Food Hydrocolloids 120 (2021) 106891

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

How pea fractions with different protein composition and purity can substitute WPI in heat-set gels

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ARTICLE INFO

Keywords:
Yellow pea
Plant protein
Mild fractionation
Heat-set gelation
Whey protein isolate

ABSTRACT

In this study we explored the gelling behaviour of a pea protein concentrate (PPC), an albumin-fraction (ALB-F) and a globulin-rich fraction (GLB-RF), in comparison with and as substitute for whey protein isolate (WPI), by small oscillatory and large amplitude oscillatory shear (SAOS and LAOS) rheology. It was found that PPC formed the firmest gels (defined as highest elastic modulus), but this gel was not as firm as a pure WPI gel. The ALB-F formed the weakest gel due to its low protein purity. For a better view on the albumin gelling behaviour, ALB-F was further diafiltrated and the albumin-enriched fraction was labelled ALB-RF. It turned out that albumins formed firmer gels per mass unit of protein than globulins. Also, the energy dissipation ratios – a measure for the plasticity of the gel – were determined as a function of strain. The ALB-RF gel showed an increase in plastic response at larger strains compared to the GLB-RF gel (40% and 10% strain, respectively). ALB-F, PPC and GLB-RF were also examined on their ability to substitute WPI in heat-set gels. It was found that ALB-F/WPI mixtures formed firm gels and were least sensitive to changes in pH and ionic strength. It also appeared that disulphide bonding plays a more important role in the ALB-F/WPI mixtures upon heat-set gelation compared to the PPC/WPI and GLB-RF/WPI mixtures. The use of pea fractions as a substitute for WPI, particularly the ALB-F, could improve the resource efficiency of pea as an ingredient source.

1. Introduction

Although dairy proteins are still widely applied in food products, plant proteins are becoming more prevalent in a variety of products, including milk-like beverages, yoghurts, cheeses, and meat analogues. The application of plant proteins in such products is facilitated by improved understanding of the differences in terms of functional behaviour (i.e. solubility, gel, emulsion, foam properties), particularly when aiming for substituting dairy proteins. In recent years numerous studies have been conducted on the physicochemical properties and functional behaviour of plant proteins, including pea protein (Ge et al., 2020; Ladjal Ettoumi, Berton-Carabin, Chibane, & Schroën, 2017; Angie Che Yan; Lam, Warkentin, Tyler, & Nickerson, 2017; Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015; Zhao, Shen, Wu, Zhang, & Xu, 2020). Recently, the potential to replace whey proteins by pea protein fractions was studied. These studies revealed that whey proteins generally form firmer gels and more stable foams and emulsions compared with pea protein (Stone et al., 2015; Wong, Vasanthan, & Ozimek, 2013). The fact that full replacement of whey protein by pea protein leads to a different functional behaviour, explains the increased interested in partial substitution of whey protein isolate (Hinderink, Münch, Sagis, Schroën, & Berton-Carabin, 2019; Ji et al., 2015; Yerramilli, Longmore, & Ghosh, 2017).

Pea proteins can be classified into globulins and albumins, with the former being more than 70% of the total protein content (Casey, Sharman, Wright, Bacon, & Guldager, 1982; Kimura et al., 2008). The globulins can be classified into legumin, vicilin and convicilin, although the latter is sometimes considered part of the vicilin subgroup. At neutral pH, legumin is mostly present as a hexamer with a molecular weight of 320–380 kDa and vicilin and convicilin as trimers of 170 and 290 kDa, respectively (Barac et al., 2010; Croy, Gatehouse, Tyler, & Boulter, 1980). Compared with whey proteins, pea globulins are larger

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https://doi.org/10.1016/j.foodhyd.2021.106891
Received 26 March 2021; Received in revised form 3 May 2021; Accepted 7 May 2021
Available online 12 May 2021

and more hydrophobic (Kornet, Veenemans, et al., 2021). The other group of pea proteins are albumins. This is a collective name for a class of proteins, including PA1 and PA2 (two types of Pea Albumin), lectin and protease inhibitors (Park, Kim, & Baik, 2010). Albumins are small and hydrophilic protein molecules, with molecular weights ranging between 4 and 26 kDa in their monomeric state (Higgins et al., 1986; Lu, Quillien, & Popineau, 2000) and are rich in cysteine (Mariotti et al., 2001). Studies on the gelling behaviour of pea albumins are scarce (Djoullah, Djemaoune, Husson, & Saurel, 2015; Djoullah, Husson, & Saurel, 2018) and studies on the heat-induced gelling behaviour of pea albumins are not available. Studies on pea globulins showed that they can form heat-induced and acid-induced gels, albeit that the resulting gels are weak (Chihi, Sok, & Saurel, 2018; Mession, Chihi, Sok, & Saurel, 2015). It was reported that heat-induced gelation is mainly driven by hydrophobic interactions and hydrogen bonding (Sun & Arntfield, 2012), and that disulphide bonding does not play a major role in pea protein gels (Kornet, Veenemans, et al., 2021; Sun et al., 2012).

The concept of enhancing functionality by (partially) substituting dairy proteins by plant proteins is not new. Studies on this subject cover a wide range of functionalities and mixtures, such as the flow behaviour of calcium caseinate and whey protein with wheat flour and soy protein isolate (Onwulata, Tunick, & Mukhopadhyay, 2014), gelling behaviour of whey protein with soy protein (Comfort & Howell, 2002; Jose, Pouvreau, & Martin, 2016; McCann, Guyon, Fischer, & Day, 2018) or less pure mixtures of soymilk and cow's milk (Grygorczyk, Duizer, Lesschaeve, & Corredig, 2014). However, most studies mentioned above make use of pea fractions with high protein purities (i.e. globulin protein isolates) as substitute for whey protein. The same was done in earlier research, where we showed that pea protein isolate could serve as a substitute for whey protein isolate by using specific fractionation routes (Kornet, Shek, et al., 2021). The advantage of such an approach is that the behaviour of the resulting mixtures is easier to understand and to predict. However, less pure fractions (that is, less processed) often have a lower environmental footprint (Lie-Piang, Braconi, Boom, & van der Padt, 2021; van der Goot et al., 2016), and equal or even better functional properties (Fuhrmeister & Meuser, 2003; Geerts, Mienis, Nikiforidis, van der Padt, & van der Goot, 2017; Kornet, Veenemans, et al., 2021; Papalamprou, Doxastakis, Biliaderis, & Kiosseoglou, 2009).

In this study we therefore examined the ability of pea fractions – fractionated to different extents and with different purities – to substitute whey protein isolate in heat-set gels. The role of pH, ionic strength and disulphide bonding in these gelled mixtures was also studied. To better understand the contribution of pea proteins in these hybrid gels, we extensively characterized the rheological behaviour of individual pea albumin and globulin gels. The insights on the use of pure and less pure pea protein fractions as WPI substitutes could improve the resource efficiency of pea as an ingredient source.

2. Materials and methods

2.1. Materials

Yellow pea seeds (*Pisum Sativum* L.) were obtained from Alimex Europe BV (Sint Kruis, The Netherlands). BiPRO whey protein isolate was obtained from Davisco (Geneva, Switersland). All chemicals were obtained from Merck (Darmstadt, Germany) and were of analytical grade. N-Ethylmaleimide (NEM) had a purity of 98% or higher.

2.2. Methods

2.2.1. Pea fractionation process

An aqueous protein fractionation process was used to obtain a pea protein concentrate (PPC), an albumin fraction (ALB-F) and a globulin-rich fraction (GLB-RF). These labels are based on protein composition analysis originating from an earlier study (Kornet et al., 2020). Pea seeds were ground into flour with an average particle size of 80 μm. The flour was dispersed in deionized water (ratio 1:10) and the pH was adjusted to 8 with aliquots of a 1 M NaOH solution. After 2 h of moderate stirring at room temperature the dispersion was centrifuged (10,000 g, 30 min, 20 °C) and the pellet was separated from the protein-enriched supernatant. Part of the supernatant was lyophilized and labelled as PPC. Further fractionation was conducted by precipitation of the pea globulins. The supernatant was brought to pH 4.5 with aliquots of a 1 M HCl solution and kept under moderate stirring at room temperature for 2 h. The precipitated globulin fraction was separated from the albumins by centrifugation (10000g, 30 min, 20 °C). The supernatant was freeze dried and labelled as ALB-F. The pellet was redispersed at pH 7 for 2 h, freeze dried and labelled as GLB-RF. A schematic overview of the full process is shown in Fig. 1.

