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Substitution of whey protein by pea protein is facilitated by specific fractionation routes

Remco Kornet [a,b], Carol Shek [b], Paul Venema [b,*], Atze Jan van der Goot [c], Marcel Meinders [a,d], Erik van der Linden [b]

[a] TiFN, P.O. Box 557, 6700, AN Wageningen, the Netherlands
[b] Laboratory of Physics and Physical Chemistry of Foods, Wageningen University, Bornse Weilanden 9, 6708, WG Wageningen, the Netherlands
[c] Laboratory of Food Process Engineering, Bornse Weilanden 9, 6708, WG Wageningen, the Netherlands
[d] Food and Biobased Research, Wageningen University and Research, 6700, AA Wageningen, the Netherlands

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ABSTRACT

In this study we investigated the effect of different aqueous fractionation processes on the suitability of pea protein isolates (PPI) to substitute whey protein isolate (WPI) in heat-set gels. We found that a milder fractionation process based on diafiltration was successful in substituting WPI, yielding similar gel strength (i.e. elastic modulus) at a range of concentrations. Three different pea protein isolates were analysed, one obtained using diafiltration (PPId), another obtained using isoelectric precipitation (PPIp), and a commercial one (PPIc) as a reference. The isolates PPIp and PPId contained mainly native proteins, whereas the proteins in PPIc were denatured. PPId had a protein solubility almost similar to that of WPI at pH 7, while PPIp and PPIc were less soluble. PPIp and PPIc had better thickening capacities, larger aggregate/particle sizes and higher viscosities compared to PPId. After heat-induced gelation all PPI's showed similar or higher gel strength than WPI between a 7–13 wt % protein concentration. Between 13 and 15 wt % PPId showed a similar gel strength compared to WPI. Above 15 wt % WPI formed the strongest gels. It was concluded that PPId can fully replace WPI up to protein concentrations of 15 wt %. For mixtures of WPI with the other PPI's, it turned out that up to half of the WPI could be replaced by any of the PPI's without compromising on gel strength. This makes us conclude that PPI is a suitable substitute for WPI in heat-set gels.

1. Introduction

Regarding the ongoing transition from dairy to plant proteins, different scientific fields and technological routes are currently explored. One route is to completely exchange dairy proteins by plant proteins. Another route is a partial replacement of dairy proteins by plant proteins, resulting in hybrid food products. The latter approach might put less constraints on the plant protein functionality, amongst others, due to the fact that synergistic functional effects can occur in such systems (Alves & Tavares, 2019; Hinderink, Münch, Sagis, Schroën, & Berton-Carabin, 2019; Jose, Pouvreau, & Martin, 2016; William Nicholas Ainis, 2017).

The potential of exchanging dairy by plant proteins depends on the functionality of the proteins. Different studies showed that mild or limited fractionation can not only yield proteins with at least similar properties than those extensively fractionated (Geerts, Mienis, Nikiforidis, van der Padt, & van der Goot, 2017; Ruiz, Arts, Minor, & Schutyser, 2016), but also require less resources (van der Goot et al., 2016). Another study found that varying the processing pH in soy protein fractionation processes can alter functional properties such as protein solubility, water holding capacity, and viscosity (Peng, Kersten, Kyriakopoulou, & van der Goot, 2020). For pea protein it was found that protein purification was unnecessary to achieve stable oil-in-water emulsions, as pea flour was able to stabilize oil-in-water emulsions equally well as pea protein concentrate (Sridharan, Meinders, Bitter, & Nikiforidis, 2020). In addition, it was found that the extent of aqueous fractionation determines the viscosity, solubility and gelling behaviour of the resulting protein-enriched ingredients. We found that pea proteins obtained through isoelectric precipitation can lead to substantially thickening of the dispersion, compared to for instance whey protein (Kornet et al., 2020). By estimating the volume to mass ratio, it was concluded that pea proteins are, at least partially, present as aggregates

* Corresponding author.
E-mail address: paul.venema@wur.nl (P. Venema).

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with a rarefied structure. Limited fractionation of pea was also found to yield pea protein concentrates with better gelling ability, compared to extensively fractionated pea protein isolate (Kornet et al., 2021). It is therefore suggested that pea can be used to derive plant protein isolates with similar functionalities as dairy proteins, making pea protein isolates suitable for replacement of dairy proteins, provided that the fractionation process is optimised for that purpose.

Generally, whey proteins form stronger gels than plant proteins, including pea protein (Wong, Vasanthan, & Ozimek, 2013). In case strong gelation is required, partial replacement of whey protein could be an approach. As such, understanding the synergistic or antagonistic effects in these plant dairy protein mixtures is relevant. Hence, there have been a number of studies that focusses on substituting an animal-derived protein, such as whey protein or casein, by a plant-derived protein. It has been reported that blending whey protein isolate (WPI) with soy protein isolate (SPI) and wheat gluten increased the viscosity of WPI (Onwulata, Tunick, & Mukhopadhyay, 2014), which could be beneficial when aiming for a thickening effect in beverages. For mixtures of micellar casein with soy protein in a 1:1 ratio, it was found that rheological behaviour (i.e. viscosity as function of temperature), was closer to soy than to casein (Beliciu & Moraru, 2011). A contrasting result was observed for heat-set gels from WPI–SPI blends, where WPI seemed to dictate the gel strength. Even so, the gel strengths generally reduced with an increased portion of soy protein (Jose et al., 2016; McCann, Guyon, Fischer, & Day, 2018) and also phase inversion has been reported (Comfort & Howell, 2002). Rheological gelling behaviour could also be influenced by homogenizing certain components, prior to gelation (Grygorczyk, Duizer, Lesschaeve, & Corredig, 2014), or by varying the gelling technique, such as sequential gelling of mixed systems (Ersch, ter Laak, van der Linden, Venema, & Martin, 2015) or acid-induced gelation (Martin, Marta, & Pouvreau, 2016; Roesch & Corredig, 2006).

Only a limited number of studies reported the heat-induced gelling behaviour or co-aggregation of mixtures from pea protein with whey protein. Previous research on salt-extracted pea and whey protein mixtures showed an increase in the elastic modulus, hardness, and minimum gelling concentration at a pea/whey ratio of 2:8 in heat-set gels, relative to pure whey protein systems. Limited enhancement was seen at pH 4 and 8, but significant synergistic enhancement was seen at pH 6 (Wong et al., 2013). Another study on heat-induced aggregation of whey and soy protein mixtures concluded that these proteins could interact, and that the ratio of soy to WPI had major impact on the type of network that was formed (Roesch & Corredig, 2005). Co-aggregation was also seen for β-lactoglobulin and pea globulins mixtures, where β-lactoglobulin seemed to dominate the sizes and molecular weights of the aggregates (Chihi, Mession, Sok, & Saurel, 2016).

In this study we use yellow pea as a model system to investigate how fractionation can facilitate the substitution of whey protein by plant protein. Three pea protein isolates are compared: one fractionated using diafiltration, another fractionated using isoelectric precipitation and a commercial pea protein isolate as a reference. The functionalities of these pea protein isolates are examined and compared to whey protein isolate. In addition, mixtures of the pea protein isolates with whey protein are studied.

2. Materials and methods

2.1. Materials

Yellow pea seeds were obtained from Alimex Europe BV (Sint Kruis, The Netherlands). WPI (BiPro, Davisco, Switzerland) and PPIc (NUTRALYS, s85 F, Roquette, France) were used as received. All chemicals and reagents were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

2.2. Methods

2.2.1. Yellow pea fractionation processes

Three different pea protein isolates were used in this research and two of them were produced in the laboratory. One protein isolate is obtained using protein precipitation (PPIp), another is purified using diafiltration (PPId) and a commercial pea protein isolate (PPIc) was used as a reference.

