition of xylanase and a consequent release of water. The addition of Shearzyme with a higher selectivity of WE-AX and Grindamyl with a higher selectivity for WU-AX allowed water savings of 2–5% (on water basis). In total, xylanase addition mainly influenced the dough consistency, G', and $J_{c,max}$ similar to an increase in water content.

Acknowledgements

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CHAPTER 3

Shear-induced starch–gluten separation at very low water content aided by xylanases

Abstract

This study examines the influence of extremely low water content on shear-induced starch–gluten separation and how endoxylanases influence the separation by releasing water associated with arabinoxylan. Shearing was performed at a water content ranging from 34% to 43.5% (w/w). It was possible to concentrate gluten to 60% protein content in local gluten clusters and most of the concentration occurred within 5 min. Contrary to higher water concentrations, a water content <40% resulted in local separation of starch and gluten but no inward migration of the gluten. The addition of an endoxylanase with a higher selectivity for water-unextractable arabinoxylans and an endoxylanase with a higher selectivity for water-extractable arabinoxylans both resulted in a significant reduction in torque. The major influence of xylanase was the release of 3–5% water (on a water basis), allowing separation at even lower water content. Furthermore, no significant differences were found between the enzymes.

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3.1 Introduction

Wheat starch and gluten are separated industrially by processes such as the Martin or the batter process [129]. These processes consume copious amounts of water and often require 10–15 L of water per kg of dry matter [119]. Alternatively, starch and gluten can be separated under more concentrated conditions by exposing wheat dough to curvilinear shear flow. Peighambardoust et al. [103] and van der Zalm et al. [134] showed that gluten can be concentrated using an in-house cone-in-cone shearing device at a water content as low as 42% (on a total basis). In this device, wheat dough is placed between an upper fixed cone and a lower rotating cone. The gluten separates from the starch following a three-step mechanism: (1) gluten aggregates form on shearing; (2) then form bigger gluten clusters that deform due to the restriction of growth; (3) and migrate to the apex of the cone [133]. The result is a gluten-enriched region at the center of the cone with up to 60% protein content [134] and a gluten-depleted region at the outside of the cone. Demixing and migration have also been reported for other polymers [2, 34, 125]. Generally, demixing and migration of polymers can occur in curvilinear shear flows when the components of the mixture differ strongly in their viscoelastic properties [73], as is the case for gluten and starch.

Shear-induced starch–gluten separation under concentrated conditions has several advantages. More concentrated conditions generally result in larger system capacity and require less energy for dehydration if the final product needs to be dried [57]. Furthermore, more concentrated conditions require less water and produce less wastewater with inherently lower treatment and disposal costs. Also, in contrast to current industrial starch–gluten separation processes, water-soluble proteins are not lost during shear-induced separation.

Besides its main components, starch and gluten, wheat flour also contains other minor components, such as the pentosan arabinoxylan (AX). AX can be divided into water-extractable (WE-AX) and water-unextractable arabinoxylan (WU-AX) with concentrations of about 0.5% and 1.5% in wheat flour, respectively [129]. WE-AX and WU-AX have been reported to negatively influence gluten network formation and agglomeration [47, 144, 145]. This can be explained by the competition between AX and gluten for water; the water-holding capacities of

AX are up to 9.9 g water/g WU-AX and 6.3 g water/g WE-AX [28]. Furthermore, WE-AX and WU-AX may negatively influence gluten formation directly by cross-linking of gluten and AX via ferulic acid [144, 145] and by steric hindrance [47, 129].

AX can be hydrolyzed using endoxylanases. In conventional starch–gluten separation, WE-AX hydrolysis has been reported to improve the agglomeration of gluten, because the hydrolysis reduces the water-holding capacity of WE-AX and thus the dough viscosity [24, 45]. However, hydrolysis of WU-AX only results in improved agglomeration when WU-AX is severely hydrolyzed to a low molecular mass [47]. Mild hydrolysis solubilizes WU-AX, which initially increases dough viscosity.

AX hydrolysis is promising regarding shear-induced starch–gluten separation, because it releases water, and possible removal of AX from the gluten might improve gluten agglomeration. Theoretically, this allows a reduction in the overall water content towards even more concentrated conditions. The approach of using reduced water content with the help of xylanases has been used before in pasta production [14]. In this study, we investigate the combination of addition of endoxylanase and extremely low water content during shear-induced starch–gluten separation using an endoxylanase with a higher selectivity for WU-AX and an endoxylanase with a higher selectivity for WE-AX.

3.2 Materials & Methods

3.2.1 Materials

Soissons wheat flour from a single wheat cultivar was obtained from Meneba (Rotterdam, The Netherlands). The water content was 14.4±0.4% and the crude protein content was 10.7±0.4% (N×5.7) (both by weight). Vital wheat gluten (Roquette) with 72.8±1.0% (N×5.7) crude protein content and 8.0±0.6% water content was obtained from Barentz BV (Hoofddorp, The Netherlands). Shearzyme 500L was kindly provided by Novozymes A/S (Bagsværd, Denmark). Shearzyme is a monocomponent endoxylanase from *Aspergillus aculeatus* with a greater selectivity for WE-AX [45]. Grindamyl POWERBake 950 was kindly supplied by Danisco A/S (Brabrand, Denmark). Grindamyl POWERBake 950 is

an endoxylanase from *Bacillus subtilis* with a greater selectivity for WU-AX [109]. The xylanases are referred to as Shearzyme and Grindamyl in the following. Tap water was used in all experiments.

3.2.2 Water and enzyme contents

Water contents of 34%, 36%, 38%, 39.5%, and 43.5% (based on total weight) were used in this study. This corresponds to Farinograph water absorptions of 29.7%, 33.8%, 38.1%, 41.5%, and 51.5% (based on 14.4% flour moisture). The upper value of 43.5% water was chosen because it represents the standard water content for shear-induced starch–gluten separation [134]. The lower value of 34% water was chosen because no full hydration of dough occurs during shearing at lower water content; some material remains powdery. It was assumed that wheat flour consists of 0.5% WE-AX and 1.5% WU-AX [129]. Enzyme-to-substrate ratios of 1:6 (0.17 g enzyme/g WE-AX) for Shearzyme and 1:9 (0.11 g enzyme/g WU-AX) for Grindamyl were used to ensure high xylanase activity and limit inhibition. The xylanase was always added to the water before adding to the wheat flour.

3.2.3 Shearing process

Shear experiments were conducted using a cone-in-cone shearing device developed in house (Wageningen University, The Netherlands), as described in detail previously [86, 103]. The shear cell was connected to a Do-corder E330 equipped with a PL2100 computer interface and controller unit (Brabender, Duisburg, Germany) to measure torque and temperature. The shear cell was heated to 40±1 °C using a circulating water flow. A sample of 100 g was prepared for each experiment with varying water content. In addition, 2% salt (on a flour basis) was added to enhance the separation [135]. The sample was loaded onto the lower cone. The upper cone was closed and a pressure of 310±10 kPa was applied. The sample was then sheared at 5 rpm for 5 min, which allowed better sample mixing. The sample was then sheared at 15 rpm for 25 min, except for the experiments on the influence of time on the protein content, when the rotational speed was 5 rpm. After the treatment, the samples

were photographed using a Canon EOS 30D camera. Then, half of the sample was frozen for microscopy analysis. From the other half of the sample, gluten clusters were gently removed, frozen, freeze-dried, and used to determine the protein content. Shear cell experiments were performed in triplicate unless otherwise stated. The shearing experiment depicted in Fig. 3.1a was performed using another shear cell cone, but with the same geometry as the shear cell cone used for the other experiments. Nevertheless, the separation was independent of the shear cell cone, as can be seen when comparing Fig. 3.1a and Fig. 3.4d.

3.2.4 Coloring of gluten clusters

To visualize the migration behavior of gluten, 12 g of vital wheat gluten, 8 g of wheat flour and 10 g of water were mixed and gluten clusters with approximately 55% protein (dry basis) were formed. These clusters were stained with 0.3% rhodamine B in 2-methoxyethanol for 1 h and subsequently rinsed with clean 2-methoxyethanol. The stained gluten clusters were placed in the middle of a fresh sample, which had been sheared for 30 s (Fig. 3.4a). Then, shearing was performed at 5 rpm.

3.2.5 Protein content

The protein content (N×5.7) of the gluten clusters was determined using the Dumas method (N analyzer FlashEA 1112 series, Thermo Scientific, Interscience). Methionine was used as a standard.

3.2.6 Microscopy

Samples were sectioned at 70 μm on a cryotome (Microm, Heidelberg, Germany) and stained with 0.1% rhodamine B in 2-methoxyethanol for 1 h. The stained sections were examined under light and polarized light using a Nikon Eclipse 80i microscope equipped with a 100-W mercury lamp and a 10× objective with 0.5 numerical aperture.

3.2.7 Statistical analysis

A two-factor analysis of variance (ANOVA) was performed to test significant differences ($\alpha = 0.05$) in shear cell torque and protein content for different water contents and xylanase addition using SPSS version 19.0. Data were normally distributed based on the Shapiro–Wilk test. Logarithmic torque values were used to test the significance. Torque trend lines as a function of the water content were fitted using a simple power law fit: $a \times x^n$. However, the primary goal was to examine the non-linear relationship between torque and water content and not to obtain the most suitable fit.

3.3 Results & Discussion

3.3.1 Visual appearance of dough

Shear-induced starch–gluten separation has been introduced at water contents from 42% to 46% [103, 106, 134]. At these water contents, shearing of wheat dough results in local gluten aggregates, which subsequently grow and migrate to the the appex of the cone. In Fig. 3.1a, the separation was reproduced at 43.5% water content. In industrial wheat separation, xylanases can be used to

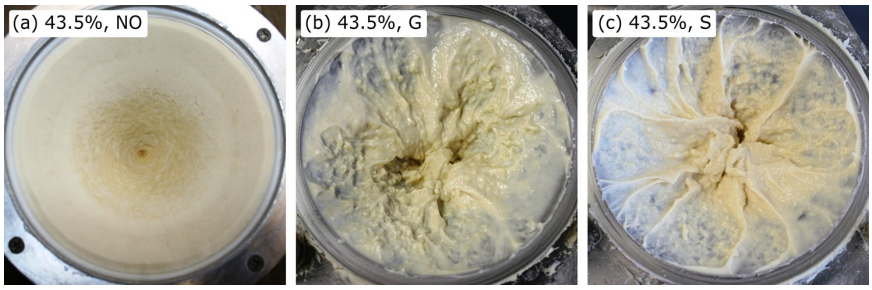

Fig. 3.1: Visual appearance of the dough just after shearing at 43.5% water content. The legends indicate the water content used. NO, no xylanase; G, Grindamyl added; S, Shearzyme added.

facilitate the separation by releasing water. Therefore, the influence of Grindamyl and Shearzyme on shear-induced starch–gluten separation was investigated (Fig. 3.1b, c). However, separation ceased to occur on addition of both xylanases. Water was visibly released and a slack and sticky dough was obtained. The

release of water had a similar effect as adding too much water to the shearing process, which was reported to have a negative influence on separation [134].

As a result of Fig. 3.1, we investigated whether separation could be induced at a lower water content after addition of enzyme. Fig. 3.2 shows the influence of water content from 34% to 38% and addition of xylanase on the visual appearance of dough after shearing. The separation process changed at lower

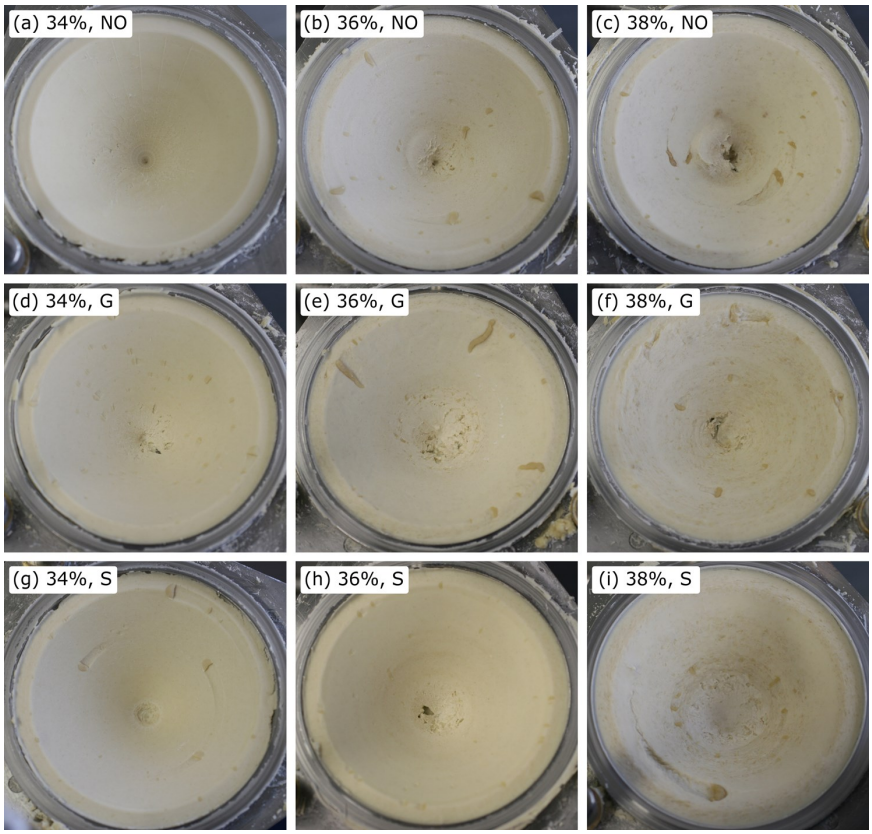

Fig. 3.2: Visual appearance of the dough just after shearing at 34–38% water content. The legends indicate the water content used. NO, no xylanase; G, Grindamyl added; S, Shearzyme added.

water content. The formation of local gluten clusters was observed without xylanases at water contents of 36% and 38% (Fig. 3.2b, c). However, these clusters no longer migrated towards the apex of the cone. At 34% water content, no gluten clusters were visible (Fig. 3.2a). The addition of xylanases also resulted in the formation of local gluten clusters without migration towards the apex of

the cone (Fig. 3.2d–i). Only small differences were observed between Grindamyl and Shearzyme, which are presumably not of technological relevance.

3.3.2 Separation mechanism at low water content

The question arises why gluten cluster migration ceases at low water content, independent of the addition of xylanase. At a water content above 40%, gluten aggregation mainly depends on the shear rate, whereas the migration of the clusters is mainly influenced by the shear stress [133, 134]. Too high shear stresses have been shown to result in the breakup of gluten aggregates [106, 133, 134]. Thus, on the one hand, low shear stresses (achieved by low shear rates) are necessary to allow the formation of gluten clusters that grow large enough to be able to deform and migrate. On the other hand, high stresses are necessary to deform these gluten aggregates, which is a prerequisite for migration.

Decreasing the water content results in larger shear stresses, which is equivalent to an increase in torque in the shearing device (Fig. 3.3). Fig. 3.3

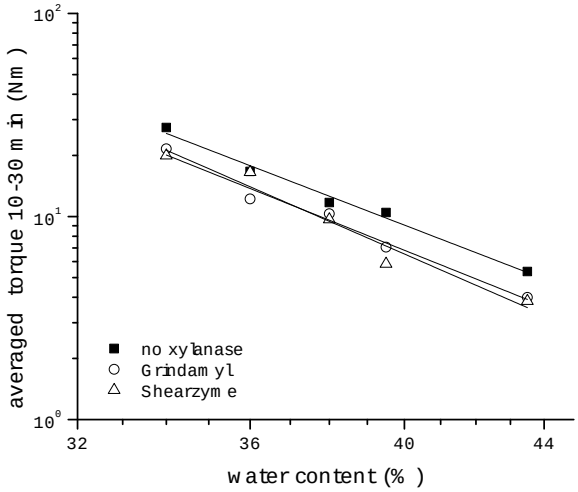

Fig. 3.3: Shear cell torque. Shearing at 15 rpm and 40 °C. Average torque within a timeframe of 10–30 min as a function of the water content. Trend lines were derived using a simple power law fit: $a \times x^n$. $R^2 > 0.96$ for all fits. Power law parameters a and n were 0.061, -5.6 for no xylanase, 0.048, -5.6 for addition of Grindamyl, and 0.030, -6.1 for addition of Shearzyme.

shows the average torques over 10–30 min shearing time, which is enough time to see the effect of arabinoxylan hydrolysis. Decreasing the water content from

43.5% to 34% resulted in a fourfold, non-linear increase in torque. The addition of Shearzyme and Grindamyl significantly reduced the torque, which is in line with Fig. 3.1 and Fig. 3.2. No significant difference in torque was found between Shearzyme and Grindamyl.

At first sight, it seems contradictory that higher shear stresses, indicated by higher torques, facilitate the formation of relatively large gluten clusters without breakage, but impede migration. Once local gluten aggregates form, they can be considered as embedded in a matrix mainly consisting of gluten (although consequently lower in content than in the initial dough) and starch. This matrix exerts a stress on the gluten aggregates that is larger with decreasing water content. However, the gluten aggregates formed also show greater resistance to extension with decreasing water content. The increase in resistance to extension with decreasing water content was demonstrated in creep tests, although at higher water contents [63, 88]. Therefore, the formation of gluten clusters can occur at water contents below 40%, remarkably, without breakage of the clusters.

But why does migration fail to appear? At higher water contents from 42% to 46%, the formation of gluten clusters is followed by deformation of these clusters and subsequent migration towards the apex of the cone [133]. However, the clusters only start deforming once their growth is restricted; the space between the upper and the lower shear cell cone determines the maximum cluster size. Consequently, van der Zalm et al. [133] showed that local gluten clusters without migration also emerged at water contents >40% when the angle between the upper and the lower cone was changed to 7.5° compared with 2.5° in this study. The gap between the two shear cell cones is considerably higher at larger cone angles, especially at the outside of the cone, and gluten clusters need to grow considerably larger before their growth is restricted and they start to extend. However, the formation of local gluten clusters at an angle of 2.5° without migration was never reported at higher water contents. Obviously, something is hindering the clusters from migrating at water contents <40%. In this study, starch–gluten separation was conducted using extremely water-depleted doughs, with the dough and especially the starch densely packed. It seems that gluten clusters cannot migrate inwards through such a dense phase. To test this assumption, we added rhodamine B-stained gluten clusters

at 38% and 43.5% water content at the beginning of the shearing process and photographed the distribution of these clusters after 5 and 15 min (Fig. 3.4). At

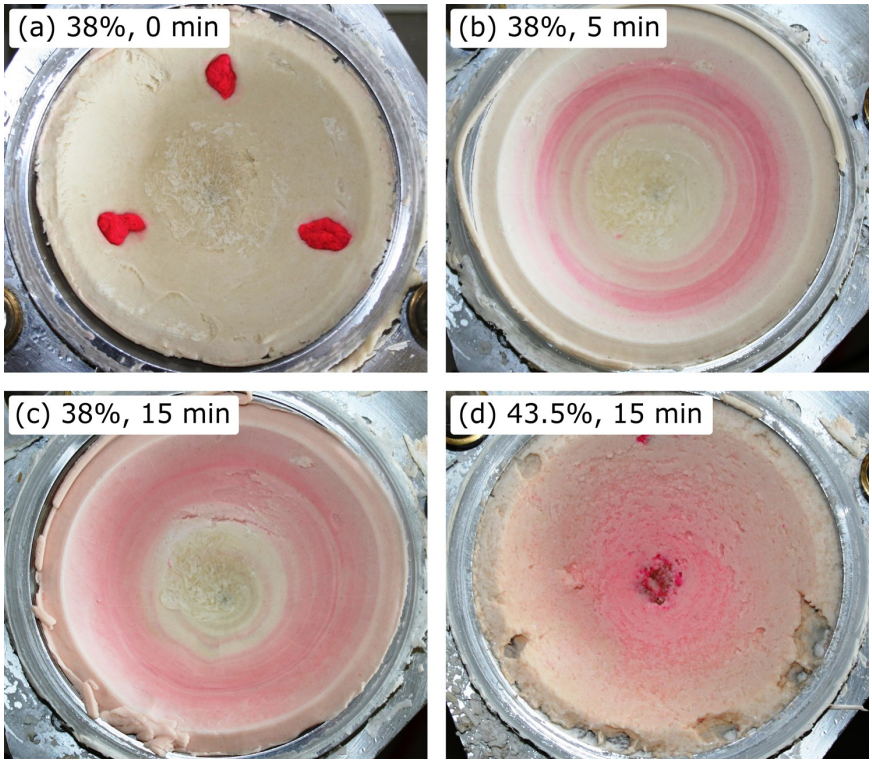

Fig. 3.4: Visual appearance of migration of gluten clusters stained with rhodamine B at 38% and 43.5% water content without addition of xylanase. The legend indicates the water content and the shearing time.

38% water content, the added gluten clusters broke within 5 min and a colored ring in the middle of the sample could be seen. After 15 min, the sample was further mixed. However, only the outer parts of the sample mixed. There was no exchange between the inner 30% of the cone and the rest. For comparison, the same experiment was performed at 43.5% water content (Fig. 3.4d). Here, inward migration of gluten towards the apex of the cone could be clearly seen. Also the protein content increased towards the apex of the cone, as shown by the more intense color.

One explanation for the absence of any mixing on the inside of the cone at low water content is the development of the shear rate along the cone. It has

been reasoned that starch granules cause the cones to not completely touch at the apex, which introduces an inhomogeneous shear field in the cone [133]; the radial shear rate is no longer constant, but gradually increases from zero at the center of the cone towards higher shear rates at the outside of the cone. Hence, presumably no mixing occurred on the inside of the cone because of the low shear rates and the dense and water-depleted dough. This explains why the gluten clusters cannot migrate through this part of the dough.

3.3.3 Protein content

Fig. 3.5 shows the influence of the water content and addition of xylanase on the protein content of the gluten clusters. The protein content is plotted as a function of the torque because the addition of xylanase reduced the viscosity of the dough and thus the torque. The addition of Shearzyme and Grindamyl

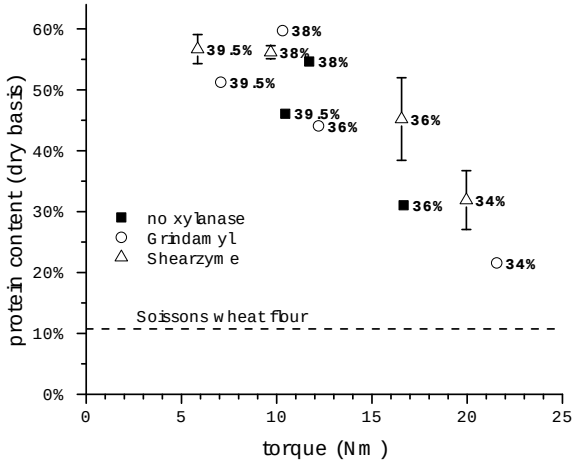

Fig. 3.5: Influence of the shear cell torque on the protein content. The legend indicates the xylanase used. The standard deviation of triplicates is given for Shearzyme. The shear cell experiments using Grindamyl and without addition of xylanase were performed in duplicate. The water content during shearing is displayed next to the symbols. The dashed line represents the protein content of Soissons wheat flour.

resulted in significantly higher protein contents compared with the xylanase-free sample at all water contents, due to the reduced viscosity; when testing the influence of both xylanases on the protein content as a function of the torque instead of the water content, no distinct differences were found compared with

the xylanase-free samples. No significant differences were observed between Shearzyme and Grindamyl regarding the protein content. The highest protein content in this study was found with 60% (dry basis) protein, which is almost as high as in commercial vital wheat gluten. For instance, the vital wheat gluten used for staining the gluten clusters in this study had a protein content of 73% (N×5.7). Decreasing the water content resulted in a decrease in the protein content. A lower water content seems to hamper the formation of the gluten network and clusters lower in protein break more easily. This also explains why no gluten cluster formation was observed at 34% water content without the addition of xylanase.

Fig. 3.6 depicts the influence of the shearing time on the protein content of the gluten clusters. Longer shearing times resulted in increased protein content.

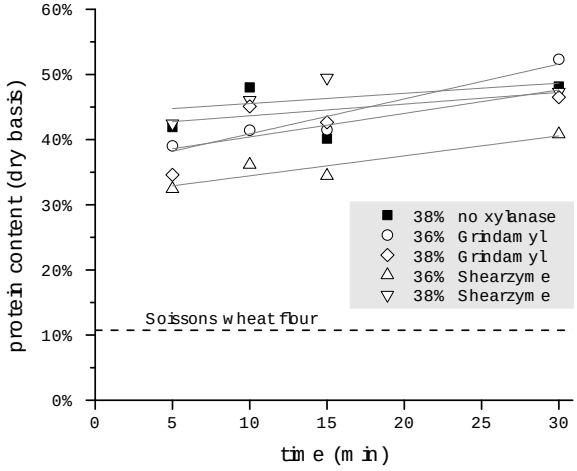

Fig. 3.6: Influence of the shearing time on the protein content. The legend indicates the water content and xylanase used. Gray lines are linear trend lines. The dashed line represents the protein content of Soissons wheat flour. Results are from single experiments.

However, most of the increase in protein content occurred in the first 5 min, showing that gluten aggregation is a relatively quick process. On average, 88% of the total increase in protein content was achieved within 5 min (relative to the protein content after 30 min). Furthermore, the protein content at 38% water was higher on average than at 36%, which is in agreement with Fig. 3.5.

