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Abstract

This study examined the thermal gelation of mixtures of laboratory-prepared pea protein isolate (PPI) and egg white proteins (EWP) at different PPI/EWP weight ratios (100/0, 75/25, 50/50, 25/75, 0/100) at pH 7.5 and 9.0. Viscoelastic and texture properties of the composite gels, along with the microstructure and molecular interactions involved in the gel network, were investigated. Except PPI-EWP 100/0 at pH 9.0, all systems gelled with increasing gel hardness, springiness and cohesiveness when EWP content increased. This phenomenon was explained by the microstructure of the gels, wherein the presence of PPI enhanced the formation of aggregates embedded in the EWP network, thus loosening it. The rheological properties of the mixed gels were primarily influenced by the EWP network, significantly involving disulfide bonds. However, upon the addition of PPI, hydrogen bonds and hydrophobic interactions predominated and the structure of the gel became more sensitive to pH as electrostatic repulsions interfered. Playing on the ratio of PPI/EWP allows for the production of gels with varying textures, and the data suggest the possibility of partially substituting egg white with pea proteins in food gel formulation.

Keywords: gelling properties, egg white protein, pea protein isolate, CLSM, dissociation agent, texture

1. Introduction

To meet the increasing demand for protein, there is a necessity to expand the range of plant-based protein products. Consequently, the use of combinations of plant and animal proteins has become increasingly appealing in the formulation of high-protein food products due to economic advantages, as well as nutritional, functional, and organoleptic properties (Chihi, Sok, & Saurel, 2018; McCann, Guyon, Fischer & Day, 2018). Egg white proteins have been extensively utilized in the food industry because of their ability to form gels with favorable nutritional and texture properties (Mine, 1995; Valverde et al., 2016; Li, Zhang et al., 2018). This natural protein mixture is rich in ovalbumin (OVA) (~54%), ovotransferrin (OVT) (~12%), ovomucoid (~11%), and lysozyme (LYS) (~3.4%) (Mine, 2002; Guha, Majumder, & Mine, 2019). The gelation of egg whites is a complex process involving protein denaturation, aggregation, and the formation of a gel network (Mine, 1995). The characteristics of egg white gels mainly depend on the medium conditions such as pH, ionic strength, and the type of salts (Croguennec, Nau, & Brulé, 2002; Nasabi, Labbafi, Mousavi, & Madadlou, 2017). The gelation of egg proteins has been described as a two-step process: i) changes in protein structure or partial denaturation; ii) additional aggregation of denatured proteins, resulting in an exponential increase in viscosity and the formation of a continuous three-dimensional network (Alleoni, 2006). During gel formation, non-covalent bonds (i.e., hydrophobic interactions during heating and hydrogen/ionic bonds during cooling) and covalent disulfide bonds develop, coordinating the aggregation of unfolded chains of polypeptides (Campbell et al.,

2003; Razi et al., 2022). In previous studies, Raikos, Campbell, and Euston (2007) reported that increasing pH and the addition of NaCl resulted in higher gelation temperatures of egg white proteins.

As an alternative to animal proteins, pulse proteins such as yellow pea (Pisum sativum L.) proteins are gaining attention due to their lower price, allergen-free, and gluten-free composition (Aluko, Mofolasayo, & Watts, 2009; Havemeier, Erickson, & Slavin, 2017; Alves & Tavares, 2019; Burger & Zhang, 2019). Pea seeds contain four main protein fractions: globulins (55-65% of total proteins), soluble in saline solutions; albumins (18-25%) soluble in water; prolamins (4-5%) soluble in hydroalcoholic solutions, and glutelins (3-4%) soluble in highly alkaline solutions (Lu et al., 2019). Pea globulins are oligomeric storage proteins, composed of legumin (11S) with a hexameric structure of 360 – 400 kDa. It contains 6 monomers (~60 kDa), linked by non-covalent interactions. Each monomer consists of an acidic polypeptide subunit of ~40 kDa and a basic subunit of ~20 kDa, connected by a disulfide bond (Barać et al., 2010; Shand, Ya, Pietrasik, & Wanasundara, 2007). Vicilin (7S) is a trimeric glycosylated protein with a molecular weight of 150 – 200 kDa, distinguished by the absence of cysteine, preventing it from participating in intramolecular or intermolecular disulfide bond formation (Shewry, Napier, & Tatham, 1995). Each monomer (~50 kDa) has two cleavage sites, possibly generating small fragments during pea seed development: α (~20 kDa), β (~13 kDa), γ (~12-16 kDa), αβ, and βγ polypeptides (Liang & Tang, 2013; Shand et al., 2007; Tzitzikas et al., 2006). A third minor 7S globulin, convicilin, is a multimeric protein of 210-290

kDa formed by weak interactions association of monomers (~71 kDa). This non-glycosylated protein has a nearly identical amino acid profile (80%) to vicilin. During the heating process, proteins undergo unfolding and aggregation until self-supporting networks are formed. Multiple types of molecular interactions, such as hydrogen bonds, dipole−dipole interactions, hydrophobic, and electrostatic interactions, are involved during the thermal aggregation and gelation of pea globulins (Sun & Arntfield, 2012; Shand et al., 2007). The contribution of disulfide bonds in these heat-induced phenomena seems limited (O'kane et al., 2004a & b; O'kane et al., 2005; Sun & Arntfield, 2012; Mession, Chihi, Sok, & Saurel, 2015).

The gelation property is one of the crucial functional aspects of proteins, providing unique textures, sensations, and flavors for food products (Zhang et al., 2019; Harfmann, 2016). While the gelation of single protein systems is well-documented, there is less available data on the gelation mechanism of protein mixtures from different sources. Nevertheless, a few studies have explored the heat-induced gels formed by combinations of egg and plant proteins, such as egg white with soy proteins (Su et al., 2015); whole egg or egg yolk proteins with soy proteins (Zhang et al., 2019); egg white with hempseed proteins (Alavi, Emam-Djomeh, & Chen, 2020). Co-gelation of pea and animal proteins has primarily involved milk proteins and has mostly focused on acid gels (Mession, Roustel, & Saurel, 2017; Ben-Harb et al., 2018; Chihi, Sok, & Saurel, 2018; Oliveira et al., 2022), with fewer studies on heat-induced gels (Wong, Vasanthan, & Ozimek, 2013; Silva, Balakrishnan, Schmitt, Chassenieux, & Nicolai, 2018). To our knowledge, no study has examined the heat-

induced gelation of egg white and pea protein mixtures. Previous research demonstrated that the presence of PPI decreased the storage modulus (G') of heated PPI-EWP mixtures during heating, and protein interactions altered the thermal denaturation temperature of OVT, LYS, and pea legumin (Kuang et al., 2023a). However, more experimental data are needed to better understand the importance of various forces in network formation in this complex system. Therefore, the present study aimed to investigate the comparative gelling, texture, and microstructure properties of composite gels based on mixed PPI-EWP at various weight ratios (100/0, 25/75, 50/50, 75/25, 0/100) at pH 7.5 and 9.0. Additionally, the intermolecular interactions involved in the heat-induced composite gels were also examined.

2. Materials and methods

2.1 Samples preparation

Fresh eggs were sourced from a local market in Dijon (France), stored at 4 °C, and utilized 15 days prior to the expiration date. The fresh liquid egg white was meticulously separated from the egg yolk and chalaza. The resulting egg white was then transferred to a beaker and gently homogenized using a magnetic stirrer for 2 hours at room temperature. The total protein content of the egg white was determined using the Kjeldahl method (N=6.25), yielding a value of 10.2% w/w on a dry basis. Pea globulins were extracted from smooth yellow pea flour (P. Sativum L.) supplied by Cosucra (Warcoing, Belgium), following the method described by Kuang et al. (2023a). The resulting protein powder, designated as PPI, contained 89% w/w

proteins on a dry basis. Prior to use, the PPI was solubilized in distilled water to achieve a 10% protein content. The dispersion was then agitated at 350-400 rpm for 3 hours at 4 °C to ensure complete hydration of the proteins. The pH of the protein suspensions was subsequently adjusted to pH 7.5 or pH 9.0 using 0.1 M HCl or NaOH before each test, without altering the dispersion's concentration. The insoluble protein fraction was considered negligible.

All other reagents and chemicals, procured from Sigma-Aldrich (St-Quentin Fallavier, France), were of analytical grade.