PPIp was obtained by a process earlier described (Kornet et al., 2020), and here denoted as process 1. In that previous study PPIp was labelled as fraction 5 and fractions 1 to 4 represented less processed fractions. In short, 10% (w/v) pea flour was dispersed in deionized water and the pH was adjusted to 8 by adding NaOH. The dispersion was stirred for 2 h and centrifuged at 10000 g for 30 min to remove solids (i.e. starch granules, cell wall material). The resulting supernatant was exposed to a protein isoelectric precipitation step, where the solution was brought to pH 4.5 and centrifuged again (10000 g, 30 min). The protein-rich pellet was re-dispersed at pH 7 for 2 h and freeze-dried afterwards.

PPId was produced using an alternative fractionation process, here denoted as process 2. Pea flour was dispersed in deionized water for 2 h, with the pH left unadjusted (~pH 6.7). Then the dispersion was centrifuged at 10000 g for 30 min and the supernatant was collected and further fractionated by diafiltration at room temperature with a SartoJet Pump (Sartorius AG, Goettingen, Germany). A flow parallel to two 5 kDa Hydrosart membrane (Sartorius AG, Goettingen, Germany) surfaces was applied with an inlet pressure of 2.2 and an outlet pressure of 1.8 bar. The cellulose based membranes were non-protein binding, and had a filter area of 2×0.1 m^2. At the start of the diafiltration process the supernatant was diluted with an equivalent amount of water. During diafiltration the filtrate, with mostly sugars and peptides, was discarded and the retentate was recirculated for five hours. To maintain diafiltration efficiency, water was added when the retentate became too concentrated, eventually leading to a total dilution factor of about 8. After diafiltration the concentrated retentate was collected and freeze-dried.

All fractionation steps were conducted at room temperature and the obtained protein-enriched solutions were frozen and freeze-dried. Dried protein isolates were stored at −18 °C. The ash content was determined by heating weighted samples to 550 °C in a furnace and weighing the ash afterwards. The protein content was calculated from the nitrogen content, measured with a Flash EA 1112 series Dumas (Interscience, Breda, The Netherlands). Nitrogen conversion factors of 5.7 for PPI and 6.38 for WPI were used. The protein recovery was defined as the recovered amount of protein in the protein isolate divided over the initial amount of protein in the flour. All subsequent measurements with the pea protein isolates were performed after re-dispersing the samples in deionized water and adjusting the pH to 7 by addition of NaOH or HCl, unless stated otherwise.

2.2.2. Solubility

The solubility of the different protein isolates in deionized water at pH 7 was determined by centrifugation. Dispersions of 2 wt % protein isolate were prepared and stirred for 2 h, after which they were centrifuged at 15000 g for 30 min. The obtained supernatants and pellets were freeze-dried. The dry matter solubility is expressed as the mass of solids in the supernatant divided by the initial mass of solids. The protein solubility was determined by dividing the mass of proteins in the supernatant over the initial mass of the solids in solution.

2.2.3. Size exclusion chromatography (SEC)

The protein composition of the pea protein isolates was determined with an Akta Pure 25 chromatography system (GE Healthcare, Diegem, Belgium) coupled to an UV detector. First a McIlvaine buffer was prepared with 10 mM citric acid, 20 mM Na2HPO4 and 150 mM NaCl, adjusted to pH 7 and filtered over 0.45 μm. Samples were prepared by

dissolving 10 g protein/L in the McIlvaine buffer and centrifuged at maximum speed for 10 min. The supernatants were transferred to HPLC vials. The samples were eluted on a Superdex 200 increase 10/300 GL column (Merck, Schnelldorf, Germany) with a range of 10–600 kDa and the McIlvaine buffer as eluent. Proteins were detected at an UV wavelength of 280 nm. For identification of the proteins based on their molecular weight, a calibration curve was prepared with molecules of known molecular weights: Aldolase, Blue Dextran, Carbonic Anhydrase, Conalbumin, Ferritin, Ovalbumin and Ribonuclease.

2.2.4. SDS-PAGE

The protein composition of the different protein isolates was determined by SDS-PAGE. Gel electrophoresis was performed using a 4–12% Bis Tris gel with a MES SDS running buffer. First, the samples were prepared by dissolving 0.1 wt % protein isolate in deionized water. For non-reducing conditions, 45 μl running buffer was added to 15 μl sample solution. For reducing conditions, 6 μl running buffer was replaced by 6 μl of a 500 mM dithiothreitol (DTT) solution. The Eppendorf tubes with solutions were vortexed and centrifuged afterwards for 5 min (Hermle Z306, 4500 rpm). The solutions, either with or without DTT, were heated to 70 °C for 10 min and allowed to cool down to room temperature afterwards. Then, 15 μl of the supernatants were loaded in each well. A marker of 2.5–200 kDa was loaded in a well at both sides of the gel. Electrophoresis was performed in a Xcell Surelock Mini-Cell for 35 min at a constant voltage of 200 V. Subsequently, the gels were stained with SimplyBlue SafeStain and washed with a 20% NaCl solution afterwards. The stained gels were scanned with a Bio-Rad GS900 gel scanner the next day.

2.2.5. Mineral composition

The mineral composition of the different protein isolates were analysed by the Chemical Biological Soil Laboratory (CBLB) of Wageningen University in The Netherlands. The freeze-dried protein isolates were first heated in a microwave in the presence of HNO_3 and concentrated HCl to destruct organic compounds. Then H_2O_2 was added and the samples were heated again to remove nitrous fumes. Subsequently, the elements in the samples could 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).

2.2.6. Differential scanning calorimetry (DSC)

The denaturation temperatures of the different protein isolates were determined using DSC. Around 30–40 mg of 10 wt % protein solutions in deionized water, adjusted to pH 7, were transferred to high volume pans. The samples were measured with a TA Q200 Differential Scanning Calorimeter (TA Instruments, Etten-Leur, The Netherlands) upon heating from 20 to 120 °C with incrementing temperature of 5 °C/min. All samples were measured in triplicate and subsequent data processing was done with TA Universal Analysis software.

2.2.7. Viscosity

After dispersing the protein isolates in deionized water and adjusted the pH to 7 with 1M NaOH and HCl, the viscosity of the protein solutions was measured with an MCR302 Rheometer (Anton Paar, Graz, Austria) combined with a sand-blasted CC-17 concentric cylinder geometry. The shear viscosity was measured as a function of shear rate varying from 0.1 to 1000 s^{-1} at 20 °C. A shear rate of 54.2 s^{-1} was selected for comparison of viscosities, as this was the minimum shear rate where all viscosities could be measured reliably. All samples were measured in duplicate.

2.2.8. Particle size analysis

Samples were prepared by dispersing 0.1 wt % of the protein isolates in deionized water and the pH was adjusted to 7 using 0.1 M NaOH or HCL. The samples were measured with a Zetasizer Ultra (Malvern, Worcestershire, United Kingdom) at 25 °C, using dynamic light scattering (DLS). The volume-based particle size distributions were obtained from the ZS Explorer software. All samples were measured in duplicate.

2.2.9. Small amplitude oscillatory shear (SAOS)

Gelation of the protein isolates, dispersed in deionized water and adjusted to pH 7, was induced by applying a temperature sweep with an MCR302 rheometer (Anton Paar, Graz, Austria). The sample was transferred to a CC-17 concentric cylinder that was sand-blasted, to prevent wall slip. With this measure taken, no sign of wall slip was observed. Solvent evaporation upon heating was prevented by placing a solvent trap on top of the outer cylinder. During the temperature sweep the samples were heated from 20 to 95 °C at a rate of 3 °C/min. The samples were kept at 95 °C for 10 min and cooled back to 20 °C with a same rate. Finally, the sample was kept at 20 °C for 5 min to verify that there was no further gel maturation. The viscoelastic response to an oscillatory imposed stress at a frequency of 1 Hz and a strain of 1% was recorded. In addition, strain sweeps were applied to confirm that the linear viscoelastic regime was not exceeded by the 1% strain applied during the temperature sweep. To study the effect of disulphide bonding by the use of a thiol-blocking agent, deionized water was replaced by a 20 mM N-Ethylmaleimide (NEM) solution, and the pH was also adjusted to 7. All samples were measured in duplicate.

