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Enzymatic hydrolysis of pea protein: Interactions and protein fractions involved in fermentation induced gels and their influence on rheological properties

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Abstract

In the light of changing nutritional trends and recommendations, yoghurt style gels from plant proteins are a promising way to incorporate relevant amounts of plant derived proteins into the diet. However, in order to attain a high level of consumer acceptance, a thorough understanding of rheological behaviour, involved protein fractions and relevant interactions is mandatory in order to later be able to customise properties of fermentation induced gels. Therefore, the aim of this study was to first characterise the type of dominating interactions within the gel network and the protein fractions involved followed by determination of the rheological properties of gels made from pea protein and pea protein hydrolysates. Results showed that the protein-protein interactions were mainly hydrophobic in nature and involved mostly the legumin fraction. A smaller contribution could be ascribed to electrostatic interactions between vicilin and the basic legumin-β chain, thus incorporating some vicilin into the gel. The interaction between vicilin and the basic legumin-β chain was influenced by modification of the molecular weight distribution via enzymatic hydrolysis. Especially hydrolysis with trypsin led to an enhanced involvement of vicilin in the gel structure due to the increased availability of legumin-β. The molecular weight distribution only had a minor impact on the rheological properties of the fermentation induced pea protein gels leading to the conclusion that in rheology the type of interactions is more important, than the protein fractions involved.

1. Introduction

The consumption of plant proteins in northern and western Europe is steadily increasing and the accompanying market is growing (European Commission, 2018). Reasons for this vary from lifestyle choices to environmental and health issues. E.g. the substitution of animal derived proteins with those from plants was shown to prevent chronic degenerative diseases (Krajcovicova-Kudlackova, Babinska, & Valachovicova, 2005) and pulses can be cultivated as catch crop, leading to environmental side benefits.

Some of the most popular vehicle foods associated with a high protein content are yoghurts and yoghurt-type products (Banovic et al., 2018). From a physicochemical point of view, yoghurt and yoghurt-type products are classified as protein gels. Gelation of proteins in general is achieved either by heat denaturation of proteins or by acidification for example via fermentation with lactic acid bacteria, as is the case in yoghurt and yoghurt-type products. Moreover, acid induced gelation requires a heating step prior to acidification in order to unfold and partially denature the protein-molecules with formation of aggregates and soluble complexes due to the exposure of more hydrophobic amino acids (e.g. (Ringgenberg, Alexander, & Corredig, 2013)). Subsequently, the gradual decrease of pH results in the neutralisation of the negatively charged amino acids, changes in the balance between electrostatic

repulsion and van der Waals interactions (Mezzenga & Fischer, 2013) and favours interaction through hydrophobic forces (Guo & Ono, 2006; Kohyama, Sano, & Doi, 1995).

Table 1 specific molecular parameters of pea globular proteins derived from literature

	parameter		reference
legumin	sedimentation coefficient	11S	
	quaternary structure	hexameric	(Croy, Derbyshire, Krishna, & Boulter, 1979; Croy, Gatehouse, Evans, & Boulter, 1980)
	molecular weight monomer	60 kDa	
	subunits	acidic α-chain (40kDa) basic β-chain (20 kDa) linked via disulphide bond	(Croy et al., 1979; Croy, Gatehouse, Evans, et al., 1980)
vicilin	sedimentation coefficient	7S	
	quaternary structure	trimeric	(Gatehouse, Croy, Morton, Tyler, & Boulter, 1981; Gatehouse, Lycett, Croy, & Boulter, 1982)
	molecular weight monomer	50 kDa	
	subunits	α+β, β+γ, α, β, γ peptides via posttranslational autolysis	(Gatehouse et al., 1981, 1982; Gatehouse, Lycett, Delauney, & Croy, 1983; Lycett, Delauney, Gatehouse, Gilroy, & Croy, 1983)
convicilin	quaternary structure	tetrameric	(Croy, Gatehouse, Tyler, & Boulter, 1980)
	molecular weight monomer	71 kDa	(Crévieu et al., 1997; Croy, Gatehouse, Tyler, et al., 1980)
	N-terminus	Highly charged	(Bown, Ellis, & Gatehouse, 1988)

It becomes obvious that gelation of protein is always based on protein – protein interactions which might be non-covalent (such as hydrogen bonds, electrostatic attractive forces and hydrophobic interactions), or covalent, like disulphide bonds. One approach to determine dominating types of interactions are gel solubility experiments. These experiments are based on the cleavage of different bonds (e.g. disulphide bonds) and the interference with different interactions (e.g. hydrophobic and electrostatic interactions) via incubation of gels in different solvents (O'Kane, Happe, Vereijken, Gruppen, & Van Boekel, 2004b; Papalamprou, Doxastakis, Biliaderis, & Kiosseoglou, 2009; Utsumi & Kinsella, 1985). Rheological measurements may be used to describe the structuring process in time-sweep experiments and to indicate product behaviour during processing and transport via frequency and amplitude sweep experiments. Especially Large Amplitude Oscillatory Shear (LAOS) measurements can indicate product behaviour during processes like mastication that are well outside the linear viscoelastic regime. Additionally, haptic sensorial properties can be derived from rheology (Akhtar, Stenzel, Murray, & Dickinson, 2005; Brückner-Gühmann, Banovic, & Drusch, 2019).

Regarding the gelation of pea protein, most publications focus on heat induced gelation of isolated pea globular fractions (e.g. (O'Kane et al., 2004b; O'Kane, Happe, Vereijken, Gruppen, & Van

Boekel, 2004a) and/or pea protein in general (e.g. (Sun & Arntfield, 2010, 2012) and most commonly only the influence of the globular fractions is discussed. Globular pea protein fractions (legumin, vicilin and convicilin) account for at least 60% of total protein (Gueguen & Barbot, 1988) and their molecular properties have been subject to extensive research in the past (table 1). The gelation mechanism for heat induced pea protein gels is mainly ascribed to hydrophobic interactions, hydrogen bonds and electrostatic interactions (e.g. (O'Kane et al., 2004b; Sun & Arntfield, 2012). The relevant types of interactions may be influenced by enzymatic hydrolysis of the protein and in turn influence rheological gel properties, as – depending on the specificity of applied enzymes – the molecular weight decreases, the amount of ionisable groups increases and previously buried hydrophobic groups become exposed (Panyam, 2002). Knowledge on customisation of rheological properties (e.g. via enzymatic hydrolysis) can in turn be very useful when tailoring haptic sensorial properties like texture or creaminess. So far the impact of hydrolysis on heat induced gels was studied with varying results: gel strength increased for soy proteins using Flavourzyme® or Alcalase® at a low degree of hydrolysis (Hrckova, Rusnakova, & Zemanovic, 2009) and oat protein gel strength was improved using Flavourzyme® or tryptic hydrolysis (Nieto-Nieto, Wang, Ozimek, & Chen, 2014). However, an impairment of gel formation for soy proteins using bromelain has also been observed (Lamsal, Jung, & Johnson, 2007). The only study investigating the effect of enzymatic hydrolysis on glucono-δ-lactone induced gelation of soy protein found that the gels have a softer texture and more syneresis with increasing degree of hydrolysis using subtilisin Carlsberg (Kuipers et al., 2005). To our knowledge, so far, the effect of hydrolysis on fermentation induced plant protein gels has not been investigated.