2.2 Gel preparation

10% (w/w) protein suspensions were prepared at pH 7.5 and 9.0 (adjusted with 0.1 M HCl or NaOH) from initial egg white protein (EWP) and stock suspensions of pea protein isolate (PPI) to obtain 100% PPI, 100% EWP, and PPI-EWP mixtures at three weight ratios (75/25, 50/50, and 25/75). The protein suspensions were transferred into glass vials and heated from 25 to 95 °C (at a rate of 5°C/min) in a water bath, then maintained at 95 °C for 15 minutes. Subsequently, the vials were cooled to room temperature in an ice bath and stored at 4 °C overnight to ensure complete gelation.

2.3 Small-strain dynamic rheology

Each protein suspension of PPI, EWP, and their mixtures at pH 7.5 or 9.0 was loaded into a rheometer MCR 302e (Anton Paar, Graz, Austria) equipped with a 50 mm parallel plate geometry. Approximately 1 mL of each sample was transferred to the

lower plate of the parallel plate geometry of the rheometer. The upper plate was lowered to achieve a gap width of 1.0 mm. A thin layer of light mineral oil was added to the well of the upper plate geometry, and a solvent trap cover was used to prevent evaporation during heating, thus maintaining a water-saturated atmosphere at the surface of the sample. The following heating protocol was applied: the sample was initially equilibrated at 25 °C for 3 min, then heated at a rate of 2 °C/min and cooled at a rate of 5 °C/min over a temperature range of 25–95–25 °C under a shear strain of 1% and a frequency of 1 Hz. Subsequently, a frequency sweep over a range of 0.01-40 Hz at 1% strain and a strain sweep from 0.01 to 100% strain at 1 Hz were performed at 25 °C. The storage modulus (G') and loss modulus (G'') were measured during temperature, strain, and frequency sweeps. The loss factor or tangent delta (tan $\delta = G''/G'$) was also calculated (calculated at 1 Hz, 1% strain), as well as the linear viscoelastic region (LVR). The LVR was calculated as described in Fig. 1 for the PPI-EWP mixture at a weight ratio of 25/75. The intersection of the two lines on both sides of the inflection point was the maximum strain without causing permanent deformation, called the yield point. Rheological data were collected for every degree change during heating and cooling. Samples were run in triplicates

2.4 Texture profile analysis

For texture profile analysis (TPA), all samples were prepared as described in section 2.2 in plastic tubes with a diameter of 40 mm (Krehalon, Deventer, Netherlands). Forty grams of sample suspensions were heated from 25 to 95 °C in a water bath and

maintained at this temperature for 15 minutes, then cooled down with ice to room temperature and stored at 4 °C overnight. Cylindrical gels with a diameter of 40 mm were sliced using a die cutter at a height of 20 mm and placed on the platform of a TA-XT Plus (Lloyd Instruments, Ametek company, UK) equipped with a 5 N load cell and a cylindrical probe with a diameter of 12 mm (SMS-P/35). TPA tests were conducted at a test speed of 0.5 mm/s, and a deformation in compression of 37.5% was applied. A time of 10 seconds was allowed to elapse between the two compression cycles. All samples were prepared in duplicate and tested twice.

The hardness, springiness, and cohesiveness of PPI-EWP gels were determined according to the method described by Bourne (Bourne, 1978). Hardness was defined as the maximum peak force during the first compression cycle. Springiness was defined as the degree of recovery of gels after decompression to their initial shape, measured by the distance of the detected height during the second compression divided by the original compression distance. Cohesiveness was calculated as the area of work during the second compression divided by the area of work during the first compression. Data were analyzed using Texture Expert software version 1.22 (Stable Micro Systems).

2.5 Confocal microscopy (CLSM)

Sample preparation and microscopy analysis were conducted following the procedures outlined by Somaratne et al. (2020a) and Kuang et al. (2023b) with minor adjustments. Four hundred microliters of each 10% protein solution at pH 7.5 or 9.0

were dispensed into 1 mL Eppendorf tubes. They were then mixed with 12 µL of 1% (w/v) Fast Green solution. Subsequently, the entire sample solution was gently loaded into the well of a chamber slide (Ibidiµ-Slide 8 well Uncoated system, Ibidi, Grafelfing, Germany). The system was covered with the provided lid and securely wrapped with Parafilm (Dispense Parafilm Through This Opening, USA) around the lid gap. Additionally, aluminum foil was used to prevent the photo-bleaching of fluorescent molecules. Finally, the systems were placed into the IBIDI system and subjected to heating as described for gel preparation in section 2.2.

The mixture gels labeled with Fast Green were visualized using a Zeiss LSM 880 Inverted confocal microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with the Airyscan detection unit. The prepared slide was positioned on a ×63 oil-immersion objective (NA = 1.4) in the thermo-regulated chamber of the microscope, set at 20°C. A He/Ne laser with a wavelength of 633 nm was employed, and images were captured using the Airyscan detector in super-resolution mode with the zoom set at 1.8.

Zen Black 2.1 (version 13.0.0.0) software was used to process the acquired datasets using the 2D mode with the default settings of the Airyscan processing function.

2.6 Gel dissolution by dissociating agents

Four different extracting reagents were used to analyze protein-protein interactions contributing to gelation. Samples were prepared according to the methods proposed by Liu & Hsieh (2008) and Chen et al. (2021) with some modifications. A 100 mM

Tris buffer solution (Tris) at pH 7.5 or pH 9.0 was used as a control (i). Tris buffer containing 8 M urea (ii), 2 M guanidinium hydrochloride (GuHCl) (iii), or 20% propylene glycol (PG) (iv) was used to extract proteins by affecting non-covalent interactions. Tris buffer containing 100 mM dithiothreitol (DTT) was used to extract proteins by reducing disulfide bonds (v). Tris buffer containing 6 M urea, 100 mM DTT, 2 M guanidinium hydrochloride (GuHCl), and 20% propylene glycol (PG) was used to extract proteins by dissociating all disulfide and non-covalent bonds as a second control (vi).

Following the protocol outlined by Chen et al. (2021), gel samples (~2.5 g), prepared as described in section 2.2, were incubated in individual extractants (~40 mL), stirred for 1 hour at 25 °C, and homogenized for 1 minute at 10000 rpm using a homogenizer (Ultra Turrax® IKA T25 Digital, IKA, Germany). The samples were then centrifuged (16000 rpm, 30 min, 4 °C). The supernatants were collected, filtered using a 0.45 μm filter, weighed, and diluted with the same extractant for protein assay. The protein content in the dilutions was measured using a commercial Coomassie Plus (Bradford) protein assay kit (λ= 660 nm) obtained from Sigma-Aldrich (St-Quentin Fallavier, France), with BSA as the standard. The solubilized protein content was then calculated as follows (equations 1 and 2):

$$Total\ protein\ solubility\ (\%) = \frac{protein\ content\ in\ gel\ supernatant\ solution}{protein\ content\ \ in\ gels} \times 100 \quad [eq.1]$$

$$Net\ protein\ solubility\ in\ dissociating\ buffer\ (\%) = total\ protein\ solubility - protein\ solubility\ in\ Tris \quad [\text{eq. 2}].$$

At least three extractions were conducted and analyzed for each sample.

2.7 Statistical analysis

Differences between samples were studied by analysis of variance (one-way ANOVA). Significance was set at $p < 0.05$. Tukey's post hoc least significant differences method was used to describe means with 95% confidence intervals. The statistical analyses were performed using Statistica software, version 12 (Tulsa, OK, USA).

3. Results and discussion

3.1 Thermal gelation and viscoelastic properties of PPI-EWP gels

Temperature sweeps were performed by small amplitude rheology to understand the sol-gel transition behavior of the different protein suspensions upon thermal treatment. Typical storage modulus (G') vs. temperature curves of 100% PPI, 100% EW, and PPI-EW mixtures at pH 7.5 and 9.0 are shown in Fig. 2A&D. The final (after cooling) G' and tan(δ) (loss factor) values of the respective protein gels were reported in Table 3.

