Making fibrous plant meat with less refined, starch-rich crops

Jan M. Bühler

Stirps caro ex vicia faba l. facta

Propositions

1. Less refined ingredients must be studied as such and not via model mixtures with similar composition using highly purified ingredients. (this thesis)

2. Control over fibre formation requires control over water distribution among ingredients. (this thesis)

3. Free public access to scientific literature will tremendously improve trust in science, especially when it is combined with teaching "scientific reading" in schools.

4. Although polar opposites, plant meat and cultured meat can, like magnets, form a strong bond if approached the right way.

5. Scientific papers written by native speakers are the most difficult to read.

6. Science is like art; there are many opinions on how to do it, but there is no single right way.

7. The term "plant meat" is neither misleading nor wrong.

8. Hiring and promoting people for their skill creates diversity of the workforce.

Propositions belonging to the thesis, entitled

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Contents

Chapter 1

Introduction

1.1 Introduction

In a time in which price and sensory appeal dictate the type of food we eat [1], the ethics and sustainability of food production are simultaneously getting more and more attention [2]. Consumers are becoming aware of the impact of their eating habits. This is evident in the surge of the number of vegetarians and flexitarians in many developed countries such as the Netherlands, reported by the "Nederlandse Vegetariersbond" [3]. Simultaneously, the same report also ascertains that meat consumption in the Netherlands is not declining, showing the gap between awareness of the implications of ones eating habits and the willingness to actually adapt ones eating habit in the long turn. Viable plant-based alternatives for real meat, such as meat analogues, are suggested to assist consumers in reducing their meat intake, which would make their diets more sustainable [4, 5]. In order to become more viable, the price of these products should decrease and the sensory appeal should be continuously improved, since these factors have a large, and often determining influence on the food choice of consumers concerns [1]. Thus, meat analogues should mimicking the entire eating experience of real meat in terms of taste, texture, smell and appearance. Kyriakopoulou et al. [6] calls achieving this mimicry the "main purpose and ultimate challenge of making meat analogues", along with making a sustainable product.

1.2 Creating fibrous structures

The fibrous structure of real meat has been identified by the scientific community as one of the key properties to focus on in the development of viable plant-based meat alternatives [7–9], and has therefore been at the center of many studies [10–18]. Extrusion, especially high moisture extrusion (HME), has been used for this purpose since the 90's [7, 19] and is currently the main process to produce meat analogues commercially. Research in the past decade revealed that another thermomechanical process called shear cell technology has strong potential as well, specifically the High Temperature Shear Cell (HTSC) [20]. Recently, the similarities and differences between both processes were described, being mixing of biopolymers, thermo-mechanical treatment, shear rates, residence time and cooling [18]. The physical principles or phenomena that cause the formation of fibrous structures are widely believed to be phase separation due to thermodynamic incompatibility of biopolymers followed by an alignment of phases under shear [21]. In HME, the material is sheared as it passes through a cooling die at the end of the extruder,

while in the HTSC the material is continuously sheared between a rotating and a stationary cone [12] or between two cylinders [22]. The process conditions in HME and HTSC are very similar, with temperatures ranging from 95 °C-140 °C and dry matter contents of 30-50 %. While the optimal rotational speed of the extruder screws depends on the extruder geometry, the rotational speed for the HTSC is typically 30 rpm. The retention time in the extruder is in the range of several minutes [23], while in the HTSC it is most often 15 minutes. The ingredients used so far are also highly similar, being Soy Protein Isolate (SPI), Pea Protein Isolate (PPI) and Wheat Gluten (WG), either in combination [20, 24] or by themselves [10, 15, 17, 20]. Next to isolates, Soy Protein Concentrate (SPC) has also been applied to achieve similar structures [12–14].

The process of making fibrous structures with the HTSC includes a mixing procedure that is somewhat different than in HME. While in HME all ingredients are fed into the extruder barrel simultaneously and continuously, the ingredients for the HTSC are combined batch-wise and step-by-step. First, the non-gluten ingredients are combined with water (eventually previously salted) and mixed until a paste or slurry forms. The mixture is then optionally left to hydrate before gluten is added. The addition of gluten is done under continuous mixing with a spatula to ensure even distribution of all ingredients. This results in a dough that is immediately placed in the HTSC. This order of mixing is important, since gluten forms a dough upon mixing with water, which makes the inclusion and hydration of any additional dry ingredients difficult.

1.3 New ingredients for meat analogues

The development of ingredients that are suitable for the formation of fibrous structures is essential for new innovations in plant-based meat analogues. Recent studies show potential of proteinaceous ingredients from sources like zein [25, 26], peanut protein [27] and rapeseed [28]. More general, protein from both oilseeds and starch bearing crops could be suitable alternatives [6]. Among the starch-rich crops, pulses stand out due to their naturally high protein content [29, 30]. Faba beans (*Vicia Faba L.*) contain up to 40 % protein [31, 32]. They can be cultivated in a wide variety of climates at low cost [33, 34] and are excellent break-crops in cereal-rich crop rotation [35]. Upon separation, pulses provide two commercially interesting ingredients, starch and protein [36]. Separation can be done via gravity sedimentation, centrifugation and filtration in wet conditions, but dry separation, such as air classification, is also

a method to make novel ingredients. A dry separation process is not only more sustainable and economical due to the lack of water, but it also results in protein fractions with different functional properties [37, 38]. As mentioned before, currently mainly protein isolates and (to some extent) concentrates are used to create fibrous structures, but interest in the use of less refined ingredients is growing. Mildly or unrefined ingredients will introduce components to the production process that are almost completely removed in isolates and commercial concentrates. In the case of faba bean and other pulses, a less refined fraction will probably contain starch, the effect of which on fiber formation is not known yet. The reasons to use less refined ingredients in meat analogues (or other food products) are ecological and economical. Less refined ingredients are more sustainable, since they use less energy and water for their production, while simultaneously having higher yields. The same arguments also make these ingredients potentially more economical. In addition, less or unrefined ingredients are at the center of "clean label", an ongoing trend of consumers longing for less processed food products [39].

1.4 Components in pulses

Starch bearing crops such as pulses consist of two major components – protein and carbohydrates. The carbohydrates mainly consist of starch, but also cellulose, dietary fibers and sugars. The composition differs depending on whether or not hulls or husks have been removed, as they are mainly composed of cellulose and dietary fibers. Starch is the energy storage in starch bearing crops and occurs in granules with a semicrystalline structure [36, 40]. This structure is made up of amylose and amylopectin, with a different ratio depending on the crop. The amylose content of pulses is typically around 40 % [41], which is higher than the amylose contents in other conventional crops. Some of these crops, for example maize, have been bred to have amylose contents below 1 % (waxy maize starch) or above 50 % (high-amylose maize starch) [36]. Amylose is a rather linear polymer consisting of 1000-10.000 glucose units, though it does have few branches. Amylopectin is made up of up to one million glucose units and highly branched. Together they form the semi-crystalline structure of starch granules, organized in lamellas and growth-rings [42]. The amylose/amylopectin composition mainly determines the gelatinization behavior of the starch and the pasting properties when mixed with water. Starch gelatinization is a multi-step process, which has been the topic of countless books chapters and review papers [43, 44]. In short, starch gelatinization occurs when starch granules are heated in the presence of sufficient water. The granules swell, amylose

leaches out and the semi-crystalline structure is lost. The starch suspension starts to thicken, forming a viscous paste. The temperature at which this occurs is called the pasting temperature and generally lies between 60°C and 80°C in excess water, while the maximum viscosity that is reached is called the peak viscosity. As swollen starch granules disintegrate, a decrease in viscosity follows, also known as Break Down [45]. Upon cooling, the viscosity increases again due to a formation of a gel network, also known as Setback [45]. Over time, starch re-crystallizes, which firms up the structure even more. This process is called retrogradation and occurs over time, in the order of minutes to hours for amylose and in the order of days for amylopectin (if at all).

Vicia Faba L.

The seeds of *vicia faba l.*, also known as faba beans, are pulses and therefore part of the legume family. They can be grown in a variety of climates at low costs [33, 34]. They are an excellent break-crop in cereal-rich crop rotation [35] and are strong in fixating atmospheric nitrogen [46, 47]. The seeds contain 20-40% protein and 38-53% starch [31, 32, 48, 49]. The protein consists mainly of the globular proteins legumin and vicilin. The starch has an amylose content of 31-37% [48, 50], with a gelation temperature of 62-64°C and a ΔH of 9.7-11.7 g J^{-1} [48] measured with DSC. Protein- and starch rich fractions can be produced via air-classification [50] without altering the properties of the components. From a nutritional point of view, Faba beans are a suitable component of a healthy human diet: Next to their high content of digestible protein and starch, faba beans are also a good source of dietary fibre (up to 20%, [49]), minerals and vitamins [51, 52]. However, faba beans contain a number of ANFs: The favism-causing vicine and convicine, tannins, trypsin-inhibitors, lectins, phytates and the flatulence-causing saccharides raffinose and stachyose [53–55].

The other main component in pulses are proteins. Those pulse proteins consist of globulins and albumins [56]. The globulins vicilin (7S) and legumin (11S) are the storage proteins of pulses and make up the major part of the protein [57]. Globulins are salt-soluble, as opposed to the water-soluble albumins, and therefore tend to end up in isolated protein ingredients produced through precipitation [29]. Proteins generally consist of amino acid chains that are folded

and combine to form hierarchical structures with 4 levels [58]. The structures are stabilized by covalent and non-covalent bonds. When protein is heated in the presence of water, these bonds break, leading to unfolding and a loss of structure. This process is called protein denaturation. Similarly to the gelatinization of starch, the process of denaturation depends on time, temperature and moisture content. The denaturation temperature of protein negatively correlates with the water content [59, 60]. The denaturation allows for aggregation and the formation of new bonds and thus new structures or networks. At sufficiently high protein concentration, this formation of new bonds can result in a gel structure [29, 61]. The rheological properties of a protein gel depend on pH, salt concentration, protein concentration and the type of protein.

1.5 Interaction of protein and starch

Starch and protein interact with each other when present in one mixture. Upon heating, these interactions might alter due to the structural changes of starch and protein when dispersed in water. This interaction can be on molecular level, especially in diluted dispersions, and on mesoscopic level. Studies on the effect of starch or protein on the thermally induced transition of the other have come to several important conclusions: Starch swelling reduces the water available for protein [62, 63], while protein (gluten) is thought to have a barrier effect on starch, reducing its swelling and delaying the gelatinization onset [64]. A proof of direct interaction in the form of hydrogen bonding between protein (amino group of the glutamine) and starch (2nd or 3rd hydroxyl of glucose) has been reported also [65]. However, starch and protein will form separate phases at high enough concentrations [66], reducing the impact of potential hydrogen bonding between starch and protein in more concentrated mixtures to an interface interaction that would mainly impact adhesion. Other interactions between starch and protein are indirect and on a phase-interaction level, e.g. exerting mechanical or osmotic pressure on one another. The direct or indirect interactions of starch and protein have implications for their application in meat analogues and other food products.

The interaction of starch and protein has been the subject of many studies. In order to determine the molecular behavior and to rule out the influence of other components, isolates are used in model studies. The results are carefully interpreted and general conclusions are drawn, but these conclusions do not always transfer directly to more complex blends or ingredients. Protein isolates are often produced by precipitation

of the protein, a method based on the (irreversible) conformational changes of the protein, resulting in (partially) denatured protein. Protein isolates therefore have functional properties, such as solubility, and emulsification and foaming ability, that differ from those of native protein present in unrefined or mildly refined ingredients. Starch is more resistant to conformational changes during its isolation process and is often able to retain its native structure. It is of course difficult to determine the functionality of a native component by itself, since the properties will always be influenced by other components present in the unrefined or mildly refined ingredient. However, in order to utilize less- or unrefined ingredients in multi-ingredient food products and production processes such as HME and HTSC, it is necessary to understand the behavior of individual components and their interaction under process conditions. This way, suitable ingredients can be selected and the window of operation can be modified accordingly.

1.6 Aim and outline of this dissertation

In this dissertation faba bean is explored as a source for new ingredients for meat analogues. The use of faba bean in meat analogues could mean introducing starch in the recipes for HME and HTSC. **It is the aim of this dissertation to explore the potential of faba bean as a new ingredient for fibrous meat analogues and to unveil the effect of using less refined ingredients.** Insights in the role of starch are vital to this endeavor, which will be obtained by investigating both model mixtures based on purified ingredients and natural blends in this dissertation.

In **Chapter 2** we investigate whether Faba bean Protein Isolate (FPI) exhibits the same suitability for use in meat analogues as other leguminous protein isolates (pea and soy) when combined with gluten. We reiterate that gluten is responsible for the formation of fibrous structures in gluten-containing recipes and hypothesize on the mechanism as well as the role of non-gluten protein in the recipe. In **Chapter 3**, we apply a dry thermal pre-treatment to a mildly refined Faba bean Protein Concentrate (FPC)and investigate the effect on the properties of protein. In **Chapter 4** we review the use of starch in meat and meat analogues, discovering that we can use exogenous starch as a functional ingredient. It will be described how endogenous starch can be transformed into a functional component. We then investigate the influence of starch on the formation of fibrous structures with gluten in **Chapter 5**, to better understand how starch can be used in meat analogue applications. Chapters 2, 3 & 5 reveal that the interaction of starch and protein with water plays an important

role in fiber formation. **Chapter 6** therefore aims to quantify the water distribution among protein and starch in mildly refined (or even unrefined) ingredients, helping us understand how these components interact with water and therefore indirectly with one another throughout the structuring process. **Chapter 7** presents a general discussion of the results obtained in all previous chapters and places those in a broader perspective. The chapter ends with an outlook for scientific challenges and new opportunities.

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Chapter 2

Apparent Universality of Leguminous Proteins in Swelling and Fiber Formation when Mixed with Gluten

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Abstract

Fibrous meat analogues can be made through shear-induced structuring from gluten in combination with a second protein. A combination of swelling experiments and shear-cell structuring was used to investigate the relation between fibrousness and the presence of a continuous gluten network for mixtures containing gluten and either pea protein, faba bean protein or soy protein. When the gluten content of the mixed gels increased, swelling of the other protein decreased proportionately. This suggested the presence of a continuous gluten network. Normalization of the swelling data resulted in an apparent master curve. The strain on the non-gluten protein was derived from the swelling data and increased with increasing gluten content. Structuring the protein mixtures in a High Temperature Shear Cell resulted in fibrous structures at gluten contents ≥ 0.5 wt/wt. The effect of gluten on swelling and fiber formation is universal for the tested proteins. We, therefore, propose that in gluten-containing mixtures, a continuous gluten network is required for the formation of fibers, while the second protein acts merely as a filler and is replaceable.

2.1 Introduction

A number of technologies exist to produce fibrous meat-like structures from food polymers [1–5]. However, effectively all industrial production relies on high-moisture extrusion (HME) cooking techniques, which are a form of thermo-mechanical processing. Despite the widespread use of extrusion in industry, the rationale required to control the final product structure is limited [3, 6]. Therefore, formulation and process development is still based mostly on empirical findings. In order to better understand the extrusion cooking process, so-called shear cells were developed [7–9]. Shear cells offer a simpler form of thermo-mechanical processing through the use of simple shear flow and heat, and can also be used to produce fibrous, meat-like structures [1, 10–12]. During shear cell processing a bio-polymer mixture is subjected to continuous shear-flow [2]. Recent investigations have yielded insights into the key process and material properties for the production of fibrous structures [13–15]. It is thought that two immiscible phases are required for fiber formation in sheared bio-polymer mixtures [16]. Tolstoguzov [17] proposed that the deformation and alignment of the dispersed phase would lead to an anisotropic structure, which implies that the dispersed phase is of importance to structure formation. Fibrous structures were obtained using various mixtures: soy/gluten [1, 3, 16, 18], soy/pectin [11], and pea/gluten [12]. For neat calcium caseinate, it was shown that air incorporation is vital to obtain a fibrous structure [14]. Also in soy-pectin mixtures, an internal air phase was found that is deformed in the shear-flow direction [11, 12]. Clearly, the knowledge base for process and formulation development is expanding rapidly, but steps still have to be taken towards the intelligent design of fibrous meat-like structures.

We have recently shown that gluten forms a continuous network when present at a sufficiently high level in (non-sheared) mixed soy protein and gluten gels [19]. Gluten protein swells to a lower level than soy protein during free swelling [1, 19, 20]. The continuous gluten network can thus limit the swelling of the soy protein in a mixed gel through a mechanical interaction [19]. The continuity of the gluten phase can, therefore, be deduced from the regime in which the swelling of soy (or another protein) is inhibited [19]. Grabowska et al. [1] showed that a fibrous structure can be made from soy protein-gluten mixtures with a shear cell, and suggested that gluten is the continuous phase [1]. We, therefore, hypothesise that in order to create fibrous structures from gluten-containing protein mixtures, gluten content needs to be high enough to form a continuous network.

We will test this hypothesis by studying the swelling and structure-formation of gluten in combination with protein isolates from either faba bean (FPI), pea (PPI) or soy (SPI). Soy and pea protein are often used as ingredients for meat analogues [21], while faba bean is just starting to gain attention in the field [22]. The rheological profile obtained during gelation of the different proteins is considered similar [23], although the relative importance of disulfide bonds seems to differ [24, 25]. For brevity, when simultaneously referring to SPI, PPI, and FPI, we will use the term 'non-gluten proteins' in the remainder of this paper. First, the maximum swelling ratio of single-phase protein gels is studied. We calculate the swelling ratio of mixed protein gels based on the swelling of single-phase gels by assuming no mechanical interaction between the protein phases. Water partitioning between the gluten and non-gluten proteins will be taken into account using Flory-Rehner theory. Subsequently, the actual swelling ratio of the mixed gels is measured and compared to the calculated values. Furthermore, the deformation of the non-gluten phase caused by the swelling is discussed using the neo-Hookean framework. The outcome of the swelling experiments will be mirrored against structure formation experiments in a shear cell using the same materials.

2.2 Material and methods

Vital wheat gluten (gluten; VITEN®, Roquette, Lestrem, France), Faba bean Protein Isolate (FPI; supplied by Ingredion, Hamburg, Germany), Pea Protein Isolate (PPI; NUTRALYS® F85G, Roquette, Vitens, Lestrem, France) and Soy Protein Isolate (SPI; Supro 500E IP, DuPont, St. Louis, MO, USA) had protein contents of 77.9 %, 84.0 %, 78.6 % and 81.7 % (dry base), respectively (Nx5.7). Sodium chloride ($NaCl$; Sigma-Aldrich, Steinheim, Germany) was of analytical grade. Mili-Q water was used for all experiments. All components were at room temperature (21°C) unless stated otherwise.

2.2.1 Preparation of single-phase gels

Single-phase gels were prepared from SPI, FPI and PPI using the protocol of Cornet et al. [19]. In short, the protein powder was dispersed in water and mixed thoroughly using a spatula. The mixture was transferred to a plastic bag and freed from air by applying a vacuum of 50 mbar for 45 s. The mixtures were left overnight at 4°C to allow for hydration. The hydrated mixtures were transferred to stainless steel gelation vessels with an internal height of 5 mm and a radius of 12.5 mm. The vessels were

hermetically sealed and submerged in a Julabo shaking water bath heated to 95°C. After 30 min the vessels were cooled in water of 15 °C for 15 min after which the gels were removed from the vessels. Gel edges were trimmed with a sharp razor and visually inspected for defects before use.

2.2.2 Mixed gel preparation

Mixed gels were prepared from mixtures of gluten with either FPI, PPI or SPI similarly to the single-phase gel preparation protocol with some adaptations. After mixing the non-gluten protein (FPI, PPI or SPI) with the water, gluten was added and mixed thoroughly through the dough. Dough and gel preparation proceeded from thereon without alterations to the protocol for single-phase gels as described in Section 2.2.1.

2.2.3 Gel washing and swelling

Gels were washed and swollen to remove any ions present and to determine their maximum level of swelling using a method of Cornet et al. [19]. In short, gels were placed in excess water (1:100 wt:wt ratio) for a period of at least 48 h until a constant gel weight was reached. The water was renewed three times during this period. After swelling, the dry matter content (DMC) was determined by oven drying for 48 h at 105°C. We will express the maximum level of swelling as the ratio between the volume of water and the volume of polymer, which for a single-phase gel is given by:

$$Q_i^I = \frac{wt_w/\rho_w}{wt_{p,i}/\rho_p} \tag{2.1}$$

wt_w is the total weight of water, $wt_{p,i}$ is the weight of protein i. ρ_w and ρ_p are the densities of water and protein and taken as 1000 $\mathrm{kg\,m^{-3}}$ and 1330 $\mathrm{kg\,m^{-3}}$, respectively. For mixed gels containing gluten, gluten was assumed to reach a constant level of swelling, absorbing 1.5 g water $\mathrm{g^{-1}}$ protein [19]. Subtracting the contribution of gluten to the swelling enabled us to calculate the swelling of the other protein in mixed gels. Note that we have previously used 2 $\mathrm{g\,g^{-1}}$, which is the value for non-heated gluten [1, 19]. The value of 1.5 $\mathrm{g\,g^{-1}}$ corresponds to that of heated gluten [19], and was considered to be a more accurate approximation. The volumetric swelling ratio of non-gluten proteins in a two-phase gel was calculated as:

$$Q_i^{II} = \frac{(wt_w - 1.5wt_{p,glu})/\rho_w}{wt_{p,i}/\rho_p} \tag{2.2}$$

With $wt_{p,glu}$ as the weight of gluten protein.

2.2.4 Dynamic vapour sorption

Water vapour sorption isotherms were determined at 25°C on an SPSX-S3-EU01508 (Project Messtechnik). Samples were dried for 24 h at a relative humidity (RH) of 0 % before increasing the RH in 10 % increments to an RH of 90 %. Equilibrium was assumed when the sample weight change was less than 0.005 % min^{-1} over a window of 10 min for a period of 120 min. The maximum step duration was set to 2000 min. Isotherms were recorded in duplicate on the protein powders, and on lyophilized, cryo-milled gels (data not shown).

2.2.5 High-temperature Shear Cell

Mixtures containing gluten and either FPI, PPI or SPI were prepared. Gluten protein fractions of 0, 0.167, 0.333, 0.500, 0.667 and 1 wt/wt were used. The mixtures were structured in a High Temperature Shear Cell (HTSC; Wageningen University, The Netherlands) [10] following the protocol previously reported by Grabowska et al. [1] with some modifications based on preliminary experiments performed in our lab. Mixtures containing FPI and PPI were prepared with a DMC of 0.375 wt/wt, while for the mixtures containing SPI a DMC of 0.300 wt/wt was used to ensure comparability with the results of Cornet et al. [19]. All samples contained 0.01 wt/wt $NaCl$. The $NaCl$ was dissolved in the water after which the non-gluten protein (FPI, PPI or SPI) was mixed in using a spatula. Gluten was added to the mixture, followed by further mixing. The doughs were immediately placed in the HTSC, which was pre-heated to 140°C. The protein blends were sheared (30 rpm; 39 s^{-1}) for 15 min at a constant temperature of 140°C. After shearing, the HTSC was cooled down in 5 min to below 60°C before opening and removing the samples. Sample structure was assessed immediately. All samples were produced in triplicate. For the swelling experiments, samples were taken from the outer edge of the sample.

2.2.6 Assessment of the fibrous structure

The samples were visually inspected for fibrous structure formation by bending them parallel to the shear flow direction. A wedge was cut from the circular sample. The wedge was bent by moving the sharp tip of the wedge towards the outer edge, resulting in a tear parallel to the shear flow direction. The bent piece was placed on a metal pin and the fracture surface was photographed. This technique reveals the potential orientation of the structure in the outer 4 cm of the sample and is similar to breaking techniques used for extruded samples to reveal fracture patterns [6, 18].

2.2.7 Statistics

Values are presented as the mean ± standard error of the mean. The number of duplicates, n, is reported with the data. Where applicable, significant differences were tested for using a one-way ANOVA with a significance level of $p<0.05$.

2.3 Theory

2.3.1 Water partitioning according to Flory-Rehner theory

The protein content at cross-linking can affect the cross-link density of a protein network. Therefore, the water partitioning in a protein mixture at gelation should be known to be able to make predictions about the swelling ratio of a mixed polymer gel. In our calculation of the water partitioning, we have assumed proteins to behave as polymers. It must be noted that in some situations it is better to consider proteins as colloids instead [26]. In the context of water sorption and gel mechanics, the assumption of proteins behaving as polymers has proven useful in numerous prior studies [19, 27, 28] and was therefore also employed here. We use the Flory-Rehner (FR) theory to describe the water partitioning in protein mixtures. This approach has previously been described in detail [19]. A brief summary of the approach is presented here, while all details can be found in the Supplementary information.

FR theory describes the swelling based on the swelling pressure, Π_{swell}, which has two contributions. The first contribution accounts for the osmotic pressure due to the mixing of polymer and solvent and is captured by the mixing pressure, Π_{mix}. The second contribution describes the pressure generated due to the deformation upon swelling and is described with the elastic pressure, Π_{elas}. Upon external compression of the gel, Π_{swell} is balanced by the external pressure Π_{ext}:

$$\Pi_{ext} = \Pi_{swell} = \Pi_{mix} - \Pi_{elas} \tag{2.3}$$

Under ambient conditions ($\Pi_{ext} = 0$), the mixing and elastic contributions must, therefore, balance each other. By solving Equation 2.3 for the moisture content in the two protein phases in a protein mixture, the water partitioning is obtained. Gluten proteins form a cross-linked network upon hydration. For the gluten phase, both Π_{mix} and $\Pi_{elastic}$ were included, while for the other proteins, only Π_{mix} was included. For further details on the approach, the reader is referred to the Supplementary Information.

2.3.2 Neo-hookean framework

In the neo-Hookean framework, deformations are relative to a reference state. This reference state can be defined as the swelling ratio at which the polymer chains are relaxed, Q_{ref}. We point out that Q is inversely proportional to the commonly used polymer volume fraction φ; the relations are, therefore, inverse compared to when using φ. van der Sman [28] showed that for several bio-polymers, φ_{ref} is proportional to the polymer volume fraction at maximum swelling, φ_0:

$$\varphi_0 = \varphi_{ref} \tag{2.4}$$

Hence, the swelling ratio at maximum swelling, Q_0, relates to Q_{ref} via:

$$Q_0 = 1.5 Q_{ref} \tag{2.5}$$

For isotropic deformations, the linear strain on the polymer network, ϵ, for a given swelling ratio, Q, is proportional to the volumetric strain, as given by the ratio between Q_{ref} and Q:

$$\epsilon \propto \left[\frac{Q_{ref}}{Q}\right]^{-1/3} \tag{2.6}$$

The polymer network is stretched when Q is greater than Q_{ref}, or compressed when Q is smaller than Q_{ref}. The polymers are non-deformed or relaxed when Q equals Q_{ref}; hence the term reference state.

2.4 Results and discussion

2.4.1 Water sorption isotherms

Water sorption isotherms were determined for faba bean protein isolate (FPI) and pea protein isolate (PPI) (Figure 2.1). The isotherm for soy protein isolate (SPI) was added for the sake of comparison. SPI has slightly higher water sorption than PPI and FPI when the water activity exceeds 0.7. Isotherms were fitted with Flory-Huggins Free Volume theory to determine the interaction parameter χ (Equation 2.12). Values for the glass transition temperature in the dry state, T_g, were taken from [29] and were 436 K for FPI and 438 K for PPI. The fitted values for χ were 0.96±0.03 and 0.90±0.02 for FPI and PPI respectively. The values are comparable to what we found previously for soy protein isolate (SPI; 0.91±0.02 [19]). The interaction parameter of gluten was determined previously as 1.16±0.04 [19]. The interaction parameters were used in Section 2.4.3 to determine the water partitioning in protein mixtures.

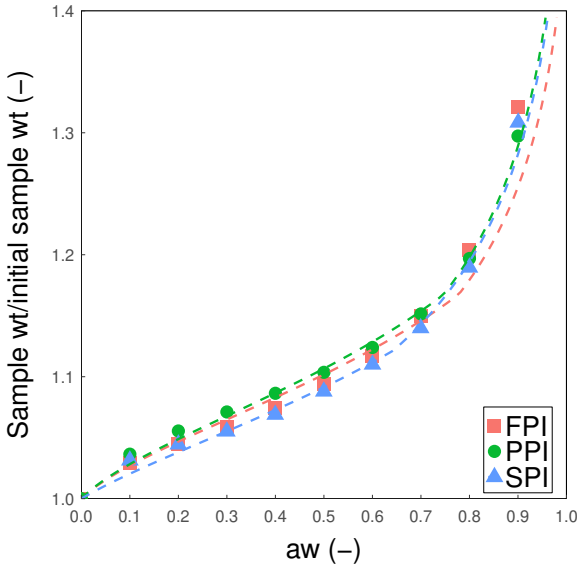

Figure 2.1: Sorption isotherms for powders of Soy Protein Isolate (SPI), Pea Protein Isolate (PPI) and Faba bean Protein Isolate (FPI) as determined at 25 °C. Fitted Flory-Huggins interaction parameters for SPI, PPI and FPI were 0.91±0.02 0.90±0.02 and 0.96±0.03 respectively. SPI data was reproduced from [19].

2.4.2 Swelling of single-phase gels

Gels were prepared from SPI, FPI, and PPI with different DMC at gelation. The gels were swollen in water until maximum swelling was achieved (Figure 2.2). Swelling decreases with increasing DMC at gelation for all tested polymers, following a power law. For SPI gels this reduction was shown to be due to an increase in cross-link density [19]; we expect this explanation also to hold for FPI and PPI. The swelling ratios of SPI and PPI are comparable, while FPI gels swell much less. Given the similar affinity for water of the different proteins (Figure 2.1), differences in cross-link density are most likely responsible for the observed differences in swelling as follows from Flory-Rehner theory (Equation 2.3).

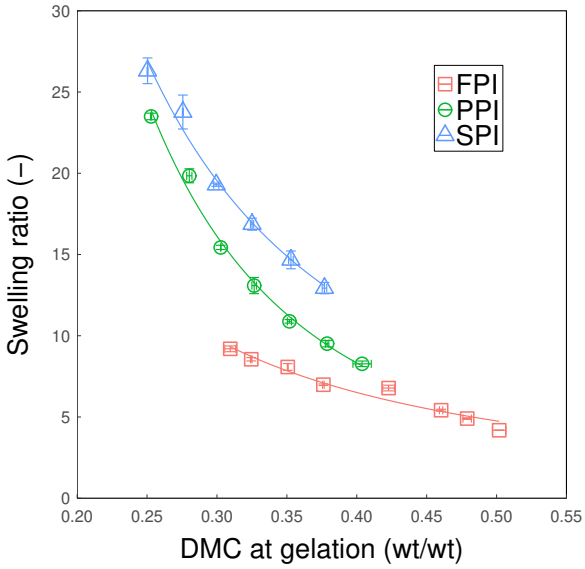

Figure 2.2: The swelling ratio of single-phase FPI, PPI, and SPI gels at maximum swelling presented as function of the dry matter content at gelation. Solid lines represent fits for $y = a(x^b)$ with $a = 1.80$, $b = -1.40$ for FPI, $a = 1.01$, $b = -2.30$ for PPI and $a = 2.35$, $b = -1.76$ for SPI. Error bars represent standard error from the mean with $n = 3$. SPI data was reproduced from [19].

2.4.3 Swelling of mixed gels

Mixed gel swelling when assuming no mechanical interaction

When assuming no mechanical interaction, the expected level of swelling for the non-gluten protein follows from the relation established for the single-phase gels, as shown in Figure 2.2. Since the swelling of single-phase gels depends on the DMC at gelation (Figure 2.2), the water partitioning in the mixed gel prior to gelation must be known to determine the level of swelling of the gel. This water partitioning was calculated using Flory-Rehner theory (Equation 2.15). Equation 2.15 was solved to arrive at the DMC in the non-gluten polymer before gelation. The expected level of swelling of the non-gluten bio-polymers is presented as the open symbols in Figure 2.3. When no mechanical interaction is assumed, all three bio-polymers show an increase in the expected level of swelling with increasing gluten content (Figure 2.3).

Mixed gel swelling with mechanical interaction

The actual experimental swelling ratios of the non-gluten phase when mechanical interactions are taken into account are presented in Figure 2.3 (closed symbols). The actual values show an opposite trend compared to when mechanical interactions are ignored, with the swelling ratio going down instead of up. This difference is due to the mechanical interaction between the gluten and non-gluten phases, as we have previously shown for SPI-gluten mixtures [19]. The reduction in swelling was the result of the continuous gluten network present. The similar qualitative behaviour shown here for PPI and FPI suggests that there is a similar mechanical interaction between gluten and the other proteins used.

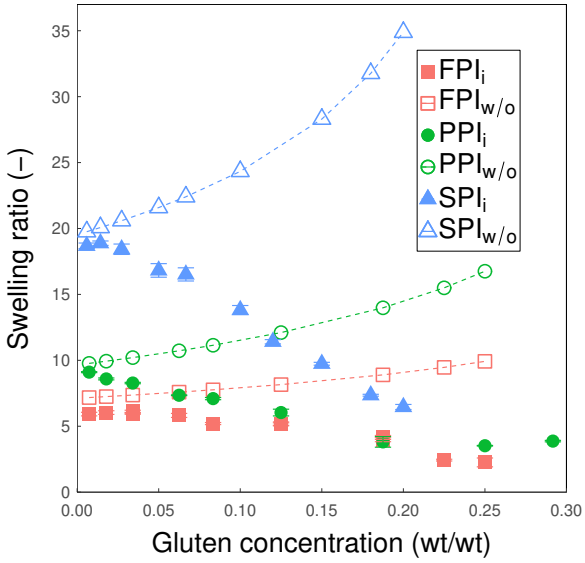

Figure 2.3: The swelling ratio of fully swollen mixed gels prepared from gluten with either FPI, PPI, or SPI as a function of the gluten concentration at gelation. Gluten swelling was subtracted used Equation 2.2 to determine the swelling ratio of the non-gluten protein phase. Open symbols represent expected swelling ratios when no mechanical interaction between phases is assumed and is based on the swelling of the single-phase gels (Figure 2.2), taking into account water partitioning. Closed symbols are measured values. For PPI and FPI a total initial DMC of 0.375 wt/wt was used; for SPI, 0.3 wt/wt was used. Error bars are standard errors from the mean with n = 3.

To identify any universality in the effect of gluten on mixed gel swelling, the relative swelling ratio of the non-gluten polymers was determined. The relative swelling ratio was calculated by dividing the swelling ratios with the swelling ratio of single-phase gels with the same initial total DMC (Figure 2.4). All three proteins show a similar *relative* reduction in swelling (Figure 2.4), despite the differences in absolute swelling (Figure 2.3). A linear regression led to a fit with $y = -2.436x + 0.955$ ($R^2 = 0.90$). Adding the type of non-gluten polymer as an independent fit parameter did not significantly improve the fit. This suggests that gluten has a similar interaction with the three different proteins used.

