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Apparent universality of leguminous proteins in swelling and fibre 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, fababean 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 $\geq$ 0.5 wt/wt. The effect of gluten on swelling and fibre 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 fibres, while the second protein acts merely as a filler and is replaceable.

1. Introduction

A number of technologies exist to produce fibrous meat-like structures from food polymers (Chiang et al., 2019; Grabowska et al., 2014; Krintiras et al., 2015; Mattice & Marangoni, 2020; Nieuwland et al., 2014). However, effectively all industrial production relies on high-moisture extrusion 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 (Chiang et al., 2019; Pietsch et al., 2019). 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 (Peighambardoust et al., 2004; van den Einde et al., 2004, 2005). 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 (Dekkers, Nikiforidis, & van der Goot, 2016; Grabowska et al, 2014, 2016; Schreuders et al., 2019). During shear cell processing a bio-polymer mixture is subjected to continuous shear-flow (Krintiras et al., 2015). Recent investigations have yielded insights into the key process and material properties for the production of fibrous structures (Dekkers, Hamoen, et al., 2018; Schreuders et al., 2020; Wang et al., 2018). It is thought that two immiscible phases are required for fibre formation in sheared bio-polymer mixtures (Dekkers, Emin, et al., 2018). Tolstoguzov (1993) 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 (Chiang et al., 2019; Dekkers, Emin, et al., 2018; Grabowska et al., 2014; Pietsch et al., 2016), soy/pectin (Dekkers, Nikiforidis, & van der Goot, 2016), and pea/gluten (Schreuders et al., 2019). For neat calcium caseinate, it was shown that air incorporation is vital to obtain a fibrous structure (Wang et al., 2018). Also in soy-pectin mixtures, an internal air phase was found that is deformed in the shear-flow direction (Dekkers et al., 2016b; Schreuders et al., 2019). Clearly, the knowledge

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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 (Cornet et al., 2020). Gluten protein swells to a lower level than soy protein during free swelling (Bot and De Bruijne, 2003; Cornet et al., 2020; Grabowska et al., 2014). The continuous gluten network can thus limit the swelling of the soy protein in a mixed gel through a mechanical interaction (Cornet et al., 2020). The continuity of the gluten phase can, therefore, be deduced from the regime in which the swelling of soy (or another protein) is inhibited (Cornet et al., 2020). Grabowska et al. (2014) 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 (Grabowska et al., 2014). 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 fababean (FPI), pea (PPI) or soy (SPI). Soy and pea protein are often used as ingredients for meat replacers (Kyriakopoulou et al., 2019), while fababean is just starting to gain attention in the field (Multari et al., 2015). The rheological profile obtained during gelation of the different proteins is considered similar (Vogelsang-O'Dwyer et al., 2020), although the relative importance of disulfide bonds seems to differ (Bühler et al., 2020; O'Kane et al., 2004). 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. Material and methods

Vital wheat gluten (gluten; VITEN®, Roquette, Lestrem, France), fababean 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.

2.1. Preparation of single-phase gels

Single-phase gels were prepared from SPI, FPI and PPI using the protocol of Cornet et al. (2020). 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. 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 2.1.

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. (2020). 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:

$$Q_i^I = \frac{wt_w/\rho_w}{wt_{p,i}/\rho_p} \tag{1}$$

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 kgm^{-3} and 1330 kgm^{-3}, respectively. For mixed gels containing gluten, gluten was assumed to reach a constant level of swelling, absorbing 1.5 *g* water g^{-1} protein (Cornet et al., 2020). 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 gg^{-1}, which is the value for non-heated gluten (Cornet et al., 2020; Grabowska et al., 2014). The value of 1.5 gg^{-1} corresponds to that of heated gluten (Cornet et al., 2020), 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:

$$Q_i^{II} = \frac{\left(wt_w - 1.5wt_{p,glu}\right)/\rho_w}{wt_{p,i}/\rho_p} \tag{2}$$

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

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).

2.5. 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; Grabowska et al., 2016) following the protocol previously reported by Grabowska et al. (2014) 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. (2020). 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.

2.6. 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 (Pietsch et al., 2016, 2019).