Before heating, all samples exhibited a relatively low G' that increased during heating due to the thermal denaturation and aggregation of the proteins, leading to the formation of a solid viscoelastic 3D protein network after cooling (tan(δ) <1), except for the 100% PPI mixture at pH 9.0, which did not form a self-supporting gel under those conditions (Fig. 2A). At this alkaline pH, far from the isoelectric point of pea protein (pI~4.5-4.8), the strong electrostatic repulsions between negatively charged

proteins prevented the formation of a cohesive protein network, resulting in only a viscous suspension for the 100% PPI sample (Kuang et al., 2023b). At pH 7.5, the 100% PPI sample gelled, but the final G' was lower than 100 Pa, significantly lower than the values obtained for the EWP-containing gels (>3000 Pa) (Table 1). Regardless of pH, mixtures containing more than 50% EWP exhibited a two-step increase in G' around 60 and 85 °C, previously attributed to the denaturation/aggregation of OVT and OVA, respectively (Kuang et al., 2023b). When EWP represented less than 50% of the mixture, a one-step increase was observed between 80 and 90 °C, consistent with the denaturation temperature of PPI globulins (Kuang et al., 2023b).

The final G' value of gelled systems significantly increased with an increased content of EWP, except for the 75/25 PPI-EWP mixture at pH 9 (Table 1). Surprisingly, this last sample presented a final G' value comparable to the 25/75 PPI-EWP sample at the same pH with a very high standard error. Moreover, the G' of this sample primarily increased during cooling (about 90-fold) compared to other samples (about 3-4-fold). These observations indicated a very unstable behavior of this mixture, having a final heterogeneous gel structure (Fig. 4 & 5) that could not be specified as a self-supporting gel network. While G' values of the 50% EWP-based gels seemed independent of pH, tan(δ) was significantly lower at pH 9.0 compared to pH 7.5, indicating a more viscous contribution at lower pH (Table 1). Tan(δ) value also decreased with an increase in EWP content and reached 0.13 and 0.11 for the 100% EWP sample at pH 7.5 and 9.0, respectively. These values were characteristic of weak

gels (tan(δ) >0.1) (Clark & Ross-Murphy, 1987), and weaker gels were formed with the gradual addition of PPI in the mixture as tan(δ) increased. The "weak" character of the gels was confirmed by the frequency sweep data presented in Fig. 2B. For all samples, G' and G'' were frequency-dependent, and both increased with increasing frequency, confirming the formation of weak viscoelastic gels (G'>G''). Physical gels are typically not crosslinked and are characterized by entanglements and weak chemical associations within the macromolecular network with a time-scale dependence upon mechanical stress (Douglas, 2018). Similar behavior was previously reported for soy- (Su et al., 2015) and oat-egg white (Ma, Yiu, & Harwalkar, 1990) protein mixtures.

Additionally, strain sweeps were performed on the final gels, and typical curves are presented in Fig. 2C. All curves exhibited a distinct linear and non-linear viscoelastic region. In the linear viscoelastic region (LVR), the gels deformed elastically, with the storage modulus (G') higher than the loss modulus (G''), indicating the gel-like nature of the samples. Beyond that region, G' decreased due to the breakdown of the network structure. Corresponding yield points (YP) were determined and reported in Table 2.

With the increased proportion of PPI in the mixture, the YP first increased up to 50% PPI (the 50/50 weight ratio sample presented a maximum at both pHs) and then decreased. This behavior could be explained by the variable structure of the gels at a microscopic level depending on the percentage of each protein type in the mixture and will be discussed in section 3.3. In general, lower YP indicates weaker connections in

298 the protein network, leading to earlier network rupture upon oscillating deformation.
299 The region of the linear response also increased with pH values, suggesting that the
300 protein gel network had more structural strength and was more elastically deformable
301 at pH 9.0, in agreement with the previous work of Handa, Takahashi, Kuroda, &
302 Froning (1998) and Alleoni & Antunes (2005). Both groups observed that the EW gel
303 hardness and elasticity were stronger at pH 9.0 than at pH 7.0. These authors
304 attributed this behavior to the increased proportion of S-OVA in egg white during
305 storage at pH 9.0, suggesting that S-OVA could improve the hardness of albumen
306 gels. Besides, more recently, Somaratne et al. (2020b) found that the hardness of egg
307 white gel at pH 9.0 was higher than that at pH 5.0, due to a more homogeneous
308 network at pH 9.0 compared to the heterogeneous protein network made of larger
309 aggregate particles at pH 5.0.
310
311 **3.2 Macrostructure of PPI-EWP gels**
312 The macrostructure of the gels was characterized by analyzing their appearance and
313 performing a texture profile analysis (TPA). The appearance of PPI-EWP gels at the
314 different weight ratios is shown in Fig. 3. Since PPI alone hardly gelled (at pH 7.5) or
315 did not gel at all (at pH 9.0), the 100% PPI samples were not presented.
316 The color of the gels obtained from the different PPI-EWP mixtures changed with the
317 increasing proportion of PPI: from pale yellow to light brown and dark brown, at pH
318 7.5 (Fig. 3A) and pH 9.0 (Fig. 3B), respectively. These color changes may be due to
319 the presence of phenolic compounds in PPI samples as suggested by Zhou, Vu &

McClements (2022) for RuBisCo gels. The color of PPI-EWP gels at pH 9.0 was darker than the those at pH 7.5, in agreement with the observations of Zhang et al. (2023) for gellan gum gels in presence of tea polyphenols.

The texture of the gels was evaluated through TPA. Hardness and springiness were typically regarded as relevant measures of gel performance (Li et al., 2018; Alavi, Emam-Djomeh & Chen, 2020). The changes in TPA parameters (hardness, springiness, and cohesiveness) of the gels are presented in Table 3.

At both pH levels, 100% EWP gels exhibited the highest gel hardness, which significantly decreased with the increasing proportion of PPI content (from 0 to 75%) in the initial mixture. This is consistent with the previous viscoelastic data where G' decreased and tan(δ) increased with increasing PPI content. A similar trend has been observed previously for egg white-hempseed protein mixtures (Alavi, Emam-Djomeh & Chen, 2020) and egg white-soy protein composite gels at higher protein concentrations (Su et al., 2015). From 50% EWP content in the sample, the hardness was higher at pH 9.0 compared to pH 7.5. This result was also consistent with lower tan(δ) and higher YP values, respectively, at pH 9.0, as observed in the previous section.

Similar effects related to gel composition were observed for springiness and cohesiveness, which decreased with higher PPI content. Both parameters represent textural qualities connected to gel elasticity and its ability to maintain an intact network structure (Handa, Takahashi, Kuroda & Froning, 1998; Fernandez-Lopez et al., 2006).

In summary, the presence of PPI modified the texture of the gels, decreasing their hardness and increasing their brittleness, as suggested by the decrease in both springiness and cohesiveness. Such results have already been described for other plant protein gels (Zhou et al., 2022), with the presence of aggregates and/or protein-protein interactions of different nature and strength.

3.3 Microstructure of PPI-EWP gels

The microstructure of PPI-EWP gels was observed using confocal microscopy. Fig. 4 shows the microscopic observations of 10% (w/w) mixed protein gels at various PPI-EWP weight ratios (0/100, 25/75, 50/50, 75/25, 100/0) at pH 7.5 and 9.0. Proteins are visible in gray and white on confocal micrographs, while pores containing the aqueous phase appear in black. It is worth mentioning that both EWP and PPI were labeled, thus preventing their discrimination in these pictures.

For the pure EWP system (Fig. 4A/a), the microstructural organization of the gel constituted of fine aggregates appeared quite different between pH 7.5 and 9.0. At pH 9.0, the EWP gel presented a denser and more homogeneous protein network than at pH 7.5, where the protein network was more porous and loosely packed. This result is similar to previously published SEM and cryo-TEM data showing granular (pH 7) vs. smooth (pH 9) EWP gel microstructure (Nyemb, et al., 2016; Clark, Kavanagh, & Ross-Murphy, 2001), and CLSM observations showing a more homogeneous structure of EWP gels at pH 9 than at pH 5 (Somaratne et al., 2020a). The different gel structures observed at both pHs may be attributed to the different behavior of

OVA and OVT during gelation at pH 7.5 and 9.0 (Nyemb, et al., 2016). At pH 7.5, OVT was close to its isoelectric point (pI) (6.5), which favored the formation of random and spherical aggregates, whereas OVA, which was far from its pI (4.5), began to form linear branched aggregates (Nyemb, et al., 2016). As a result, in this case, the egg white gel was made up of a variety of aggregated structures: dispersion of OVT spherical aggregates in the protein network of OVA linear branched aggregates. Van der Plancken et al. (2006) highlighted that the net protein charge and the electrostatic repulsions were greatly enhanced at pH 9, and the activation energy barrier required to unfold the protein was lowered. In this case, the proteins tended to unfold to form a homogeneous protein network rather than spherical aggregates (Clark, Kavanagh, & Ross-Murphy, 2001).

