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# Protein-Protein Interactions and Structure of Heat-Set Gels Based on Pea Protein and Egg White Mixtures

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1 **Protein-protein interactions and structure**  
2 **of heat-set gels based on pea protein and**  
3 **egg white mixtures**

4

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7

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13

14 **Abstract**

15 This study examined the thermal gelation of mixtures of laboratory-prepared pea  
16 protein isolate (PPI) and egg white proteins (EWP) at different PPI/EWP weight ratios  
17 (100/0, 75/25, 50/50, 25/75, 0/100) at pH 7.5 and 9.0. Viscoelastic and texture  
18 properties of the composite gels, along with the microstructure and molecular  
19 interactions involved in the gel network, were investigated. Except PPI-EWP 100/0 at  
20 pH 9.0, all systems gelled with increasing gel hardness, springiness and cohesiveness  
21 when EWP content increased. This phenomenon was explained by the microstructure  
22 of the gels, wherein the presence of PPI enhanced the formation of aggregates  
23 embedded in the EWP network, thus loosening it. The rheological properties of the  
24 mixed gels were primarily influenced by the EWP network, significantly involving  
25 disulfide bonds. However, upon the addition of PPI, hydrogen bonds and hydrophobic  
26 interactions predominated and the structure of the gel became more sensitive to pH as  
27 electrostatic repulsions interfered. Playing on the ratio of PPI/EWP allows for the  
28 production of gels with varying textures, and the data suggest the possibility of  
29 partially substituting egg white with pea proteins in food gel formulation.

30

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

33

## 34 **1. Introduction**

35 To meet the increasing demand for protein, there is a necessity to expand the range of  
36 plant-based protein products. Consequently, the use of combinations of plant and  
37 animal proteins has become increasingly appealing in the formulation of high-protein  
38 food products due to economic advantages, as well as nutritional, functional, and  
39 organoleptic properties (Chihi, Sok, & Saurel, 2018; McCann, Guyon, Fischer & Day,  
40 2018). Egg white proteins have been extensively utilized in the food industry because  
41 of their ability to form gels with favorable nutritional and texture properties (Mine,  
42 1995; Valverde et al., 2016; Li, Zhang et al., 2018). This natural protein mixture is  
43 rich in ovalbumin (OVA) (~54%), ovotransferrin (OVT) (~12%), ovomucoid (~11%),  
44 and lysozyme (LYS) (~3.4%) (Mine, 2002; Guha, Majumder, & Mine, 2019). The  
45 gelation of egg whites is a complex process involving protein denaturation,  
46 aggregation, and the formation of a gel network (Mine, 1995). The characteristics of  
47 egg white gels mainly depend on the medium conditions such as pH, ionic strength,  
48 and the type of salts (Croguennec, Nau, & Brulé, 2002; Nasabi, Labbafi, Mousavi, &  
49 Madadlou, 2017). The gelation of egg proteins has been described as a two-step  
50 process: i) changes in protein structure or partial denaturation; ii) additional  
51 aggregation of denatured proteins, resulting in an exponential increase in viscosity  
52 and the formation of a continuous three-dimensional network (Alleoni, 2006). During  
53 gel formation, non-covalent bonds (i.e., hydrophobic interactions during heating and  
54 hydrogen/ionic bonds during cooling) and covalent disulfide bonds develop,  
55 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 (Barac 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:  $\alpha$  (~20 kDa),  $\beta$  (~13 kDa),  $\gamma$  (~12-16 kDa),  $\alpha\beta$ , and  $\beta\gamma$  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

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

87 The gelation property is one of the crucial functional aspects of proteins, providing  
88 unique textures, sensations, and flavors for food products (Zhang et al., 2019;  
89 Harfmann, 2016). While the gelation of single protein systems is well-documented,  
90 there is less available data on the gelation mechanism of protein mixtures from  
91 different sources. Nevertheless, a few studies have explored the heat-induced gels  
92 formed by combinations of egg and plant proteins, such as egg white with soy  
93 proteins (Su et al., 2015); whole egg or egg yolk proteins with soy proteins (Zhang et  
94 al., 2019); egg white with hempseed proteins (Alavi, Emam-Djomeh, & Chen, 2020).  
95 Co-gelation of pea and animal proteins has primarily involved milk proteins and has  
96 mostly focused on acid gels (Mession, Roustel, & Saurel, 2017; Ben-Harb et al.,  
97 2018; Chihi, Sok, & Saurel, 2018; Oliveira et al., 2022), with fewer studies on heat-  
98 induced gels (Wong, Vasanthan, & Ozimek, 2013; Silva, Balakrishnan, Schmitt,  
99 Chassenieux, & Nicolai, 2018). To our knowledge, no study has examined the heat-