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 $p<0.05$.

3. Theory

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 (Buell, 2017). In the context of water sorption and gel mechanics, the assumption of proteins behaving as polymers has proven useful in numerous prior studies (Boire et al., 2013; Cornet et al., 2020; van der Sman, 2015) 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 (Cornet et al., 2020). 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}:

$$\Pi_{ext} = \Pi_{swell} = \Pi_{mix} - \Pi_{elas} \tag{3}$$

Under ambient conditions ($\Pi_{ext} = 0$), the mixing and elastic contributions must, therefore, balance each other. By solving Equation (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.

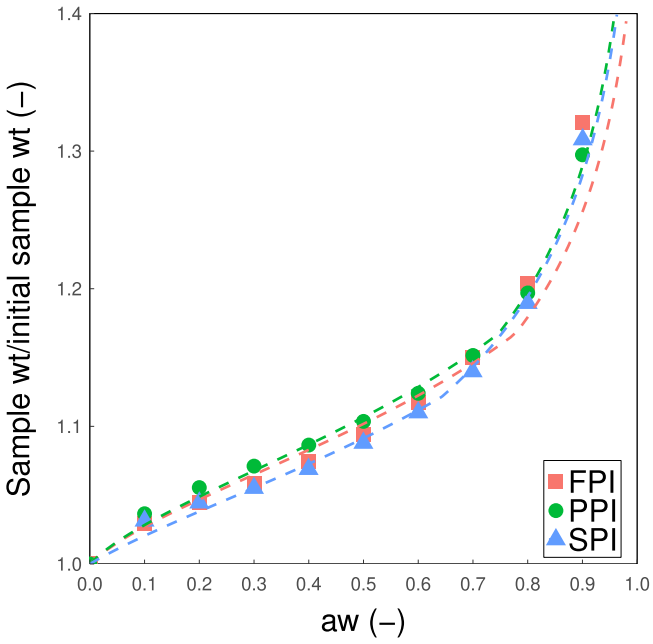

Fig. 1. Sorption isotherms for powders of soy protein isolate (SPI), pea protein isolate (PPI) and fababean protein isolate (FPI) as determined at 25 °*C*. Fitted Flory-Huggins interaction parameters for SPI, PPI and FPI were 0.91±0.02 0.90±0.2 and 0.96±0.03 respectively. SPI data was reproduced from Cornet et al. (2020).

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 (2015) showed that for several bio-polymers, ϕ_{ref} is proportional to the polymer volume fraction at maximum swelling, ϕ_0:

$$\phi_0 = \phi_{ref} \tag{4}$$

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

$$Q_0 = 1.5 Q_{ref} \tag{5}$$

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:

$$\varepsilon \propto \left[\frac{Q_{ref}}{Q}\right]^{-1/3} \tag{6}$$

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.

4. Results and discussion

4.1. Water sorption isotherms

Water sorption isotherms were determined for fababean protein isolate (FPI) and pea protein isolate (PPI) (Fig. 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 S(6)). Values for the glass transition temperature in the dry state, T_g, were taken from Derbyshire et al. (1976) 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 Cornet et al., 2020). The interaction parameter of gluten was determined previously as 1.16± 0.04 (Cornet et al., 2020). The interaction parameters were used in Section 4.3 to determine the water partitioning in protein mixtures.

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 (Fig. 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 (Cornet et al., 2020); 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 (Fig. 1), differences in cross-link density are most likely responsible for the observed differences in swelling as follows from Flory-Rehner theory (Equation (3)).

4.3. Swelling of mixed gels

4.3.1. 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 Fig. 2. Since the swelling of single-phase gels depends on the DMC at gelation (Fig. 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 S(9)). Equation S(9) 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 Fig. 3. When no mechanical interaction is assumed, all three bio-polymers show an increase in the expected level of swelling with increasing gluten content (Fig. 3).