Fig. 4 E/e shows the microstructure of heated PPI at pH 7.5 and 9.0, respectively. At pH 9.0, protein particles and small aggregates were poorly interconnected (indicating no gel formation as previously mentioned), whereas at pH 7.5, a denser protein network with gel-like properties was observed. Additionally, larger particles were observed at pH 7.5 (Fig. 4E), whereas only spaced small particles were apparent at pH 9.0 (Fig. 4e). The higher repulsive force between protein particles at high pH, as indicated previously, could explain the formation of smaller aggregates with insufficient interconnections to form a solid network.

Different structures were observed for the three mixed protein systems at both pHs (Fig. 4B/b to D/d). For the 25/75 PPI-EWP gels at pH 7.5 (Fig. 4B), large irregular-shaped aggregates (>10 μm) were formed, surrounded by a white homogeneous

protein network. It was assumed that this homogeneous network was formed by egg protein since egg white was predominant in the mixture, and the appearance of this domain resembled that observed for 100% EWP gels. With 50% of PPI in the mixtures (Fig. 4C), more spherical aggregates with black holes were present, and the surrounding network area decreased. With a higher concentration of PPI in PPI-EWP mixtures, the gel structure appeared more heterogeneous, forming random protein clusters of smaller size and irregular shape (Fig. 4D). In contrast, PPI-EWP gels at pH 9.0 (Fig. 4b-d) exhibited some differences. When EWP was the dominant component (Fig. 4B-C), the gel showed some large aggregates (~10-20 μm) resembling brain-like structures, surrounded by a continuous network similar to pure EW gel. When PPI comprised 50% of the mixtures, the gel contained numerous protein clusters of smaller size. When PPI was the dominant component, the irregular clusters were dispersed in a less well-defined continuous phase. Similar observations regarding mixed gels were previously reported by Kornet et al. (2020), who found that whey protein-PPI gels contained large clusters with a high pea protein content. Silva, Cochereau, Schmitt, Chassenieux, & Nicolai (2019) demonstrated that mixtures of micellar caseins and PPI at pH 5.8 formed gels with protein clusters, whereas more homogeneous gels were obtained for individual proteins. McCann et al. (2018) and Roesch & Corredig (2005) observed a discontinuous network in soy protein-whey protein gels at a total protein concentration of around 6%, indicating phase separation, while Gómez-Mascaraque & Pinho (2021) found a microgel structure between soy and whey protein gels.

It's worth noting that, as evident from the structure of the aforementioned mixed gels, the network structure of these gels was not as dense as that of egg white, with the formation of large clustered aggregates which did not exist in the pure PPI systems. In the mixtures, it was assumed that the EWP could form the basic architecture of the protein network, and that gelation was accompanied by the formation of protein aggregates, which could be either pure PPI aggregates or mixed aggregates consisting of pea globulins and some EWP. Particularly, positively charged LYS can form complexes with pea proteins (Kuang et al., 2023a). The total or partial phase separation between EWP and PPI could be caused by depletion or thermodynamic incompatibility effects (Tolstoguzov, 1995 & 2003; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). Although thermodynamic incompatibility is commonly described between food proteins and polysaccharides, these phase separation phenomena could occur between proteins of different natures with a favorable effect of denaturation (Polyakov, Grinberg, & Tolstoguzov, 1997). In our systems, these phenomena would undoubtedly be amplified by the lower gelation temperature of OVT. Indeed, our group has previously shown (Kuang et al., 2023b) that a first gel point appeared at a temperature < 59°C in egg white-based systems, with this early gelation attributed to OVT. The primary gel network thus formed would be prone to excluding the other protein particles that formed later during heating, primarily consisting of the nascent pea protein aggregates that would reassemble into large clusters. The differences in gel structure noted at pH 9.0 would be due to a greater difficulty for pea proteins to associate due to the repulsive forces between protein particles at this pH. Indeed,

smaller aggregates would form in this case with less ability for interconnection. These results were consistent with the decrease of G' observed in strain sweep tests, and TPA parameters when the proportion of PPI increased in the mixtures. The aggregates observed in CLSM could weaken the primary EWP network, thus explaining the changes in gel texture. It could be assumed that the concentration effect resulting from phase separation phenomena between proteins could increase the interconnections within the dominant EWP network, while more protein clusters affected the continuity of the network and weakened the gel. This phenomenon could also explain the maximum observed for YP in strain weep experiments presented in section 3.2. Micro-phase separation first extended the elastic deformability region for low proportions of PPI in the mixtures, whereas less EWP concentration in the continuous network at higher proportions of PPI negatively affected the gel's elastic strength.

3.4 Intermolecular interactions involved in PPI-EWP gels

Typical protein gels can be stabilized by both non-covalent and covalent forces. Chang & Chen (2000) illustrated that hydrophobic interactions, disulfide bonds, and hydrogen bonds stabilize heated EWP gels. To evaluate the type of interactions involved in PPI-EWP mixture-based gels at pH 7.5 and 9.0, a dissociation approach was investigated and compared with the predicted effects. The utilization of urea, propylene glycol, DTT, and guanidinium-HCl as dissociating agents allowed us to assess interactions between proteins in various gels. Table 4 summarizes the reported

effects of urea, DTT, propylene glycol, and guanidinium-HCl on hydrogen bonds, disulfide bonds, and hydrophobic interactions, respectively.

3.4.1 Effect of dissociating agents on 100% PPI- and 100% EWP- gels

Fig. 5 shows the percentage of proteins that were solubilized by the dissociating agents for both PPI and EWP gels at both pH 7.5 and 9.0.

Dissolution of gels in 100 mM Tris buffer (used as a control) allows us to understand which fraction of the protein system is dissociated in the absence of any dissociating agent. It could be hypothesized that this solubility corresponds to protein particles not bound to the gel network or that certain interactions were weakened by the buffer, releasing some part of the protein material. Tris ($C_4H_{11}NO_3$) is a very polar molecule with one amine and three hydroxyl groups (a weak base) and a pKa of 8.3, close to the two pH values studied. At a concentration of 100 mM, the properties of the molecule could affect hydrogen and ionic bonds, which would explain the partial protein dissociation from the gels in this buffer. The 100% EWP gel was poorly dissociated in this buffer (approximately 4% at both pH values), and the solubility increased to approximately 21% for the 100% PPI gel at pH 7.5 and 55% at pH 9.0. This assumes that whereas most of the EWP was strongly retained in the gel network, PPI is more easily released into the solution, especially at pH 9.0 where their high electronic charge may favor disruption of hydrogen and ionic bonds by the Tris buffer.

Regardless of the type of dissociating agent (including the control) and regardless of the pH, the amount of total protein dissociated from the 100% PPI gel was always

much higher than that from the 100% EWP gel (Fig. 5A/a vs 5B/b), suggesting fewer or weaker interactions in PPI gels than in EWP gels. The remaining protein in the gel represents the protein material that is still interacting despite the presence of dissociating agents. This means that other interactions (covalent bonds, ionic interactions) not affected by the dissociating agents could be involved or that the intrinsic solubility of the released particles was insufficient. Moreover, new interactions created between released particles could lead to their precipitation. The efficiency of the agent in dissolving the gel should be therefore considered with caution and used here for comparative purposes. Consequently, we have considered that the more the gel was dissolved in the presence of a chemical agent, the more the agent was able to affect the corresponding interactions and release soluble protein particles.

In 100% PPI gel, urea, guanidinium-HCl, and DTT significantly increased the quantity of solubilized protein regardless of the pH (Fig. 5A&a). This suggests that hydrophobic interactions, hydrogen bonds, and to a lesser extent, disulfide bonds were involved in PPI gels and were more easily disrupted in gels at pH 9.0 than at pH 7.5. These results are consistent with those of Sun & Arntfield (2012), who mentioned that hydrophobic interactions and hydrogen bonds were mainly involved in heat-induced pea protein gelation with 0.3 M NaCl at pH 5.65, while disulfide bonds played a lesser role in gel formation. Tanger, Müller, Andlinger, & Kulozik (2022) confirmed that the main protein interactions in pea protein gels were non-covalent regardless of pH and ionic strength.