The highest protein contents in this study ranged between 55% and 60% (Fig. 3.5). This is in agreement with previous shear cell studies at higher water contents [103, 134]. In these studies, several process parameters such as the water content, the temperature, the rotation rate, and the processing time were changed. These experiments suggest that there is a threshold above which gluten cannot be concentrated further when inducing shear under concentrated conditions.

The remaining 40% of the gluten clusters with the highest protein content consists mainly of starch. To get more insight into the structure of the gluten clusters, we stained the gluten phase using rhodamine B and made use of the birefringence of starch granules under polarized light (Fig. 3.7). Fig. 3.7a–d compares the structure of gluten clusters at 38% water content with Grindamyl, Shearzyme and without addition of xylanase and at 36% water content with addition of Shearzyme. The gluten clusters mainly consisted of a gluten network with starch granules embedded in this network. Starch has been reported to be physically entrapped in the gluten network [112, 118]. Nevertheless, we always observed sections inside the clusters that were high in starch and sections without starch. Therefore, starch and gluten also separated on a small scale. Furthermore, we observed no major differences in the microscopic appearance of the gluten clusters when xylanase was added.

The starch distribution in Fig. 3.7a–d provides some information on the maximum attainable protein content of local gluten clusters. The starch granules are embedded in the gluten network and can only be removed through deformation of the gluten clusters by applying shear stress. However, the gluten clusters themselves are embedded in the dough phase, which mainly consists of starch. Therefore, it is likely that shearing of the gluten clusters results in equilibrium, whereby starch granules are removed from the clusters but new starch granules are also embedded again. This is supported by the fact that the protein content was rather independent of the shearing time after 5 min. Overall, this would explain why the highest protein content during shear-induced starch–gluten separation has not yet exceeded 60%.

Fig. 3.7e gives an impression of the starch distribution in a whole gluten cluster. Fig. 3.7f shows the effect of shearing on a dough at 34% water content

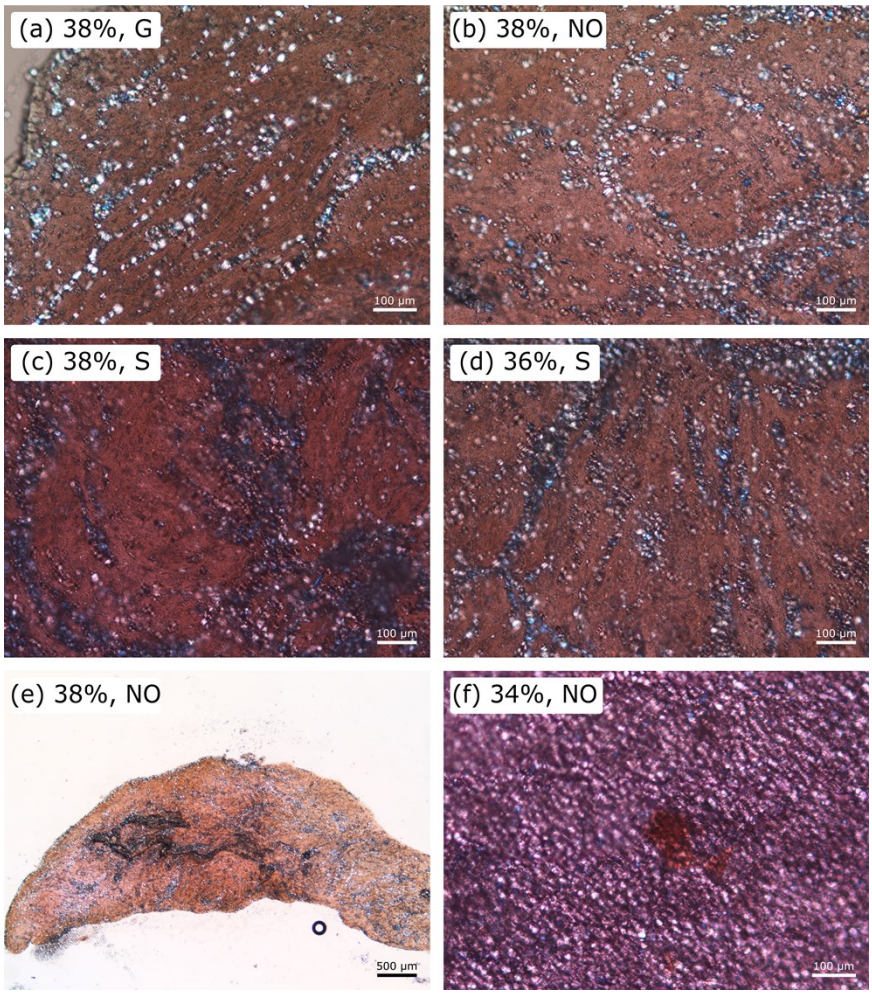

Fig. 3.7: Gluten clusters under light and polarized light. Starch granules illuminate under polarized light. Rhodamine B-stained gluten appears red. The legends indicate the water content used. NO, no xylanase; G, Grindamyl added; S, Shearzyme added. Image (e) was taken using a 2× objective. All other images were taken using a 10× objective.

without addition of xylanase. As mentioned earlier, no visible separation was observed here. Nevertheless, some small gluten sections were found using the microscope.

3.3.4 Influence of xylanase

We studied the influence of Shearzyme and Grindamyl on shear-induced starch–gluten separation to answer two questions: (1) Does AX hydrolysis release water and does the consequent reduction in viscosity facilitate the separation process? (2) Does a possible removal of any direct interaction between gluten and AX improve gluten agglomeration and result in higher protein content in the gluten clusters? The release of water was clearly visible (Fig. 3.1 and Fig. 3.2) and confirmed by changes in torque (Fig. 3.3). This is in line with Wang et al. [144, 145]. The consequent reduced viscosities allowed separation at even lower water content (Fig. 3.2). However, no strong evidence was found that Shearzyme and Grindamyl influenced the formation of gluten clusters other than the release of water. Although small differences in the visual appearance of the gluten clusters were observed (Fig. 3.2), we did not find any increase in protein content with the addition of xylanase. The increase in protein content at a constant water content (e.g. 38%) can be explained by the reduced torque (Fig. 3.5).

Even though Shearzyme and Grindamyl behaved similarly in many aspects, Shearzyme has a greater selectivity for WE-AX and Grindamyl has a greater selectivity for WU-AX [45, 109]. As mentioned earlier, mild hydrolysis of WU-AX results in the solubilization of WU-AX and increased viscosity. On prolonged hydrolysis, the molecular weight of this solubilized WU-AX reduces further, which reduces the viscosity again. Therefore, higher torque values are theoretically expected in the case of WU-AX hydrolysis using Grindamyl, at least at the beginning of the shearing process. However, the averaged torque within 10–30 min was independent of the xylanase used (Fig. 3.3), just like the torque at the beginning of the reaction (not shown). The overall visual appearance of the dough samples was rather independent of the type of enzyme used (Fig. 3.2), and no significant difference in the protein content of the clusters was found (Fig. 3.5). This differs from conventional starch–gluten separation, where the hydrolysis of WE-AX improves the separation to a much larger extent than hydrolysis of WU-AX [24, 45], because of the opposite influence of WU-AX and WE-AX hydrolysis on the viscosity. One reason for this difference could be that starch–gluten separation in the shear cell was performed at very concentrated conditions. Thus, the effect of WU-AX hydrolysis on the water-holding capacity,

with consequent reductions in viscosity and torque (Fig. 3.3), was much larger than the effect of solubilizing WU-AX, if solubilization even occurred at this low water content.

3.3.5 Concluding remarks on practical implications of xylanases

We investigated the modification of shear-induced starch–gluten separation from a wheat dough by enzymatically hydrolyzing arabinoxylans at very low water content. For this, a xylanase with a higher specificity for water-unextractable arabinoxylan (Grindamyl) and another xylanase with a higher specificity for water-extractable arabinoxylan (Shearzyme) were evaluated. It was shown that enzyme addition at increased water content negatively influenced the separation. Therefore, we concluded that shear-induced separation only benefits from addition of xylanase at a water content <40%.

The combined effects of the water content and hydrolysis of arabinoxylan on the torque can be described using a power law equation (Fig. 3.3). This can be used to estimate possible water reductions by using xylanase in starch–gluten separation at very low water content. Shearzyme allowed water reductions of 3.2–4.8% (from 34% to 43% water) and Grindamyl a water reduction of 4.1% (all reductions on a water basis).

The use of either xylanase led to a significant increase in the protein content of the clusters of around 5.6% (on a dry basis) when using 36–38% water in the dough. This could be attributed to the release of water by arabinoxylan hydrolysis, and the consequent reduction in the viscosity of the dough.

Although gluten clusters can be formed at water contents <40%, it was not possible for the gluten clusters to migrate towards the apex of the cone. However, migration and large-scale separation of starch and gluten are not necessarily required to separate starch and gluten. Local separation might be an interesting first step in separation of starch and gluten. In a next process step, the gluten and the starch could be further separated. We generally observed that the gluten clusters showed higher resistance to disruption and breakage than the gluten-depleted regions in the wet and the dry state. Hence, the gluten clusters could be separated by mechanical force. Here, dehydration might ease the separation.

Alternatively, dry fractionation might be a possibility to further separate the dried shear cell samples [119].

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CHAPTER 4

Influence of high solid concentrations on enzymatic wheat gluten hydrolysis and resulting functional properties

Abstract

Enzymatic hydrolysis at increased solid concentrations is beneficial with regard to energy and water consumption. This study examines the influence of the solid concentration on the enzymatic hydrolysis of wheat gluten and the resulting functional properties of the hydrolysate. Wheat gluten was mildly hydrolyzed at a solid concentration varying from 10% to 60% to degrees of hydrolysis (DH%) ranging from 3.2% to 10.2%. The gluten was susceptible to hydrolysis at all solid concentrations but the hydrolysis rate was influenced by increasing solid concentrations. Size-exclusion high-performance liquid chromatography revealed an increase in the ratio of peptides with a molecular mass >25 kDa for solid concentrations of 40% and 60%. The water solubility increased on hydrolysis and was independent of the solid concentration during proteolysis. The foam stability was not influenced by the solid concentration at low DH%. At DH% higher than 8%, high solid concentrations increased the foam stability, which might be related to the presence of more peptides with a molecular mass >25 kDa. In addition, we found increased reactor productivity. The results show the potential of hydrolyzing wheat gluten at high solid concentrations, which could lead to large savings for water and energy use when applied industrially.

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4.1 Introduction

Wheat gluten, the major protein of wheat, is a valuable ingredient in the breadmaking industry. Moreover, it is a plant protein, renewable, and abundantly available at moderate prices. Although native wheat gluten is an excellent bread improver, its further application in the food industry is limited. Native wheat gluten is scarcely water-soluble close to its isoelectric point at pH 6–7. In addition, it has low emulsifying activity and does not produce steady foams [111]. One possibility to overcome these limitations is to hydrolyze wheat gluten. Mild hydrolysis at low solid concentrations has been demonstrated to enhance the water solubility at neutral pH [70, 143]. Furthermore, foam capacity increases and foam stability decreases [80, 92, 142].

Wheat gluten can be hydrolyzed either chemically at high or low pH or by means of enzymes. Acid hydrolysis can be applied easily and is the most commonly used method to hydrolyze gluten in industry [22]. However, acid hydrolysis can lead to undesired side reactions, may result in the degradation of essential amino acids [33], and can lead to products with too much salt [22]. Enzymatic hydrolysis is a milder and more specific process with few undesirable side reactions. Furthermore, enzymes are biodegradable and can remain in the final product when inactivated.

Protein hydrolysis is usually performed at low solid concentrations. The highest solid concentrations used for enzymatic wheat gluten hydrolysis at neutral pH range from 13% to 22% [22, 84, 117]. At low solid concentrations, wheat gluten forms a fluid-like dispersion with all the gluten dispersed in the liquid. For higher solid concentrations, wheat gluten forms a dough with high viscosity and elasticity. This increased viscosity may lead to insufficient mixing and can be highly energy consuming in conventional stirring tanks [72]. Therefore, low solid concentrations are preferred industrially because of the manageability of the system. In addition, water plays an important role as the solvent for the enzyme and as the transport medium for the substrate and the product; thus, a reduced water concentration might lead to diffusional limitations. Water can also act as a reactant and this role can be influenced by reduced water activity at higher solid concentrations.

The use of a higher concentration, however, has several advantages. First, it results in larger system capacity either by a higher throughput in existing equipment or by a reduced system volume [131]. Second, less energy is required to heat and cool the slurry during the hydrolysis reaction and the consequent drying of the product, thus reducing the energy usage and costs for processing. Furthermore, downstream processing is facilitated by higher product concentrations [94]. Third, a high solid concentration during hydrolysis requires less water and produces less wastewater with inherently lower treatment and disposal costs.

Few studies have investigated enzymatic protein hydrolysis at high solid concentrations. Krause et al. [71] examined the chymosin-catalyzed hydrolysis of β-casein at 1% and 50% (w/v) dry mass concentration. Hydrolysis was strongly limited at 50% (w/v), which was explained by the competition between the enzyme and the substrate for the reduced amount of water available. Enzymatic hydrolysis of whey protein isolate and egg white protein has been investigated for concentrations up to 30% (w/v) and 35% (w/v), respectively [16, 113]. In both cases, hydrolysis occurred at higher substrate concentrations but a lower hydrolysis rate was observed with increasing substrate concentration. Several publications report the same effect for the hydrolysis of biomass in biofuel production [72]. The decreased hydrolysis rate appears to be an intrinsic effect of increasing solid concentrations and its cause is not yet fully understood. To the best of our knowledge, wheat gluten hydrolysis at high solid concentration has never been reported in the literature. Moreover, for proteins in general, the influence of the solid concentration during proteolysis on the functional properties of the resulting hydrolysates has not been described in the literature so far.

In this study, enzymatic wheat gluten hydrolysis was examined using solid concentrations ranging from 10% to 60%. The aim was to study whether the solid concentration during proteolysis affects the functional properties of the resulting wheat gluten hydrolysates. Solubility and foam stability tests were carried out and the molecular mass distribution was evaluated. The potential reductions in the use of water and energy are discussed.

4.2 Materials & Methods

4.2.1 Materials

Vital wheat gluten (Roquette) with 72.8±1.0% crude protein content (N×5.7) and 8.0% water content (all by weight) was obtained from Barentz BV, Hoofddorp, The Netherlands. Protease from *Aspergillus oryzae* (Flavourzyme 500 LAPU/g) from Novozymes was purchased from Sigma-Aldrich, Germany. Flavourzyme contains a mixture of endo- and exoproteases [38]. Milli-Q water was used in all experiments.

4.2.2 Hydrolysis reaction

The hydrolysis reactions were carried out using a 200-mL double-walled glass vessel connected to a water bath. Each experiment was conducted using a total mass of 100 g, varying the solid concentrations from 10% to 60% (w/w), and an enzyme-to-substrate ratio of 1:100 (w/w). The hydrolysis temperature was 50 °C. Before hydrolysis, the vital wheat gluten powder was allowed to stand for 5 min at the reaction temperature. The enzyme was first mixed with the water before adding it to the substrate. The double-walled glass vessel was closed with a clasp to avoid evaporation of water and stirred using an overhead stirrer at 40 rpm. The reaction was conducted under floating pH conditions. After hydrolysis, all samples were inactivated for 10 min at 95 °C in a water bath. The samples were then cooled down, frozen, and freeze dried. The freeze-dried samples were ground and stored for further analysis.

4.2.3 Degree of hydrolysis (DH%)

The degree of hydrolysis (DH%) was measured by the *o*-phthaldialdehyde (OPA) method according to Nielsen et al. [99], with minor modifications: 1.25 mg/mL wheat gluten hydrolysate was suspended in 12.5 mM sodium tetraborate decahydrate plus 2% (w/w) sodium dodecyl sulfate (SDS) according to Wang et al. [142], mixed for 60 min and then centrifuged at 3900 × *g* for 15 min. The resulting supernatant was used for analysis. The DH% was calculated using the

following formula:

$$DH\% = \frac{(Serine\text{-}NH_2 - \beta)/\alpha}{h_{tot}} \tag{4.1}$$

where

$$Serine\text{-}NH_2 = \frac{A_{hydr} - A_{OPA}}{protein\left[\frac{g}{l}\right]} \cdot \frac{mM\ serine}{A_{serine} - A_{OPA}} \tag{4.2}$$

and $Serine\text{-}NH_2$ is meqv serine-NH_2 per gram of protein. A_{hydr} is the absorbance of the wheat gluten hydrolysate sample, A_{OPA} is the absorbance of the blank OPA reagent, and A_{serine} is the absorbance of the serine standard. $h_{tot} = 8.3$ and $\alpha = 1$ [1]. A value of 0.16 was measured for β. The calculated DH% was the mean of four determinations.

4.2.4 Process conditions

Table 4.1 shows the process conditions used for the various wheat gluten hydrolyses. Hydrolysates were obtained in a DH% range from 3.2% to 10.2%. We only measured the DH% after completion of the hydrolysis reaction. Online DH% measurement methods, such as the pH-stat method, were not suitable for higher solid concentrations, because most native samples had the consistency of a dough.

4.2.5 Size-exclusion high-performance liquid chromatography

Wheat gluten hydrolysate (2.5 mg/mL) was suspended in 12.5 mM sodium tetraborate decahydrate plus 2% (w/w) SDS, stirred overnight, and then centrifuged at 3900 × g for 15 min. The resulting supernatant was injected onto a TSKGel G2000 SWXL (300×7.8 mm) column and eluted with 70% / 30% / 0.1% water / acetonitrile / trifluoroacetic acid at a flow rate of 1.0 mL/min and detected at a wavelength of 214 nm. All experiments were performed in duplicate. The molecular mass distribution was calculated according to molecular mass markers based on carbonic anhydrase (29,000 Da), α-lactalbumin (14,100 Da), aprotinin (6510 Da), insulin (5700 Da), bacitracin (1420 Da), and phenylalanine (165 Da). The standard calibration curve was: $log_{10}MW = 7.1268 - 0.452t_r$ where t_r is the retention time. Based on the

Table 4.1: Sampling parameters: solid concentration, hydrolysis time, and DH%; errors represent standard deviation of DH% measurements.

Solid concentration	Hydrolysis time (min)	DH%
10%	45	3.2±0.2%
10%	55	4.4±0.2%
10%	105	6.2±0.6%
10%	130	8.4±0.3%
10%	155	10.2±0.4%
20%	60	3.0±0.1%
20%	75	4.9±0.2%
20%	165	9.1±0.3%
40%	60	4.5±0.2%
40%	150	6.7±0.4%
40%	200	9.4±0.2%
60%	110	3.8±0.2%
60%	145	5.5±0.2%
60%	360	8.8±0.3%
60%	420	9.2±0.2%

molecular mass, the results were divided into the following classes: <2 kDa, 2–10 kDa, 10–25 kDa, and >25 kDa.

4.2.6 Solubility

Wheat gluten hydrolysate (25 mg/mL) was suspended in water and mixed for 60 min at ambient temperature using a Heidolph MultiReax at 1500 rpm and then centrifuged at 3900 × g for 15 min. The resulting supernatant and pellet were freeze-dried and weighed. The nitrogen content of both freeze-dried samples was determined using the Dumas method (N analyzer FlashEA 1112 series, Thermo Scientific, Interscience) with methionine as a standard. The nitrogen solubility index (NSI) was taken as the fraction of soluble nitrogen to total nitrogen. All experiments were performed in duplicate. It has been shown that wheat gluten solubility is independent of the pH within a pH range of 4–10 for DH% greater than 5% [70] and scarcely affected within a pH range of 6–8 for DH% of 1.3–1.9% [23]. Therefore, the pH was not adjusted during the solubility experiments. Nevertheless, the pH remained between 6 and 7 for all solubility experiments carried out.

4.2.7 Foam stability

Wheat gluten hydrolysate was suspended in water at ambient temperature, mixed for 60 min using a Heidolph MultiReax at 1500 rpm and then centrifuged at 3900 × g for 15 min. Thirty milliliters of the resulting supernatant were poured into a glass column (12 cm in height, 6 cm in diameter) with a porous metal disk (20–30 μm pore diameter). A foam height of 12 cm was created by bubbling nitrogen at a constant rate of 400 mL/min into the hydrolysate solution. The foam height was recorded visually after 5 and 15 min. Two sets of experiments were conducted to determine the foam stability. First, 4.25 mg/mL wheat gluten hydrolysate was suspended in water. Therefore, the amount of soluble protein during the foaming measurements varied for different DH% values. Because foam stability is mainly determined by soluble protein, a second set of foam stability tests was performed using a similar soluble protein concentration. Therefore, wheat gluten hydrolysate was suspended in water in such an amount to achieve a concentration of water-soluble protein of 2.0 mg/mL. The foam stability results are means of four determinations.

4.3 Results & Discussion

4.3.1 Influence of the solid concentration on the hydrolysis reaction

The solid concentration affects the state of wheat gluten–water mixtures to a significant extent. Fig. 4.1 shows these differences for native and hydrolyzed wheat gluten at solid concentrations of 10%, 40%, and 60%. Native wheat gluten formed a suspension in the presence of aggregated gluten lumps for a solid concentration of 10%; a solid concentration of 40% and 60% resulted in a dough (Fig. 4.1a–c). At 60%, parts of the powder remained dry. It should be emphasized that native wheat gluten is transformed from a suspension state to a dough state at a solid concentration range of 10–40%. At 20%, a suspension was still formed but the gluten lumps showed a high tendency to aggregate further. Nevertheless, these aggregates could be disrupted easily compared with breaking wheat gluten dough at higher solid concentrations. At solid concentrations of

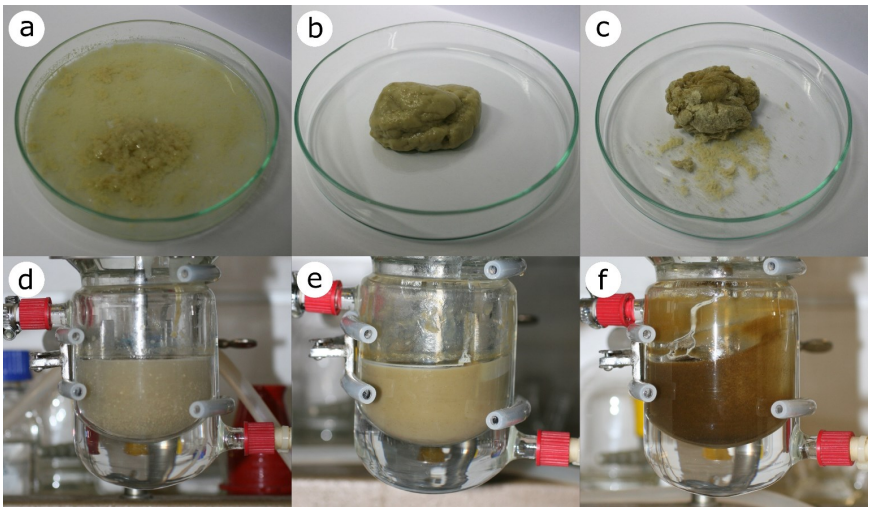

Fig. 4.1: Influence of the solid concentration and enzymatic hydrolysis on the visual appearance of wheat gluten. (a–c) Native wheat gluten with a solid concentration of 10%, 40%, and 60%, respectively. (d–f) Hydrolyzed wheat gluten with a solid concentration of 10%, 40%, and 60%, and DH% of 8.4%, 9.4%, and 9.2%, respectively.

30% and 35%, native wheat gluten started forming a dough. However, not all the water was held by the solid phase and some free water remained visible. No free water was observed at a solid concentration of 40%. This is in accordance with the literature where a water-holding capacity of native vital wheat gluten of 1.45 g water/g solid is reported [19]. In this study, a high solid concentration is defined as the solid concentration at which native vital wheat gluten forms a dough and all the water is held by the solid phase. This applied for solid concentrations of 40% and higher.

The transition from a suspension to a dough also resulted in an increase in viscosity. This increased viscosity impeded the mixing of the system during hydrolysis. Moreover, the smaller part of the solid that remained dry at 60% solid concentration (Fig. 4.1c) was inaccessible to the enzyme at the beginning of the hydrolysis reaction. Nevertheless, the wheat gluten remained susceptible to enzymatic hydrolysis at all solid concentrations investigated despite the fact that the initial conditions changed. Once the hydrolysis started, liquefaction occurred shortly afterwards. Within 20 min, most of the initial solid phase dispersed in the water phase and a homogenous slurry was formed at all solid concentrations.

Furthermore, an insoluble lump, most likely consisting of starch, remained after termination of the hydrolysis reaction for solid concentrations below 60%. The hydrolysis of wheat gluten was accompanied by a decrease in viscosity. This decrease in viscosity has also been reported in the literature [7]. Fig. 4.1d–f shows the changes in appearance on enzymatic hydrolysis for the different solid concentrations in a DH% range of 8.4–9.4%.

Table 4.1 shows the influence of the hydrolysis time on the DH% for different solid concentrations. A DH% of 4.5% was reached within 75 min for solid concentrations of 10%, 20%, and 40%; the differences in reaction time were minor. For higher DH%, these differences became progressively greater. For a solid concentration of 60%, a strong increase in reaction time was observed for all DH%. Compared with a solid concentration of 10%, a more than twofold increase in reaction time was required to achieve a DH% of 3.8%, and a threefold increase to achieve a DH% of 9.2%. Nevertheless, the increase in reaction time at 60% compared with 10% is in contrast to a sixfold increase in hydrolyzed substrate.