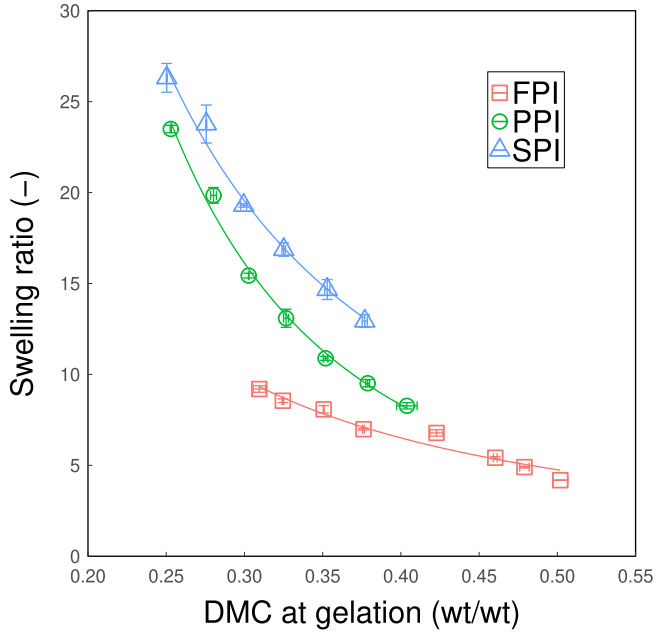

Fig. 2. The swelling ratio of single-phase FPI, PPI, and SPI gels at maximum swelling presented as function of the dry matter content at gelation. Solid lines represent fits for $y = a(x^b)$ with $a = 1.80$, $b = -1.40$ for FPI, $a = 1.01$, $b = -2.30$ for PPI and $a = 2.35$, $b = -1.76$ for SPI. Error bars represent standard error from the mean with $n = 3$. SPI data was reproduced from Cornet et al. (2020).

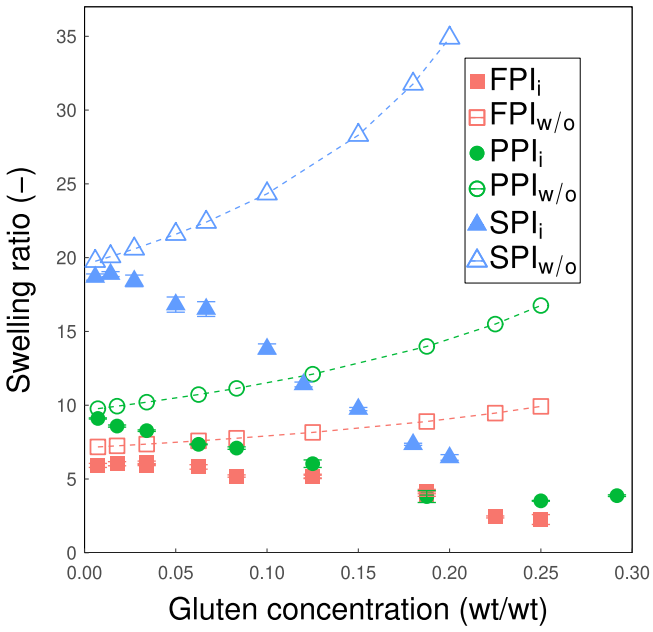

Fig. 3. The swelling ratio of fully swollen mixed gels prepared from gluten with either FPI, PPI, or SPI as a function of the gluten concentration at gelation. Gluten swelling was subtracted used Equation (2) to determine the swelling ratio of the non-gluten protein phase. Open symbols represent expected swelling ratios when no mechanical interaction between phases is assumed and is based on the swelling of the single-phase gels (Fig. 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.

4.3.2. 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 Fig. 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 (Cornet et al., 2020). 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.

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 (Fig. 4). All three proteins show a similar *relative* reduction in swelling (Fig. 4), despite the differences in absolute swelling (Fig. 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.