On the contrary, in 100% EWP gels, only urea and DTT exhibited a significant effect on total protein solubilization for both pHs (Fig. 5 B&b), suggesting the predominance of hydrophobic and disulfide bonds in these gels. This result is consistent with the previous work of Huang et al. (2019) and Wang et al. (2020), who found that disulfide bonds involved in egg white gel outnumbered the hydrophobic effect. Jin, Chen, Zhang, & Sheng (2021) also reported that disulfide bonds play the primary role in heat-induced EWP gel formation, followed by hydrophobic interactions, hydrogen bonds, and ionic bonds, regardless of the duration of the heating time.

The simultaneous application of the four dissociating agents showed an overwhelming increase in protein solubilization for all samples, indicating the synergistic effect of the dissociating agents regardless of the pH and the type of gel. This reflects the interdependence of the different types of interactions involved.

At pH 9.0, the quantity of protein dissociated from both the 100% PPI gel (more accurately described as a coagulum in this case) and the 100% EWP gel was generally higher than at pH 7.5 (Fig. 5 A/B vs 5a/b), suggesting the presence of a greater amount of i) low-energy interactions and/or ii) proteins not associated with the protein network at pH 9.0. Indeed, previous microscopic observations of the 100% PPI system at pH 9.0 (Fig. 4e) showed mainly small and poorly interconnected protein aggregates that were more susceptible to solubilization. This observation is consistent with the findings of Tanger et al. (2022), who reported that 15% pea protein isolate suspensions formed an entangled colloidal suspension rather than a continuous gel

network at pH 9.0 and 0.9 M NaCl. In comparison, the protein solubility of 100% EWP gels at pH 9.0 remained low in all cases (≤11.5% for urea at pH 9.0). This suggests that even if some interactions are affected by the chemical agents, the gel particles released remain insufficiently soluble, indicating the presence of strong interactions. The EWP gels, therefore, remained particularly insoluble even when the agents were used simultaneously, with only 35.8% of proteins solubilized. Finally, adding all the chemical agents simultaneously did not lead to complete solubilization of the gel except in the case of PPI at pH 9.0, with a total solubility reaching 97.5% in this instance (bearing in mind that no self-supporting gel was formed under these conditions).

3.4.2 Effect of dissociating agents on PPI-EW mixed gels

The protein solubility of PPI-EWP mixed gels at different weight ratios and pHs (7.5 and 9.0) increased in the presence of dissociating agents as the proportion of PPI protein in mixed gels increased (Fig. 6 A & B).

As observed for 100% PPI gels, the protein solubility in mixed gels was generally higher at pH 9.0 than at pH 7.5. Mixed gels rich in EWP (such as PPI-EWP 50/50 and PPI-EWP 25/75) remained particularly insoluble even when the agents were used simultaneously. However, these gels were especially sensitive to urea and DTT, indicating the significant role of hydrogen bonds, hydrophobic interactions, and disulfide bonds in the structure of these mixed gels.

On the contrary, when mixed gels were rich in PPI (PPI-EWP 75/25), urea was the most efficient dissociating agent (47.8% ± 0.9), followed by guanidinium-HCl (29% ± 0.5) at pH 7.5 (Fig. 6A). This result suggests a combination of non-specific and lower energy interactions, similar to the case of 100% PPI gels, with a dominance of hydrogen and hydrophobic bonds.

No significant effect of propylene glycol (PG) was observed regardless of the sample or pH. PG disrupts hydrophobic interactions but enhances hydrogen bonds and electrostatic interactions by lowering the dielectric constant of the solvent and reducing the energy barrier to protein-protein interactions sufficiently to enable structure formation (Ustunol et al., 1992; Utsumi & Kinsella, 1985). This agent may be ineffective because its effect on hydrogen bonds could be masked by the TRIS-HCl buffer effect. Overall, protein solubility increased significantly as the proportion of PPI increased in the system regardless of the chemical agent. The mixed gels exhibited an intermediate behavior between the 100% EWP and 100% PPI systems regarding chemical dissociation. Thus, EWP-based gels were weakly dissociable up to the 50/50 ratio, indicating that EWP played a dominant role in the structure of the gels, consistent with the CLSM observations (Fig. 4). In all cases, the gels at pH 9.0 were more dissociable than at pH 7.5, as the higher pH promoted more repulsive forces within the protein network during gel formation due to the higher protein charge at a more alkaline pH.

4. Conclusion: proposition of a mechanism for gelation of EWP-PPI

mixtures

Combining the results of chemical dissociation, texture, microscopy, and dynamic rheology data, we propose the following mechanism regarding the heat-induced gelation of PPI-EWP mixtures at pH 7.5 and 9.0. Indeed, we hypothesize that the heat-set gels obtained from the PPI-EWP mixtures consist of a primary network of egg white proteins containing large aggregates of pea proteins or mixed PPI-EWP, induced by a phase separation phenomenon. This is suggested by the observations of gel microstructure that show a continuous protein network, very similar to the pure EW system, where irregular protein clusters, of varying sizes, are embedded. This hypothesis could be reinforced as the viscoelastic data indicate a first gelation point around 55 °C when heating the protein mixtures containing at least 50% EWP, which is attributed to OVT denaturing at lower temperatures than the other proteins. At higher temperatures (>60 °C), the denaturation of other proteins leads to the formation of large protein aggregates, which are supposed to be induced by thermodynamic incompatibility, depletion, and/or steric exclusion phenomena. These aggregates probably mainly involve pea globulins as such aggregates are not present in pure EWP systems, even if the contribution of other EW proteins (OVA, LYS…) in formed aggregates cannot be excluded. The smaller size of the dispersed protein particles at pH 9 compared to pH 7.5 could be explained by higher repulsive forces between proteins at a more alkaline pH, limiting self-association phenomena. Moreover, the viscoelastic data and texture parameters show that weaker, less rigid, and cohesive gels are formed when the proportion of PPI increases in the initial

protein suspensions. This trend could be first explained by the simultaneous decrease in EWP concentration that affects the tightness of the continuous protein network, primarily constituted of EW proteins. Furthermore, the different nature of the interactions formed during the aggregation/gelation process comparing EWP and PPI is also highlighted by the gel solubilization tests using different dissociating agents. EW gels are hardly solubilized compared to PPI gels, indicating more extended and stronger interactions within the protein network. The molecular interactions evaluated for EWP gels, comparable at both studied pH levels, are dominated by disulfide bonds and to a lesser extent by hydrophobic interactions and hydrogen bonds. In the case of PPI systems, the contribution of disulfide bonds is found to be lower compared to hydrogen bonds and hydrophobic interactions. The solubilization of PPI gels is also easier at pH 9, confirming that the protein network is less "associated" in this case due to more repulsive forces between highly charged proteins at elevated pH levels. This study provides a deeper understanding of the gelation properties of hybrid protein systems and will contribute to enhancing the design of composite protein ingredients or new plant-based food products.

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606 Figure 1: Strain intersection (yield point) of PPI-EWP mixtures at a weight ratio of
607 25/75 at pH 7.5. Lines 1 and 2 are the regression lines used to calculate the yield
608 point.

609 Figure 2. (A, D) The storage modulus of PPI-EWP gels (100/0 in ◆, 75/25 in ▼,
610 50/50 in ▲, 25/75 in ●, 0/100 in ■) during heating from 25 to 95°C, then cooling to
611 25°C at 2°C/min (1Hz, 0.1% strain) at pH 7.0 and 9.5; (B, E) Changes in storage (full
612 symbols) and loss modulus (empty symbols) with frequency after cooling PPI-EWP
613 gels (25°C, 0.1% strain) at pH 7.0 and 9.5; (C, F) changes in storage and loss modulus
614 with increasing shear strain (25°C, 1 Hz) at pH 7.0 and 9.5.