4.3.2 Molecular mass

Size-exclusion high-performance liquid chromatography experiments were conducted to reveal the influence of the solid concentration during proteolysis on the molecular mass distribution of the wheat gluten hydrolysates (Fig. 4.2). Fig. 4.2a shows the impact of hydrolysis on the molecular mass distribution for a solid concentration of 10%. According to expectations, the average molecular mass decreased on hydrolysis as indicated by decreasing ratios of peptides >10 kDa. These results are similar to those of Wang et al. [143] who found around 90% of peptides were in a molecular mass range between 5 and 15 kDa for papain-catalyzed wheat gluten hydrolysis in a DH% range of 3–10%. Linarès et al. [80] concluded that most peptides had a molecular mass of 15–25 kDa for DH% below 5%.

The influence of the solid concentration on the molecular mass for hydrolysates with a DH% of 3.8–5.5% is demonstrated in Fig. 4.2b. No noticeable differences were observed for solid concentrations of 10% and 20% in all molecular mass classes in contrast to solid concentrations of 40% and

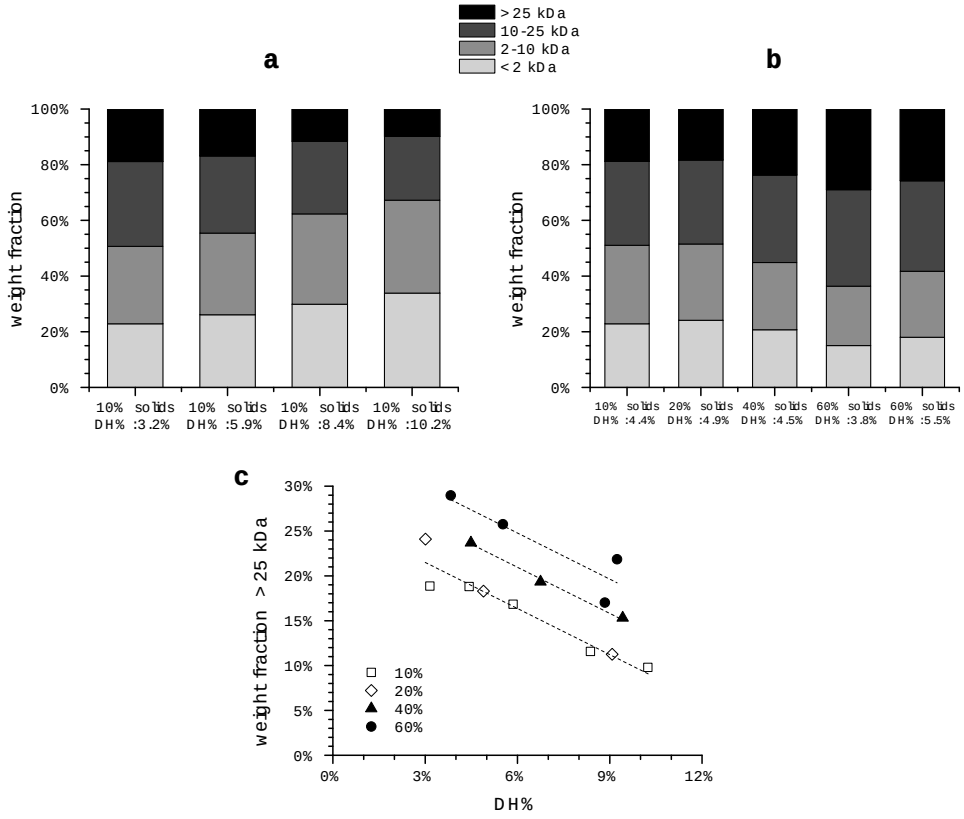

Fig. 4.2: Influence of the solid concentration during proteolysis on the molecular mass of wheat gluten hydrolysates soluble in water plus 2% (w/w) SDS. (a) Influence of the DH% on the molecular mass at a solid concentration of 10%. (b) Influence of the solid concentration on the molecular mass in a DH% range of 3.8–5.5%. (c) Influence of the solid concentration and the DH% on the weight fraction of peptides >25 kDa. Horizontal lines are drawn to guide the eye.

60%, for which an increasing concentration of peptides with a molecular mass of 10–25 kDa and >25 kDa was found concomitant with a decrease in peptides <10 kDa. More than 25% of all peptides had a molecular mass >25 kDa at a solid concentration of 60% and a DH% of 5.5%; this figure was only 18.8% at a solid concentration of 10% and a DH% of 4.4%. Fig. 4.2c shows the influence of the solid concentration and the DH% on the weight fraction of peptides with a molecular mass >25 kDa. It can be seen that increasing DH% resulted in a decrease in peptides with a molecular mass >25 kDa. In addition, high solid

concentrations resulted in a higher weight fraction of peptides with a molecular mass >25 kDa than low solid concentrations at any DH% investigated.

The increased weight fraction of peptides with a molecular mass >25 kDa is most likely related to the increase in viscosity at high solid concentrations. A higher viscosity influences the mass transfer of the enzyme and the substrate and it influences the mass transfer of the products away from the reactive site of the enzyme. Roberts et al. [110] examined the effective diffusivity of gadolinium-labeled bovine serum albumin (BSA) in high-solid cellulose suspensions. BSA thereby acted as a model protein approximating cellulase. The authors demonstrated decreasing effective diffusivities of BSA for increasing solid concentrations. Hence, if the enzyme mixture and the substrate are less mobile in the high-solid wheat gluten system discussed here, which could lead to enzyme attack at the outer parts of the gluten molecules, then this would lead to a higher number of shorter peptides and longer peptides compared with a system with a low solid concentration.

4.3.3 Solubility

Fig. 4.3 depicts the influence of the DH% on the nitrogen solubility index (NSI) for different solid concentrations. The wheat gluten solubility increased during

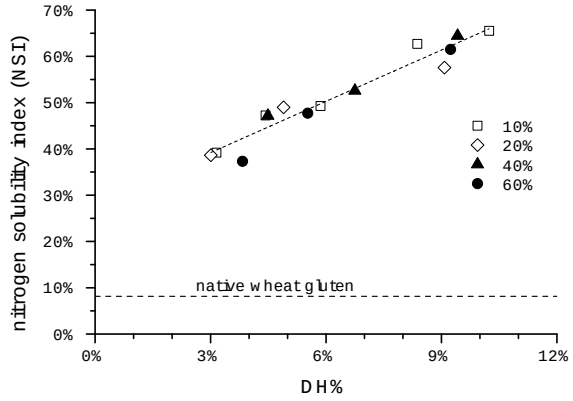

Fig. 4.3: Influence of the DH% on the NSI for different solid concentrations. The standard deviation was maximal 1.6%. The lower dashed line represents the NSI of native vital wheat gluten. The legend indicates the solid concentration during proteolysis.

hydrolysis and depended on the DH%. The NSI increased from 8.1% for native wheat gluten to 34.8% at a DH% of 3.0% and to 65.5% at a DH% of 10.2%. Furthermore, the results suggest that the solid concentration during proteolysis did not affect the water solubility of the wheat gluten hydrolysates.

4.3.4 Foam stability

Fig. 4.4 depicts the influence of the solid concentration during proteolysis on the foam stability. Fig. 4.4a shows the foam stability of 2.0 mg/mL gluten solutions

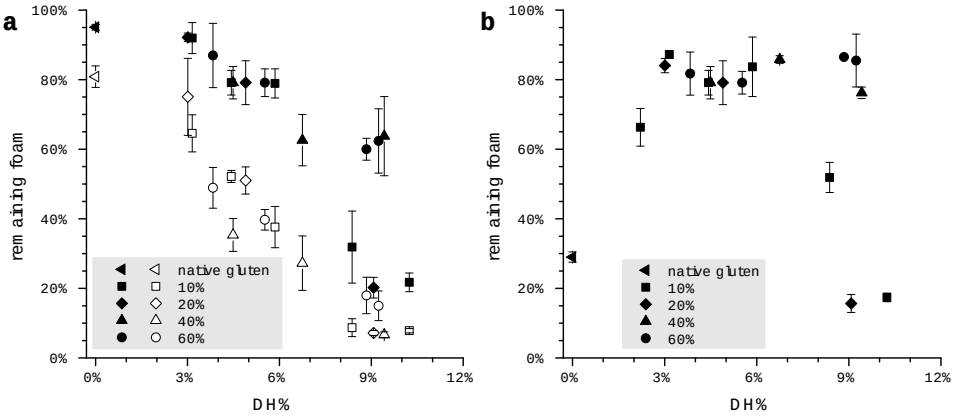

Fig. 4.4: Effect of the solid concentration during proteolysis and the DH% on foam stability. The legend indicates the solid concentration during proteolysis or the usage of native vital wheat gluten, respectively. Closed and open symbols represent foam remaining after 5 and 15 min, respectively. Insoluble parts were removed before the measurements. Error bars represent the standard deviation of four measurements. (a) Foam stability with a constant protein solution of 2.0 mg/mL wheat gluten after centrifugation. (b) Foam stability at a constant hydrolysate concentration of 4.25 mg/mL wheat gluten before centrifugation.

after 5 and 15 min. The foams were most stable for native wheat gluten; the stability decreased with increasing DH% independently of the solid concentration and the time. These results are in line with other studies. Linarès et al. [80] reported no significant influence of the DH% on the half drainage times for constant soluble concentrations in a DH%-range of 0.5–4.7%. This is comparable with the results in this study, where more than 78% of the foams remained after 5 min up to a DH% of 5.9%. Decreasing foam stabilities for increasing DH% were reported for higher DH% ranges [37, 70].

The foam stability was independent of the solid concentration at DH% <7.0%, but did depend on the solid concentration at higher DH%. The foam stability was lower for wheat gluten hydrolysates prepared with solid concentrations of 10% and 20%, whereas hydrolysates from solid concentrations of 40% and 60% led to more than 60% of the foam remaining after 5 min. These differences in foam stability were greatly reduced after 15 min. The difference in foam stability is probably caused by the presence of a higher weight fraction of peptides >25 kDa with increasing solid concentrations, which is beneficial for stabilizing foams. Wang et al. [142] separated wheat gluten hydrolysates at a DH% of 2.6% using ultrafiltration, and found that foam stability decreased with peptide size. The stability of the foam noticeably diminished, especially for peptide sizes below 50 kDa, in agreement with a study by Popineau et al. [108].

Fig. 4.4b demonstrates the foam stability after 5 min with a constant mass of hydrolysate suspended in water. The concentration of solubilized wheat gluten increases with the DH% as illustrated in Fig. 4.3. Native wheat gluten thus showed poor foam stability due to the low solubility. Foam stability was enhanced up to a DH% of 3.2% for increasing DH% and remained constant up to a DH% of 5.9% with more than 79% of the foam remaining after 5 min. For wheat gluten hydrolysates with a DH% higher than 5.9%, the stability of the foam depended on the solid concentration during proteolysis, confirming the result in Fig. 4.4a. Increased foam stability with increasing DH% at constant hydrolysate concentrations has been reported up to a DH% of 4.7% [80]. Fig. 4.4b shows that there is an optimum DH% at which the solubility of the protein is enhanced and the foam stability has not yet deteriorated. The result is a foam that is more than twice as high as that obtained with native wheat gluten after 5 min. At low DH%, the solubility of the hydrolysates is not sufficient; at high DH%, peptides show low foam-stabilizing properties.

4.3.5 Industrial potential of high-solid wheat gluten hydrolysis

Increasing the solid concentration is relevant for new industrial applications that aim to save water and energy. The water consumption, heat of vaporization, and reactor productivity were estimated to compare wheat gluten hydrolysis at low and high solid concentrations on a larger scale. The evaporation energy of

Table 4.2: Water consumption, heat of vaporization, and reactor productivity for wheat gluten hydrolysis at solid concentrations ranging from 10% to 60%; the reactor productivity assumes a final DH% of 4.5% for wheat gluten hydrolysates.

Solid concentration	Water consumption (l/kg gluten)	Heat of vaporization (MJ/kg gluten)	Reactor productivity (kg gluten/m^3h)
10%	9.0	20.3	112.3
20%	4.0	9.0	189.5
40%	1.5	3.4	446.4
60%	0.7	1.5	352.2

water is 2257 MJ/kg. For reactor productivity, hydrolysis times of 55, 67, 60, and 120 min were assumed for solid concentrations of 10%, 20%, 40%, and 60%, respectively, to reach a DH% of 4.5%; the enzyme-to-substrate ratio was 1:100. This was based on the findings in Table 4.1. Furthermore, the density of wheat gluten was calculated to be 1290 kg/m3 [50]. Table 4.2 illustrates the water consumption, the heat of vaporization, and the reactor productivity for solid concentrations of 10–60%.

Hydrolyzing wheat gluten at a solid concentration of 10% requires 9 L of water per kg of gluten, whereas at a solid concentration of 60% 0.7 L of water per kg of gluten are consumed. In other words, more than 92% of water can be saved. Furthermore, the heat of vaporization reduces in the same percentage. One possibility to dry wheat gluten hydrolysates is spray drying [22]. However, an energy efficiency of 50% has to be taken into account for wheat gluten drying [119]. It is clear that a higher solid concentration is always favorable regarding water and energy consumption. However, an optimum solid concentration was found for reactor productivity. Although the reactor productivity data give only an estimation of the actual reactor productivity, they show that the highest productivity occurs at a solid concentration of 40%. This can be explained by the observed increase in reaction time for increasing solid concentrations above 40%. A higher reaction rate, and therefore higher reactor productivity, can be achieved by increasing the enzyme load at a solid concentration of 60%. However, mixtures of endo- and exoproteases are expensive, which affects the cost of the process to a large extent [38]. Nevertheless, high solid concentrations show

great potential with regard to lowering the consumption of water and energy and increasing reactor productivity compared with low solid concentrations of 10% and 20%.

4.4 Conclusions

Wheat gluten is commonly hydrolyzed enzymatically at solid concentrations below 20%. The findings of this study show that wheat gluten can be hydrolyzed at solid concentrations as high as 60%. The solubility of the resulting wheat gluten hydrolysates was independent of the solid concentration during proteolysis. Foam stability was only affected at higher DH%. This can be explained by changes in the molecular mass distribution. Hydrolysis at high solid concentration leads to enhanced reactor productivity and shows potential for water and in particular for high energy savings compared with a solid concentration of 10%. Increased reaction times at very high solid concentrations result in optimum productivity at 40% solids.

Acknowledgements

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CHAPTER 5

Factors impeding enzymatic wheat gluten hydrolysis at high solid concentrations

Abstract

Enzymatic wheat gluten hydrolysis at high solid concentrations is advantageous from an environmental and economic point of view. However, increased wheat gluten concentrations result in a concentration effect with a decreased hydrolysis rate at constant enzyme-to-substrate ratios and a decreased maximum attainable degree of hydrolysis (DH%). We here identified the underlying factors causing the concentration effect. Wheat gluten was hydrolyzed at solid concentrations from 4.4% to 70%. The decreased hydrolysis rate was present at all solid concentrations and at any time of the reaction. Mass transfer limitations, enzyme inhibition and water activity were shown to not cause this hydrolysis rate limitation up to 50% solids. However, the hydrolysis rate limitation can be, at least partly, explained by a second-order enzyme inactivation process. Furthermore, mass transfer impeded the hydrolysis above 60% solids. The addition of enzyme after 24 h at high solid concentrations scarcely increased the DH%, suggesting that the maximum attainable DH% decreases at high solid concentrations. Reduced enzyme activities caused by low water activities can explain this DH% limitation. Finally, a possible influence of the plastein reaction on the DH% limitation is discussed.

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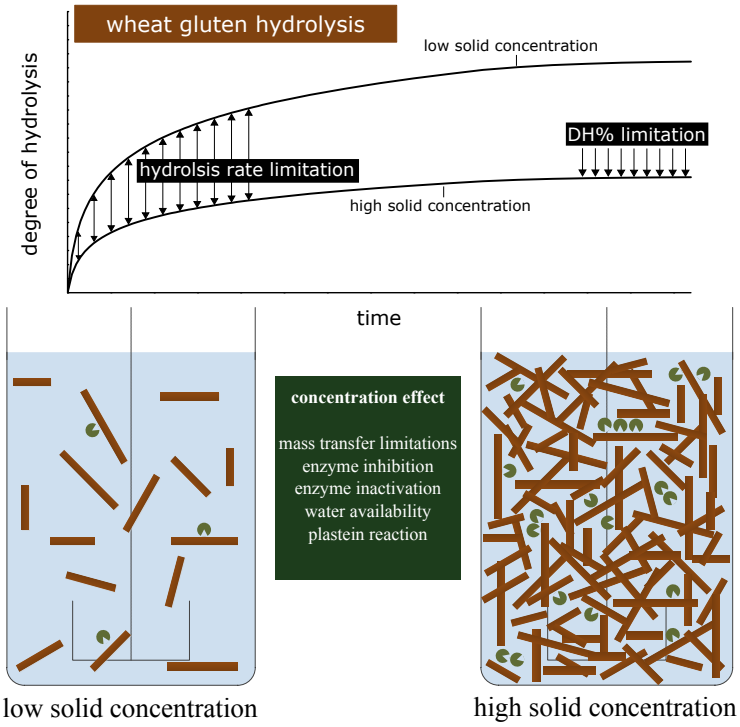

Fig. 5.1: Chapter 5 – graphical table of contents.

5.1 Introduction

Wheat gluten is a high-value plant protein, available in abundance as a by-product of the wheat starch production. Although native wheat gluten is an excellent bread improver, its insolubility in water at neutral pH rather limits further applications in the food industry. Therefore, enzymatic hydrolysis is a mild and selective process to increase the water solubility and to alter other functional properties of wheat gluten, such as the foaming and emulsifying properties [57, 70]. In practice, wheat gluten hydrolysates are used as e.g. savory flavoring agents, in sports nutrition, and for the production of bioactive peptides [13, 51].

Currently, wheat gluten is hydrolyzed at rather low solid concentrations. In literature, the highest wheat gluten concentrations reported ranged between 17–

22% for enzymatic hydrolysis at neutral pH [22, 84]. Hydrolyzing wheat gluten at higher solid concentrations, though, provides several benefits. A higher solid concentration results in smaller reactors and equipment, reducing capital costs [93]. Furthermore, less energy is required for the process and the consequent dehydration of the product, thus reducing the processing energy usage and costs. Finally, hydrolyzing at a high solid concentration requires less water and produces less wastewater with inherently lower treatment and disposal costs.

On the downside, high-solid hydrolysis also creates new challenges. One major effect, here denoted as concentration effect, signifies lower degrees of hydrolysis (DH%) at higher wheat gluten concentrations. The concentration effect has also been referred to as solids effect [72] and has been reported for several biopolymeric materials including lignocellulose [72] , cellulose [110], starch [6], whey protein [16], and wheat gluten [57].

Several factors must be taken into account as possible causes of the concentration effect. These are: (1) Mass transfer limitations: A higher solid concentration results in a higher viscosity and therefore higher diffusion times, especially for substrates that cannot be dissolved completely. The enzyme, substrate and product diffusion can be hindered, leading to a non-homogeneous distribution of enzyme and substrate [110]. Furthermore, the ability of the enzyme to diffuse is reduced above a critical substrate concentration, leading to decreased reaction rates [20]. (2) Enzyme inhibition: Increasing solid concentrations can favor product inhibition, since the diffusion of the product away from the reactive site can be impeded [59] and the total amount of product increases. Some studies revealed the detrimental influence of product inhibition at high solid loadings for olive tree biomass and softwood [17, 123]. Other studies, though, excluded product inhibition as a cause of the concentration effect [16, 72, 110, 130]. (3) Enzyme inactivation might increase with solid concentration. Additionally, a higher enzyme concentration in the case of proteases could enhance the inactivation of the enzyme. (4) Water availability: Increasing solid concentrations and progressive hydrolysis result in a decline in water activity and thus water availability. This can result in lower enzyme activities. (5) The water activity influences the thermodynamic equilibrium of the hydrolysis reaction where higher water activities favor

hydrolysis while lower water activities favor peptide synthesis (see e.g. [140]). Furthermore, in this study, the high-solid wheat gluten hydrolysis is performed in a concentration range similar to the range where the plastein reaction takes place. In the plastein reaction, water-insoluble higher molecular mass peptides are formed out of water-soluble peptides in the presence of proteases.

In a previous study, we showed that wheat gluten can be hydrolyzed at high solid concentrations without detrimental changes in the water solubility and the foam stabilizing properties, while the highest productivity was at 40% solids [57]. In this paper, we report on the underlying factors impeding wheat gluten hydrolysis at high solid concentrations. Here, high-solid wheat gluten hydrolysis will be defined as the solid concentration at which native wheat gluten forms a dough and no free liquid is present, which implies a solid concentration of 40% or higher.

5.2 Materials & Methods

5.2.1 Materials

Native vital wheat gluten (Roquette) with 74.0±0.5% crude protein content (N×5.7 [102]) and 8.0±0.6% water content (all by weight) was obtained from Barentz BV, Hoofddorp, The Netherlands. Protease from *Aspergillus oryzae* (Flavourzyme 500 LAPU/g) from Novozymes was purchased from Sigma-Aldrich, Germany. Flavourzyme contains a mixture of endo- and exoproteases [38]. Milli-Q water was used in all experiments.

5.2.2 Hydrolysis reaction

The hydrolysis was carried out using 200-mL double-walled glass vessels connected to a water bath. Each experiment was conducted using a total mass of 150 g reaction mixture and varying solid concentrations from 4.4% to 70% (w/w). The solid concentrations, enzyme concentrations, and enzyme-to-substrate ratios (w/w) used are depicted in Table 5.1.

Only the protein fraction of native wheat gluten was considered as substrate when calculating the enzyme-to-substrate ratios. Furthermore, the water present

Table 5.1: Solid concentration, enzyme concentration (U/g reaction mixture), and enzyme-to-substrate ratio (w/w) of performed experiments. Flavourzyme 500 U/g according to manufacturer.

Solid conc.	4.4%	13.3%	20%	30%	40%	50%	60%	70%
Enzyme conc.								
1.4		1:40						
2.7	1:6.7	1:20	1:30	1:45	1:60	1:75	1:90	1:105
5.4					1:30			
8.1		1:6.7	1:10	1:15	1:20	1:25	1:30	
32.1					1:5			

in the wheat gluten powder was taken into account when calculating the solid concentration. The hydrolysis temperature was 50 °C. The enzyme was mixed with the water prior to addition to the substrate. The double-walled glass vessel was closed with a clasp to avoid evaporation of water and stirred using an overhead stirrer at 40 rpm. After reaction, the enzyme present in the mixture was inactivated by heating at 95 °C for 10 min in a water bath. The samples were then cooled, frozen, and freeze-dried. The freeze-dried samples were ground and stored for further analysis. Hydrolysis reactions were conducted under floating pH conditions. Nevertheless, we measured the pH of some samples after 24 h. The pH always remained within the suitable pH range of Flavourzyme from 5–8 [98].

5.2.3 Degree of hydrolysis (DH%)

The DH% was measured by the o-phthaldialdehyde (OPA) method as described previously [57], with minor modifications: The amount of suspended wheat gluten hydrolysate was varied between 0.5–1 mg/mL to avoid spectrophotometer absorbance values above 1.5 for samples with a high DH%. The calculated DH% was the mean of two determinations.

5.2.4 Water activity (a_w)

The water activity was measured after 3 h and 24 h of hydrolysis using an Aqualab TE water activity meter (Decagon Devices Inc., Pullman, WA, USA). The water activity was measured in triplicate at the maximum possible temperature

of 40 °C. The reproducibility was ±0.002 a_w units. Samples for water activity measurements were not inactivated to avoid possible changes on the water activity during the inactivation.

5.2.5 Sorption isotherms

Adsorption isotherms were measured using the dynamic vapor sorption (DVS) elevated temperature system (SMS Ltd, London, UK). The measurements were carried out at 50 °C with a sample mass of 10–15 mg freeze-dried hydrolysate. The samples were considered to be at equilibrium at a given water activity, once the sample weight varied for less than 0.002%/min within a timeframe of 30 min or when a maximum measuring time of 600 min was reached.

To determine the total water uptake in the sorption isotherms, it is necessary to know the moisture contents of the freeze-dried hydrolysates at ambient temperature and humidity. These were measured in duplicate using the Sartorius Moisture Analyzer MA45 at 130 °C. The moisture contents were 11.7%, 13.0%, 14.8%, and 17.5% for hydrolysates hydrolyzed at 60%, 40%, 20%, and 13.3% solids, respectively. The reproducibility was ±0.3%.

Furthermore, it was investigated whether freeze-drying, enzyme inactivation, and the enzyme itself influenced the sorption isotherms. Otherwise, a clear comparison between native and hydrolyzed wheat gluten is difficult, since native wheat gluten contained no Flavourzyme, was not heat-treated after hydrolysis, and not freeze-dried. To investigate the influence of all these factors, enzyme at a concentration of 8.1 U/g reaction mixture was dispersed in water and inactivated for 10 min at 95 °C. Afterwards, this inactivated water-enzyme mixture was mixed with native wheat gluten at 60% solids and immediately inactivated again for 10 min at 95 °C. The sample was subsequently freeze-dried and used for DVS analysis. The moisture content was 10.5%.