An albumin-rich fraction (ALB-RF) was obtained by diafiltration, using the albumin-containing supernatant after isoelectric precipitation (ALB-F). The supernatant was diafiltrated at room temperature with a SartoJet Pump (Sartorius AG, Goettingen, Germany). An inlet pressure of 2.2 bar was applied to create a flow parallel to two 2 kDa Hydrosart membrane surfaces (Sartorius AG, Goettingen, Germany), and the

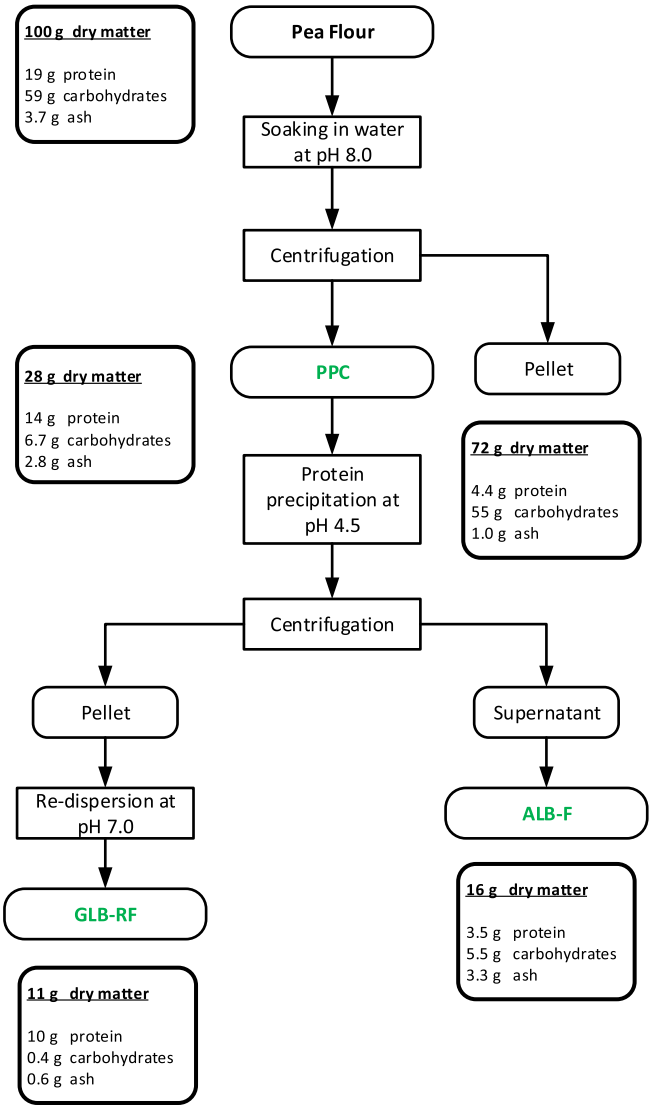

Fig. 1. Schematic overview of the aqueous fractionation process used to obtain a pea protein concentrate (PPC), an albumin-fraction (ALB-F) and a globulin fraction (GLB-RF). A dry matter based mass balance is shown within the scheme, with the major fraction constituents shown (percentages are given in Table 1).

resulting outlet pressure was 1.5 bar. The filtrate was discarded and the retentate was recirculated, while the decrease in filtrate conductivity was monitored. Filtration was stopped once the filtrate reached a conductivity of <50 μs/cm. Throughout the diafiltration process, water was added to the retentate, resulting in an eventual dilution factor of 7.5. The collected retentate was freeze-dried and labelled ALB-RF.

The ash content was determined by weighing the remainder of the sample after overnight heating in a furnace at 550 °C. The protein content was calculated from the nitrogen content that was measured with a FLASH EA 1112 series Dumas (Interscience, Breda, The Netherlands), using a nitrogen conversion factor of 5.7. All protein contents were expressed on dry matter basis.

2.2.2. Mass balance

A mass balance was compiled from the protein content and protein recovery data multiple fractionation processes (n = 4). The total recovered mass was calculated for PPC, ALB-F and GLB-RF, using the mass balance equation (Eq. (1)).

The mass balance reads:

$$x_{p,\,pf}\varphi_{pf} = x_{p,\,p1}\varphi_{p1} + x_{p,\,p2}\varphi_{p2} + x_{p,\,s2}\varphi_{s2} \tag{1}$$

Where x is the mass fraction (-) and φ the mass (g) with subscripts for protein (p), pea flour (pf), the pellet after first centrifugation (p1), the supernatant after first centrifugation (s1), the pellet after second centrifugation (p2) and the supernatant after the second centrifugation (s2).

The amount of total dry matter in the pea flour fraction is determined by:

$$m_{dm,pf} = \left(1 - x_{w,\,pf}\right)\varphi_{pf} \tag{2}$$

The fraction (ζ) protein in the dry matter of the pea flour (purity) was obtained from:

$$\zeta_{p,pf} = \frac{m_{p,pf}}{m_{dm,pf}} = \frac{x_{p,\,pf}\varphi_{pf}}{\left(1 - x_{w,\,pf}\right)\varphi_{pf}} = \frac{x_{p,pf}}{\left(1 - x_{w,\,pf}\right)} \tag{3}$$

The recovered mass percentage of each fraction can be calculated using the equation below, where pea protein fraction (ppf) can be s1, p2 or s2.

$$dry\ matter\ recovery\ (\%) = \frac{\varphi_{ppf}}{\varphi_{pf}} * 100\% \tag{4}$$

The recovered mass of the first pellet (m_{p1}) was approximated by subtracting the mass of the first supernatant (m_{s1}) from the initial mass of pea flour (m_{pf}).

The protein recovery in each fraction can be calculated using Eq. (5).

$$protein\ recovery\ (\%) = \frac{x_{p,\,ppf}\varphi_{ppf}}{x_{p,\,pf}\varphi_{pf}} * 100\% \tag{5}$$

The total mass of each fraction was multiplied with the ash and carbohydrate content mass fractions to obtain the ash and carbohydrate content in the fraction. The protein content was measured for each batch (four times), the carbohydrate content was based on one batch, while ash content was determined of at least two batches. The results from carbohydrate analysis were published before (Kornet et al., 2020), and the method has been described there. Regardless of the number of batches, the compositional analyses themselves were always performed in duplicate or triplicate.

2.2.3. Conductivity measurements

The conductivity of the protein mixtures was measured with a CO 3000 L conductivity meter (VWR International, Leuven, Belgium) at 20 °C. The measured conductivities were used to calculate the ionic strengths – expressed as the equivalent of a NaCl molar concentration – using Eq. (6) (Butré, Wierenga, & Gruppen, 2012).

$$Ionic\ strength\ (NaCl\ equiv\ M) = 1.02 \cdot 10^{-5} \cdot conductivity\ \left(\mu s.cm^{-1}\right) \tag{6}$$

2.2.4. Mineral composition

The samples were first heated in a microwave together with HNO_3 and concentrated HCl to destruct organic compounds. Subsequently, H_2O_2 was added and the samples were heated again to remove nitrous fumes. Calcium, copper, iron, potassium, magnesium, sodium, phosphor and zinc could now be detected and quantified by Inductively Coupled Plasa Atomic Emission Spectroscopy (ICP-AES) with a Thermo iCAP-6500 DV (Thermo Fischer Scientific, Cambridgeshire, United Kingdom). The chloride content was estimated by subtracting the total mineral mass from the ash content.

2.2.5. Sample preparation

The pea fractions were dispersed in deionized water in concentrations of 15 wt % dry matter and the pH was adjusted to 3.8, 5.0 or 7.0 with aliquots of 1 M NaOH and 1 M HCl solutions. The initial ionic strength of the dispersions – calculated from the conductivities – was increased to 50 mM, 100 mM and 200 mM using NaCl. For determining the contribution of disulphide-bonding a 20 mM N-Ethylmaleimide (NEM) solution was prepared as a solvent for the protein dispersion. After dispersing, the protein was solubilized under mild agitation at room temperature for 2 h.

2.2.6. Small amplitude oscillatory shear (SAOS) rheology

The gelling behaviour of the dispersed pea fractions was studied by measuring their linear viscoelastic response upon heating and cooling with an MCR302 rheometer (Anton Paar, Graz, Austria). Such temperature sweeps can provide insight in the gelatinization behaviour of starch (Singh & Kaur, 2004; Vallons & Arendt, 2009) or the gelling behaviour of proteins (Monteiro & Lopes-da-Silva, 2019; Tang, 1993). The rheometer was equipped with a sand-blasted CC-17 concentric cylinder geometry. To prevent solvent evaporation during heating, a solvent trap was placed on top of the cylinder. The dispersions were first heated to 95 °C, then kept at 95 °C for 10 min, and cooled with to 20 °C. Both the heating and cooling rate was 3 °C/min. The temperature was kept constant at 20 °C for another 5 min. The storage (G′) and loss moduli (G″) were recorded under constant oscillation at 1 Hz and 1% strain amplitude. After the temperature sweep, a frequency sweep was applied within the LVE (linear viscoelastic) regime at 1% strain deformation and a logarithmic increase from 0.01 to 10 Hz. Gel firmness was defined as the G' value after heat treatment.