4.3.3. Deformation of the non-gluten phase

The similar relative reduction in swelling ratio and clear dependence

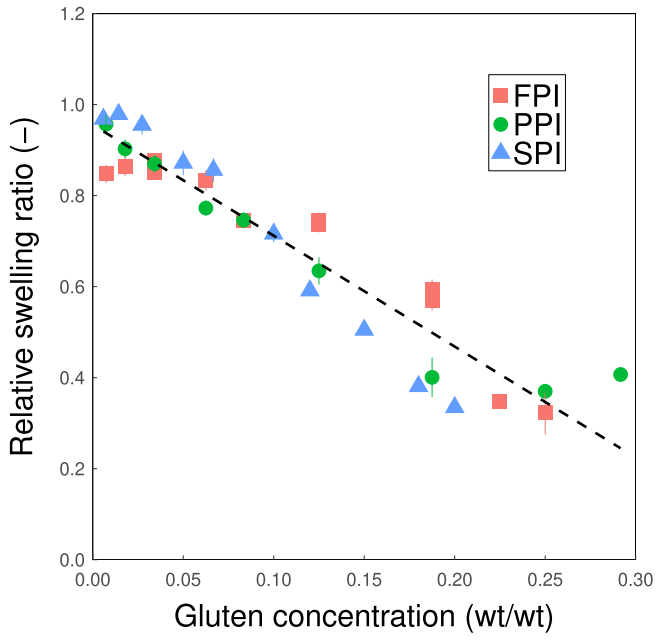

Fig. 4. Measured levels of non-gluten swelling (FPI, PPI, and SPI; Fig. 3) were divided by the swelling of their respective single-phase gel with the same DMC. The obtained relative swelling is expressed as function of the gluten fraction of total protein ($n = 3$). Dashed line is a linear fit ($y = -2.436x + 0.955$), with $R^2 = 0.90$.

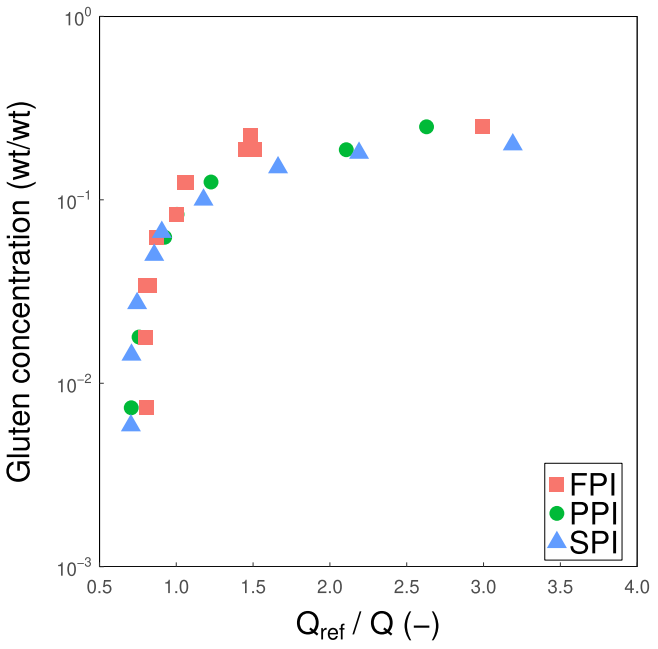

Fig. 5. Approximation of the master curve as shown by van der Sman (2015), with Q_{ref}/Q as a measure for deformation and gluten concentration as a measure for Π_{ext}. Q_{ref} follows from Equation (5) and Fig. 3.

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 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 (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 S(9)), the expected swelling ratios when no mechanical interaction is assumed are known (open symbols; Fig. 3). By using Equation (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; Fig. 3) into Equation (6).

van der Sman (2015) 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 (Cornet et al., 2020). 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 (2015) (Fig. 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 (2015). 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 Fig. 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 (Fig. 3). Due to the balance between the swelling pressure and the external pressure (or gluten pressure), these differences are limited on a relative scale (Fig. 4). This balance may only be there for materials with a similar Flory-Huggins interaction parameter, as is the case here. Fig. 5 shows that gluten interacts in a similar way with the three different 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 (Cornet et al., 2020). 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. (2020) (0.69) and van der Sman (2015) (1.5) was attributed to possible inhomogeneities in cross-link density (Cornet et al., 2020). For the sake of completeness, we have included Fig. S1 in the Supplementary information, where this alternative relation is used.