Moreover, while in the area of fermentation induced plant protein gels in general considerable amounts of research exist on soy protein (e.g. (Cheng, Thompson, & Brittin, 1990; Donkor, Henriksson, Vasiljevic, & Shah, 2007; Ferragut, Cruz, Trujillo, Guamis, & Capellas, 2009; Karleskind, Laye, Halpin, & Morr, 1991; Yazici, Alvarez, & Hansen, 1997), other protein sources such as oat (Brückner-Gühmann et al., 2019), lupine (Hickisch, Bindl, Vogel, & Toelstede, 2016) and pea (Klost & Drusch, 2019) have been less investigated so far but are believed to follow the general acid induced gelation process outlined above. With regard to pea proteins we previously described its ability to form fermentation induced self-supporting gels and proposed a two-step gelation process that consists of the formation of an overall percolated network structure followed by condensation of smaller aggregates (Klost & Drusch, 2019). However, there is no specific knowledge on the type and ratios of interactions participating in the formation and stabilisation of these gels or on the influence of hydrolysis on gel properties.

Therefore, the focus of this study is to investigate protein fractions and relevant interactions involved in fermentation induced gelation of pea protein. It was expected, that enzymatic hydrolysis with different enzymes would change electrostatic and hydrophobic properties of pea protein, in turn influencing rheological gel properties giving indications for future customisation of haptic sensorial properties.

2. Materials and Methods

2.1. Materials

Pea protein concentrate (LOT-Nr.: 16041801) (78% protein) was obtained from IGV (Institut für Getreideverarbeitung) GmbH, Nuthetal, Germany. Materials and chemicals used for SDS-PAGE were purchased from BioRad Laboratories GmbH (München, Germany), and all other chemicals were purchased from Carl Roth GmbH + Co.KG (Karlsruhe, Germany), Merck and Sigma Aldrich (Darmstadt, Germany). The lactic acid culture (YoFlex®) was kindly provided by Chr. Hansen, Hoersholm, Denmark and contained *S. thermophilus* and *L. bulgaricus.* The enzymes were kindly provided by Novozymes (Bagsværd, Denmark): Protamex® (Batch: PW2A1135), Alcalase® 2.4 L FG (Batch: PLN05508), trypsin (Formea RTL 1200 BG, Batch: PF130006).

2.2. Hydrolysis of pea protein

Enzymes (trypsin, Protamex® and Alcalase®) with different specificities were chosen to generate different peptide profiles. Slurries of pea protein concentrate in distilled water were prepared considering the volume of NaOH needed for the pH stat method, so that all samples had a 10% (w/w) protein concentration at the end of the hydrolysis. The pH value of slurries was adjusted to pH 8 with 0.1 M NaOH before the start of hydrolysis. Due to the buffering capacity of the proteins, the adjustment of the pH took around 30-45 min, and was performed with constant agitation while heating the sample at 50 °C. Hydrolysis was performed to a degree of hydrolysis (DH) 1 at pH 8 and 50°C using the pH-stat method with an automated titrator (902 Titrando, Metrohm AG, Herisau, Switzerland). The required amount of NaOH to reach DH 1 was calculated from:

$$B = \frac{DH \cdot \alpha \cdot mp \cdot h_{tot}}{N_b \cdot 100\%} \quad (1)$$

where B is the volume of base consumption [mL], N_b is the molarity of the base [M], α is the average degree of dissociation of the α-NH groups [-], h_{tot} is the total number of peptide bonds in the substrate [meqv/g protein], and mp is the mass of protein used [g] (Adler-Nissen, 1986). After the required volume of NaOH was added, hydrolysis was terminated by quickly transferring the sample to a water bath at 80 °C for 30 min. During this heating step, 5,3% saccharose was added to act as a supplement for the lactic acid bacteria later on. Subsequently the protein slurry was cooled down and homogenised (Panda Plus, Niro Soavi, Germany) at 800 bar and 1 pass and directly processed for fermentation without a freeze drying step. Additionally, hydrolysates without added sugar were prepared following the same protocol. Those hydrolysates were not fermented but freeze dried directly after homogenisation and stored at 4°C until further use.

2.3. SDS-PAGE of freeze-dried hydrolysates

The molecular weight distribution of the freeze-dried hydrolysates was carried out by SDS-PAGE on 12% Criterion™ TGX™ Gel with 26 wells (BioRad Laboratories GmbH, München, Germany). Running of the gels was done according to the BioRad Bulletin #4110001. 10 µL of sample (0.1% protein in sample buffer, reducing conditions (Biorad 2xLaemmli sample buffer, Cat# 161-0737) with added dithiothreitol (DTT)) were applied to the gel alongside lanes of the molecular weight marker (PageRuler™ Prestained Protein Ladder, Cat# 26616, ThermoScientific). Running buffer was Biorad 10xTris/Glycine/SDS (Cat# 161-0732). The gels were photographed, and the bands were transformed to peaks using the inverted intensity of the green channel generated via graphics → RGB profile plot plugin (open source software ImageJ 1.52d (Schneider, Rasband, & Eliceiri, 2012)). Bands were appointed to individual protein fractions by estimating their position in relation to the molecular weight marker and reference values from literature.