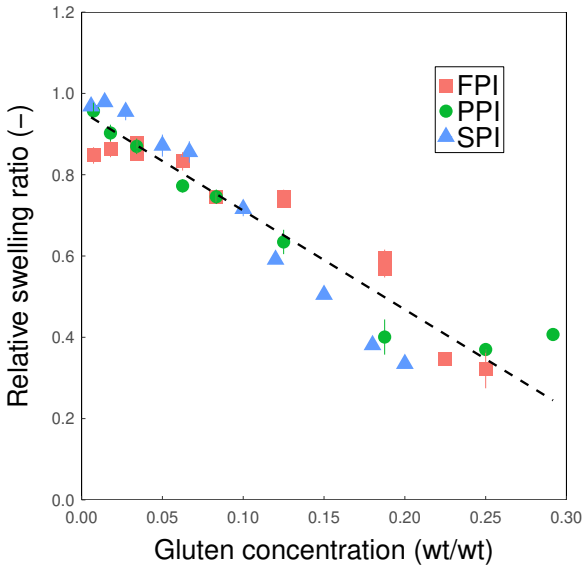

Figure 2.4: Measured levels of non-gluten swelling (FPI, PPI, and SPI; Figure 2.3) were divided by the swelling of their respective single-phase gel with the same DMC. The obtained relative swelling is expressed as function of the gluten fraction of total protein ($n = 3$). Dashed line is a linear fit ($y = -2.436x + 0.955$), with $R^2 = 0.90$.

Deformation of the non-gluten phase

The similar relative reduction in swelling ratio and clear dependence on gluten concentration suggest a universal underlying mechanism. To better understand the apparent universality of the effect of gluten content on mixed gel swelling we will

discuss the deformation of the non-gluten phase. As explained in Section 2.3.2, deformation is relative to a reference state, Q_{ref}. The value of Q_{ref} is directly related to the maximum swelling ratio, Q_0, via $Q_{ref} = Q_0/1.5$ (Equation 2.5). Due to the mechanical interaction with gluten, the maximum swelling ratio (Q_0) cannot be reached. However, since the water partitioning between the gluten and non-gluten phases before gelation was determined (Equation 2.15), the expected swelling ratios when no mechanical interaction is assumed are known (open symbols; Figure 2.3). By using Equation 2.5, we obtain the values for Q_{ref} for the different proteins and gluten concentrations. The strain on the non-gluten phase is then obtained by entering Q_{ref} and the experimental values for Q (closed symbols; Figure 2.3) into Equation 2.6.

van der Sman [28] constructed a master curve of network deformation (φ/φ_{ref} or equivalently Q_{ref}/Q) versus Π_{ext} normalized with the cross-link density of the gel and identified two distinct regimes. Furthermore, it was concluded that when $Q > Q_{ref}$ the elastic pressure dominates the swelling behaviour, and when $Q < Q_{ref}$ the mixing pressure dominates. The pressure applied by gluten, Π_{gluten}, is known to increase with increasing gluten content of the gel [19]. Gluten content can therefore be considered as proportional to the externally applied pressure, Π_{ext}. By plotting the gluten content as a function of Q/Q_{ref} we obtain an approximation of the master curve as presented by van der Sman [28] (Figure 2.5). Two different slopes can be observed, with a transition around $Q = Q_{ref}$, in line with the master curve shown by van der Sman [28]. This suggests that the same transition from elastic to mixing pressure dominated behaviour might also occur in these gels.

The pressure exerted by gluten on the non-gluten phase depends on the deformation of gluten. Since gluten forms a continuous network in the mixed gels, its deformation must be proportional to the swelling of the non-gluten phase. The apparent master curve in Figure 2.5 suggests this effect might already be captured, which can be qualitatively explained based on the effect of the elastic modulus on the swelling and deformability of polymer networks. A polymer network with a low elastic modulus will swell more but is also more easily deformed than a network with a higher elastic modulus. The greater deformability results in a larger absolute reduction in swelling compared to a network with a higher modulus, and vice versa (Figure 2.3). Due to the balance between the swelling pressure and the external pressure (or gluten pressure), these differences are limited on a relative scale (Figure 2.4). This balance may only be there for materials with a similar Flory-Huggins interaction parameter, as is the case here. Figure 2.5 shows that gluten interacts in a similar way with the three different

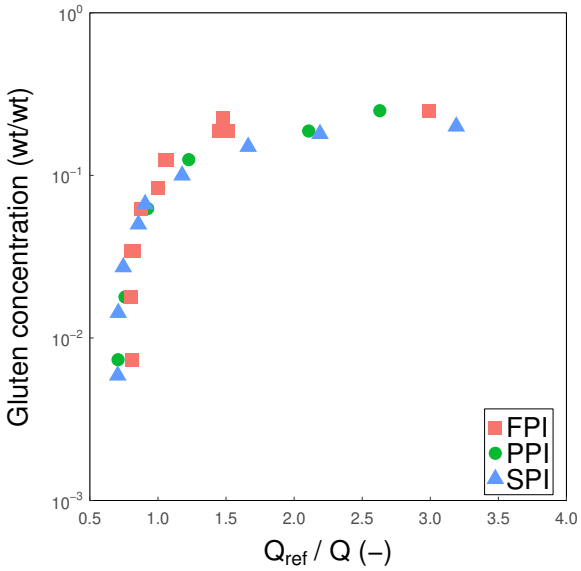

Figure 2.5: Approximation of the master curve as shown by van der Sman [28], with Q_{ref}/Q as a measure for deformation and gluten concentration as a measure for Π_{ext}. Q_{ref} follows from Equation 2.5 and Figure 2.3.

proteins used. This suggests that the different protein phases in the mixed gels are arranged in the same way.

It must be noted that the relation of Q_{ref} and Q_0 in SPI/WG gels was found to be $Q_{ref} = 0.69Q_0$ in a previous study [19]. Using this relation causes a horizontal shift and dilation but results in a comparable curve, and therefore does not impede the interpretation made above. Our results do not allow us to select one or the other value. The discrepancy between the value found by Cornet et al. [19] (0.69) and van der Sman [28] (1.5) was attributed to possible inhomogeneities in cross-link density [19]. For the sake of completeness, we have included Figure S1 in the Supplementary information, where this alternative relation is used.

Swelling of sheared samples

The gluten-containing mixtures were also swollen after processing in a shear cell. This will relate the shear structuring experiments to the swelling experiments and

reveal any effect of thermo-mechanical treatment on swelling. Absolute swelling ratios of the sheared single-phase SPI and PPI gels were significantly higher than of the non-sheared gels; FPI was significantly lower (see caption to Figure 2.6). We note that the difference in swelling between the sheared and non-sheared samples in absolute terms are limited. For sheared SPI and PPI gels, the relative swelling as a function of gluten content shows a negative trend, as was also observed for the non-sheared samples (Figure 2.4). The gradual reduction in swelling was less extensive but suggests a similar effect of gluten for SPI and PPI. The smaller effect of gluten on swelling in the sheared samples may be due to anisotropy of the gluten phase. The anisotropy could modify the mechanical interaction between the gluten and non-gluten phase. This could lead to anisotropic and potentially higher swelling of the non-gluten phase. However, anisotropy in swelling was not observed. Differences in processing temperature offer an alternate explanation for the observed differences in swelling. The effect of gluten content on the swelling of the sheared FPI gels is different from SPI and PPI, with an increase in swelling ratio with gluten content. The sheared FPI samples suffered from skin formation while the SPI and PPI samples did not (Figure S2). The skin appeared to be harder than the bulk of the material, hindering the swelling of the sample. Skin formation also seemed to reduce with increasing gluten content. Hence, the swelling ratio of the pure FPI sample was probably affected the most. Since the effect of the skin is not constant, its effect on swelling cannot be differentiated from that of the gluten content. Since the relative swelling ratio (insert in Figure 2.6) is a function of the swelling ratio of the sample without gluten, the values of FPI can not be compared directly with those of the other proteins. Still, based on the observations for SPI and PPI we believe that gluten also has a mechanical interaction with the non-gluten proteins after processing in a shear cell.

2.4.4 Structure formation under shear

To study the relation between the presence of a continuous gluten network and the formation of fibrous structures, sheared gels were produced from FPI, PPI and SPI in combination with gluten using a HTSC. The same compositions were used as for the mixed gels (Sec. 2.4.3). Gluten content will be indicated as a weight fraction of the total protein content. The sheared gels were bent in the parallel direction to the shear flow direction to visualize any fiber formation using a method similar to [6, 18] (Figure 2.7). Shearing of pure FPI, PPI and SPI doughs without gluten resulted in visually homogeneous gels with no orientation in the shear flow direction. Addition of 0.167 wt/wt gluten had no effect on the structure of either the FPI or SPI sample. The PPI sample showed a rough surface after bending without visible orientation or

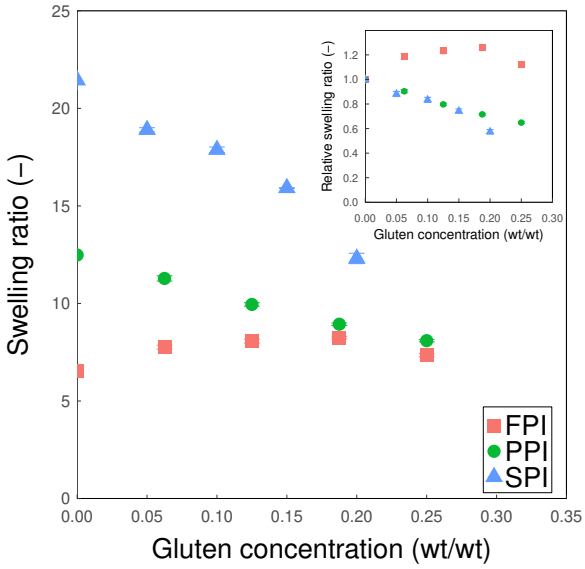

Figure 2.6: The swelling ratio of fully swollen, sheared mixed gels prepared from gluten with either FPI, PPI, or SPI as a function of the gluten concentration at gelation (main figure). Gluten swelling was subtracted used Equation 2.2 to determine the swelling ratio of the non-gluten protein phase. For PPI and FPI a total initial DMC of 0.375 wt/wt was used; for SPI, 0.3 wt/wt was used. The measured levels of non-gluten swelling of sheared samples (FPI, PPI, and SPI) were divided by the swelling of their respective single-phase sheared gel with the same DMC. The obtained relative swelling is expressed as a function of the gluten fraction of total protein (insert). Error bars are standard errors from the mean with n = 3.

fibers. At 0.33 wt/wt gluten both FPI and PPI mixtures showed structures orientated in the shear flow direction, but no fibers were observed. The SPI mixture showed no orientation at all. For all ingredients fibers appeared only when at least 0.5 wt/wt gluten was added. The individual fibers in the FPI sample containing 0.5 wt/wt gluten appear to be thicker than in the corresponding PPI and SPI samples, although the fibers become thinner for all three proteins as the gluten content approaches 1 wt/wt.

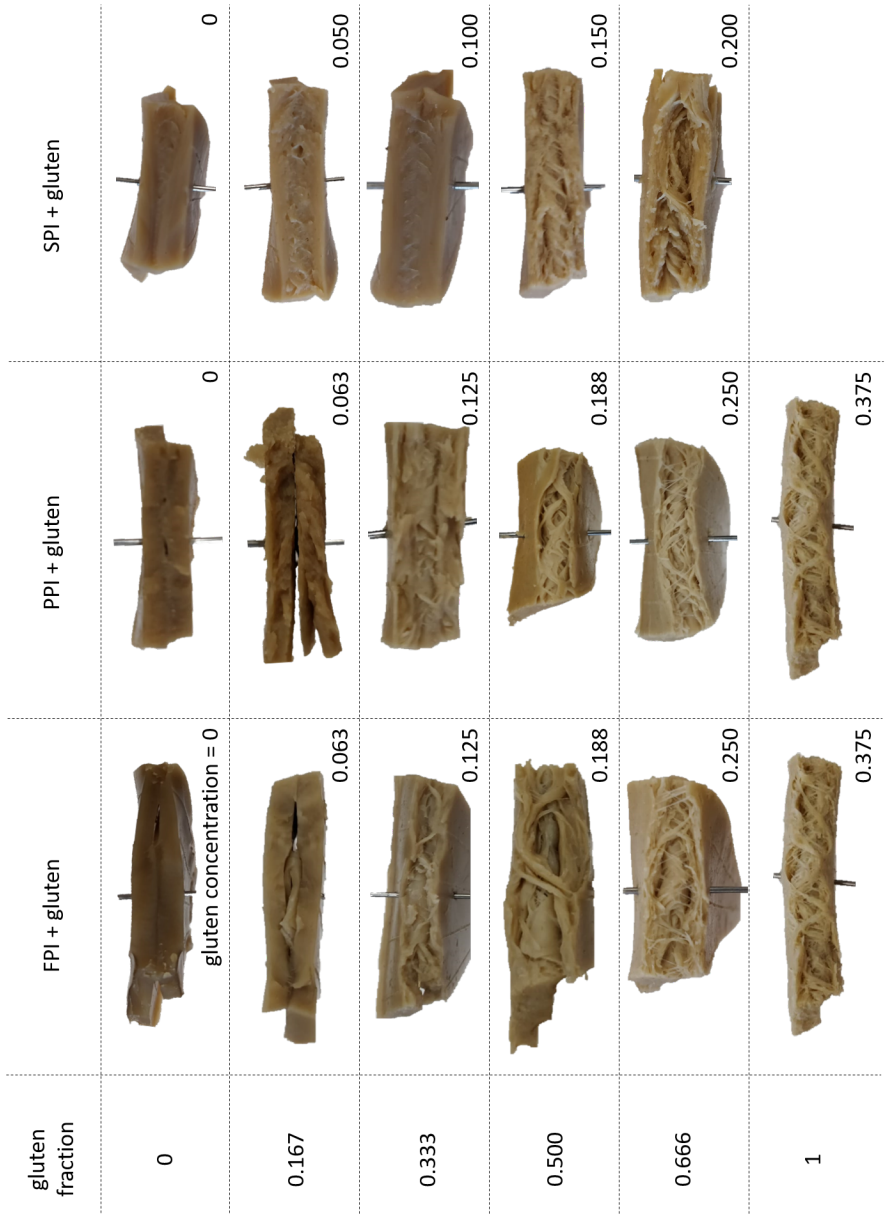

Figure 2.7: Photographs of the macro-structures obtained after structuring FPI-gluten, PPI-gluten and SPI-gluten mixtures in a shear cell. The left column indicates the gluten fraction of total protein (wt/wt), while the numbers presented with the images indicate actual the actual gluten concentration (wt/wt). The shear cell was operated at 140°C for 15 min at a shear rate of 30 rpm (39 s^{-1}). Structures were prepared in duplicate; these are representative images of the structure found in the outer 4 cm of the sample. Each sample has a width of approximately 5 cm ($n = 2$).

2.4.5 General discussion

We investigated the swelling and shear cell structuring of gluten mixed with SPI, PPI or FPI. Single-phase SPI, PPI, and FPI gels were swollen until equilibrium and used to predict the swelling of the gluten-containing mixed gels. These predictions assumed no interaction between the gluten and non-gluten phases and suggested an increase in non-gluten swelling ratio with increasing gluten content (Figure 2.3). Experimental measurements of mixed gel swelling showed, however, that there was an interaction between the gluten and non-gluten phases, which resulted in a decreased swelling ratio. The absolute levels of swelling differed between protein sources. After normalization with gels of the pure secondary protein phase, seemingly universal behaviour was seen regardless of protein source and absolute swelling ratio (Figure 2.4). Based on our previous study on SPI–gluten mixtures, the interaction can be attributed to the presence of a continuous gluten network [19]. This seemingly universal behaviour indicates that gluten might interact in a similar manner with other proteins as well, although this requires further investigation. Further analysis of the deformation of the polymer network revealed behaviour similar to that reported by van der Sman [28] and underlined the similarity of the interaction with gluten between the different proteins (Figure 2.5). Furthermore, the two apparent master curves obtained suggest that the gluten–non-gluten composites have similar structures for the different proteins. However, additional experiments are necessary to confirm the origin of the universality.

We initially hypothesised there to be a percolation threshold above which a continuous network would be present. This would have been indicated by a sudden reduction in swelling ratio when the gluten content surpassed this threshold. The approximately linear reduction in swelling ratio starting from low gluten contents onward does not support this hypothesis and implies that there is an interaction already at low gluten contents. Our shear cell experiments showed that when gluten is the main component ($\geq$ 0.5 wt/wt) fibrous structures can be made with all of the proteins used (Figure 2.7). This level of similarity and interchangeability between protein sources has not been seen before in shear cell processing. Previous studies using SPI and PPI in combination with gluten also found fibers at gluten fractions of 0.5 wt/wt [12, 16]. In contrast, earlier studies reported the presence of fibers in sheared SPI–gluten gels at lower gluten fractions (>0.2 wt/wt) [1, 2]. However, direct comparison with the present study is impeded by the lower DMC and processing temperatures used in the mentioned studies (0.3 wt/wt and 95°C), along with the lack of visual representations of the formed structures. Grabowska et al. [1] also reported the formation of fibers

using only hydrated gluten (0.3 wt/wt). However, since this was accompanied by hysteresis, the moisture content cannot be compared directly with the current study. We note that recent studies also show an apparent shift towards higher gluten content formulations to produce fibrous structures.

A recent rheological study by Schreuders et al. [12] showed that gluten is a continuous or bi-continuous phase during shear treatment at high temperature (120-140°C) when combined with PPI or SPI. The exact structure of the composite was found to depend on the processing conditions and raw material used. Micro-graphs of mixed SPI-gluten gels taken by Dekkers et al. [16] using confocal scanning laser microscopy (CSLM) show that gluten can be present in elongated domains at relatively low concentrations (gluten fraction = 0.06 wt/wt; total DMC 0.30 wt/wt). Lucas et al. [30] studied the micro-structure of gluten networks in wheat flour doughs in more detail and developed a system to classify the different microstructures. Some of the observed structures had very thin gluten strands. These thin gluten strands were associated with a more inter-connected and branched network. Possibly, such a continuous gluten network forms at low gluten fractions when gluten is combined with another protein, as indicated by the reduction in non-gluten swelling at low gluten fractions (Figure 2.4). Such a low-volume but interconnected gluten phase could still limit the swelling of the non-gluten phase, but might not be visible upon inspection of the macro-structure of the sheared material due to its limited volume fraction. However, the gluten concentration above which a continuous gluten network forms cannot be determined based on our results.

The exact mechanism by which fiber are formed is still unclear [31]. However, a dominant hypothesis on how polymer systems form fibrous structures under shear flow is based on the deformation and solidification of a two-phase system [17, 32, 33]. The dispersed phase is thought to deform and align, resulting in mechanical anisotropy. Grabowska et al. [1] already suggested that gluten forms the continuous phase in SPI-gluten based fibrous structures. SPI was, therefore, considered the dispersed phase, contributing to the fiber formation. In mixed (non-sheared) SPI-gluten gels, gluten is thought to form a continuous network that entraps SPI [19]. However, Schreuders et al. [15] showed that under shear flow a structure with gluten being co-continuous with the second bio-polymer can occur, which was concluded based on their rheological behaviour. The presence of a bi-continuous network structure is not in line with the hypothesis of a dispersed and deformed phase [11, 17].

Our results suggest that the non-gluten phase is not essential to fiber formation as fibers were also obtained in the absence of a second protein, as shown in Figure 2.7.

Hydrated gluten could be considered a two-phase system with glutenin and gliadin making up the two respective phases [27]. Hence, hydrated gluten alone could already fulfill the requirement of a dispersed and continuous phase. This could explain why fibers can be produced from hydrated gluten alone. Furthermore, commercial gluten ingredients also contain starch residues, which could act as a second (or third) phase. Addition of limited amounts of a second bio-polymer (≤ 0.5 wt/wt) still resulted in the formation of fibers. When gluten was no longer the main continuous component, no fibers were formed. We, therefore, propose that in gluten-containing mixtures, gluten is primarily responsible for the formation of fibers, while the second polymer acts merely as a filler. Addition of a second polymer can still be useful though, as our results showed that by varying the amount of second polymer one can modulate the thickness of the fibers and extent of fibrillation.

We note that the formation of a fibrous structure does not only depend on formulation; process parameters such as temperature and shearing time are key and will need to be adjusted [1, 11, 12]. Nevertheless, a continuous gluten network seems essential to achieve a fibrous structure.

2.5 Conclusion

We have studied the swelling of single-phase gels from faba bean protein, pea protein, and soy protein, as well as mixed gels in combination with gluten. Analysis of the swelling of gluten-containing mixed gels suggested that gluten applies an external pressure that limits non-gluten swelling. This was attributed to the formation of a continuous gluten network. Normalizing the level of swelling with that of a single-phase gel resulted in seemingly universal behaviour between the three studied polymers, regardless of DMC and absolute level of swelling. Shear structuring with a High Temperature Shear Cell resulted in the formation of fiber structures when gluten was the main protein component. Hydrated gluten also forms fibers without a second bio-polymer present. This suggests that the choice of the non-gluten bio-polymer could extend beyond the bio-polymers used in this study. These insights could benefit future investigations into the use of novel ingredients for use in meat analogue products.

Supplementary information

Mixing pressure

We have used free volume Flory-Huggins theory to describe Π_{mix}:

$$\Pi_{mix} = \frac{RT}{\nu_w}\left[\ln(1-\varphi) + \varphi(1-\frac{1}{N}) + \chi\varphi^2 + F(\varphi)\right] \tag{2.7}$$

Here, R is the universal gas constant, T is the absolute temperature, v_w is the molar volume of water, φ is the polymer volume fraction, $F(\varphi)$ accounts for the additional sorption due to excess elastic energy stored in glassy polymeric materials [34, 35] and N is the ratio of the molar volumes of water and polymer. Since the molar volume of protein is very large compared to that of water, the term $\frac{1}{N}$ is effectively zero, simplifying the equation. χ is the effective Flory-Huggins interaction parameter and captures the polymer-water affinity. χ is composition dependent and therefore depends on φ [36]:

$$\chi = \chi_0 + (\chi_1 - \chi_0)\varphi^2 \tag{2.8}$$

χ_0 and χ_1 are the interaction parameters under dilute and concentrated conditions respectively. Since water is a theta solvent for proteins in the limit of low protein concentrations, χ_0 is 0.5 [28]. Heat can induce protein unfolding, which can affect its water sorption as indicated by a change in χ_1 [37]. We recently found that for SPI and gluten, the value of χ_1 is the same before and after heating [19]. The production of commercial protein isolates often involves intensive processing, which generally renders the proteins denatured. Therefore, we assume that the same holds for FPI and PPI. Hence, χ_1 was considered constant. $F(\varphi)$ was calculated based on van der Sman and Meinders [36]:

$$F(\varphi) = \begin{cases} 0 & \text{if } T \geq T_g \\ -M_w y_s^2 \frac{\Delta C_{p,w}}{RT}\frac{dT_g}{dy_s}\frac{T-T_g}{T_g} & \text{if } T \leq T_g \end{cases} \tag{2.9}$$

with

$$\frac{dT_g}{dy_s} = -\frac{\Delta C_{p,s}\Delta C_{p,w}(T_{g,w} - T_{g,s})}{(y_w\Delta C_{p,w} + y_s\Delta C_{p,s})^2} \tag{2.10}$$

and T_g was calculated according to Couchman-Karasz [38]:

$$T_g = \frac{y_w\Delta C_{p,w}T_{g,w} + y_s\Delta C_{p,s}T_{g,s}}{y_w\Delta C_{p,w} + y_s\Delta C_{p,s}} \tag{2.11}$$

M_w represents the molar weight of water, $\Delta C_{p,i}$ is the change in heat capacity over the glass transition, $T_{g,i}$ is the glass transition temperature of the pure material, and y_i represent the weight fractions of polymer and water as denoted with subscripts s and w, respectively. $\Delta C_{p,s}$ was taken as 0.425 kJ K^{-1}, which appears to be universal for bio-polymers [36, 37, 39]. The additional term introduced by the free volume extension, $F(\varphi)$, is equal to zero in the rubbery regime ($T > T_g$). Hence, $F(\varphi)$ was equal to zero when determining the water partitioning. The water activity a_w, relates to Π_{mix} via:

$$\ln(a_w) = \frac{v_w \Pi_{mix}}{RT} \tag{2.12}$$

Elastic pressure

We describe Π_{elas} using the phantom network model:

$$\Pi_{elas} = -G_{ref}[\tilde{\varphi}^{1/3} - \frac{\tilde{\varphi}}{2}] \tag{2.13}$$

G_{ref} is the elastic shear modulus in the reference state. The reference state refers to the composition at which the polymers in the network are relaxed, and thus experience neither compressive nor extensional stress [40]. $\tilde{\varphi}$ is a measure for network deformation relative to the reference state φ_{ref}:

$$\tilde{\varphi} = \frac{\varphi}{\varphi_{ref}} \tag{2.14}$$

Water partitioning in a bio-polymer mixture

The elastic properties of the non-gluten protein phases depends on the moisture content at gelation. Therefore, the hydration properties of the gel also depend on the moisture content at gelation. At the moment of gelation, the water is assumed to have partitioned between the gluten and non-gluten protein according to thermodynamic equilibrium. To calculate the water partitioning, the gluten was considered a cross-linked network [41, 42], while this was not the case for the other proteins (soy, pea, and faba bean). Hence, FR theory was used to describe the hydration of gluten (Equation 2.13 and 2.7 [19]), while regular FH theory was used for the hydration of the other protein phase (Equation 2.7). We have recently used this approach to describe water partitioning between soy protein and gluten [19] and will use the same approach here. Thus, the water partitioning in the hydrated protein mixture before gelation can be found by solving:

$$\Pi_{mix,i}(\varphi_i) = \Pi_{mix,gluten}(\varphi_{gluten}) - \Pi_{elas,gluten}(\varphi_{gluten}) \tag{2.15}$$

with i as either SPI, FPI, or PPI. The elastic properties of gluten were taken as $G_{ref} = 0.17$ MPa and $\varphi_{ref} = 0.023$ [19]. These values were determined previously [19] by fitting Equation 2.15 to the water partitioning data presented by Dekkers et al. [43]. They used time-domain nuclear magnetic resonance (TD-NMR) to determine the water partitioning in hydrated mixtures of soy and gluten with DMCs between 25 and 45 %. The water partitioning determined by Cornet et al. [19] using Equation 2.15 was in reasonable agreement with the aforementioned TD-NMR data. We note that this does not necessarily impart any physical meaning to these elastic properties. The value of φ_{ref} is not physically attainable by swelling gluten gels, which might explain the high value of G_{ref} compared to experimental values for G ($\sim$0.07 MPa; [19]). Nonetheless, Equation 2.15 is considered to provide a reasonable approximation of the water partitioning.
The composition of the gluten phase and phase i will depend on the amount of water added to the proteins at the moment of hydration. Denoting the weight fraction of water in the hydrated mix as wt_w, the protein weight fractions as $wt_{p,i}$ and the water partition coefficient as P, the polymer volume fractions of the two phases read:

$$\varphi_i = \frac{wt_{p,i}/\rho_p}{wt_{p,i}/\rho_p + (P \cdot wt_w)/\rho_w}$$

$$\varphi_{gluten} = \frac{wt_{p,gluten}/\rho_p}{wt_{p,gluten}/\rho_p + ((1-P) \cdot wt_w)/\rho_w} \tag{2.16}$$

ρ_p and ρ_w are the densities of polymer and water, and taken as 1330 kg m^{-3} and 1000 kg m^{-3}. The water partitioning coefficient P is obtained by simultaneously solving Equation 2.15 and 2.16. Equation 2.15 was solved using the bisection method and varying the partition coefficient P.

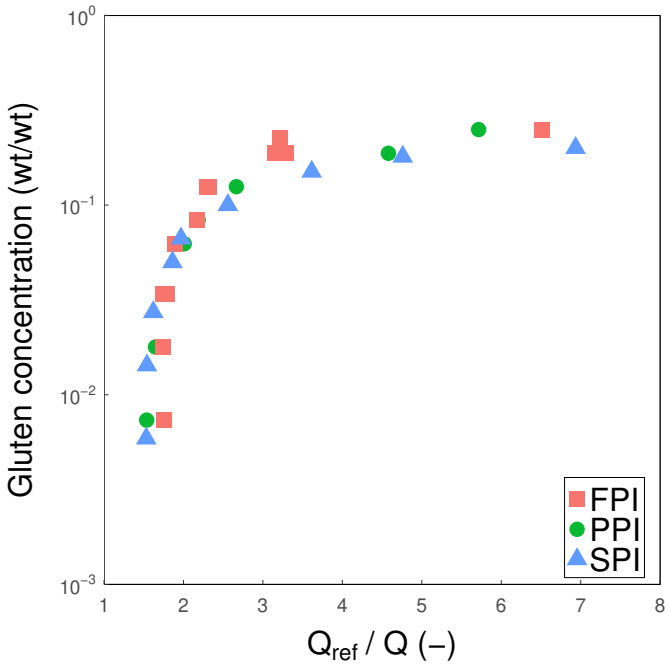

Figure S1: Approximation of the master curve as shown by van der Sman [28], with Q_{ref}/Q as a measure for deformation and gluten concentration as a measure for Π_{ext}. Q_{ref} follows from $Q_{ref} = 0.69Q_0$ [19] and Figure 2.3.

(a)

(b)

Figure S2: Representative image of a FPI – gluten sample after shear structuring. The darker areas had a skin (Figure S2a). Representative image of a sample after shear structuring, without a skin (Figure S2b).

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Chapter 3

Modifying Faba Bean Protein Concentrate Using Dry Heat to Increase Water Holding Capacity

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3.1 Abstract

We investigated the effect of dry-heat treatment on the properties of faba bean protein concentrate using soy protein concentrate as a benchmark. While soy protein—widely used as an ingredient in meat replacers—is recovered through a wet fractionation, protein recovery from starch bearing pulses like faba bean can be done via dry fractionation. This process does not require drying or heating steps and therefore, keeps the original protein functionality intact. This results in differences in properties such as water binding capacity of the protein fraction. Faba bean protein concentrate was dry-heated at temperatures from 75–175 °C, which resulted in higher water-holding capacity and less soluble protein, approaching values of soy protein concentrate. These changes were due to partial denaturation of protein, changing the structure of the protein, and exposing hydrophobic sites. This led to protein aggregation, as observed by light microscopy. Only noncovalent bonds caused the decrease of solubility of dry-heated faba bean protein concentrate. We conclude that dry-heating of dry fractionated faba bean protein can change the functional properties of the protein fraction to desired properties for certain applications. The effect is similar to that on soy, but the underlying mechanisms differ.

3.2 Introduction

In recent years, both research and governing organizations have stressed the need for a protein transition [1]. Currently, soy is the most extensively researched and commercially used crop for plant proteins [2, 3]. Soy protein products are widely applied in Asian cuisine and in novel products such as meat analogues. However, a successful protein transition comprises diversification of protein sources. Faba bean could be another plant-based protein source next to soy. It can be grown in colder areas such as northern Europe [4], and is an excellent break crop for wheat-heavy growing cycles [5]. Currently, faba beans are mostly applied in feed applications, but show great potential to be applied in food as well [6], as faba bean protein has good nutritional quality [7]. Faba bean protein can be obtained through dry or wet fractionation, resulting in faba bean protein concentrate (60 % protein [8]) or isolate. Faba bean protein has been used in pasta production to increase protein content [9–11] without negatively influencing product quality when replacing up to 30 % of traditional ingredients. Further applications could include baking [12, 13] and use as binders and nutritional enhancers in meat products [14]. It is also used in meat analogue products such as Beyond Sausages (Beyond Meat, El Segundo, USA), Pulled Oats (Gold And Green, Helsinki, Finland), or Crab Free Cakes (Good Catch, New York City, NY, USA). In these products, faba bean is an additive to increase protein content or part of a bean blend [15]. Nevertheless, the current industrial applications are still limited, and therefore faba bean is mostly consumed as an unprocessed side dish [6].

To expand the use of faba bean protein in, for example, meat analogues, its functional properties should be similar to those of soy. Soy is mostly used for structuring applications because of its characteristic functional properties, such as high water-holding capacity (WHC) and good gelling behavior, fat absorption, and emulsification capacities [16]. WHC has been established as a crucial property and is one of the properties often used to describe the functionality of ingredients of meat replacers [17–21]. It has been shown that higher WHC leads to better structuring behavior. Therefore, it was chosen as the focus point of this study. During the purification process, soy protein undergoes a dry-heating or drying step. This impacts water-holding capacity and makes soy suitable for meat replacers [17]. Furthermore, extraction and precipitation conditions can also influence structural and functional properties of soy and faba bean protein [22, 23].

Dry fractionation, such as air classification, can be used to create fractions from faba bean that are enriched in protein or starch, as opposed to soy, in which protein is concentrated through a wet process. Dry fractionation is less energy intensive since no water is added, and therefore does not need to be removed, making this process more sustainable and economical [8, 24]. Therefore, the protein-rich ingredients are not heated during purification, allowing them to maintain their native state and functionality [8]. Dry processed faba bean proteins have a high solubility in water [24], a low water-holding capacity [25], and the doughs prepared from faba bean proteins are sticky [9], making them difficult to handle [26]. Consequently, the heating step is identified as a potential solution to the low applicability of faba bean protein in meat replacers and possibly other structured food products caused by the less suitable properties for structuring after dry fractionation. Previous studies on dry or mild fractionation processes for faba beans and other legumes often focus on other applications such as emulsions and foams, where high solubility, high emulsification capacity, and high foam stability are required [24, 27]. A dry-heat treatment has already been applied by Petitot et al. [10] to change the properties of pasta that contained faba bean protein. They report a stronger structure of the faba bean fortified pasta after a dry-heat treatment and possible Maillard reactions. However, to the best of our knowledge, no investigation was dedicated to explore the possibilities of dry-heating as a tool to improve functional properties such as water-binding capacity of faba bean or to unravel the mechanisms that cause this change in functional properties.

In this research, the influence of a dry-heat treatment on the functional properties important for structuring of faba bean protein concentrate are investigated. The results are compared to a soy protein concentrate. Further, the underlying mechanisms causing the change in functional properties are identified and compared to those in soy protein concentrate.