4.3.4. *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

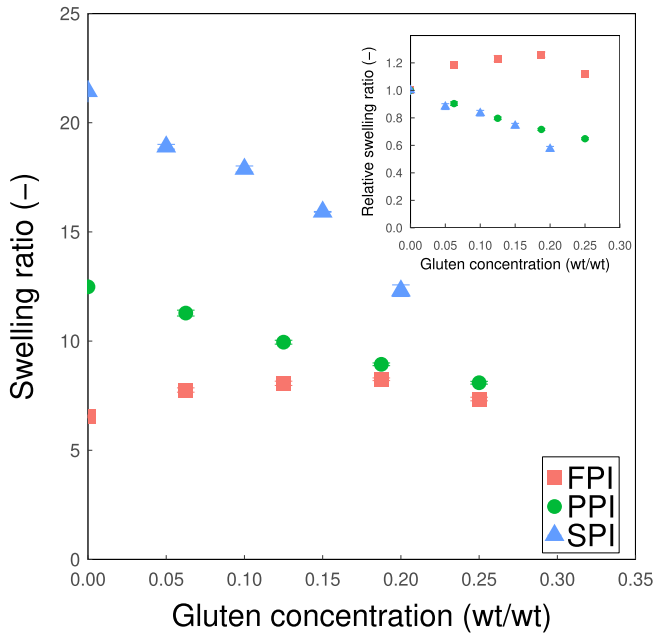

Fig. 6. The swelling ratio of fully swollen, sheared mixed gels prepared from gluten with either FPI, PPI, or SPI as a function of the gluten concentration at gelation (main figure). Gluten swelling was subtracted used Equation (2) to determine the swelling ratio of the non-gluten protein phase. For 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.

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 Fig. 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 (Fig. 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 (Fig. 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 Fig. 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.

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 High Temperature Shear Cell (HTSC). The same compositions were used as for the mixed gels (Sec. 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 fibre formation using a method similar to (Pietsch et al., 2016, 2019) (Fig. 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 fibres. At 0.33 wt/wt gluten both FPI and PPI mixtures showed structures orientated in the shear flow direction, but no fibres were observed. The SPI mixture showed no orientation at all. For all ingredients fibres appeared only when at least 0.5 wt/wt gluten was added. The individual fibres in the FPI sample containing 0.5 wt/wt gluten appear to be thicker than in the corresponding PPI and SPI samples, although the fibres become thinner for all three proteins as the gluten content approaches 1 wt/wt.

4.5. General discussion

We investigated the swelling and shear cell structuring of gluten mixed with soy, pea, or fababean protein isolate (SPI, PPI, 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 (Fig. 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 (Fig. 4). Based on our previous study on SPI–gluten mixtures, the interaction can be attributed to the presence of a continuous gluten network (Cornet et al., 2020). 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 (2015) and underlined the similarity of the interaction with gluten between the different proteins (Fig. 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

Fig. 7. Photographs of the macro-structures obtained after structuring FPI-gluten, PPI-gluten and SPI-gluten mixtures in the High Temperature Shear Cell (HTSC). Numbers in left column indicate gluten weight fraction (wt/wt), while numbers next to the samples indicate actual overall gluten concentration (wt/wt). The HTSC 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 outer 4 *cm* of the sample. Each sample has a width of approximately 5 *cm* ($n = 2$).

gluten is the main component ($\geq$ 0.5 wt/wt) fibrous structures can be made with all of the proteins used (Fig. 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 fibres at gluten fractions of 0.5 wt/wt (Dekkers, Emin, et al., 2018; Schreuders et al., 2019). In contrast, earlier studies reported the presence of fibres in sheared SPI–gluten gels at lower gluten fractions (>0.2 wt/wt) (Grabowska et al., 2014; Krintiras et al., 2015). 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. (2014) also reported the formation of fibres 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. (2019) 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, Emin, et al. (2018) 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. (2018) 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 (Fig. 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 fibre are formed is still unclear (Cornet et al., 2021). 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 (Dekkers, Boom, & van der Goot, 2018; Tolstoguzov, 1993; Tolstoguzov et al., 1974). The dispersed phase is thought to deform and align, resulting in mechanical anisotropy. Grabowska et al. (2014) already suggested that gluten forms the continuous phase in SPI-gluten based fibrous structures. SPI was, therefore, considered the dispersed phase, contributing to the fibre formation. In mixed (non-sheared) SPI-gluten gels, gluten is thought to form a continuous network that entraps SPI (Cornet et al., 2020). However, Schreuders et al. (2020) 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 (Dekkers, Nikiforidis, & van der Goot, 2016; Tolstoguzov, 1993).