5.2.6 Size-exclusion high-performance liquid chromatography

Size-exclusion HPLC experiments were performed using a Thermo Surveyor Plus HPLC system (Thermo Scientific) equipped with a PDA detector. Wheat gluten hydrolysate (2.5 mg/mL) was suspended in 12.5 mM sodium tetraborate

decahydrate plus 2% (w/w) SDS, stirred overnight, and then centrifuged at 3900 × *g* for 15 min. The resulting supernatant was injected onto a TSKGel G2000 SWXL (300×7.8 mm) column and eluted with 70% / 30% / 0.1% water / acetonitrile / trifluoroacetic acid at a flow rate of 1.0 mL/min and detected at a wavelength of 214 nm. All experiments were performed in duplicate. The molecular mass was determined using molecular mass markers based on carbonic anhydrase (29,000 Da), α-lactalbumin (14,100 Da), aprotinin (6510 Da), insulin (5700 Da), bacitracin (1420 Da), and phenylalanine (165 Da).

5.3 Results

5.3.1 The concentration effect

The influence of the solid concentration on the DH% during enzymatic wheat gluten hydrolysis is depicted in Fig. 5.2. The DH% increased with the reaction

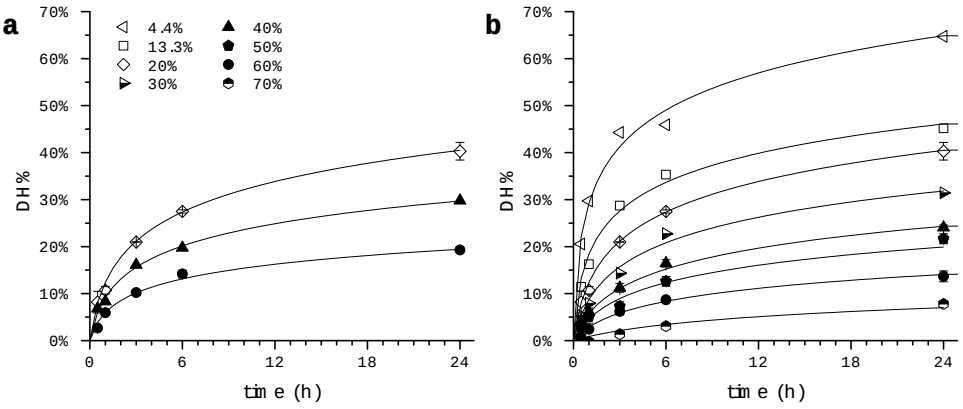

Fig. 5.2: Influence of the solid concentration on the increase in DH% over time. (a) Constant enzyme-to-substrate ratio of 1:30. (b) Constant enzyme concentration of 2.7 U/g reaction mixture. Standard deviations are presented where experiments have been performed in triplicate. The legend indicates the solid concentration during proteolysis and is valid for Fig. (a) and (b). Lines are drawn to guide the eye.

time for all samples within 24 h, but this leveled off towards a plateau. Between 24 h and 48 h the increase in DH% was less than 2%. Furthermore, increasing the solid concentration led to a reduced DH% at every time of the hydrolysis reaction for a constant enzyme-to-substrate ratio of 1:30 (Fig. 5.2a) and thus

also for a constant enzyme concentration of 2.7 U/g reaction mixture (Fig. 5.2b). The highest DH% reached during this study was 65% at a solid concentration of 4.4% after 48 h.

5.3.2 Addition of water and enzyme

Fig. 5.3 shows the influence of changes in the water and enzyme concentration on the DH% after 24 h. Hydrolysis reactions were performed for a total of 48 h.

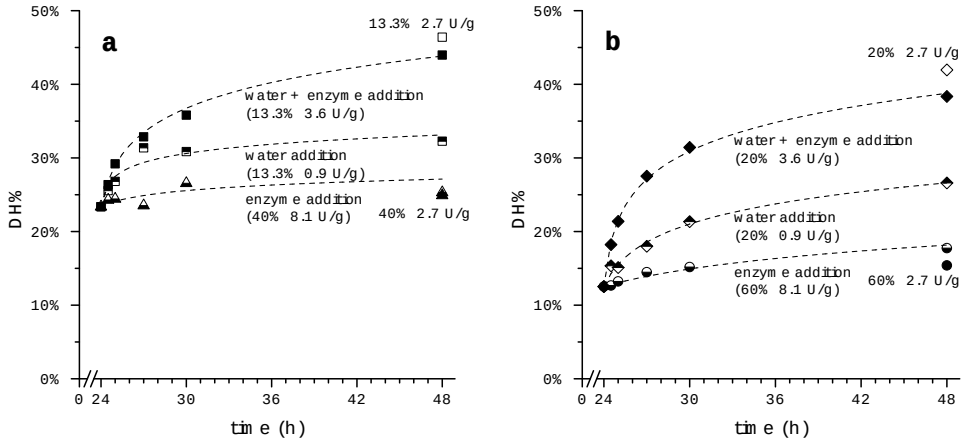

Fig. 5.3: Influence of dilution and additional enzyme addition on the hydrolysis reaction. Initial reactions were all performed with 2.7 U/g reaction mixture and a solid concentration of (a) 40% and (b) 60%. Water addition: Threefold dilution. Enzyme addition: Enzyme addition of 5.4 U/g reaction mixture. Water + enzyme addition: Threefold dilution and enzyme addition of 2.7 U/g reaction mixture. Data in brackets show resulting solid and enzyme concentration. For comparison, 13.3%, 20%, 40%, and 60% 2.7 U/g show the DH% after 48 h when the reaction was initially started with the respective solid and enzyme concentration. All other process conditions besides water and enzyme addition were not altered throughout the whole 48 h.

After 24 h either water, enzyme, or water and enzyme were added. All other initial conditions were not altered. The addition of 5.4 U/g reaction mixture at 40% solids increased the DH% from 23.4% after 24 h to 25.3% after 48 h only (Fig. 5.3a). A threefold dilution, which also resulted into a threefold reduction of the enzyme concentration, resulted in a higher increase in DH% from 23.4% to 32.3% after 48 h. The combination of enzyme and water addition led to the highest increase in DH%. Here, the DH% after 48 h was similar to the DH% obtained when initially hydrolyzing wheat gluten at 13.3% solids and

2.7 U/g reaction mixture. Fig. 5.3b displays the same procedure for 60% solids. A threefold dilution again resulted in a higher increase in DH% than the addition of enzyme. Furthermore, the combined addition of enzyme and water led again to the highest increase in DH%.

5.3.3 Molecular mass distribution

The molecular mass distributions of selected gluten hydrolysates are shown in Fig. 5.4. Samples with a low DH% showed the highest peak after 5.8 min

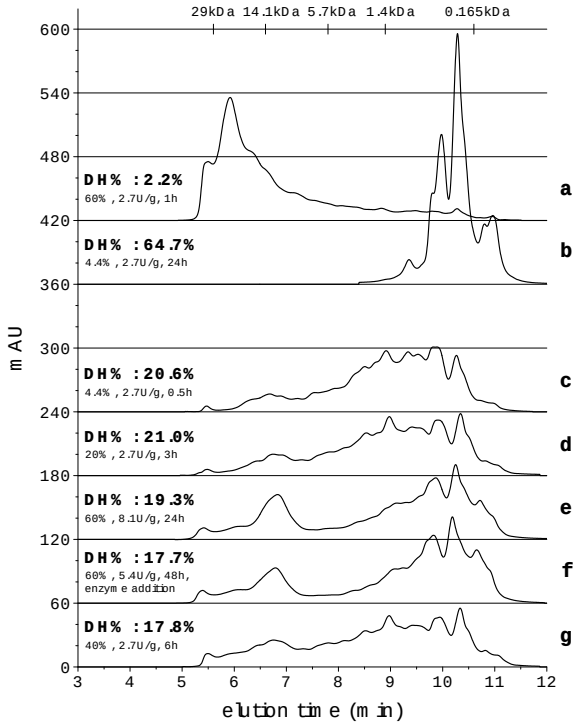

Fig. 5.4: Molecular mass distributions of selected gluten hydrolysates. Curves were modified in a way that all have the same area under the curve. The legend indicates DH%, solid concentration, enzyme concentration, and time of sampling.

elution time (Fig. 5.4a), representing high molecular mass fragments. The sample with the highest DH% obtained in this study had the maximum peak after 10.3 min (Fig. 5.4b), representing individual amino acids and oligopeptides. When comparing samples with similar DH%, the molecular mass distributions

were rather independent of the solid concentration up to 40%, but at 60% solids a distinct peak after 6.7 min (indicative of fragments of around 14 kDa) was observed (Fig. 5.4c–g). This higher peak after 6.7 min at 60% solids was reproducible and not a product of peptide aggregation, since the addition of 6M urea resulted in the same peak area (curve not shown).

5.3.4 Water activity and sorption isotherm

The relation between wheat gluten hydrolysis at different solid concentrations and the water activity and sorption isotherms is depicted in Fig. 5.5. Differences

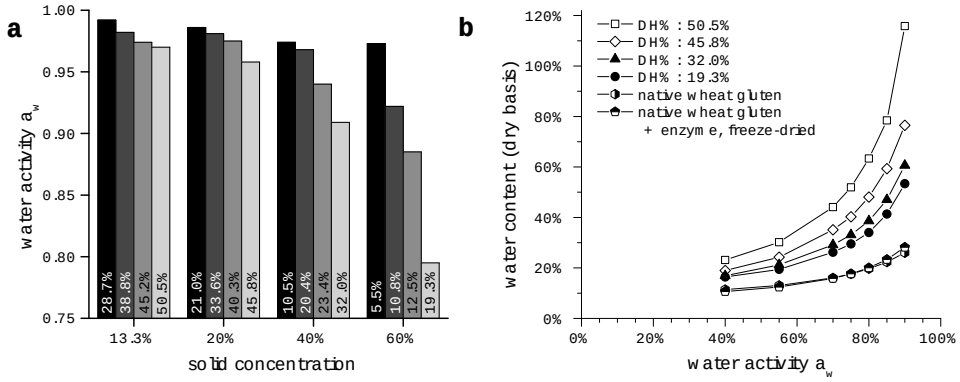

Fig. 5.5: (a) Influence of the DH% on the water activity at different solid concentrations. Percentages in bars show the DH%. (b) Sorption isotherms obtained by DVS for native wheat gluten and wheat gluten hydrolysates. Analyzed samples were hydrolysates after 24 h, hydrolyzed at 13.3%, 20%, 40%, and 60% solids, respectively (from high DH% to low DH%).

in the water activity (a_w) were minor for 13.3% and 20% solids and a_w remained higher than 0.95 even at DH% above 45% (Fig. 5.5a). At 40% and 60% solids, the water activity decreased noticeably upon hydrolysis to 0.909 at a DH% of 32.0% for 40% solids and to 0.795 at a DH% of 19.3% for 60% solids. The sorption isotherms shown in Fig. 5.5b reveal a higher equilibrium water content of the hydrolyzed samples compared to native wheat gluten at all water activities investigated. Furthermore, a higher DH% resulted in higher water contents. The increase in equilibrium water content for the hydrolyzed samples was independent of the inactivated enzyme present in the hydrolysate, the inactivation process, and the freeze-drying of the samples. To test this, the DVS

result of native wheat gluten was compared with the DVS results of freeze-dried native wheat gluten that contained inactivated enzyme - the DVS results were similar at all a_w.

5.4 Discussion

In wheat gluten hydrolysis the concentration effect can be divided into a hydrolysis rate limitation and a DH% limitation (Fig. 5.6). The hydrolysis rate

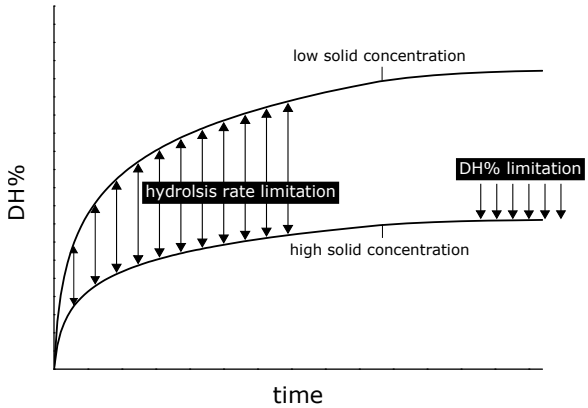

Fig. 5.6: Schematic illustration of the concentration effect. The concentration effect refers to a decreased hydrolysis rate at constant enzyme-to-substrate ratios and a decreased maximum attainable DH%.

limitation refers to decreased reaction rates at higher solid concentrations and constant enzyme-to-substrate ratios meaning that the difference in DH% between low and high solid concentration increases over time (Fig. 5.2 and Fig. 5.6). The DH% limitation refers to a decreased maximum attainable DH% at higher solid concentrations, which could not be corrected for by adding more enzyme or increasing the reaction time (Fig. 5.3 and Fig. 5.6). Whereas the hydrolysis rate limitation is most pronounced at the beginning of the hydrolysis reaction, the DH% limitation impedes the hydrolysis at the end of the reaction. Below, we will evaluate the possible influencing factors regarding the hydrolysis rate limitation and the DH% limitation.

5.4.1 Mass transfer limitations

Native wheat gluten assumes the consistency of a dough at 30–40% solids, which is characterized by a high viscosity, difficult mixing and slow diffusion of enzyme, water and substrate. Nevertheless, liquefaction already occurs at the initial stages of the hydrolysis [57]. Therefore, the highest mass transfer limitations at higher solid concentrations would be expected in the beginning of the hydrolysis, which would then affect the reaction rate. Fig. 5.7 shows the influence of the solid concentration on the total conversion at constant enzyme concentrations as function of time. Here, it was considered that the total amount of cleaved peptide bonds is equal to the DH% (i.e. the percentage of cleaved bonds) multiplied by the total amount of substrate. Fig. 5.7 demonstrates that

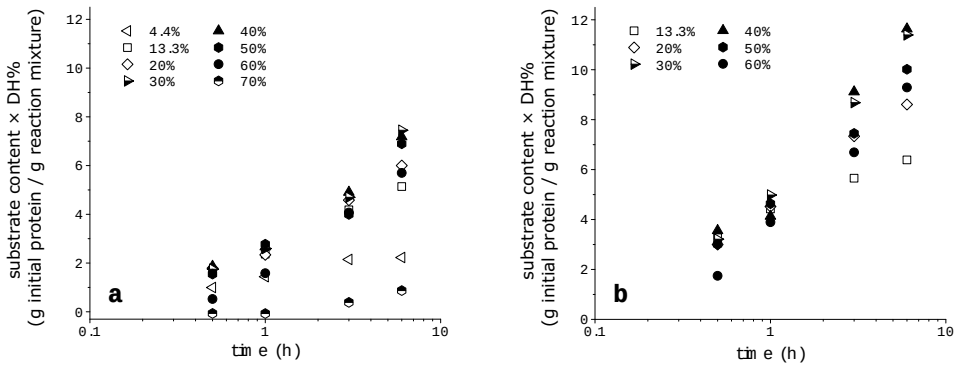

Fig. 5.7: Influence of the solid concentration on the total number of cleaved peptide bonds at constant enzyme concentrations of (a) 2.7 and (b) 8.1 U/g reaction mixture. The y-axis is proportional to the total number of cleaved peptide bonds. The legend indicates the solid concentration during hydrolysis. The reader is referred to Fig. 5.2 for standard deviations of triplicate experiments.

the enzyme activity at a constant enzyme concentration was almost independent of the solid concentration up to 50% solids at 2.7 and 8.1 U/g reaction mixture: the total number of bonds cleaved remained almost constant. The reduced number of peptide bonds cleaved at 2.7 U/g reaction mixture for 4.4% solids after 1 h and for 13.3% solids after 6 h can be explained by substrate exhaustion: the DH% for 4.4% solids after 6 h was already 45.9%; even at a theoretical DH% of 100%, the number of peptide bonds cleaved would have been lower than at

all other studied solid concentrations. At 8.1 U/g reaction mixture substrate exhaustion occurred earlier.

At 60% solids the total amount of cleaved peptide bonds was slightly lower and at 70% solids the total conversion decreased drastically. It can therefore be concluded that mass transfer limitations are not responsible for the hydrolysis rate limitation up to 50% solids. However, mass transfer impeded the hydrolysis above 60% solids. This is in line with Cheng and Prud'homme [20], who stated that the diffusion of the enzymes controls the process above a certain substrate concentration. Similarly, Hodge et al. [59] showed that enzymatic lignocellulose hydrolysis was impaired by mass transfer limitations above a certain solid concentration.

5.4.2 Enzyme inhibition

Enzyme inhibition has been reported for wheat gluten hydrolysis using Flavourzyme [51]. Generally, enzyme inhibition originates from the fact that the enzyme forms complexes with the product or the substrate thereby reducing the active enzyme concentration. The extent of this effect increases with decreasing enzyme-to-inhibitor ratios, as shown for example in the case of β-glucosidase inhibition in cellobiose hydrolysis [151]. Therefore, enzyme inhibition will have a more pronounced effect at low enzyme concentrations. For example, a low enzyme concentration and a long hydrolysis time will only result in the same DH% as a high enzyme concentration and a short hydrolysis time, if no inhibition takes place. This is schematically shown in Fig. 5.8a for two different solid concentrations. If inhibition takes place, the DH% will be lower at lower enzyme concentrations (Fig. 5.8b). Fig. 5.8c depicts the influence of the initial enzyme concentration multiplied by the reaction time on the DH% for different solid and enzyme concentrations during gluten hydrolysis. The approach to exclude enzyme inhibition in concentrated systems by comparing conversion with enzyme concentration and hydrolysis time has been used previously by Kristensen et al. [72]. We can see that for each separate solid concentration, the curve with different enzyme ratios (filled, half-filled and empty symbols) is the same, which indicates that there was no inhibition in the range studied. Of course, the curve for each solid concentration still differs from that for other

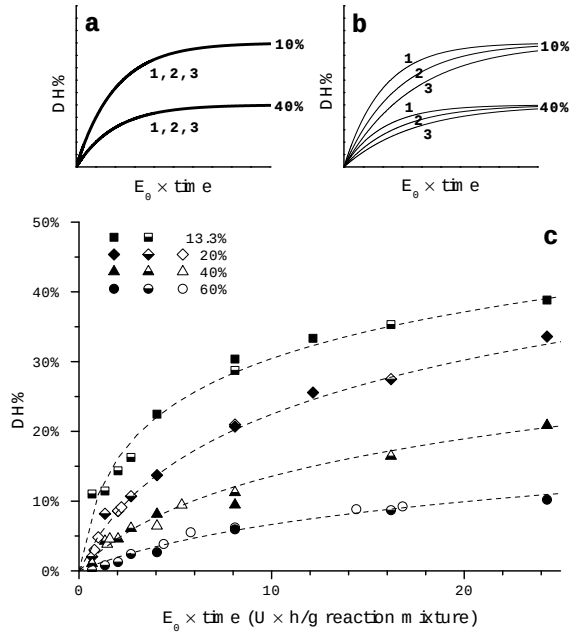

Fig. 5.8: DH% as a function of the enzyme concentration multiplied by the reaction time. Schematic curves when (a) no enzyme inhibition and (b) enzyme inhibition takes place for (1) high, (2) medium, and (3) low enzyme concentration at solid concentrations of 10% and 40%. (c) Wheat gluten hydrolysis. The legend indicates the solid concentration during hydrolysis. Enzyme concentrations of 8.1 (filled symbols) and 2.7 (half-filled symbols) U/g reaction mixture. Corresponding enzyme-to-substrate ratios are shown in Table 5.1. Empty symbols are taken from Hardt et al. [57] and represent 0.8, 1.6, and 2.4 U/g reaction mixture at 20%, 40%, and 60% solids, respectively. One can see that the enzyme concentration does not influence the DH% as function of E_0 × time, which is in accordance with Fig. (a). Lines are drawn to guide the eye.

solid concentrations, consistent with the hydrolysis rate limitation. Therefore, enzyme inhibition does not explain the hydrolysis rate limitation.

5.4.3 Enzyme inactivation

Fig. 5.3 shows that some enzyme was still active after 24 h, since the DH% increased upon further water addition. However, some enzyme inactivation took place during the hydrolysis reaction, since the DH% after 48 h was at least 10% higher upon a combined enzyme and water addition compared to when only water was added. A higher increase in DH% would have been expected in the

case that no inactivation was present. Instead, the increase in DH% leveled off upon water addition after 30 h.

Fig. 5.8c gives information on the order of inactivation. Since the DH% is independent of the enzyme concentration multiplied by hydrolysis time, there is either no enzyme inactivation present or the enzyme inactivation follows second-order kinetics. Thus, given the fact that Fig. 5.3 indicates that some inactivation took place, Fig. 5.8c implies that the inactivation is a second-order process. Protease inactivation rate has indeed been shown and modeled as second-order process [53, 87]. This has been explained for whey protein hydrolysis with Alcalase to be via free enzymes attacking enzyme-substrate complexes [53]. Since the hydrolysis in general is considered to be a process that is first order, and if the enzyme inactivation rate follows second-order kinetics, then a hydrolysis rate limitation must be observed at constant enzyme-to-substrate ratios: increasing enzyme concentrations result in stronger inactivation of the enzyme than the increase in hydrolysis rate.

Fig. 5.7 already suggests that the enzyme activity was independent of the solid concentration up to 50% solids for constant enzyme concentrations of 2.7 and 8.1 U/g reaction mixture. Based on this, Fig. 5.9 depicts the total amount of hydrolysis per enzyme concentration (calculated as the substrate concentration multiplied by the DH% and divided by the initial enzyme concentration) as a function of the initial enzyme concentration. The y-axis is proportional to the total number of cleaved peptide bonds per enzyme molecule. The number of peptide bonds cleaved per initial enzyme concentration decreased with increasing initial enzyme concentration and was independent of the solid concentration. Only after 3 h the influence of substrate exhaustion at 13.3% and 20% solids could be observed. Thus, we may conclude that protease self-inactivation is an important mechanism in the reduction of enzyme activity. Furthermore, we may conclude that the hydrolysis rate limitation is not directly related to the solid concentration, but rather to the higher enzyme concentration generally used. A highly concentrated hydrolysis system should therefore be operated at enzyme concentrations as low as possible, to avoid excessive enzyme inactivation. This obviously has consequences for the volumetric productivity – careful optimization

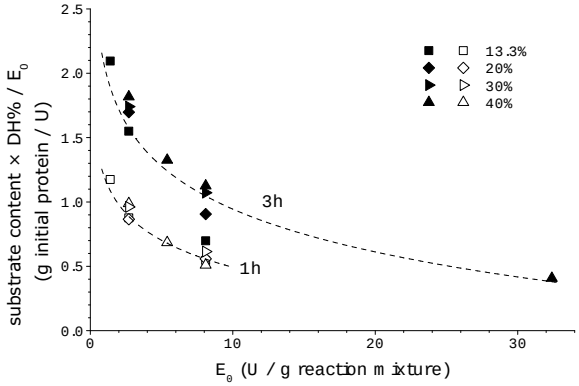

Fig. 5.9: Influence of the initial enzyme concentration on the total amount of hydrolysis per initial enzyme concentration after 1 h and 3 h. The legend indicates the solid concentration during hydrolysis.

between avoidance of enzyme inactivation and high volumetric productivity is therefore important.

5.4.4 Water availability

Water is important as a reactant in the hydrolysis reaction. When the solid concentration is increased, the amount of water available for reaction decreases. The availability of water is described by its activity (a_w). Upon wheat gluten hydrolysis, a_w decreases, not only since water reacts with the peptides, but also because more molecules having charged groups (free amino acids and short peptides) are produced, which will bind more water. Many microbiological and enzymatic processes cease or slow down considerably at a_w below 0.9 [120]. In non-protein systems, it has been shown that replacing water by other solvents reduced the enzyme activity for different oxidases [75], alcohol dehydrogenase [65], and cellulase [115] already at a_w below 0.98.

Considering Fig. 5.7, it can be concluded that the water activity cannot explain the hydrolysis rate limitation up to 50% solids because here the enzyme activity was found to be independent of the solid concentration. This does not contradict results reported in literature since the hydrolysis rate limitation was already present at the beginning of the hydrolysis reaction where the water activity was still high despite being somewhat lower for higher solid

concentrations. Nevertheless, the reduced a_w depicted in Fig. 5.5a might impact the DH% limitation, where a_w was below 0.91 for 40% and 60% solids. The sorption isotherms of native wheat gluten in Fig. 5.5b give further insight in the influence of the water activity on the hydrolysis reaction. It should be noted that the sorption isotherms were determined using the freeze-dried samples, so the water contents are not exactly equal to the water contents during hydrolysis. The sorption isotherms are in line with other studies regarding native wheat gluten, (e.g. [139]) and similar to other protein hydrolysates [21, 62]. Fig. 5.5b illustrates that an increase in DH% is only possible with a reduction in a_w. For example, an increase in DH% at 55% water content (dry basis) from 19.3% to 50.5% reduces a_w from about 0.9 to about 0.75. Taking together the reduced a_w observed during wheat gluten hydrolysis and the fact that a lower a_w noticeably decreases the enzyme activity, we postulate that the lack of water reduces the enzyme activity to such an extent that the hydrolysis practically ceases at high solid concentrations. This conclusion is supported by the fact that the addition of fresh enzyme after 24 h only slightly increased the DH% whereas dilution resulted in a far higher increase in DH% (Fig. 5.3). Therefore, the DH% limitation could be explained by changes in water activity. This argumentation is also supported by Selig et al. [120], who showed that the formation of soluble species during concentrated lignocellulose hydrolysis reduced a_w and thus the enzyme activity. The authors also stated that stabilizers commonly present in commercial enzyme mixtures, e.g. glycerol, sorbitol, and simple sugars, further depress a_w, which led to the conclusion to keep the enzyme concentration as low as possible. This is in line with Fig. 5.9 and the findings in this study.