3.3 Materials and Methods

3.3.1 Materials

Faba bean protein concentrate (FPC, VITESSENCE® Pulse 3600) was obtained from Ingredion (Hamburg, Germany). Soy protein concentrate (SPC, ALPHA® 8 IP) from Solae (St. Louis, MO, USA) was used as a reference. Their protein content was measured with the Dumas method, using a Nitrogen analyzer, FlashEA 1112 series, (Thermo Scientific, Breda, The Netherlands) and a protein conversion factor of 5.71

[28, 29]. FPC had a protein content of 0.60 $g\,g^{-1}$, SPC 0.675 $g\,g^{-1}$. Further, the producer reports 0.031 $g\,g^{-1}$ fat, 0.16 $g\,g^{-1}$ carbohydrates, 0.11 $g\,g^{-1}$ dietary fibers, and 0.05 $g\,g^{-1}$ ash for FPC. All chemicals used were bought from Merck (Breda, The Netherlands).

3.3.2 Dry-Heating

Heating was done dry, in a hot air oven (Heratherm, Thermo Scientific, Breda, The Netherlands) at 75 °C, 100 °C, 120 °C, 130 °C, 150 °C, 160 °C, and 175 °C for 60 min. The protein concentrate powders were spread on a tray as an approximately 5–10 mm-thick layer. The tray was placed in the preheated oven and removed after 60 min. The dry-heated protein concentrates were subsequently placed in a desiccator to cool to room temperature and placed in a closed container for storage before further analysis. Dry heating at all temperatures decreased the water content from 0.0725 $g\,g^{-1}$ to less than 0.01 $g\,g^{-1}$.

3.3.3 Water-Holding Capacity and Overall Solubility

To determine the WHC and the solubility of protein and non-protein fractions of the protein concentrates, a method previously reported by Geerts et al. [17] was used. 0.02 $g\,g^{-1}$ dispersions were made from the protein concentrates. The dispersions were shaken at room temperature overnight before centrifugation at 10,000 g for 30 min at 20 °C (Lynx centrifuge, Thermo Scientific, Breda, The Netherlands). Supernatant was discarded and the mass of the wet pellet was recorded. After freeze-drying, the mass of the dry pellet was also recorded. The solubility of the material was determined according to Equation (3.1), $WHC_{overall}$ was determined according to Equation (3.2), and $WHC_{insoluble}$ was determined according to Equation (3.3).

$$solubility = \frac{m_{dry\ powder} - m_{dry\ pellet}}{m_{dry\ powder}}, \tag{3.1}$$

$$WHC_{overall} = \frac{m_{wet\ pellet} - m_{dry\ pellet}}{m_{dry\ powder}}, \tag{3.2}$$

$$WHC_{insoluble} = \frac{m_{wet\ pellet} - m_{dry\ pellet}}{m_{dry\ pellet}}, \tag{3.3}$$

in which $m_{dry\ powder}$ is the mass of the overall added dry powder, $m_{wet\ pellet}$ is the mass of the pellet after centrifugation and before drying, and $m_{dry\ pellet}$ is the mass

of the pellet after centrifugation and drying. Since the full material will eventually be used in a final application, the WHC is defined per overall powder instead of per protein. The WHC defined per insoluble material is used to identify the effect of solubility on WHC. Furthermore, protein content of the pellets was measured by the Dumas method, using a Nitrogen analyzer, FlashEA 1112 series (Thermo Scientific, Breda, The Netherlands) and a protein conversion factor of 5.71 to determine protein solubility.

3.3.4 Light Microscopy

An upright microscope Axioscope (Carl Zeiss Microscopy, LLC, Oberkochen, Germany) with a camera was used to inspect the samples on particle scale. Samples were prepared as a 0.02 $\mathrm{g\,g^{-1}}$ dispersion in milliQ water and shaken overnight at room temperature. Subsequently, one droplet of the sample was placed on a microscope glass slide, which was covered with a cover slip. The differences between the non-dry-heated and dry-heated samples were examined by determination of the size of the protein aggregates. Images were taken with 10× and 40× magnification.

3.3.5 SDS-PAGE

A reducing SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was performed on all samples. Dispersions of 4 $\mathrm{mg\,L^{-1}}$ of powder were made in water, shaken overnight, then analyzed using SDS-PAGE. 2-Mercaptoethanol was used as a reducing agent in the SDS-PAGE sample buffer. Ready-made BioRad running buffer as well as BioRad precast tris/glycine gels were used. Coomassie BioSafe stain was used to stain the protein bands.

3.3.6 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was used to determine the state of protein denaturation. Dispersions with 0.15 $\mathrm{g\,g^{-1}}$ dry matter in milliQ water were created and analyzed immediately. About 0.05 g of sample was weighed into High Volume Pans (100 µL, TA Instruments, New Castle, USA) while the weight was accurately recorded and placed in the DSC (DSC-250, TA Instruments). The pans with samples were equilibrated at 20 °C until the temperature was constant, then heated with a ramp of 5 $\mathrm{°C\,min^{-1}}$ to 125 °C. After cooling, the cycle was repeated once more per sample to verify irreversible denaturation of the protein. Onset temperature and peak height were determined using TRIOS software (TA Instruments). Enthalpy is expressed per total sample mass.

3.3.7 Analysis of Protein–Protein Interaction

Protein–protein interactions were analyzed using a method previously described in [30], with certain changes. A 0.2 mol L^{-1} sodium phosphate buffer (SPB) was used to disrupt electrostatic interactions. The same buffer with the addition of 17.3 mmol L^{-1} sodium dodecyl sulfate (SDS) and 8 mol L^{-1} urea were used to disrupt electrostatic interactions, hydrophobic interactions, and hydrogen bonds. Further, disulfide bonds were disrupted by adding 10 mmol L^{-1} dithiotreitol (DTT) to the second buffer. A total 10 mL of buffer and 0.1 g of protein was mixed and vortexed for 30 s and placed in a rotator for one hour. Afterwards, the solutions were centrifuged for 30 min at 10,000 g at 20 °C. The absorption of the supernatant was measured at 280 nm in a Beckman DU720 UV-VIS spectrophotometer (Woerden, The Netherlands). A calibration curve was made by a series of dilutions of Bovine serum albumin dissolved in the specific buffers in the appropriate range. All three buffers were adjusted to pH 6.9 before they were used on the same day they were prepared. Significant difference of the measured values was determined by 1-way ANOVA followed by a Tukey Test with $p < 0.01$.

3.3.8 Hydrophobicity of Insoluble Protein

To measure the hydrophobicity of the insoluble protein, an adaption of a method described by Chelh et al. [31] was used. Sample solutions of 0.02 g g^{-1} were shaken in a rotator for six hours and stored overnight in a fridge. The solutions were centrifuged at 10,000 g for 30 min. The supernatant was discarded and pellets were dried for 30 min by placing the tubes upside down on tissue paper. The amount of insoluble protein in the pellets was calculated by measuring and subtracting soluble protein content. This was used to ensure a constant concentration of insoluble protein in the assay. A total of 200 µL of a 1 mg mL^{-1} bromophenol blue (BPB) solution was added to 1 mL of protein dispersion containing 5 mg mL^{-1} of insoluble protein and mixed by a vortex before it was shaken for 15 min in an Eppendorf shaker at 600 rpm. The pH was adjusted to 6.9 for all samples, including the blank. After mixing, the solutions were centrifuged in an Eppendorf 5424 centrifuge (Eppendorf, Nijmegen) for 30 min at 12,000 g. The supernatant and a control of milliQ water with 200 µL BPB solution was diluted at a ratio of 1:10 and measured in a Beckman DU720 UV-VIS spectrophotometer (Woerden, The Netherlands) at 595 nm against a blank of milliQ water. The amount of BPB bound was calculated according to Equation (3.4).

$$BPB_{bound}\ (\mu g\,mg^{-1}) = \frac{200\mu g\ *\ (Absorbance_{control}\ -\ Absorbance_{sample})}{Absorbance_{control}\ *\ 5\ mg} \quad (3.4)$$

3.3.9 Determination of Reducing Sugars

To determine the amount of reducing sugars, a PAHBAH-assay was performed, as described by Lever [32]. To create the PAHBAH-reagent, 0.1 g of 4-Hydroxybenzhydrazide was added to 2 mL of a 0.5 mol L^{-1} HCl solution. Before use, 8 mL of a 0.5 mol L^{-1} NaOH solution was added. A glucose standard curve in the range 150–750 µg mL^{-1} was used. 10 % solutions of protein concentrate were made and treated following the same procedure as for determination of WHC, of which the supernatant was used. 200 µL of PAHBAH-reagent was added to 10 µL of supernatant in a 96-Well Elisa Microplate in different dilutions. The microplate was heated for 35 min at 70 °C on a shaker. Absorbance was measured at 405 nm using a Tecan reader 511 (Tecan Benelux).

3.3.10 Determination of Free Amino Groups

The amount of free amino acids present in the soluble protein was measured by OPA (o-Phthaldialdehyde) assay [33]. To obtain OPA-reagent, 3.81 g of borax and 100 mg SDS were dissolved in 80 mL of water, and 80 mg of OPA dissolved in 2 mL of ethanol was added. After dissolving the OPA, 88 mg of DTT was added and the solution was filled up to 100 mL. The solution was filtered using a 0.45-µm filter. A L-serine calibration curve of 50–200 mg L^{-1} was used. Samples were made and treated following the same procedure as for determination of WHC, of which the supernatant was used. 1.5 mL of OPA-reagent was mixed with 200 µL of supernatant. The samples were measured after 3 min at 340 nm using a Beckman DU720 UV-VIS spectrophotometer (Woerden, The Netherlands). The number of free amino groups per raw material was adjusted for protein solubility.

3.3.11 Statistical Analysis

Statistical analysis was performed using R in RStudio. The number of repetitions (n) is reported with the results. Significant differences of the measured values were determined by 1-way ANOVA followed by a Tukey Test with $p < 0.05$ unless stated otherwise. All differences discussed are significant, unless stated otherwise. Furthermore, significant differences are indicated by different small letters in the figures.

3.4 Results & Discussion

3.4.1 Influence of Dry-Heating on Solubility and Water Holding

The overall WHC of non-dry-heated FPC was 1.25 $g\,g^{-1}$, less than half of the value of SPC (3.53 $g\,g^{-1}$, Figure 3.1, left). While dry-heating at 75 °C and 100 °C did not alter the overall WHC, dry-heating at higher temperatures led to a significant increase in WHC. Substantial increases are seen for samples treated at 150 °C and 175 °C, reaching values of 3.10 $g\,g^{-1}$.

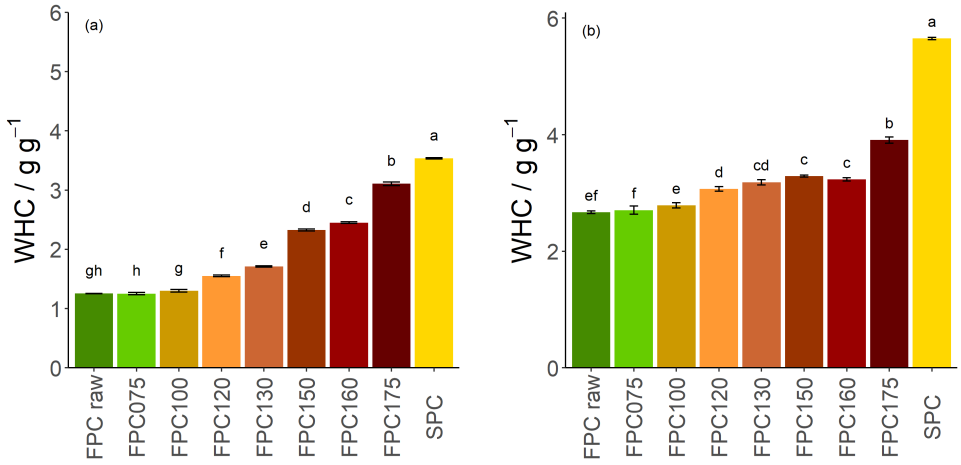

Figure 3.1: (**a**) Water-holding capacity of the overall powder (Equation (3.2)) and (**b**) water-holding capacity of the insoluble fraction (Equation (3.3)) of dry-heated and non-dry-heated faba bean protein concentrate (FPC) as well as commercial soy protein concentrate (SPC). $n = 3$. Water-holding capacity (WHC) of FPC powder dry-heated at up to 100 °C did not show any significant difference from non-dry-heated FPC powder. FPC powder dry-heated at higher temperatures showed an increase in WHC, with 150 °C and 175 °C having the largest effect. The WHC of the insoluble fraction only increased slightly after heat treatment, with the exception of 175 °C (right).

To understand this change in WHC, the solubility of FPC was investigated. The amount of soluble material was initially 0.53 $g\,g^{-1}$ for the non-dry-heated sample, remaining unchanged after dry-heating at 75 °C and 100 °C, and only slightly decreasing after dry-heating at 120 °C and 130 °C. However, after dry-heating at

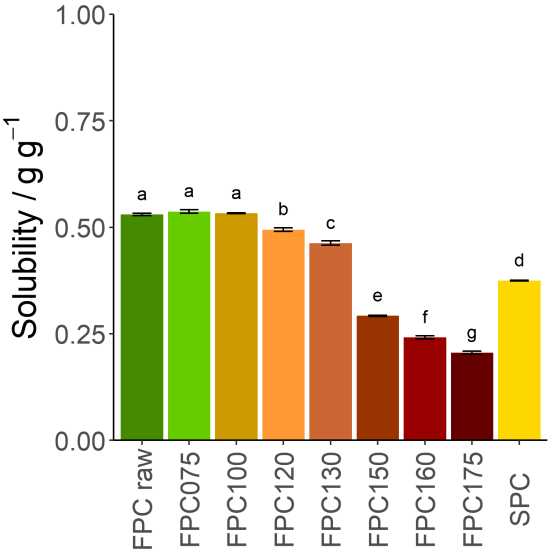

Figure 3.2: Solubility of the material of dry-heated and non-dry-heated FPC and commercial SPC. n = 3. Dry-heating at 75 °C and 100 °C had no influence on the solubility. Dry-heating at 120 °C and 130 °C slightly lowered solubility; while dry-heating at 150 °C, 160 °C, and 175 °C cut solubility in half. SPC is shown as an industry reference.

150 °C or more, it decreased to 0.29 g g^{-1}, lower than the solubility of SPC (Figure 3.2). The WHC of the insoluble fraction only increased by 23.2 % after dry-heating at 150 °C (Figure 3.1 right), whereas the WHC of the overall dry matter increased by 86.4 %. As the soluble protein fraction has no WHC, the increase of the insoluble protein fraction is the main cause of the increase in WHC of FPC dry-heated at 150 °C. At 175 °C, the change in overall WHC cannot be explained by insolubility only. An explanation for the decrease in solubility of heat-treated FPC could be a change of the protein structure due to degradation leading to aggregation of proteins. Considering the composition of the FPC, sugars or starches could have had an influence on the solubility of the protein fraction, e.g., through an intermolecular reaction, such as Maillard reactions. To study the underlying mechanisms, three samples were selected: non-dry-heated, mildly dry-heated (100 °C), and severely dry-heated (150 °C) FPC. Raw FPC and SPC were also further analyzed as blank and industry reference.

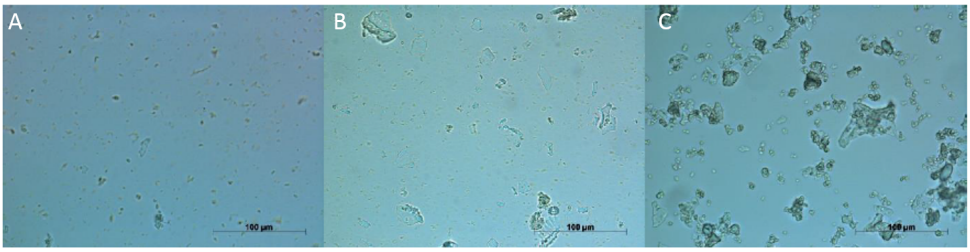

Figure 3.3: Light microscopy pictures of 0.02 $g\,g^{-1}$ dispersions of dry-heated and non-dry-heated FPC. Samples dry-heated at 150 °C (**C**) showed larger particles than non-dry-heated FPC (**A**) and FPC dry-heated at 100 °C (**B**).

3.4.2 Light Microscopy

To visualize protein aggregation, dry-heated and non-dry-heated samples were observed using light microscopy. Figure 3.3 shows a 40x magnification of the non-dry-heated (A) and dry-heated samples at 100 °C (B) and 150 °C (C). For the non-dry-heated sample, black spots below 10 µm were observed, with no particles above 20 µm. After dry-heating at 100 °C and 150 °C, the number of larger aggregates increased, with sizes above 20 µm. Dry-heating at 100 °C resulted in fewer particles in the sample, with multiple particles below 10 µm. Samples dry-heated at 150 °C contained more particles above 20 µm and 50 µm, while the small particles that were observed in the first two samples were hardly found. Therefore, it is an indication that dry-heating at 100 °C and 150 °C led to aggregation of particles, and the effect was stronger at the higher temperature.

3.4.3 Molecular Weight of Protein Subunits

To determine the change in molecular weight of the protein subunits of legumin and vicilin in FPC, SDS-PAGE was performed (Figure 3.4). For non-dry-heated FPC as well as FPC dry-heated at 100 °C, bands corresponding to convicilin (60 kDa), vicilin (46-55 kDa), α-legumin (38-40 kDa), and β-legumin (23 kDa) were seen [34]. The bands for α- and β-legumin were less intense after dry-heating at 150 °C, while the bands for vicilin and convicilin disappeared. Instead, indistinguishable bands larger than 250 kDa appeared at the top of the column. SPC showed bands corresponding to the subunits of glycinin and β-conglycinin [35]. SDS-PAGE showed aggregation of proteins. It also showed that no peptide bonds were broken due to dry-heating, as the subunits of the FPC proteins stayed intact and no bands appeared at the bottom

of the column.

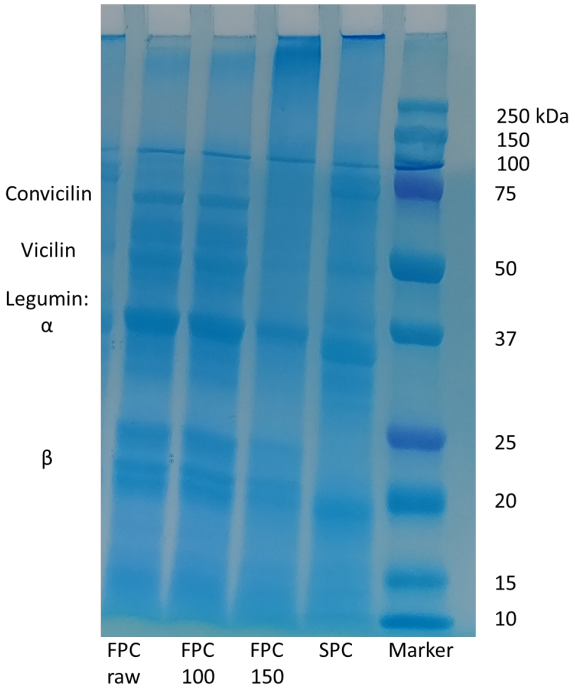

Figure 3.4: SDS-PAGE of non-dry-heated and dry-heated FPC. SPC is shown as a reference. Non-dry-heated and dry-heated at 100 °C FPC showed bands for convicilin, vicilin, α-legumin, and β-legumin. FPC dry-heated at 150 °C showed the legumin bands less pronounced, while the vicilin and convicilin bands were not detectable. Instead, indistinguishable bands larger than 250 kDa appeared. For soy, the subunits for glycinin and β-conglycinin were visible.

3.4.4 Nonenzymatic Browning Reactions

During heat treatments, coloration reaction such as caramelization of sugars or Maillard reactions can occur. As mentioned in Section 3.4.1, Maillard reactions could affect the solubility of proteins [36–38]. In Figure 3.5, the number of reducing sugars per total dry matter is shown. FPC dry-heated at 100 °C had 8 % less reducing sugars than non-dry-heated FPC. Dry-heating at 150 °C caused a more pronounced

reduction of reducing sugars by 0.26 mmol g^{-1}. This illustrates that caramelization occurred.

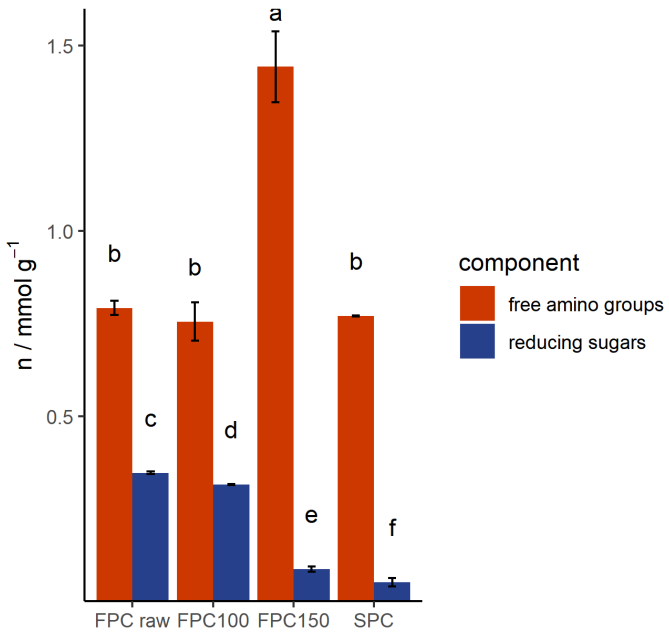

Figure 3.5: Number of reducing sugars and free amino groups in mmol g^{-1} dry matter of dry-heated and non-dry-heated FPC and commercial SPC. $n = 3$. The number of reducing sugars decreased with increasing dry-heating temperature, while the number of free amino groups increased after dry-heating at 150 °C. SPC is shown as an industry reference.

The number of free amino groups measured in the non-dry-heated FPC (0.79 mmol g^{-1}) was in line with the generally expected amount of free amino groups containing amino acids in FPC (lysin and arginine, 0.62 mmol g^{-1})[39]. The loss of free amino groups can be used as a measure of Maillard reactions [36, 40, 41]. As reducing sugars react with free amino groups during Maillard reactions, a similar decrease in both the free amino groups and reducing sugars was expected if Maillard reactions occurred. However, the number of free amino groups did not decrease, but increased by 0.65 mmol g^{-1} after dry-heating at 150 °C (Figure 3.5). The increase of free amino groups might have been caused by a change in protein conformation, which

could have caused more exposure of the free amino groups. Protein hydrolyzation is unlikely, according to the results of the SDS-PAGE.
Thus, no direct evidence of Maillard reactions was found. However, as Maillard reactions occur mostly in wet conditions [42], the decrease of reducing sugars is probably attributed to caramelization. Since caramelization only involves sugars and not proteins, its effect on protein aggregation is negligible. Further, the decrease in reducing sugars limits Maillard reactions in any final processing steps that involve heating.

3.4.5 Protein–Protein Interactions

Different selective agents were used to solubilize the protein to find the type of bonds stabilizing the protein and causing lower solubility after dry-heating. In sodium phosphate buffer (B1), electrostatic interactions were disrupted by the phosphate buffer, which led to a solubility of the non-dry-heated FPC of 1.53 $\mathrm{g\,g^{-1}}$ (Figure 3.6). In sodium phosphate buffer + SDS + urea (B2), noncovalent bonds such as H-bonds and hydrophobic interactions were also disrupted. This increased the solubility of non-dry-heated FPC significantly to 1.71 $\mathrm{g\,g^{-1}}$, illustrating the presence of noncovalent bonds. By addition of DTT (B3), disulfide bonds were also broken. The solubility of non-dry-heated FPC in B3 was not significantly different than in B2. The solubility of FPC dry-heated at 100 °C showed no significant difference to the non-dry-heated FPC in any of the buffers. Solubility of FPC dry-heated at 150 °C was lower than that of non-dry-heated FPC in B1, but not in B2 or B3. Since no increase was detected from B2 to B3 for any material, disulfide bonds do not contribute to the insolubility of FPC. The similar trend for solubility in water showed that in FPC, only noncovalent bonds were affected by dry-heating at 150 °C and contributed to the insolubility of these samples. This is in line with the findings of Zheng et al.[43], who showed that purified legumin from faba bean formed aggregates after a heat treatment that were stabilized by noncovalent bonds.

Solubility of SPC increased from B1 to B2 and to B3 (Figure 3.6), showing that both noncovalent and covalent bonds keep it from solubilizing. Similar trends have been reported for SPI [44], SPC [30], and wheat gluten [45, 46]. Chen et al. [47] state that the importance of noncovalent bonds outweighs that of covalent bonds after extrusion of SPI. This is in line with the findings of Liu and Hsieh [48], who found that disulfide bonds did not play an important role for solubility of SPI and SPC as such, but caused insolubility after extrusion.

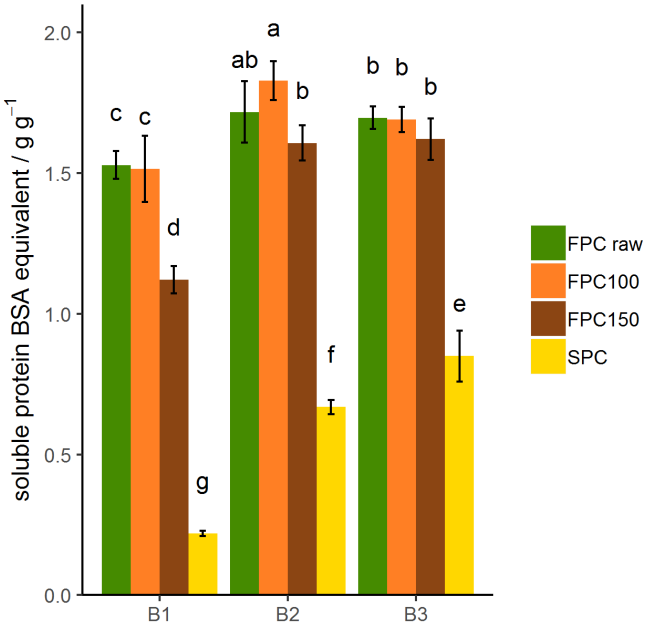

Figure 3.6: Solubility of protein dry-heated and non-dry-heated FPC as well as commercial SPC after disrupting electrostatic interactions (B1), noncovalent interactions (hydrophobic and electrostatic interactions and hydrogen bonds) (B2), and covalent and noncovalent interactions (B3). $n = 9$. Solubility is expressed as BSA equivalent per gram FPC or SPC. Significant differences are indicated by different lowercase characters ($p < 0.01$). Samples dry-heated at 150 °C showed a significant difference in solubility in B1 compared to non-dry-heated FPC. Solubility of dry-heated and non-dry-heated FPC increased from B1 to B2, but not from B2 to B3. Solubility of SPC increased from B1 over B2 to B3.

The amino acid composition of SPC [49] and faba bean [39] are comparable. In fact, the largest difference lies with Arginine, of which faba bean has 25 % more than SPC. All other amino acids are within 0.01 $\mathrm{g\,g^{-1}}$ of overall protein. However, it is possible that they have different bonds that stabilize the protein, as they are organized in different structures: vicilin and legumin in faba bean; and glycinin and β -conglycinin in soy. Vicilin lacks the disulfide bond forming the amino acid cystein [50–52] compared to β-conglycinin [53, 54], whereas legumin from faba bean is very similar to glycinin [55–57]. This explains the limited influence of disulfide bonds on the solubility of faba bean protein concentrate. Further, the lack of disulfide bonds presents an explanation for the difference in solubility and also overall WHC between raw FPC and SPC. The inherent inability of faba bean proteins to form disulfide bonds is also a potential reason why dry-heat treatment only increased the WHC of FPC, but did not close the gap to SPC.

Additionally, hydrophobicity of insoluble particles was determined by the amount of BPB bound. As can be seen in Figure 3.7, dry-heating FPC at 100 °C did not affect the amount of BPB bound and therefore hydrophobicity. SPC bound the same amount of BPB as non-dry-heated FPC, coincidentally. However, FPC dry-heated at 150 °C bound 55 % more BPB than non-dry-heated FPC, showing that the treatment increased the hydrophobicity of the insoluble fraction. This suggests that the hydrophobic sites on the inside of the protein were exposed by (partial) denaturation.

3.4.6 Denaturation of Protein

DSC measurements were conducted to determine the extent of the protein denaturation due to dry-heating. Figure 3.8 is an example of a DSC thermogram of the performed measurements for every treatment and ingredient. For all samples, an endothermic peak was observed in the first run. The second run showed no peaks for all samples. An overview of the curve analysis of the experiments performed in triplicate can be found in Table 3.1. The T_d found for non-dry-heated FPC at a dry matter content of 0.15 $\mathrm{g\,g^{-1}}$ was 93.2 °C, with a ΔH of 0.92 $\mathrm{J\,g^{-1}}$. The enthalpy ΔH as well as the peak temperature T_d decreased for samples dry-heated at 150 °C. Since less heat was needed to denature these samples, they must have been partially denatured by the dry-heating treatment. Similar findings have been reported by Arntfield and Murray [58], who also found a decrease in ΔH necessary for denaturation of faba bean concentrate after wet-heat treatments. They concluded that partial and complete, irreversible denaturation occurred due to treatment at 85

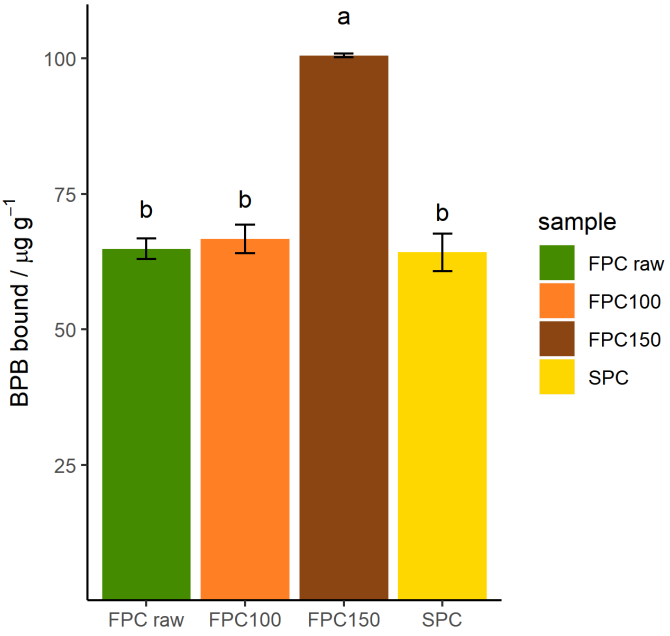

Figure 3.7: Hydrophobicity as determined by amount of bromophenol blue (BPB) bound to insoluble particles of dry-heated and non-dry-heated FPC as well as commercial SPC. $n = 3$. FPC dry-heated at 150 °C bound more BPB than all other samples.

°C and 95 °C, respectively. This is in line with the T_d found by them, 88 °C, and in this research, 93.2 °C. Shevkani et al. [59] found T_d ranged from 82.7 °C to 85.5 °C for different varieties of faba bean protein isolates, Sosulski et al. [60] found $T_d = 91$ °C for faba bean protein flour, all at comparable dry matter contents. The differences in T_d can be explained by differences in amino acid composition of the used faba bean varieties or the state of protein due to processing history resulting in a different protein structure [61, 62]. It is hypothesized that the partial denaturation caused by dry-heating at 150 °C changed the structure of the proteins so that more hydrophobic sites were exposed, causing the proteins to aggregate and become less soluble in water. This hypothesis is supported by the results of the analysis of protein–protein interactions using different buffer systems and the hydrophobicity study on insoluble proteins.

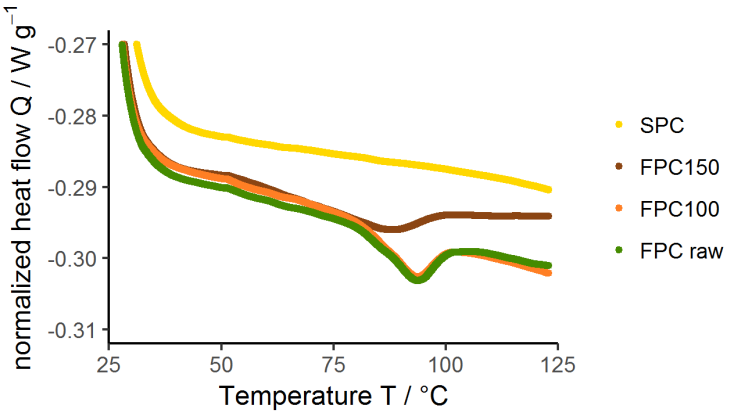

Figure 3.8: Thermograms of 0.15 g g^{-1} dispersions of dry-heated and non-dry-heated FPC as well as commercial SPC, produced with Differential Scanning Calorimetry (DSC). A ramp of 5 °C min^{-1} was used and each sample was measured in two cycles. The second cycles did not show any peaks for any sample.

Table 3.1: Average peak temperature and enthalpy of protein denaturation of dry-heated and non-dry-heated FPC. $n = 3$. Values obtained using the TA Instruments software TRIOS. Dry-heating at 150 °C shifted the peak to a lower temperature and reduced the enthalpy to a third of the value of non-dry-heated FPC.

	T_d/°C	+/−/°C	ΔH/J g^{-1}	+/−J g^{-1}
FPC raw	93.2	0.2	0.92	0.05
FPC100	93.6	0.5	0.96	0.03
FPC150	88.8	0.6	0.32	0.08

3.5 Conclusions

Dry-heating can be a useful tool trying to bridge the gap between the functional properties of mild fractionated plant proteins such as faba bean protein concentrate and conventionally processed SPC. In this study, it was shown that it had a similar positive effect on WHC of FPC as it did for SPC, Dry heating is a tool to control the functional properties of FPC. Increased heating leads to higher WHC and lower solubility. These changes make the FPC a potential ingredient to replace soy in

applications like meat replacers. However, soy still has a higher WHC. While the aggregation/insolubility of FPC after dry-heating was caused by hydrophobic interactions and hydrogen bonds, SPC was further stabilized by disulfide bridges. The absence of these disulfide bridges in FPC explains its higher solubility, and therefore, potentially, the remaining difference in WHC with soy. Finally, this study shows that when choosing ingredients for meat replacers, not only protein content and source but also (thermal) processing history should be taken into consideration.

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Chapter 4

Starch in Plant-based Meat Analogues

A New Approach to Using Endogenous Starch from Cereals and Legumes

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Abstract

This review discusses the use of starch in plant-based meat analogues. Starch is often added to meat and meat analogues as a functional ingredient. The function of starch in those applications is investigated to be able to describe how it affects the production and structuring process as well as product properties. Often modified starch is used in these products, because of its improved functionality compared to native starch. Next to that, starch can also be present in meat analogues as part of other ingredients, *e.g.* when using legume or cereal meals or concentrates. It is discussed if this endogenous starch can have similar functionality and if not, whether it can be modified in a similar way as industrially modified starch. We propose a new perspective on endogenous starch, demonstrating options for in-situ modifications and promoting the use of less refined and therefore more sustainable ingredients in fibrous meat analogues.