2.2.7. Large amplitude oscillatory shear (LAOS) rheology

The non-linear rheological behaviour of the heat-set gels was studied by LAOS measurements, with oscillating strain at a frequency of 1 Hz and a temperature of 20 °C, and strain amplitude increasing logarithmically from 0.1% to 1000%. The end of the LVE regime was expressed as the critical strain, which was the strain amplitude at which the elastic modulus had decreased to 90% of its original plateau value.

The oscillating strain, stress and shear rate signals were recorded for each imposed sinusoidal strain amplitude. The resulting data was used to construct Lissajous plots. The elastic and viscous stress contributions were calculated using the Rheocompass Software (Anton Paar, Graz, Austria).

The area enclosed in a Lissajous curve is equivalent to the energy dissipated per unit volume during one oscillatory strain cycle. The ratio of dissipated energy over the energy dissipated by a perfect plastic material is termed the energy dissipation ratio (Schreuders et al., 2021). This energy dissipation ratio ($\varnothing$) is calculated using Eq. (7) with the loss modulus (G'') and the maximum stress (σ_{max}) at a given strain amplitude (γ_0) (Ewoldt, Winter, Maxey, & McKinley, 2010). The energy dissipation ratio was plotted as a function of strain amplitude, to visualize the main information from the Lissajous curves in a compact manner.

$$\varnothing = \frac{E_d}{(E_d)_{pp}} = \frac{\pi G'' \gamma_0}{4\sigma_{max}} \quad (7)$$

2.2.8. Statistical analysis

All samples were prepared in duplicate and thereafter measured. The samples were prepared from one batch of pea fractions, and the mass balance was based on four batches. The mean values are shown and the standard deviations are given as a measure of error. In case of a range of datapoints with good reproducibility, a representative curve was selected. Claims regarding significant effects were supported by a Welch's unequal variances *t*-test performed in R, applied on independent samples. Significance was concluded when $P < 0.05$.

3. Results

3.1. Fractionation efficiency

Three pea fractions were produced by aqueous fractionation and exposed to different extents of fractionation (Fig. 1). The first fraction is a pea protein concentrate (PPC) as obtained via the supernatant after the first centrifugation step at pH 8. An additional centrifugation at pH 4.5 of PPC resulted in an albumin-fraction (ALB-F) in the supernatant and a globulin-rich fraction (GLB-RF) in the pellet. The efficiency of the fractionation processes is reflected in the protein recovery of the resulting fractions (Eq. (5)). Limited fractionation yields PPC with a protein recovery of 74% (±3.2%). The remaining 26% is lost in the pellet. Upon further fractionation – where albumins and globulins are separated – the combined protein recovery is 71% (19% for ALB-F and 52% for GLB-RF, with standard deviations of 1.5 and 7.3%, respectively). This shows that only 3% of the proteins are lost upon isoelectric precipitation. A schematic representation of the process is shown in Fig. 1. Also, a mass balance is included, which is based on the data from four batches.

The pea protein fractions used in this study were chosen because they could be obtained by applying (part of) a conventional wet fractionation process. Following this process to different extents yielded protein fractions with different purities. Fewer fractionation steps are beneficial in terms of environmental footprint and energy or water usage. First, more of the protein present in pea is used in less refined fractions. A better use means that a lower quantity of raw material needs to be cultivated and processed. This will give the biggest advantage as main resource use in primary production of crops. In addition, further processing requires more water, energy and in some cases chemicals. For example, the further fractionation towards GLB-RF requires around 25L water per kg of protein in addition. Less water use generally means less energy costs for drying (Lie-Piang et al., 2021).

3.2. Composition of the pea fractions

Previously, it was found that isoelectric precipitation induces a separation between albumins and globulins (Kornet et al., 2020). This separation is based on the characteristic of globulins precipitating at pH 4.5 (Barać, Pešić, Stanojević, Kostić, & Čabrilo, 2015), and of the albumins remaining soluble (Higgins et al., 1986; Schroeder, 1982; Yang et al., 2020).

Table 1 provides an overview of the composition of the pea fractions. The major constituent of PPC and GLB-RF is protein, more specifically pea globulin. Although the pea albumins end up in the ALB-F, the protein content of this particular pea fraction is only 21 wt %. This is a result of the low albumin content in pea seeds, which is less than 30% of the total protein content (Casey et al., 1982; Kimura et al., 2008) and the presence of soluble non-protein components in pea, which also end up in the supernatant in this process. Until now, the ALB-F is often considered a by-product from a globulin fractionation process, not suitable for use in food products. However, the economic potential of this fractionation process could be enhanced if ALB-F could serve as a functional ingredient, for example as whey proteins substitute.

Table 1
Composition of yellow pea, the pea fractions and of whey protein isolate. All quantities are expressed in grams per 100 g of dry matter. The results originate from Kornet et al. (2020) and Kornet, Shek, et al. (2021). All samples were measured at least in duplicate and the numbers in superscript represent the standard deviations.

	Protein Content	Total carbohydrate content	Starch or starch derivative content	Ash content
Pea	18.8 $^{\pm 0.2}$	59.0 $^{\pm 2.1}$	48.8 $^{\pm 1.7}$	3.6 $^{\pm 0.3}$
PPC	51.4 $^{\pm 0.8}$	23.6 $^{\pm 0.1}$	3.6 $^{\pm 0.2}$	11.7 $^{\pm 0.3}$
ALB-F	21.1 $^{\pm 0.2}$	34.9 $^{\pm 2.1}$	6.0 $^{\pm 0.0}$	20.8 $^{\pm 0.4}$
GLB-RF	87.3 $^{\pm 1.0}$	3.4 $^{\pm 0.6}$	0.3 $^{\pm 0.1}$	6.1 $^{\pm 0.0}$
WPI	100 $^{\pm 1.0}$	–	–	1.2 $^{\pm 0.2}$

The major pea fraction impurities are carbohydrates and ash (Table 1). Previous research (Kornet et al., 2020) showed that over 90% of the carbohydrates in the pea fractions were present in the form of oligosaccharides – probably raffinose and stachyose – and the remainder as small polysaccharides of around 3 kDa. These soluble sugars were found to have minor influence on viscosity (Kornet et al., 2020), and probably also on gelling behaviour. Minerals on the other hand – which quantity is reflected by the ash content – can affect functional properties. Therefore, the mineral content was analysed into more detail (Table 2). The pea fractions are relatively abundant in K^+ and P^{3+}, which probably originates from phytic acid and K-phytate. The former is commonly found in pea cotyledons where phosphate is stored (Lott, Goodchild, & Craig, 1984). Phytic acid is also considered to be an anti-nutrient, as it chelate minerals and consequently reduce their bioavailability (Zhou & Erdman, 1995). Also Mg^{2+} and Ca^{2+} are quite abundantly present in the pea fractions, while Cu^+, Zn^{2+} and $Fe^{2+/3+}$ are only present in minor quantities. The relatively high Na^+ content of the pea fractions is a direct result from the pH adjustment using NaOH during the fractionation process. Except from Na^+, the GLB-RF contains significantly less minerals than PPC and ALB-F. Compared with the pea fractions, WPI shows even lower quantities of all minerals, except from Ca^{2+}.

3.3. Gelation of pea fractions and whey protein isolate

The rheological heat-induced gelling properties of WPI and the different pea fractions were characterised before measuring mixtures of those samples. Fig. 2 shows that WPI gels have the highest G′, followed by PPC, GLB-RF and ALB-F. The gels were all standardized on 15 wt %. The differences between these G′ values – used as a measure for gel firmness – cannot be explained by the differences in protein content. For instance, PPC has a higher G′ than GLB-RF, but the protein contents are 51% and 87%, respectively. Hence it is the type of protein that accounts for different gelling behaviour. Fig. 2 shows an abrupt sol/gel transition around 80 °C for WPI, which is close to the β-lactoglobulin denaturation temperature (JI Boye & Alli, 2000). The pea fractions show a more gradual increase in G′, which starts at lower temperatures. The gelling onset of GLB-RF begins at a temperature of around 60 °C. PPC starts to gel at around 65 °C, and ALB-F shows an increase in G′ at around 70 °C. The order of the onset of gelation is consistent with previous protein denaturation measurements (Kornet, Veenemans, et al., 2021), but the gelling onset temperatures are lower than the denaturation onset temperatures (77, 82 and 74 °C for PPC, ALB-F and GLB-RF, respectively). This could be related to differences in heating rate between the rheology and the differential scanning calorimetry (DSC) measurement. Furthermore, PPC and GLB-RF show a G′ increase at both the heating and cooling stage, whereas the G' of ALB-F and WPI increases mostly upon heating.