Our results suggest that the non-gluten phase is not essential to fibre formation as fibres were also obtained in the absence of a second protein, as shown in Fig. 7. Hydrated gluten could be considered a two-phase system with glutenin and gliadin making up the two respective phases (Boire et al., 2013). Hence, hydrated gluten alone could already fulfill the requirement of a dispersed and continuous phase. This could explain why fibres 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 ($\geq$ 0.5 wt/wt) still resulted in the formation of fibres. When gluten was no longer the main continuous component, no fibres were formed. We, therefore, propose that in gluten-containing mixtures, gluten is primarily responsible for the formation of fibres, 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 fibres 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 (Dekkers, Nikiforidis, & van der Goot, 2016; Grabowska et al., 2014; Schreuders et al., 2019). Nevertheless, a continuous gluten network seems essential to achieve a fibrous structure.

5. Conclusion

We have studied the swelling of single-phase gels from fababean 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 fibre structures when gluten was the main protein component. Hydrated gluten also forms fibres without a second biopolymer present. This suggests that the choice of the non-gluten biopolymer 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.

Author contribution

Steven H. V. Cornet: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization, Co-first author.

Jan M. Bühler: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization, Co-first author.

Raquel Goncalves: Validation, Investigation.

Marieke E. Bruins: Supervision, Writing – Review & editing.

Ruud G. M. van der Sman: Supervision, Writing – Review & editing.

Atze Jan van der Goot: Supervision, Writing – Review & editing, Funding Acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary materials

Theory

Mixing pressure

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

$$\Pi_{mix} = \frac{RT}{\nu_w}\left[\ln(1-\varphi) + \varphi\left(1-\frac{1}{N}\right) + \chi\varphi^2 + F(\varphi)\right] \tag{S1}$$

Here, R is the universal gas constant, T is the absolute temperature, ν_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 (Leibler and Sekimoto, 1993; Vrentas and Vrentas, 1991) 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 (van der Sman and Meinders, 2011):

$$\chi = \chi_0 + (\chi_1 - \chi_0)\varphi^2 \tag{S2}$$

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 (van der Sman, 2015). Heat can induce protein unfolding, which can affect its water sorption as indicated by a change in χ_1 (van der Sman, 2012). We recently found that for SPI and gluten, the value of χ_1 is the same before and after heating (Cornet et al., 2020). 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 (2011):

$$F(\varphi) = \begin{cases} 0 & \text{if } T \geq T_g \\ -M_w y_s^2 \frac{\Delta C_{p,w}}{RT}\frac{dT_g}{dy_s}\frac{T-T_g}{T_g} & \text{if } T \leq T_g \end{cases} \tag{S3}$$

with

$$\frac{dT_g}{dy_s} = -\frac{\Delta C_{p,s}\Delta C_{p,w}\left(T_{g,w} - T_{g,s}\right)}{\left(y_w \Delta C_{p,w} + y_s \Delta C_{p,s}\right)^2} \tag{S4}$$

and T_g was calculated according to Couchman-Karasz (Couchman and Karasz, 1978):

$$T_g = \frac{y_w \Delta C_{p,w} T_{g,w} + y_s \Delta C_{p,s} T_{g,s}}{y_w \Delta C_{p,w} + y_s \Delta C_{p,s}} \tag{S5}$$

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 kJK^{-1}, which appears to be universal for bio-polymers (van der Sman, 2012; van der Sman and Meinders, 2011; Pouplin et al., 1999). 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:

$$\ln(a_w) = \frac{v_w \Pi_{mix}}{RT} \tag{S6}$$

Elastic pressure

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

$$\Pi_{elas} = -G_{ref}\left[\widetilde{\varphi}^{1/3} - \frac{\widetilde{\varphi}}{2}\right] \tag{S7}$$