2.4. Fermentation

The fermentation of the samples was performed at 43 °C for 18 h using a thermophilic yoghurt culture (YoFlex®). This culture was added to the homogenised pea protein slurry and subsequently filled into disposable rheology beaker for the concentric cylinder system CC27 (Cat No 3716, Anton Paar, Graz, Austria) and 10 ml centrifuge tubes for fermentation. After the fermentation, the gels were stored for 24 h at 4 °C before performing the gel characterisation. Additionally, the fermentation process was tracked in a rheometer using a concentric cylinder system (Physica UDS 200 and MCR 502, Anton Paar, Graz, Austria, Z3DIN (measuring bob radius = 12.5 mm, measuring cup radius = 13.56 mm, gap length = 37.5 mm) and CC27 (measuring bob radius = 13.33 mm, measuring cup radius = 14.46 mm, gap length = 40 mm) respectively, f = 1 Hz, $\gamma = 0.1\%$) and via pH monitoring (Lab 865, blueline 14 electrode, SI Analytics, Xylem, USA). Storage modulus G' and loss factor tan δ were used to compare the obtained gels at the end of fermentation.

2.5. Syneresis of fermentation induced pea protein gels

Samples of 10 g were introduced in three 12 mL centrifuge tubes and fermented under the same conditions as explained above. After resting for 24 h at 4 °C the supernatant was weight without a preceding centrifugation step. Syneresis was calculated as the weight of liquid released, in relation to the total weight of sample in the tube.

2.6. Gel solubility

0.3 g of each gel without supernatant were weight and put into 2 mL Eppendorf caps. 1.5 mL of the following solutions were used as solvents: 0.5 M NaCl, 1.5% SDS, 0.1 M dithiothreitol (DTT), 20% propylene glycol (PG), distilled water. All the solutions were previously adjusted to pH 4.7 with 0.1 M HCl/NaCl. This method is based on gel solubility experiments by O'Kane,et al., 2004b; Utsumi & Kinsella, 1985, with modifications. The samples were left on an agitator with gentle agitation for 6h at room temperature. After agitation, samples were centrifuged at 10,000 x g for 15 min at room temperature. The protein content in the supernatant was quantified with the Dumas method and calculated as percent of the protein content of the undissolved gel. Additionally, the molecular weight distribution in the supernatants was analysed by SDS-PAGE as described above.

2.7. Rheological characterisation of fermentation induced pea protein gels

The frequency and amplitude sweeps of the pea protein hydrolysate gels were carried out after resting the gels for 24 h at 4 °C using the Anton Paar MCR 502 with a concentric cylinder system (CC27, as described above). For both sweeps, storage modulus G' and loss modulus G'' were measured and plotted in double logarithmic plots against frequency and amplitude respectively. Additionally, the slope of G' vs. frequency (dlogG'/dlogf) was calculated from the double logarithmic plot of frequency sweeps. Frequency sweeps were performed at 10 °C and an amplitude that was found to be within the linear viscoelastic regime, $\gamma = 0.1\%$, for frequency values from 0.01 to 10 Hz. For the amplitude sweeps with SAOS and LAOS measurements oscillations were performed at 10 °C, $\omega = 1$ rad·s^{-1} and $\gamma = 0.01\%$ to 1010%. For further evaluation of the amplitude sweeps, results were plotted in Lissajous plots (Ewoldt, Hosoi, & McKinley, 2008). Within the linear viscoelastic regime, the shape of an elastic Lissajous plot (stress τ vs deformation γ) can vary from a straight line for purely elastic materials, over an ellipse for viscoelastic materials to a circle for purely viscous materials, while the shape of the viscous Lissajous plot (τ vs. shear rate $\dot{\gamma}$) follows the opposite behaviour. Beyond the linear viscoelastic regime, the shapes of the Lissajous plots become distorted. Numerical information can be obtained from the stiffening (S-factor)- and thickening (T-factor) ratios defined by Ewoldt et al., 2008:

$$S \equiv \frac{G'_L - G'_M}{G'_L} \quad (2)$$

where G'_L and G'_M are the large strain elastic modulus (secant line) and minimum strain elastic modulus (slope of the elastic Lissajous plot at zero strain), respectively. And

$$T \equiv \frac{\eta'_L - \eta'_M}{\eta'_L} \quad (3)$$

where the η'_L and the η'_M are the large-rate dynamic viscosity (secant line) and the minimum rate dynamic viscosity (slope of the viscous Lissajous plot at zero shear rate), respectively.

2.8 Statistic evaluation

Statistic evaluation was performed by one way ANOVA followed by Tukey postHoc test ($\alpha = 0.05$) for G' and tan δ at the end of fermentation. A two way ANOVA followed by Tukey postHoc test was performed for statistical evaluation of gel solubility. Standard deviation is given in tables and represented as error bars in figures. All statistical evaluation was performed using OriginPro 9G software (OriginLab, Northhampton, USA).

3. Results and Discussion

3.1. Molecular weight distribution

Fig. 1 shows the molecular weight distributions of the unhydrolyzed pea protein and the three different hydrolysates. The unhydrolyzed protein displays the typical molecular weight profile of pea protein as can be derived from table 1. Despite showing a molecular weight just above 70 kDa we associate the band marked "con" with convicilin. This is in agreement with findings from (Adal et al., 2017). The pronounced bands at ~50 kDa and ~30 kDa correspond to the monomer of vicilin and the α+β fraction of vicilin respectively. Moreover, many of the fractions between 25 and 20 kDa and below 19 kDa may also be various smaller vicilin fragments including the β+γ fraction and individual α, β and γ fractions, as vicilin is prone to post translational proteolysis (Gatehouse et al., 1982). The third main pea globulin is legumin, which in SDS-PAGE under reducing conditions appears as two peaks at 38 and 40 kDa (acidic α-chain) and ~20 kDa (basic β-chain). Application of different enzymes let to different molecular weight patterns (fig. 1). Hydrolysis with Protamex® mainly degraded the larger vicilin fractions, and some of the convicilin as can be seen by the decrease in the height of the corresponding peaks while the legumin fractions were almost unaffected. In contrast, trypsin cleaved mainly the legumin-α chain as well as the vicilin α+β and the convicilin and generally led to a wider distribution of peptides including a larger proportion of peptides < 17 kDa as can be seen from the more elevated baseline. The pronounced degradation of legumin-α and convicilin can be ascribed to the specificity of trypsin, that mainly cleaves at legumin-α and convicilin (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010). In addition to being less