The final average G′ of the WPI gel is around 7 kPa (±2 kPa). PPC shows a G′ average value of around 2 kPa (±0.6 kPa), which is higher compared to the other pea protein gels. GLB-RF forms a soft solid with a

Table 2
The mineral composition (g/kg) of yellow pea, the dried pea fractions and whey protein isolate. The results for pea, ALB-F and WPI were also previously reported (Kornet, Shek, et al., 2021).

	Ca^{2+}	Cu^{+}	$Fe^{2+/3+}$	K^{+}	Mg^{2+}	Mn^{2+}	Na^{+}	P^{3+}	Zn^{2+}
Pea	0.62	0.01	0.06	10.4	1.07	0.01	0.01	4.53	0.04
PPC	1.89	0.02	0.12	24.0	2.72	0.03	4.43	11.6	0.10
ALB-F	3.33	0.03	0.00	47.3	5.02	0.04	10.0	8.16	0.16
GLB-RF	0.44	0.01	0.22	2.2	0.41	0.02	16.4	14.9	0.04
WPI	0.88	0.00	0.00	0.4	0.06	0.00	6.31	0.61	0.00

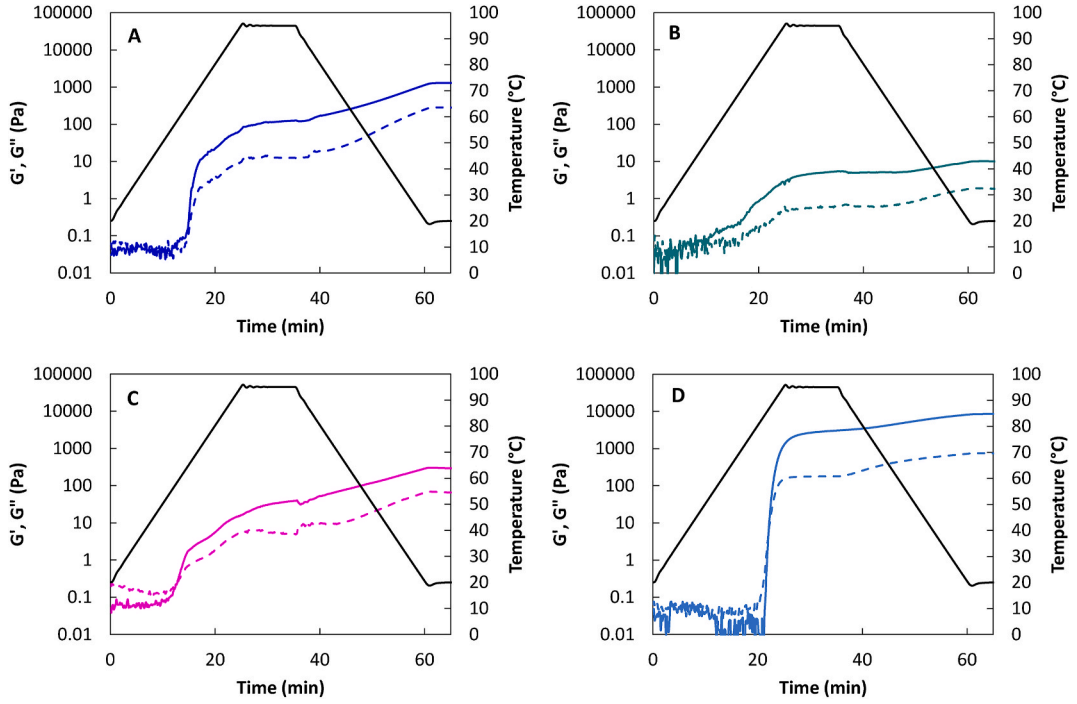

Fig. 2. Temperature sweeps of PPC (A), ALB-F (B), GLB-RF (C) and WPI (D). The samples were dispersed in concentrations of 15 wt % dry matter and adjusted to pH 7. The G' (solid line) and G" (dashed line) response was recorded over time. All samples were measured at least in duplicate and a representative curve was selected.

paste-like texture, which is also reflected in a lower G′ value of 300 Pa (±40 Pa). ALB-F shows the lowest G′ of around 8 Pa (±2 Pa). Although ALB-F behaves like a weak solid (where G' > G"), the appearance after heat treatment is that of a dispersion with large protein particles. The higher gel firmness of the PPC, compared with the purer GLB-RF, has been previously studied and can be attributed to different factors, of which isoelectric precipitation is the most important one (Kornet, Veenemans, et al., 2021). In that particular study, it was also found that PPC was more ductile than GLB-RF, evidenced by a later transition from elastic to viscous behaviour upon large deformation. Since, the dry matter in dispersion was kept constant at 15 wt %, the protein content varied between the samples. GLB-RF contains 87% protein (i.e. globulins), and it can be assumed that the gelling behaviour is dictated by the globulins. The gelling behaviour of albumins is not very clear, as the ALB-F shows a low G', probably as a result of the low protein content.

For a better comparison on the gelling behaviour of albumins with globulins – the ALB-F was further fractionated by diafiltration to increase the purity to 53.5 wt % (dry matter basis). This new fraction was

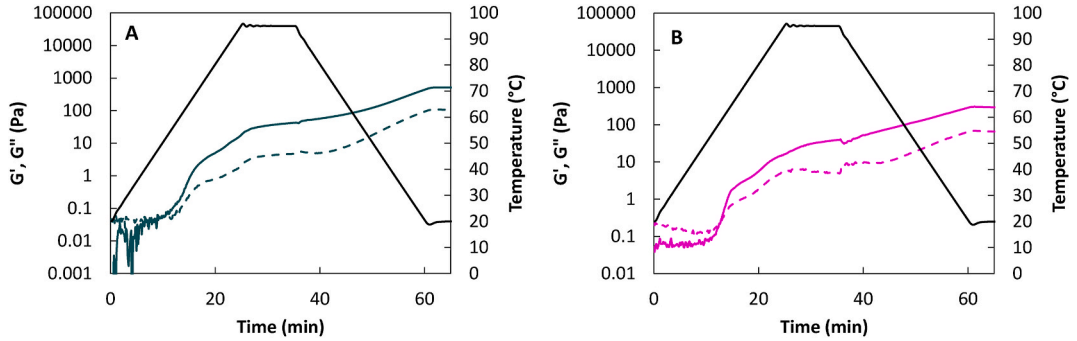

Fig. 3. Temperature sweeps of an albumin-rich fraction (ALB-RF) (A) and a globulin-rich fraction (GLB-RF) (B). The samples were dispersed in concentrations of 15 wt % dry matter and adjusted to pH 7. The G' (solid line) and G" (dashed line) response was recorded over time. All samples were measured at least in duplicate and a representative curve was selected.

labelled albumin-rich fraction (ALB-RF). Also here, it is assumed that protein controls the gelling behaviour, as the impurities are mostly small polysaccharides (with molecular weights smaller than 2 kDa) and minerals (Kornet et al., 2020).

The dry matter of the samples was kept constant at 15 wt % (13 wt % and 8 wt % protein for GLB-RF and ALB-RF, respectively). Fig. 3 shows that the gelation of ALB-RF and GLB-RF both start around 50 °C, but the increase of ALB-RF is more gradual than GLB-RF. At the end of the temperature sweep, albumins form slightly firmer gels than globulins, with G' values of 545 Pa (±46 Pa) and 324 Pa (±43 Pa), respectively, despite of the difference in protein purity (53.5% versus 87.3%). This implies that albumins are better gelling agents per mass of protein than the globulins. The differences in non-linear rheological behaviour were also studied.