4.1 Introduction

In the last decades meat analogues have gained interest on a global scale, with predictions for greater growth in the coming years [1–5]. As a result, more products are appearing in the market that mimic meat or offer protein-rich alternatives to the consumers. One of the biggest challenges in producing these products is creating the adequate texture, flavor and color [6]. The success of making those structures depends on both the ingredients used and the process to make the products [7]. An often applied combination of ingredients consists of protein (often from wheat and legumes), fat or oil, binding agents, flavors and color agents [8]. Frequently also starch is used as an ingredient in commercially available meat analogues and real meat products, though mostly applied in low quantities. Table 4.1 shows the application of starch in meat analogues and processed meat products currently on the market. In these products, starch is added as a minor ingredient next to other purified ingredients, such as protein isolates or concentrates. Starch often acts as a filler and can increase yield or modify water holding [9]. Such purposely added starch is called exogenous starch and can come from a different source than the protein. Exogenous starch can be modified to improve its functionality even further. The choice of starch is based on the functionality and availability.

Another reason to incorporate starch in meat analogues, next to functionality, is sustainability. Endogenous starch, as opposed to exogenous starch, is naturally present as a component that remains to a certain extend in an ingredient after purification. The current focus of the industry is on the use of purified protein ingredients, *e.g.* Pea Protein Isolates (PPI), in which the endogenous starch is removed completely. As evident from Table 4.1, this approach sometimes requires even the later addition of exogenous, potentially modified starch for functionality [10]. Consecutive removal and addition of starch seems contradictory and inefficient from a sustainability point of view, as this requires a lot of energy and leads to high material losses. This is especially true for crops that are considered to have an important role in the transition towards a more plant-based diet, and for which no current industrial use exists for the starch. Typical examples are mung beans, cowpea, faba beans and other pules. Losses in the form of (starch) waste streams could be avoided by using these ingredients in un- or less-refined form. Therefore, the use of such less refined ingredients is far more sustainable and economic, especially in the situation where the individual components are re-combined into a new ingredient mix to achieve the desired composition and functionality [11]. However, the functionality of the components has to be considered as well when evaluating their suitability for

food products [12].

Based on the information above, it becomes clear that the need for more sustainable food (ingredients) poses the following research questions: Is it possible to use endogenous starch as a functional component? If not, is it possible to modify starch that is present in a mildly refined protein-rich fraction in order to functionalize it? This review aims to contribute to the answers by summarizing the current research on the role of starch in meat analogues. The processes to structure plant proteins considered here are extrusion cooking, gel formation processes, as well as the newly emerging High Temperature Shear Cell (HTSC) technology [13]. We first describe the general role of starch in foods and modification routes. After that, the interactions of starch and proteins are described. Then we show how starch is used as a functional ingredient when it is purposely added to meat products. Furthermore, we discuss the effect of exogenous starch on protein gelation and extrusion processing for meat analogues. Next we lay out the known effects of endogenous starch on structure formation of protein gels. Lastly, we propose a new perspective on endogenous starch as a possible functional ingredient and introduce concepts to functionalize it.

Table 4.1: Collection of meat and meat analogues on the market that contain starch

Application	Starch	Meat/ Vegetarian/ Vegan	Ref.
Albert Heijn Hamburger	Potato	Meat	[14]
Albert Heijn Runderbraadworst	Potato	Meat	[15]
Albert Heijn Shoarmareepjes	Potato	Meat	[16]
Albert Heijn Biologische Rundergehaktballetjes	Potato	Meat	[17]
GoodBite Vers Gehakt	Potato, corn, wheat	Vegetarian	[18]
GoodBite Hamblokjes	Potato, corn, wheat	Vegetarian	[19]
Quorn Meatless Nuggets	Wheat starch	Vegetarian	[20]
Garden Gourmet Schnitzel	Wheat flour, corn	Vegetarian	[21]
Vivera Kaasschnitzel	Wheat	Vegetarian	[22]
Vivera Wokreepjes	Wheat	Vegetarian	[23]
Albert Heijn Stukjes Als Van Kip	Wheat	Vegetarian	[24]
De Vegetarische Slager Visvrije Tonyn	Unknown	Vegetarian	[25]
De Vegetarische Slager MC2 Burger	Wheat	Vegetarian	[26]
Moving Mountains Burger	Wheat	Vegan	[27]
Moving Mountains Sausage	Wheat	Vegan	[28]
Beyond burger	Potato	Vegan	[29]
Vivera Krokante Schnitzel	Wheat	Vegan	[30]
Vivera Balletjes	Potato	Vegan	[31]
Vivera Steak	Wheat	Vegan	[32]
Vegan Zeastar Zalmon Sashimi	Tapioca	Vegan	[33]
Gardein Beefless Strips	Wheat	Vegan	[34]
Gardein Beefless Tips	Potato	Vegan	[35]
Gardein Chicken Strips	Potato	Vegan	[36]
Tofurky Chick'n	Corn	Vegan	[37]
Like Schnitzel	Corn, potato	Vegan	[38]

4.2 Exogenous Starch

4.2.1 Modified starch

Native starch is used widely in foods as a thickener or stabilizer. However, it also has less favorable properties, which are limited solubility in cold water, loss of viscosity and thickening power after cooking, high tendency to retrogradation, low shear resistance and thermal resistance [39]. Therefore, starch is often modified to tune its functional and physicochemical properties towards the food application [39, 40]. A large variety of reviews and books are available on the modification and functionalization of starch [9, 39–44]. Starch modifications can be on chemical and physical basis. Physical modifications are *e.g.* dry-heating, agglomeration and granulation, multiple deep freezing and thawing and pre-gelatinization. The latter can be achieved by drum drying, causing the pasting behavior of the starch to change: The starch swells and pastes at room temperature, while the viscosity at higher temperatures as well as the viscosity after cooling is reduced (Figure 4.1). These thermo-mechanical treatments applied to native starch result in so called "functional native starches" that are not required to carry an E-number and are potentially considered "clean label" [9, 45]. Despite being created to increase consumer trust in food labels and manufacturing practices, E-numbers seem to do the opposite [46–48]. Therefore, physical modifications can be a powerful tool to functionalize starch for the use in meat analogues and improve the product quality without decreasing consumer trust in the product. The food industry is well aware of these trends, evident by the patents filed in this field [49–51] and products on the market such as Ingredion's NOVATION product line [52] or Cargills SimPure [53], to name a few.

Chemical modifications can alter the properties of starch even further. Chemical modifications include hydrolysis, oxidation, esterification, etherification, crosslinking, hydroxypropylation and acetylation. Hydrolysis can be achieved by enzymatic or acid hydrolysis leading to a breakup of the starch molecules into dextrin, maltose and glucose. The main effect of (partial) hydrolysis is a strongly reduced viscosity when in solution. Esterification results in increased viscosity, due to the fact that the gelatinization temperature is lowered and the tendency to form a gel is reduced [39]. Esterified starch is used as an emulsion stabilizer and for encapsulation, and can also be used to partially replace fat in emulsion based food products [55, 56], such as plant-based sausages. As a thickening agent in foods, etherified or cross-linked starch is used frequently. Both modifications lead to decreased solubility of starch, by adding inter- and intramolecular bonds, strengthening and stabilizing the starch

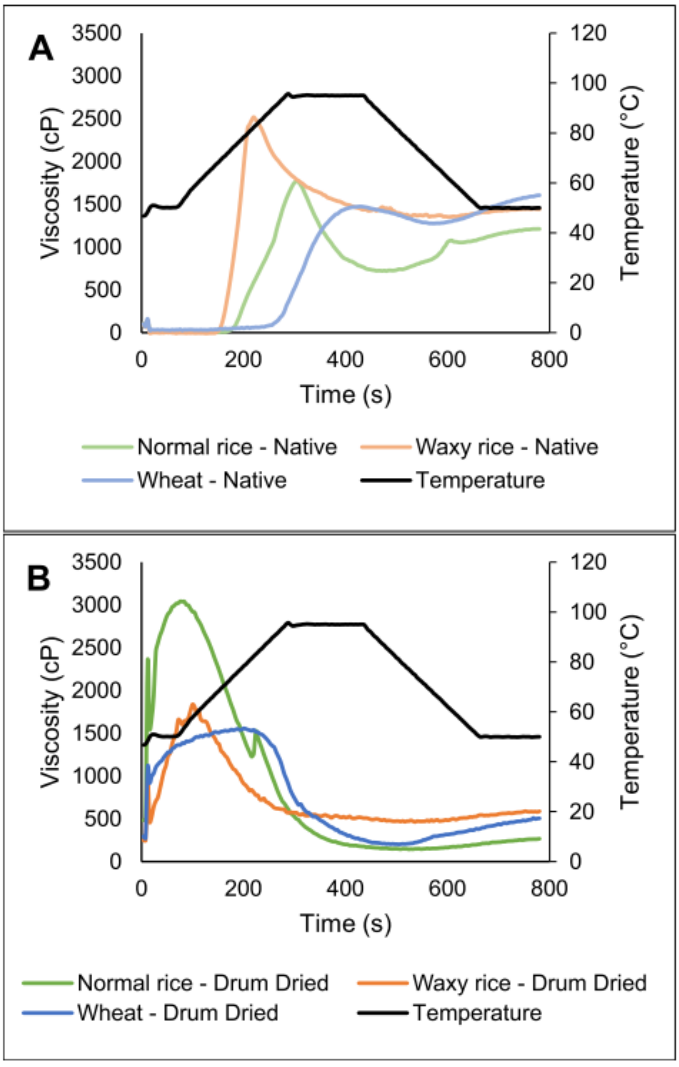

Figure 4.1: RVA profiles of (A) native and (B) drum dried normal rice, waxy rice and wheat starch. From Hayes et al. [54]. Reproduced with permission from Food Research International, Elsevier Ltd.

polymers. Cross-linked starch is increasingly resistant to high and low temperatures and pH, however also come with smaller swelling volume [40]. Hydroxypropylation is a modification of starch based on etherification with propylene oxide in the presence of an alkaline catalyst. Hydroxypropylated starch improves freeze-thaw stability and decreases gelatinization and pasting temperatures [57, 58]. In acetylated starch, hydroxyl groups are replaced with acetyl groups, increasing the viscosity as well as the solubility [59].

4.2.2 Interaction of Starch and Protein

Interactions between starch and proteins are widely studied to understand the properties of products containing both components. The interaction of protein and starch can occur on multiple scales: Associative interactions of the molecules [60], steric hindrances [61], competitive hydration [62] and phase behavior [63]. Heterotypic associative interactions of molecules, *i.e.* interactions of molecules of different types, are thermodynamically less favorable than homotypic interactions, *i.e.* interactions of molecules of the same type. However, it is still possible for heterotypic associative molecular interactions to occur, *e.g.* at the interface of two liquid phases or within the phases themselves as they might contain multiple components. These interactions include the formation of ordered heterotypic junctions (similar to homotypic junction zones in single component polysaccharide gels), electrostatic attraction between negatively charged polysaccharides (polyanions) and proteins below their isoelectric point (polycations), and formation of Maillard reaction complexes between proteins and reducing sugars [60]. Interactions can also be more indirect in multiphase products. Especially when added in higher concentration, starch and protein might form separate phases with little molecular interaction, although interaction can occur through differences in water binding [64]. Therefore, the addition of starch can influence the macro- and micro-structure of protein gels and products.

To create food products with starch and protein, powders containing plant-based protein and possibly starch are mixed with water, resulting in a wet biopolymer blend. Almost all biopolymer blends form a two-phase system under certain conditions [60, 63, 65]. At low concentration the dispersion can split into two co-existing phases after initial full mixing because of thermodynamic effects that drive phase separation. The latter leads to two phases that are enriched in one polymer and depleted in the other. The latter mostly occurs at low concentrations. For example, a carbohydrate concentration of 1 % and a protein concentration of 5 % are enough to form a two-phase system [66]. The concentrations used for meat

analogues usually well exceed these values, with protein concentrations >30 % and carbohydrate concentrations above 2 % [7, 67, 68]. Thus, these materials likely form a multi-phase blend with limited molecular interaction, but steric hindrance and competitive hydration influence gelling behavior of starch and protein reciprocally. For example, the onset temperature of starch gelatinization becomes higher upon the addition of protein [62, 69], while paste viscosity decreases [69–71]. Eliasson [72] researched the water migration during thermal processing of gluten and starch and calculated the amount of water associated with the gluten based on the enthalpy change of the starch gelatinization. Gluten forms a network already at ambient temperatures and thereby hinders starch gelatinization [62]. Li et al. [73] investigated thermal behavior of soy protein isolate and corn starch blends at 30 % - 70 % dm. They concluded that there was no significant chemical interaction between the protein and the starch, since the high concentration of the samples (50 % dmc) favors a phase separated matrix. They further deduced that the starch restricted protein denaturation and protein restricted starch gelatinization indirectly.

4.2.3 Starch in meat and comminuted meat products

The use of starch in meat products has been extensively researched over the last decades [74–76]. It is used as a filler in comminuted meat products, like sausages or meat patties. Such fillers are non-meat ingredients, which help bind water and are usually good bulking agents. Starch is considered a good bulking agent and is added to bind water [74], which would otherwise be exuded from the product. If added as pre-gelatinized starch, the ability to absorb water can even be increased further [76]. In its pre-gelatinized form, starch binds water at lower temperature, which is favorable as it prevents water loss upon heating of the meat [74]. The water binding properties of starch further enable the reduction of the caloric content of *e.g.* sausages [77]. Starch can retain the sensory and textural properties of products, *e.g.* by increasing the firmness and strength of the product when animal fats are replaced by vegetable oils, improving the lipid profiles towards higher contents of unsaturated fats [76]. Furthermore, it was shown that the presence of starch in meat emulsions results in a more compact and stronger heat-induced protein matrix [75, 78]. The reported advantages of employing starch in meat products are low cost and good control over functional properties such as cold swelling capacity, water solubility and rheological properties through physical or chemical modification [76]. The examples in Table 4.2 show that the applicability of starch in meat products has already been widely researched and might therefore provide understanding of the potential role of starch in meat analogues.

Table 4.2: Types of starch investigated for their functionality in application in meat and meat analogues in literature

Modification	Source	Variety	Added amount / % w.b.	Added to	Function in application	Meat/ Vegetarian/ Vegan	Ref.
none	unknown	resistant	4	Sausage	replace or reduce fat	meat	[79]
extruded with wheat gluten	corn		10-20	Beef Burger	reduce cooking loss, improve sensory attributes	meat	[80]
partially pre-gelatinized	tapioca		0-3	Beef burger	replace or reduce fat	meat	[81]
none	potato	native	2.8	Poultry Meat Batter	reduce cooking loss	meat	[82]
low cross-link and mid to high substitution modification	potato	native	2.8	Poultry Meat Batter	reduce cooking loss	meat	[82]
none	tapioca	native	2.8	Poultry Meat Batter	reduce cooking loss	meat	[82]
low cross-link and mid to high substitution modification	tapioca	modified	2.8	Poultry Meat Batter	reduce cooking loss	meat	[82]
Stabilized	waxy corn	modified	1.5	Chicken rolls	increase yield	meat	[9]
stabilized, cross-linked	tapioca	modified	1.5	Chicken rolls	increase yield	meat	[9]
none	rice	native	1.5	Chicken rolls	increase yield	meat	[9]
none	waxy rice	native	1.5	Chicken rolls	increase yield	meat	[9]
unknown	corn	modified	3-7	Meat analogues	bind water	vegetarian	[83]
pre-gelled	potato	modified	5	Meat analogues	replace or reduce fat	vegetarian	[83]
none	wheat	native	2.8	Extruded meat analogue (based on peanut protein)	improve texture	vegan	[84]
cross-linked	tapioca	modified	1-7	Extruded meat analogue (based on soy protein)	replace gluten	vegan	[85]
hydroxypropylated	unknown	modified	1-7	Extruded meat analogue (based on soy protein)	replace gluten	vegan	[85]

4.2.4 Starch in meat analogues

As pointed out in 4.2.3, starch is often used in processed meat products for its water binding ability, its influence on textural properties or as a bulking agent. Therefore, one would expect potential to use starch in meat analogues in a similar manner. Literature on the use of starch in meat and meat analogues suggests that especially modified starch is used. As is evident from Table 4.1, starch is indeed added to commercial meat analogues, with Table 4.2 showing that this is done most likely for similar reasons as it is done in meat products. Starch is successfully being used as an ingredient in meat analogues to modify the sensory as well as the textural properties. This is due to the rheological properties of starch at different temperatures (Figure 4.1). When starch gelatinizes, viscosity increases drastically, followed by a drop in viscosity over time and shear. Upon cooling, the viscosity increases again, allowing starch to contribute to *e.g.* the hardness of a product. Different starches have different pasting profiles, depending on their amylose and amylopectin content and the origin of the starch [86], as well as any modification. Pre-gelatinized or "Cold Water Swelling Starches" increase the viscosity of a product already before heating, while simultaneously taking up more water.

While the effects on sensory properties such as hardness, chewiness and springiness can be partially explained by the rheological properties of different starches combined with the polymer blending law [60, 87], the knowledge about the effect of starch on structure formation is merely empirical. This is due to the fact that the exact mechanism of structure formation is not yet fully understood [88]. It seems that the effects depend on the origin of the starch, the moisture content and the other ingredients in the mixture used for processing. The influence of starch on structure formation could be based on indirect interactions, *e.g.* by changing the water content of the protein through different water binding, leading to changes in the rheological properties of the phase responsible for structure formation. As starch also undergoes significant changes during thermal processing (swelling, gelling, degrading, setting), it could also cause a steric hindrance to the formation of a protein matrix that is able to form a fibrous structure. However, all the studies mentioned have an inductive approach, and the assumptions are not tested. Deductive studies on this matter do not exist yet. Furthermore, the rheological behavior of starch itself could influence the formation of fibrous structures. Several studies suggest that the rheological properties of the individual phases are crucial for fiber formation in the HTSC [89–91]. There is little knowledge about the rheological properties at conditions relevant for the HTSC or high moisture extrusion (HME). Starch pasting curves such as in (Figure 4.1) are

measured at lower temperatures, lower pressure, lower shear and lower concentration. At higher temperatures, shear rates and pressure, starches tend to disintegrate. In addition, starch generally exhibits shear-thinning behavior [92, 93], which makes the prediction of rheological properties of starch even more difficult. Pasting curves can give an indication about the rheological behavior of the starch, but studies designed to elucidate the properties of starches under the relevant conditions are necessary.

Textural properties

Textural properties such as hardness, chewiness and springiness are of importance for meat analogues to mimic the eating experience of meat. There are studies that investigate the influence of starch on these textural properties of protein extrudates [84, 94, 95]. Zhang et al. [94] investigated the influence of 20 % (d.b.) starch from different sources on the textural properties of extruded soy protein isolate/wheat gluten mixtures (50 % moisture, d.b.). They report variations of the degree of fibrousness between the investigated starch sources, connecting the different thermal transition properties (including peak temperature, enthalpy changes) of the starch sources with the extrusion response parameters, such as Specific Mechanical Energy (SME) and die pressure. They conclude that the thermal transition properties of the starch affected the textural properties of the extrudates by influencing the extrusion response parameters. Through fitting the physicochemical properties of extruded casein/wheat starch mixtures based on extrusion trials, it was found that the addition of 50 % starch gave a maximum compression force of the extrudate when using 28 % moisture and 194 °C in the extruder [95]. For all moisture contents and temperatures studied (126 °C<T<194 °C, 18 %<moisture<32 %), wheat starch addition first increased the firmness of the product, while a further addition lowered the firmness again. The authors attribute this to the formation of bonds between the starch and the hydrophilic groups of the protein, made possible by the structural modification of both biopolymers caused by the high temperatures in the extruder. They claim that these bonds were responsible for the limited hydration capacity of the protein, a higher initial viscosity and the dense and rigid structures they observed after addition of starch. A study on Soy Protein Isolate (SPI) based meat analogues, designed to find hydrocolloids suitable to replace gluten in the recipe, found that the addition of 1-4 % hydroxypropyl starch and cross-linked starch decreased the hardness, gumminess, springiness and cohesiveness of the extruded meat analogues [85]. The changes in textural properties were found to correlate well with the rheological data they obtained directly from the extruder. The starches were not found to be suitable analogues for gluten in the recipes, unlike blends of other hydrocolloids. Another

study [96] also found that the addition of amylopectin to PPI decreased hardness, springiness and chewiness of the extrudates, while amylose had the opposite effect, underlining that the effect of starch also depends on the composition and origin of the starch. This means that it is possible to adjust several textural properties of meat analogues by adding the right amount of the right starch, depending on the desired outcome. Generalizing the effect of starch on textural properties is difficult, especially since the effect is indirect via the extrusion response parameters, as pointed out by Zhang et al. [94]. The aforementioned polymer blending law can give indications of the effect of a starch on the textural properties if the rheological properties of the starch and the other ingredients at process conditions are known. Generally speaking, firmness can be increased or decreased by addition of amylose or amylopectin rich starches, respectively. Modified starches could be able to deliver tailor made textural properties for meat replacing properties (Section 4.2.1). There are also a number of publications describing extrusion experiments that use starch as an ingredient, but do not specify the purpose or do not report on the influence of starch [97, 98].

Fibre formation

When it comes to whole cut meat analogues, aimed to resemble whole cuts of meat, fibrousness and a meat-like structure are key factors for consumer acceptance of those products [99–104]. To create plant-based products with such a fibrous texture, some sort of processing has to be done, such as extrusion or shearing in a HTSC. In both processes, plant-based, protein-rich powders are combined with water, heat and shear are applied and the material is cooled down [13, 105]. In extrusion, the fibrousness is sometimes expressed as "degree of texturization", quantified as the ratio of the force needed to cut a sample lengthwise and crosswise of the direction of extrusion [94, 106, 107]. A similar measurand exists for samples prepared in the HTSC, called the anisotropic index [13, 68]. Here, the ratio of the tensile strength parallel and perpendicular to the direction of the shear flow is used to quantify the fibrousness. Generally speaking, there is still a limited number of methods to evaluate the structure of meat replacing structures, especially when it comes to quantifying fibrousness [108]. A lack of standard procedure makes quantitative comparison of studies difficult.

The mechanism behind fiber formation in these materials is still debated in literature [68, 88–90, 97, 104, 109–117]. Next to process parameters such as dry matter content, processing time, temperature and pressure, the origin and composition of the ingredients are of utmost relevance as well. Many studies focus on protein-protein interactions and polymerization [115, 118, 119], but also the influence of carbohydrates

is investigated [7]. Dekkers et al. [120] *e.g.* added pectin in a soy protein isolate dough to aid fiber formation. The effect of starch on fiber formation and textural properties of extruded protein has been investigated by Walsh et al. [80]. The study revealed that a fibrous texture can be achieved with blends of whey protein and starch at starch contents from 20 – 40 %. They report that starch was also responsible for the increase of water holding they observed. Contrary to that, Zhang et al. [84] recently showed that the addition of native wheat starch had a negative effect on the fibrousness of peanut protein extrudates. Furthermore, the addition of starch lowered the chewiness and hardness of the extrudates. The outcomes were explained by considering that wheat starch promoted the aggregation of protein, supposedly "(...)breaking the intramolecular disulfide bonds, enhancing the hydrophobic interactions and increasing the apparent viscosity to stabilize the newly formed conformation." [84]. Similar results were obtained for other polysaccharides in this study, but the effect of starch was found to be the highest. Other polysaccharides, such as sodium alginate, actually promoted the formation of fibrous structures [84]. A study on extrusion based meat analogues made of potato protein, oat protein, wheat bran, potato dietary fibers and potato starch showed that potato starch addition had no effect on the microstructure observed within different sections of the extruder when added at 25 to 50 % (d.b.) [121]. The shape of the protein aggregates was considered here as a measure for the deformation and therefore as an indicator for fiber formation. It was found that potato starch did not affect the size of protein aggregates formed but did decrease the size of (dietary) potato fiber aggregates. The aforementioned recent study by Chen et al. [96] investigated the influence of 10 % (d.b.) amylose and amylopectin on HME of PPI. They conclude that amylopectin promotes formation of a fibrous structure (even though the difference in fibrous degree was not statistically significant), while amylose does not.

4.3 Endogenous Starch

Nowadays, interest in the use of ingredients derived from pulses and legumes in meat analogues is increasing. Those pulses and legumes contain starch, part of which, depending on the method of extraction, remains in the fractionated protein ingredients. Generally speaking, the first steps in making protein-enriched ingredients from pulses and legumes are dehulling and subsequent milling. Since no starch and/or protein is lost during the milling step and no heating is applied, flours still contain high amounts of endogenous starch with native functionality. Further purification can be done either by more conventional methods (*e.g.* acid precipitation) to obtain

highly purified protein isolates, or by applying novel fractionation processes such as air classification, which result in protein-rich fractions that only contain a limited amount of starch (*e.g.* less than 2 % [122]). Berghout et al. [123] illustrate the trade-off between purity and yield and therefore resource efficiency for traditional purification methods. Applying novel fractionation processes, especially dry fractionation such as air classification, is less energy intensive due to the lack of drying steps and therefore also more resource efficient in regards to water use. Schutyser et al. [10] suggest the use of a combination of dry and aqueous fractionation to increase purity while maintaining some benefits of the dry fractionation. Tailoring fractionation routes to produce fractions with the desired functional properties (so-called functional fractions) for the intended application could be a key to increase the sustainability of meat analogues [124]. In order to determine the desired functional fractions for meat analogues, it is crucial to understand the effect of endogenous starch on structuring applications such as HME and HTSC structuring.

The use of endogenous starch in structuring processes for meat analogues has not been investigated, to the best of our knowledge. In case of protein isolates and concentrates, endogenous starch is often seen as a contaminant or unwanted component. Aguilera et al. [125], for example, report that starch in peanut flour interfered with texture formation in low moisture extrusion. There are, however, multiple studies on endogenous starch in low moisture extrusion, usually applied for snacks and cereals, but also for production of Texturized Vegetable Protein (TVP). Though they have a much lower moisture content (a very significant parameter in food structuring), these findings could still give indications for the use of endogenous starch in HME or the HTSC. Most studies on the role of endogenous starch focus on flours instead of protein concentrates or isolates [125–132].

It is noteworthy that the use of endogenous starch and starch in general can also have negative aspects. On the one hand it poses process-related challenges, such as possibly increased Maillard reaction due to increased sugar contents in less refined fractions or less consistency in ingredient quality and specification. On the other hand, it can have an effect on nutritional quality of the product, since less refined fractions of *e.g.* faba bean that include endogenous starch also include the flatulence causing oligosaccharides raffinose and stachyose [133], as well as other anti-nutritional factors (ANFs) that can limit protein and starch digestions [134]. Furthermore, when the two are present in their native form as starch granules and protein bodies, organized in a tightly packed matrix, protein can compromise the digestibility of starch [135] and *vice versa* [136].

4.3.1 Endogenous starch in protein gels

Pelgrom et al. [122] studied the gelatinization behavior of mildly refined fractions of yellow pea. They found that gel strength increased with higher starch concentration. Similar results were found for gels prepared of purified soy protein and wheat starch as well as from purified lentil protein and lentil starch [69, 137]. Furthermore, Pelgrom et al. [122] show that gels prepared from purified starch had higher gel strength compared to those prepared from dry processed fractions for a given starch concentration. This was explained by the presence of protein and fiber that weaken the starch network by forming dispersed domains.

4.3.2 Endogenous Starch in Extrusion

Several studies report on extrusion of pulse and legume flours, with most of them focusing on low moisture extrusion [125–132]. In general, presence of starch in the raw material contributes to a higher pasting viscosity as a result of its amorphous gel phase [127]. However, extrusion barrel temperatures and SME levels highly influence the behavior of starch in a dense, protein-rich blend. The effect is difficult to predict. On one hand, high temperatures or SME levels can increase starch degradation, thus reducing the melt viscosity in the extruder barrel. On the other hand, it was found that increased extrusion temperature led to increased cooking of starch and therefore a better expansion of whole pinto bean meal extrudate [126].

Jebalia et al. [128] compared the morphology and mechanical behavior of extruded pea flour to those of extruded mixtures of pea starch and PPI. Both products consisted mostly of amorphous starch after extrusion. They found that for samples processed with low SME, starch domains were surrounded by a protein matrix. At higher SME levels, starch formed a continuous matrix around protein aggregates, which is likely related to the higher percentage of starch in comparison to protein. Furthermore, they report a difference in size of the protein aggregates, with the pea flour exhibiting smaller and more dispersed protein domains than the PPI - starch mixtures with less dispersed but larger protein domains. Additionally, the protein domains of the PPI - starch mixtures were elongated in the direction of the extrusion flow. Similar morphologies were observed by Kristiawan et al. [129] for low moisture extrusion of pea flour at low SME levels. It was further shown that at increased SME levels the extruded material formed a bi-continuous network of protein aggregates and starch molecules [129, 138]. They suggest that different states, sizes and continuities of protein aggregates can lead to various protein-starch morphologies.

Orientation of dispersed phase domains in the direction of shear flow was previously found to be an important parameter for fiber formation in HTSC structuring [68, 139]. As mentioned earlier, protein domains in extruded starch-protein blends were found to be oriented in the flow direction, while protein domains in extruded pea flour composites showed no orientation [128]. If we assume that elongation of the dispersed phase is a prerequisite for the formation of fibers with the HTSC technology or HME, it is expected that it is not possible to transform untreated pea flour into fibrous materials successfully. The authors further describe that extrudates from pea flour were more brittle than extrudates from starch-protein blends. This is explained by the fact that pea flour extrudates had smaller protein domains and therefore a larger interfacial area. As protein and starch domains have limited interaction, an increased interfacial area leads to easier breaking. Kristiawan et al. [129] suggest that a higher interfacial area favors bubble nucleation and therewith expansion of the extrudates when producing snacks from pulses. This would imply that pea flour extrudates would have a higher expansion rate than starch-protein blend extrudates. In low moisture extrusion of faba bean flour, bubble nucleation was found to be promoted by the formation of linkages between starch molecules, resulting in a larger expansion than reference products [127]. From previous research we know that air bubbles can enhance fibrousness in plant protein blends structured by HTSC technology [90, 139]. Addition of starch to these blends might lead to increased air bubble nucleation and thus a more defined fibrous structure. However, one must keep in mind that air is introduced through expansion in extrusion, whereas in HTSC technology it is introduced as a foam. As described above, the structuring properties of a flour is often not identical to that of a blend of protein isolate and starch with similar overall composition. Here, the processing needed to make pure starch and protein isolate probably explains the differences in structuring properties. Protein isolate and starch have undergone wet purification steps, acid precipitation and subsequent spray drying, which alters the functional properties of the components [10, 140, 141]. The difference in functionality is expected to influence the fiber formation potential. This finding also indicates that we need alternative routes to translate results from exogenous starch systems to endogenous starch-containing materials.

4.3.3 Wheat gluten as endogenous starch source

Wheat gluten is often used to make fibrous, meat-like structures. Especially in HTSC technology, functional properties of wheat gluten have shown to be very important [13, 88, 90, 114]. Starch is often overlooked as a remaining component in protein ingredients. One of these protein ingredients is wheat gluten. According to suppliers

there is approximately 10 % starch still present in the wheat gluten isolate [142]. However, studies on structuring of wheat gluten usually do not comment on the presence or effect of starch. As we know from exogenous starch, the addition of a small amount can affect textural and viscoelastic properties. Therefore, it is highly likely that the starch present in wheat gluten also affects textural and viscoelastic properties of the full system.

4.4 New Approaches

As described in the previous sections, starch is added to meat and meat analogues to increase product yield, bind water and modify texture and structure. At the same time, meat analogues are often based on highly refined plant proteins, which have been depleted in all other components, including starch. The isolates are eventually modified individually and recombined to make a product with the desired properties. To lower the environmental impact of meat analogues, it would be better to use less refined plant protein ingredients, while maintaining quality of the end product [10, 11, 124]. A consequence of less refinement is higher inclusion levels of other components, including starch. This might pose problems, but also creates opportunities as well, due to the richer composition. As pointed out, starch is used in meat analogues already. The main issue is that the presence of native starch does not generally improve the structuring properties. It explains why mostly modified starch is used in meat and meat analogues.

Up to now, modifications of starch are mainly performed for purified starch. To maintain the product quality when moving from products made from mixtures consisting of highly purified ingredients to those made from less refined ingredients, new pathways to functionalize the components inside the ingredients have to be developed. These new pathways could utilize similar treatments as are currently used for purified starch or protein, only applied to more complex, multi-component ingredients. When applying these pathways to mild-fractionated starch or protein enriched products, these modifications will not only influence the functional properties and molecular structure of the starch, but also those of the other components present. This might especially be influential for proteins present in a starch or protein concentrate. This again could be utilized, as proteins retain their native functionality in mildly refined fractions [122]. The isolates applied in industry exhibit vastly different functional properties due to the conditions during the isolation process, and are thus less prone to fine-tuning [10, 140, 141], as explained in 4.3.2.

The physical treatments used to functionalize starch isolates, such as freezing and thawing, drum drying, annealing and agglomeration are controlled by temperature, moisture content and pressure. The same parameters can potentially be used to control pre-treatments of less refined ingredients and even target single components within one ingredient. For this, the behavior and interaction of the components needs to be understood, which can be achieved when studying those components in the multi-component ingredient. Figure 4.2 illustrates this by showing a thermogram of an air classified faba bean fraction containing mainly starch and protein. Depending on the moisture content of the material, the temperature at which the changes occur, such as starch gelatinization or protein denaturation, can be controlled and even separated. The latter is possible, because the denaturation temperature of proteins depends on the moisture content over a wide range [143, 144], while the temperature of the initial starch gelatinization is less dependent on moisture content; only the degree of the initial gelatinization changes with moisture content [145–147]. The presence of other components can also influence the available moisture of the individual components in such a multi-component ingredient. Therefore, the distribution of moisture among all components also has to be understood [148].

So far, only a limited number of studies on functionalizing less refined ingredients exist, such as moisture heating of aqueous fractionated soy [149, 150] to increase viscosity and lower solubility of the protein, or dry heat treatment of air classified Faba bean Protein Concentrate (FPC) to increase water holding [151]. Geerts et al. [149] show that thermal treatment is a route to improve structuring properties of a soy concentrate. Results with FPC suggest similar improvement. Apart from physical modifications, also chemical or enzymatic modifications could be applied to less refined ingredients. Hydrolysis for example, which is often applied to modify starch, leads to cleavage of proteins into smaller peptides and thereby gradually alters their functionality in terms of i.e. water holding capacity and gelling properties. Nieto-Nieto et al. [152] investigated the gelling properties of partially hydrolyzed oat protein and show that the formed gels exhibit similar mechanical strength and water holding capacity as gels produced from egg white. However, peptic hydrolysis of soy protein isolates resulted in poor gelling properties [153].