615 Figure 3: Photographs of PPI-EWP gels at the different weight ratios at pH 7.5 (A)
616 and pH 9.0 (B).

617 Figure 4. CLSM images visualizing the microstructure of PPI-EWP protein gels
618 (0/100 A/a, 25/75 B/b, 50/50 C/c, 75/25 D/d; 100/0 E/e) at pH 7.5 (left) and pH 9
619 (right) (magnification x63)

620 Figure 5. Effect of different dissociating agents on total protein solubilization from
621 100 % PPI gel (A/a) or 100 % EWP gel (B/b) at pH 7.5 (A/B) and pH 9.0 (a/b).
622 Control: 100 mM Tris-HCl.

623 Figure 6. Effect of different dissociating agents on total protein solubilization from
624 PPI-EWP mixed gels at pH 7.5 (A) and 9.0 (B). Control: 100 mM Tris-HCl.

625

626

627 Tables:

628

629 Table 1: Final G' and tan (δ) of PPI-EWP gels at the different weight ratios and pH

630 after temperature sweep (0,1% strain, 1 Hz frequency).

PPI-EWP ratio	G' (Pa)		Tan (δ)	
	pH 7.5	pH 9.0	pH 7.5	pH 9.0
0/100	15115 ± 632^{aA}	14446 ± 413^{aA}	0.135 ± 0.002^{aA}	0.115 ± 0.001^{aB}
25/75	7284 ± 192^{bA}	7204 ± 281^{bA}	0.138 ± 0.001^{aA}	0.118 ± 0.003^{aB}
50/50	4725 ± 324^{cA}	4182 ± 440^{cA}	0.151 ± 0.002^{bA}	0.134 ± 0.004^{bB}
75/25	3446 ± 331^{cA}	9237 ± 3249^{bB}	0.157 ± 0.003^{bA}	0.158 ± 0.008^{cA}
100/0	97 ± 4^{d}	no gel	0.227 ± 0.005^{c}	no gel

631 All data were given as mean ± SD (n ≥3). Means in a column bearing the same lowercase letter are not

632 significantly different (p<0,05). Means in a row with the same uppercase letter are not significantly

633 different (p<0,05).

634

635 Table 2. Yield point (%) of PPI-EWP gels at the different weight ratios at pH 7.5 and

636 9.0

PPI-EWP ratio	yield point (%)	
	pH 7.5	pH 9.0
0/100	5.5 ± 0.1^{a}	16.6 ± 0.6a
25/75	9.7 ± 0.6b	41.6 ± 5.4b
50/50	11.4 ± 0.6b	52.3 ± 2.0b
75/25	3.9 ± 0.2a	9.7 ± 3.8a
100/0	5.6 ± 0.3a	no gel

637 All data were given as mean ± SD of triplicate measurements. Means in a column bearing the same

638 letter are not significantly different (p<0,05).

639

640 Table 3. Parameters of texture profile analysis (TPA) of PPI-EWP gels at the different

641 weight ratios at pH 7.5 and 9.0.

PPI-EWP	pH 7.5	pH 9.0

	Hardness /N	Cohesiveness	Springiness	Hardness /N	Cohesiveness	Springiness
0/100	3.10±0.21[a]	0.73±0.02[a]	0.95±0.04[a]	3.90±0.08[a]	0.75±0.01[a]	0.93±0.02[a]
25/75	2.78±0.17[a]	0.67±0.02[a]	0.92±0.04[ab]	3.45±0.16[a]	0.74±0.01[ab]	0.91±0.03[ab]
50/50	1.69±0.03[b]	0.58±0.02[b]	0.84±0.00[ab]	1.84±0.08[b]	0.71±0.00[bc]	0.89±0.01[ab]
75/25	0.79±0.00[c]	0.59±0.02[b]	0.80±0.01[b]	0.51±0.01[c]	0.67±0.01[c]	0.81±0.01[b]

642 Different superscripts in each column represent a significant difference ($p<0.05$).

643

644 Table 4. Effect of various reagents on molecular forces existing in protein structures.

	Non-covalent bonds			Covalent bond	References
	Ionic effect/ Electrostatic interaction	Hydrophobic interaction	Hydrogen bond	Disulfide bond	
Dithiothreitol (DTT)				Disrupt	Rüegg & Rudinger (1977), Léger & Arntfield (1993), Sun & Arntfield (2012),
Guanidinium-HCl (GuHCl)	Disrupt	Weaken	Disrupt		Tanford (1968), Léger & Arntfield (1993), Sun & Arntfield (2012)
Propylene glycol (PG)	Promote	Disrupt	Promote		Tanford (1962), Ustunol et al. (1992), Utsumi & Kinsella (1985)
Urea		Disrupt	Disrupt		Gordon & Jencks (1963), Uruakpa & Arntfield (2006), Ustunol et al. (1992), Nozaki & Tanford (1963)

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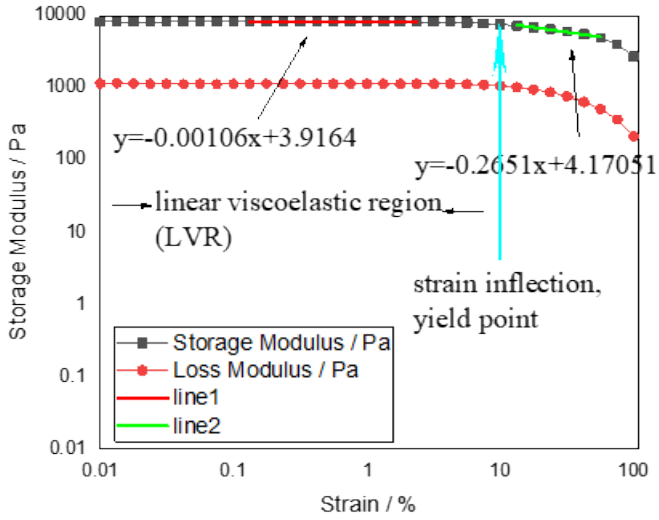

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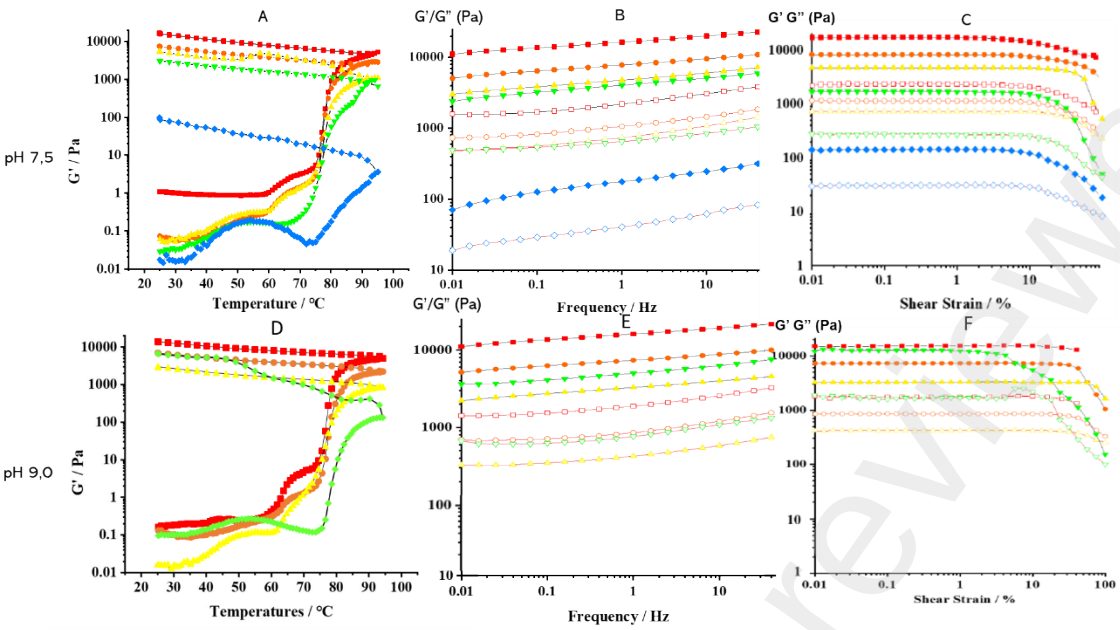