Fig. 4 shows Lissajous plots of ALB-RF and GLB-RF both within and beyond the linear viscoelastic (LVE) regime. The limit of the LVE regime was found by determining the critical strain γ_c (i.e. the strain amplitude at which the G′ plateau value is reduced by 10%), which was 3.5% (±0.4%) and 1.8% (±0.1%), respectively, indicating that ALB-RF is more ductile than GLB-RF. Within the LVE regime, at a strain amplitude of 1%, the Lissajous plots had an elliptical shape, which indicates predominant linear viscoelastic behaviour. At 25% strain amplitude this elliptical shape showed deflections at maximum deformation in the case of ALB-RF, which indicates intracycle stiffening. At this strain amplitude, GLB-RF already showed plastic behaviour, with an initial rigid response (steep stress increase from left bottom corner) followed by flow (horizontal part where stress is quite constant), and recovery (top right corner). A similar response was seen for ALB-RF, but at a higher strain amplitude of 159%. At this strain amplitude GLB-RF already behaved almost fully viscous. Above 159%, ALB-RF showed self-intersections (i.e. lines from forward and backward oscillation cross-over at maximum strain) in the viscous Lissajous plot, which is a general indication of extreme nonlinearity being that existing stress is unloaded more quickly than new deformation is accumulated (Ewoldt & McKinley, 2010). This implies reformation of crosslinks within the timescales of the oscillatory deformation (Duvarci, Yazar, & Kokini, 2017). GLB-RF showed almost purely viscous behaviour at strain amplitudes of 318% and 798%, indicated by the near-circular shape and the increased curve area. The integrated area inside the Lissajous plots reflects the energy dissipation, and thus the level of viscous response. The ratio of observed dissipation over perfect plasticity, termed the dissipation ratio, is a measure for the material's plasticity. To visualize the main information from the Lissajous plots in a compact way, the energy dissipation ratio was plotted as function of strain amplitude (Fig. 5). Also, far beyond the LVE regime ALB-RF behaved more elastic, as Fig. 5 shows a steep increase in dissipation ratio at around 10% strain amplitude for GLB-RF, whereas ALB-RF only started to increase around 40% strain amplitude. In conclusion, pea albumins form firmer gels and better resist deformation compared to pea globulins.

The gel properties are probably dictated by the proteins, because protein is the major component (53.5 wt %) and the impurities are mostly oligosaccharides and salts (Kornet et al., 2020). To get a better insight on the behaviour of the protein structure – and potentially detect the contribution of components based on relaxation times – the gels were exposed to a frequency sweep. Fig. 6 shows the G′ and G" frequency dependency of ALB-RF and GLB-RF over a frequency range of 0.1–10 Hz.

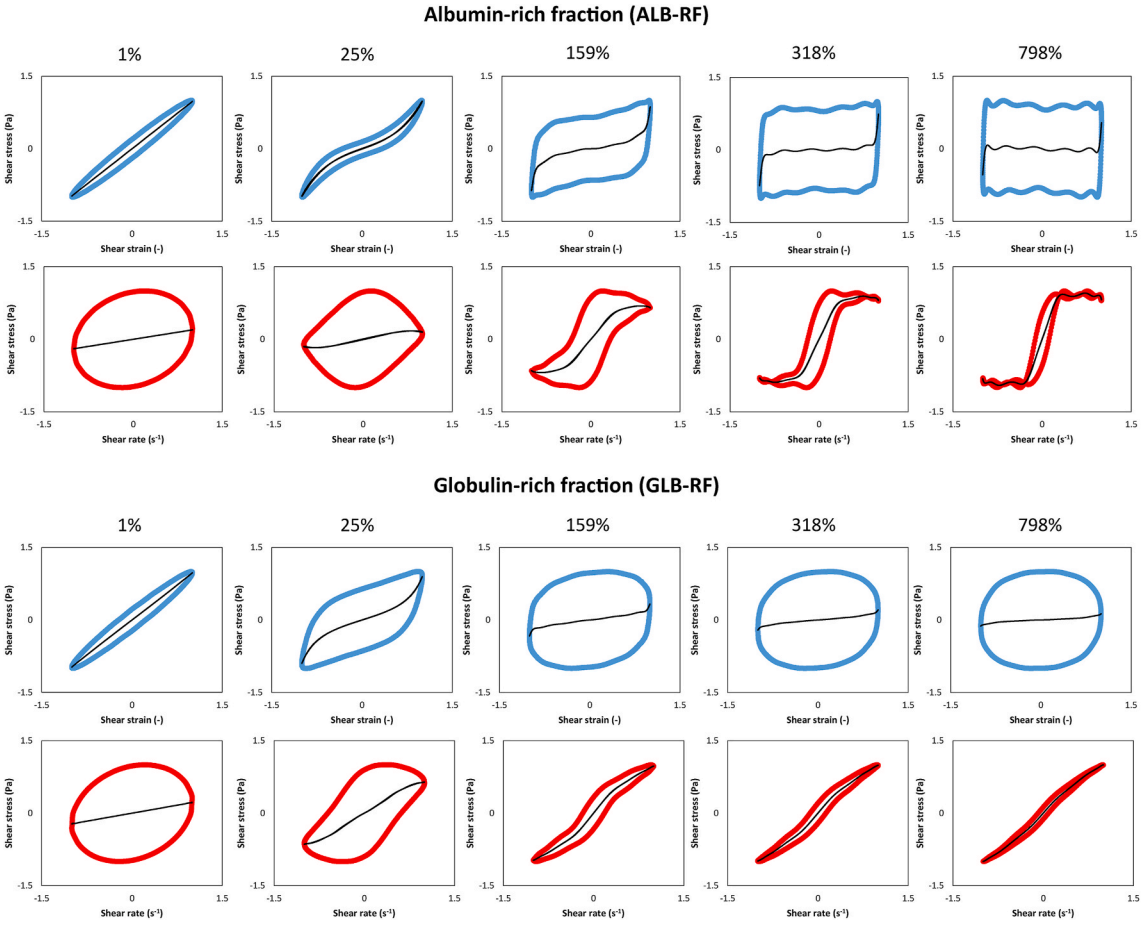

Fig. 4. The response to deformation of ALB-RF and GLB-RF gels (15 wt % dry matter, pH 7), visualized by elastic Lissajous plots of stress versus strain (▬) and viscous Lissajous plots of stress versus strain rate (▬). The normalized stress responses to oscillatory deformations at 1, 25, 159, 318, 798% are shown. The black line represents the elastic stress contribution. All samples were measured at least in duplicate and a representative curve was selected.

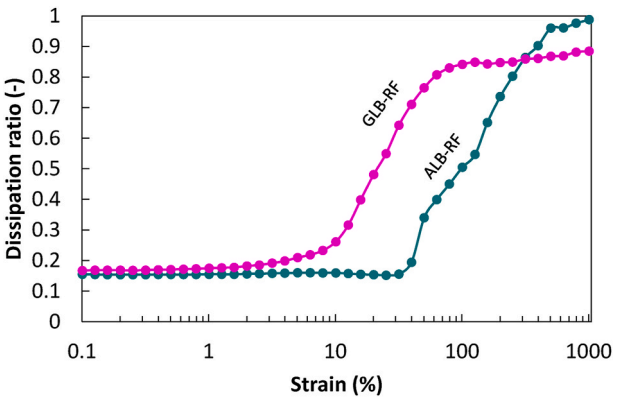

Fig. 5. Energy dissipation ratio of ALB-RF (—) and GLB-RF (—) heat-set gels, prepared from a dispersion with 15 wt % pea fractions, adjusted to pH 7. All samples were measured at least in duplicate and a representative curve was selected.

The G′ at frequencies below 0.1 Hz could not be studied due to low signal to noise ratios of the rheometer in these regions. From 0.1 to 10 Hz the G′ and G" remain quite constant with increasing frequency. This weak G' dependency on frequency of both ALB-RF and GLB-RF gels indicate that either the system is highly elastic or that the whole frequency range is high enough to make important network cross-links seem permanent (Clark, 1991). Such weak frequency dependency was also observed for commercial and salt-extracted pea protein isolate gels (Sun & Arntfield, 2010) and WPI heat-set gels (Lorenzen & Schrader, 2006).

So far, pea albumins received less attention than pea globulins in scientific research. Although globulins may be able to form firmer gels if they were fractionated more mildly, it is remarkable that pea albumins – normally underutilized in a fractionation process – can compete with conventionally fractionated globulins in the context of heat-induced gelation. This also stresses the potential of using an ALB-F by-product as a functional ingredient or even as a WPI substitute.

3.4. Gelling behaviour and gel properties of pea fraction – WPI mixtures

Mixtures from pea fractions and WPI were prepared in ratios of 1:3, 2:2 and 3:1 with a total dispersed mass fraction of 15 wt %. Fig. 7 shows the G′ of heat-set WPI gels in a concentration range of 9–21 wt % (blue line). Note that to clearly demonstrate the effect of a decreasing WPI content in the pea fraction/WPI mixtures the horizontal axis in Fig. 7 was inverted. The dashed lines represent mixtures of WPI with the pea fractions in different ratios and a constant total mass of 15 wt %. Gels made from the different pea fractions all showed lower G′ values than the gels containing only WPI. Even small amounts of WPI in the pea fractions (1:3) yielded higher G′ values than the pea fractions themselves. Fig. 7 shows that the G′ can reduce by a factor 10, with decreasing proportion of WPI in the GLB-RF/WPI mixtures. The inclusions of PPC and ALB-F led to an increase in G′ at different ratios. Such a synergistic effect was not observed in a previous study (Kornet, Shek, et al., 2021), where WPI was replaced by pea protein isolates (PPI). An overview on how the pea fractions in this study relate to the previously studied PPI in the context of WPI substitution (1:3 ratio) is shown in Table 3. Although the tan δ values – a measure for the solid-like behaviour of the gels – show small differences between pea fractions and protein isolates, larger variation in G′ is observed for the pea fractions compared with pea protein isolates. Also, higher G' values can be reached with PPC and ALB-RF as substitute, compared with the different pea protein isolates. Although the pea protein isolates were versatile in their behaviour (i.e. viscosity, gelling, solubility) (Kornet, Shek, et al., 2021), they behave more similar in substitution with WPI, compared with the differently processed pea fractions.