We therefore conclude that mild-fractionated ingredients have great potential in terms of sustainability and, in combination with tailored pre-treatments, interesting functionality. The challenges along the way, such as the interaction of components, the distribution of water or the lack of functionality can be solved by investigating the ingredients as the multi-component mixtures that they are.

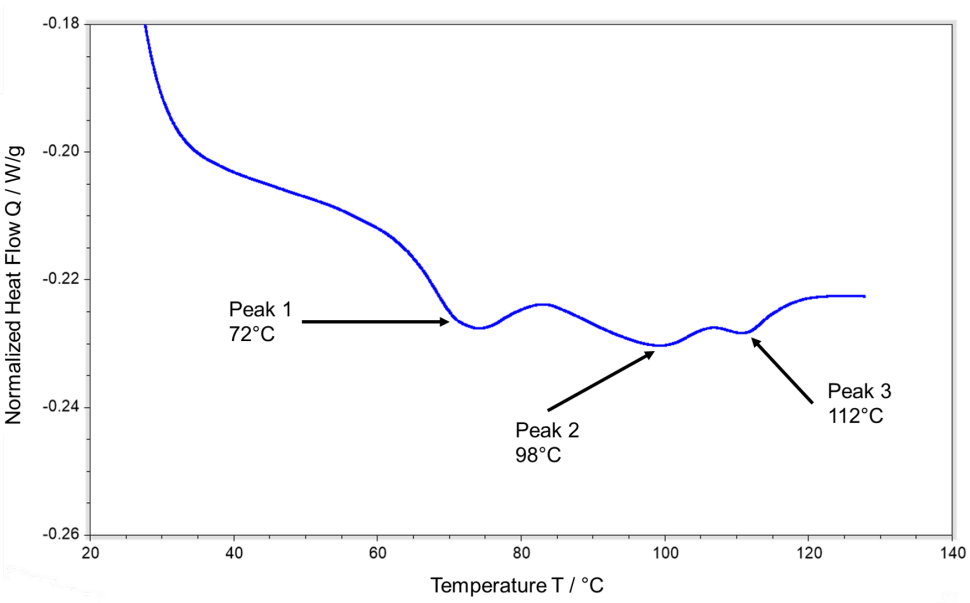

Figure 4.2: DSC curve of starch-rich faba bean fraction (air classified), 45 % dm, 5 °C min^{-1} heating rate. Peak 1 represents the initial gelatinization of starch, Peak 2 represents the denaturation of vicilin and Peak 3 represents the denaturation of legumin

4.5 Conclusion

Starch takes different roles in meat and meat analogues. It acts as a functional ingredient or filler that is added to the food product to improve textural properties or to bind water. Simultaneously, it alters the gelling properties of proteins and often decrease fiber formation in HME or the HTSC. The effect of starch depends on the properties of the different types of starch, ranging from particle size, water holding capacity and solubility to thermal properties such as pasting profile and viscosity. Furthermore, starch can interact with protein on a molecular level and on a macroscopic level through phase interaction. All those properties can differ depending on the source of protein and starch, the concentration used and the processing history of those components.

The literature on the effect of endogenous starch on protein gels and meat analogues is rather limited. Besides, the findings from studies of exogenous starch are

not directly transferable, as the functional properties of starch and protein are altered in the separation and other processing steps. It implies that studies on model systems cannot directly be translated to the properties of multi-component ingredients obtained via mild fractionation. It is therefore necessary to further explore the structuring potential of materials that contain endogenous starch, such as concentrates and flours from legumes. To increase the functionality of endogenous starch, modification treatments commonly used to improve properties of starch isolates such as pre-gelatinization or potentially chemical modifications could be applied to the less refined raw materials as well. Thermal pre-treatments could be a promising way to functionalize less refined raw materials, as there are potential synergies with the functionalization of protein in the same materials. Great progress can be made in terms of quality, sustainability and cost-efficiency of meat analogues by designing fractionation processes for protein- and starch-rich crops specifically for meat analogues and combining them with the aforementioned treatments to tune the functional properties of the fractions.

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Chapter 5

Fibrous Structures from Starch and Gluten

This chapter is based on:

J.M. Bühler, A.J. van der Goot, M.E. Bruins (2021). Fibrous Structures from Starch and Gluten. submitted.

Abstract

Starch is added to meat analogues for binding and water holding. In this study, we investigate whether starch can have an additional role as a structuring agent. Therefore, different types of starch were combined with wheat gluten at various amounts and sheared in a High Temperature Shear Cell to determine how starch influences the structuring behaviour of gluten-starch blends. The starches were chosen based on their diverse amylose contents, leading to different technological properties. Remarkable differences were found between the starches investigated. The addition of Amioca starch (containing 1 % amylose) had a strong negative influence on the ability of gluten to form fibres. Maize starch (25 % amylose) and Hylon VII (68 % amylose) formed fibrous materials up to high starch additions. Pre-gelatinizing of Maize starch increased the ability of gluten-starch mixtures to form fibrous structures even further. The influence of the different types of starch on the hardness, deformability and stiffness of the sheared samples was also assessed, revealing a spectrum of achievable properties through the addition of starch. Most remarkable was the formation of a material with anisotropy in Young's modules. This anisotropy is also found in chicken meat, but not in protein-based fibrous materials. Furthermore, it was observed that pre-gelatinization of starch facilitated fibre formation. A similar effect of pre-gelatinizing the starch was found when using Faba bean Meal with added wheat gluten where fibrous structures could even be formed in a recipe that previously failed to produce such structures without pre-treatment. This suggests that results obtained in this work can be extrapolated to starch-containing flours.

5.1 Introduction

In a world with an ever-growing population, meat analogues are gaining more and more momentum to contribute to a more sustainable diet and this momentum is not expected to stop [1–5]. The number of products on the market mimicking meat and delivering protein-rich alternatives is increasing. To make these products more attractive, they should closely resemble the fibrous structure found in real meat [6, 7]. In general, a fibrous structure is regarded as a necessity for consumers to accept plant-based meat analogues as a viable alternative for real meat [8–10]. Fibrous structures in plant-based products can be created using the combination of heat and shear, either with an extruder or with a High Temperature Shear Cell (HTSC). Generally, these products are made from dry, plant-based, protein-rich ingredients combined with water. The exact mechanism of how these fibrous structures are created is still not fully understood, but consensus is that a multi-phase mixture with specific properties is necessary [11–14]. The properties influencing the process have been heavily researched [10, 15–17] along with the role of ingredients, e.g. protein-protein interaction [18–20] or the influence of some carbohydrates [21, 22]. Currently, intensive fractionation/separation processes are used to purify protein-rich crops for plant-based meat analogues. However these processes take away (part of) the positive effects on sustainability of replacing animal products with plant-based products. Therefore, a transition from purified ingredients to functional fractions will be necessary in the future [23]. To investigate the possibilities of using starch-rich crops, especially pulses, in meat analogues, it is important to understand the influence of starch in these protein/starch composites. The exact mechanism of the effects of starch on fibre formation is not very well explored. For the HTSC, the influence of starch has not been investigated to our knowledge. The effect of starch on protein extrudates has been studied to some extent, focusing on product properties such as hardness, chewiness and springiness but also fibrous degree [24–28]. However, these studies do not explore the mechanism of the influence of starch on fibre formation [29].

In this study, we explore the role of starch in fibre formation in the HTSC. Special emphasis will be on the opportunities to utilize the unique gelling behavior of starch to modify product properties. We investigate the influence of three different types of starch on fibre formation, tensile strength, deformability and stiffness of gluten-starch blends. The starches used are chosen for their different amylose contents, leading to different technological properties: A low amylose starch (Amioca, 1 %, AS), an intermediate amylose starch (Maize Starch, 25 %, MS) and a high amylose starch

Table 5.1: Composition of the raw materials

	Dry matter/ %	**Protein content / %**	**Starch content / %**
FM	-	28.6*	38.5†
WG	93.9‡	74.6§	12.6¶
MS	91.9‡	-	>97‖
AS	93.5‡	-	>97‖
HVII	92.0‡	-	>97‖

(Hylon VII, 68 %, HVII). Their characteristics are also heavily studied and reasonably well reported in literature. Furthermore, we pre-gelatinized the intermediate amylose starch and used it in the same way as the other starches. Finally we test our findings in a recipe with gluten and Faba bean Meal (FM), an exemplary unrefined, starch-rich crop. FM was pre-gelatinized and processed in the HTSC in its native and pre-treated form in combination with gluten to transfer the findings on isolated starch to a less refined ingredient. Faba bean was chosen as it is a starch-rich crop, the protein of which is suitable for the formation of fibrous structure in the HTSC when combined with gluten [13].

5.2 Materials and Methods

5.2.1 Materials

Vital wheat gluten (WG) was obtained from Roquette (Lestrem, France) and had a protein content of 74.6 % w.b. (Kjeldahl, N-conversion factor = 6.25) and a dry matter content of 93.9 %. Amioca starch (AS), Maize starch (MS) and Hylon VII (HVII) were provided by Ingredion (Hamburg, Germany) and had an amylose content of 1 %, 25 % and 68 %. Faba bean Meal (FM) was also provided by Ingredion (Hamburg, Germany) and had a protein content of 28.6 % (Dumas, N-conversion factor = 5.7). The composition of the materials is listed in Table 5.1. Sodium chloride (NaCl) was acquired from Sigma-Aldrich (Zwijndrecht, The Netherlands). All components were kept at room temperature unless stated otherwise.

*Dumas (Nx5.7)
†Megazyme Starch Kit
‡determined via oven drying
§Kjeldhal (Nx6.25)
¶Chromatography sugar analysis
‖Product data sheet Ingredion

5.2.2 Pre-gelatinization

Pregelatinized Maize Starch (PMS) and pre-heated FM were obtained by creating 33.3 % (w.b.) MS or FM slurries in milliQ water, placing it in a sealed plastic bottle and heating it for 60 min at 90 °C in a shaking water bath. The obtained gel was kept in the sealed bottle at 4 °C until use, but at least for 24 h. The dry matter content of PMS was determined to be 33.1 % (w.b.) using oven drying at 105 °C for 48 h.

5.2.3 High-temperature Shear Cell

Doughs containing demineralized water, NaCl, gluten and starch (MS, PMS, AS or HVII) or FM were prepared. The doughs were prepared following a protocol previously reported by Grabowska et al. [30], with some modifications to the temperature used based on findings of Cornet et al. [13] and to the dry matter content based on preliminary experiments performed on gluten doughs in our lab. The overall dry matter content for the samples containing starch was kept constant at 40 % (w.b.), while the gluten and added starch content were varied to obtain doughs with 0 – 70 % starch (d.b.). For the samples containing FM, the overall dry matter content was also 40 %, with a WG:FM ratio of 1:1. The moisture content of the powders was taken into account when calculating the dry matter content. All doughs contained 1 % (w.b.) NaCl, which was dissolved in demineralized water. The appropriate amounts of FM or starch (MS, PMS, AS or HVII) were added and stirred by hand with a spatula until homogeneous. Gluten was added, followed by further mixing. The doughs were immediately placed in the HTSC, which was pre-heated to 140 °C. The doughs were sheared for 15 min and 30 rpm (39 s^{-1}) at 140 °C. The HTSC was cooled down to 60 °C before opening and removing the sample. The samples were placed in plastic bags, sealed and allowed to cool to room temperature before further analysis. The sample without starch was repeated on every experiment day to monitor the reproducibility of the results throughout the experiment.

5.2.4 Assessment of the fibrous structure

The fibrous structure of the samples was assessed visually following a bending procedure for HTSC samples previously described by Cornet et al. [13], revealing the potential orientation of the structure in the outer 4 cm of the sample.

5.2.5 Tensile strength analysis

The stiffness (Young's Modulus), maximum tensile strength (true fracture stress) and maximum deformation (fracture strain) in parallel and perpendicular direction to the shear flow were obtained performing tensile tests with a texture analyzer (TA.XTplusC, Stable Micro Systems, Surrey, UK). The tests were performed closely following a protocol previously described by Schreuders et al. [17]. In short, six dog-bone shaped tensile bars were cut from each HTSC sample, three perpendicular and three parallel to the shear flow direction. The tensile bars were clamped in the texture analyzer with a distance of 15.5 mm between the clamps. A uniaxial tensile test was performed with a displacement rate of 1 $\mathrm{mm\,s^{-1}}$. The force-displacement curves obtained were used to determine the true Hencky's stress (σ) and strain (ε), which are defined as

$$\varepsilon(t) = ln\frac{h(t)}{h_0} \tag{5.1}$$

$$A(t) = \frac{h_0}{h(t)} * A_0 \tag{5.2}$$

$$\sigma(t) = \frac{F(t)}{A(t)} \tag{5.3}$$

where h_0 is the initial length of the sample (15.5 mm), $h(t)$ is the length of the sample at the time of fracture, A_0 is the initial cross section area of the sample, $A(t)$ is the cross section area of the sample at time of fracture and $F(t)$ is the force at time the time of fracture. The volume of the tensile bar is assumed to remain constant throughout the measurement. The fracture stress (σ_f) and fracture strain (ε_f) are defined as σ and ε at the time of fracture, which is defined as the first substantial decrease of the force in the force-displacement curve. The Young's modulus is taken as slope of the initial, linear part of the σ over ε curve ($\sim$0.05-0.15 of the fracture strain).

5.3 Results

5.3.1 Fibrous structure

To study the effect of starches on fibre formation, we tested different starches at different concentrations in gluten-starch blends. Gluten doughs with increasing amounts of Maize Starch (MS), Pre-gelatinized Maize Starch (PMS), Amioca Starch (AS) or Hylon VII (HVII) were sheared in the HTSC. The sheared samples were bent in parallel to the shear flow direction, as described by Cornet et al. [13] to visualize any fibre formation (Figure 5.1). The starch content was increased until no fibrous structure was visible anymore or the sample did not gel properly.

Samples without added starch exhibited a fibrous structure, meaning that short and long fibres being oriented in the shear flow direction were visible upon bending the sample. This fibrous structure is used as a point of reference for all samples with added starch. The fibrous structure faded with an increased amounts of MS, PMS and HVII in the blend. The nature of the fibrous structure noticeably changed when $\geq$ 20 % MS was added, as the number of fibres seemed to decrease and they became less distinguishable. At 40 % MS, the fibrous structure was hardly noticeable anymore, while the sample containing 50 % MS was too soft to break upon bending and therefore no fibrous structure could be observed. Samples containing HVII also showed a noticeably different fibrous structure, which became less visible at 30 % HVII and disappeared at higher HVII contents. Contrary to the sample containing 50 % MS, the samples containing 50 % HVII broke upon bending, but exhibited no fibrous structure. The addition of HVII also appeared to make the samples more stiff and brittle when bending. Samples containing any amount of PMS appeared to have smaller fibres in size, but they were still noticeable at higher added starch contents of up to 60 % PMS. The maximum amount of starch that could be added without completely losing the fibrous structure (S_{max}) was 30 %, 40 % and 60 % for HVII, MS and PMS respectively. The addition of AS gave very different results: Samples containing >5 % AS did not gel properly and could therefore not be analyzed further with the exception of 8 % AS. Remarkably, a samples containing 8 % AS still exhibited a fibrous structure similar to that of samples without added starch, though softer and falling apart easier.

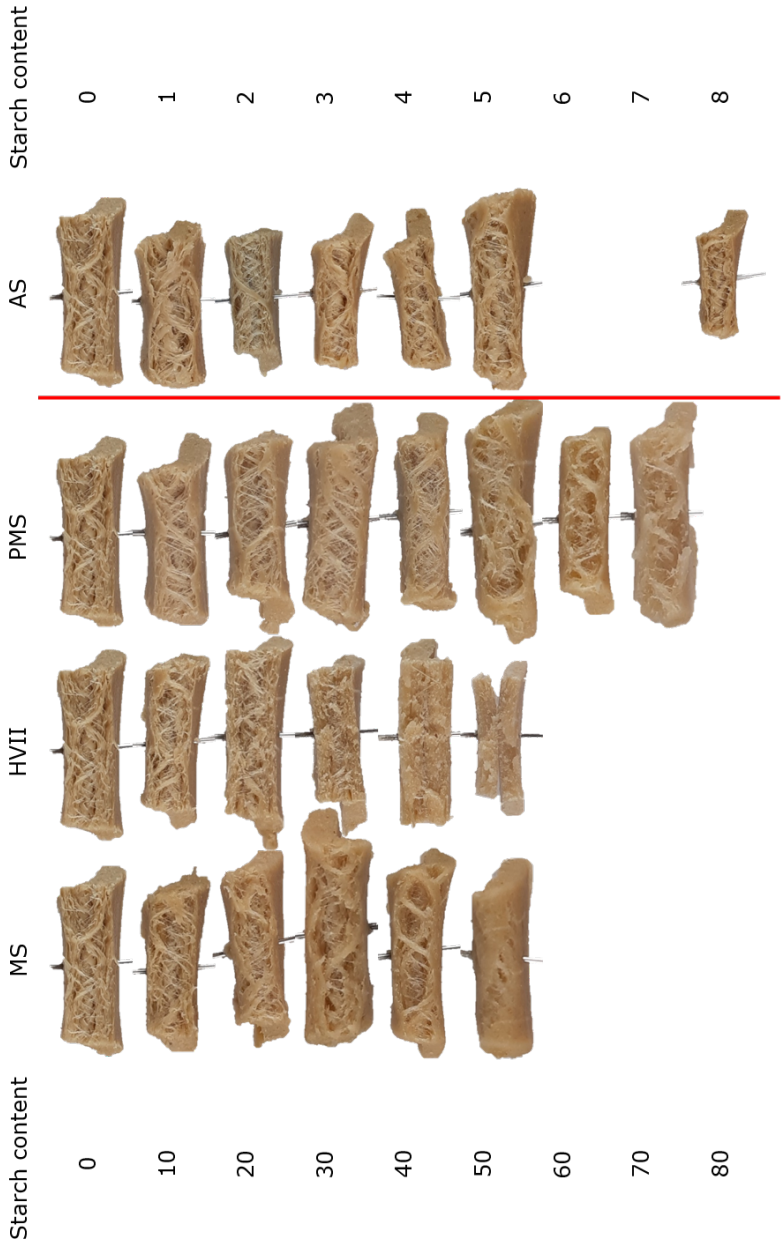

Figure 5.1: Photographs of the macro-structures after shearing MS, HVII, PMS or AS in combination with gluten in the HTSC. Numbers in the left column indicate the amount of added starch (d.b.). The amount of added AS was 10-fold smaller, ranging from 1-8 %. All samples had a dry matter content of 40 % (w.b.). Each sample had a width of approximately 5 cm.

5.3.2 Fracture Strain ε_f

The fracture strain ε_f was obtained from the tensile strength analysis and is used as a measure for the deformability of the sheared samples. It is displayed in Figure 5.2. The average ε_f of the pure WG reference was 0.64 in parallel and 0.37 in perpendicular direction. It is therefore anisotropic, meaning that this property depends on the direction it is measured in, or that it is not the same in every direction. The overall values were higher than those of chicken meat, obtained by Schreuders et al. [17], which were also not anisotropic. The addition of MS caused an increase of the ε_f in both parallel and perpendicular direction, after a small decrease at low MS addition. At S_{max} (40 %), the ε_f in both directions was slightly higher than the reference. The loss of fibrous structure at MS contents >40 % is not represented by the anisotropy of the deformability, since one would expect less anisotropy when there is no structural orientation. The increased deformability could be an explanation for the fact that the bending test was not able to reveal a fibrous structure at 50 % MS: The material was not deformed enough to fracture in a way that fibrous structure became visible.

The effect of the addition of PMS is peculiar: The increase of ε_f in the perpendicular direction was less than for the addition of MS, while in parallel direction it was much larger and did not show the initial decrease seen for MS. This increased the anisotropy of the ε_f up to an addition of 50 % PMS. This was not expected, since the composition of this material is identical to the composition of MS. However, at S_{max} (60 %), the ε_f in parallel direction decreased sharply, arriving at values comparable to the addition of 60 % MS. This shows that via pre-processing, the structuring potential of materials can be altered.

Addition of up to 5 % AS resulted in a decrease of ε_f in a similar manner to the initial decrease upon addition of MS. Samples containing higher amounts of AS were not suitable for tensile strength analysis, with the exception of the 8 % AS sample in parallel direction.

The addition of HVII resulted in a decrease of ε_f in both parallel and perpendicular direction. Unlike for the addition of MS, the decrease persisted also at higher amounts of HVII. The ε_f at S_{max} (20 %) in both directions was lower than the reference, while the anisotropy was also reduced. This is in line with the observation that the samples appeared to become more brittle with the addition of HVII. HVII was the only starch that brought the deformability of the samples closer to that of cooked chicken meat.

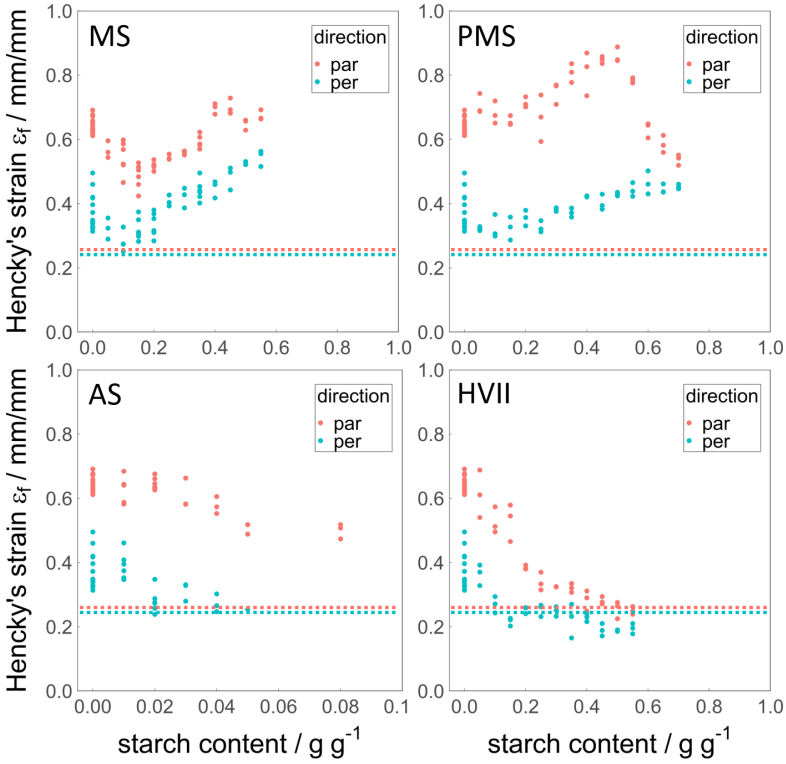

Figure 5.2: Fracture strain ε_f in parallel (red) and perpendicular (blue) direction over the added amount of MS, PMS, AS and HVII (d.b.). The horizontal dotted lines represent the values for cooked chicken meat, obtained by Schreuders et al. [17]. Note that the x-axis for AS has a different range than the others (0-0.1 g g^{-1} instead of 0-1 g g^{-1})

5.3.3 Fracture Stress σ_f

The fracture stress σ_f was obtained from the tensile strength analysis and is used as a measure for the hardness of the sheared samples. It is displayed in Figure 5.3. The average σ_f of the pure WG reference was 0.34 MPa in parallel and 0.09 MPa in perpendicular direction and therefore also anisotropic. This was overall higher than the fracture stress of cooked chicken meat [17], though the anisotropy is comparable.

The σ_f in perpendicular direction decreased due to an addition of up to 10 % MS,

above which it increased again, returning to a similar value at an addition of 55 % MS as the reference. The σ_f in parallel direction decreased rapidly due to an addition of up to 15 % MS. Above 15 % MS, σ_f in parallel direction remained constant, apart from some fluctuation at higher MS addition. As a result, the anisotropy of σ_f remained rather constant upon addition of MS. There was no indication of a loss of fibrous structure at S_{max} (40 %). Overall, an addition of 15 % MS or more resulted in a similar σ_f than that of cooked chicken meat.

The effect of the addition of PMS on σ_f in perpendicular direction was the same as that of the addition of MS. In parallel direction, the pre-gelatinization modified the effect the starch had on σ_f: The values increased on average but remained scattered up to an addition of 50 % PMS, above which a sharp decrease was observed. The difference between MS and PMS in the range of 5-50 % added starch is remarkable. The anisotropy of σ_f in this range of added PMS was larger than for any other material or property, but was reduced at higher amounts of added PMS. Therefore, there is an indication of structure change at S_{max} (60 % PMS). It is possible that starch becomes the continuous phase at that concentration. The values of σ_f for MS and PMS in both parallel and perpendicular direction were remarkably similar for the corresponding S_{max} (40 % MS, 60 % PMS).

The σ_f in parallel and perpendicular direction decreased due to the addition of small amounts of AS, comparable to the decrease due to addition of MS. Larger amounts of added AS led to samples that were too weak to perform the tensile strength analysis on. It seems that their σ_f decreased so much that they fractured under their own weight.

In contrast to the other starches, the addition of HVII led to an increase of σ_f in perpendicular direction, reaching a value similar to the initial σ_f of the reference in parallel direction. In parallel direction, the σ_f first decreased with the addition of HVII, reducing the anisotropy to a minimum. Above 25 % added HVII, the σ_f in both directions increased with a similar slope, keeping the anisotropy limited. This is an indication of structure change away from fibres at S_{max} as determined by the visual observation.

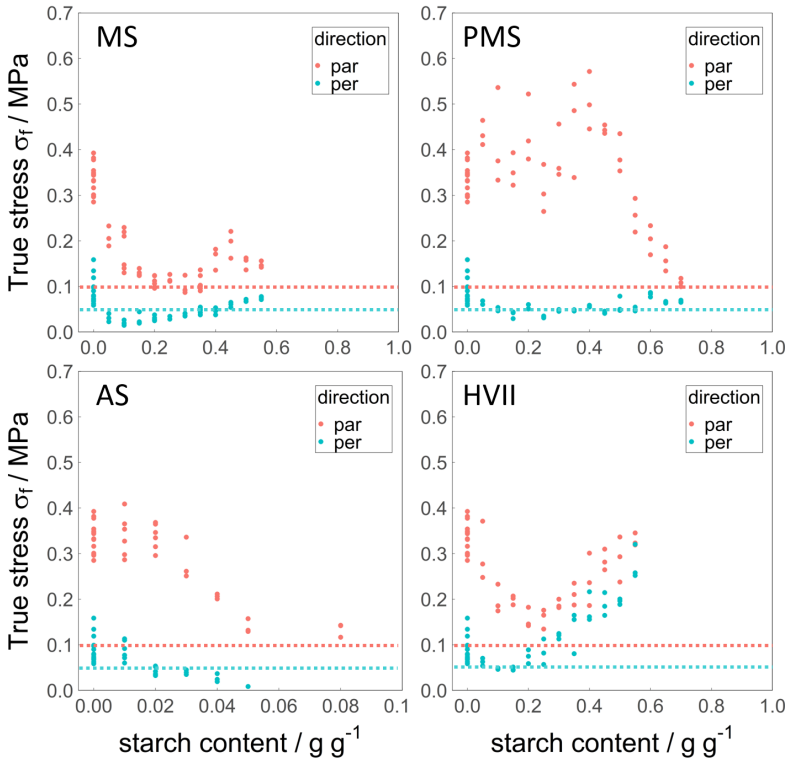

Figure 5.3: Fracture stress σ_f in parallel (red) and perpendicular (blue) direction over the added amount of MS, PMS, AS and HVII (d.b.). The horizontal dotted lines represent the values for cooked chicken meat, obtained by Schreuders et al. [17]. Note that the x-axis for AS has a different range than the others (0-0.1 $\mathrm{g\,g^{-1}}$ instead of 0-1 $\mathrm{g\,g^{-1}}$))

5.3.4 Young's Modulus

The Young's modulus was obtained from the tensile strength analysis and is used as a measure for the stiffness of the sheared samples. This property, unlike the fracture stress σ_f or the fracture strain ε_f, gives information about the initial deformation of the sample. It is displayed in Figure 5.4. The average Young's modulus of the pure WG reference was 299 Pa in parallel and 209 Pa in perpendicular direction and is therefore less anisotropic than σ_f or ε_f. Furthermore, the Young's modulus of the reference was scattered, particularly in perpendicular direction. The fibrous structure observed is inhomogeneous by nature, and especially the initial deformation

in perpendicular direction could have been influenced by local differences in fibre thickness. The Young's modulus in parallel direction of the gluten reference was quite similar to that of cooked chicken meat [17], while it was much higher than cooked chicken meat in perpendicular direction. The anisotropy of the Young's modulus of cooked chicken meat was remarkably high.

Samples containing any amount of MS showed a lower Young's modulus than the reference. The Young's modulus in parallel direction decreased with increasing addition of MS, while it was constant and lower for the perpendicular direction. There is no indication of a loss of fibrous structure at S_{max} (40 %). In contrast to this, addition of PMS led to a more gradual decrease of the Young's modulus in perpendicular direction, while in parallel direction it did not alter the Young's modulus up to additions of 50 % PMS. Therefore, the anisotropy of the Young's modulus was largest at 40 % PMS. At this point, the Young's modulus closest resembled that of cooked chicken meat. At S_{max} (60 %), the Young's modulus in parallel and perpendicular direction were similar, and therefore did not accurately predict the loss of fibrous structure at higher PMS addition. Addition of AS caused a decrease in Young's modulus in parallel and perpendicular direction alike, matching the values obtained for the addition of MS in the similar range (5 %).

The addition of HVII had the largest effect on the Young's modulus. It increased in parallel and perpendicular direction, reaching values up to 8 times higher than the reference. Due to the uniform increase, the anisotropy decreased and was lost, with values overlapping in parallel and perpendicular direction. This confirms the observation made upon bending: addition of HVII made the sample more stiff.

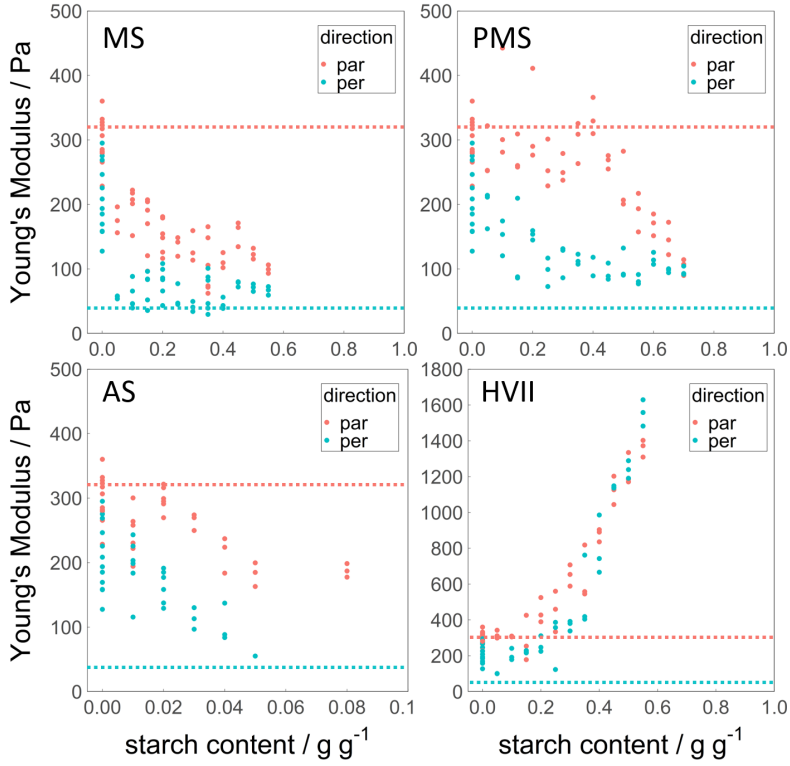

Figure 5.4: Young's modulus in parallel (red) and perpendicular (blue) direction over the added amount of MS, PMS, AS and HVII (d.b.). The horizontal dotted lines represent the values for cooked chicken meat, obtained by Schreuders et al. [17]. Note that the x-axis for AS (0-0.1 g g^{-1} instead of 0-1 g g^{-1} and the y-axis of HVII (0-18000 Pa instead of 0-500 Pa) have a different range than the others.

5.3.5 Faba bean

In order to apply the findings about the influence of starch on the formation of fibrous structures to less refined ingredients, gluten was combined with untreated FM or pre-heated FM and processed in the HTSC. The sample containing 50 % untreated FM did not show any fibrous structure or orientation in the shear flow direction, much like the sample containing the same amount of MS (Figure 5.5). The sample was soft and appeared grainy but homogeneous. The sample containing 50 % pre-gelatinized FM had a fibrous structure and was soft and inhomogeneous. It seems that the

pre-treatment had a similar effect on the FM as on the MS, resulting in a fibrous structure where there was none before.

Figure 5.5: Photographs of the macrostructure after shearing gluten with untreated MS and FM as well as pre-gelatinized MS and FM in the HTSC. All samples had a dry matter content of 40 % (w.b.), with a gluten content of 50 % (d.b.). Each sample has a width of approximately 5 cm.

5.4 Discussion

Three native starches (MS, AS and HVII) and one pre-gelatinized starch (PMS) were added to WG at different ratios and processed in the HTSC to determine the structuring behavior of starch-gluten blends and their rheological properties. Potential factors influencing the effect of the added starches are pH and salt content, which both depend on the purification process of the commercial starches. The pH range (pH 3.5-7 in 20 % aqueous suspension) and salt content (<500 mg/100g) provided by the manufacturer are rather broad for all three starches. The pH of 5-10 % aqueous solution was measured to be 4.6, 4.3 and 4.8 and the conductivity 175 $\mu S\,cm^{-1}$, 112 $\mu S\,cm^{-1}$ and 34 $\mu S\,cm^{-1}$ for AS, MS and HVII. The pH of the doughs after addition of gluten was determined for selected samples and was consistent at pH 6.1-6.2. The pH of these doughs is dominated by the gluten protein. The conductivity does seem to differ between starch types, but is insignificant when considering the conductivity of the NaCl added, which is roughly 14000 $\mu S\,cm^{-1}$ for 1 % NaCl in water [31]. Based on these measurements, the influence of pH and salt content does not seem to be a determining factor.

5.4.1 Effect of native starch on sheared samples

The three native starches that were used all contained different amylose and amylopectin content. AS mainly contains amylopectin, and less than 1 % amylose, HVII contains mostly amylose and much less amylopectin, while MS has an intermediate amylose/amylopectin content in between the two others. These differences can largely explain the rheological behavior of the starch-gluten blends: AS and MS have similar pasting or pasting onset temperatures (Table 5.2) of 70-76 °C. The final viscosity of MS is higher than that of AS, due to the increase in amylose content. The effect of added MS and AS on the measured tensile strength, deformability and stiffness was quite similar in the range where they could both be measured. Li et al. [32] and Ai et al. [33] investigated the gel strength of, among others, low amylose maize starch, maize starch and high amylose maize starch after heating at 95 °C and storage. Both low and high amylose maize starch did not form gels in their experiments. It is likely that in our study, the almost pure amylopectin in AS was unable to form a gel, leading to a loss of structure and making samples to soft to analyze. Hylon VII has a higher pasting onset temperature of 89.72-94.8 °C (Table 5.2), causing it to not paste completely during the RVA procedure. The final viscosity of HVII is therefore substantially lower. Since the high temperature used in this paper most likely allowed a complete pasting of HVII, the final viscosity is likely higher due to the higher amylose content. Carvalho et al. [34] also captured this phenomenon. The recrystallization of amylose upon cooling after complete pasting caused stiffness; the deformability decreased and the hardness increased.