100 induced gelation of egg white and pea protein mixtures. Previous research  
101 demonstrated that the presence of PPI decreased the storage modulus ( $G'$ ) of heated  
102 PPI-EWP mixtures during heating, and protein interactions altered the thermal  
103 denaturation temperature of OVT, LYS, and pea legumin (Kuang et al., 2023a).  
104 However, more experimental data are needed to better understand the importance of  
105 various forces in network formation in this complex system. Therefore, the present  
106 study aimed to investigate the comparative gelling, texture, and microstructure  
107 properties of composite gels based on mixed PPI-EWP at various weight ratios  
108 (100/0, 25/75, 50/50, 75/25, 0/100) at pH 7.5 and 9.0. Additionally, the intermolecular  
109 interactions involved in the heat-induced composite gels were also examined.

110

## 111 **2. Materials and methods**

### 112 **2.1 Samples preparation**

113 Fresh eggs were sourced from a local market in Dijon (France), stored at 4 °C, and  
114 utilized 15 days prior to the expiration date. The fresh liquid egg white was  
115 meticulously separated from the egg yolk and chalaza. The resulting egg white was  
116 then transferred to a beaker and gently homogenized using a magnetic stirrer for 2  
117 hours at room temperature. The total protein content of the egg white was determined  
118 using the Kjeldahl method ( $N=6.25$ ), yielding a value of 10.2% w/w on a dry basis.  
119 Pea globulins were extracted from smooth yellow pea flour (*P. Sativum* L.) supplied  
120 by Cosucra (Warcoing, Belgium), following the method described by Kuang et al.  
121 (2023a). The resulting protein powder, designated as PPI, contained 89% w/w

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

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

130

## 131 **2.2 Gel preparation**

132 10% (w/w) protein suspensions were prepared at pH 7.5 and 9.0 (adjusted with 0.1 M  
133 HCl or NaOH) from initial egg white protein (EWP) and stock suspensions of pea  
134 protein isolate (PPI) to obtain 100% PPI, 100% EWP, and PPI-EWP mixtures at three  
135 weight ratios (75/25, 50/50, and 25/75). The protein suspensions were transferred into  
136 glass vials and heated from 25 to 95 °C (at a rate of 5°C/min) in a water bath, then  
137 maintained at 95 °C for 15 minutes. Subsequently, the vials were cooled to room  
138 temperature in an ice bath and stored at 4 °C overnight to ensure complete gelation.

139

## 140 **2.3 Small-strain dynamic rheology**

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



144 lower plate of the parallel plate geometry of the rheometer. The upper plate was  
145 lowered to achieve a gap width of 1.0 mm. A thin layer of light mineral oil was added  
146 to the well of the upper plate geometry, and a solvent trap cover was used to prevent  
147 evaporation during heating, thus maintaining a water-saturated atmosphere at the  
148 surface of the sample. The following heating protocol was applied: the sample was  
149 initially equilibrated at 25 °C for 3 min, then heated at a rate of 2 °C/min and cooled  
150 at a rate of 5 °C/min over a temperature range of 25–95–25 °C under a shear strain of  
151 1% and a frequency of 1 Hz. Subsequently, a frequency sweep over a range of 0.01-  
152 40 Hz at 1% strain and a strain sweep from 0.01 to 100% strain at 1 Hz were  
153 performed at 25 °C. The storage modulus ( $G'$ ) and loss modulus ( $G''$ ) were measured  
154 during temperature, strain, and frequency sweeps. The loss factor or tangent delta ( $\tan$   
155  $\delta = G''/G'$ ) was also calculated (calculated at 1 Hz, 1% strain), as well as the linear  
156 viscoelastic region (LVR). The LVR was calculated as described in Fig. 1 for the PPI-  
157 EWP mixture at a weight ratio of 25/75. The intersection of the two lines on both  
158 sides of the inflection point was the maximum strain without causing permanent  
159 deformation, called the yield point. Rheological data were collected for every degree  
160 change during heating and cooling. Samples were run in triplicates

161

## 162 **2.4 Texture profile analysis**

163 For texture profile analysis (TPA), all samples were prepared as described in section  
164 2.2 in plastic tubes with a diameter of 40 mm (Krehalon, Deventer, Netherlands).