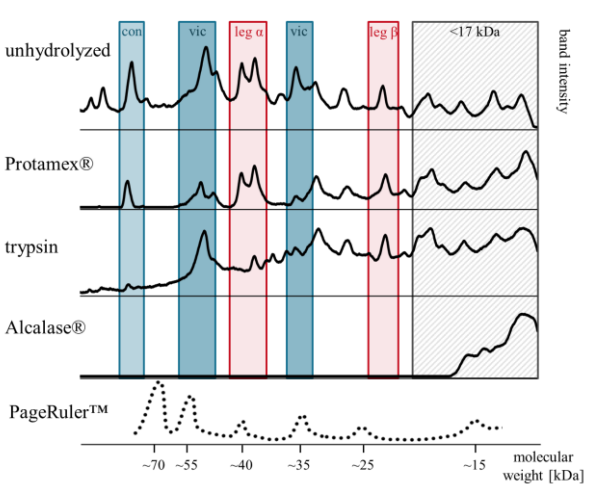

Figure 1: Molecular weight distribution of pea protein and pea protein hydrolysates obtained by enzymatic hydrolysis with Protamex®, trypsin and Alcalase® determined by SDS-PAGE under reducing conditions.
(con = convicilin, vic = vicilin, leg = legumin)

accessible, legumin-β contains less arginine and lysine than legumin-α (13 Arg and 9 Lys in legumin-β vs. 35 Arg and 13 Lys in legumin-α) as counted from the UniProtKB database (Bateman et al., 2017), and convicilin contains a highly charged N-terminus (Bown et al., 1988), making it also a favourable substrate for trypsin. Both, hydrolysis with Protamex® and trypsin did not affect the legumin-β. Resistance of the 12S-β fraction of oat protein was previously related to its burial at the interior of the 12S protein structure (Nieto-Nieto et al., 2014).

The third enzyme investigated in this study was **Alcalase®** (fig. 1). Alcalase® cleaved nonspecifically (Doucet, Gauthier, Otter, & Foegeding, 2003) and left only peptides smaller than 17 kDa.

3.2. Fermentation and syneresis

The gels made from unhydrolyzed protein can be considered as reference samples. At the end of fermentation, the elastic modulus G' was 5260 ± 203 Pa, the value for tan δ = 0.15 ± 0.01 clearly indicated a mainly elastic behaviour and syneresis after resting for 24 h was low (0.53 ± 0.19%). Table 2 shows storage modulus G' and loss factor tan δ at the end of fermentation and syneresis after 24 h of gel storage for all samples. G' decreased in the order unhydrolyzed, Protamex®, trypsin, Alcalase®. Despite being in the same order of magnitude, G' of the gels made from tryptic hydrolysate was significantly lower than the storage moduli of gels from unhydrolyzed protein and Protamex® hydrolysates. However, all three gels showed similar tan δ indicating similar viscoelasticity and can be described as weak gels (Sun & Arntfield, 2010). Syneresis follows an opposite trend to G' at the end of fermentation which in turn may be related to the increasing content of smaller peptides in the respective hydrolysates. When considering the protein fractions present after hydrolysis (mainly legumin in Protamex® hydrolysates and mainly vicilin in tryptic hydrolysates), our results are to some extend contradictory to those found by Mession, Chihi, Sok, & Saurel, 2015, who described higher values in G' for acid induced vicilin gels, than for pea protein or legumin gels, with legumin reaching the lowest values. However, in our study protein concentration was much higher (10% vs. 3.5/4%), which may have led to different behaviour, as Kohyama & Nishinari, 1993 described higher storage moduli for soy 11 S proteins than soy 7 S proteins at protein concentrations above 5%. Similar results with higher G' values for 11S protein than 7S proteins were also found in heat induced soy protein gels (Renkema, Knabben, & van Vliet, 2001).

Table 2 Characteristics of gels prepared from pea protein and pea protein hydrolysates obtained by enzymatic hydrolysis with Protamex®, trypsin and Alcalase®: storage modulus G',end and loss factor tanδ,end (f = 1 Hz and γ = 0.1%) at the end of fermenation, syneresis of fully set of gels.

Sample	G'_{end} [Pa]	$\tan\delta_{end}$ [-]	syneresis [%]
no enzyme	5260^{a} ± 203	0.15^{a} ± 0.01	0.53 ± 0.19
Protamex®	4860^{a} ± 455	0.16^{a} ± 0.01	2.81 ± 0.27
trypsin	3650^{b} ± 711	0.15^{a} ± 0.01	$5.64^{\#}$
Alcalase®	$^{*}7^{c}$ ± 004	0.34^{b} ± 0.09	14.70 ± 1.80

Values are mean of triplicate determination and the corresponding standard deviations.
#owing to insufficient data, this is the mean of a duplicate determination
Different letters represent significant differences (α=0.05) as determined by one way ANOVA followed by Tukey postHoc test.
**despite tan δ<1, this sample was more like a thick protein dispersion than a gel and is therefore not considered in further rheology experiments.*

In contrast to the first three gels, samples from Alcalase® showed very low values of G' (7 ± 4 Pa) alongside significantly higher values of tan δ (0.34 ± 0.09). Similar values were previously reported for acid induced pea legumin gels below their least gelling concentration and were ascribed to coagulation rather than gelation (Mession et al., 2015). Nieto-Nieto et al., 2014 found similar results for

heat induced gelation of oat protein hydrolysates, where Alcalase® hydrolysed protein was only able to gel at pH 9. Furthermore, in our study high syneresis (table 2) and visual assessment also supports the assumption of coagulation and sedimentation instead of gelation. C. Wang & Damodaran, 1990 reported a minimum molecular weight of 23 kDa for globular proteins to be able to gel at all, therefore at maximum molecular weight of 17 kDa gelation is unlikely. For these reasons, we conclude, that Alcalase® hydrolysates were unable to form self-supporting gels under the conditions of our study and therefore this hydrolysate was considered any further.

3.3. Interactions and gel fractions

The following section covers the influence of different types of interactions on the formation of fermentation induced pea protein gels. Additionally, those types of interactions may be influenced by the decrease in individual protein fractions, the increase in the number of ionisable groups and the increased exposure of previously buried hydrophobic groups after hydrolysis.