Table 5.2: Properties of Amioca, Maize Starch and Hylon VII from literature. It is important to remark that the properties in this table are measured at lower temperatures than used in our experiments, and HVII does not yet pastes fully, causing the reported low viscosity measurements for HVII.

	Amioca	**Maize**	**Hylon VII**
amylose / %	<1	27	68
pasting temp. (RVA) / °C	72.68 [35]	76.12 [35]	-
pasting temp. (DSC) / °C	71.98 [36]	73.81 [36]	89.72 [36]
	71.2 [32]	70.1 [32]	-
onset temp. (RVA) / °C	70.2 [34]	72.3 [34]	94.8 [34]
final visc. (RVA) / mPa s	2349 [34]**	2546 [34]**	46 [34]**
	2191 [35]	3153 [35]	<1 [35]
water retention capacity at 90 °C / g/100g	20.40 [36]	11.19 [36]	4.48 [36]

Fiber formation is believed to be related to the technological properties of the ingredients, including water holding capacity [37, 38] and rheological properties such as viscosity [10, 30, 39, 40]. The starches investigated in this study differ in amylose/amylopectin content, which leads to differences in these technological properties. Fibre formation is therefore affected by the amylose/amylopectin content of the starch: Fiber formation is easily disturbed by AS addition, as it does not form a gel after cooling. The inability of amylopectin to form a gel upon cooling [32, 33] caused the samples to fall apart. Furthermore, amylopectin degrades under high temperature and shear [41], potentially drastically lowering its viscosity. This could have had a lubricating effect preventing proper shearing of the remaining gluten part. The large effect of low addition levels of AS can be attributed to the fact that AS takes up relatively large amounts of water (Table 5.2), adding to its volume. While non-gluten ingredients do not actively contribute to the formation of fibrous structures in gluten containing blends and therefore act as fillers [13], they can prevent the formation of fibrous structures if they are not able to form a strong enough gel. Amylose disturbed fiber formation less, although too much amylose (in case of HVII) apparently also prevented gluten from forming a fibrous structure as the high amylose gel became too stiff. Furthermore, the low water retention capacity of HVII (Table 5.2) could have diluted the gluten phase in such a way that it prevented fibre formation. As mentioned earlier, previous studies on the fibre formation in the

**calculated from their data

HTSC assumed that the rheological properties of the ingredients have an influence on fibre. Dekkers et al. [42] claimed that the rheological properties of the "phases" should be similar, while Schreuders et al. [40] hypothesized that a bi-continuous network is a requirement for fibre formation, the existence of which is governed by the rheological properties of the phases. Cornet et al. [13] concluded that a continuous gluten network was essential in order to create a fibrous structure. There, a gluten content of at least 50 % was necessary to create a fibrous structure in combination with leguminous protein isolates. This is in line with our results for native starches. Potentially, the stiff and therefore less deformable nature of the amylose-rich HVII after cooling also influenced the breaking behavior, breaking the gluten fibres while bending the sample. An optimum of the softness of amylopectin and the stiffness of amylose seems to be necessary to ensure proper embedding of the gluten fibres during shearing and bending. A high amount of amylopectin disrupted fibre formation during shearing (lubrication effect) and after cooling (low gel strength), while a high amount of amylose disrupted fibre formation after cooling (breaking behavior).

5.4.2 Effect of pre-gelatinization

Pre-gelatinizing MS prior to mixing with gluten and processing in the HTSC mainly modified the stiffness, strength and deformability in parallel direction of the shear flow, while the values in perpendicular direction remained close to those of MS. This is likely not an additive effect but an indirect effect of PMS on the WG and the fibrous structure. The properties measured in the parallel direction can be regarded as the properties of the gluten fibres. Pre-gelatinized starch takes up more water at room temperature than native starch. WG therefore had limited water available when mixed with the PMS slurry in the sample preparation. As gluten already forms a network when mixed with water at room temperature, the limited water content of the WG resulted in a more dense WG network. This could have led to stronger, stiffer and more deformable WG fibres, even at high amounts of PMS addition. In perpendicular direction the gluten dominates at low starch contents and determines the properties. As starch content increases, the adhesion of gluten/starch (which depends on the type of starch) starts to play a role, decreasing strength and deformability. At higher starch contents, the starch dominates the properties, in case of HVII this led to a low deformability, though an increased strength. This is why the effect of pre-gelatinization, so the difference between MS and PMS, mainly manifested in the parallel direction. The small differences in perpendicular direction could stem from the different conditions during gelatinization of the starch: while the starch in the PMS was allowed to gel freely with only water present, the starch

in MS was competing with the gluten for water and was sterically hindered by the gluten network during swelling and gelatinization. The anisotropy of the Young's modulus largely increased with pre-gelatinization. This made the samples containing PMS match the Young's modulus of chicken strips the best. Such a resemblance of a real meat texture has not been reported before. The use of pre-gelatinized starch could be a unique option to advance the development of meat analogues that actually resemble real meat.

5.4.3 Application in Fababean Meal

The sample containing 50 % FM did not form a fibrous structure, likely due to limited water uptake of FM at room temperature which diluted the gluten phase. FM had a starch content of 38.5 %, resulting in an added starch content of 19.25 % in the sheared samples. This falls in the range of the starch contents tested that could form fibrous products. The starch of FM (40 % amylose) has a higher amylose content than MS (27 %), but lower than HVII (68 %), so on one hand one would expect it to form a fibrous structure up to an addition between 30 % and 40 %. On the other hand, the gluten content in the sheared FM samples was 50 %, a recipe that also failed to produce fibrous structures with MS and HVII. FM also contains native protein, which is generally regarded to be less suitable for fibre formation in the HTSC [38, 43]. Furthermore, Li et al. [32] found that faba bean starch has a much higher gel strength than maize starch, even though their amylose content, branch-chain-length distribution as well as average degree of polymerization was comparable. This suggests that the differences between starches are not fully captured by their amylose/amylopectin composition. Pre-gelatinizing the MS modified the water uptake of the starch at room temperature, resulting in a denser gluten network and a fibrous structure at the same amount of added starch. This concept turned out to be also applicable to less pure ingredients than commercial starch, since the same effect of pre-gelatinization was observed for FM. This shows that less refined materials, thought to be less suitable for the formation of fibrous structures, can be modified "in-situ", so without separation of components. This approach was previously suggest by Bühler et al. [44]. This proof of concept is likely to be expandable to other starch-rich crops, such as peas or lentils.

5.5 Conclusions

The effect of the addition of starch on the structure formation of gluten in the HTSC was investigated. It is possible to add substantial amounts of native starch (up to 40 %) to gluten and still obtain a fibrous structure. With the addition of starch, textural properties of the samples change depending on the type and amount of starch, which can result in textural properties not previously seen for protein blends. The difference in amylose/amylopectin content of the starches lead to different technological properties such as viscosity and water uptake, thereby affecting fibre formation and textural properties. Pre-gelatinization of the starch enhances the ability of starch/gluten blends to form fibrous products, allowing addition of up to 60 % of starch. It also leads to an increased anisotropy of the Young's modulus, resembling chicken meat. This effect, and the higher maximum addable amount of PMS, are attributed to the higher water uptake of PMS at room temperature, which increases the density of the gluten network and therefore strengthens it. This concept was applied to FM, a unrefined material containing a considerable amount of starch. It was shown that not only can starch can be used to move the properties of plant-based meat analogues closer to those of real meat, but unrefined, starch-rich crops can be modified "in-situ" to become suitable for the production of fibrous meat analogues.

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Chapter 6

Quantifying Water Distribution between Starch and Protein in Doughs and Gels from Mildly Refined Faba Bean Fractions

This chapter is based on:

J.M. Bühler, A.J. van der Goot, M.E. Bruins (2021). Quantifying Water Distribution between Starch and Protein in Doughs and Gels from Mildly Refined Faba Bean Fractions. submitted.

Abstract

The development of novel and sustainable food products, such as cheese- and meat analogues, requires a better understanding of the use of less refined ingredients. We investigated the distribution of water between the protein and starch phase of doughs and gels made from air-classified faba bean fractions by developing a method suited for investigation of such multi-component ingredients. The moisture contents of the protein and starch phases in the dough were determined using a method based on partial sorption isotherms of mixed doughs of protein- and starch-rich fractions at high water activity. Water content of the protein phase is higher than that of the starch phase in dough, showing that protein takes up more water than starch at room temperature. Also, the moisture content of the protein phase in the gels was calculated using a model based on the denaturation temperature of legumin. From the experiments and the modelling, it became evident that the moisture content of the protein phase in the gel is lower than the moisture content of the protein phase in the dough, showing the importance of considering moisture migration from the protein to the starch during heating.

6.1 Introduction

To make attractive new food products, we have to understand how sensory properties of the product depend on components and their interactions with water. This understanding is especially important in soft-solid products like cheeses and meat but even more so for the plant-based alternatives of those products. Thus far, the products are mainly based on soy, pea or wheat, but interest in the use of other raw materials is increasing. Faba beans are considered as one of the promising new crops to provide healthy plant proteins [1]. It is therefore taken as an exemplary material of a starch-containing crop in this study.

Meat and cheese analog products are characterized by having a dry matter content of around 20 to 40 % in the water phase, which gives high water activity. Besides, multiple aqueous phases can be present in those products. The water content in each phase of such materials has a large influence on properties such as gelling behavior and thereby on the properties of the final product. Thus far, research on the influence of starch on protein gelation and vice-versa, as well as research on the influence of water content on product properties of soft-solid products, is often limited to the use of pure components, meaning isolates [2–7]. The use of less refined ingredients would improve the sustainability and cost efficiency of these products, for example meat analogues [8–10]. In order to use less refined ingredients for making food products, the interactions of the components present in less refined ingredients needs to be understood. This can only be achieved by performing experiments directly on the less refined ingredients instead of mixtures of highly purified ingredients, where protein and starch structure and therefore functionality have been modified during the purification process applied to make the ingredients [11].

Starch gelatinization is a clear example of the effect of water content on food properties. Although the initial gelatinization of starch occurs at a gelatinization temperature independent of the moisture content, the magnitude of gelatinization at said temperature decreases with the water content [12–14]. In case of incomplete gelatinization of starch, a second transition occurs at higher temperatures. This transition is the melting of starch crystals, and the melting temperature depends on the available water [14]. Additives that modify the water activity (and therefore the water availability) also influence the gelatinization parameters of starch [15]. Similarly, the gelling parameters of protein are influenced by the available water as well. Barker [16] found a linear correlation between the decrease of the denaturation temperature of egg albumin and the moisture content. Since then, similar relations

have been established for many proteins, including vicilin and legumin in faba bean [17]. As the gelling properties of protein and the gelatinization properties of starch both depend on the available moisture, determining this available moisture is essential to understand the behavior of food products that contain both starch and proteins. In a mixed dough or gel, starch and protein are often seen as individual phases that do not mix on a molecular level due to thermodynamic incompatibility [18–21]. Due to different affinities for water, the available moisture in these two phases will differ from the overall moisture content, which is an average value of the different moisture contents in the different phases. Often this water distribution is not measured, but obtained via fitting the water distribution between phases in a biopolymer blend or assumed from the overall moisture content when describing for example rheological properties by applying the polymer blending law to starch-protein mixtures or mixed protein blends [3, 22–24]. In addition, Dekkers et al. [2] developed a method based on TD-NMR to determine water distribution in mixed protein gels. However, it requires the availability of and information about the pure components used to make the mixed gels. For many food products, it is not possible to obtain the components in pure form, especially in the same physical state as in the product. For example, a purification process might involve a heating step, which changes properties of starches and proteins, which can change the distribution of water among them. The use of model mixtures of protein isolates and starch isolates will therefore not accurately reflect the behavior of real ingredients [11].

The aim of this research is to determine how starch and protein content influence the water distribution in doughs and gels that are prepared from mixtures of mildly refined ingredients. For this, we developed new methods for the determination of the water distribution in doughs at room temperature and for gels at elevated temperatures that do not depend on pure components that are otherwise difficult to obtain in the same, native form in which they are present in the mildly refined fractions. The method for the doughs combines fundamental principles of sorption isotherms with mass balances. Modelling is used to calculate the contribution of each pure component, which is then transferred into a sorption isotherm for the pure component at high water activity ($a_w > 0.96$). This high a_w is relevant when considering products like meat and cheese analogues that have a moisture content between 40 and 80 % [25–27]. However, this range of water activity is rarely investigated, causing a limitation of available data to compare to for both faba bean [28, 29] and starch in general [30]. The method to determine the distribution of moisture among the phases of gels at elevated temperatures is based on the denaturation temperature of the protein, measured with DSC. These methods enable us to understand the changes in water distribution

due to a thermal treatment under the relevant conditions. These changes might have implications for research on the properties of such mixed doughs and gels that depend on the water distribution, for example determining and predicting sensory attributes of protein/starch gel products (meat analogues, cheese analogues, sauces, dairy analogues).

Faba bean Meal (FM) is taken as an exemplary material. FM can be dry fractionated, delivering protein and starch enriched fractions with native properties. This ensures that neither protein nor starch are denatured or otherwise influenced by the fractionation method, therefore retaining their native functionality. Furthermore, FM contains enough starch to allow a starch content range in mixed doughs of 0.09 – 0.66 $g\,g^{-1}$ (db.) while ensuring that protein content is high enough to allow the detection of its denaturation.

6.2 Materials & Methods

Faba bean Meal HOMECRAFT®Pulse 3101 (FM) was supplied by Ingredion (Hamburg, Germany). FM had a protein content of 29 % (N-conversion factor = 6.25). FM was air classified using a Hosokawa Multi Mill (Alpine, Augsburg, Germany). The classifier wheel speed was set to 7000 rpm and the air flow to 70 $m^3\,h^{-1}$. The composition of the protein-rich fine fraction (Protein Fraction - PF) and the starch-rich coarse fraction (Starch Fraction - SF) is shown in Table 6.1.

Table 6.1: Composition of PF and SF in mass %. Starch content was determined using MegaZyme Starch Kit, protein content was determined using DUMAS (N-conversion factor = 6.25). Moisture content was determined using oven drying at 105 °C until the weight remained constant.

	Starch content / %	**Protein content / %**	**Moisture content / %**
PF	8.5	58.8	8.2
SF	66.3	15.5	6.79

PF and SF were mixed at different ratios to vary starch content (9-70 % d.b.). Starch content will be expressed in $g\,g^{-1}$ as w/w % on d.b., unless specified otherwise. Mixtures were combined with distilled water to create doughs with different moisture content (36-66 % wet basis), for which the moisture content of the powders was taken into account. Moisture content or dry matter content are expressed in $g\,g^{-1}$ on wet

basis, unless specified otherwise. Doughs were stored in vacuum-sealed bags at 4 °C for 48 h to allow water distribution to reach equilibrium.

6.2.1 Water activity

Water activity (a_w) of the doughs was measured using an Aqualab TDL water activity meter (METER Group, PUllman, USA). Approximately 3 grams of sample were used for each measurement. The measuring temperature was set to 25 °C with a deviation of 0.05 °C, ensuring constant measuring conditions.

6.2.2 Moisture content

Moisture content (mc) of all doughs was determined by oven drying. Dough samples were dried in aluminum cups at 105 °C for 24 hours. Moisture content was calculated according to Equation 6.1:

$$mc = \frac{m_{wet} - m_{dry}}{m_{wet}} \tag{6.1}$$

Where m_{wet} is the weight of the wet sample and m_{dry} is the weight of the dry sample. Moisture contents are always expressed on a wet basis (wb.), unless specified otherwise.

6.2.3 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) was used to determine the denaturation temperature of the proteins and the degree of starch pasting. Dough samples were degassed in a ultrasonic water bath for 15 minutes. 60 mg was transferred to High Volume Pans (100 µl, TA Instruments, New Caste, USA). The pans were placed in the DSC (DSC-250, TA Instruments, New Caste, USA) where they were first equilibrated at 20 °C until the temperature was constant. Samples were then heated with a ramp of 5 °C min^{-1} to 160 °C. After cooling, the cycle was repeated. TRIOS software was used to analyze the obtained thermograms and to identify the peak temperatures of the protein denaturation and the enthalpy change of the initial peak of starch pasting (G peak). ΔH was obtained by adjusting for the amount of starch present in the sample using

$$\Delta H_{Starch} = \frac{\Delta H}{(1 - mc) * sc} \tag{6.2}$$

where ΔH is the enthalpy change per overall sample mass in $\mathrm{J\,g^{-1}}$, mc is the overall moisture content in $\mathrm{g\,g^{-1}}$ (wb.) and sc is the starch content in $\mathrm{g\,g^{-1}}$ (db.).

6.2.4 Modeling and statistics

All measurements are shown as individual data points in the graphs. Multiple Linear Regression was used to fit the data in R (Version 3.6.1). For the dough, the model Equations 6.3-6.6 were used:

$$mc = a * sc + d \tag{6.3}$$

$$mc = a * a_w + d \tag{6.4}$$

$$mc = a * a_w + b * sc + d \tag{6.5}$$

$$mc = a * a_w + b * sc + c * a_w * sc + d \tag{6.6}$$

where mc is the overall moisture content of the sample in $\mathrm{g\,g^{-1}}$ (wb.), a_w is the water activity, sc is the starch content in $\mathrm{g\,g^{-1}}$ (db.) and a, b, c and d are model parameters. For the model of the gel, the same method was used with the model Equations 6.7 - 6.10.

$$T_d = e * sc + h \tag{6.7}$$

$$T_d = e * mc + h \tag{6.8}$$

$$T_d = e * mc + f * sc + h \tag{6.9}$$

$$T_d = e * mc + f * sc + g * mc * sc + h \tag{6.10}$$

where T_d is the denaturation temperature of legumin in °C, mc is the overall moisture content of the sample in $\mathrm{g\,g^{-1}}$ (wb.), sc is the starch content in $\mathrm{g\,g^{-1}}$ (db.) and e, f, g and h are model parameters. The residuals of each fit were checked for correlation in R. The summary tables of each model can be found in the Supplementary information. The best fit was chosen based on the value of R^2_{adj} and the significance of the independent variables (p-value).

6.3 Theory

Starting point of the method to determine the distribution of moisture among phases of a starch-protein blend at room temperature presented in this paper is the fact that the water activity of such a blend is determined by both the overall moisture content and the composition of the dough [31] (Figure 6.1). The assumption of full phase

separation between starch and protein makes it possible to use the sorption isotherm of the blend to obtain the isotherms of the individual phases [32]. The moisture content of the individual phases in the dough can then be read from the sorption isotherm of the individual phases at the measured a_w of the dough. At moisture contents higher than 40 %, the water activity of the product is between 0.9 and 1 and their relation can be approximated well by a straight line. The shape of the sorption isotherm of starch and protein only deviates from a linear relation at $a_w < 0.95$ [33, 34]. This simple method to determine the water available to starch and protein can however only be carried out when the mixture is in equilibrium. Therefore, it is not suitable at high temperatures due to evaporation and reactions taking place (*e.g.* gelation). To determine the distribution of moisture at higher temperatures, a method based on the denaturation temperature of the protein is proposed. As the denaturation temperature of protein depends on the moisture content of the protein phase, the first can be used to determine the latter if the relation is known. We model the relation by extrapolating the measured denaturation temperature of protein of the mixed gels to a protein phase containing no starch, yielding the moisture content of the protein phase in the mixed gel.

Next to the denaturation temperature of protein, the gelatinization of starch can also be monitored. If sufficient water is available, starch granules fully gelatinize at the initial gelatinization temperature [35, 36], resulting in the peak of gelatinization (G peak) [14] in a DSC thermogram. Under low moisture conditions, the amorphous regions of the starch granule are not fully hydrated and hence the crystalline regions initially stay partly intact. The crystalline regions of the starch granule then melt at a higher temperature, also resulting in a peak in the DSC thermogram (*M1* peak: high temperature, low moisture melting peak) [12, 37]. The position of this peak depends on the amount of water that can be taken up by the granules during the initial gelatinization. When no water is available, only the *M1* peak is present [35]. According to Tananuwong and Reid [14], the sum of the enthalpy change of the G and *M1* peak is independent of the overall moisture content. At high moisture contents, the peak temperature of the *M1* peak decreases in a magnitude that it overlaps the G peak. Here, this fact is used to take the enthalpy change of the G peak of the starch phase (ΔH) as a measure of the moisture content of starch in the dough.

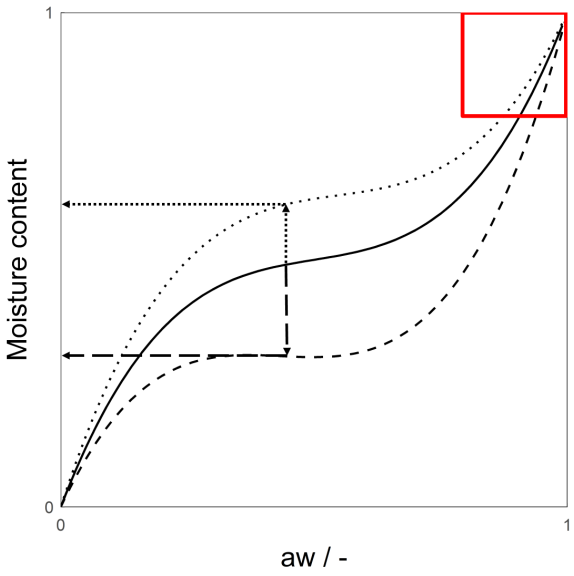


Figure 6.1: Sketch of sorption isotherms of protein (dotted line), starch (dashed line) and a mixture of the two (solid line), adapted from Barbosa-Cánovas et al. [31]. Arrows indicate the method to obtain the moisture content of the individual phases in the mixture. The red square indicates the area of the curve that is investigated in this study.

6.4 Results & Discussion

6.4.1 Moisture distribution in starch-protein doughs at room temperature

In this study, protein-rich fractions (PF) and starch-rich fractions (SF) were mixed to obtain doughs that vary in protein and starch content. Besides, water addition was used to change the water contents. Since PF and SF used in this study were obtained via dry fractionation, it is assumed that starch granules will remain intact [38]. This prevents mixing with the other components on a molecular level and starch can therefore be regarded as a separate phase. The other phase consists of proteins and other components such a fibres and sugars. Also these components will bear native properties, due to the lack of heating in dry fractionation. Despite the presence of the other components, we will describe the latter phase as protein phase, and study how the water will distribute between the starch and this so-called protein phase [4].

The overall moisture content (mc) and water activity (a_w) of 155 dough samples with different starch and protein contents and added amounts of water were measured (Figure 6.2) at 25 °C. The data obtained represents the section of the sorption isotherm where the shape of the curve becomes asymptotic [39], indicated by the red square in Figure 6.1. The partial sorption isotherm therefore becomes linear at these high moisture contents. Therefore, the data in Figure 6.2 shows linear relations for each starch concentration linking water activity to moisture content. The slopes differ depending on the starch content, leading to a larger difference in a_w at the lower moisture contents than at the highest moisture content.

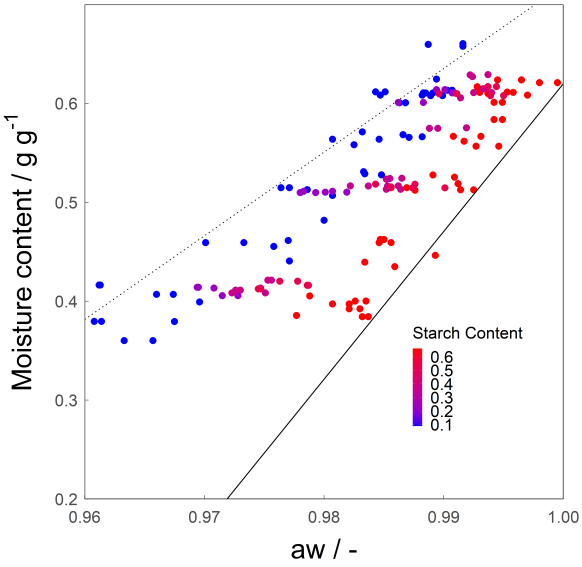


Figure 6.2: Partial sorption isotherms showing the influence of starch content of the correlation between overall moisture content and water activity. The dotted line represents the protein phase (Equation 6.12), the solid line the starch phase (Equation 6.13).

The potential influence of the starch content on the correlation between the moisture content and the water activity can be incorporated in a model via Equation 6.3 - 6.6. It is important to note that the dry based protein content (pc) and the dry based starch content (sc) of the samples can be expressed as $sc + pc = 1$. This means that protein content was not varied independently of starch content and is therefore not used as a independent variable. Equation 6.6 was found to give the best fit based on

the highest R^2_{adj}, while the p-value was below 0.001 for all variables used, showing that they are significant. Summary tables of all fitted models can be found in the Supplementary information (Tables S1-S4). Fitting resulted in the following equation describing the relation between water activity, starch content and moisture content in starch-protein doughs:

$$mc = 8.46 * a_w - 6.58 * sc + 6.48 * a_w * sc - 7.74 \tag{6.11}$$

where mc is the overall moisture content of the sample in $\mathrm{g\,g^{-1}}$ (wb.), aw is the water activity, and sc is the starch content in $\mathrm{g\,g^{-1}}$ (db.). This optimized fit resulted in $R^2_{adj} = 0.89$. The investigated range is limited to the moisture content relevant for protein/starch gel products such as meat- and cheese analogues.

The moisture content of the protein phase as well as the starch phase in a sample at a given water activity can be calculated from Equation 6.11 by setting the starch content or respectively the protein content to zero. This yields the moisture content of the protein phase in the dough ($mc_p(dough)$, Equation 6.12) and the moisture content of the starch phase in the dough ($mc_s(dough)$, Equation 6.13):

$$mc_p(dough) = 8.46 * a_w - 7.74 \tag{6.12}$$

$$mc_s(dough) = 14.94 * a_w - 14.32 \tag{6.13}$$

where $mc_i(dough)$ is the moisture content of the protein or starch phase in the dough in $\mathrm{g\,g^{-1}}$ (wb.) and a_w is the water activity (-) of the sample. These equations are given as lines in Figure 6.2. The linear relation from Equation 6.11 is supported by the fact that the shape of the sorption isotherm of starch and protein only deviates from a linear relation at lower a_w, according to literature: In a study on several raw legume flours (chickpea, lentil and yellow pea), sorption isotherms were measured, for all of which this deviation only occurred at $a_w < 0.8$ and $mc < 0.13$ $\mathrm{g\,g^{-1}}$ (wb.) [34]. For corn starch the relation seems to be linear at $a_w > 0.9$ and $mc > 0.15$ $\mathrm{g\,g^{-1}}$ (wb.) at 30 °C [40]. All samples used in the further analysis of this paper had an a_w of over 0.97.

In Figure 6.3, the calculated moisture content in the protein phase at room temperature is shown over the overall moisture content for all used starch contents (db.). The moisture content of the protein phase in the dough increases with starch content, depending on the overall moisture content. At lower moisture contents (0.47 $\mathrm{g\,g^{-1}}$), an increase of the starch content from 0.09 to 0.7 $\mathrm{g\,g^{-1}}$ (d.b.) leads to an

increase in moisture content of the protein phase of 0.11 $g\,g^{-1}$ (22 %). The same increase in starch content at higher overall moisture content (0.61) only causes an increase of 0.07 $g\,g^{-1}$ (9 %). As the overall moisture content increases and the a_w approaches 1, the sample approaches a regime of excess water, where a change in starch content will not have an influence on the moisture content in protein, as both the moisture content in starch and protein will be equal to the overall moisture content.

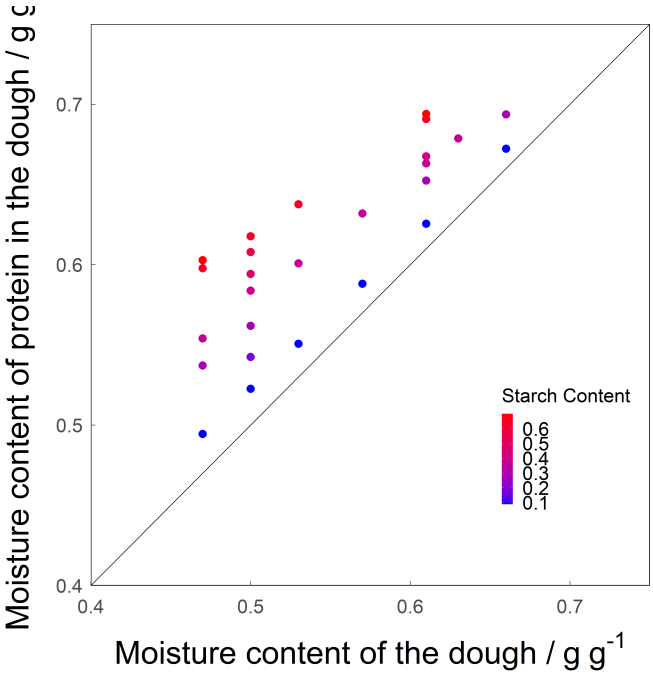


Figure 6.3: Modelled moisture content in the protein phase in the dough ($mc_p(dough)$) over the moisture content of the dough. The solid line represents where the moisture content of the dough and the moisture content of the protein phase in the dough would be the same.

6.4.2 Moisture distribution in starch-protein mixtures at increasing temperature

DSC thermograms of starch/protein blends

The starch gelatinization as well as the protein denaturation were analyzed using DSC. From the thermograms of the DSC (Figure 6.4) we can gain information about changes that occur, such as the peak temperature (T) and the enthalpy change (ΔH) associated with the reaction. Figure 6.4 shows three exemplary thermograms, one with a low $mc_s(dough)$ (red), one with an intermediate $mc_s(dough)$ (green) and one with a high $mc_s(dough)$ (blue). There are three peaks visible in all three thermograms. The first peak is the initial gelatinization of starch, also called the *G* peak [14], which occurs when starch is heated above the pasting temperature in the presence of excess water [35, 36]. For most starches this peak lies between 60 °C and 80 °C [41]. The second peak, around 90 °C, was credited to the denaturation of vicilin [17]. The third peak was credited to the denaturation of legumin at around 106 °C [17]. DSC measurements of starch can also give rise to another peak (*M1*), especially at low moisture content, which represents the melting of the starch. This peak was not identified in this research.

Starch gelatinization

For the samples analysed, the *G* peak (the excess water gelatinization peak) occurred at 73.4 °C (± 2.4 °C) (data not shown). The peak temperature did not correlate with the overall moisture content or the moisture content of starch in the dough (Equation 6.13) ($R^2_{adj} < 0.1$), as is expected from literature (see Section 6.1). van der Sman and Meinders [42] modelled the initial gelatinization of starch using free volume extension of Flory-Huggins theory and constructed a state diagram for starch, which shows limited influence of the mass fraction of water (y_w) (which is equivalent to $mc_s(dough)$) on the gelatinization temperature for $y_w > 0.3\ \mathrm{g\,g^{-1}}$. They do, however, show a linear relation of the melting temperature of starch to the mass fraction of water, which describes the peak temperature of the *M1* peak. In this paper, the *M1* peak could not be identified, as its position (T) and size (ΔH) depend on the moisture content and it can overlap with the denaturation peak of vicilin and potentially also legumin. The possible effects of this on the interpretation of the denaturation temperature of legumin (T_d) are discussed towards the end of Section 6.4.2.

In Figure 6.5, the ΔH over the overall moisture content of the dough is shown. There

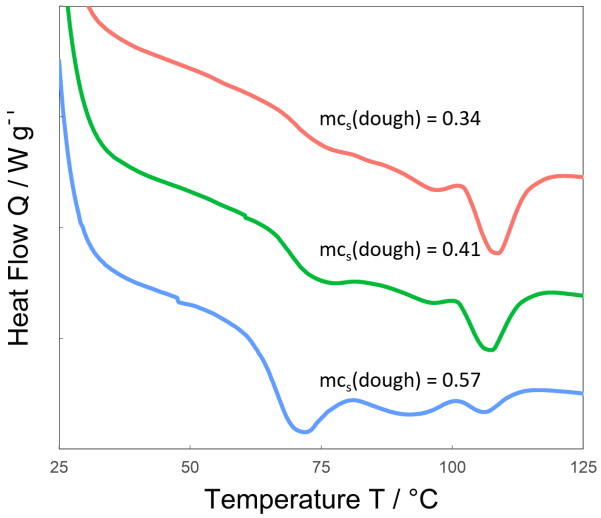
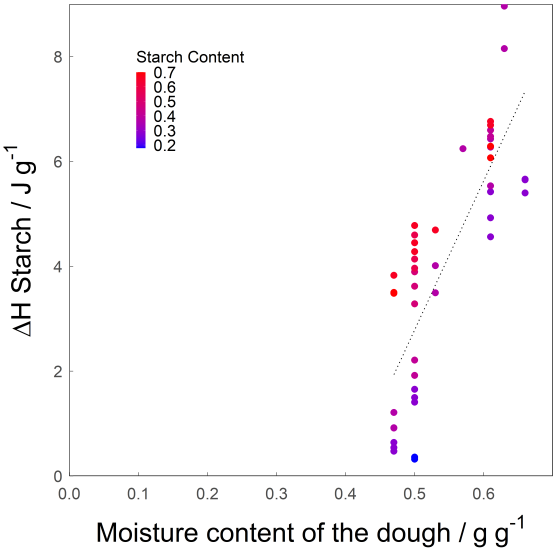


Figure 6.4: Exemplary DSC thermograms of three samples with (red) 0.28 g g^{-1} starch content (d.b.), 0.51 g g^{-1} moisture content resulting in a $mc_s(dough)$ = 0.34 g g^{-1}, with (green) 0.37 g g^{-1} starch content (d.b.), 0.53 g g^{-1} moisture content resulting in a $mc_s(dough)$ = 0.41 g g^{-1}, and with (blue) 0.66 g g^{-1} starch content (d.b.), 0.61 g g^{-1} moisture content resulting in a $mc_s(dough)$ = 0.57 g g^{-1}. Curves are offset in y-direction to display all three graphs in one figure without overlapping. Tick-marks on y-axis represent steps of 0.02 W g-1. All curves shown are in the range of -0.28 to -0.20 W g-1. Please note that the Heat Flow Q shown here is only normalized for the overall mass, not the mass of starch in the sample.