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 (Quesada-Pérez et al., 2011). $\widetilde{\varphi}$ is a measure for network deformation relative to the reference state φ_{ref}:

$$\widetilde{\varphi} = \frac{\varphi}{\varphi_{ref}} \tag{S8}$$

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 (Attenburrow et al., 1990; Ng and McKinley, 2008), while this was not the case for the other proteins (soy, pea, and fababean). Hence, FR theory was used to describe the hydration of gluten (Equation S7 and S1 (Cornet et al., 2020)), while regular FH theory was used for the hydration of the other protein phase (Equation S1). We have recently used this approach to describe water partitioning between soy protein and gluten (Cornet et al., 2020) and will use the same approach here. Thus, the water partitioning in the hydrated protein mixture before gelation can be found by solving:

$$\Pi_{mix,i}(\varphi_i) = \Pi_{mix,gluten}\left(\varphi_{gluten}\right) - \Pi_{elas,gluten}\left(\varphi_{gluten}\right) \tag{S9}$$

with i as either SPI, FPI, or PPI. The elastic properties of gluten were taken as $G_{ref} = 0.17\ MPa$ and $\varphi_{ref} = 0.023$ (Cornet et al., 2020). These values were determined previously (Cornet et al., 2020) by fitting Equation S9 to the water partitioning data presented by Dekkers et al. (2016a). 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. (2020) using Equation S9 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 (~0.07 MPa; (Cornet et al., 2020)). Nonetheless, Equation S9 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:

$$\begin{aligned}\varphi_i &= \frac{wt_{p,i}/\rho_p}{wt_{p,i}/\rho_p + (P \cdot wt_w)/\rho_w} \\ \varphi_{gluten} &= \frac{wt_{p,gluten}/\rho_p}{wt_{p,gluten}/\rho_p + ((1-P)\cdot wt_w)/\rho_w}\end{aligned} \tag{S10}$$

ρ_p and ρ_w are the densities of polymer and water, and taken as 1330 kgm^{-3} and 1000 kgm^{-3}. The water partitioning coefficient P is obtained by simultaneously solving Equation S9 and 5.0.3. Equation S9 was solved using the bisection method and varying the partition coefficient P.

Supplementary data

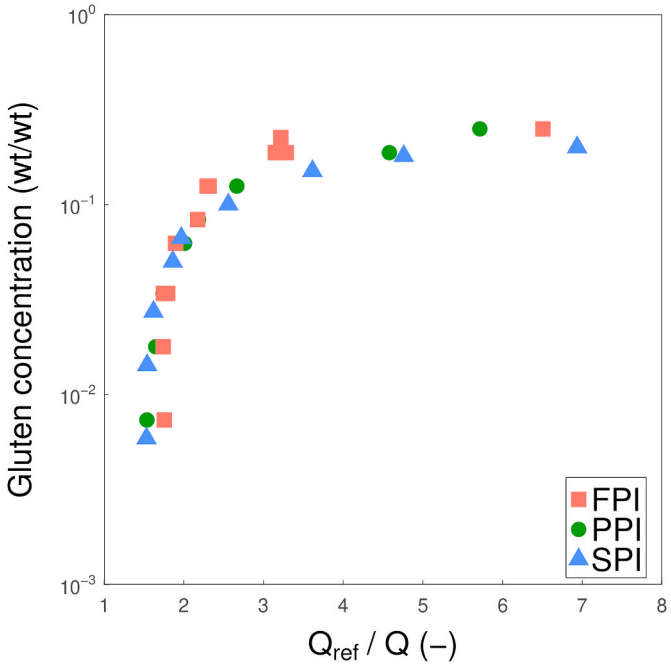

Figure S1. Approximation of the master curve as shown by van der Sman (2015), with Q_{ref}/Q as a measure for deformation and gluten concentration as a measure for Π_{ext}. Q_{ref} follows from $Q_{ref} = 0.69Q_0$(Cornet et al., 2020) and Figure 3.

(a)

(b)

Figure S2. Representative image of a fababean protein isolate – 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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