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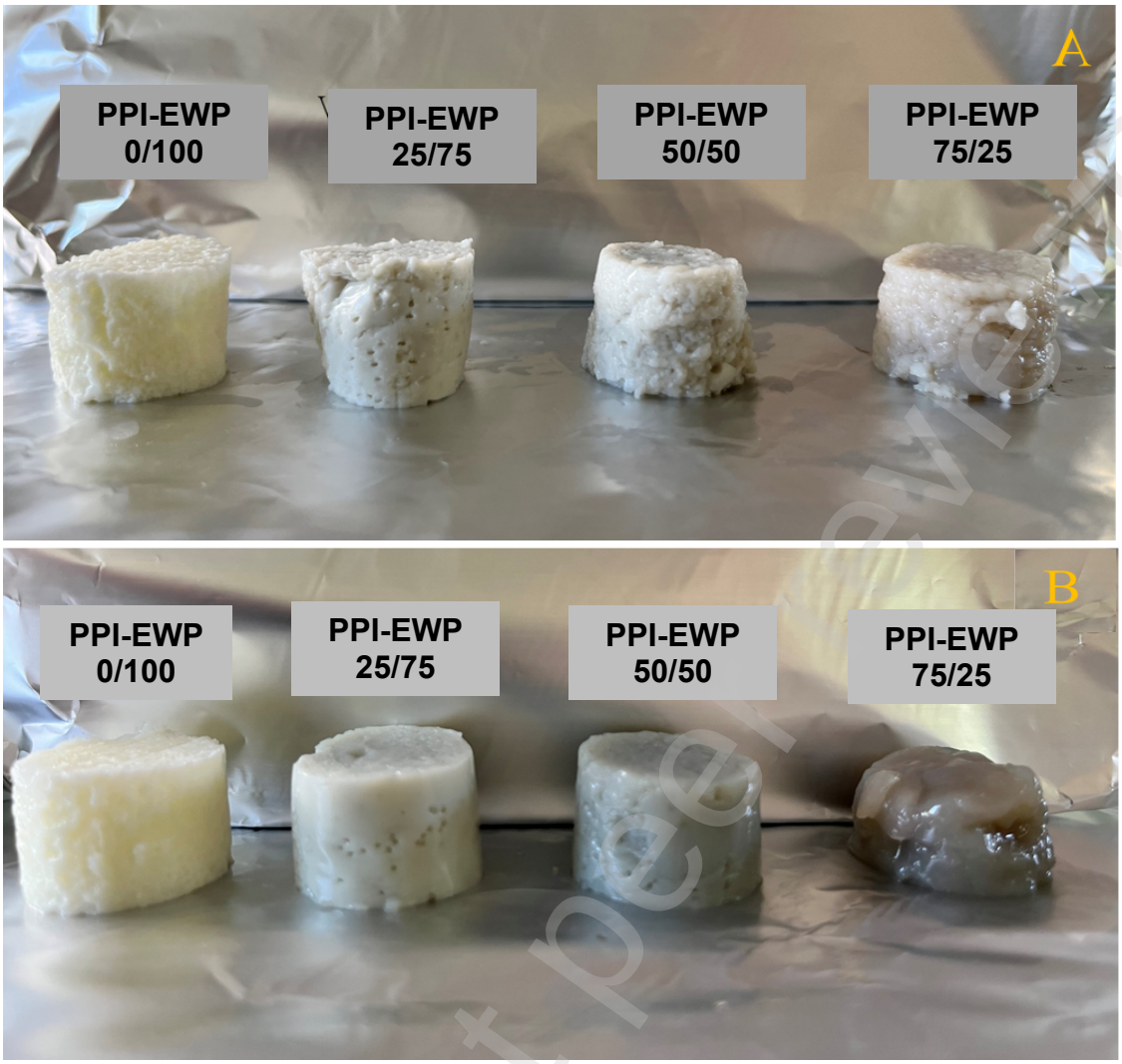

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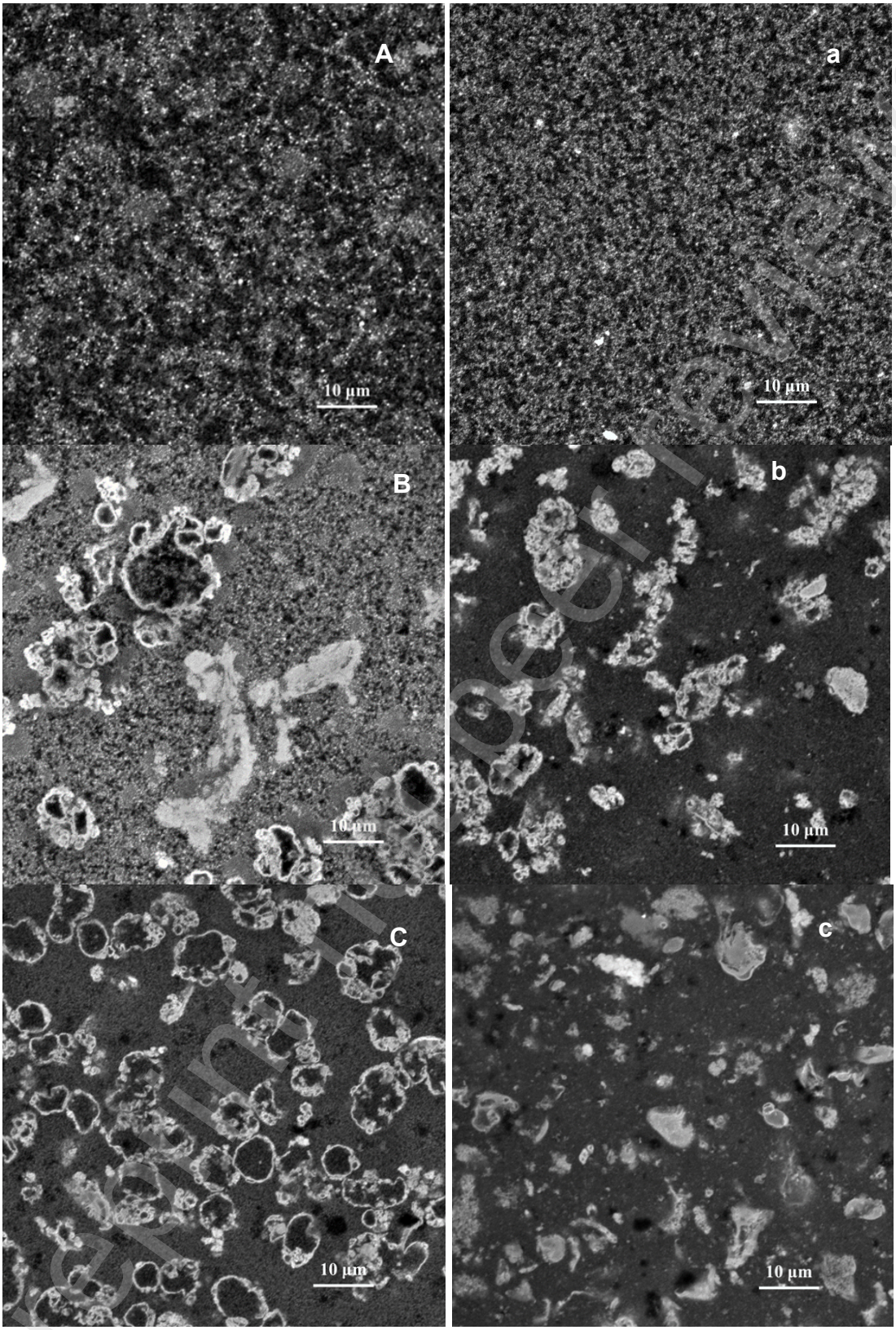