In gel solubility experiments (fig 2), aliquots of gels made from hydrolysed and unhydrolyzed pea protein were incubated in various solvents followed by centrifugation and analysis of the supernatants. 0.5 M NaCl solution acts by screening the charges of the proteins, hence reducing the working range of its electrostatic interactions by reducing the Debye double layer (Delahaije, Gruppen, Giuseppin, & Wierenga, 2015). This will result in a decrease of the attractive electrostatic interactions that might be present between proteins. SDS acts as an anionic surfactant, thus reducing the amount of hydrophobic interactions (O'Kane, 2004). DTT acts as a reducing agent, disrupting the disulphide bonds that might be present in the gels and PG acts by enhancing the hydrogen bonds, and the electrostatic interactions, but may decrease the hydrophobic contributions (Utsumi & Kinsella, 1985). There was no significant influence of the applied enzymes on gel solubility. But, all gels incubated in SDS showed a significantly increased gel solubility (α=0.05) and incubation in NaCl increased the gel solubility compared to incubation in PG and DTT, while there was no significant difference between the reference (H_2O) and the gels incubated in PG, NaCl and DTT (fig 2). Literature has described interaction in heat set gels to be hydrophobic, electrostatic and via hydrogen bonds (e.g. (O'Kane et al., 2004b; Sun & Arntfield, 2012). In contrast, our results indicate interactions in the fermentation induced gel network to be mainly hydrophobic in nature regardless of enzymatic treatment. This is not unexpected as the final pH-value of all gels is close to the isoelectric point of the pea protein and therefore net charge is low.

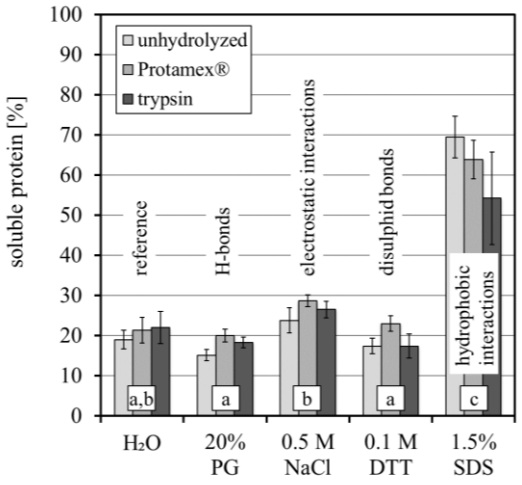

Figure 2: Gel solubility of gels prepared from pea protein and pea protein hydrolysates obtained by enzymatic hydrolysis with Protamex® and trypsin in different solvents. (H2O=distilled water, PG=propylene glycol, NaCl=sodium chloride, DTT= dithiothreitol, SDS= sodium dodecyl sulfate). Error bars represent the standard deviations of triplicate determinations.

In order to further investigate the contributions of the different types of interactions, and to look into the protein fractions involved, SDS-PAGE of the supernatants was performed. From differences in molecular weight profiles, it can be deducted, which protein fractions participate in the gel structure via which type of interactions.

No differences could be found between the molecular weight profiles in H_2O, PG and DTT (results for PG and DTT are therefore not shown), thus leading to the assumption, that neither hydrogen- nor disulphide-bonds play a relevant role in the formation of the gel structure. Concerning disulphide-bonds, this was expected, as acid induced gels are usually formed by non-covalent interactions (Grygorczyk & Corredig, 2013; Kohyama et al., 1995). As a result, fig. 3 only shows molecular weight profiles of supernatants from gels incubated in H_2O, NaCl and SDS.

Fig. 3a shows the molecular weight profiles of the gel from unhydrolyzed protein. In the bottom row, the soluble protein fraction in water is shown. The most apparent difference between the water-soluble fractions of the gel (fig 3a) and the original molecular weight profile of the pea protein (fig 1) is the complete lack of legumin fractions, which leads to the conclusion, that the legumin is a major contributor in building the gel network. Moreover, the height of the 50 kDa vicilin peak is decreased and the 30 kDa vicilin peak is missing, implying that some vicilin participates in the gel network as well. Similar dominance of legumin was previously described by Nieto-Nieto et al., 2014 who found a large influence of oat 12S α-subunits on the heat induced gelation of oat protein. The last apparent difference between the molecular weight profile of the soluble fraction in water and that of the protein before fermentation is a very large increase in peptides smaller than 17 kDa. Since these peptides are smaller than 23 kDa (C. Wang & Damodaran, 1990) we conclude, that these peptides are too small to have a relevant impact on the gelation properties. These findings are also in agreement with the findings for the Alcalase® hydrolysates that showed no relevant gelation ability (table 2) and only contained peptides smaller than 17 kDa (fig 1). Moreover, similar behaviour was also reported for pepsin hydrolysates of oat protein, where only small peptides remained after hydrolysis (Nieto-Nieto et al., 2014).