The rheological parameters used in this study to describe the gels are the storage modulus (G′), loss modulus (G″) and the loss factor tan δ (G”/G′). G’ and G” represent the elastic and viscous portion of the viscoelastic behaviour and tan δ described the ratio of these two portions. A material can be considered a solid when tan $\delta < 1$ and a strong solid when tan $\delta \ll 1$.

2.2.10. Covalent labelling of WPI

WPI was covalently labelled with fluorescein isothiocyanate (FITC) based on a method described earlier (Sağlam, Venema, de Vries, & van der Linden, 2013). First a WPI solution of 1 wt % in 0.1 M carbonate buffer (pH 9) was prepared. Then another solution of 0.4 w/v % FITC solution in DMSO was made. Subsequently, 50 μl of the FITC solution per mL of WPI solution was slowly added upon gentle stirring. The sample was incubated in the dark for 6h and after incubation the WPI solution was dialysed using dialyses membranes with 12–14 kDa pore size. Dialysis was performed in the dark at 4 °C for ~72h and water was refreshed once a day. The solution was then freeze-dried and the powder was stored in the dark at −18 °C. The covalently labelled WPI is further referred to as WPI-FITC.

2.2.11. Confocal laser scanning microscopy (CLSM)

Protein solutions were prepared by dissolving 15 wt % protein isolate and the pH was adjusted to 7 with 1 M HCl or NaOH. The proteins in the single PPI and WPI solutions were labelled non-covalently to Rhodamin B using a final concentration of 0.0003% of the fluorescent dye. PPI was labelled in the same way for the combined systems with WPI. Subsequently, WPI was added to these solutions in final ratios of 1:3, 2:2 and 3:1, where 1 wt % of the WPI was replaced by WPI-FITC. After 2h of solubilization the protein solutions were transferred to sealed glass chambers (Gene frame 65 μl adhesives, Thermo Fisher Scientific, United Kingdom) and heated in a water bath at 95 °C for 15 min and cooled back to room temperature afterwards. The microstructures were visualized using a Leica SP8X-SMD confocal microscope (Leica, Amsterdam, The Netherlands), coupled to a white light laser. A dry objective (10x, 0.40) and water immersion objectives (20x, 0.70 and 63x, 1.20) were used for magnification. For the PPI samples labelled to Rhodamin B, the laser excitation wavelength and the filter emission wavelength were 540 nm and 580 nm, respectively. For the combined samples imaging was performed in sequential mode. Rhodamin B was now excited at 561 nm and the emitted signal was detected between 570 and 790 nm. FITC was excited at 488 nm and the signal was acquired between 500 and 570 nm.

2.2.12. Statistical analysis

All measurements were conducted at least in duplicate. The mean values are shown and the standard deviations are given as a measure of error. Claims regarding significant effects were supported by a Welch's unequal variances *t*-test performed in R, applied on independent samples (i.e. at least two different PPI batches). Significance was concluded when $P < 0.05$.

3. Results and discussion

3.1. General characterization

Table 1 shows the protein content, protein recovery and solubility of the different protein isolates. The protein contents are from the protein isolate batches used in this study, whereas the recovery and solubility are averages of multiple extraction processes ($n \geq 2$). In a previous study it was found that the carbohydrate content of the protein isolates was typically below 4 wt %, and were mainly present as small sugars (Kornet et al., 2020). In most cases there is a trade-off between purity and recovery in plant protein extraction (Loveday, 2020), but here PPId displays both a higher purity and a higher protein yield. The reason for a higher purity and yield of PPId is that both the globulins and albumins are retained. The protein composition of the PPI's will be discussed in more detail in the next section.

3.1.1. Pea protein isolate compositions

The protein composition of the different pea protein isolates was studied by size exclusion chromatography (SEC). Pea contains two major groups of proteins, which are globulins and albumins. Globulins comprise legumin (11S), vicilin (7S) and convicilin (7-8S). The latter is highly homologous with vicilin, but contains an extended N terminus (Barac et al., 2010; O'Kane, 2004; O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004a). At pH 7 legumin is mainly present as a hexamer with a molecular weight of 320–380 kDa. These hexamers consist of six subunits that are non-covalently bound, with each subunit consisting of an acidic and basic subunit. At pH 7 Vicilin is mainly present as trimer of ~170 kDa and convicilin in its native form has a molecular weight of 280–290 kDa. The latter can be present as homo- or heterotrimers with convicilins and vicilins (Barac et al., 2010; Croy, Gatehouse, Tyler, & Boulter, 1980; Lam, Can Karaca, Tyler, & Nickerson, 2018). Pea albumin (PA) comprises a group of proteins, including PA1, PA2, lectin, lipoxygenases and protease inhibitors (Park, Kim, & Baik, 2010). PA1 and PA2 are most abundant and are commonly present as dimers. PA1 dimers are comprised of PA1a and PA1b and have a combined molecular weight of 10 kDa. PA2 can be subdivided in PA2a and PA2b and form homodimers with molecular weights of 53 kDa and 48 kDa respectively (Higgins et al., 1986).

The two peaks in Fig. 1 that are denoted as albumins are only present in PPId and correspond to PA2 (left) and PA1 (right), with retention volumes of 20.5 and 22.9 mL respectively. These albumins are hydrophilic (Lu, Quillien, & Popineau, 2000) and remain soluble upon isoelectric precipitation (Yang et al., 2020), which is why they are absent in PPIp and PPIc. The three globulin peaks correspond to legumin, convicilin and vicilin with retention volumes of 15.5, 16.5 and 17.6 mL respectively. They appear for PPId and PPIp, but not for PPIc. The latter only shows a peak at a lower retention volume of 12.7 mL, which corresponds to a molecular weight of ~2700 kDa. This single peak indicates that nearly all globulins in PPIc are aggregated. This is likely to be an underestimation, as larger aggregates were filtered out before bringing the samples on the column. This is in line with the SDS-PAGE profiles. Fig. 2a shows the gel where all non-covalent bonds are broken by the addition of SDS. Fig. 2b shows the gel where also the disulphide bonds are broken by the addition of DTT. The presence of globulin bands in Fig. 2a indicate that aggregates observed in Fig. 1 are formed from non-covalently bound pea globulins. It has been reported that the legumin acidic subunit (40 kDa) and basic subunit (20 kDa) are covalently linked by one or more disulphide bonds (Gatehouse, Croy, & Boulter, 1980). This is also confirmed by the band at 60 kDa visible in Fig. 2a but not visible in Fig. 2b, where disulphide bonds were broken by DTT. Fig. 2 also confirms the previous statement that PPId contains pea albumins, whereas PPIp and PPIc only contain globulins.

Table 1
Protein content, protein recovery, overall solubility and protein solubility of the protein isolates. Protein recovery is defined as the percentage of protein that was recovered in the PPI after fractionation. Dry matter and protein solubility are defined as the percentage of dry matter or protein that remained in the supernatant after centrifugation at pH 7. The recovery and solubility of PPIp and PPId are the averages of ≥2 fractionation processes. The numbers in superscript represent the standard deviations.