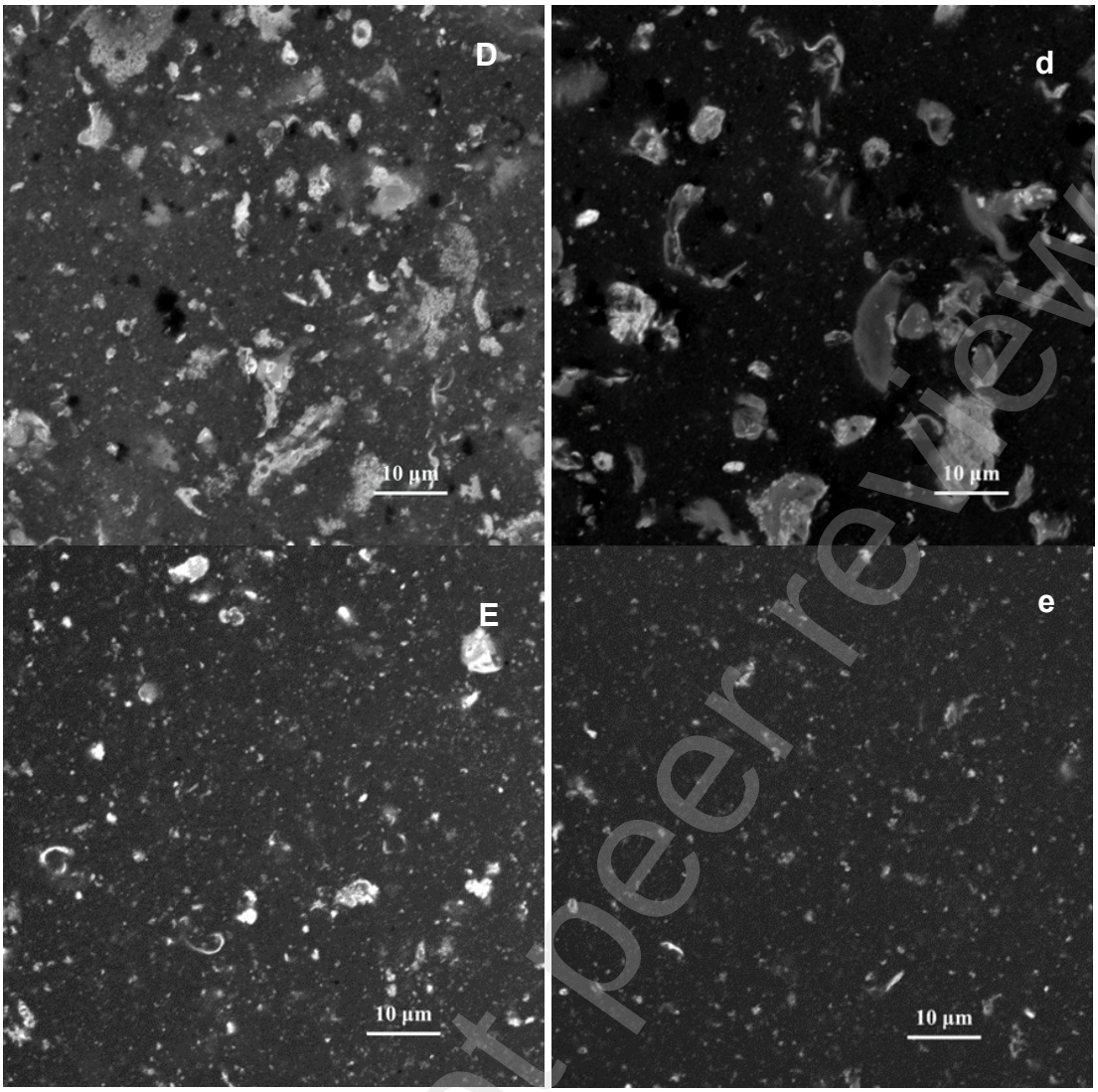

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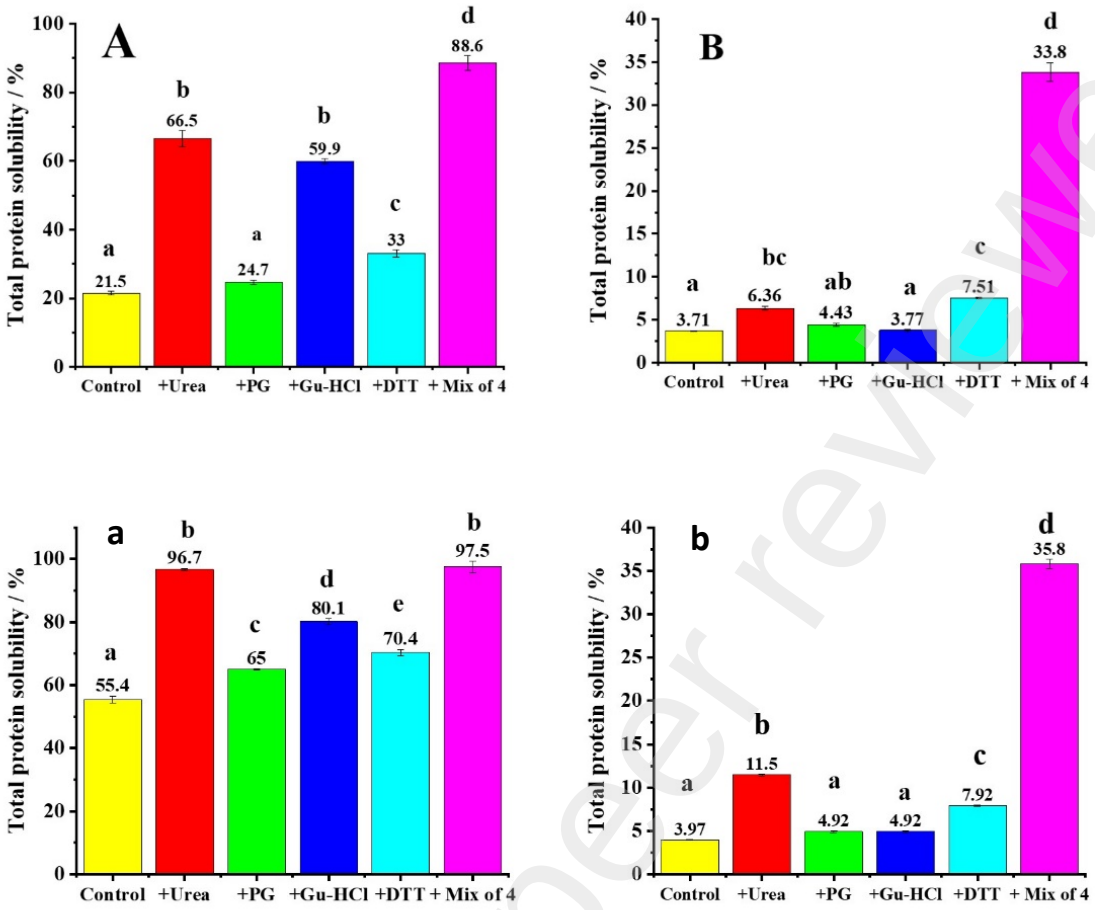

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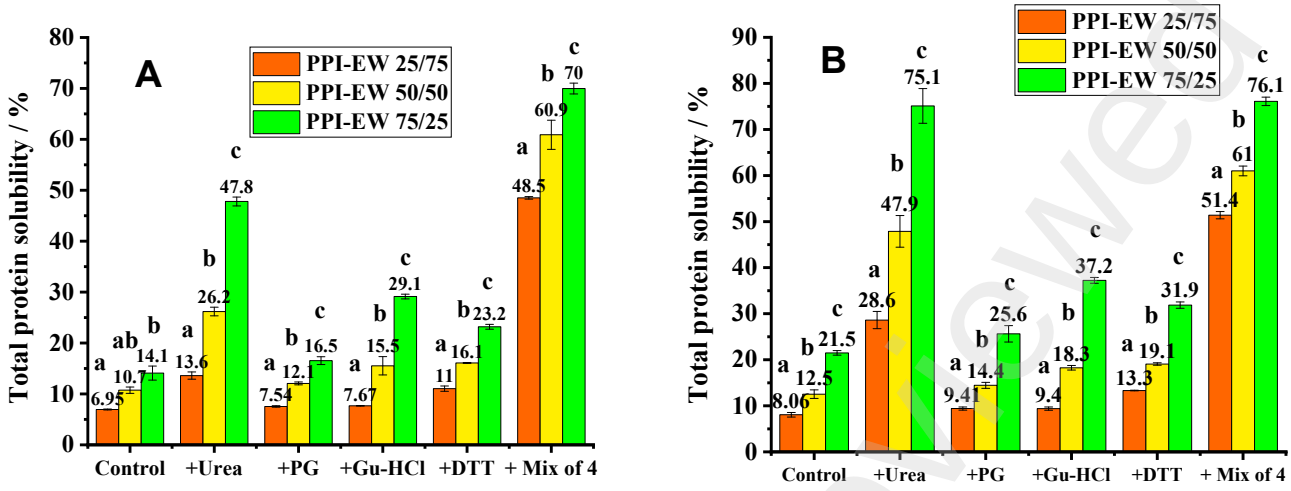

Figure 6.