5.4.5 Plastein reaction

Enzymatic hydrolysis is a reversible reaction where the equilibrium is strongly shifted towards hydrolysis at high water activities. Usually, very low water activities are necessary to shift the equilibrium towards “classical” peptide synthesis (see e.g. [140]), which were not observed in this study (Fig. 5.5). Nevertheless, peptide “resynthesis” can occur at higher water activities by means of the plastein reaction. The plastein reaction is characterized by the production of water-insoluble peptides when exposing water-soluble hydrolyzed

peptides to proteases at elevated substrate concentrations, and has been attributed to several causes: condensation, transpeptidation, and physical forces [150]. However, merely in the case of condensation, the overall amount of free amino groups reduces, which in terms of wheat gluten hydrolysis would be equivalent to a lower DH%. A reduction in free amino groups has indeed been reported e.g. for casein hydrolysate using Alcalase at substrate concentrations above 30% [153] and for bovine serum albumin hydrolysates using α-chymotrypsin at substrate concentrations above 40% [26]. Peptide formation by Flavourzyme during porcine hemoglobin hydrolysis has also been suggested [18]. Since the plastein reaction is taking place in the pH and temperature range, and at solid concentrations similar to 40% and 60% solids used in this study, it is possible that the plastein reaction also occurred during wheat gluten hydrolysis at high solid concentrations. The prerequisite is a sufficiently high DH%, where the peptides are small enough to be susceptible to the plastein reaction. The observed peak at 6.7 min elution time that was partly obtained at 40% and especially at 60% solid concentration (Fig. 5.4) could be an indication that indeed a repolymerized residue formed through the plastein reaction. Nevertheless, the observed peak could also be peptide residues inaccessible to hydrolysis and caused by mass transfer limitations.

5.5 Conclusions

We studied the factors that cause two central phenomena in concentrated enzymatic wheat gluten hydrolysis: (1) the hydrolysis rate limitation, leading to a lower reaction rate at higher concentrations and constant enzyme-to-substrate ratios, and (2) the DH% limitation, which implies that the maximum attainable DH% is reduced as well. Wheat gluten could be enzymatically hydrolyzed at high solid concentrations up to 50% without a reduction in enzyme activity at constant enzyme concentration. Only at a high solid concentration above 60%, the reaction rate decreased due to mass transfer limitations. The hydrolysis rate limitation up to 50% solids cannot be explained by enzyme inhibition or changes in water activity, but seems to be mostly caused by second-order enzyme auto-inactivation rate due to the higher enzyme concentrations used.

The DH% limitation, we believe, can be explained by reduced enzyme activities due to a decline in water activity.

The results show that wheat gluten can be hydrolyzed to high DH% of at least 20% at solid concentrations up to 60%. However, since the enzyme activity per enzyme concentration decreases with higher enzyme concentrations, future research in high-solid (gluten) hydrolysis should focus on improving the enzyme performance at higher enzyme concentrations.

Acknowledgements

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CHAPTER 6

Starch facilitates enzymatic wheat gluten hydrolysis

Abstract

Wheat gluten can be hydrolyzed by either using (vital) wheat gluten or directly from wheat flour. This study investigates the influence of the presence of starch, the main component of wheat, on enzymatic wheat gluten hydrolysis. Wheat gluten present in wheat flour (WFG) and vital wheat gluten (VWG) were hydrolyzed at constant protein concentrations, but subsequently 5.6 times higher amounts of wheat flour. Nevertheless, WFG hydrolysis at 40% total solids resulted in significantly higher degrees of hydrolysis (DH%) than VWG hydrolysis at 7.2% solids. This difference increased to up to 4.5% in 6 h and diminished again for longer reaction times. Possible differences in the gluten composition and the presence of albumins and globulins in wheat flour could not explain the difference in DH% because the addition of starch to VWG increased the rate of hydrolysis similarly. Instead, it was concluded that starch granules impede gluten aggregation, which facilitates the hydrolysis. At higher solid concentrations of up to 70% wheat flour, the positive effect of starch disappeared, because WFG hydrolysis was hindered by mass transfer limitations and lower water activities.

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Hardt, N. A., R. M. Boom, and A. J. van der Goot. Starch facilitates enzymatic wheat gluten hydrolysis.

6.1 Introduction

Wheat gluten, the major protein of wheat, is a high-value, modestly priced plant protein, available at increasing amounts [31]. Native vital wheat gluten is an excellent bread improver and also used to texturize foods [3]. However, native vital wheat gluten is scarcely water-soluble at neutral pH, which limits further applications in the food industry. Therefore, enzymatic hydrolysis is used as a mild process to increase the water solubility at neutral pH and to alter other functional properties, such as its foaming and emulsifying properties [57, 70, 80]. In practice, wheat gluten hydrolysates are used as savory flavoring agents, in sports nutrition, and for the production of bioactive peptides, among others [13, 51].

Hydrolysis of wheat gluten is performed using isolated (vital) wheat gluten (VWG). Thus, a pre-process step is required to separate gluten from wheat flour in processes such as the Martin or the batter process. These separation processes consume copious amounts of water and often require 10 to 15 L of water per kg of dry matter [119], which has to be removed afterwards. Alternatively, wheat gluten can be hydrolyzed when still present in the wheat flour (WFG) [12, 13], thereby omitting the separation step, or separation can take place after gluten hydrolysis. Currently, mild proteolysis of wheat flour is performed in the baking industry with the aim to "weaken" the gluten network and to improve the handling properties of dough [12, 149]. Other possible applications of wheat flour hydrolysates are cereal-based drinks [100].

Previously, we showed that vital wheat gluten can be hydrolyzed at solid concentrations of up to 60% at increased reactor productivity compared to the conventionally used concentration of 20% solids [57]. It is therefore interesting to investigate the role of the high starch content in wheat flour (around 80%, dry basis [8]) on the hydrolysis process, since starch replaces water in WFG hydrolysis compared to VWG hydrolysis at constant protein concentration. Starch in wheat flour is present as starch granules. Since wheat starch granules absorb up to 50% of its dry weight of water [52], high starch concentrations might induce mass transfer limitations or reduce the water activity, which would reduce the enzyme activity. On the other hand, starch granules might also facilitate the hydrolysis:

Native wheat gluten shows a strong tendency to aggregate in the presence of water at neutral pH close to its isoelectric point, which then introduces limitations to the transfer of the enzyme and the hydrolysis products. While hydrated vital wheat gluten forms gluten aggregates of up to some centimeter length [57], the gluten aggregates in the presence of wheat starch in wheat flour batters are millimeter-sized [129] and thus easier accessible for the protease.

In this study, we therefore compare high-solid wheat flour hydrolysis with vital wheat gluten hydrolysis. First, the influence of starch on wheat gluten hydrolysis is investigated and the molecular mass distributions and the protein solubility of the hydrolysates are analyzed. Second, the influence of varying wheat flour concentrations from 20% to 70% (w/w) on protein hydrolysis is studied.

6.2 Materials & Methods

6.2.1 Materials

Ibis wheat flour with 12.6±0.4% (N×5.7, Dumas method) crude protein content and 13.1±0.5% water content (all by weight) was obtained from Meneba (Rotterdam, The Netherlands). Vital wheat gluten (Roquette) with 73.5±1.3% crude protein content and 8.9±0.6% water content was obtained from Barentz BV (Hoofddorp, The Netherlands). Wheat starch with 10.5±0.7% water content was obtained from Sigma-Aldrich, Steinheim, Germany. Two commercial protease mixtures from *Aspergillus oryzae* (Flavourzyme ≥ 500 LAPU/g) and from *Bacillus licheniformis* and *Bacillus amyloliquefaciens* (Protamex ≥ 1.5 AU-NH/g) were purchased from Sigma-Aldrich, Steinheim, Germany. Flavourzyme is a mixture of endo- and exopeptidases with mainly exoproteolytic activity [4] and Protamex is an endoprotease [96]. Borosilicate glass beads with 1 mm diameter and glass beads with 5 mm diameter were obtained from Sigma-Aldrich, Steinheim, Germany. Milli-Q water was used in all experiments unless stated otherwise.

6.2.2 Gluten washing

Ibis wheat flour (200 g) was mixed with 120 g of tap water and allowed to rest for 15 min. Then, the dough ball was gently kneaded, placed in a 500-mL beaker full of tap water at ambient temperature, and allowed to stand for 5 min. This step was repeated three times. Afterwards, the remaining starch was continuously washed out under running tap water until the dough ball mass had approximately reduced to 16–18% of the initial mass. The dough ball was then freeze-dried, ground, and used for hydrolysis. The average crude protein and water content of the self-washed Ibis gluten were 86.6±1.1% and 1.9±0.2%, respectively.

6.2.3 Hydrolysis reaction

The hydrolysis reaction was carried out using 200-mL double-walled glass vessels connected to a water bath. Each experiment was conducted with a total mass of 150 g reaction mixture. Table 6.1 shows the process conditions used for hydrolysis at 5.8% protein concentration. All hydrolysis reactions were performed

Table 6.1: Sampling parameters for standard hydrolysis reactions.

Source	Added wheat flour or gluten (g)	Added water (g)	Added wheat starch (g)	Added enzyme (g)	Solid conc.	Protein conc.
vital wheat gluten (VWG)	11.84	138.16		0.087	7.2%	5.8%
wheat flour (WFG)	69.04	80.96		0.087	40%	5.8%
VWG + starch	11.84	83.17	54.99	0.087	40%	5.8%
self-washed Ibis gluten (SWG)	10.05	139.95		0.087	6.6%	5.8%

at an enzyme-to-substrate ratio of 1:100 (w/w). Only the protein fraction was considered as substrate when calculating the enzyme-to-substrate ratio. The hydrolysis temperature was 50 °C. The enzyme was mixed with the water prior to

addition to the substrate. The double-walled glass vessel was closed with a clasp to avoid evaporation of water and stirred using an overhead stirrer at 40 rpm. After reaction, the enzyme was inactivated by heating at 95 °C for 15 min in a water bath. The samples were then freeze-dried, ground and stored for further analysis. Hydrolysis reactions were conducted under floating pH conditions. Standard experiments with Flavourzyme were performed in quadruplicate and with Protamex in triplicate, except for all reactions with the self-washed gluten, which were performed in duplicate. Experiments investigating the influence of the protein concentration on hydrolysis were single experiments.

6.2.4 Degree of hydrolysis (DH%)

The DH% was measured by the o-phthaldialdehyde (OPA) method as described previously [57], with minor modifications: The amount of suspended hydrolysate was varied between 0.6–0.9 mg protein/mL to avoid spectrophotometer absorbance values above 1.5 for samples with a high DH%. Thus, the mass of the added wheat flour hydrolysate powder was approximately 5.5 times higher than for the vital wheat gluten hydrolysate powder. The calculated DH% was the mean of two determinations. The protein concentration of the dried hydrolysates changed after hydrolysis compared to the native powders owing to small differences in the moisture content. Crude protein contents were 74.0±1.2% for vital wheat gluten, 14.2±0.2% for wheat flour, 79.9±1.8% for self-washed Ibis gluten, and 14.3±0.6% for vital wheat gluten plus starch hydrolysates, respectively.

6.2.5 Size-exclusion high-performance liquid chromatography

Size-exclusion HPLC experiments were performed using a Dionex UltiMate 3000 HPLC system (Thermo Scientific) equipped with a PDA detector. Hydrolysate (2.5 mg protein/mL) was suspended in 12.5 mM borax plus 2% (w/w) SDS, stirred overnight, and then centrifuged at 3900 × *g* for 15 min. The resulting supernatant was injected onto a TSKGel G2000 SWXL (300×7.8 mm) column and eluted with 70% / 30% / 0.1% water / acetonitrile / trifluoroacetic acid at a flow rate of 1.0 mL/min and detected at a wavelength of 214 nm. All

experiments were performed in duplicate. The molecular mass was determined using molecular mass markers based on carbonic anhydrase (29,000 Da), α-lactalbumin (14,100 Da), aprotinin (6510 Da), insulin (5700 Da), bacitracin (1420 Da), and phenylalanine (165 Da).

6.2.6 Solubility

Hydrolysate (10 mg protein/mL) was suspended in water and mixed at ambient temperature for 60 min using a Heidolph MultiReax at 1500 rpm and then centrifuged at 3900 × g for 15 min. The resulting supernatant and pellet were freeze-dried and weighed. The nitrogen content of both freeze-dried samples was determined using the Dumas method (N analyzer FlashEA 1112 series, Thermo Scientific, Interscience) with methionine as a standard. The nitrogen solubility index (NSI) was taken as the fraction of soluble nitrogen to total nitrogen. It has been shown that wheat gluten solubility is independent of the pH within a pH range of 4–10 for DH% greater than 5% [70]. Therefore, the pH was not adjusted during the solubility experiments. Nevertheless, the pH remained between 6 and 7 for all solubility experiments carried out.

6.3 Results

6.3.1 Hydrolysis reaction

Fig. 6.1a depicts the influence of the reaction time on the DH% for gluten present in wheat flour (WFG) and vital wheat gluten (VWG). All experiments were performed at a constant protein concentration of 5.8% (w/w), implying that WFG was hydrolyzed at 40% solids, whereas VWG was hydrolyzed at 7.2% solids (Table 6.1). Hydrolysis of WFG resulted in higher DH% than VWG hydrolysis at all reaction times and for both proteases used. Hydrolyses using Flavourzyme were performed four times to test the significance of the difference in DH%, and t-tests revealed a significantly ($\alpha = 0.05$) higher DH% for WFG after 0.5 h, 3 h, and 6 h. Hydrolyses with Protamex were performed in triplicate and t-tests revealed significantly higher DH% for WFG after 1 h, 3 h, and 6 h. Generally, the use of Flavourzyme resulted in higher DH% compared to the use of Protamex, which can

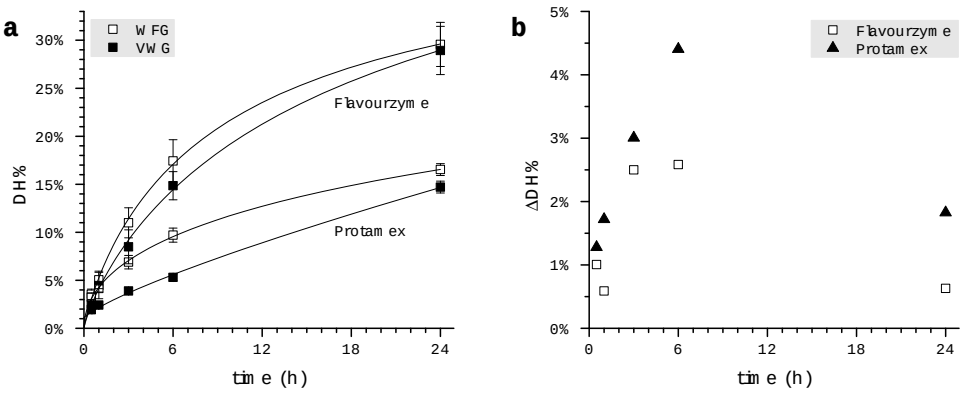

Fig. 6.1: (a) Hydrolysis of gluten in wheat flour (WFG, 40% solids, empty symbols) and vital wheat gluten (VWG, 7.2% solids, filled symbols) hydrolysis as a function of time. Standard deviations are given for quadruplicate Flavourzyme (upper curves) and triplicate Protamex (lower curves) experiments. Lines are drawn to guide the eye. (b) The difference in DH% between WFG and VWG as a function of time.

be explained by the exo-activity of Flavourzyme. Fig. 6.1b reveals an increasing difference in DH% between WFG and VWG up to 6 h for Flavourzyme and Protamex. This difference became less at longer reaction times. The maximum absolute difference was 2.5% for Flavourzyme and 4.4% for Protamex, being 29% and 83% relative increase.

To investigate the influence of the gluten source, hydrolysis reactions were performed, in which either starch was added to vital wheat gluten (VWG + starch) or self-washed gluten (SWG) from the Ibis wheat flour was used (Fig. 6.2). Similar to Fig. 6.1a, hydrolysis of VWG + starch resulted in higher DH% than VWG alone and hydrolysis of WFG resulted in higher DH% than SWG for Flavourzyme and Protamex. In all cases, this difference increased up to 6 h and then decreased again, similar to Fig. 6.1b.

6.3.2 Molecular mass distribution

Fig. 6.3 depicts the molecular mass distributions of WFG, VWG + starch, and VWG hydrolysates after 3 h, 6 h, and 24 h. The most noticeable difference between WFG and VWG after 3 h for Flavourzyme and Protamex is the higher peak for VWG hydrolysates after 6.9 min elution time, representing peptides

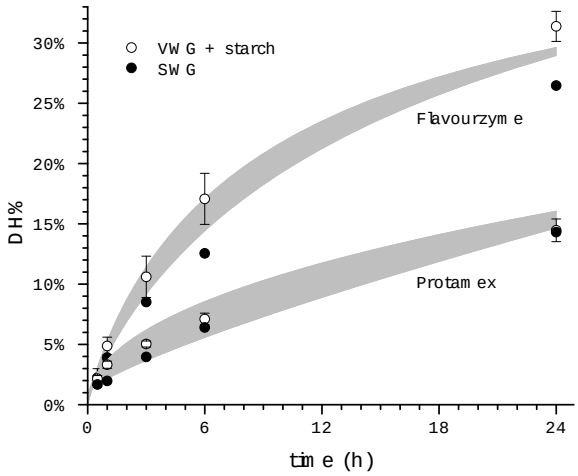

Fig. 6.2: Hydrolysis of VWG + starch and self-washed Ibis gluten (SWG) as function of the reaction time for Flavourzyme (upper curves) and Protamex (lower curves). The upper and lower border of each gray area indicate the DH% of WFG and VWG, respectively from Fig. 6.1a.

with molecular masses of around 10 kDa. For longer elution times and smaller peptide sizes, the peak was higher for WFG, which is in line with the higher DH% in Fig. 6.1a. In the case of Flavourzyme, the difference in the molecular mass distributions between WFG and VWG became smaller after 6 h and 24 h. In the case of Protamex, a difference between WFG and VWG hydrolysates remained after 6 h and 24 h (see peaks after 9.3 and 10.3 min).

The molecular mass distribution of VWG + starch was in-between the molecular mass distributions of VWG and WFG after 3 h and 6 h. After 24 h, the difference between VWG + starch and VWG was negligible. The differences in the molecular mass distributions between Flavourzyme and Protamex (especially the peak after 10.6 min) can be explained by the exo-activity of Flavourzyme.

6.3.3 Solubility

Fig. 6.4 depicts the influence of the DH% on the nitrogen solubility index (NSI) for WFG, VWG + starch, and VWG for Flavourzyme (Fig. 6.4a) and Protamex (Fig. 6.4b). The solubility increased with higher DH%, which agrees with other studies [57, 70]. The highest solubility was 89% for both Flavourzyme and

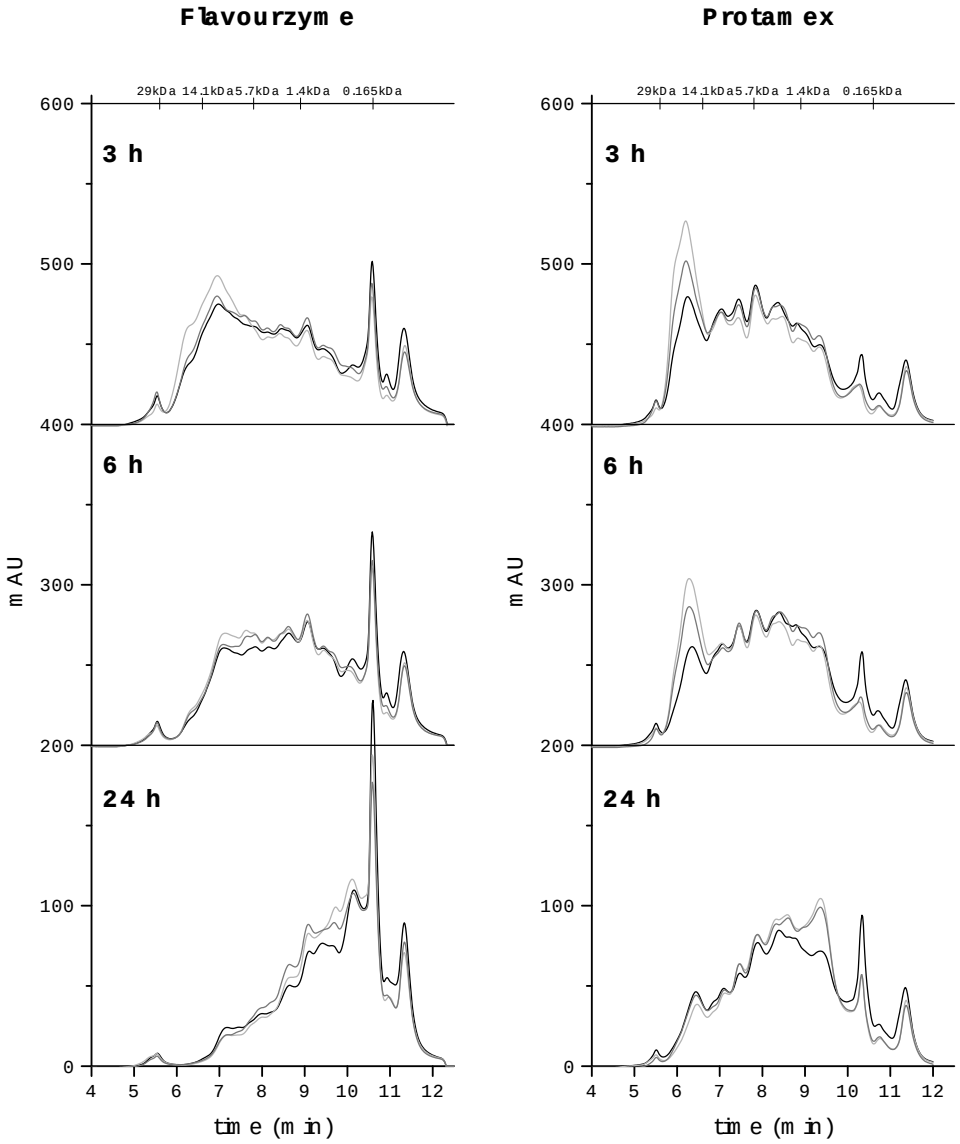

Fig. 6.3: Molecular mass distributions of vital wheat gluten (VWG, light gray), VWG + starch (dark gray), and gluten in wheat flour (WFG, black) after 3 h, 6 h, and 24 h for Flavourzyme (left) and Protamex (right). Molecular mass distributions are averages of triplicate or quadruplicate hydrolysis reactions. Curves were modified in a way that all have the same area under the curve.

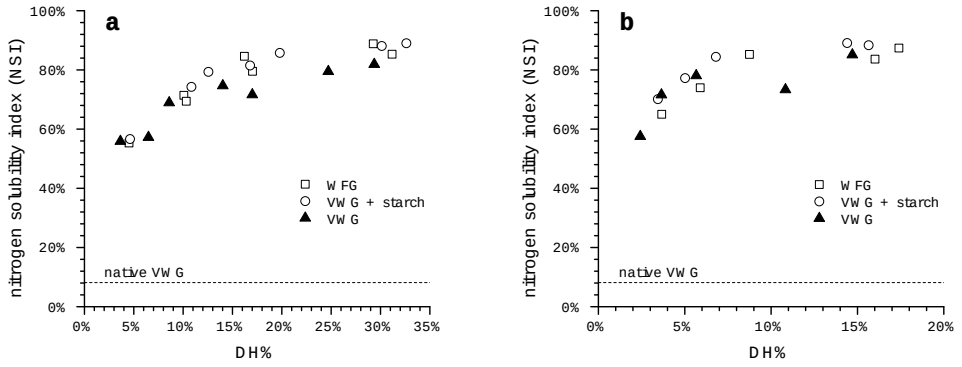

Fig. 6.4: Influence of the DH% on the nitrogen solubility index (NSI) for different gluten sources for (a) Flavourzyme and (b) Protamex. The lower dashed line represents the NSI of native vital wheat gluten. The legend indicates the gluten source. Data points are single experiments, taken from two different hydrolysis reactions for every gluten source.

Protamex, but in case of Flavourzyme a higher DH% was needed to obtain this solubility value. The effect on solubility was independent of the gluten source for WFG and VWG + starch for both proteases. The use of Flavourzyme resulted in a VWG solubility that was approximately 5–10% lower compared to WFG and VWG + starch at DH% above 10%, though the mechanism behind this observation is not clear. For Protamex no such difference was found.

6.3.4 Influence of the solid content

Fig. 6.5 illustrates the influence of the protein concentration on the DH% for VWG and WFG after 3 h, 6 h, and 24 h, using Flavourzyme to investigate the influence of high wheat flour concentrations. The protein concentration of 5.8% represents the data given in Fig. 6.1. Generally, the DH% for WFG decreased stronger with increasing protein concentration than the DH% for VWG. This effect was stronger at longer hydrolysis times. Therefore, the positive effect of starch on wheat gluten hydrolysis diminished at higher solid concentrations.