	Protein content (g/100 g d.m.)	Protein recovery (%)	Dry matter solubility (%)	Protein solubility (%)
PPIc	78.7 $^{\pm 1.0}$	–	32.2 $^{\pm 1.9}$	28.1 $^{\pm 1.3}$
PPIp	83.0 $^{\pm 0.7}$	52 $^{\pm 7.3}$	77.2 $^{\pm 8.8}$	79.4 $^{\pm 8.0}$
PPId	88.3 $^{\pm 3.3}$	63 $^{\pm 2.1}$	91.4 $^{\pm 4.0}$	94.0 $^{\pm 8.0}$
WPI	100 $^{\pm 1.0}$	–	100 $^{\pm 0.8}$	100 $^{\pm 0.8}$

3.1.2. Mineral composition

The mineral composition of the protein isolates is shown in Table 2. It can be observed that PPId is particularly rich in the multivalent ions such as Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}. PPId is also high in the monovalent ion K^{+}, but substantially lower in Na^{+} compared to PPIc and PPIp. The high potassium content present in the pea seed is retained in process 2 and probably bound to phytate. K-phytate is readily water soluble and may be discarded in process 1 during the precipitation step (Brown, Heit, & Ryan, 1961; Crean & Haisman, 1963). The higher phosphorus content origins from phytic acid, which serves as a phosphorus storage during seed dormancy (Samotus, 1965). Its ability to chelate divalent ions such as Ca^{2+} and Mg^{2+} (Crean et al., 1963) can also explain the higher contents of these minerals in PPId, as phytic acid remains in process 2 and is discarded in process 1. The high sodium contents of PPIc and PPIp can be explained by the use of NaOH for pH adjustments in the precipitated isolates. The mineral composition of WPI is similar to what has been reported elsewhere (Cornacchia, Forquenot de la Fortelle, & Venema, 2014).

3.1.3. Protein nativity

Differential scanning calorimetry (DSC) was conducted to determine whether the proteins were still native after processing. The resulting temperatures of denaturation onset and denaturation peak as well as the heat enthalpies are shown in Table 3. Protein denaturation of WPI starts at 63.6 °C (±0.37) and the peak denaturation temperature is observed at 76.2 °C (±0.02). A shoulder is visible (i.e. flatter slope followed by a steeper slope) in the denaturation peak, which is in line with what has been reported elsewhere (Fitzsimons, Mulvihill, & Morris, 2007). The shoulder starting at 63 °C and the endotherm that is centred at around 75 °C can be assigned to denaturation of α-lactalbumin and β-lactoglobulin respectively (Boye & Alli, 2000). PPIc does not show any endothermic peaks, suggesting complete denaturation. The fractionation conditions used to obtain PPIc are not known, but different studies reported that commercial pea protein isolate is generally denatured and display low solubilities (Adebiyi & Aluko, 2011; Sun & Arntfield, 2010; Taherian et al., 2011). Those observations are consistent with ours and is expected to be caused by harsh processing conditions (i.e. higher temperatures, pH changes, isoelectric precipitation). The lab-extracted PPIp and PPId still contain native protein, evidenced by clear denaturation peaks. The denaturation peaks shown for PPIp and PPId are rather similar. These single peaks comprise the thermal effects of both vicilin and legumin denaturation, based on literature in which it was reported that denaturation of those proteins occur at 71.8 °C and 87 °C respectively (O'Kane et al., 2004a; O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004b). The heat enthalpies for the protein denaturation in PPIp

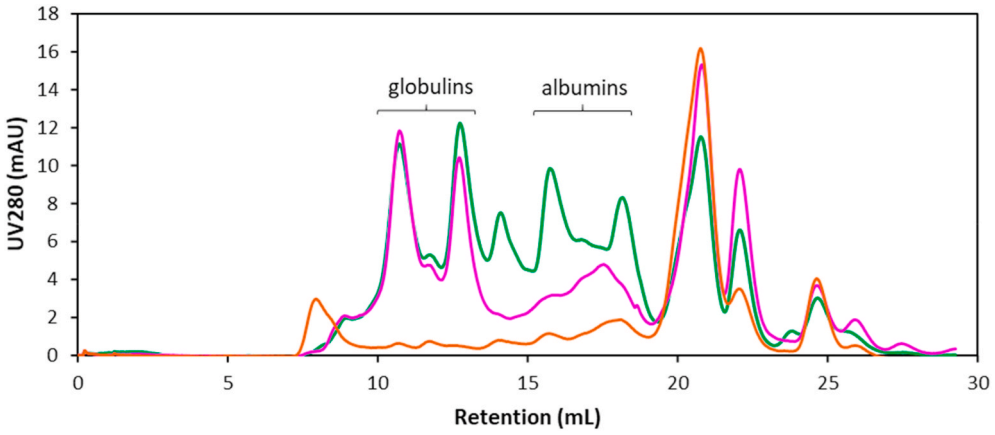

Fig. 1. SEC chromatogram of PPIp (—), PPIc (—) and PPId (—) with UV detection at 280 nm as function of retention volume.

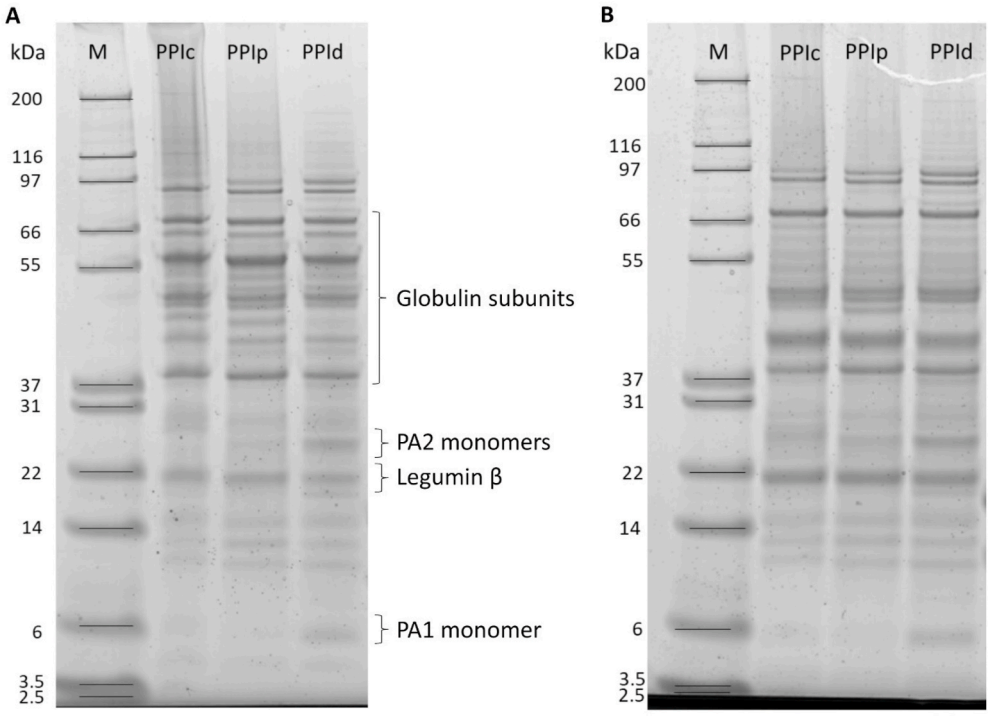

Fig. 2. SDS-PAGE profiles of the pea protein isolates under non-reducing conditions (a) and reducing conditions (b). Lane M indicates the protein marker from 2.5 to 200 kDa. Identification of the bands is based on multiple studies (Gatehouse et al., 1980; Higgins et al., 1986; O'Kane, 2004; Rubio et al., 2014).

Table 2
The total ash content and mineral composition (g/kg) of the pea seed and dried protein isolates.

	Ash	Ca^{2+}	Cu^{+}	$Fe^{2+/3+}$	K^{+}	Mg^{2+}	Mn^{2+}	Na^{+}	P^{3+}	Zn^{2+}
Pea	33	0.62	0.01	0.06	10.4	1.07	0.01	0.01	4.53	0.04
PPIc	36	0.69	0.02	0.12	4.1	0.78	0.02	10.3	9.83	0.08
PPIp	46	0.44	0.01	0.22	2.2	0.41	0.02	16.4	14.9	0.04
PPId	59	2.09	0.04	0.24	14.4	4.25	0.06	1.37	18.6	0.11
WPI	12	0.88	0.00	0.00	0.4	0.06	0.00	6.31	0.61	0.00

and PPId are quite similar, with 9.0 J/g (±0.4) and 8.0 J/g (±0.9), respectively. It can be concluded that the precipitation and diafiltration processes yield proteins that are still native and show similar denaturation temperatures.