The high G′ of the WPI/ALB-F mixture suggests a synergistic effect, as the measured G′ is higher than the proportional sum of the G′ of the pea fractions and WPI gels. Such a synergistic effect could be caused by increased interactions (e.g. disulphide bonding), between the same type of proteins or between whey proteins and pea proteins. It could also be related to the minerals present in the pea fractions, as increased NaCl concentrations was found to have a positive effect on the G' of WPI gels (Hussain, Gaiani, Jeandel, Ghanbaja, & Scher, 2012). The effect of salt, as well as the role of disulphide bonding, will be discussed in the next sections.

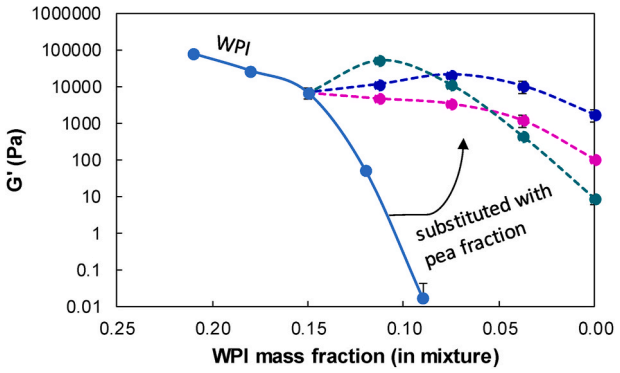

Fig. 7. G′ of heat-set gels produced from mixtures of pea protein concentrate (PPC), albumin fraction (ALB-F) and globulin fraction (GLB-RF) with whey protein isolate (WPI). The dashed lines are a guide for the eye, and represent mixtures of PPC (—), ALB-F (—) and GLB-RF (—) with WPI in 1:3, 2:2 and 3:1. Also the G′ of pure pea fractions (0% WPI) and pure WPI (—) as function of WPI mass fraction are shown. All gels were produced from dispersions with a total of 15 wt % dry matter, adjusted to pH 7. The samples were measured at least in duplicate and error bars represent the standard deviations. Note that the WPI mass fraction (on the horizontal axis) was inverted.

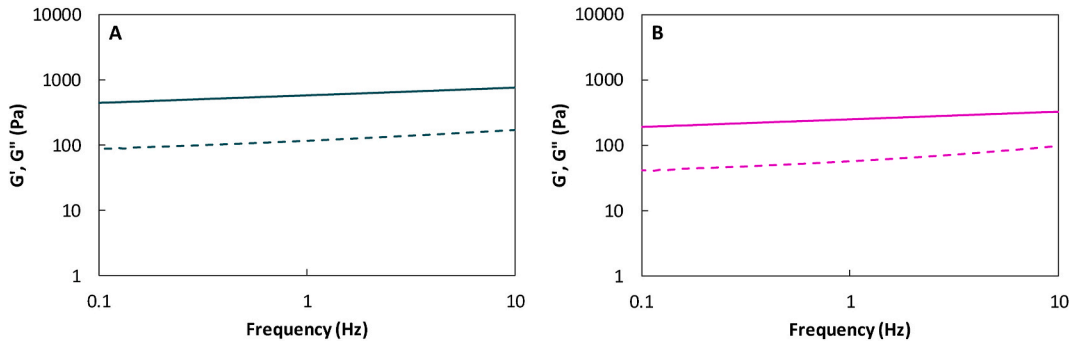

Fig. 6. Frequency sweeps of an albumin-rich fraction (ALB-RF) (A) and a globulin-rich fraction (GLB-RF) (B). The samples were dispersed in concentrations of 15 wt % dry matter and adjusted to pH 7. The G' (solid line) and G" (dashed line) response was recorded over time. All samples were measured at least in duplicate and a representative curve was selected.

3.5. The pH and ionic strength sensitivity of substituted WPI gels

Gelling properties of proteins are influenced by pH and ionic strength. A pH close to the isoelectric point (pI) facilitates protein aggregation, due to reduced electrostatic repulsion. The pI of whey proteins is 5.2 (Ju & Kilara, 1998) and the pI of pea globulins is between 4 and 5 (Joyce Boye, Zare, & Pletch, 2010; M.L. Chihi, Mession, Sok, & Saurel, 2016; A. C. Y. Lam, Can Karaca, Tyler, & Nickerson, 2016). Fig. 8 shows a G′ increase for PPC and GLB-RF mixtures with WPI at pH 5.0 and 7.0 at low temperatures. Even at the very onset of the temperature sweep they display solid-like behaviour at the measured frequency (1 Hz), as G' > G" (or tan δ < 1). It is possible that at lower frequencies the G′ would decrease, which would indicate a viscoelastic response, rather than the presence of an elastic network. These results may also suggest the presence of a weak network of aggregates formed around the pI of the proteins. Such behaviour (i.e. G' > G" with f = 1 Hz) was not observed for WPI (Shiroodi & Lo, 2015), but was observed for the GLB-RF with an initial tan δ (= G"/G′) of 0.59 (±0.07), potentially indicating solid-like behaviour. However, the sudden drop in G′ around 80 °C in Fig. 8 has not been seen in any of the pea fraction or WPI gels, which means that it is a property of the mixture. The temperature at which the decrease in G′ occurs is similar to the gelling onset of WPI (Ainis, Ersch, & Ipsen, 2018; Kornet, Shek, et al., 2021). A similar sudden drop in G' was seen for heat-set gels from Bambara groundnut and WPI mixtures at pH 5. An initially formed gel network – facilitated by electrostatic attraction around the pI – was disrupted around the denaturation temperature of β-lactoglobulin (Diedericks, Stolten, Jideani, Venema, & Linden, 2021). This suggests that WPI denaturation causes breakdown of a network initially formed, and that denaturation has a temporary adverse effect on the gelation process.

At pH 7, the gelling onset starts at higher temperatures, around 70 °C for mixtures with PPC and around 80 °C for mixtures with GLB-RF. The mixtures of ALB-F with WPI (Fig. 8) show different behaviour. Except from a slightly earlier gelation onset, the curves at pH 3.7, 5.0 and 7.0 are quite similar, and so are the final G′ values of the gels. ALB-F compares favourably with the other pea fractions as a WPI substitute. It does not only give firmer gels, but also appears quite insensitive to pH changes in the range of pH 3.8 to 7.0. This could be an advantage in food formulations with an acidic pH, such as mayonnaise, cheese and yoghurt. The insensitivity of the ALB-F/WPI mixture, as well as its high G', could be a result of the solubility of albumins at acidic pH. An additional explanation for the small pH-effect could be the high mineral (i.e. salt) content, present in ALB-F (Table 1). Like pH, salt screens the charges of protein, and greatly reduces the effect of electrostatic repulsion. To verify this, the influence of salt on the three mixtures was studied.

The effect of ionic strength on the heat-set gelation of WPI itself has been the subject of different studies. It has been reported that at pH's away from the isoelectric point, the concentration at which gels can be formed (i.e. critical gel concentration) decreases with increasing ionic strength. At pH 7 the critical gel concentration decreases from around 55 g/L to 10 g/L when the ionic strength increased from 0.02 to 0.09 M NaCl, where after the critical gel concentration did not decrease further (Renard & Lefebvre, 1992). Also, the heat-set gel strength of WPI increases with increasing $CaCl_2$ or NaCl concentrations (Hermansson, 1972; Lorenzen et al., 2006; Schmidt, Illingworth, Ahmed, & Richter, 1978; Schmidt, Illingworth, Deng, & Cornell, 1979). Fig. 9 shows the G′ of the pea fraction/WPI mixtures as function of ionic strength. The initial ionic strengths varied between the mixtures, with ALB-F/WPI mixtures already starting at 88 mM (NaCl equivalent). The PPC/WPI and GLB-RF/WPI mixtures had an initial ionic strength of 37 and 30 mM (NaCl equivalent), based on their conductivities. An increase of the ionic