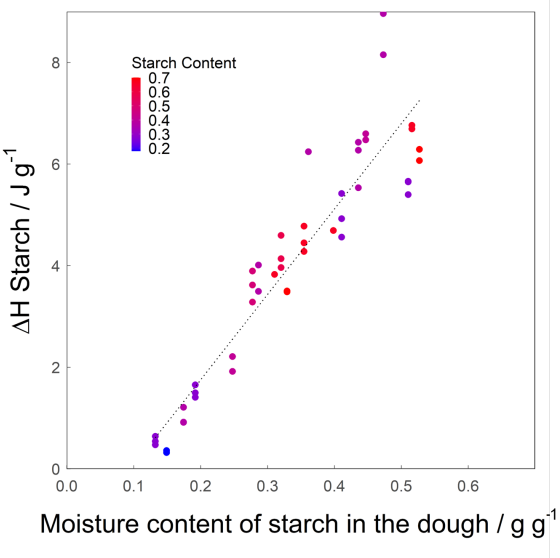
is no clear correlation of the two, resulting in an R^2_{adj} of the linear regression of 0.63. It is quite obvious, however, that ΔH depends on the starch content as well, since the high starch content samples (red) are underestimated by the linear regression, while the low starch content samples are mostly overestimated (purple, blue). Therefore, in Figure 6.5b, ΔH is shown over $mc_s(dough)$, which is derived from Equation 6.13 which in turn depends on the overall moisture content and the starch content of the sample (Equation 6.11). The use of a linear regression to correlate ΔH_{Starch} and $mc_s(dough)$ yields an R^2_{adj} of 0.87. This underlines that Equations 6.12 and 6.13 give a reasonable approximation of the distribution of moisture between the protein and starch phase. It is important to note that at high $mc_s(dough)$ starch always fully gelatinizes during

the initial gelatinization and therefore ΔH becomes constant [43]. This most likely occurs outside the regime of $mc_s(dough)$ investigated here, as [44] and [45] found a ΔH of 13.4 ± 0.6 $\mathrm{J\,g^{-1}}$ and 13.4 ± 2.0 $\mathrm{J\,g^{-1}}$ for wheat starch at a moisture content of 0.91 $\mathrm{g\,g^{-1}}$ and 0.9 $\mathrm{g\,g^{-1}}$ respectively. The extrapolation of the current data set to $mc_s(dough) = 0.9\ \mathrm{g\,g^{-1}}$ yields a ΔH of 14.68 $\mathrm{J\,g^{-1}}$, indicating the validity of our data and the linear correlation of $mc_s(dough)$ and ΔH. For samples with low moisture content of starch in the dough, the starch does not fully gelatinize at the temperature of the G peak but undergoes further transition at higher temperatures. The degree to which starch gelatinizes at this temperature depends on the initial water content of the starch [14], or in this case $mc_s(dough)$. At $mc_s(dough)$ below 0.3 $\mathrm{g\,g^{-1}}$, no gelatinization occurred at the initial gelatinization temperature.

This also suggests that the often described swelling of starch granules before or during initial gelatinization [46, 47] does not affect the moisture content of the protein phase or starch phase. Since the moisture content of the starch phase remains the same during swelling, the swelling of starch granules is caused by a migration of water within the starch phase. It is therefore likely that the moisture content of the starch phase consists of an extra-granular and an intra-granular water population, changing to only an intra-granular water population through swelling [14].



(a)



(b)

Figure 6.5: The enthalpy of the initial starch gelatinization (ΔH_{Starch}) over (a) the overall moisture content of the dough and (b) the modelled moisture content of starch in the dough ($mc_s(dough)$). Dotted lines are linear fits of ΔH_{Starch} with (a) mc and (b) $mc_s(dough)$ as variables, resulting in $R^2_{adj} = 0.63$ and $R^2_{adj} = 0.87$, respectively.

Protein denaturation temperature of mixed starch-protein gels

As mentioned in Section 6.4.2, three peaks were found in the thermograms depending on starch and moisture content. The second and third peak at around 90 °C and 106 °C respectively represent vicilin and legumin [17]. As the third peak was the most distinct and with good resolution, it was chosen to represent the denaturation of protein. Besides, the effect of moisture content on denaturation temperature (T_d) seems to be comparable for vicilin and legumin in the studied range [17], and the composition of the protein in the protein phase is unlikely to change.

Figure 6.6 shows the denaturation temperature of legumin (T_d) against the overall moisture content of the sample. By changing the starch content, the denaturation temperature of legumin decreased by maximally 5.12 °C, when using blends with an overall moisture content of 0.47 $\mathrm{g\,g^{-1}}$.

The potential influence of the starch content on the correlation between the moisture content and T_d can be incorporated in a model via Equation 6.7 - 6.10. Equation 6.9 was found to give the best fit based on the highest R^2_{adj}, while the p-value was below 0.001 for all variables used in this model, showing that they are significant. Equation 6.10 resulted in the same R^2_{adj}, but the p-values show that not all variables are significant. Summary tables of all fitted models can be found in the Supplementary information (Tables S5-S8). Fitting resulted in the following equation describing the relation between moisture content, starch content and the denaturation temperature of legumin in starch-protein gels:

$$T_d = -64.16 * mc - 3.40 * sc + 143.15 \tag{6.14}$$

where T_d is the denaturation temperature of legumin in °C, mc is the overall moisture content of the sample in $\mathrm{g\,g^{-1}}$ (wb.) and sc is the starch content in $\mathrm{g\,g^{-1}}$ (db.), resulting in an $R^2_{adj} = 0.88$.

Similarly to the approach used to determine the $mc_p(dough)$, setting sc to 0 after solving for mc yields the moisture content of the protein phase in the gel ($mc_p(gel)$):

$$mc_p(gel) = \frac{T_d - 143.15}{-64.16} \tag{6.15}$$

where $mc_p(gel)$ is the moisture content of the protein phase in the gel in $\mathrm{g\,g^{-1}}$ (wb.) and T_d is the denaturation temperature of legumin in °C. This relation is shown in Figure 6.6 by the dashed blue line. Furthermore, the values of denaturation

temperature of legumin found by [17] using DSC are shown as well (open symbols). The relation of the moisture content of faba bean protein and these values can be split in two regimes: a linear decrease of T_d with increasing moisture content until 0.6 g g^{-1} and a constant T_d above 0.6 g g^{-1}. Here we found a similar dependency of the denaturation temperature on moisture content in the protein phase as reported by Arntfield et al. [17]. They do, however, differ from our results in temperature, which could be due to possible differences in origin of the raw materials and the purification process used by them. In our paper, a less refined material is used, leaving the possible influence of other components like sugars, fibres and salts on the denaturation temperature. The purification of the faba bean protein used by Arntfield et al. [17] could have partially denatured the protein, causing the shift of the curve to lower temperatures observed here. Most likely, the T_d of mildly refined faba bean fractions also does not decrease for moisture contents above the maximum moisture content used in this study.

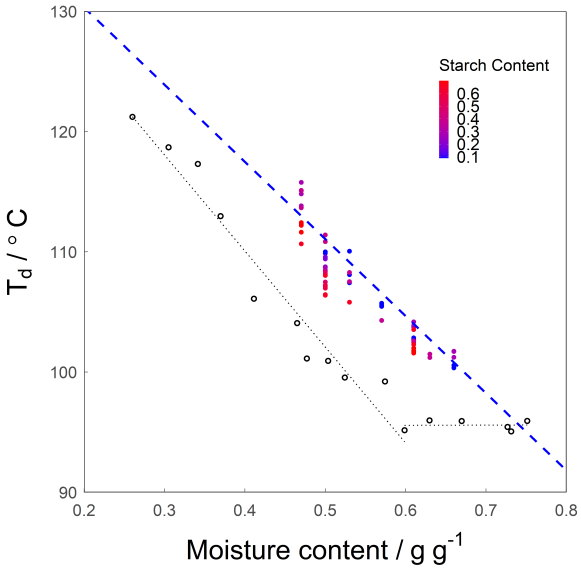


Figure 6.6: T_d measured using DSC over the overall moisture content of the sample (wb.). The open black symbols show data for legumin in faba bean protein isolate, taken from Arntfield et al. [17]. The blue dashed line is T_d for sc = 0, according to Equation 6.14, the black dashed lines are linear regressions of the data reproduced from [17] in the range where T_d depends on mc and where T_d remains constant irrespective of mc.

In Figure 6.7, the modelled moisture content in the protein phase at denaturation is shown over the overall moisture content for all starch contents used (db.). Moisture content in protein at denaturation increases with starch content. At overall moisture contents $> 0.6\ \mathrm{g\,g^{-1}}$ the trend becomes less evident. This either means that the influence of starch on the moisture content of protein at denaturation is limited at high moisture contents (as is the case at room temperature), or that the influence of moisture content in protein on denaturation temperature becomes smaller at overall moisture contents $> 0.6\ \mathrm{g\,g^{-1}}$. The latter was also found to be true by Arntfield et al. [17], who showed that the decrease of denaturation temperature of legumin with increasing moisture content levels off at $1.5\ \mathrm{g\,g^{-1}}$ (db.), which is equal to $0.6\ \mathrm{g\,g^{-1}}$ (wb.).

It is important to note that the starch melting peak (*M1* peak) was not identified in this research. It potentially overlaps with the T_d peak and interferes with the analysis. Hoseney et al. [37] and Tananuwong and Reid [14] show that the *M1* peak is rather broad and not a sharp peak like the *G* peak or the peaks for protein denaturation observed in this study. Such overlap is most likely at the lowest overall moisture content and starch content, as these conditions lead to the lowest values of $mc_s(dough)$), which in turn lead to a high *M1* temperature. However, this also decreases the overall enthalpy of the starch gelatinization (since there is less starch in the sample), thereby decreasing the influence of the *M1* peak on the analysis of the denaturation peak of legumin and likely making this overlap less relevant. There is, however, the possibility of relevant overlap of the legumin peak and the *M1* peak at low moisture contents ($0.47\ \mathrm{g\,g^{-1}}$) and intermediate starch contents (purple points), as these conditions lead to a relatively low $mc_s(dough)$, causing the *M1* peak to increase in height and appear at higher temperatures, and also lead to a smaller peak for the denaturation of legumin, as there is less protein present as the starch content increases. This could be the cause for the deviation of these samples from the rest of the data, observed in Figure 6.6 and Figure 6.7.

6.4.3 Comparing water distribution in doughs and gels

The comparison of the moisture content in the protein phase in the ingredients studied at room temperature (Figure 6.3) with the moisture content in the protein phase at denaturation (Figure 6.7) revealed that the latter is lower by up to 21.15 %. The difference between them is larger for lower moisture contents and increases with starch content. Therefore, moisture had migrated from the protein phase to the starch phase during the heating process. A similar conclusion was drawn by Eliasson [4] after

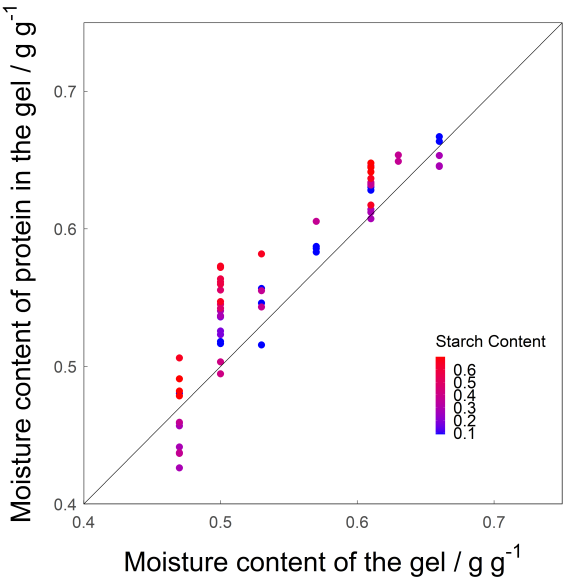


Figure 6.7: Modelled moisture content in the protein phase in the gel ($mc_p(gel)$) over the moisture content of the gel. The solid line represents where the moisture content of the gel and the moisture content of the protein phase in the gel would be the same.

studying the influence of wheat gluten on the gelatinization of wheat starch, using isolated ingredients. Since we have established that the moisture content of protein at room temperature is not influenced by the swelling of starch granules during their initial gelatinization (G peak), the redistribution of water must occur later, at higher temperatures. We suggest that this occurs during starch melting, which takes place at those higher temperatures. This is represented by the *M1* peak that most likely overlaps with the denaturation peaks of vicilin and legumin and can therefore not be detected. Melting of starch modifies the affinity of starch for water, creating a force "pulling" the water to the starch phase. Simultaneously, denaturation of protein exposes hydrophobic sites, creating a force "pushing" the water to the starch phase.

6.5 Conclusions

The distribution of water between the starch and protein phase in doughs and gels of mixed mildly-refined fractions of faba bean flour was determined in a moisture content range relevant for soft-solid foods such as plant-based meat and cheese alternatives. Quantification of the moisture content distribution was achieved by applying models based on established concepts such as sorption isotherms, mass balances and the moisture dependence of starch gelatinization and protein denaturation. The model based on sorption isotherms was used to determine the water distribution in a dough at room temperature, while the model based on the moisture dependence of protein denaturation determines the water distribution at elevated temperatures when protein denatures and gelatinizes. In the dough, protein takes up between 0.12 $\mathrm{g\,g^{-1}}$ and 0.28 $\mathrm{g\,g^{-1}}$ more water than starch. The amount of water associated with the protein phase in the gel is up to 0.12 $\mathrm{g\,g^{-1}}$ less than in the dough, showing the amount of water that migrated from the protein phase to the starch phase upon heating. The enthalpy change of the initial starch gelatinization correlates reasonably well with the calculated moisture content of the starch phase in the dough. Therefore, no or limited redistribution of moisture occurs between the starch and protein phase during the initial starch gelatinization, but at higher temperatures, during the melting of starch crystals.

This paper shows that it is possible to derive properties of components present in a flour or enriched fraction without having the components as pure ingredients. Such an approach will become more important when focusing on the use of enriched fractions rather than pure ingredients in future food applications. These findings have implications for the further development and design of production processes for aforementioned plant-based meat and cheese alternatives. The methods presented here can be used to predict and control ingredient properties that determine process parameters such as processing temperature. Furthermore, they underline the limitations of using overall moisture content without understanding water uptake and distribution in the individual components, all of which becomes even more important when using less refined ingredients.

Supplementary information

Summary Tables for the models of the dough

Table S1: Summary of model based on Equation 6.3. $R^2 = 0.00$, $R^2_{adj} = 0.00$

Term	Coefficient	Std. Error	t value	Pr(>|t|)
intercept	0.52838	0.01275	41.43	<2e-16
sc	-0.0206	0.02954	-0.697	0.487

Table S2: Summary of model based on Equation 6.4. $R^2 = 0.66$, $R^2_{adj} = 0.66$

Term	Coefficient	Std. Error	t value	Pr(>|t|)
intercept	-7.3749	0.4558	-16.18	<2e-16
aw	8.0216	0.463	17.32	<2e-16

Table S3: Summary of model based on Equation 6.5. $R^2 = 0.88$, $R^2_{adj} = 0.88$

Term	Coefficient	Std. Error	t value	Pr(>|t|)
intercept	-9.53081	0.30446	-31.3	<2e-16
aw	10.2824	0.31118	33.04	<2e-16
sc	-0.1896	0.01155	-16.41	<2e-16

Table S4: Summary of model based on Equation 6.6. $R^2 = 0.90$, $R^2_{adj} = 0.89$

Term	Coefficient	Std. Error	t value	Pr(>|t|)
intercept	-7.7394	0.4491	-17.231	<2e-16
aw	8.4609	0.4576	18.488	<2e-16
sc	-6.5802	1.2474	-5.275	4.52E-07
aw:sc	6.4814	1.265	5.123	9.03E-07

Summary Tables for the models of the gel

Table S5: Summary of model for T_d based on Equation 6.7. $R^2 = 0.00$, $R^2_{adj} = 0.00$

Term	Coefficient	Std. Error	t value	Pr(>|t|)
intercept	107.2473	1.1068	96.897	<2e-16
sc	-0.7564	2.6465	-0.286	0.776

Table S6: Summary of model for T_d based on Equation 6.8. $R^2 = 0.86$, $R^2_{adj} = 0.85$

Term	Coefficient	Std. Error	t value	Pr(>|t|)
intercept	141.1	1.795	78.61	<2e-16
mc	-62.665	3.274	-19.14	<2e-16

Table S7: Summary of model for T_d based on Equation 6.9. $R^2 = 0.88$, $R^2_{adj} = 0.88$

Term	Coefficient	Std. Error	t value	Pr(>|t|)
intercept	143.1475	1.7248	82.995	<2e-16
mc	-64.1607	3.0064	-21.341	<2e-16
sc	-3.3958	0.9188	-3.696	0.000476

Table S8: Summary of model for T_d based on Equation 6.10. $R^2 = 0.88$, $R^2_{adj} = 0.88$

Term	Coefficient	Std. Error	t value	Pr(>|t|)
intercept	144.445	3.372	42.835	<2e-16
mc	-66.541	6.107	-10.895	9.40E-16
sc	-7.073	8.248	-0.858	0.395
mc:sc	6.81	15.177	0.449	0.655

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Chapter 7

General discussion

7.1 Introduction

Consumer awareness of ethical concerns about food production is rising, while price and sensory appeal remain the deciding factors in food choice [1]. Plant-based meat analogues, commonly associated with less ethical concern and more sustainability, offer consumers a solution to reduce their meat intake. To be a viable solution, meat analogues should mimic the eating experience of real meat as much as possible, including the fibrous structure, without sacrificing the sustainability advantages. For the latter, it is necessary to diversify the ingredient portfolio of plant meat to prevent monocultures [2]. Besides, the processing intensity for making the ingredients should be minimized [3]. This can be achieved by using starch bearing crops such as faba bean. **It was therefore the aim of this dissertation to explore the potential of faba bean as a new raw material for fibrous meat analogues and to unveil the effect of using less refined ingredients.** Since less refinement implies the introduction of starch in the recipes for plant meat, the role of starch has to be investigated. The first part of the aim was studied in Chapter 2 and 3, while the other chapters had a focus on the role of starch.

In **Chapter 2** we discovered that blends of gluten with Faba bean Protein Isolate (FPI) behave similarly as gluten blends with Pea Protein Isolate and Soy Protein Isolate (PPI and SPI). Just as with PPI and SPI, FPI forms a two-phase blend when mixed with gluten. Gluten restricts the swelling capacity of the other protein phases in those blends by forming a continuous structure around them. Gluten is solely responsible for the formation of fibrous structures in the studied recipes. In **Chapter 3** we showed that the water holding capacity (WHC) of Faba bean Protein Concentrate (FPC) can be increased through a dry heat treatment of the powder. This increase could enhance the potential of FPC to be used as ingredient for meat analogues. The increase in WHC was caused by partial denaturation of the protein, leading to agglomeration due to hydrophobic interactions and hydrogen bonds. In **Chapter 4** we reviewed the literature on starch in meat and meat analogues and found that exogenous starch is added to meat analogues in small quantities to increase yield or to prevent cooking loss. More generally stated starch is used to bind water. While knowledge on the effect of endogenous starch is still lacking, there is potential to use the starch naturally present in starch bearing crops as a functional component, especially through "in-situ" modification without prior separation. In **Chapter 5** we revealed that native starch can be used in a similar way as non-gluten protein isolates to form fibrous structures in combination with gluten. Pre-gelatinization of starch increased the ability of the starch-gluten blends to form fibrous products. The type of

starch as well as the pre-treatment of the added starch strongly influenced the tensile properties of the product, resulting in a unique variety of achievable textures. The "in-situ" pre-gelatinization of starch in Faba bean Meal (FM) promoted the formation of fibrous structures in combination with gluten, accompanied by textural properties so far unachievable with protein. In **Chapter 6** the water contents in the starch and protein phases in doughs with varying starch/protein content were determined using modeling of partial sorption isotherms at high water activity. The doughs were made by mixing dry fractionated fractions of native FM. The distribution of water in gels with varying starch/protein contents was determined using a model based on the denaturation temperature of legumin. The water content of the protein phase in the gel was lower than the water content of the protein in the dough, suggesting that water migrated from the protein phase to the starch phase during heating. A further analysis of the gelatinization of starch via DSC revealed that the migration of water occurred after the initial gelatinization of starch.

When overviewing all results described in this dissertation, we see that gluten is responsible for the formation of fibrous structures, while other ingredients act as fillers in gluten-containing recipes. These fillers can be non-gluten protein such as FPI, but also starch. Starch can help deliver a range of mechanical properties that has not been seen in meat analogues with protein, especially with respect to small deformation properties. Furthermore, we have demonstrated that starch can be modified by simple pre-gelatinization to become more suitable for the use in meat analogues, while protein can be modified by a dry heat treatment. We also show that it is possible to use unrefined starch bearing crops such as FM to make fibrous structures in combination with gluten. This reveals that the structuring potential of starch is at least equally high as that of proteins. The application of starch as an ingredient for fibrous products will further benefit from an understanding of the properties of starch and protein in the multi-component blends or unrefined ingredients they are part of. The water content of the individual components determines important properties such as gelatinization or gelation temperature as well as rheological properties. We used modeling of partial sorption isotherms and denaturation temperature to determine the water content of starch and protein in such ingredients without having access to the pure components, allowing us to study the components without modifying these properties through harsh conditions during purification.

7.2 Reflection on the work

The starting point of this dissertation were the recipes for fiber formation using SPC or blends of SPI and gluten in the High Temperature Shear Cell (HTSC) from Grabowska et al. [4, 5], Dekkers et al. [6]. Directly replacing SPI or SPC in these recipes with equivalent faba bean fractions did not result in fibrous structures. In order to produce fibrous structures from 1:1 blends of FPI (and also PPI) and gluten, the dry matter content (dmc) had to be increased from 30 % to 37.5 % (Chapter 2). Commercially available faba bean ingredients, such as FM and FPC, did not produce fibrous structures in combination with gluten at 37.5 % dmc. We concluded that the composition of the ingredients and the therefore resulting functionality of the ingredients was not suitable for fiber formation as such. The high starch content and the native functionality of the protein in FM and FPC were identified as possible hindrances. The literature study on the use of starch in meat analogues (Chapter 4) revealed that when starch is added as a functional ingredient to meat analogues in research or the industry, it is often in a modified form. To reach the aim of this dissertation and unveil the potential of faba beans as an ingredient for fibrous meat analogues, the understanding of the native components in faba beans, including starch, and their effect on fiber formation in the HTSC became the focus point to find ways to modify their functionality accordingly.

The traditional way of studying the effect of certain ingredients, here starch and protein, is through using model blends obtained by combining FPI and faba bean starch isolate at different ratios. In order to determine the differences between highly purified isolates and less or even unrefined ingredients, we could have applied conventional and well documented methods to measure general functional properties of plant proteins, such as solubility, emulsification, foaming and gelling. This would have resulted in a sound comparisons of properties of ingredients, but might have turned out to be less relevant to understand fiber formation in the HTSC using less refined ingredients. Our approach of using isolates as well as less refined ingredients allowed us to arrive at more relevant conclusions on the use of all types of ingredients, as well as better understand how the functionality of less refined ingredients can be altered. Furthermore, we combined well-known experimental methods (WHC, solubility, texture analysis, DSC) with less conventional experimental methods (Hydrophobicity of insoluble protein, determining relative gel swelling ratio, partial sorption isotherm) to measure, model and predict the properties of the relevant components under the relevant conditions and to gain insights in the mechanisms involved (formation of continuous gluten network, partial protein denaturation,

dilution/concentration of gluten, migration of moisture at process temperature). This approach does not only allow the industry to directly apply our findings in real industrial applications, but it also further strengthens our understanding of the ingredient interactions and the process of fiber formation. This shows that applied research can lead to general conclusions.

7.3 Faba beans as novel ingredients

Throughout this dissertation we showed that faba beans and fractions thereof can be used in gluten-containing recipes to produce fibrous structures upon processing in the HTSC. This can be achieved by modifying either the recipe (dmc), the composition of the ingredients (purification) or the components "in-situ" (thermal treatment). When combined with gluten, faba bean serves the same role as soy or pea, with comparable resulting product properties. The use of pre-gelatinized starch in less purified fractions has the potential to also deliver new properties that could help in mimicking the structure and sensory experience of meat. However, less refined faba bean fractions have a strong "plant" or "beany" flavor. Thermal pre-treatments similar to the one applied in Chapter 3 are known to reduce the beany flavor [7]. It was also noticed (though not measured or reported) that the smell and taste disappeared or lessened during the HTSC experiments with FM and FPC. Samples made with isolate had a less beany odor and taste than all other samples. It is necessary to mask the natural flavors of plant-based ingredients, but that could be done in combination with the introduction of meaty flavors in plant meat. Alternatively, differences in flavor can be solved through marketing efforts, moving away from meat-like flavors to a new category of naturally flavored plant meat.

Another point of attention is that several anti-nutritional factors (ANFs) are present in faba bean. These ANFs include, but are not limited to, the favism causing vicine and convicine [8], tannins, as well as the flatulence causing oligosaccharides raffinose and stachyose [9]. It is expected that processing faba bean in the HTSC has a similar reducing effect on the ANFs as extrusion, due to the similarity of the two processes. Interestingly, the gene responsible for the synthesis of vicine and convicine, the most important ANFs in faba bean, was recently identified, along with an insertion sequence that inactivates said gene [10]. Besides, breeding of faba bean led to varieties low in tannins [11] and other ANFs. Therefore, the issues stemming from these ANFs could be solvable through traditional or modern breeding methods including genetic modification. A recent literature review from Rahate et al. [7] finds bioprocessing and

extrusion to be the most effective treatments to combat ANFs in faba bean.

7.4 Ingredients for fiber formation: The role of gluten, pectin and zein

In Chapter 2 we concluded that gluten is responsible for fiber formation and the non-gluten protein acts as a filler that does not contribute to fiber formation. In Chapter 5 we showed that this also applies to native and pre-gelatinized starch. In both chapters, we confirmed that gluten has to form a continuous network to form a fibrous product. It is likely that the proteins glutenin and gliadin in gluten are mainly responsible for the formation of fibrous structures: one provides elasticity while the other provides viscosity [12]. It has previously been speculated that they could form a two-phase system that causes the formation of fibrous structure upon shearing [13], based to the phase separation of glutenin and gliadin in severely diluted samples [14]. Such a phase separation seems less likely in samples containing 30-50 % dry matter such as meat analogues. Studies on extrusion of pea protein isolate speculate that phase separation can occur, but not due to incompatibility of different proteins, but by spinodal phase separation [15, 16]. This separation leads to a protein-rich and a water-rich phase form upon cooling in the extruder die, which is a first step towards the formation of a fibrous structure. Similar findings were reported for extruded SPI by means of an interesting method [17]: SPI extrudates were freeze-dried to remove the water-rich phase, while maintaining the structure of the sample. An X-ray measurement reveals the (now evaporated) water-rich phase as cavities. A combination of this approach with X-ray images of fresh extrudates or HTSC samples to expose potential air-bubbles in the samples [18–20] would determine if a similar mechanism causes the formation of fibrous structures in gluten samples.

Previous experiments on fibrous structures from gluten in the HTSC included a hydration period for the non-gluten ingredients prior to the addition of gluten. This hydration period was omitted for all samples prepared in this dissertation, due to the low viscous paste that was created by mixing water and FM, FPC or starch. The hydration period was deemed unnecessary, as the materials were sufficiently and homogeneously hydrated. The concept of hydrating the samples could however be interesting when it is applied after the addition of gluten. Hydration of the gluten-containing dough could modify its viscoelastic properties, *e.g.* by increasing the number of disulfide bonds and therefore the crosslink density of the gluten phase ("gluten development"). A key question is then whether the number

of disulfide bonds formed prior to the thermomechanical treatment will influence the viscoelastic properties of the sample during the thermomechanical treatment. However, modification of the mixing procedure and including hydration periods of different lengths prior to thermomechanical treatment in the HTSC could give insights in the importance of disulfide bonds (and therefore the structure formed by gluten at room temperature) for fiber formation.

There are other plant-based ingredients that were used to successfully produce fibrous structures in the HTSC: Soy- and Rapeseed Protein Concentrate (SPC, RPC) [5, 21] as well as blends of SPI and pectin [22]. The deformation of dispersed pectin domains is thought to cause the fibrous structure in these recipes. Other plant protein concentrates, such as FPC, do not contain substantial amounts of pectin, which might be an explanation that those concentrates do not form fibrous structures in the HTSC without the addition of gluten. The polysaccharides in faba bean are mainly starch and dietary fibers. There is a difference in the appearance of fibrous structures with gluten and with pectin: Fibers in gluten-containing samples are thin, long and numerous (this dissertation), while fibers in pectin-containing samples appear short and thick (images from Jia et al. [21], Dekkers et al. [22]). Furthermore, pectin-containing samples are less deformable than gluten containing samples, suggesting that their fibers are less elastic. It is important to note that the pectin content in the mentioned recipes is low (2.2 %, [22]). The fact that gluten is able to form certain fibrous structures without the addition of other components apart from water and without shearing and heating suggests that the mechanism for fiber formation in gluten containing samples is different from that in pectin containing samples. However, as mentioned in the review paper in Chapter 3 and the materials section of Chapter 4, commercial wheat gluten contains a substantial amount of starch ($\sim$12 %). The endogenous starch in gluten could have a similar role as the pectin had in SPI/pectin mixtures, and therefore be the cause of the fibrous structure in gluten extrudates or HTSC samples. This hypothesis could be tested by removing starch from gluten by multiple washing steps, potentially combined with the addition of an amylase to break down starch, and processing the resulting mass in the HTSC at appropriate conditions.

Zein is another component that has potential to form fibrous structures in the HTSC without gluten, which we therefore briefly explored. It is one of the major proteins in corn, which is extracted from Corn Gluten Meal (CGM). Zein is thus a byproduct of corn starch production [23]. Not only the name is similar to wheat gluten, but also the properties are thought to be similar, at least partially [24]. This explains why

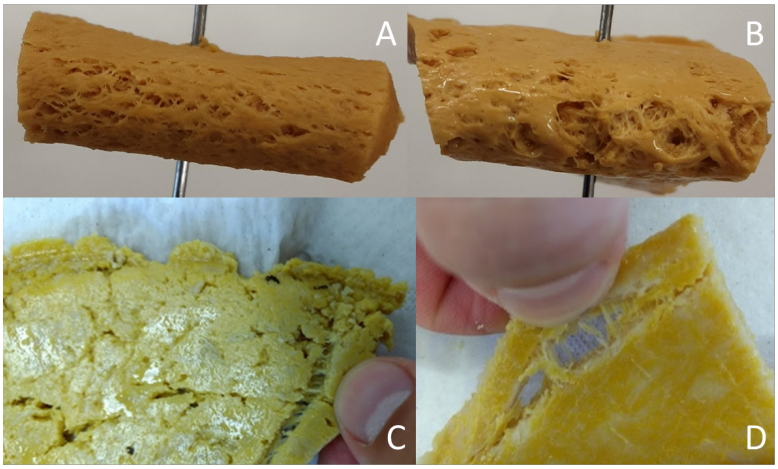

Figure 7.1: Blends of (A) zein and starch in aqueous ethanol (10 w.t. %), (B) zein and pectin in water, (C) zein and SPI with a ratio of 3:2 and (D) 2:3, processed in the HTSC.

zein is used in gluten-free bread doughs [25, 26]. The same viscoelastic properties that make it suitable for this application also bear the potential for it to be used as a successful ingredient in fibrous meat analogues and possibly an alternative for gluten [27]. Mattice and Marangoni [28] demonstrated that it is possible to produce fibrous materials from zein and soy using different techniques. They produced zein gels with different structures by electrospinning, anti-solvent precipitation or mechanical elongation, followed by incorporation in a soy gel by hand. The texture of samples containing mechanically elongated zein showed statistical to chicken meat. This suggests that zein is able to form fibrous structures upon deformation. We explored the potential of zein forming fibrous structures in the HTSC in various combinations, a selection of which can be seen in Figure 7.1.

Zein formed short and thin fibers when mixed with starch and aqueous ethanol solution and processed in the HTSC at 140 °C. Zein is not soluble, nor does it swell in water, but it can do so in aqueous ethanol, which is why this solvent mixture was used to aid with gelation. However, the use of ethanol of course renders the resulting product less suitable for consumption. Nevertheless, the outcomes of those experiments were interesting. More pronounced, but irregular fibers were observed in a sample containing zein, pectin and water. Texture analysis revealed that samples with pectin and zein in water had similar textural properties as samples with gluten

in water, apart from having a higher Young's modulus. Samples with aqueous ethanol solution had much higher tensile stress and strain than those containing gluten. Furthermore, a distinct anisotropy of textural properties was observed, the stress and strain being 2-4 times higher when measured in parallel to the shear flow direction compared to the perpendicular direction. Samples with blends of zein and SPI produced inhomogeneous products, clearly showing large individual SPI and zein domains. Nonetheless, small fibers were observed in these samples as well. In combination with the right protein or polysaccharide, zein could be a suitable replacement for gluten regarding the production of fibrous meat analogues using the HTSC. Mejia et al. [24] and Fevzioglu et al. [29] suggest that addition of a protein capable of increasing the stability and relaxation time of zein doughs could result in a more suitable gluten replacement. Non-protein components such as starch or pectin could fulfill that role.

We demonstrated that zein has the ability to produce fibrous structures in the HTSC. However, it has to be combined with other ingredients to yield structures suitable for food products. A higher water uptake of zein would also improve its structuring potential. Lastly, the fractionation process of zein is rather harsh, so a milder fractionation process could be considered. A larger starch fraction could also increase water uptake.

7.5 Towards less refined ingredients: opportunities and challenges

The application of less refined ingredients in fibrous meat analogues is not only a challenge for researchers, but also for ingredient manufacturers and food producers. Commercial food ingredients are often isolated and modified to keep their properties within a specific range and to ensure constant quality. Many commercial food ingredients are designed and advertised to have a very specific functionality, often for general applications. Food producers can then select and combine a multitude of isolated or concentrated ingredients based on the functionality they desire for their final food product. This way of working relies on the ingredient manufacturer to deliver the advertised properties in a constant manner. The ingredient manufacturer in turn entrusts the decision process and the assembly of products to the food producer. For either side, offering or requesting native blends with unexpected and synergistic interactions throughout the production process seems like a big risk.

Both food producers and ingredient manufacturers have an uncharted wealth of experience when it comes to ingredient or component interactions in food products. This knowledge can be used to take a step back and asses production processes with the goal of re-designing the ingredients used in meat analogue manufacturing. In this dissertation, we attempted to do so for fibrous meat analogues produced in the HTSC. The list of plant-based ingredients that are suitable for fiber formation comprises mainly isolates. We discovered that gluten is mainly responsible for the formation of fibrous structures, other non-gluten proteins act as fillers. These fillers need to fulfill a number of requirements, being that they

- take up a certain amount of water to allow gluten to form a continuous network, allowing a higher overall water content in the final product to maintain commercial viability;
- have a high enough modulus to transfer shear forces to gluten to allow it to be deformed in the HTSC;
- form a gel hard enough to maintain sample integrity after cooling;
- form a gel soft and deformable enough to allow gluten fibers to stretch and be revealed upon bending.