3.2. Viscosity of the protein isolates

In this section we discuss the viscosities (at a shear rate of 54.2 s^{-1}) and particle size distributions of the protein isolates. The viscosity is a relevant functionality and can give an indication on the protein voluminosity or state of aggregation. Fig. 3a shows that PPIc has the highest viscosity per mass of protein, followed by PPIp, and the lowest viscosity

Table 3
Denaturation onset temperatures, denaturation peak temperatures and endothermic heat enthalpies of the protein isolates heated from 20 to 120 °C. The samples were measured in triplicate and standard deviations are shown in superscript.

Protein isolate	T_{onset} (°C)	T_d (°C)	ΔH_d (J/g protein)
WPI	63.6 $^{\pm 0.37}$	76.2 $^{\pm 0.02}$	11.7 $^{\pm 0.6}$
PPId	70.5 $^{\pm 0.66}$	82.5 $^{\pm 0.13}$	8.0 $^{\pm 0.9}$
PPIp	73.0 $^{\pm 0.29}$	82.9 $^{\pm 0.11}$	9.0 $^{\pm 0.4}$
PPIc	-	-	-

is seen for PPId. Fractionation processes that include pH changes (i.e. solubilization at pH 8 and precipitation at pH 4.5) and higher temperature, expected to be applied to PPIc, enhance the viscosity. These higher viscosities for PPIc and PPIp are also consistent with the particle size distributions, shown in Fig. 3b. The aggregates observed in PPIc and PPIp comprise both soluble and insoluble aggregates, and are probably a result of isoelectric precipitation (Cui et al., 2020; Kornet et al., 2020; Tanger, Engel, & Kulozik, 2020). Inherent to isoelectric precipitation is that protein-protein interactions are induced at a net charge of around zero. These interactions may be partially irreversible upon re-dispersion at neutral pH. Moreover, phytic acid present in pea could contribute to the formation of aggregates, as they can bind to proteins below pH 5 (Carnovale, Lugaro, & Lombardi-Boccia, 1988; Maga, 1982). Aggregates in PPIc and PPIp have a higher effective volume than single protein molecules. A higher volume leads to more friction and hence increases viscosity. Aggregates also explain the lower solubility of the isolates shown in Table 1. WPI and PPId contain fewer aggregates and are more soluble than PPIc and PPIp. In summary, fractionation processes in which pH and temperature are varied, yield protein isolates with lower solubilities, higher viscosities and larger protein aggregates. PPId that was obtained using diafiltration, shows a viscosity, solubility and particle size distribution most similar to WPI.

3.3. Gelling behaviour of the protein isolates

3.3.1. Elastic moduli after heating

Fig. 4a shows that the elastic modulus (G′) is higher for PPId than PPIp and PPIc in a protein concentration range of 7–17 wt%. Even at protein concentrations of 11 wt % PPIp, PPIc and PPId already behave as weak solid materials with loss factors (tan δ) of 0.325, 0.302 and 0.203 respectively. In Fig. 4a, WPI shows a strong increase in G′ at a protein concentration of 11 wt %, which identifies the gelling concentration at pH 7. Below this gelling concentration G′ shows a steep increase with concentration. The concentration dependencies beyond the gelling concentration of WPI is rather similar to PPIp and PPIc, but the G′ at the gelling concentration (13 wt %) is much higher for WPI and is caused by an abrupt sol/gel transition (as shown later in Fig. 6b). This is related to the type of network being formed. Gelling of WPI involves disulphide-mediated polymerization, which occurs when heating WPI above 85 °C at a pH between 3 and 7 (Monahan, German, & Kinsella, 1995). For the pea protein isolate gels it is claimed that disulphide bonding does not play a major role (Sun & Arntfield, 2012). We verified the role of disulphide bonding by using 20 mM of the thiol-blocking agent N-Ethylmaleimide (NEM). Fig. 4b shows the temperature sweeps with and without NEM for WPI and PPId. For WPI, preventing disulphide bonding

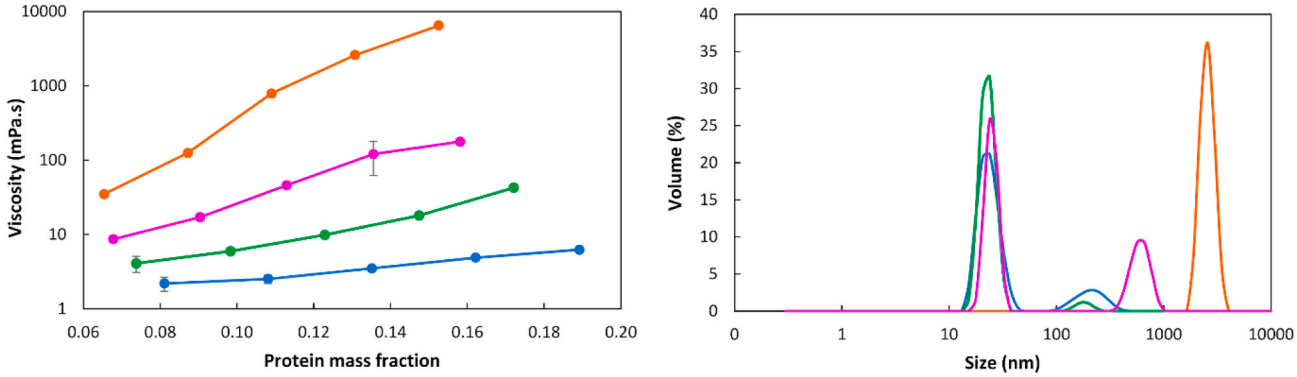

Fig. 3. Viscosity (at a shear rate of 54.2 s-1) as function of protein mass fraction (a) and particle size distributions of WPI (—), PPIp (—), PPIc (—) and PPId (—) solutions, measured at pH 7 (b). Standard deviations are presented as error bars. The viscosity was measured at 52.4 s^{-1}; the minimum shear rate where all viscosities could be measured accurately.

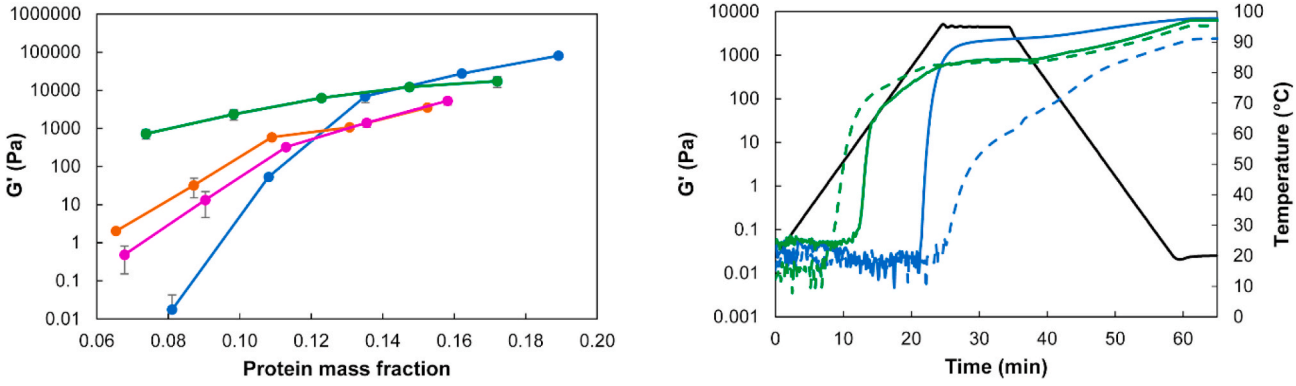

Fig. 4. Elastic moduli (G′) of the heat-set gels from WPI (—), PPIp (—), PPIc (—) and PPId (—), measured at 1% strain and 1 Hz, as function of protein mass fraction (a). Temperature sweeps of 15 wt % WPI (—) and PPId (—) with (dashed line) and without (solid line) the thiol-blocking agent NEM (b). Samples were measured at least in duplicate and standard deviations are presented as error bars in 4a. Representative curves are shown in 4b.