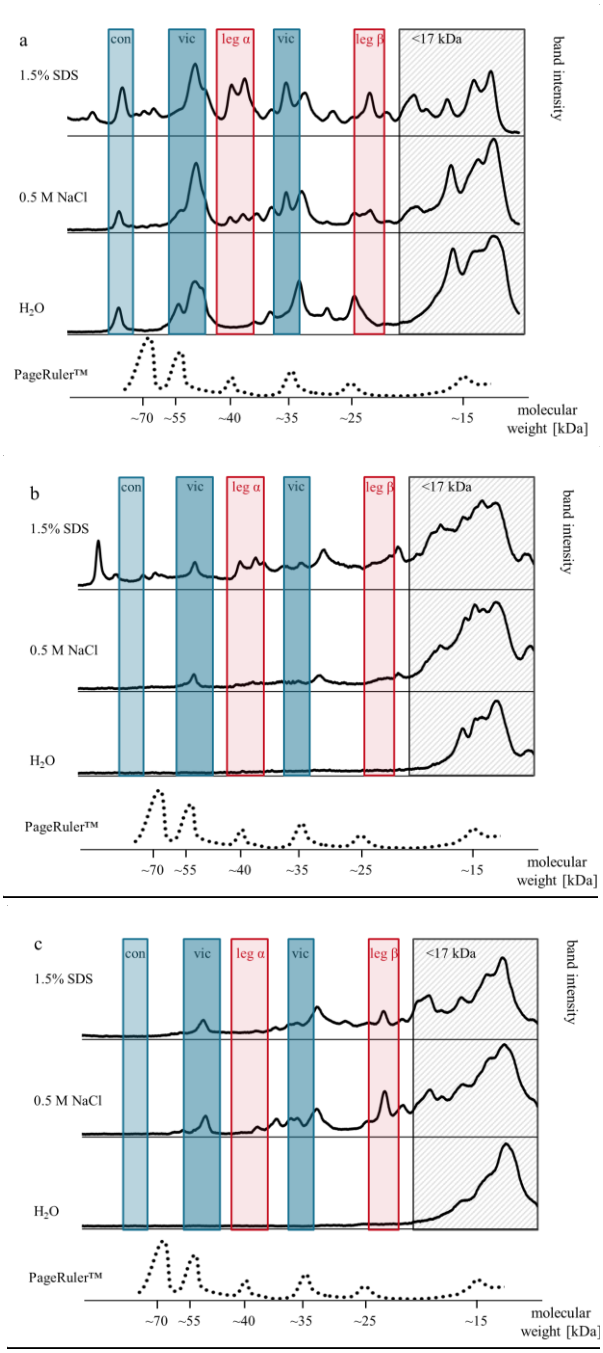

Figure 3 Molecular weight distribution of supernatants from gel solubility experiments in H2O, NaCl and SDS of gels from pea protein (a) and pea protein hydrolysates obtained by enzymatic hydrolysis with Protamex® (b) and trypsin (c) determined by SDS-PAGE under reducing conditions. (con = convicilin, vic = vicilin, leg = legumin)

Fig. 3a shows the soluble protein fraction in NaCl solution. Here, both vicilin peaks are clearly present again, with the 50 kDa fraction being very pronounced. It has been previously described for soy, that upon heating the basic subunit of the 11S fraction and the 7S fraction form a soluble complex via electrostatic interactions (Damodaran & Kinsella, 1982; German, Damodaran, & Kinsella, 1982). Moreover, similar results were found in the acidification of soy milk, where the basic subunit of the 11S fraction together with the β-subunits of the 7S fraction were not part of the soluble fraction at pH-values

below 5.9 (Ringgenberg et al., 2013). This is in full agreement with our results, where vicilin was released upon disturbing the electrostatic interactions via charge screening upon addition of NaCl (Delahaije et al., 2015) and confirms the assumption, that electrostatic interactions have a relevant contribution in network stabilisation by incorporating some of the vicilin. Furthermore, this assumption is supported by a significantly lower solubility in PG than in NaCl, as PG may enhance electrostatic interactions while NaCl interferes with them. Finally, in SDS (fig. 3a), all fractions seen in fig. 1 are present, with the only difference being a larger fraction at molecular weights smaller than 17 kDa. This increase in the number of smaller peptides again confirms the assumption, that these fractions do no integrate in the gel, while some of the larger fractions remain undissolved even in SDS (gel solubility 69.5±5.2%).

A slightly different picture arises, when considering the H_2O soluble fractions of gels from Protamex® and tryptic hydrolysates (fig. 3b and c): from these gels only fractions smaller than 17 kDa were dissolved. In case of the Protamex® hydrolysate, this may be due to the fact, that the 50 kDa vicilin fraction was already cleaved prior to fermentation. Considering the tryptic hydrolysate, the interaction between legumin-β and the vicilin would have been favoured by the increased accessibility of the legumin-β following the degradation of legumin-α by tryptic hydrolysis. These assumptions are mirrored in the NaCl soluble fractions (fig. 3b and c). The Protamex® hydrolysate only showed a very small increase in fractions >17 kDa, as mainly vicilin (which is already degraded during hydrolysis) would be released under these conditions. On the other hand, the NaCl soluble fraction of the trypsin hydrolysates showed a clear increase in vicilin, as well as in legumin-β fractions, again due to the disruption of the electrostatic interactions between the two. Due to the lack of legumin-α (that is usually bound to the legumin-β via a disulphide bond), the legumin-β becomes dislodged from the gel more easily.

Fig. 3b, shows the SDS soluble fractions of gel made from Protamex® hydrolysates. In general, all fractions shown in fig. 1 can be recovered. However, as already observed for the gel made from unhydrolyzed protein (fig. 3a) the proportion of peptides smaller than 17 kDa is also increased. This increase is more pronounced in gels from Protamex® and tryptic hydrolysates (fig. 3c) than in those from unhydrolyzed protein due to the development of small peptides during hydrolysis. In the Protamex® hydrolysates the absence of legumin-β and the small peaks for legumin-α in the molecular weight profile indicate, that due to lack of sufficient amounts of vicilin more insoluble legumin-β complexes are formed (German et al., 1982). In the molecular weight profile of the SDS soluble fraction of gels made from tryptic hydrolysates, fewer and less pronounced peaks are visible, compared to the NaCl soluble fraction. This agrees with the mechanism proposed for the interaction between legumin-β and vicilin, that is most pronounced in tryptic hydrolysates and may not be disrupted by SDS. Despite the influence of electrostatic interactions with regards to legumin-β – vicilin complexes, it needs to be kept in mind, that the hydrophobic interactions still dominate all pea protein gels regardless of hydrolysis, and should therefore have the largest impact on the functional properties.

3.4. Rheology of fermentation induced pea protein gels

After resting the gels for 24 h at 4 °C, more extensive rheological tests were performed to characterise the gels beyond the structuring process and to account for rearrangements during cooling. To this purpose, frequency and amplitude sweeps were performed. From frequency sweeps the slopes (dlogG'/dlogf) of G' were calculated while amplitude sweeps were used to investigate small and large amplitude rheological behaviour. The slopes (dlogG'/dlogf) of G' from frequency sweeps (fig. 4a) were 0.13 which is slightly lower, but still similar to results found in previous work (Klost & Drusch, 2019) and can be ascribed to the high protein content of the gels. In amplitude sweeps (fig. 4b), according to the classification made by Hyun, Kim, Ahn, & Lee, 2002, the shape of G' and G'' over strain shows an