The small decrease in DH% for VWG in Fig. 6.5 is related to a concentration effect present in protein hydrolysis: the DH% decreases for higher protein concentrations and at constant enzyme-to-substrate ratio. This concentration effect has been reported for protein concentrations above 10% in wheat gluten

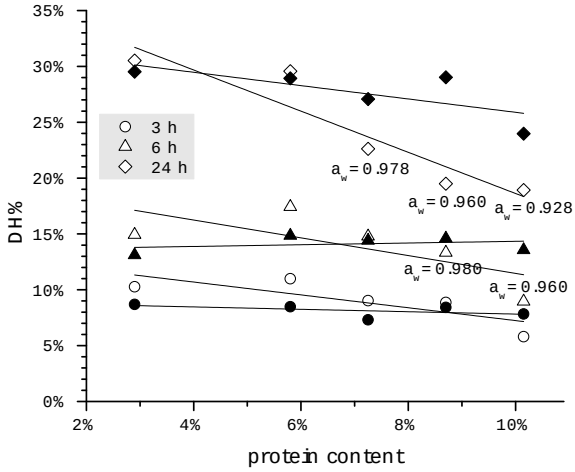

Fig. 6.5: Influence of wheat flour (empty symbols) and vital wheat gluten (filled symbols) concentrations on the DH%. Flavourzyme and a constant enzyme-to-substrate ratio of 1:100 (w/w) were used. Wheat flour concentrations were 20%, 40%, 50%, 60%, and 70%, respectively. Vital wheat gluten concentrations were 3.6%, 7.2%, 9.0%, 10.8%, and 12.6%, respectively. For comparison, the DH% is plotted as a function of the protein concentration. The legend indicates the reaction time. The water activity is shown only for $a_w \leq 0.980$. Lines are linear trend lines.

hydrolysis [56] and above 1% in whey protein hydrolysis [16], and is thus also present at relatively low wheat gluten concentrations of 3–10%.

6.4 Discussion

The initial hydrolysis rate of gluten in wheat flour (WFG) hydrolysis is higher than in vital wheat gluten (VWG) hydrolysis at similar protein concentration (Fig. 6.1). Also the initial hydrolysis rate of VWG + starch is higher than of VWG (Fig. 6.2). The higher initial hydrolysis rates are interesting, because proteases account for a large part of the costs in industrial hydrolysis [38] and increases in DH% in hydrolysis reactions are of a logarithmic nature. Hence, an increase of the hydrolysis rate either reduces the amount of enzyme needed and thus the costs, or reduces the reaction time. Below, different factors will be evaluated, that could explain the difference in DH% for WFG and VWG observed in Fig. 6.1.

6.4.1 Influence of starch, protein and pH

Native VWG forms bigger aggregates than native gluten in its starch matrix (WFG). As mentioned, hydrated vital wheat gluten forms gluten aggregates of up to some centimeter length [57], while gluten aggregates in wheat flour batters are millimeter-sized [129]. We observed the same in this study, where a homogeneously looking slurry was formed for WFG and VWG + starch hydrolysis from the beginning, but big gluten lumps formed in VWG and SWG hydrolysis, and the slurry only appeared homogeneous after 2–3 h of hydrolysis. We therefore hypothesize that the presence of non-proteinaceous components, mainly starch, hinders the aggregation of the water-insoluble gluten; the presence of starch granules keeps the size of the wheat gluten patches small, while the starch granules sterically prevent the aggregates from merging at the same time. Smaller aggregates sizes are equivalent to an increase in the overall surface area. Especially at the beginning of the reaction, when (native) wheat gluten is water-insoluble, the smaller aggregates can be better accessed by the enzyme than the larger aggregates. This easier access of the enzyme then results in a higher hydrolysis rate. At longer reaction times, the solubility increased (Fig. 6.4), the aggregates disappeared, and with it the positive effect of the starch granules on gluten hydrolysis.

This conclusion is supported by the decreasing difference in DH% between WFG and VWG between 6 h and 24 h (Fig. 6.1b) and by the decreasing difference in the molecular mass distribution between VWG and VWG + starch between 6 h and 24 h (Fig. 6.3), showing that the difference in DH% originates from a kinetic effect: the reduced hydrolysis rate can be compensated by a longer reaction time.

Other factors could also be responsible for the difference in DH% for WFG and VWG observed in Fig. 6.1. In the following it will be explained why they cannot solely explain this difference:

(a) WFG and VWG originate from different gluten sources (Ibis wheat flour and Roquette vital wheat gluten) with possibly different compositions in gliadin and glutenin and different gliadin/glutenin-ratios. However, Fig. 6.2 shows higher DH% for VWG + starch than for VWG and higher DH% for WFG than

for SWG. Furthermore, Fig. 6.3 indicates that the molecular mass distribution is independent of the gluten source used and just a function of the DH% and the enzyme used. Hence, the gluten source did not cause the difference in the hydrolysis rate.

(b) Wheat flour contains 15% albumins that are water-soluble and 7% globulins that are soluble in dilute salt solutions [8], and which are not present in VWG. It has been stated that soluble wheat proteins are easier to degrade than insoluble proteins [7]. Therefore, the higher DH% for WFG could be induced by the albumins and globulins. However, Fig. 6.2 shows again that this effect cannot solely be explained by the globulins and albumins present in WFG, because higher DH% were obtained for VWG + starch than for VWG without starch, and no protein was detected in the added starch using the Dumas method. Furthermore, the protein solubility was just a function of the DH% but independent of WFG or VWG + starch (Fig. 6.4).

(c) Differences in the pH can also not explain the difference in DH%. We measured the pH of some samples after 24 h. The pH always remained above 4 for Flavourzyme and Protamex and was approximately 0.5 higher for VWG than for WFG due to the differences in the water content. Since the optimum pH range for Flavourzyme is stated to be between 4 and 8 and for Protamex between 6 and 8 [97], if at all there was a difference in enzyme activity, then a higher enzyme activity would be expected for VWG than for WFG hydrolysis.

(d) The DH% and molecular weight distributions were measured by first dissolving the gluten hydrolysate in 12.5 mM borax plus 2% (w/w) SDS. The solubility of the VWG and WFG hydrolysates in this medium was tested to exclude the possibility that differences in solubility influenced the DH% and molecular mass measurements. After 6 h, when the difference in DH% between WFG and VWG was the highest, 97.9% of WFG and 98.9% of VWG were soluble. Therefore, the samples were nearly completely soluble in borax plus SDS.

6.4.2 Influence of the solid content

Fig. 6.1 and Fig. 6.4 compare the hydrolysis of vital wheat gluten (VWG) and gluten in wheat flour (WFG) at 5.8% protein concentration, i.e. 40% wheat

flour and 7.2% VWG. Given the fact that starch enhanced the hydrolysis, we believe that the starch at 40% wheat flour (i.e. approximately 30% starch) did not induce any mass transfer limitations. This is in line with a previous study, where no mass transfer limitations were observed in high-solid VWG hydrolysis up to 50% solids [56]. Similar observations were done in a high-solid cellulose hydrolysis, where it was shown that the addition of 15% (w/w) water-insoluble, non-hydrolyzable dextrans had no effect on the hydrolysis of 5% cellulose slurries [120], which is comparable to water-insoluble, non-hydrolyzable starch in gluten hydrolysis.

However, the hydrolysis will be hindered by the starch content at some concentration. For instance, hydrolysis of 100% wheat flour as an extreme is not possible, because the enzyme cannot diffuse to the substrate (mass transfer limitations) and no water is available as reactant (low water activity). Therefore, Fig. 6.5 demonstrates that the positive effect of starch on wheat gluten hydrolysis diminishes at higher wheat flour concentrations. Similar to high-solid VWG hydrolysis [56] mass transfer limitations can explain why the positive effect of starch disappeared after 3 h and 6 h above 60% wheat flour. Additionally, we believe that the reduced water activity further impedes WFG hydrolysis at longer reaction times. Water activities below 0.98 can already reduce that enzyme activity [56]. Therefore, samples with $a_w \leq 0.980$ are denoted in Fig. 6.5. The water activity reduced especially at longer reaction times and higher wheat flour concentrations down to 0.928 at 70% wheat flour after 24 h. This explains why VWG hydrolysis became already favorable at 7.3% protein (i.e. 50% wheat flour; 9% VWG) after 24 h.

6.4.3 Prevention of aggregation

If the difference in DH% in Fig. 6.1 originates from reduced gluten aggregation, then the hydrolysis rate in wheat gluten hydrolysis can be theoretically improved independent of the starch. One option could be the usage of more sophisticated mixing devices that disrupt gluten aggregates, such as kneaders, extruders, or static mixers. Another alternative could be the use of additives other than starch. Therefore, we conducted duplicate VWG hydrolyses with Flavourzyme where either 1-mm or 5-mm glass beads were added. Higher DH% compared to VWG

hydrolysis were measured after 1 h and 3 h (1.6% and 1.8% higher, respectively) for 1-mm glass beads addition. For longer reaction times and for the 5 mm glass beads no difference in DH% was observed. Hence, the effect of glass beads was less pronounced than the effect of starch. The reason for this could be related to the higher density of the glass beads. Another explanation could be the larger sizes of the beads compared to the starch granules (<0.1 mm). Then, the positive effect of additives would diminish with increasing size. Nevertheless, the 1-mm glass beads confirm the results obtained with the starch granules, being that prevention of gluten aggregation can be a route to facilitate the hydrolysis of wheat gluten. The concept of adding an inert filler that is easy to separate from the hydrolyzed mixture seems to be an industrially feasible route.

6.5 Conclusions

We studied the influence of the presence of starch on wheat gluten hydrolysis. Wheat gluten in wheat flour (WFG) was hydrolyzed at 5.6 times higher solid concentrations but at the same protein concentrations than vital wheat gluten (VWG). WFG hydrolysis at 40% solids resulted in higher DH% than VWG hydrolysis at 7.2% solids. Furthermore, higher DH% were obtained when hydrolyzing VWG with added starch, compared to VWG alone and for WFG hydrolysis compared to the washed gluten fraction of the wheat flour. This showed that differences in the gluten composition and the presence of albumins and globulins in wheat flour cannot explain the difference in DH%. Instead, we concluded that starch granules facilitated the hydrolysis reaction by hindering the gluten aggregation. The final composition of the hydrolysates, though, was just a function of the enzyme used and the DH%, but not influenced by the starch. At higher solid concentrations up to 70% wheat flour, the positive effect of starch disappeared, because WFG hydrolysis was hindered by mass transfer limitations and lower water activities.

The results show that the understanding and prevention of wheat gluten aggregation is important in order to improve wheat gluten hydrolysis, and could be a drive for mixer design or the use of additives other than starch.

Acknowledgements

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CHAPTER 7

General discussion

7.1 Outline

In the following general discussion, the main findings of the preceding chapters are summarized. Then, outcomes will be generalized by comparing the effects of different proteases on concentrated wheat gluten hydrolysis. Afterwards, the findings of this thesis are used to compare the current status of starch–gluten separation and gluten hydrolysis with proposed separation and hydrolysis processes utilizing reduced water contents; the focus is on improvements in water, energy, and reactor volume usage. In the end, future prospects in high-solid wheat gluten hydrolysis are briefly discussed.

7.2 Findings

Chapters 2 and 3 describe the possibility of removing water associated with arabinoxylan to make it available for other wheat components. In **chapter 2**, wheat dough rheology at very low water content is studied. Decreasing the water content from 43.5% (w/w) to 34% results in a non-linear increase in the dough consistency, elastic modulus G', and a decrease in the maximum creep compliance $J_{c,max}$ of 1–2 orders of magnitude. This is related to a reduction of the free water, and consequently, a reduced plasticizing effect of the water. The mechanism of hydration, however, is not affected as indicated by $tan\ \delta$ and the elastic strain J_{el} being independent of the water content. The addition of two xylanases, Grindamyl and Shearzyme, results in a decrease in the dough consistency and G' and an increase in $J_{c,max}$. The main influence of both xylanases is attributed to the release of water, but hardly any influence on $tan\ \delta$ and no influence on J_{el} are observed, confirming that the mechanism of hydration is hardly influenced by the water availability.

In **chapter 3**, the influence of xylanases on the shear-induced starch–gluten separation is tested with water contents ranging from 34% to 43.5%. Shearing after addition of xylanase results in a slurry without any separation at the standard water content of 43.5%. As a result, water contents below 40% are used, which results in the local formation of gluten clusters with and without xylanase usage. However, no inward migration of these patches occurs, which is opposed to shear-induced separation at 43.5% water without xylanase. Nevertheless, gluten

clusters with up to 60% protein (N×5.7) are formed. Chapters 2 and 3 show that the amount of water released through xylanases corresponds to about 2–5% water on a total water basis.

Chapters 4, 5, and 6 report on wheat gluten hydrolysis at high solid concentrations (≥ 40% solids). **Chapter 4** describes the influence of high solid concentrations on enzymatic wheat gluten hydrolysis and the resulting functional properties of the gluten hydrolysates. It is possible to hydrolyze wheat gluten up to 60% solids (w/w). The water solubility of the dried hydrolysates at similar degrees of hydrolysis (DH%) is independent of the solid concentration during the hydrolysis, just like the foam stability at DH% below 8%. At DH% higher than 8%, high solid concentrations even increase the foam stabilizing properties of the resulting hydrolysates, which is related to the presence of more peptides with a molecular mass >25 kDa. Furthermore, an increase in solid concentration results in an increase of the volumetric productivity.

Chapter 4 also shows that the hydrolysis rate decreases at higher solid concentrations and constant enzyme-to-substrate ratios. The factors causing this hydrolysis rate limitation are investigated in **chapter 5**. It is shown that enzyme inhibition, the water activity, and mass transfer limitations do not cause the hydrolysis limitation up to 50% solids. However, the hydrolysis rate limitation can be explained by a second-order enzyme auto-inactivation rate along with the higher enzyme concentrations used. The hydrolysis rate further decreases at solid concentrations above 50% due to mass transfer limitations. Furthermore, the addition of enzyme after 24 h at high solid concentrations hardly increases the DH%, suggesting that the maximum attainable DH% decreases at high solid concentrations. This DH% limitation is explained by a reduced enzyme activity due to a decline in water activity.

In **chapter 6**, we compare high-solid wheat flour hydrolysis with vital wheat gluten hydrolysis. For doing so, wheat gluten present in wheat flour is hydrolyzed, thereby omitting the starch–gluten separation step. At a constant protein concentration of 5.8% (w/w), the protease activity is higher for wheat flour hydrolysis (at 40% total solids) than for vital wheat gluten hydrolysis (at 7.2% total solids) in the initial 6 h of hydrolysis, despite the high starch content

in wheat flour and consequently lower water content. This is related to the strong aggregation of vital wheat gluten at low DH%, which is impeded by the starch granules. After 6 h, the difference in DH% diminishes again. At wheat flour concentrations above 50% solids and for longer reaction times, the positive effect of starch on gluten hydrolysis disappears. This is explained by mass transfer limitations and reduced water activities in the wheat flour slurry or dough, respectively.

7.3 The importance of the enzyme concentration

In chapter 4, we show that wheat gluten can be hydrolyzed at solid concentrations up to 60% without detrimental changes in the functional properties of the hydrolysate. In chapter 5, we show that no mass transfer limitations occur up to 50% solids (Fig. 5.7). In conclusion, hydrolyzing gluten at 50% instead of 20%, as commonly done in industry [22, 84], is possible and has advantages regarding the water and energy consumption and the volumetric productivity (chapter 4).

Nevertheless, the rate of hydrolysis decreases with increasing solid concentration at constant enzyme-to-substrate ratio, implying that the difference in DH% between low and high solid concentration increases over time. This means that longer reaction times are needed at higher solid concentrations to reach the final DH%, or that more enzyme is needed to keep the reaction time constant. We conclude in chapter 5 that this hydrolysis rate limitation is caused by the higher enzyme concentration, rather than the higher solid concentration used. To check whether this is a general feature of gluten hydrolysis, we investigated more commercial enzyme mixtures: Protamex, Neutrase 0.8 L, and Alcalase 2.4 L (Fig. 7.1). The left-hand side of Fig. 7.1 shows the hydrolysis rate limitation for Protamex, Neutrase, and Alcalase. Again, higher solid concentrations resulted in lower DH% at constant enzyme-to-substrate ratios. Hydrolysis with Protamex, Neutrase, and Alcalase all three resulted in a lower DH% compared to Flavourzyme (Fig. 5.3), which can be explained by the specificity of the proteases. Alcalase, Neutrase, and

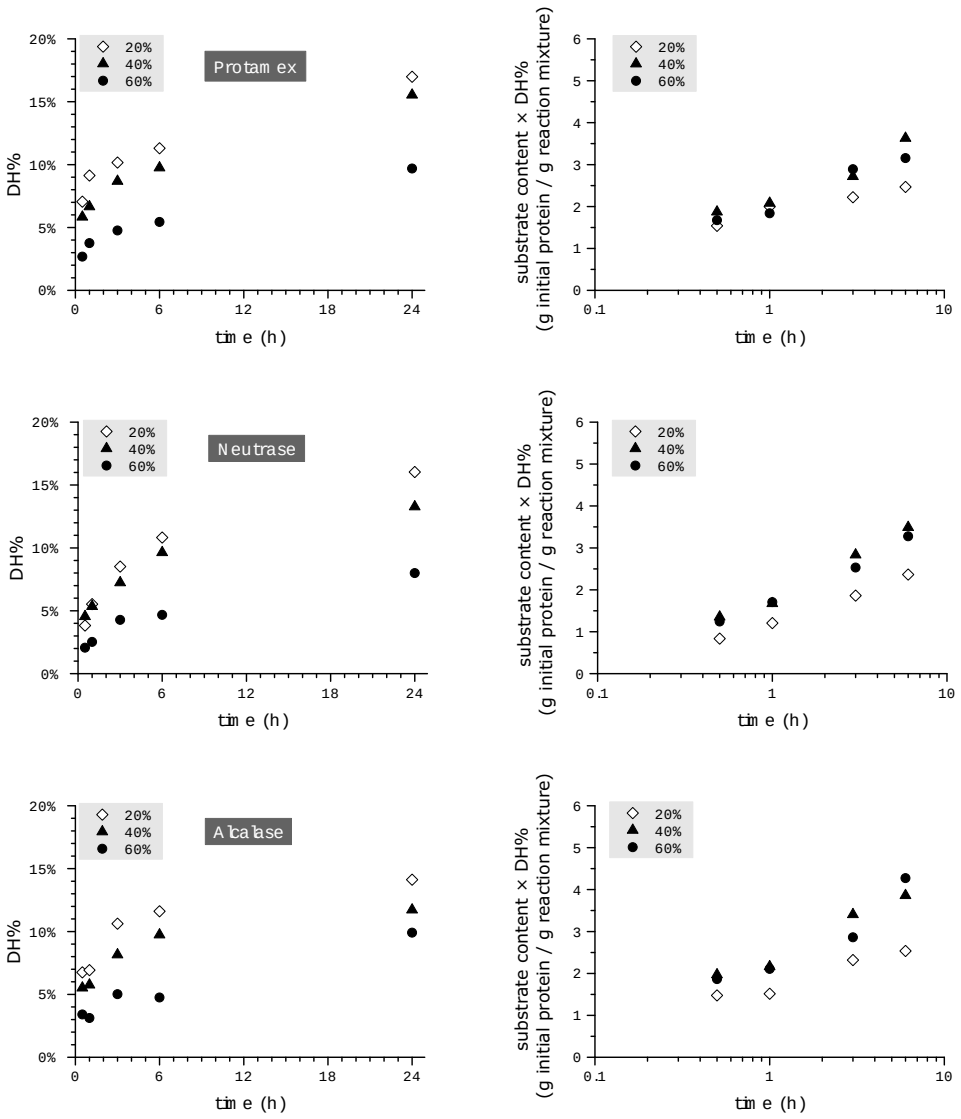

Fig. 7.1: The hydrolysis rate limitation at a constant enzyme-to-substrate ratio of 1:30 (left) and the influence of the solid concentration on the total conversion (right) for Protamex, Neutrase, and Alcalase. The legend indicates the solid concentration during hydrolysis. Results were obtained in the same way as described in section 5.2.2 for Flavourzyme.

Protamex are endoproteases [96], while Flavourzyme is a mixture of endo- and exopeptidases with mainly exoproteolytic activity [4].

The right-hand side of Fig. 7.1 shows the influence of the reaction time on the total conversion, calculated as the substrate content × DH% at a constant enzyme concentration of 0.81 g enzyme/g reaction mixture. This total conversion is proportional to the total number of cleaved peptide bonds. Similar to Flavourzyme (Fig. 5.7), the enzyme activity was not affected by higher solid concentrations up to 40%. In fact, the amount of peptide bonds cleaved was even lower at 20% solids for Protamex, Neutrase, and Alcalase, which can be explained by substrate exhaustion. At 60% solids, the enzyme activity decreased, supposedly again due to mass transfer limitations, which is in line with the results obtained for Flavourzyme.

Fig. 7.1 and Fig. 5.7 show lower total conversions for lower and also for higher solid concentrations up to 6 h of hydrolysis. Fig. 7.2 shows the same trend for the initial hydrolysis rate as a function of the solid concentration for Flavourzyme at enzyme concentrations of 2.7 and 8.1 U/g reaction mixture (Fig. 7.2a) and for Protamex, Neutrase, and Alcalase at 2.7 U/g reaction mixture (Fig. 7.2b). The initial hydrolysis rate here refers to the conversion after 0.5 h hydrolysis. The results in Fig. 7.2 are in line with Cheng and Prud'homme [20], who demonstrated three concentration regions for high-solid guar hydrolysis: at very low concentrations (1) the reaction rate is controlled by the substrate or solid concentration (first-order), at intermediate concentrations (2) the conversion is controlled by the enzyme cleavage kinetics (zero-order), and at very high concentrations (3) the diffusion of the enzyme molecules reduces so much that it controls the process (mass transfer limitations). The same trend is observed for Flavourzyme at two different enzyme concentrations. For Protamex, Neutrase, and Alcalase (Fig. 7.2b) only three data points of single experiments are available for each respective protease, which makes such a clear distinction in concentration regions difficult. Nevertheless, the initial hydrolysis rate increased up to 40% solids and then reduced again for all three proteases.

Although it has been questioned whether the standard Michaelis-Menten model $V = V_{max} \cdot \frac{S_m}{S_m + k_m}$ completely applies in high-solid hydrolysis [72], the zero-order concentration regime number 2 in Fig. 7.2a relates to $S >> k_m$,

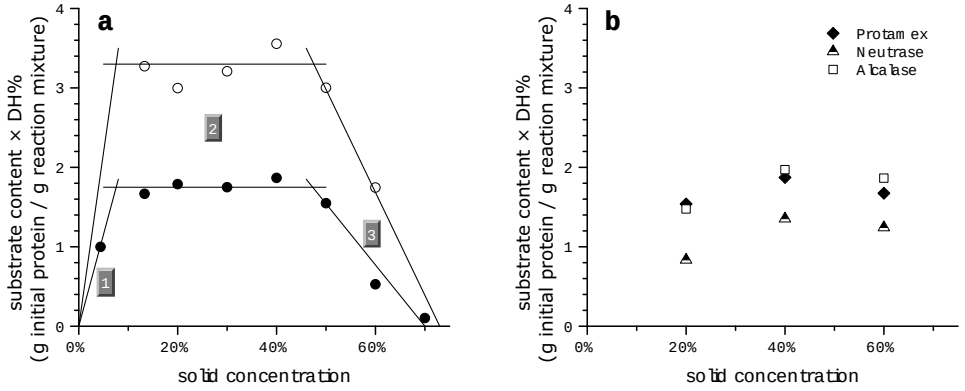

Fig. 7.2: Initial hydrolysis rate as function of the solid concentration. Initial hydrolysis rate here refers to the conversion after 0.5 h hydrolysis. (a) Flavourzyme hydrolysis at 2.7 (filled symbols) and 8.1 (empty symbols) U/g reaction mixture. The solid concentration regions 1, 2, 3 are marked according to Cheng and Prud'homme [20]. For further details see the text. (b) Protamex, Neutrase, and Alcalase hydrolysis at 0.81 g enzyme/g reaction mixture. The legend indicates the protease used.

where V becomes V_{max}. In this zero-order regime, the influence of the enzyme concentration on high-solid hydrolysis can be investigated without the influence of the solid concentration. Therefore, Fig. 7.3 depicts the total amount of hydrolysis per enzyme molecule (calculated as the substrate content multiplied by the DH% and divided by the initial enzyme concentration) as a function of the initial enzyme concentration for Protamex, Neutrase, Alcalase, and Flavourzyme. Fig. 7.3 is an extension of Fig. 5.9 with more proteases. Higher initial enzyme concentrations result in a reduced total amount of hydrolysis per enzyme molecule for Protamex, Neutrase, and Alcalase, which has been related to protease self-inactivation in case of Flavourzyme (chapter 5). This decrease cannot be compensated by adding more substrate. This suggests that a lower total amount of hydrolysis per enzyme molecule at higher enzyme concentrations is a generic property in gluten hydrolysis for most or all proteases. Furthermore, it implies that the hydrolysis rate limitation in wheat gluten hydrolysis is caused by the higher enzyme concentration, independent of the enzyme used. The same argumentation does not hold true for the solid concentration. Here, the total conversion per initial solid concentration (i.e. the DH%) will also decrease with higher solid concentrations (Fig. 5.2b). However,

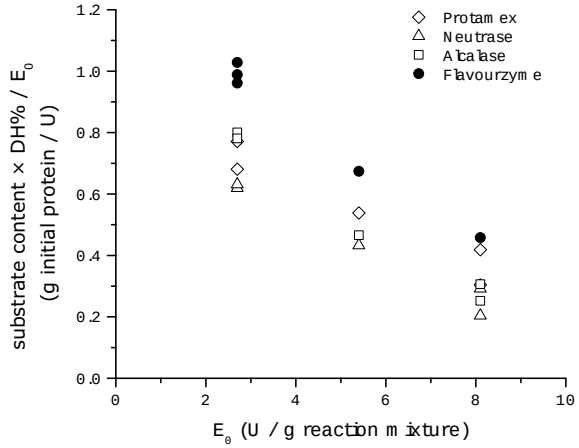

Fig. 7.3: Influence of the initial enzyme concentration on the total amount of hydrolysis per initial enzyme concentration after 1 h. The legend indicates the enzyme used. Solid concentrations were 40–60% for Protamex, Neutrase and Alcalase, and 30–50% for Flavourzyme. Enzyme concentration was 0.81 g enzyme/g reaction mixture. Results were obtained in the same way as for Flavourzyme as described in section 5.2.2.

this decrease can be compensated to some extent by adding more enzyme (see appendix A.3, Fig. A.3).