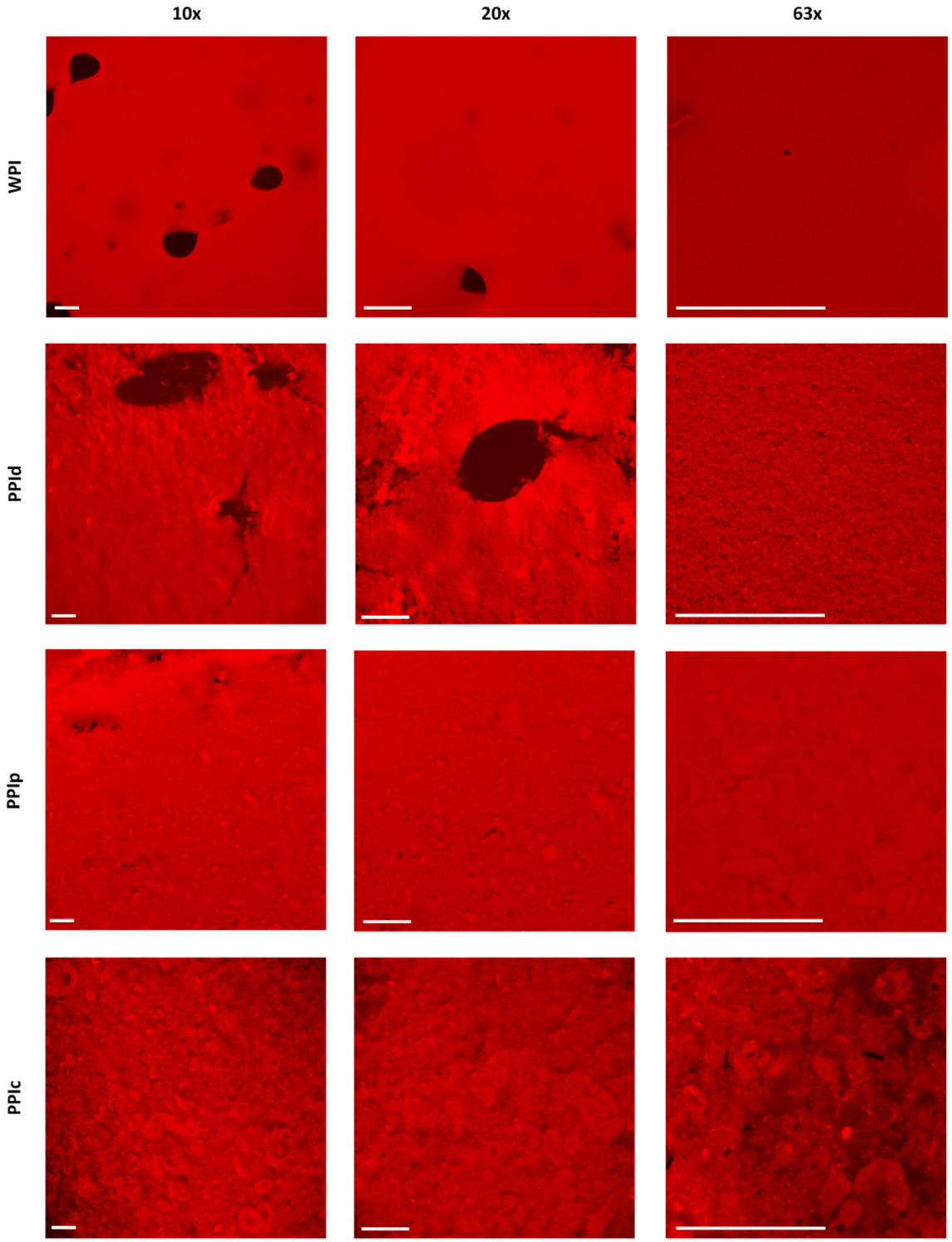

Fig. 5. CLSM images visualizing the microstructures of heat-set gels from the protein isolates (15 wt %, pH 7) at three magnifications, with protein shown in red. The white scale bar represents 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

results in a different viscoelastic behaviour during heating. During the first heating stage gelling is virtually absent and G′ only starts to increase during the 10 min holding time at 95 °C and upon cooling. It appears that disulphide bonding affects the kinetics of gelation mostly, and to lesser extent the G′ after heat treatment. PPId is less affected by the presence of a thiol blocking agent. The final G′-values are similar, although the gelation of PPId with NEM starts slightly earlier compared to the PPId without NEM. PPIp with NEM showed the same trend as the one without (data not shown in Fig. 4b). The small difference between PPIp and PPId may be caused by the albumins present in the latter, as pea albumins are more abundant in sulphur groups than pea globulins (Schroeder, 1984). In conclusion, disulphide bonding is a major contributor to the gelation of WPI at pH 7, whereas disulphide bonding does not play a major role for PPI gels, independent of the fractionation method applied.