overall strain thinning behaviour of the gels with a decrease of G' and G'' from γ = 1%. The elastic Lissajous plots (shear stress τ against shear strain γ) at this strain (Fig. 5a) exhibit shapes close to an ellipse which is characteristic for the linear viscoelastic regime. As the amplitude increases, the ellipse starts to widen, as a result of G' decreasing faster than G''. Around γ = 10%, a crossover occurs between G' and G'' (fig. 4b) and the Lissajous plots clearly deviate from an ellipse, indicating that the measured storage and loss moduli are not representative of the mechanical response anymore due to the presence of higher harmonics in the oscillation spectrum. As γ increases, the Lissajous curves turn into a squared box shape. This is due to an initial elastic response within each oscillation cycle, where the stress sharply increases, reaching a yield point after which the gel starts to flow (nearly horizontal part). This is a typical shape for yield stress response (Ewoldt, Winter, Maxey, & McKinley, 2010). When the shear rate decreases to 0 as the maximum strain is reached, interactions recover, and the same response is observed in the opposite direction, resulting in the square shape. This box shape has been observed before in bulk rheology of food products like tuna myofibrillar protein (Liu, Bao, Xi, & Miao, 2014), debranched waxy rice starch (Precha-Atsawanan, Uttapap, & Sagis, 2018) or waxy maize starch pastes (B. Wang, Wang, Li, Wei, & Adhikari, 2012). The viscous Lissajous plots (Fig. 5b) obtained plotting the stress τ against the strain rate $\dot{\gamma}$ also showed non-linear behaviour when γ is increased, resulting in an S-shape as seen in previous studies (Domenech & Velankar, 2015; Precha-Atsawanan et al., 2018). This behaviour was further quantified using the shear stiffening (S-Factor) and shear thickening (T-Factor) ratios (Fig. 6). The S-Factor is close to 0 until γ = 1.6%, indicating that the tangent line at minimum and the secant line at maximum strain, have the same slope. This is because in the linear viscoelastic regime, $G' = G'_M = G'_L$ (Ng, McKinley, & Ewoldt, 2011) as only the first harmonic contributes to the signal. Above γ = 1.6%, higher harmonics start to appear in the oscillation pattern, and the S-factor deviates from 0. The increase of the S-factor indicates intra cycle stiffening. This behaviour of intra cycle strain stiffening has been addressed before (Mermet-Guyennet, Gianfelice de Castro, & Habibi, 2014), and should not be confused with the shear thinning response seen above for the overall behaviour of the gels (fig. 4b). The strain stiffening is a result of G'_M being lower than G'_L, when the slope of the tangent line at minimum strain is lower than the slope of the secant line at maximum strain. A similar behaviour occurs for the T-Factor at γ = 1.6% with initial positive values due to $\eta'_M < \eta'_L$, but decreases below 0 at γ > 160%, indicating shear thinning of these gels, as observed also in fig. 5b with the S-shape of the curves. The absolute values reached by the T-Factor are larger than the S-Factor, and therefore indicate that the gels exhibit a softening/thinning behaviour (Precha-Atsawanan et al., 2018).

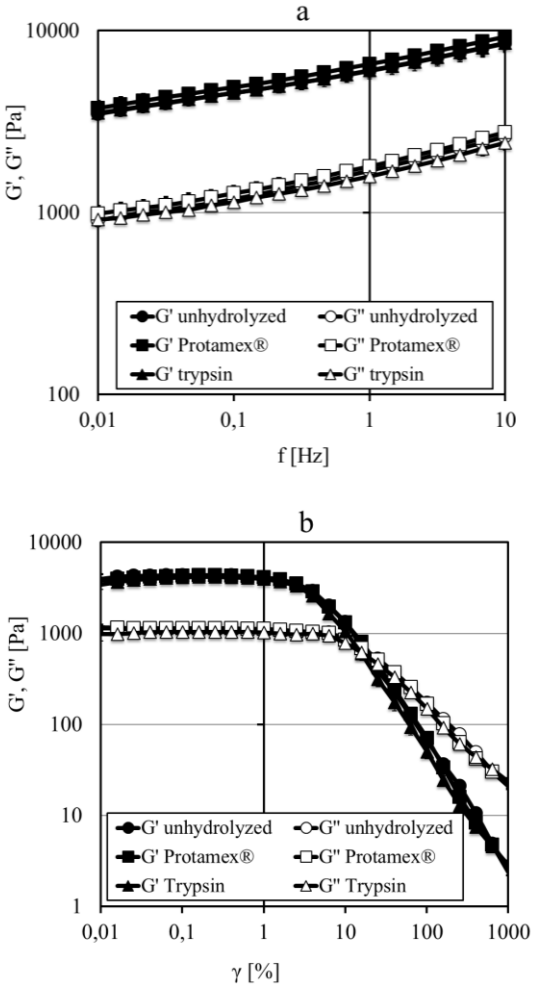

Figure 4: Storage modulus G' and loss modulus G'' from Frequency- (a) and amplitude- (b) sweeps of fully set of gels prepared from pea protein and pea protein hydrolysates obtained by enzymatic hydrolysis with Protamex® and trypsin.