The hydrolysis rate limitation described in this thesis does not only seem to be a generic effect in wheat gluten hydrolysis, but also a generic effect for high protein concentrations at constant enzyme-to-substrate ratios in general. Butré et al. [16] showed similar results regarding the hydrolysis rate limitation for whey protein hydrolysis. The finding that the hydrolysis rate limitation is related to the enzyme and not the substrate indeed suggests that the results of this thesis for high-solid wheat gluten hydrolysis can be extended to other protein systems as well.

7.4 Separation and hydrolysis at high solid contents – comparison of water, energy and volume consumption

7.4.1 Qualitative comparison

This thesis describes the separation of wheat starch and gluten and the subsequent hydrolysis of gluten at concentrated conditions. But what do the results of this thesis imply when comparing the current standard industrial separation and hydrolysis with the findings of this study? Fig. 7.4 shows Sankey diagrams to compare the standard process (Fig. 7.4a) with a shear-induced starch–gluten separation with a subsequent hydrolysis at 50% solids (Fig. 7.4b) and the hydrolysis of wheat gluten in wheat flour at 40% solids with a subsequent separation (Fig. 7.4c). The key figures are given in the following text to demonstrate the main effect. Besides, only the main components in wheat flour and derived products thereof are considered, being gluten, starch and water. An overview of all other assumptions and calculations is given in the appendix A.4.

Fig. 7.4a shows the standard process in industry, which requires the addition of 2.6 L water per kg of wheat flour [132]. Here, typically a dough or batter is formed after which additional water is added to wash out the starch. The resulting streams consist of: (a) The gluten stream with approximately 80–85% gluten (on a dry basis), pentosans, starch, and lipids. Approximately 20% of the added water ends up in the gluten stream [132]. (b) The A-starch stream contains >99% starch (on a dry basis) and 30% of the added water. (c) The rest stream contains pentosans, water-soluble proteins, B-starch, and 50% of the added water. All three streams are dried after separation. The dried A-starch stream can be used further e.g. for starch hydrolysis. The dried rest stream is used as feedstuff [90]. The dried gluten stream in Fig. 7.4a is rehydrated and hydrolyzed at 20% solids, which represents the solid concentration commonly used in industry [22, 84]. Subsequently, the wheat gluten hydrolysate is dried.

Fig. 7.4b shows the separation and hydrolysis of wheat gluten at concentrated conditions. Starch and gluten are separated by shear at 42.7% water (i.e. 0.5 L

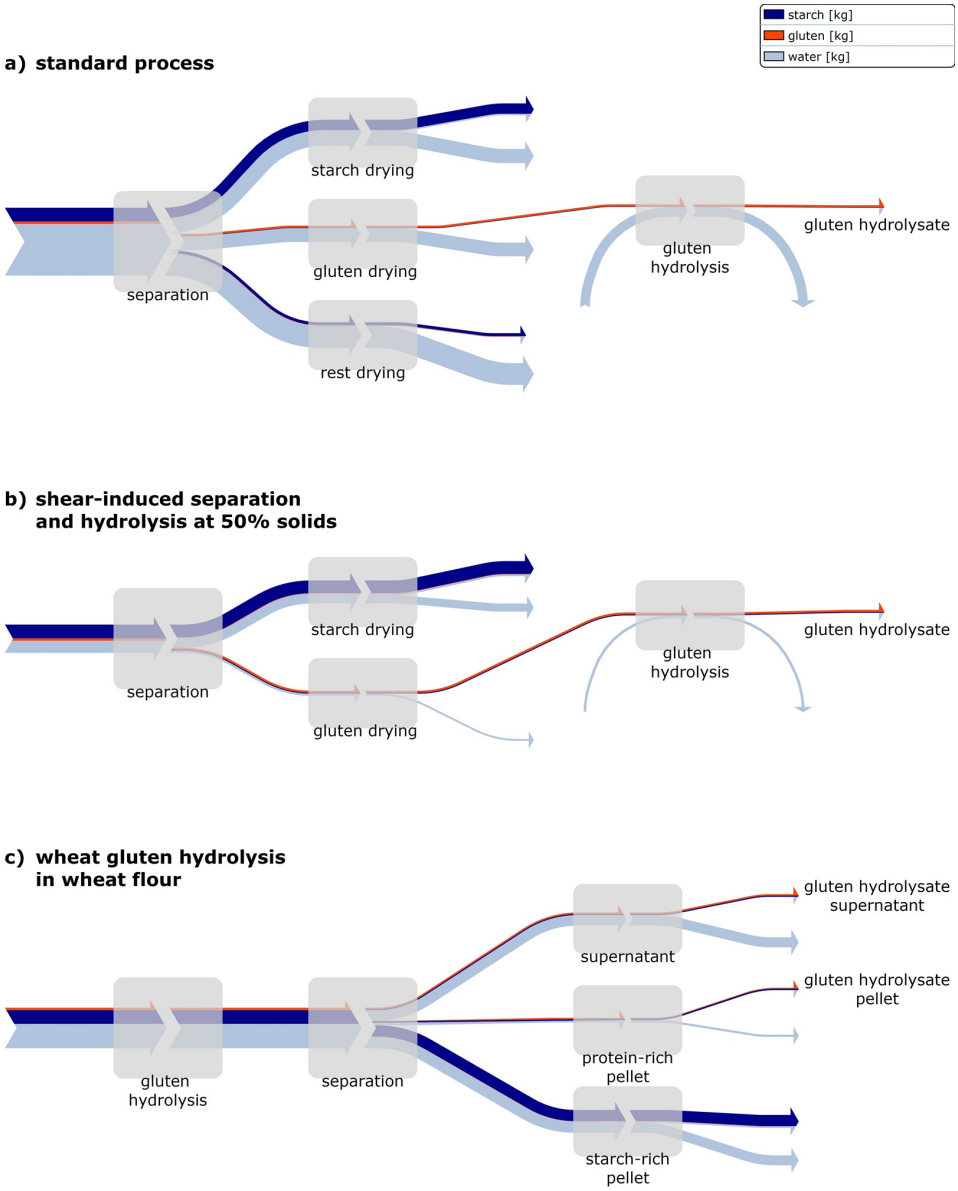

Fig. 7.4: Sankey diagrams. Schematic overview of the water consumption for: (a) standard starch–gluten separation and gluten hydrolysis at 20% solids, (b) shear-induced starch–gluten separation and gluten hydrolysis at 50% solids, and (c) high-solid wheat gluten hydrolysis in wheat flour at 40% solids with a subsequent separation. See text and appendix A.4 for assumptions made.

water addition per kg dry matter). It is assumed that gluten can be concentrated up to 60% protein (on a dry basis, Fig. 3.5), that 80% protein is recovered and that consequently the starch-rich layer contains 3.1% protein. After drying, the gluten-rich phase is hydrolyzed at 50% solids, which is the highest solid content without any mass transfer limitations (chapter 5).

Fig. 7.4c shows the direct hydrolysis of wheat gluten in wheat flour, thereby making the pre-separation step superfluous. Instead, starch and gluten are separated after hydrolysis using a hydrocyclone. The wheat gluten is hydrolyzed at 40% solids, where centrifugal separation of the starch and the hydrolysate is possible. After centrifugal separation, the supernatant contains 49.7%, the protein-rich pellet 30.6%, and the starch-rich pellet 2.0% protein, respectively (Table 7.1).

Table 7.1: Protein content and yield after centrifugal separation of starch and hydrolyzed wheat gluten. Hydrolysis was performed with Flavourzyme for 1 h and an enzyme-to-substrate ratio of 1:100. The picture shows a wheat flour hydrolysate after centrifugal separation with the supernatant, protein-rich pellet, and starch-rich pellet from top to bottom.

	protein content (on a wet basis)	protein yield
supernatant	49.7%	59.3%
protein-rich pellet	30.6%	31.0%
starch-rich pellet	2.0%	9.6%

7.4.2 Quantitative comparison

Table 7.2 shows the water, energy and volume consumption to quantitatively compare the standard process, shear-induced separation and hydrolysis at 50% solids, and high-solid wheat flour hydrolysis outlined in Fig. 7.4. The DH% of the gluten hydrolysate is assumed to be 5%. Since this thesis focuses on wheat gluten, Table 7.2 shows the water, energy, and volume consumption in the separation process in relation to the gluten. For this, only the mass of water, gluten and starch entering the gluten stream are considered, which is known for all three processes introduced. For instance, the water consumption in the standard process is the water entering the gluten drying process divided by

Table 7.2: Water, energy, and volume consumption for the standard process, shear-induced separation and hydrolysis at 50% solids, and high-solid wheat flour hydrolysis. High-solid wheat flour hydrolysis considers supernatant and protein-rich streams as gluten streams. Hydrolysis times for hydrolysis to DH% 5%. See appendix A.4 for other assumptions made.

	time		water consumption		energy consumption		volume consumption	
	separation	hydrolysis	separation	hydrolysis	separation	hydrolysis	separation	hydrolysis
	h	h	$\frac{\text{L}}{\text{kg gluten}}$	$\frac{\text{L}}{\text{kg hydrolysate}}$	$\frac{\text{MJ}}{\text{kg gluten}}$	$\frac{\text{MJ}}{\text{kg hydrolysate}}$	$\frac{\text{m}^3}{\text{tonne gluten/h}}$	$\frac{\text{m}^3}{\text{tonne hydrolysate/h}}$
standard process (Fig. 7.1a)	1.25	1.25	7.1	4.9	35.2	24.7	10.0	7.4
shear-induced separation and hydrolysis at 50% (Fig. 7.1b)	0.5	1.75	1.1 (-84%)	1.3 (-73%)	6.5 (-81%)	7.6 (-69%)	1.1 (-89%)	4.6 (-39%)
high-solid wheat flour hydrolysis (Fig. 7.1c)	1		5.8 (-51%)		29.8 (-50%)		7.4 (-58%)	

the mass of separated gluten. Since it is unlikely that the starch is not utilized after separation, an overview of the volume, water, and energy consumption considering the whole wheat flour is given in the appendix A.4 (Table A.3). Although the comparison in Table 7.2 can only give an estimation of the actual water, energy, and volume consumption in a real process, the data is meaningful when comparing the different processes:

Water consumption: To separate the gluten from the starch and subsequently hydrolyze it at 20% solids, 7.1 L/kg gluten and 4.9 L/kg gluten hydrolysate are needed. For shear-induced separation and hydrolysis at 50% solids, these figures reduce by 84% and 73%, respectively. For high-solid wheat flour hydrolysis, 6.2 L/kg gluten hydrolysate are required for hydrolysis and separation. This figure reduces by 51% compared to the separation and the hydrolysis in the standard process (7.1 L/kg gluten + 4.9 L/kg hydrolysate).

Energy consumption: The standard process consumes 35.2 MJ/kg gluten during separation and 24.7 MJ/kg hydrolysate during hydrolysis. Since only short reaction times are necessary for hydrolysis until DH% 5%, the drying of the gluten, and gluten hydrolysate consumes most of the energy and the mixing and heating energy is less than 25% of the total energy. Therefore, the savings in energy consumption for shear-induced separation and hydrolysis at 50% solids and high-solid wheat flour hydrolysis are closely related to the savings in water consumption.

Volume consumption: The standard process requires 10.0 m^3 to separate and 7.4 m^3 to hydrolyze a tonne of gluten per hour. Again, this figure is smaller in shear-induced separation and hydrolysis at 50% solids, even though a longer hydrolysis times is required at 50% solids. The savings in volume consumption in high-solid wheat flour hydrolysis are 58%.

Table 7.2 shows the volume consumption for a DH% of 5% with relatively short hydrolysis times up to 105 min. For higher DH%, the hydrolysis time necessary in the different processes varies more and a different picture regarding the volume consumption is given. This is shown in Table 7.3 for hydrolysis up to DH% 15%, which requires hydrolysis times up to 24 h at 50% solids. It takes approximately 24 h to hydrolyze to DH% 15% at 50% solids because the

Table 7.3: Volume consumption for the standard process, shear-induced separation and hydrolysis at 50% solids, and high-solid wheat flour hydrolysis. High-solid wheat flour hydrolysis considers supernatant and protein-rich streams as gluten streams. Hydrolysis times for hydrolysis to DH% 15%. See appendix A.4 for other assumptions made.

	time		volume consumption	
	separation	hydrolysis	separation	hydrolysis
	h	h	$\frac{m^3}{\text{tonne gluten/h}}$	$\frac{m^3}{\text{tonne hydrolysate/h}}$
standard process (Fig. 7.1a)	1.25	6	10.0	35.6
shear-induced separation and hydrolysis at 50% (Fig. 7.1b)	0.5	24	1.1 (-89%)	62.6 (75%)
high-solid wheat flour hydrolysis (Fig. 7.1c)	5		37.0 (-19%)	

concentration effect described in chapter 5 plays a prominent role at higher DH% and solid concentrations. As a result, higher volumes are needed to hydrolyze one tonne gluten per hour. Regarding high-solid wheat flour hydrolysis, the volume consumption is only 19% lower when hydrolyzing to DH% 15%. Because separation of starch and gluten is a rather fast process (1.25 h [46]), long hydrolysis times will favor the standard process over high-solid wheat flour hydrolysis regarding the volumetric productivity.

7.4.3 Shear-induced separation and hydrolysis at 50% solids

Fig. 7.4 and Table 7.2 show the great economic and environmental potential of shear-induced starch–gluten separation and hydrolysis at 50% solids. However, some challenges remain: while we believe that hydrolysis at 50% solids could be readily performed in existing equipment, shear-induced starch–gluten separation is performed in batch on lab scale to this date. However, the

Table 7.4: Water, energy, and volume consumption for shear-induced separation at 42.7% water and shear-induced separation with xylanase at 38% water. Separation time = 0.5 h. See appendix A.4 for assumptions made.

	water content	water consumption	energy consumption	volume consumption
		$\frac{\text{L}}{\text{kg wheat flour}}$	$\frac{\text{MJ}}{\text{kg wheat flour}}$	$\frac{\text{m}^3}{\text{tonne wheat flour/h}}$
shear-induced separation (Fig. 7.1b)	42.7%	0.50	3.7	0.61
shear-induced separation and with xylanase	38%	0.39 (-23%)	4.0 (8%)	0.56 (-9%)

transition from batch to continuous is not trivial. For example, a material inlet and an outlet would have to be created, which should influence the shear flow in the system minimally [132]. Another drawback of shear-induced separation is the lower protein concentration in the final gluten product: up to 60% (N×5.7) compared to 72% in the gluten from the standard process. Therefore, the final hydrolysate will either be lower in protein content than current gluten hydrolysates commercially available, or another concentration step is required to increase the protein content. We would like to point out, though, that protein contents of 60% might be sufficient in many food applications and higher protein contents not necessary. For instance, in the case of adding the gluten hydrolysate to cereal products high in starch, the higher starch content in the hydrolysate is not necessarily detrimental. In addition, it was shown that the functionality of the shear-induced separated gluten is comparable or improved compared to commercially available gluten [136].

In chapter 3, we investigated shear-induced starch–gluten separation aided by xylanases to test the possibility of separating at even more concentrated conditions. In Table 7.4, the water, energy, and volume consumption for such a xylanase-aided separation at 38% water is compared to shear-induced separation at 42.7% water without xylanase. Xylanase addition reduces the water consumption from 0.50 to 0.39 L water/kg wheat flour (i.e. by 23%).

However, the volume consumption only reduces by 9%, because only the amount of water changes but not the amount of wheat flour. The energy consumption even increases by 8% because the lower water content of 38% leads to an increased viscosity and mixing input (Fig. 3.3). Thus, given the fact that enzymes are costly, the advantages in water and volume consumption need to be compared carefully to the enzyme costs and higher energy consumption.

7.4.4 High-solid wheat flour hydrolysis

Fig. 7.4 and Table 7.2 show water, energy, and volume savings of 50–58% for high-solid wheat flour hydrolysis compared to the standard process when hydrolyzing to DH% 5%. Higher savings are possible using shear-induced separation and hydrolysis at 50% solids. However, high-solid wheat flour hydrolysis has the advantage that it could be readily applied in existing equipment, whereas shear-induced starch–gluten separation is only available on lab scale so far.

High-solid wheat flour hydrolysis benefits especially from short reaction times, because it makes use of the increased initial hydrolysis rate (chapter 6). Furthermore, starch–gluten separation is a relatively fast process. In Table 7.2 it is assumed that the separation requires 1.25 h in the standard process [46]. This implies that hydrolysis times longer than 1.25 h in high-solid wheat flour can be considered as detrimental regarding the volume consumption because hydrolysis and separation are combined. Therefore, we suggest high-solid hydrolysis as an alternative to the standard process for short reaction times from the perspective of the volumetric productivity.

A drawback of high-solid wheat flour hydrolysis is the relative impurity of the protein hydrolysate. The supernatant stream does not only contain the hydrolysate but also other water-soluble or dispersible components, such as pentosans and damaged starch. At a DH% of 5% the dried supernatant had a protein content of 49.7% (on a wet basis) (Table 7.1). This protein content can be further increased in a post-process step. However, similar as stated for the shear-induced separation, a protein content of 50% might already be sufficient in many food applications. The fact that all the components of the supernatant stream are water-soluble make them suitable for application in sports drinks, in special medical diets, or as a supplement in other drinks to enhance the nutritional

and functional properties [25]. Furthermore, the protein-rich pellet also has a relatively small protein content of 30.6% and might require an additional concentration step.

7.5 Future prospects – new scientific challenges and possible applications

In the future, it is interesting to further investigate high-solid wheat gluten hydrolysis. From the results presented in this thesis, it can be concluded that improvements of wheat gluten hydrolysis at high solid concentrations can be achieved by preventing auto-inactivation or minimizing the enzyme concentration. The design of specific proteases with no or low specificity for its own kind might be a future option to decrease protease auto-inactivation. Alternative paths to improve high-solid wheat gluten hydrolysis could also lead towards protease immobilization, which also prevents protease auto-inactivation.

Section 7.4 showed the environmental and economic benefits of concentrated systems. The scientific challenges are to develop further understanding that allows the application of these concentrated systems on a larger scale. For instance, (a) to scale up the shear-induced starch–gluten separation, preferably to a continuous process, or (b) perform high-solid enzymatic wheat gluten hydrolysis at larger scales and extend the understanding to other protein systems.

In addition, it is worthwhile to make further use of the properties high-solid protein systems offer. In chapter 5, it was discussed that the plastein reaction might take place at high-solid gluten hydrolysis and impedes hydrolysis. The plastein reaction is characterized by the production of water-insoluble peptides when exposing water-soluble, hydrolyzed peptides to a protease at elevated substrate concentrations. Therefore, it was tested, whether plastein products form for wheat gluten hydrolysates (Fig. 7.5). Wheat gluten was hydrolyzed to DH% 20% using Flavourzyme and DH% 10% using Alcalase and Protamex. Subsequently, the plastein reaction was conducted by exposing the freeze-dried and ground supernatant to freshly added protease at high solid concentration. The protease during hydrolysis and the plastein reaction were always the same.

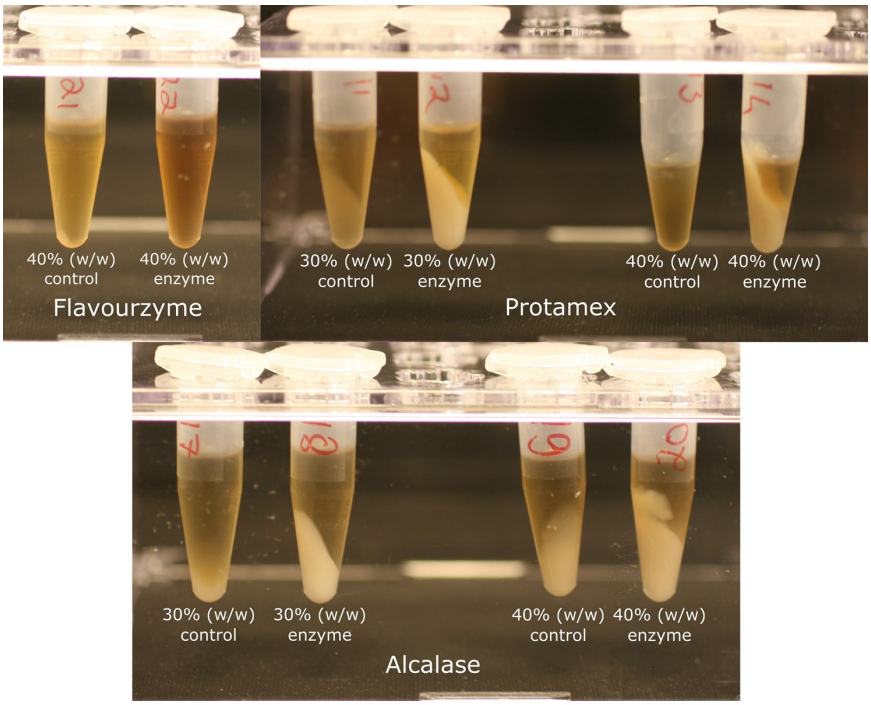

Fig. 7.5: Plastein formation after 24 h. The same proteases were used for the hydrolysis and the plastein reaction. Only the freeze-dried supernatant (water-soluble) of the hydrolysis reaction was used for the plastein reaction. The legend shows the hydrolysate concentration during the plastein reaction for the enzyme and control sample.

Fig. 7.5 shows no evidence for plastein formation for Flavourzyme, probably due to the exo-activity of Flavourzyme. It has been suggested that short peptides, such as di- and tripeptides, are not suitable for the plastein reaction [150]. However, plastein formed for Alcalase and Protamex and more plastein formed for the protease sample than for the control sample. This suggests that plastein also forms during high-solid hydrolysis using Alcalase and Protamex. In the future, we should not only focus on improving high-solid hydrolysis with respect to the lower hydrolysis rate compared to low-solid hydrolysis. Instead, we should also try to make use of the unique opportunities that the high-solid systems have to offer. For instance, high-solid hydrolysis could be used to derive specific peptides, such as shown for the plastein reaction.

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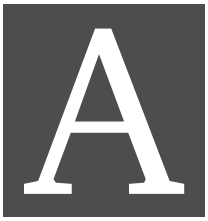

Appendix

A.1 two-factor ANOVA – detailed results

Table A.1: Two-factor analysis of variance (ANOVA) – **influence of the enzyme** for samples without xylanase and samples with Grindamyl and Shearzyme. If $\alpha \leq 0.05$ then two parameters (i.e. no xylanase, Grindamyl or Shearzyme) were significantly different. Experiments were performed at water contents from 34% to 43.5–44.8%. See chapter 2 for details.

G'^{*}	no xylanase	Grindamyl	Shearzyme
no xylanase	–	0.000	0.000
Grindamyl	0.000	–	0.727
Shearzyme	0.000	0.727	–

tan δ	no xylanase	Grindamyl	Shearzyme
no xylanase	–	0.000	0.173
Grindamyl	0.000	–	0.000
Shearzyme	0.173	0.000	–

$J_{c,max}{}^{*}$	no xylanase	Grindamyl	Shearzyme
no xylanase	–	0.000	0.000
Grindamyl	0.000	–	0.415
Shearzyme	0.000	0.415	–

$J_{el}{}^{**}$	no xylanase	Grindamyl	Shearzyme
no xylanase	–	0.336	0.548
Grindamyl	0.336	–	0.508
Shearzyme	0.548	0.508	–

[*] log G' and $J_{c,max}$-values were used.
[**] ANOVA performed at water contents below 40%.

Table A.2: Two-factor analysis of variance (ANOVA) results – **influence of the water content** for samples without xylanase and samples with Grindamyl and Shearzyme. If $\alpha \leq 0.05$ then the water content had a statistically significant influence on the respective parameter (i.e. G', *tan* δ, $J_{c,max}$, or J_{el}). Experiments were performed at water contents from 34% to 43.5–44.8%. See chapter 2 for details.

	G'^{*}	*tan* δ	$J_{c,max}{}^{*}$	J_{el} <40%**	J_{el} all**
no xylanase	0.000	0.044	0.000	0.204	0.000
Grindamyl	0.000	0.000	0.000	0.150	0.000
Shearzyme	0.000	0.001	0.000	0.001	0.000
all***	0.000	0.055	0.000	0.004	0.000

[*] log G' and $J_{c,max}$-values were used.
[**] *<40%:* ANOVA performed at water contents below 40% and *all:* performed at all water contents up to 43.5%
[***] ANOVA performed using all data points (no xylanase, Grindamyl, and Shearzyme).