The difference between the G′ of PPId and PPIp was tested for

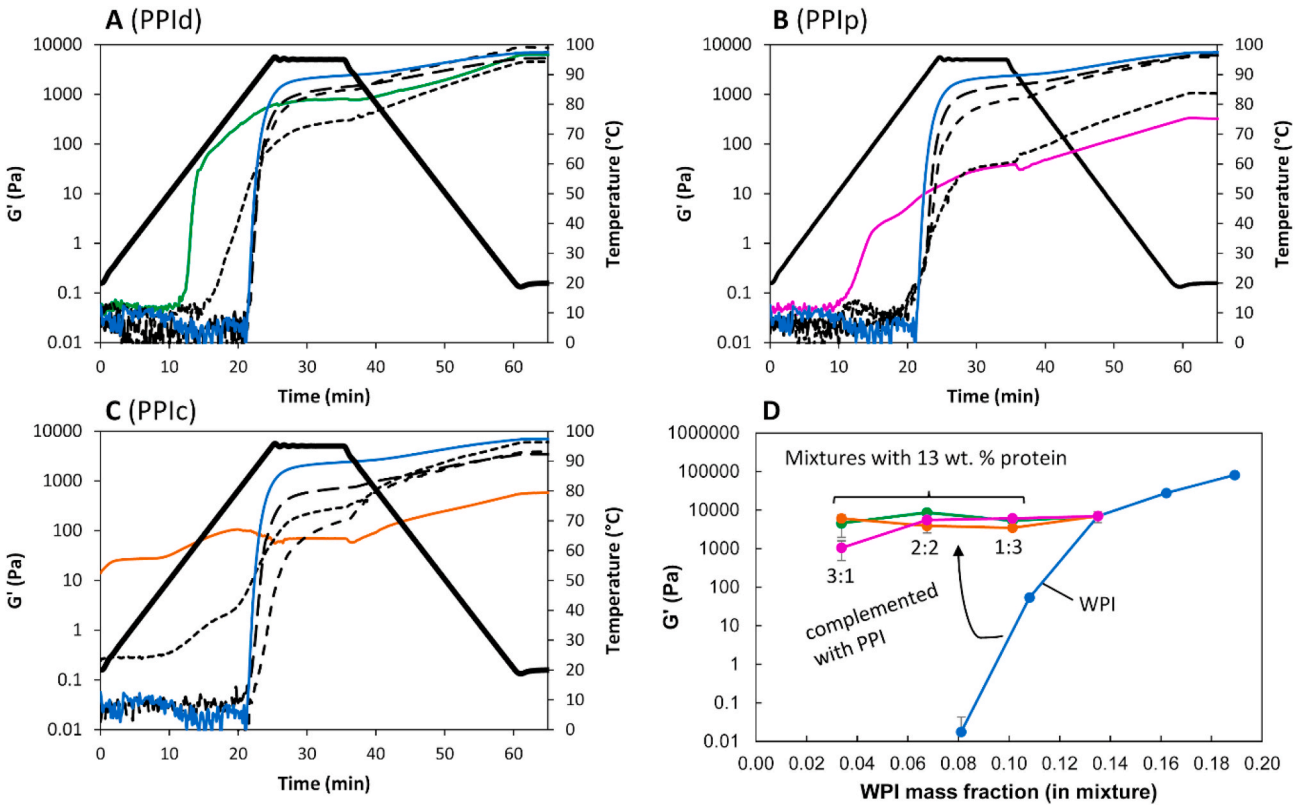

Fig. 6. Temperature sweeps of 15 wt % pea and whey protein mixtures at pH 7. The dashed line interval length is in incrementing order PPI: WPI (3:1, 2:2, 1:3) with WPI (—), PPIp (—), PPIc (—) and PPId (—) (a–c). G′ of the gels as function of whey protein concentration in the mixtures with a total of 15 wt % protein isolate, where the blue line represents the pure WPI gels (d). Samples were measured at least in duplicate and representative curves are shown (6a-c) or standard deviations are shown with error bars (6d).

significance at a concentration of 15 wt %, by measuring the G' ($n \geq 4$) of PPI from multiple fractionation processes ($n \geq 2$). Process 1 and 2 yielded PPIp and PPId gels with significantly ($P < 0.05$) different G'. In previous research it was found that isoelectric precipitation reduces the capacity of pea protein to form strong gels (Kornet et al., 2021), which is probably related to the formation of protein aggregates, as discussed before in section 3.1 and displayed in Fig. 3b. Protein aggregates are more abundantly present in PPIp and PPIc. Upon heating, these aggregates have less interaction sites per volume of protein compared to non-aggregated protein, which impairs the gelling capacity. PPId shows very limited aggregation and also forms gels with higher G' throughout the concentration range tested. How these differences are reflected in the microstructures is discussed in next section.

3.3.2. Microstructure

The gels produced from 15 wt % dry matter were further characterized by analysing their microstructures using confocal laser scanning microscopy (CLSM). Fig. 5 shows that the microstructure of the WPI gel is homogeneous, which is indicated by the lack of variation in contrast. It has been reported that whey protein, particularly β-lactoglobulin, forms gels after heating at pH > 6 and low salt content (~0.1%), due to the formation of long, fine strands (Langton & Hermansson, 1992). Another study (Mulvihill, Rector, & Kinsella, 1990) showed that coarser gels are formed with increasing salt content. Fig. 5 shows a homogeneous WPI gel without any particles at microscale, which is related to the low salt content in the systems.

There are major differences in gel microstructures between the pea protein isolates. PPId form the most homogeneous gels at microscale. PPIp forms a more heterogenous gel with larger protein particles (5–10 μm) that contain higher quantities of protein than the surrounding, as indicated by the higher intensity of red. Even larger particles (10–100 μm) are seen for PPIc. These particles probably correspond to the largest PPIc particles of the size distribution shown in Fig. 3b. The heterogeneity and larger particles probably weaken the gelled systems of PPIp and PPIc, as the protein within these particles cannot actively contribute to a space-spanning network. This is consistent with their lower G' values, as discussed in section 3.2.1.

3.4. Gelling behaviour of pea and whey protein mixtures

3.4.1. Elastic moduli upon heating

WPI was combined with the three PPI's in ratios of 1:3, 2:2 and 3:1 and the gelation behaviour of those mixtures was studied. Fig. 6a–c show the temperature sweeps of mixtures with PPId, PPIp and PPIc (black dashed lines) as well as the single PPI and WPI systems (coloured lines). A few conclusions can be drawn with respect to the gelling behaviour of PPI-WPI mixtures.

Firstly, Fig. 6a–c show that the final G′ values of the 2:2 and 1:3 mixtures, represented by the black lines with longer intervals, are similar to the G′ values of a pure WPI gel. For PPIc and PPId this is even true for the 3:1 ratio. This implies that at least half of the WPI can be replaced by any of the PPI's without compromising on the G′ of the gel at the conditions studied (pH 7, 13 wt % protein). This is also visualized in Fig. 6d, where the G′ remains fairly constant for the combined systems with PPIc and PPId, where WPI is substituted up to 75%. It shows that pea protein isolate is a suitable substitute for whey protein isolate, as it can maintain the G′ of WPI even when half or more is replaced.

Secondly, with increasing WPI concentrations the gelation onset moves from ~60 °C to 80 °C. As discussed in section 3.1.3, pea protein starts to denature around 72 °C with a peak at 83 °C, whereas WPI starts to denature at 64 °C and shows a peak at 76 °C. The gelation onset temperature is higher than the denaturation temperature. This indicates

that denaturation of α-lactalbumin is not sufficient to induce gelation. Denaturation of β-lactoglobulin, starting at 75 °C (Boye et al., 2000), is required for WPI to form a gel. In case of PPI, reaching its denaturation onset temperature (~70 °C) is sufficient to increase the G'. The higher gelation onset temperature of WPI, relative to its lower denaturation temperature (Table 3), is also related to the neutral pH and low ionic strength (Fitzsimons et al., 2007). The ionic strength is estimated to be around 60 mM, based on the mineral content shown in Table 2. This is likely to be an overestimation as not all minerals may be present as ions in solution (e.g. Ca^{2+} can be bound to α-lactalbumin). At 80 °C it is evident that disulphide bonding plays a major role in the sol/gel transition, as indicated by Fig. 4b. There the presence of a thiol-blocking agent inhibits this abrupt sol/gel transition. The observed gelation temperature for WPI is consistent with another study (Monahan et al., 1995) where gelation of whey proteins was observed to start at 80 °C at pH 7. The authors claimed that this temperature was required to sufficiently unfold whey proteins and induce disulphide bonding and hydrophobic interactions, and ultimately form a gelled network. For the mixtures of PPI and WPI analysed in this study, it appears that the gelation onset of dispersions with WPI concentrations ≥50% is similar to that of 100% WPI. This implies that WPI aggregation, mediated by disulphide bonding and hydrophobic interactions, is essential for these combined proteins to form a gelled network.

Thirdly, the initial G′ before heating decreases with increasing WPI concentrations in the mixtures with PPIc and to lesser extent PPIp. For these protein isolates it was observed that the G′ is higher than the G″, even before heating (G" not shown here). This would indicate some kind of network already present. In mixtures with more WPI than PPI the pea protein aggregates are diluted to such extent that the G' before heating is as low as it is for pure WPI. When less than 50% of the protein is WPI, the pea proteins in PPIc and PPIp preserve the capacity to form some type of network that is able to store energy upon deformation.

Overall, the mixtures with different PPI's approach the rheological behaviour of pure WPI. This is true for any of the PPI's until 50% substitution and for PPId and PPIc even up to 75% substitution. Also the final gel strength (i.e. elastic modulus) of these mixtures is similar to that of a WPI gel. These findings make us conclude that PPI is a suitable substitute for WPI in heat-set gels.