In Chapter 2 we concluded that gluten should form a continuous network to form a fibrous product. This does not automatically result in a dispersed non-gluten ingredient, as blends of PPI and gluten formed fibrous structures after processing in the HTSC and exhibit signs of a bi-continuous network [30]. Nevertheless, it seems that the addition of higher amounts of any ingredient (protein, starch, blends or native blends) causes a loss of fibrous structure. Possible reasons are that the large volume fraction of the non-gluten ingredient prevents gluten from forming a continuous structure or that the (potentially bi-continuous) non-gluten ingredient has a low modulus at process temperature, acting as a lubricant that prevents gluten from deforming upon shearing in the shear cell. Furthermore, properties of the non-gluten ingredient after processing, especially cooling, are also of importance. If an ingredient does not form a (strong enough) gel, it will compromise the integrity of the product, causing it to fall apart (*e.g.* Amioca Starch, Chapter 5). If the gel formed is too hard and brittle, the fibrous structure of the gluten will not be revealed upon bending (*e.g.* Hylon VII Starch, Chapter 5).

In addition to ingredient requirements, there are ingredient properties that would be desirable when producing meat analogues., which are

- no strong vegetable or plant flavor;
- no anti-nutritional factors;
- additional nutritional or health benefits;
- broad availability;
- low cost;
- low level of processing;
- no material waste streams;
- low energy consumption during production.

The isolates used in former HTSC recipes tick all the boxes for requirements and some of the desired properties (water uptake, flavor, anti-nutritional factors, availability). However, they are likely to be outperformed by un- or less refined ingredients in terms of additional nutritional or health benefits (due to removal of dietary fibers and secondary plant metabolites) [31], have higher production cost, a higher level of processing by default, a lower yield and higher energy consumption during production [32–34]. None of these can be improved significantly by continuing to apply isolates. In contrast, less refined ingredients have lower energy consumption during processing, higher yield, lower production cost and a lower level of processing by definition.

In conclusion, the use of isolates or unrefined ingredients both has specific advantages and drawbacks. It should be noted that isolates have been studied more widely than unrefined ingredients. It can therefore be expected that additional research has strong potential to overcome the process-related issues of unrefined ingredients. The uncharted wealth of experience of ingredient manufactures and food producers could and should streamline this development. While the use of less refined ingredients for meat analogues is favorable in terms of sustainability, and many issues regarding the process can be overcome, the impact on nutritional quality and taste could be a bigger hurdle. As of right now, less refined faba beans are not suitable as a main ingredient for meat analogues due to the ANFs and the natural flavor. Other starch bearing legumes, *e.g.* pea, are to be favored due to their lower ANF content and more pleasant natural flavor. If the nutritional challenges are overcome, less or unrefined faba bean becomes a suitable ingredient for meat analogues. Faba bean Protein

Isolate (FPI) is already a suitable alternative for PPI and SPI as an ingredient in meat analogues.

7.6 New insight in protein-starch blends

7.6.1 The importance of water distribution

The water content influences the thermal transition a component undergoes during heating: The denaturation temperature of protein decreases with increasing water content, the degree of initial gelatinization of starch increases with water content and the temperature as well as the amount of starch "melting" decreases with water content (Figure 7.2). At sufficient water content, protein denaturation temperature will not decrease further and degree of initial starch gelatinization will be at 100 %, while starch melting will entirely disappear. When the water content becomes very low, protein denaturation temperature will reach a plateau value, starch melting temperature will increase until the maximum is reached at a water content of 0, while starch gelatinization will not occur at low water content. Within the range of the water contents used this dissertation, the temperature of the initial starch gelatinization will not change with the water content (Chapter 6).

The fact that the temperature at which the initial gelatinization of starch occurs does not change with the water content was also found in an experiment performed on the same doughs that were used in Chapter 6 with a hot-stage microscope with polarized light. This microscope was used to determine the temperature, where the loss of birefringence occurred, which marks the loss of the crystalline structure of the starch granules (Figure 7.3). The temperature was increased at a rate of 10 °C min^{-1} and images were recorded with a frame rate of 3 fps. The number of granules in each image was counted using an image analysis script in R. As granules started to paste, they lost birefringence and became invisible under the microscope with polarized light. In Figure 7.4 A the percentage of pasted granules is shown over the temperature. The temperature at which the percentage of pasted granules reached 40 % was considered the pasting temperature, which is shown over the starch content (d.b.) of the samples in Figure 7.4 B. The water content of the starch phase increases with the starch content, as was shown in Chapter 6. Therefore, we conclude that the pasting temperature of the starch did not alter with a change of starch content and water content in the starch phase. The deviation in the data (Figure 7.4) correlates with the time of day of the experiment: The experiments performed in the afternoon resulted in a higher pasting temperature than those performed in the morning. This

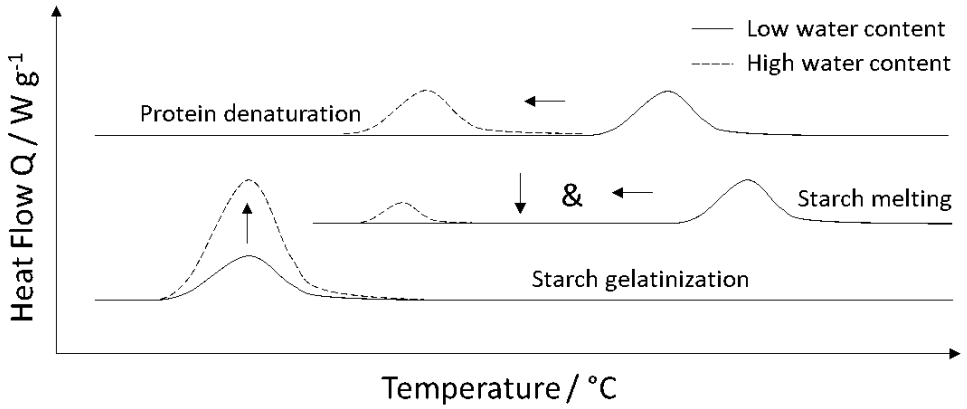

Figure 7.2: Representative DSC graphs of starch gelatinization, starch melting and protein denaturation. Horizontal lines represent the possible baseline of a DSC measurement, solid-line peaks represent reactions at low water contents and dashed-line peaks represent reactions at elevated water contents. Arrows indicate the difference in heat flow or peak temperature due to the increase of water content of the corresponding reaction.

suggests that the method needs to be developed further.

When multiple components are present in an ingredient, or they are combined as part of a blend, water will distribute among them until a thermo-dynamic equilibrium is reached. This equilibrium depends on the components affinity for water. Native starch generally takes up less water than protein at room temperature. Components therefore influence each other's water content, which in turn influences their thermal transitions and rheological properties. However, the thermal transitions themselves change the structural conformation of a component, which changes its affinity for water. Starch takes up water during the gelatinization, and denaturation of protein also changes its affinity for water. These effects influence the distribution of water throughout the process. Thermal transitions and mass transfer through diffusion do not happen instantly but are time dependent. This means that the kinetics of these transitions have to be considered as well when understanding the distribution of water in multi-component blends during thermal processing.

Before samples are placed in the HTSC, water, salt, starch and protein are mixed

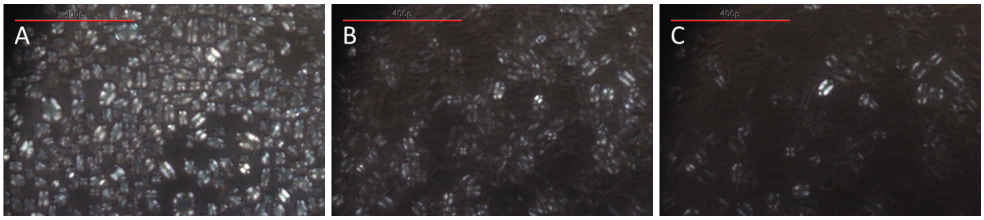

Figure 7.3: Images of air-classified, starch-rich FM at 44 °C (A), 68 °C (B) and 71 °C (C). Series of images was analyzed, and the temperature at which a defined percentage of starch granules lost birefringence was recorded. No differences in this temperature were observed for all water, starch and protein contents.

together in that order. Water will distribute among these components at room temperature. The ingredients are then placed into the pre-heated shear cell, which will heat the blend quickly to the final process temperature. A fast heating rate might not allow starch to swell and absorb water before protein denaturation occurs (Figure 7.5), because there is not enough time to reach an equilibrium at the temperature of the initial starch gelatinization. Therefore, the distribution of water at room temperature becomes more relevant for structuring processes in the HTSC. The upscaling of the technology to industrial applications will likely slow down the heating rate of the blend, thereby increasing the importance of understanding the changes in water distribution throughout the process.

The order of mixing is also of importance, especially when gluten is involved. The ability to form a network at room temperature immediately when mixed with water makes it necessary for gluten to be added last. Otherwise, even distribution of water within the non-gluten phase, be it starch or protein, is hard to achieve, which leads to dry lumps of non-gluten ingredients embedded in the gluten network. When the mixture is allowed to reach an equilibrium, it could counter this effect, though the resulting dough would be less homogeneous than if gluten is added last. Additionally, allowing the mixture to sit will also lead to "gluten development", possibly leading to different properties of the gluten phase. Previous studies on the HTSC mention a hydration period for the non-gluten ingredients in water, while stressing that gluten was added just before the sample was placed into the HTSC [4, 6, 20].

It is possible to control the water content of components in native ingredients and multi-component blends. If we consider the addition of starch to gluten from Chapter 5, we know that we do not obtain a fibrous structure at 50 % added MS. If we

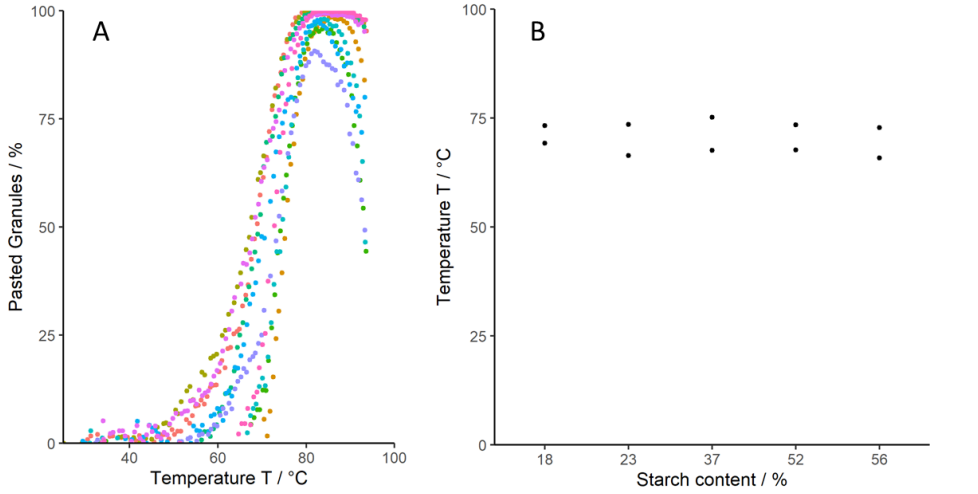

Figure 7.4: (A) Percentage of pasted granules over the temperature of the hot stage microscope, counted by image analysis in R. Colors indicate separate samples, with randomly assigned colors. (B) Pasting temperature over the starch content (d.b.) of the samples.

consider the impact of a starch content of 50 % on the water content of the protein phase in Chapter 6, we know that it increases the water content of the protein phase to 70-65 %. Pure gluten did not form fibrous structures in the HTSC at these water contents (unpublished results). Grabowska et al. [4] did observe fibrous structures in gluten samples with this water content, however, they processed at lower temperatures and used a different batch of gluten with a higher protein content (83 % d.b.). A decrease of the overall water content of the starch/gluten blend to 50-55 % would lower the water content of the gluten phase to 60 %, which should allow fiber formation. Alternatively, the starch could be replaced by a starch with a higher water uptake at room temperature *e.g.* "cold water swelling starches", modified by thermal pre-treatment [35]. However, a higher water uptake increases the volume fraction of the starch that limits the ability of the gluten to form a continuous network which prevents formation of a fibrous structure.

There is a general challenge in investigating these properties in multi-component blends. The isolates that are typically combined to create a multi-component

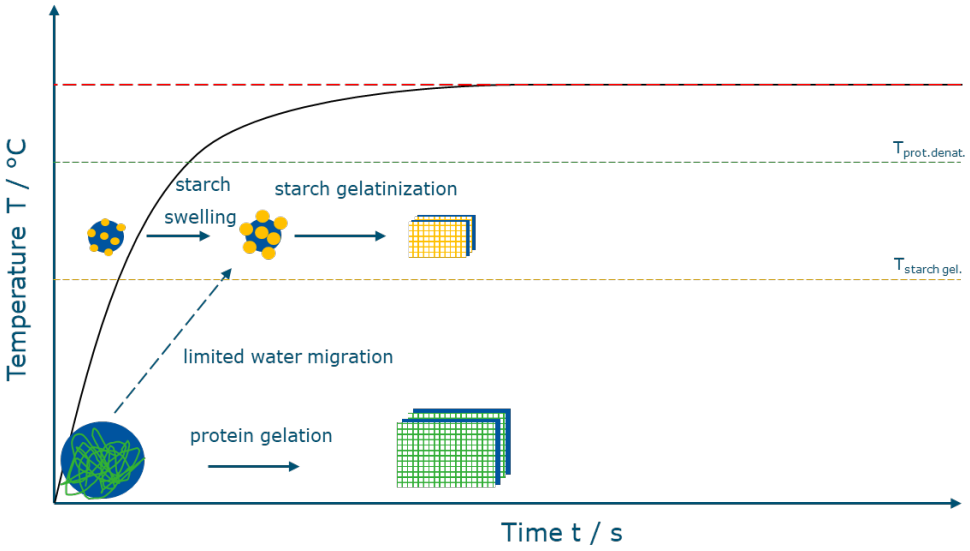

Figure 7.5: Approximation of the temperature profile within the HTSC with corresponding reactions of starch and protein. The fast heating rate within the (pre-heated) HTSC and the presence of protein most likely does not allow starch to swell and gelatinized as it normally would, resulting in a starch phase with lower water content and volume fraction.

blend undergo a harsh treatment which can lead to modification of their structural conformation and therefore their affinity for water [36]. This could lead to results that are not the same for a material with the same composition of native components. This demonstrates that it is necessary to study the distribution of water in multi-component blends with native components. Dry fractionation can be applied to starch bearing crops to create fractions that are rich in either protein or starch without changing their structural conformation. These fractions can be used to create natural blends with varying starch and protein contents.

7.6.2 Thermal pre-treatments to improve functionality of ingredients

The interaction of the components with water largely determines the product properties and the process in general. Next to choosing specific ingredients to achieve a certain composition and water distribution, we can apply pre-treatments

to the ingredients to modify the protein or starch within. A dry heat treatment of protein leads an increase in WHC due to partial denaturation. Pre-gelatinization of starch through wet heat treatment leads to an increased water uptake at room temperature. Both modifications are achieved through a thermal pre-treatment, though the water content differs. A wet pre-treatment of protein at high temperatures causes an irreversible gelation and aggregation of protein, therefore requiring a drying and milling step to achieve proper mixing prior to product assembly. Such a wet pre-treatment was investigated by Peng et al. [37], who found that it improved the functionality of soy protein for the use in meat analogues. A dry heat treatment of starch does not lead to pre-gelatinization but, if temperatures are high enough, to melting or even degradation of starch. If done correctly, these treatments can be used to improve the functionality of single or multi-component ingredients (Maize Starch - MS, Faba bean Protein Concentrate - FPC). Pre-gelatinization allowed the filler material (starch) to take up more water at room temperature, which changed its rheological properties. This resulted in an anisotropy of the Young's modulus that had not been reported for plant-based HTSC samples before and is close to that of chicken meat. This demonstrates that pre-treatment of isolated or concentrated ingredients can help to produce more viable plant-based meat analogues.

The increased anisotropy of the Young's modulus of HTSC samples containing gluten and pre-gelatinized MS (Chapter 5) is worth another look. The graphs of the texture analysis show two regimes over the addition of native starch: An initial decrease followed by an increase, which can be interpreted as two separate regimes. This allows a new interpretation of the results: In the first regime, the bulk properties are dominated by the properties of gluten. They show a decrease, because the addition of native starch increases the water content of the gluten phase, lowering the density of the gluten phase. As starch content increases further, the properties of the starch start to become more dominant, moving the bulk properties closer to those of pure starch. In the first regime of the graphs of added PMS, something extraordinary occurs: starch does not seem to have an influence in parallel direction. The stress, strain and Young's modulus in parallel direction do not decrease, but remain the same or even increase compared to the pure gluten sample. This is likely due to the increased water uptake of the PMS at room temperature, keeping the water content of the gluten phase the same or even decreasing it as PMS content increases. Therefore, the tensile properties of the gluten phase remain the same or become even stronger. In the second regime, where the properties of the starch become more relevant, the strain, stress and Young's modulus in parallel direction exhibit a drastic drop, almost matching the values at the same amount of added native MS. In perpendicular direction, the

graphs of all three properties of PMS resemble that of MS more closely over both regimes. The reason why the effect is different in parallel and perpendicular direction is not known, though it could be that the measurements in perpendicular direction also depend on the adhesion between the fibers and therefore on the adhesion between the starch and gluten phase, which might not be affected by the pre-gelatinization of the starch. As a result, the Young's modulus of the samples increases in parallel direction while it slowly decreases in perpendicular direction over the addition of PMS, increasing the anisotropy of the Young's modulus to a value higher than for pure gluten. In other words, addition of PMS weakens the adhesion of fibers (like MS), while maintaining the water content and therefore the textural properties of the gluten phase (unlike MS). This underlines that understanding the effect of water distribution among phases and controlling it is vital to understanding and controlling fiber formation.

Thermal pre-treatment becomes a far more powerful tool when applied to unrefined ingredients. Unrefined ingredients might include native components not suitable for the intended application, making it difficult to use unrefined ingredients in large quantities. The ability to modify these native components "in-situ" opens up new opportunities. For example, FM was able to form fibrous structures in combination with gluten after a thermal pre-treatment. When FM is heated in water, the starch within is gelatinized, which allows it to take up a large amount of water. This has the same effect as lowering the overall water content in the recipe to decrease the water content of the gluten phase, with the added economic benefit of using less dry matter. Low overall water contents resulted in dry products, which is not sensorially appealing. Pre-gelatinization could therefore also improve the sensorial properties of the product, by incorporating more water. To make further savings in terms of energy and water use, such a pre-treatment should be done immediately preceding product assembly in the HTSC without drying or storing in between (Figure 7.6).

The reason that thermal pre-treatments can affect a thermo-mechanic process such as the HTSC lies with the kinetics of the thermally induced transitions and the consequential mass transfers. The pre-treatment allows the target component to undergo the desired transition under controlled conditions, provided that no other transitions occur at lower temperatures. The right water content and time-temperature profile of the pre-treatment can ensure that starch fully gelatinizes (Figure 7.6) or protein only partially denatures but does not gel, allowing proper mixing at a later stage. The same transitions eventually also occur in the HTSC, but the fast heating rate as well as the presence of other components within the blend

processed in the HTSC do not allow for a selective starch gelatinization or protein denaturation and aggregation (Figure 7.5). A de-coupled starch (pre-)gelatinization and product assembly in the HTSC allows the transitions to occur independently of one another. After product formation and therefore the eventual formation of a fibrous structure, another heat treatment should not interfere with the structure of the product, since protein gelation is irreversible. Gelatinized starch, though, undergoes significant structural changes upon cooling and storage, which could influence the eating experience of the end consumer depending on the method of preparation.

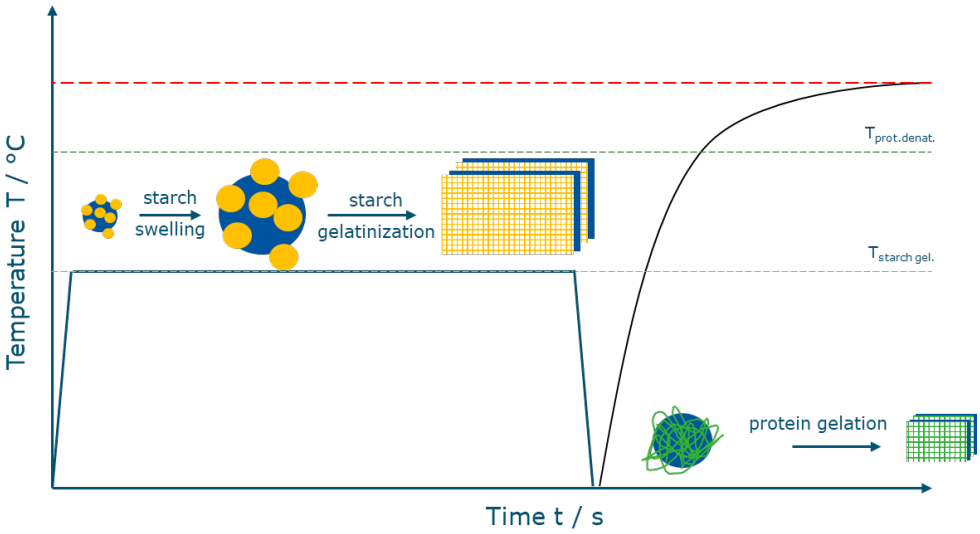

Figure 7.6: Approximation of the temperature profile of a pre-treatment and consecutive processing in the HTSC with corresponding reactions of starch and protein. De-coupled starch gelatinization and product assembly in the HTSC allows the transitions to occur independent of one another, changing the distribution of water in the dough that is placed in the HTSC. The increased water uptake of the starch after pre-treatment and consequential gelatinization leaves less water for the protein to absorb. This could allow a higher water content to be used in the HTSC, along with a higher starch content.

7.7 View on the research field

To accommodate the preferences and expectations of meat analogue consumers, food manufacturers need to be able to modify the fibrous structure of their products at their will. This means controlling visual properties such as the number, length and thickness of fibers; textural properties such as radial and axial strength and elasticity of fibers; and their effect on overall sensory product properties such as bite, mouthfeel and breakdown. The current quest of researchers and manufacturers for a more diverse and sustainable ingredient portfolio is commendable, and in this dissertation, we showed that ingredients from faba bean could be transformed into fibrous products when mixed with gluten. However, gluten is still essential for the creation of fibrous structures in these products, so it is most important to determine how gluten forms those structures in the HTSC. New ingredients suitable for fiber formation are currently found through trial and error, based on educated guesses. The understanding of the mechanism of fiber formation in more depth would dramatically improve the quality of those guesses. Attempts have been made to describe and understand the mechanism of fiber formation, some of which have been mentioned already. Generally speaking, they are all based on the deformation of two or more separate phases. In case of gluten, this two-phase system could consist of a) gliadin and glutenin, b) a gluten- and a water-rich phase or c) a gluten- and a starch-rich phase. A next step could be the use of model systems of the mentioned components to determine which is true. The results can then be verified in more sophisticated recipes.

Regardless of how a two- or multi-phase blend comes to be, it is important to understand the circumstances under which such a blend can form a fibrous structure upon shearing. Numerous studies have investigated process temperature as well as water content, but so far results were rarely transferable to ingredients other than those investigated in these studies. In her dissertation “Structuring pea towards meat analogues”, Schreuders offers a hypothesis on the formation of fibrous structures based on the compatibility of the viscosity and elasticity of the gluten phase and a pea or soy phases, which orients on the Grace curve [38] which describes droplet elongation or break-up of droplets. It is necessary to study the rheological behavior of ingredients at the relevant conditions to build on this hypothesis. Therein it is important to take the correct distribution of water among the phases into account, especially since this depends on the ingredients and their processing history, as well as the process itself (Chapter 5 & Chapter 6).

Once we know which components in gluten doughs form the two phases in the blend and which conditions allow them to form fibrous structures, we can set a window of operation in terms of recipe composition and process parameters, which will act as a base for future experiments. Other ingredients can be added to modify sensory or textural properties, increase the nutritional value or improve the overall sustainability of the product. These added ingredients will modify the window of operation of fiber formation, which can be predicted once the mechanism of fiber formation is understood. New ingredients that can induce the formation of a fibrous structure (such as pectin and zein), and therefore replace gluten, can be explored if we know what we are looking for.

7.8 Closing remarks

From a technological point of view, faba bean products (FM, FPC and FPI) are, or can be modified to become suitable ingredients for fibrous meat analogues produced in the HTSC. Future research needs to find ways to make them suitable in terms of nutritional quality and taste. Next to protein, starch is a component in faba beans that can be used as an ingredient in fibrous meat analogues. It provides textural properties that were previously unattainable with protein blends, such as an increased anisotropy of the Young's modulus. The rich composition of faba beans can be fully utilized by applying them in less refined form. Less refined ingredients are promoted for their sustainability, but hardly tested for use in meat analogues. We demonstrated that functionality of ingredients derived from mild or no fractionation can be tuned towards meat analogue applications via specific modifications of target components in-situ, even without prior purification. It shows the importance of studying faba beans and other raw materials for meat analogues in a more holistic way, with science and industry looking at the whole material instead of individual components only.

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Summary

The reduction of meat consumption is considered an effective way to increase the sustainability of one's diet. Meat analogues can help consumers to eat less meat and more plant-based foods. It is often suggested that meat analogues should mimic the texture and taste of real meat as much as possible in order to be an adequate replacement. The texture, more specifically the fibrous structure of real meat, can be re-created from plant-based ingredients in thermo-mechanical processes such as High Moisture Extrusion (HME) or in the High Temperature Shear Cell (HTSC). Currently, mainly proteins from soy, wheat and to a lesser extent pea are used in meat analogues, which are obtained after intense fractionation processes. However, it is now recognized that new and less processed ingredients have to be incorporated to prevent monocultures and to increase the sustainability of meat analogues. In this dissertation, we aim to explore the potential of faba beans as a new ingredient for fibrous meat analogues and unveil the effect of using less refined ingredients. A consequence of the latter is the introduction of more starch to the final product.

In Chapter 2, we described the suitability of Faba bean Protein Isolate (FPI) as an ingredient in gluten-containing fibrous meat analogues and compared it to Soy Protein Isolate (SPI) and Pea Protein Isolate (PPI). Swelling experiments on samples processed in the HTSC revealed a universal interaction of gluten and the legume proteins, where the gluten formed a continuous network and exerted a pressure on the legume phase, which prevented it from swelling. Furthermore, we found that gluten formed fibrous structures on its own, while SPI, PPI and FPI did not. We concluded that gluten was responsible for the formation of fibrous structures and the non-gluten protein acted as a filler. A gluten content of $\geq$50% (d.b.) was necessary to form fibrous structures, which confirmed that gluten needs to form a continuous network to form a fibrous structure.

To increase sustainability of the ingredients, we moved from isolates to concentrates. However, less refined raw ingredients sometimes lack the alleged functional properties for making fibrous food products. A high water holding capacity (WHC) is considered one of the functional properties relevant for fiber formation in the HTSC. In Chapter 3, we therefore studied the WHC of Faba bean Protein Concentrate (FPC), a mildly refined faba bean product. We found that the WHC of FPC was lower than that of Soy Protein Concentrate, but could be increased via a dry heat treatment. The dry heat treatment partially denatured the native protein in FPC and thereby increased the hydrophobicity of the protein. This led to aggregation, a lower protein solubility in water and finally an increased WHC of FPC.

Next to protein, faba beans contain a significant amount of starch. In Chapter 4 we reviewed the literature on the use of starch in meat and meat analogues. We discovered that purified starch is used as a functional ingredient in meat and meat analogues, often in modified form. We called this exogenous starch, because it is added to the recipe. Starch that is endogenously present in the original matrix of less refined or whole crop ingredients is rarely added deliberately, minimizing the reviewable literature findings in this regard. We proposed a new approach in which endogenous starch can be modified 'in-situ", using conventional starch modification processes as a route to obtain functional ingredients. The effect of starch on textural properties such as hardness and springiness of fibrous products has been investigated empirically, while deductive studies on the effect of starch on the mechanism of fibre formation are still lacking.

This gap in the literature was taken as motivation for the work described in Chapter 5, where we explored the effect of starch on the formation of fibrous structures in the HTSC when mixed with gluten. We found that low amylose content starch (Amioca Starch - AS) disrupted fibre formation due to its inability to form a gel, while intermediate amylose content starch (Maize Starch – MS) and high amylose content starch (Hylon VII) could be applied in the same way as protein isolates. Pre-gelatinizing MS improved its structuring potential and resulted in remarkable textural properties, most notably a large anisotropy of the Young's modulus that was previously not seen with protein ingredients.

Chapters 2, 3 & 5 revealed that the interaction of the ingredients with water played a large role in the formation of fibrous structures. In Chapter 6 we therefore quantified the water content of the protein and the starch phase of doughs at room temperature and gels at high temperatures. The samples were made from air-classified faba bean fractions, but we modelled the water content of the individual starch and protein water

phases. For the doughs, data for modelling was extracted from the partial sorption isotherms of doughs containing different amounts of starch, protein and water. The water content of the protein phase in the gels was modelled using the denaturation temperature of legumin. We found that starch takes up less water than protein at room temperature in the doughs. The often described swelling of starch before initial gelatinization did not increase the water content of the starch phase. However, upon protein gelation, the starch had absorbed more water, thereby lowering the water content in the protein phase. We concluded that the water had migrated from the protein to the starch phase after the initial gelatinization of starch but before or during the gelation of protein.

The dissertation was concluded with a general discussion of the results in Chapter 7. We reviewed the role of gluten as an agent of the formation of fibrous structures and revealed new insights regarding zein as a possible alternative. We discussed the use of faba beans as novel ingredient, as well as the opportunities and challenges that stem from using less refined ingredients. A list of ingredient requirements for fillers in fibrous structures was presented, along with a list of desirable properties. We highlighted the importance of water distribution in protein-starch blends and suggested thermal pre-treatments as a tool to improve the functionality of ingredients. To round out the dissertation, a new approach in the search for suitable new ingredients for fiber formation was proposed.

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About the author

Jan Bühler was born on June 11th 1991, in Herdecke, Germany. He grew up in Dortmund, attended the Lieberfeld Grundschule and Goethe-Gymnnasium, where he obtained the german Abitur in 2010. In 2007/2008 he spent a year in the USA, attending Sullivan High School, IL. After finishing school, he did a voluntary year of community service. In 2011, Jan started studying bioengineering at the Karlsruhe Institute of Technology (KIT), where he obtained a BSc. degree in 2015 with a thesis on the influence of process parameters on the stability of colloid-mill homogenized pulpy fruit juice. He continued his studies at the KIT and finished his MSc. degree in 2017 with a thesis on the influence of defined thermal and mechanical treatment on the molecular structure of soy proteins at high-moisture extrusion-like conditions. During his studies, he did internships at hte GmbH in Heidelberg, Germany, and at the Nestlé PTC in Singen, Germany. In 2017, Jan continued his studies as a PhD candidate in the project Plant Meat Matters at Wageningen University & Research.

List of publications

This dissertation

S.H.V. Cornet*, **J.M. Bühler***, R. Gonçalves, M.E. Bruins, R.G.M. van der Sman, A.J. van der Goot (2021). Apparent universality of leguminous proteins in swelling and fibre formation when mixed with gluten. *Food Hydrocolloids, 120,* 106788.

J.M. Bühler, B.L. Dekkers, M.E. Bruins, A.J. van der Goot (2020). Modifying Faba Bean Protein Concentrate Using Dry Heat to Increase Water Holding Capacity. *Foods, 9, (8),* 1077.

J.M. Bühler, M. Schlangen, A.C. Möller, M.E. Bruins, A.J. van der Goot (2021). Starch in plant-based meat replacers - A new approach to using endogenous starch from cereals and legumes. *Starch - Stärke*, 2100157.

J.M. Bühler, A.J. van der Goot, M.E. Bruins (2021). Fibrous Structures from Starch and Gluten. submitted.

J.M. Bühler, A.J. van der Goot, M.E. Bruins (2021). Quantifying Water Distribution between Starch and Protein in Doughs and Gels from Mildly Refined Faba Bean Fractions. submitted.

Other scientific publications

V.L. Pietsch, **J.M. Bühler**, H.P. Karbstein, M.A. Emin (2020). High moisture extrusion of soy protein concentrate: Influence of thermomechanical treatment on protein-protein interactions and rheological properties. *Journal of Food Engineering, 251,* 11-18.

*These authors share first authorship

Overview of completed training activities

Discipline specific activities	
Courses	
Microscopy & Spectroscopy in Food and Plant Science, VLAG, the Netherlands	2018
Science and Technology for Meat Analogues, WUR, the Netherlands	2018
Winterschool of Physical Chemistry, Han-sur-Less Winterschool, Belgium	2019
Food proteins, VLAG, the Netherlands	2019
School of Rheology, KU Leuven, Belgium	2019
Conferences	
Thermodynamics and Phase Transitions in Food Processing, WUR, the Netherlands	2018
Science and Technology for Meat Analogues, WUR, the Netherlands	2018
EFFoST^{+}	2019
International Symposium on Food Rheology & Structure^{+}, ETH, Switzerland	2019
15th Virtual Plant-Based Foods & Protein Ingredients Summit*§, Bridge2Food	2020
Science and Technology for Meat Analogues*§, WUR	2021
ProcessNet Fachgruppetreffentreffen Lebensmittelverfahrenstechnik*§, DECHEMA e.V.	2021
New Food Conference§, ProVeg e.V.	2021
General courses	
PhD Week, VLAG, the Netherlands	2018
PhD Workshop Carousel, WGS, the Netherlands	2019
Introduction to R, VLAG, the Netherlands	2019
Applied statistics, VLAG, the Netherlands	2019
climateKIC stage 1, EIT, the Netherlands	2019
climateKIC stage 2, EIT, the Netherlands	2019
Optional activities	
Preparation of research proposal	2017
PhD trip to Canada, FPE	2018
Weekly group meetings at Food Process Engineering, FPE	2017-2021
Journal club on Food Structuring, Food Structuring PhD students	2018-2021

*oral presentation $^{+}$poster presentation §online

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a) Vicia faba l.
b) Leaves of vicia faba l.
c) Pods of vicia faba l.
d) Open pods with beans
e) Beans of vicia faba l.
f) Flour of vicia faba l.
g) Beaker and spatula
h) High Temperature Shear Cell
i) Pancake of vicia faba l.
j) Fibrous structure of vicia faba l.
k) Plant meat of vicia faba l.