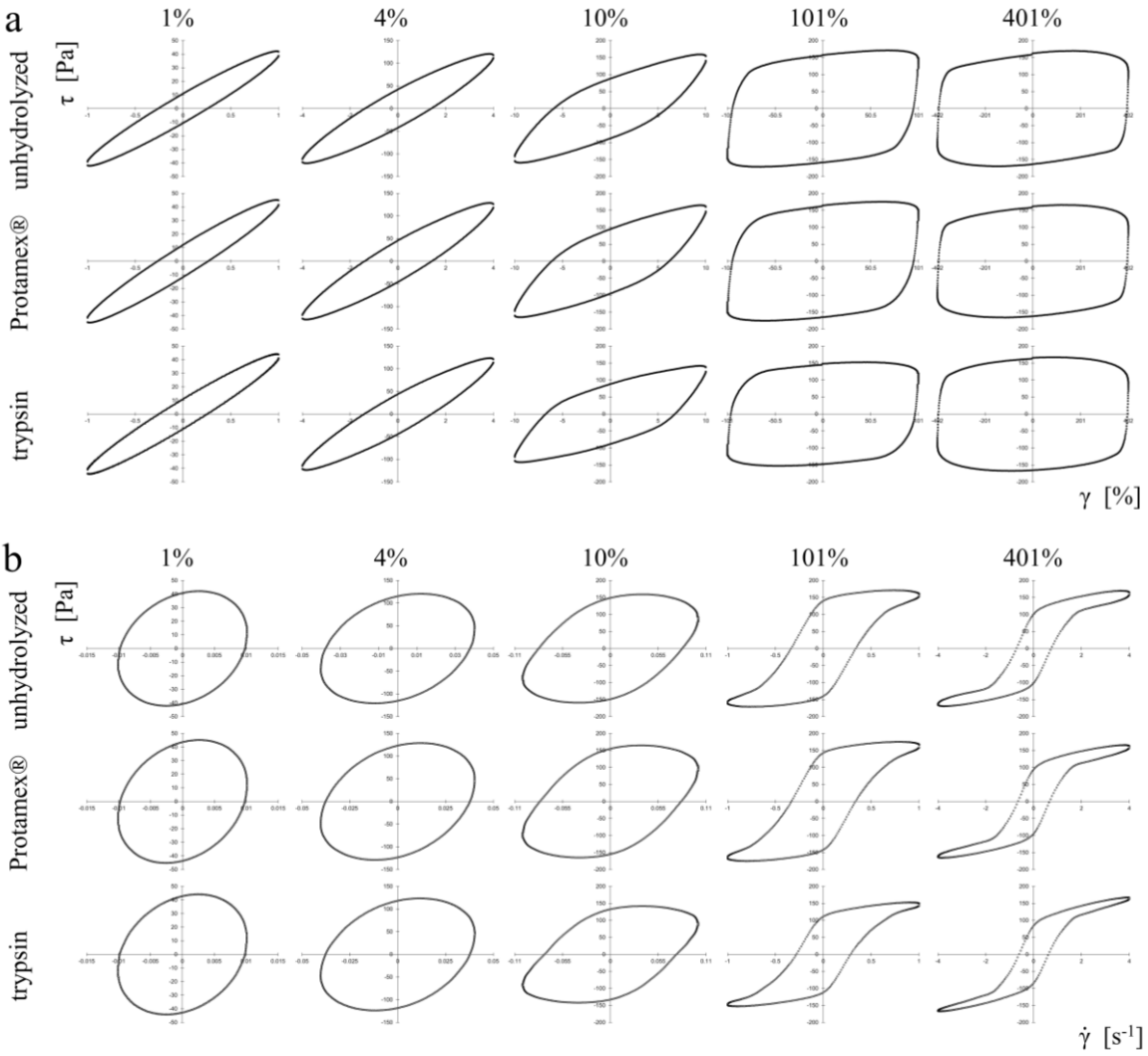

Figure 6: Lissajous plots of fully set of gels prepared from pea protein and pea protein hydrolysates obtained by enzymatic hydrolysis with Protamex® and trypsin: (a) elastic curves of stress τ versus strain γ, (b) viscous curves of stress τ versus shear rate "γ"

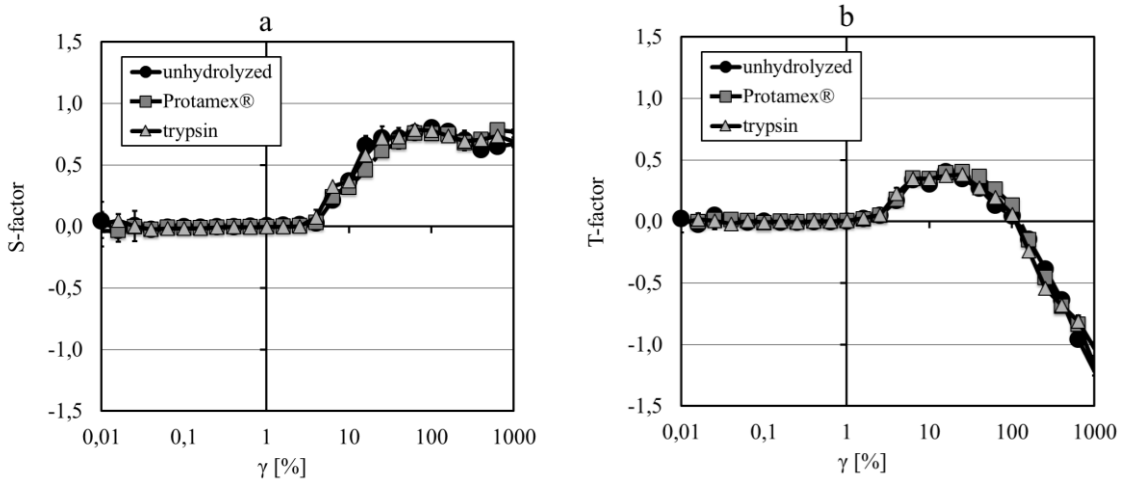

Figure 5: Shear stiffening ratio (S-factor) (a) and shear thickening ratio (T-factor) (b) of gels prepared from pea protein and pea protein hydrolysates obtained by enzymatic hydrolysis with Protamex® and trypsin.

After resting at 4 °C for 24 hours, all three gels showed similar G' and G'' values. This may be due to different types of rearrangements in the different gels during resting, and to experimentally necessary discarding of supernatants before further rheological measurements. As decrease in G' after fermentation was complementary to the increase in syneresis (table 2), the discarding of the supernatants would have increased the protein concentration in the remaining gels which may in turn have contributed to the alignment of G' values. Moreover, all gels showed identical behaviour in all further rheological tests (figs. 4-6), indicating a prevailing influence of the type of interactions over the influence of the protein fractions involved.

Conclusions

The results from this study show, that upon fermentation pea protein forms mainly legumin-gels that are stabilised via hydrophobic interactions. However, part of the vicilin gets incorporated in the protein gels via electrostatic interactions with the legumin-β chain. This effect is promoted, if tryptic hydrolysis is applied to the protein prior to fermentation, while hydrolysis with Protamex® inhibits this effect due to degradation of vicilin. Peptides smaller than 17 kDa, were not incorporated into the gel network, thus explaining the inability of Alcalase® hydrolysates to form a gel network.

Nevertheless, the stabilisation of the gel networks is dominated by hydrophobic interactions, with no influence of the enzymatic treatment. This is reflected by the lack of differences in rheological properties between gels from differently pre-treated protein. The slopes (dlog G'/dlogf) of G' from the frequency sweeps were 0.13 for all gels and can be ascribed to the high protein content of the gels, while in amplitude sweeps, the shapes of G' and G'' show an overall strain thinning behaviour. In large amplitude oscillatory shear experiments between amplitudes of $\gamma = 1.6\%$ and $\gamma = 160\%$ all gels show intra cycle strain stiffening behaviour while at amplitudes above $\gamma = 160\%$ intra cycle shear thinning prevails, indicating a softening/thinning behaviour.

This study helped to obtain a more thorough understanding of rheological behaviour, involved protein fractions and relevant interactions in fermented pea protein gels and therefore contributes to the development of strategies for the customisation of such gels for use in pea protein-based yoghurt alternatives with a high consumer acceptance.

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CRediT authorship contribution statement:

M. Klost: Conceptualization, Supervision, Writing - original draft, Writing - review & editing, Formal analysis, Investigation, Data curation, Visualization. G. Giménez-Ribes: Writing - original draft, Writing - review & editing, Formal analysis, Investigation, Data curation, Visualization. S. Drusch: Supervision, Writing - review & editing, Funding acquisition, Resources.

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