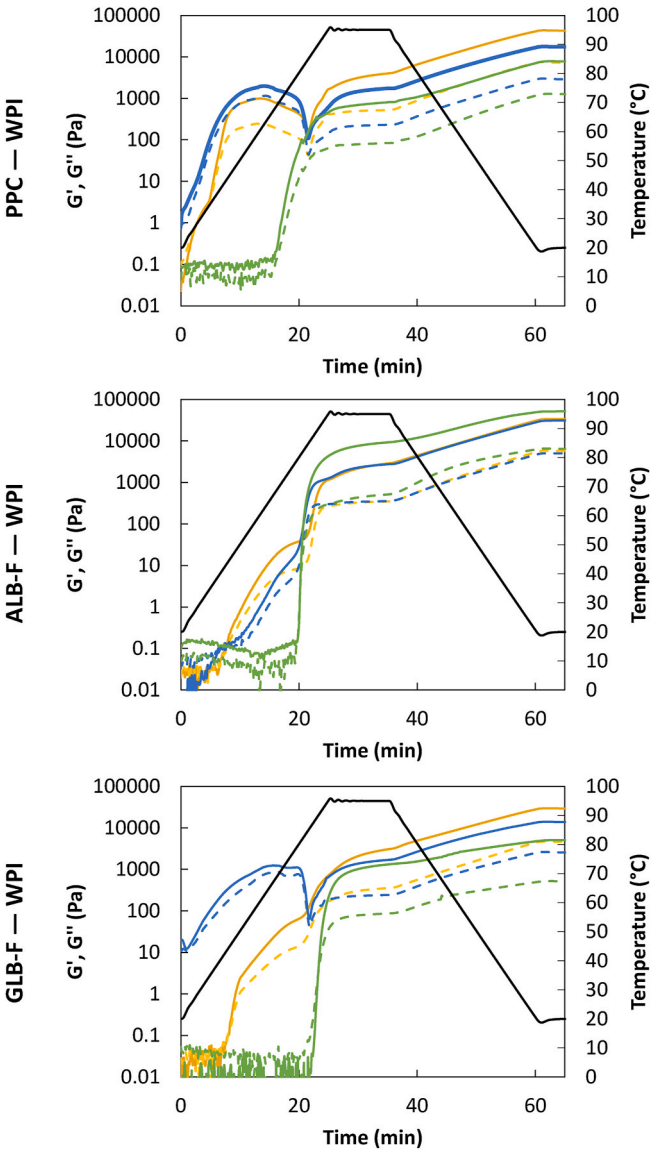

Fig. 8. Mixtures of pea protein concentrate (PPC), albumin fraction (ALB-F) and globulin fraction (GLB-RF) with whey protein isolate (WPI) at pH 3.8 (—), 5.0 (—) and 7.0 (—). The pea and whey proteins were mixed in a ratio of 1:3. G′ is represented by a solid line and G'' by a dashed line. All samples were measured in duplicate and a representative curve is shown.

Table 3
Comparison between the elastic moduli (G′) and dissipation factors (tan δ (= G"/G')) of WPI combined with PPC, ALB-F and GLB-F and with the previously studied pea protein isolates PPIp (precipitated), PPId (diafiltrated) and PPIc (commercial) (Kornet, Shek, et al., 2021) in a 3:1 ratio. Please note that GLB-RF and PPIp followed the same fractionation process, but that different pea fraction and WPI batches are compared.

	PPC	ALB-RF	GLB-RF	PPIp	PPId	PPIc
G' (kPa)	6.4 $^{\pm 0.0}$	54.4 $^{\pm 2.0}$	4.9 $^{\pm 0.3}$	6.1 $^{\pm 0.4}$	5.3 $^{\pm 0.5}$	3.5 $^{\pm 0.1}$
Tan δ	0.11 $^{\pm 0.00}$	0.13 $^{\pm 0.00}$	0.10 $^{\pm 0.00}$	0.11 $^{\pm 0.00}$	0.17 $^{\pm 0.01}$	0.12 $^{\pm 0.00}$

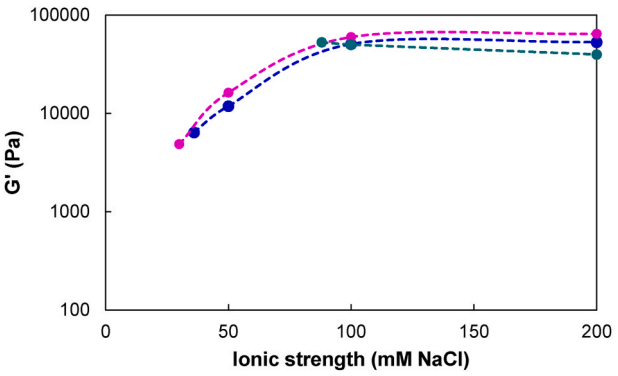

Fig. 9. The G′ of heat-set gels from mixtures of PPC (—), ALB-F (—) and GLB-RF (—) with whey protein isolate (WPI) in a 1:3 ratio as function of salt concentration. The initial ionic strength of the mixtures was calculated from the measured conductivities. NaCl was added to increase the ionic strengths to 50, 100 and 200 mM NaCl. The total dry matter content was 15 wt % and the dispersions were adjusted to pH 7 prior to gelation. All samples were measured in duplicate and average G′ values are shown. The error bars, representing standard deviations, were smaller than the data points.

strength to 100 mM NaCl led to a G′ increase for PPC/WPI and GLB-RF/WPI mixtures. This ionic strength range could not be measured for the GLB-RF/WPI mixtures, as the initial ionic strength was too high. From 100 mM NaCl onwards, the G′ of all mixtures showed a small decrease with increasing ionic strength. This result suggests that the initial high salt content in ALB-F plays a major role in the gel firmness of the mixture, as for the mixtures with PPC and GLB-RF the G′ mostly increases up to 100 mM NaCl. A similar effect was reported for WPI gels, where the critical concentration was affected by ionic strength till around 100 mM NaCl (Renard et al., 1992). It is thus plausible that the effect of ionic strength on WPI has a large impact on the behaviour of the mixtures. This makes us conclude that after standardization on ionic strength, all three pea fractions could serve equally well as WPI substitute at pH 7 in terms of gel firmness, leading to G′ values of around 50 kPa.

3.6. Covalent interactions in pea – whey proteins mixtures

In addition to weaker physical forces (i.e. electrostatic interactions, hydrophobic interactions and hydrogen bonding), covalent intermolecular disulphide bonding can also contribute to gelation, through formation of permanent chemical crosslinks within a gelled network (Dickinson & Chen, 1999). For WPI it has been reported that disulphide bonding plays an important role in gelation, both heat-induced (Shimada & Cheftel, 1989; Visschers & de Jongh, 2005) and acid-induced (Alting, Hamer, de Kruif, & Visschers, 2000). For pea globulins, disulphide bonding plays a limited role in the gel formation and hence has little to no effect on the elastic modulus after gelation (Kornet, Veenemans, et al., 2021; O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004; Sun et al., 2012). The role of disulphide bonding may however be affected by fractionation – more specifically by alkaline extraction – as the thiol pKa value of most cysteine residues range between 8 and 9 (Jensen, Hansen, & Winther, 2009). Above the pKa, deprotonated thiols can participate in sulfhydryl-disulfide exchange reactions. The pea fractions in this study were all exposed to alkaline extraction, but it is noted that a different fractionation route may influence the occurrence of disulphide bonding upon heat-set gelation.

The ability of pea albumins to form disulphide bonds has not been studied before, possibly because these proteins only comprise less than 30% of the total seed protein. However, PA1 and PA2 albumins collectively contain about 50% of the total sulphur amino acids in the pea seed (Higgins et al., 1986). Hence, we elaborate further on disulphide bonding in pea albumin in this study. Fig. 10 shows that the resulting gel firmness was significantly lower ($P < 0.05$), with a G′ value of 128 Pa (±37 Pa) when disulphide bonding was inhibited, compared with 540 Pa (±34 Pa) for the ALB-RF without NEM. The initial gelling behaviour upon heating was not affected, but upon cooling the G′ increase was less pronounced. This means that disulphide bonding affects heat-set gelation of pea albumins at the conditions studied. Also, WPI showed reduced gelling in the presence of NEM. The abrupt sol/gel transition, typical for WPI, changed into a more gradual G′ increase upon heating. However, a steeper increase was seen upon cooling, which eventually led to an average G′ value of 2.4 kPa. This is lower compared to the situation without NEM, where the G′ of WPI reached an average value of 7 kPa. The major difference between WPI and ALB-RF, with respect to the contribution of disulphide bonding, is the stage of the temperature cycle where G' increases. When disulphide bonding is inhibited, it is mostly upon heating that gelation is affected for WPI, whereas for ALB-RF it is mostly upon cooling. This difference in the stage at which disulphide bonding plays a role, is not necessarily temperature-dependent, but could also be time-dependent (i.e. disulphide bonding starts later in ALB-RF than in WPI).