3.4.2. Microstructure

Fig. 7 shows the microstructures of gels containing both PPI and WPI. The yellow to orange coloured regions represent PPI and the green

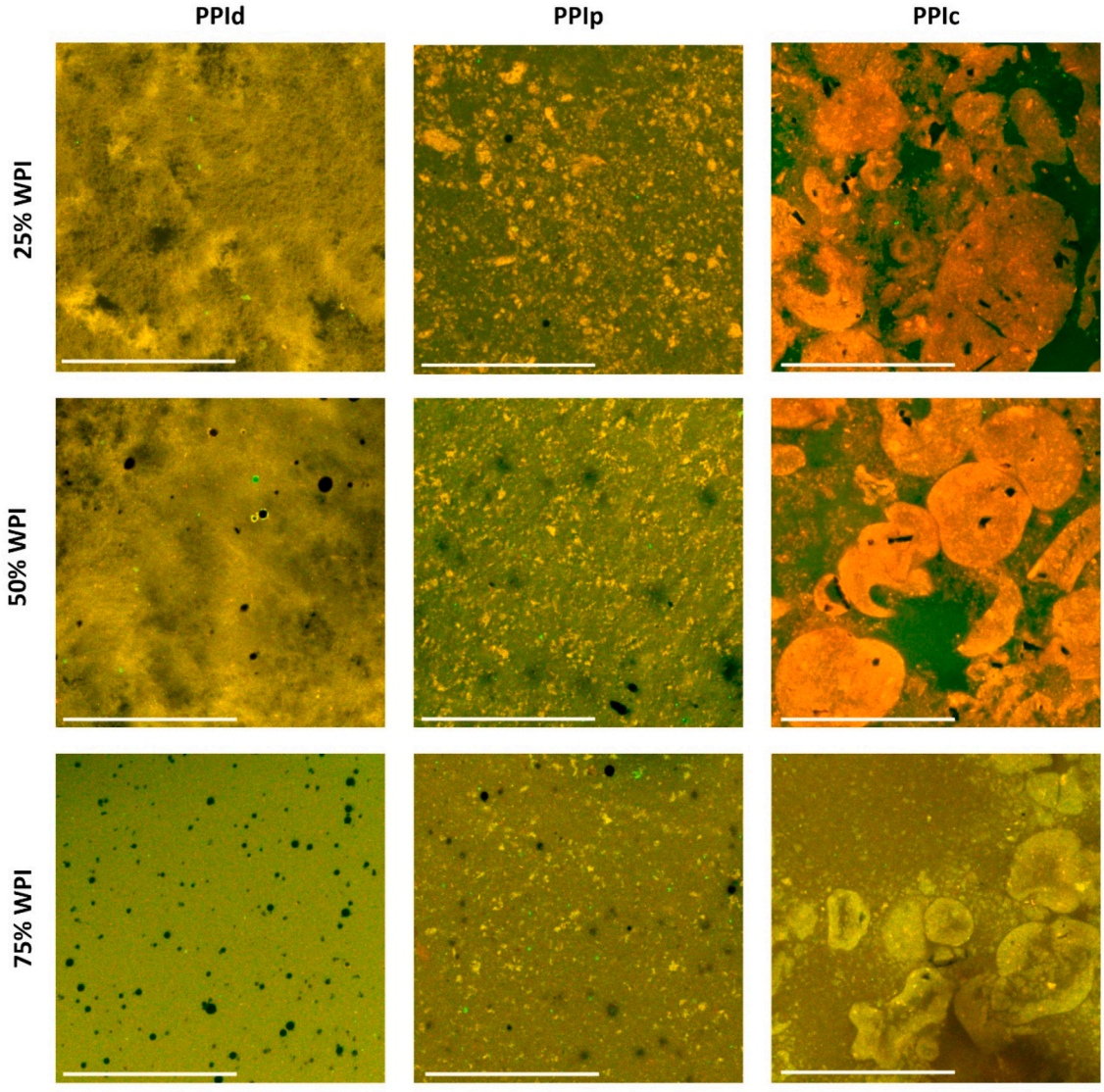

Fig. 7. CLSM images visualizing the microstructures of the pea - whey protein mixtures at 63x magnification. PPI is visualized by higher intensities of red and WPI by higher intensities of green. The white scale bar represents 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

regions represent WPI. Rhodamine B was used to label the pea protein, as it binds to hydrophobic patches of the protein (Bartasun, Cieśliński, Bujacz, Wierzbicka-Woś, & Kur, 2013; Ersch et al., 2016), and hence is expected to have higher affinity for pea than for whey protein due to its hydrophobic nature (Kornet et al., 2021). Colour intensities between pea and whey protein could vary between images and hence these images should not be used for any type of quantification. However, the images provide insight on the distribution of pea and whey proteins in the gel, where higher intensities of green correspond to regions with higher concentrations of WPI and higher intensities of red correspond with higher concentrations of PPI.

The mixtures of WPI with PPId form homogeneous gel structures at microscale, which show great similarities with homogeneous structures obtained for gels containing only PPI or WPI. (Fig. 5). The mixtures with PPIp show micro-phase separation (Fig. 7), with clusters ranging from 5 μm (25% PPI) to 20 μm (75% PPI). These clusters have relatively high pea protein concentrations and are dispersed in a continuous matrix that is relatively low in pea protein. For PPIc-WPI mixtures, large clusters up to ~100 μm, high in pea protein concentration are seen. For these samples, it was noted that the gel was heterogeneous and that there were also regions with smaller clusters. The regions with smaller clusters looked similar to the case of PPIp and WPI mixtures with 75% WPI (Fig. 7).

Fig. 7 shows both proteins distributed on a micrometre scale. There are a few possibilities how these gels containing both PPI and WPI behave at nanoscale, explaining the gelation behaviour. In the mixtures with PPI, whey protein could form a continuous network with pea protein (aggregates) incorporated. A similar observation was made for mixed gels from soy and whey protein (Comfort et al., 2002; McCann et al., 2018) In those studies, it was concluded that whey protein formed the primary protein network with soy protein incorporated as particulate fillers. It is also possible that WPI interacts and co-aggregates with PPI. We hypothesize that this co-aggregation takes place when WPI is mixed with PPId, which contains mostly small protein molecules that are highly reactive at their gelation onset (Fig. 6a). For future research, it would be relevant to study the molecular interactions between whey and pea proteins to understand or even predict the gel network nano-structure of such plant-dairy protein gels. Furthermore, this research could be extended towards proteins from other pulses (e.g. chickpea, lupin, lentil), as there are resemblances between the proteins and their fractionation processes. Finally, our current research could inspire food producers to consider the history of fractionation processes that have led to a given protein fraction of plant based matter, in order to be able to tailor the replacement of animal protein ingredients by plant based protein ingredients, while at the same time ensuring minimal processing energy usage with a maximum of (multi-) functionality.

4. Conclusion

In this study we showed that protein fractionation processes have major impact on the functional bulk behaviour of pea proteins. Harsher processing (i.e. pH shifts, higher temperatures) yields protein isolates with lower solubility, higher viscosities and lower elastic moduli of the gels. In view of replacing whey (i.e. animal-based) proteins, one can optimize the protein fractionation process of plant based proteins by using diafiltration instead of precipitation. In the case of pea protein, this yields a plant protein isolate that approaches the functional behaviour of whey protein isolate. Diafiltrated pea protein isolate has comparable solubility and viscosity as whey protein isolate. In heat-set gels it actually can replace whey protein isolate in forming gels with similar strength. When it comes to partial replacement it is less crucial which type of fractionation process is used, as for different pea protein isolates WPI dominates the rheological behaviour at concentrations above 50% WPI.

Author statement

Remco Kornet: Conceptualization, Methodology, Investigation, Validation, Formal Analysis, Visualization, Writing – Original Draft. **Carol Shek:** Methodology, Investigation. **Paul Venema:** Supervision, Methodology, Conceptualization, Writing – Review & Editing. **Atze Jan van der Goot:** Conceptualization, Writing – Review & Editing. **Marcel Meinders:** Conceptualization, Funding Acquisition, Writing – Review & Editing. **Erik van der Linden:** Conceptualization, Project Administration, Funding Acquisition, Resources, Supervision, Writing – Review & Editing.

Declaration of competing interest

We confirm that this work is original and has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. All the authors have approved the manuscript and agree with submission to your esteemed journal. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. The authors have declared that no competing interests exist.

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