A.2 Farinograph mixing profiles

Fig. A.1 depicts the Farinograph mixing profiles at water contents from 34% to 44.8% without enzyme and for Grindamyl and Shearzyme addition.

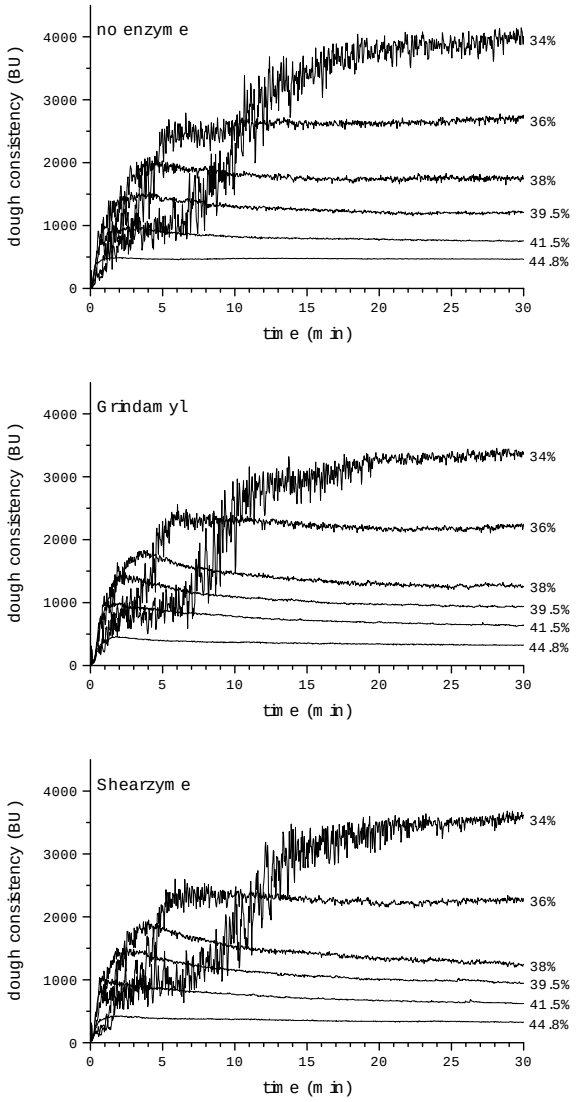

Fig. A.1: Farinograph mixing profiles at water contents from 34% to 44.8% without enzyme and for Grindamyl and Shearzyme addition. Percentages next to curves show used water content. Farinograph experiments were performed according to AACC-method 54-21. Supplementary of Fig. 2.1.

A.3 Total conversion per initial substrate as function of the initial substrate

Fig. A.2 illustrates the initial hydrolysis rate of the total amount of hydrolysis per initial solid concentration (i.e. the DH%) as function of the initial solid concentration.

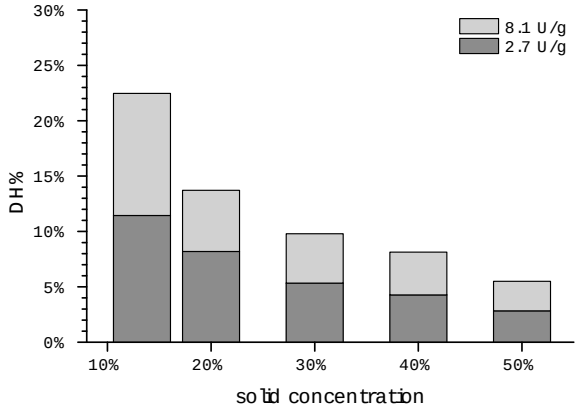

Fig. A.2: Initial hydrolysis rate of the total amount of hydrolysis per initial solid concentration (i.e. the DH%) as function of the initial solid concentration. Initial hydrolysis rate here refers to the conversion after 0.5 h hydrolysis using Flavourzyme. The legend indicates the enzyme concentration used.

A.4 Assumptions for starch–gluten separation and wheat gluten hydrolysis

Table A.3 depicts the water, energy, and volume consumption considering the whole wheat flour. Table A.3 is similar to Table 7.1, where the water, energy, and volume consumption related to the gluten are considered.

Table A.3: Water, energy, and volume consumption in the separation process, considering the whole wheat flour for standard process, shear-induced separation and hydrolysis at 50% solids, and high-solid wheat flour hydrolysis. Hydrolysis times for hydrolysis to DH% 5%.

	time	water consumption	energy consumption	volume consumption
	h	$\frac{\text{L}}{\text{kg wheat flour}}$	$\frac{\text{MJ}}{\text{kg wheat flour}}$	$\frac{\text{m}^3}{\text{tonne wheat flour/h}}$
standard process (Fig. 7.1a)	1.25	2.6	22.0	4.2
shear-induced separation and hydrolysis at 50% (Fig. 7.1b)	0.5	0.5 (-81%)	3.7 (-83%)	0.6 (-85%)
high-solid wheat flour hydrolysis (Fig. 7.1c)	1	1.2 (-56%)	6.5 (-71%)	1.9 (-55%)

Table A.4 shows the **general assumptions** for the standard process, shear-induced starch–gluten separation and gluten hydrolysis at 50% solids, and high-solid wheat flour hydrolysis with a subsequent separation. Only starch, gluten, and water are considered. All other minor ingredients are neglected and included in the starch fraction. Drying times were neglected in the comparison.

Table A.4: General assumptions for starch–gluten separation and wheat gluten hydrolysis.

parameter	figure	source
separation temperature	30 °C	
evaporation temperature separation	80 °C	
hydrolysis temperature	50 °C	
evaporation temperature hydrolysis	60 °C	[54]
water evaporation energy	2.257 MJ/kg water	
heat capacity water	$4.2 \cdot 10^{-3}$ MJ/kg water	
heat capacity gluten	$0.365 \cdot 10^{-3}$ MJ/kg water	[91]
energy efficiency	50%	[119]
density starch	1510 kg/m^3	[50]
density gluten	1290 kg/m^3	[50]
protein content wheat flour	13%	
starch content wheat flour	73%	
water content wheat flour	14%	
enzyme-to-substrate ratio	1:100	

Table A.5 shows the **specific assumptions for the standard separation and hydrolysis process**. In case of starch dehydration, most of the water is removed using hydrocyclones. This was ignored in the comparison.

Table A.5: Specific assumptions standard separation and hydrolysis process.

parameter	figure	source
protein content gluten fraction	72%	
starch content gluten fraction	18%	
water content gluten fraction	10%	
protein content starch fraction	0.3%	[54]
starch content starch fraction	87.7%	[54]
water content starch fraction	12%	[129]
water addition separation	2.6 L/kg wheat	[132]
water addition hydrolysis	4.0 L/kg dry matter	chapters 4 & 5
recovery starch	75%	[90, 132]
recovery gluten	80%	[90, 132]
water content starch fraction before drying	60%	[90, 132]
water content gluten fraction before drying	85%	[90, 132]
mixing energy separation	0.05 MJ/kg batter	
mixing energy hydrolysis	0.05 MJ/kg slurry	
separation time	1.25 h	[46]
hydrolysis time to DH% 5%	1.25 h	chapters 4 & 5
hydrolysis time to DH% 15%	6 h	chapters 4 & 5

Table A.6 shows the **specific assumptions for the shear-induced separation and high-solid hydrolysis at 50% solids**.

Table A.6: Specific assumptions shear-induced separation and high-solid hydrolysis at 50% solids.

parameter	figure	source
protein content gluten fraction	60%	chapter 3
starch content gluten fraction	30%	
water content gluten fraction	10%	
protein content starch fraction	2.3%	[134]
starch content starch fraction	85.7%	
water content starch fraction	12%	[129]
water addition separation	0.5 L/kg wheat	[132]
water addition hydrolysis	1.0 L/kg dry matter	chapters 4 & 5
recovery gluten	80%	
water content gluten fraction before drying	43%	chapter 3, [132]
mixing energy separation	0.332 MJ/kg dough	[105]
mixing energy hydrolysis	0.15 MJ/kg slurry	
separation time	0.5 h	chapter 3
hydrolysis time to DH% 5%	1.75 h	chapters 4 & 5
hydrolysis time to DH% 5%	24 h	chapter 5
water addition separation (incl. xylanase)	0.39 L/kg wheat	
mixing energy separation (incl. xylanase)	0.664 MJ/kg dough	chapter 3, [105]
w.c. gluten fraction before drying (incl. xylanase)	38%	chapter 3

Table A.7 shows **specific assumptions for the high-solid wheat flour hydrolysis**.

Table A.7: Specific assumptions high-solid wheat flour hydrolysis.

parameter	figure	source
protein content gluten fraction	58%	Fig. 7.1
starch content gluten fraction	42%	
water content gluten fraction	10%	
protein content starch fraction	2%	Fig. 7.1
starch content starch fraction	86%	
water content starch fraction	12%	[129]
water addition	1.15 L/kg wheat	chapter 6
recovery gluten (supernatant)	59%	Fig. 7.1
recovery gluten (supernatant + protein-rich pellet)	90%	Fig. 7.1
recovery starch	75%	
water cont. gluten fraction before drying (supernatant)	80%	
water cont. gluten fraction before drying (spnt & pellet)	73%	
water content starch fraction before drying	50%	
mixing energy separation & hydrolysis	0.08 MJ/kg slurry	
separation & hydrolysis time to DH% 5%	1 h	chapter 6
separation & hydrolysis time to DH% 15%	5 h	chapter 6
hydrocyclone separation time	neglected	
hydrocyclone energy & hydrolysis	0.0216 MJ/kg slurry	[138]

Fig. A.3 shows the gluten, starch, and water masses used for the Sankey diagram comparison in section 7.4. Besides the shown masses, Fig. A.3 is the same as Fig. 7.4.

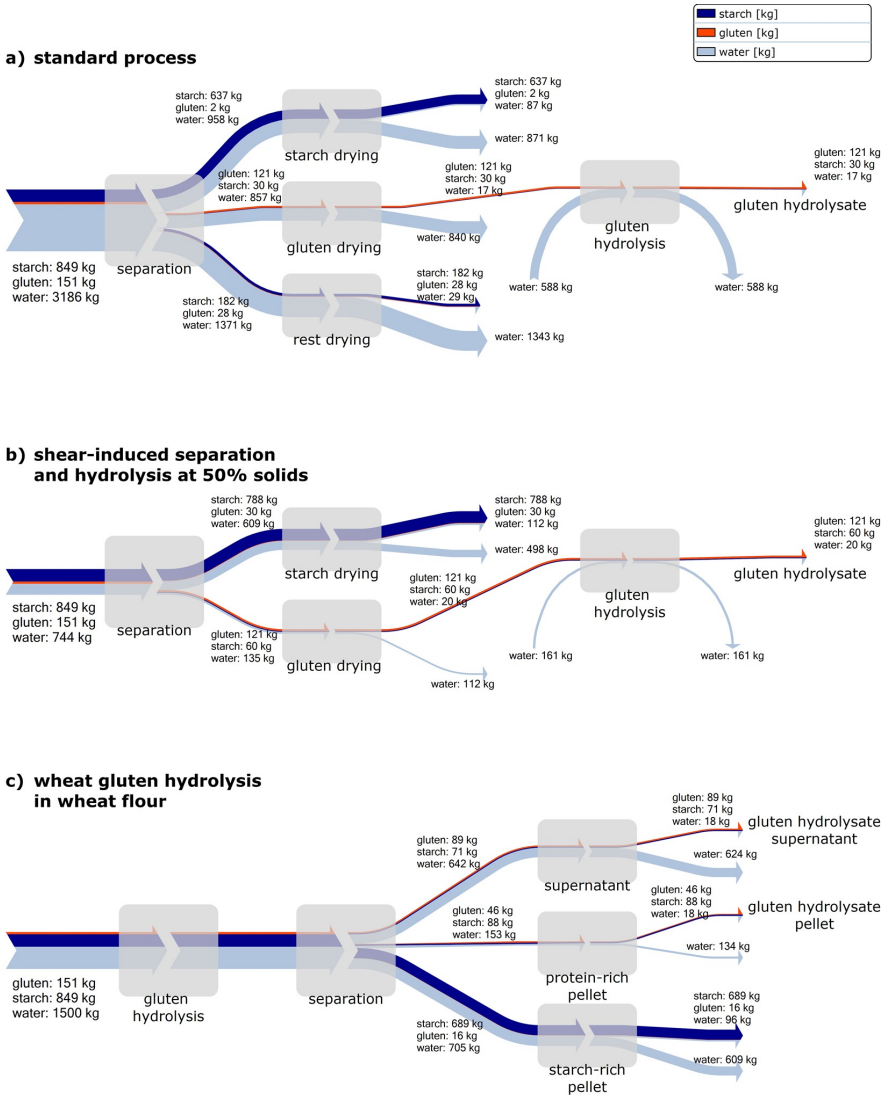

Fig. A.3: Sankey diagrams. Schematic overview of the water consumption including the gluten, starch, and water masses for: (a) standard starch–gluten separation and gluten hydrolysis at 20% solids, (b) shear-induced starch–gluten separation and gluten hydrolysis at 50% solids, and (c) high-solid wheat gluten hydrolysis in wheat flour at 40% solids with a subsequent separation.

Summary

Summary

The food industry is one of the largest water consumers in industry. Using large amounts of water, however, is undesirable from an environmental point of view because freshwater is a scarce good in many regions of the world and undesirable from an economic point of view because high water loadings require high amounts of energy for dehydration and signify high amounts of wastewater.

This thesis uses wheat, one of the major crops in human nutrition, to study the influence of low water concentrations on two relevant processes in wheat processing:

1. **The separation of starch and gluten**. Separation is often performed using 10–15 L water per kg dry matter. Instead, starch and gluten can be separated by inducing shear using 0.5 L water per kg dry matter. In this thesis we make use of xylanases to hydrolyze arabinoxylan present in wheat, thereby releasing the water associated with arabinoxylan. In doing so, shear-induced starch–gluten separation is performed at even more concentrated conditions. The influence of arabinoxylan hydrolysis in wheat dough at low water contents is studied in chapters 2 and 3.

2. **The hydrolysis of gluten**. Hydrolysis is currently performed using approximately 4 L water per kg dry mater. In this thesis we perform gluten hydrolysis at solid concentrations of up to 70%, thereby investigating the changes in the hydrolysis reaction and the functionality of the resulting hydrolysates. Wheat gluten hydrolysis at low water contents is studied in chapters 4, 5 and 6.

This thesis consists of seven chapters. **Chapter 1** gives a general introduction of the thesis. In **chapter 2**, wheat dough rheology at low water contents below 40% and the influence of xylanases is studied. A reduction in water content from 43.5–44.8% (representing optimal Farinograph water absorption) to 34% (the lowest water content where a dough forms) results in a non-linear increase in the dough consistency, elastic modulus G', and a decrease in the maximum creep compliance $J_{c,max}$ of 1–2 orders of magnitude. Addition of xylanases has the same effect on the dough consistency, G' and $J_{c,max}$ as an increase in water

content of 2–5% (on a water basis). *Tan* δ is hardly and J_{el} not influenced by xylanase addition showing that the influence of xylanases on the mechanism of hydration is negligible.

In **chapter 3**, shear-induced starch–gluten separation with the help of xylanases is studied at water contents from 43.5% to 34%. Addition of xylanases at the standard water content of 43.5% results in a slurry without any separation. As a result, lower water contents are used. At water contents below 40%, the local formation of gluten clusters is observed with and without xylanases addition. However, opposed to shear-induced separation at 43.5% water without xylanase, the gluten patches do not migrate to the center of the cone because of the densely packed dough and an inhomogeneity in the shear field. Nevertheless, gluten clusters can be concentrated up to 60% (N×5.7) protein. Similar to chapter 2, xylanase addition allows water savings of 3–5% (on a water basis).

Chapter 4 introduces enzymatic wheat gluten hydrolysis at high solid concentrations and describes the influence of high-solid hydrolysis on the resulting functional properties of the gluten hydrolysates. Wheat gluten can be hydrolyzed at solid concentrations of up to 60% (w/w). The water solubility of the dried hydrolysates is independent of the solid concentration during hydrolysis, just like the foam stabilizing properties at degrees of hydrolysis (DH%) below 8% At DH% above 8%, high solid concentrations even increase the foam stabilizing properties of the resulting hydrolysates, which is related to the presence of more peptides with a molecular mass >25 kDa. Furthermore, an increase in solid concentration results in an increase of the volumetric productivity.

Despite the advantages of high-solid gluten hydrolysis, we also observe lower hydrolysis rates in high-solid gluten hydrolysis compared to low-solid gluten hydrolysis at constant enzyme-to-substrate ratios. The factors causing this hydrolysis rate limitation are investigated in **chapter 5**. It is shown that enzyme inhibition, the water activity, and mass transfer limitations do not impede the hydrolysis up to 50% solids. However, the hydrolysis rate limitation can be explained by a second-order enzyme auto-inactivation rate along with the higher enzyme concentrations used. At solid concentrations above 50%, the

hydrolysis rate further decreases due to mass transfer limitations. Furthermore, the addition of enzyme after 24 h at high solid concentrations hardly increases the DH%, suggesting that the maximum attainable DH% decreases at high solid concentrations. This DH% limitation is explained by a reduced enzyme activity due to a decline in water activity.

Based on the findings in chapters 4 and 5, a direct hydrolysis of gluten present in wheat flour at high solid concentrations is investigated in **chapter 6**, thereby omitting the starch–gluten separation. At a constant protein concentration, the protease activity is higher for wheat flour hydrolysis (at 40% total solids) than for vital wheat gluten hydrolysis (at 7.2% total solids) in the initial 6 h of hydrolysis, despite the high starch content in wheat flour and consequently lower water content. This is related to the starch granules in wheat flour, preventing the aggregation of (native) gluten. At wheat flour concentrations above 50% and for longer reaction times the positive effect of starch disappears. This is explained by mass transfer limitations and reduced water activities in the wheat flour slurry or dough, respectively.

Chapter 7 summarizes and generalizes the main findings of this thesis and compares the current status in starch–gluten separation and gluten hydrolysis with the concentrated separation and hydrolysis processes developed in this study. Water and energy savings of at least 50% are possible when separating and hydrolyzing at concentrated conditions. In the end, future prospects in high-solid wheat gluten hydrolysis are briefly discussed.

Samenvatting

In de levensmiddelenindustrie wordt veel water verbruikt, wat ongewenst is vanwege het feit dat dit niet duurzaam is. Zoet water is tegenwoordig een schaarse grondstof in veel delen van de wereld. Ook is het gebruik van veel water ongewenst vanuit een economisch perspectief, omdat water meestal weer verwijderd moet worden door middel van droogprocessen die energie-intensief zijn. Bovendien wordt veel afvalwater geproduceerd. In dit proefschrift wordt een onderzoek beschreven naar mogelijke waterbesparingen bij het industrieel verwerken van tarwe, één van de grootste voedingsgewassen. De waterbesparingen worden bereikt door het verwerken van tarwe bij een hogere concentratie droge stof uit te voeren. Twee processen zijn hierbij onderzocht:

1. **De scheiding van zetmeel en gluten.** Bij het huidige industriële scheidingsproces van tarwe in zetmeel en gluten wordt 10 tot 15 L water per kg droge stof gebruikt. Recent onderzoek toonde echter aan dat zetmeel en gluten ook gescheiden kunnen worden in een proces op basis van afschuiving, waarin slechts 0,5 L water per kg droge stof nodig is. In dit proefschrift is onderzocht of door middel van enzymatische hydrolyse van arabinoxylanen extra water vrij gemaakt kan worden dat anders door deze polysacchariden wordt geabsorbeerd. Op deze wijze zou het op afschuiving gebaseerde scheidingsproces met nog minder toegevoegd water kunnen worden uitgevoerd. Het effect van de hydrolyse van arabinoxylanen is beschreven in hoofdstukken 2 en 3.

2. **De hydrolyse van gluten.** Momenteel wordt voor de hydrolyse van gluten ongeveer 4 L water per kg droge stof gebruikt. In dit proefschrift is deze hydrolyse van gluten uitgevoerd bij veel hogere droge-stofconcentratie (tot wel 70% droge stof). De veranderingen in de reactie en de functionaliteit van de gevormde hydrolysaten zijn daarbij uitgebreid onderzocht en beschreven in hoofdstukken 4, 5 en 6.

Dit proefschrift bestaat uit 7 hoofstukken. Hoofdstuk 1 geeft een algemene inleiding. In **hoofdstuk 2** wordt het reologisch gedrag van deeg met weinig water beschreven evenals de veranderingen in reologisch gedrag die ontstaan

door toevoeging van xylanase. Een verlaging in watergehalte van 43,5–44,8 (wat de optimale Farinograaf waterhoeveelheid is) naar 34% (het laagste watergehalte waarbij nog een deeg gevormd wordt) resulteerde in een niet lineaire toename in deegconsistentie, elastische modules G' en een afname van de maximale kruip $J_{c,max}$ die kon oplopen tot 90% van de oorspronkelijke kruipwaarde. Het toevoegen van xylanase had een vergelijkbaar effect op G' en $J_{c,max}$ als het toevoegen van 2 tot 5% extra water. De $tan\ \delta$-waarde is nauwelijks en de J_{el} is niet beïnvloed door het toevoegen van xylanase, wat aangeeft dat de invloed van xylanase op de mechanisme van deeghydratatie verwaarloosbaar is.

Hoofdstuk 3 beschrijft hoe de ”shear-induced” (door afschuiving veroorzaakt) zetmeel-glutenscheiding wordt beïnvloed door xylanase. Het toevoegen van xylanase aan deeg met 43% vocht (de standaard hoeveelheid) gaf een slurry waarin geen scheiding werd waargenomen. Daarom werd het vochtgehalte verlaagd. Onder de 40% vocht werden lokaal glutenclusters gevormd zowel met als zonder xylanase toevoeging. Echter, in tegenstelling tot shear-induced scheiding bij 43,5% vocht zonder xylanase, migreerden de glutenclusters niet naar het midden van de kegel, vanwege de dichte pakking van het deeg en inhomogeniteit in het stromingsveld. Niettemin, het eiwitgehalte in de glutenclusters kon oplopen tot 60% (N×5,7). Net als in hoofdstuk 4 kon het vochtgehalte met ongeveer 3 tot 5% verlaagd worden na xylanasetoevoeging om vergelijkbare effecten te verkrijgen.

Hoofdstuk 4 introduceert enzymatische hydrolyse bij hoge drogestofconcentraties en beschrijft de invloed van deze hoge concentratie op de hydrolyse en de verkregen functionele eigenschappen van de glutenhydrolysaten. Tarwegluten bleken te kunnen worden gehydrolyseerd tot 60% droge stof. De wateroplosbaarheid van de gedroogde hydrolysaten hangt niet af van de drogestofconcentratie tijdens hydrolyse, net als de schuimstabilisatie-eigenschappen van hydrolysaten met een hydrolysegraad lager dan 8%. Boven de 8% werden zelfs een hogere schuimstabiliteit gevonden. Dit effect werd toegeschreven aan de aanwezigheid van peptiden met een molecuulgewicht boven de 25 kDa. Verder resulteerde een hoger drogestofgehalte in een verhoogde volumetrische productiviteit.

Naast de genoemde voordelen vonden we ook lagere hydrolysesnelheden bij hoge drogestofconcentraties. De factoren die deze verlaging veroorzaakten zijn bestudeerd in hoofdstuk **hoofdstuk 5**. Het bleek dat enzyminhibitie, de wateractiviteit en transportlimitaties de reactie niet hinderden tot een drogestofconcentratie van 50%. Echter de hydrolysesnelheid kon lager worden doordat het enzym zichzelf inactiveerde. Boven de 50% nam de hydrolysesnelheid verder af door transportlimitatie. Verder nam de graad van hydrolyse na 24 uur nauwelijks nog toe, zelfs niet na extra enzymdosering, wat suggereerde dat de maximaal haalbare graad van hydrolyse afneemt bij hogere drogestofgehalten. Dit laatste kon worden verklaard door een afname van de wateractiviteit.

Op basis van de bevindingen in hoofdstukken 4 en 5 is de hydrolyse van gluten in tarwebloem onderzocht bij hoge concentraties in **hoofdstuk 6**. Bij een zelfde eiwitconcentratie bleek de enzymactiviteit hoger bij hydrolyse van eiwit in tarwebloem (40% droge stof) dan in vitale gluten (7,2% droge stof) in de eerste 6 uur van de hydrolyse. Dit opmerkelijke resultaat werd verklaard doordat de zetmeelkorrels in bloem de agglomeratie van gluten vermindert. Bij een concentratie hoger dan 50% en bij langere reactietijden verdween het positieve effect van zetmeel. Dit werd verklaard door transportlimitaties en een lagere wateractiviteit in het hoog geconcentreerde deeg.

Hoofdstuk 7 geeft een samenvatting van de belangrijkste bevindingen van het proefschrift en plaatst deze in een breder perspectief van de huidige industriële praktijk van zetmeel-gluten scheiding en de glutenhydrolyse. Als de resultaten uit dit proefschrift vertaald kunnen worden naar een industriële schaal, dan zijn water- en energiebesparingen tot wel 50% realiseerbaar indien erbij de hoogst mogelijke drogestofconcentratie geproduceerd gaat worden. Als laatste zijn de toekomstperspectieven van het bewerken van hoog geconcentreeerde tarweglutenhydrolyse beschreven.

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