For the mixtures, disulphide bonding plays a role in the gelation kinetics and gel firmness also, as shown in Fig. 11. The PPC/WPI mixtures showed a more gradual increase, upon NEM addition, in the heating stage. Also, the average G′ value of the gel is lower (2 kPa) than without NEM (12 kPa). This indicates that disulphide bonding is an essential contributor to gelation of the PPC – WPI mixture. Disulphide bonding may occur both between whey proteins, and whey proteins and pea albumins. In the latter case, a larger difference would be expected in the ALB-F/WPI mixture. Here, the average G′ values reduced from 50 kPa to 9 kPa in the presence of NEM. This large reduction in G′ indicates that disulphide bonding plays a crucial role in these mixtures indeed, and makes it plausible that disulphide bonding occurs between WPI and ALB-F constituents, as the G′ reduction in the WPI gel itself was much lower. For the GLB-RF – WPI mixtures a relatively small effect of NEM was seen. The average G' value reduced from 5 kPa to 3 kPa. This small

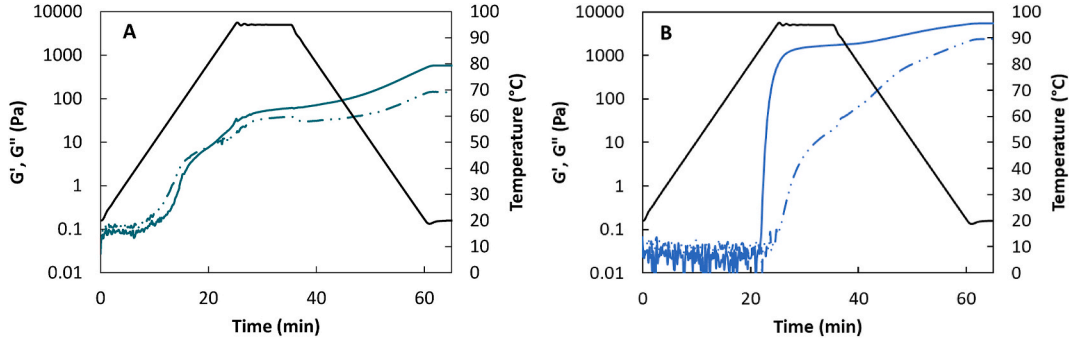

Fig. 10. Heat-induced gelation of ALB-RF (A) and WPI (B) with disulphide bonding (solid line) and without (dashed line). Prior to gelation the dispersions were standardized on 15 wt % dry matter and adjusted to pH 7. All samples were measured in triplicate and a representative curve was selected.

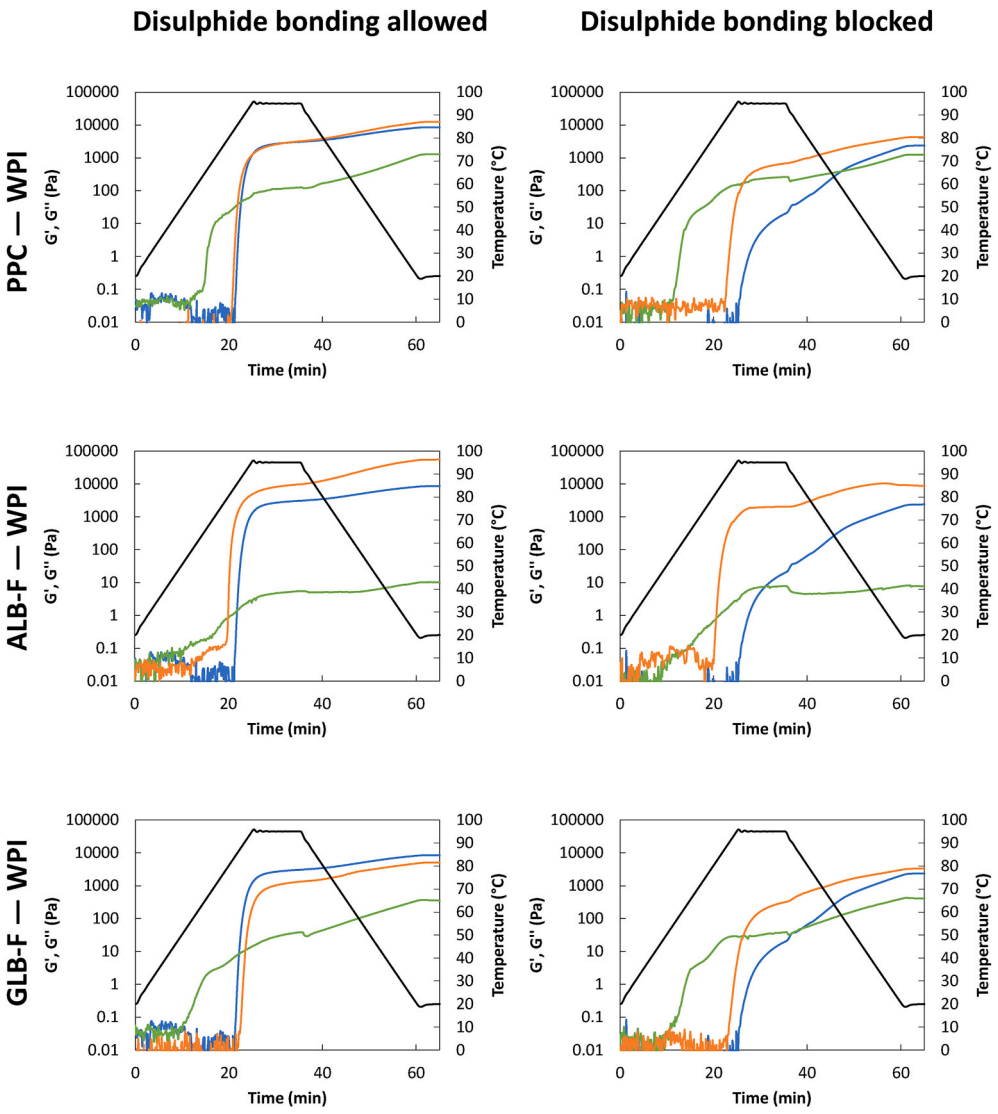

Fig. 11. Temperature sweeps of the pea fractions (PPC, ALB-F and GLB-RF) (), WPI () and mixtures of pea fraction and WPI in a 1:3 ratio (), 15 wt % dispersed at pH 7. In the left situation disulphide bonding was allowed to occur and in the right situation disulphide bonding was blocked by the thiol blocking agent N-Ethylmaleimide. All samples were measured in duplicate and a representative curve was selected.

reduction (36%) is probably a result of the inhibition of whey protein disulphide bonding, since NEM hardly affected globulin gelation.

4. Conclusion

In this study we evaluated the pea albumin and globulin gelling behaviour, both as individual gelling agents and in mixtures with whey protein isolate. By doing so, the ability of pea protein fractions to replace whey protein in heat set gels was quantified. It was found that the pea protein concentrate – a mixture of albumins and globulins – and the albumin-rich fraction formed firmer gels per mass of protein than the globulin-rich fraction, but none of the pea fractions studied were able to provide a similar gel firmness as whey proteins. When part of whey protein isolate was substituted by the pea fractions, it turned out that mixtures of albumins with whey proteins showed the most stable gelling behaviour over a range of pH and ionic strengths. The other fractions also gave high gel firmness, but were more sensitive to pH and ionic strength changes. The finding with albumins is especially interesting as this fraction is currently an underutilized by-product from the conventional pea protein fractionation process. The use of this by-product as a WPI substitute will enhance the resource efficiency of peas. These results may guide both researchers and food manufacturers, to optimize plant protein fractionation processes and to bring most value to legumes such as pea, as a source for functional ingredients.

CRediT authorship contribution statement

Remco Kornet: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Visualization, Writing – original draft. **Simone Penris:** Methodology, Investigation. **Paul Venema:** Supervision, Conceptualization, Writing – review & editing. **Atze Jan van der Goot:** Conceptualization, Writing – review & editing. **Marcel B.J. Meinders:** Conceptualization, Funding acquisition, Writing – review & editing. **Erik van der Linden:** Conceptualization, Project administration, Funding acquisition, Resources, Supervision, Writing – review & editing.

Acknowledgements

The authors want to thank Helene Mocking-Bode and Irene van den Hoek for their contribution in obtaining the pea protein fractions. This research project is organised by and executed under the auspices of

TiFN, a public - private partnership on precompetitive research in food and nutrition. The authors have declared that no competing interests exist in the writing of this publication. Funding for this research was obtained from Unilever Research and Development Wageningen., Nutricia Research B·V., Bel S.A., Pepsico Inc., and the Top sector Agri&Food.

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