165 Forty grams of sample suspensions were heated from 25 to 95 °C in a water bath and

166 maintained at this temperature for 15 minutes, then cooled down with ice to room  
167 temperature and stored at 4 °C overnight. Cylindrical gels with a diameter of 40 mm  
168 were sliced using a die cutter at a height of 20 mm and placed on the platform of a  
169 TA-XT Plus (Lloyd Instruments, Ametek company, UK) equipped with a 5 N load  
170 cell and a cylindrical probe with a diameter of 12 mm (SMS-P/35). TPA tests were  
171 conducted at a test speed of 0.5 mm/s, and a deformation in compression of 37.5%  
172 was applied. A time of 10 seconds was allowed to elapse between the two  
173 compression cycles. All samples were prepared in duplicate and tested twice.  
174 The hardness, springiness, and cohesiveness of PPI-EWP gels were determined  
175 according to the method described by Bourne (Bourne, 1978). Hardness was defined  
176 as the maximum peak force during the first compression cycle. Springiness was  
177 defined as the degree of recovery of gels after decompression to their initial shape,  
178 measured by the distance of the detected height during the second compression  
179 divided by the original compression distance. Cohesiveness was calculated as the area  
180 of work during the second compression divided by the area of work during the first  
181 compression. Data were analyzed using Texture Expert software version 1.22 (Stable  
182 Micro Systems).

183

## 184 **2.5 Confocal microscopy (CLSM)**

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

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

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

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

205

## 206 **2.6 Gel dissolution by dissociating agents**

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

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

218 Following the protocol outlined by Chen et al. (2021), gel samples (~2.5 g), prepared  
219 as described in section 2.2, were incubated in individual extractants (~40 mL), stirred  
220 for 1 hour at 25 °C, and homogenized for 1 minute at 10000 rpm using a homogenizer  
221 (Ultra Turrax® IKA T25 Digital, IKA, Germany). The samples were then centrifuged  
222 (16000 rpm, 30 min, 4 °C). The supernatants were collected, filtered using a 0.45 µm  
223 filter, weighed, and diluted with the same extractant for protein assay. The protein  
224 content in the dilutions was measured using a commercial Coomassie Plus (Bradford)  
225 protein assay kit ( $\lambda = 660$  nm) obtained from Sigma-Aldrich (St-Quentin Fallavier,  
226 France), with BSA as the standard. The solubilized protein content was then  
227 calculated as follows (equations 1 and 2):

$$228 \quad \begin{aligned} & \text{Total protein solubility (\%)} \\ & = \frac{\text{protein content in gel supernatant solution}}{\text{protein content in gels}} \times 100 \quad [\text{eq.1}] \end{aligned}$$

229 *Net protein solubility in dissociating buffer (%) = total protein solubility –*  
230 *protein solubility in Tris [eq. 2].*

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

## 232 **2.7 Statistical analysis**

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

## 239 **3. Results and discussion**

### 240 **3.1 Thermal gelation and viscoelastic properties of PPI-EWP gels**

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

247  
248 Before heating, all samples exhibited a relatively low  $G'$  that increased during heating  
249 due to the thermal denaturation and aggregation of the proteins, leading to the  
250 formation of a solid viscoelastic 3D protein network after cooling ( $\tan(\delta) < 1$ ), except  
251 for the 100% PPI mixture at pH 9.0, which did not form a self-supporting gel under  
252 those conditions (Fig. 2A). At this alkaline pH, far from the isoelectric point of pea  
253 protein ( $pI \sim 4.5-4.8$ ), the strong electrostatic repulsions between negatively charged

254 proteins prevented the formation of a cohesive protein network, resulting in only a  
255 viscous suspension for the 100% PPI sample (Kuang et al., 2023b). At pH 7.5, the  
256 100% PPI sample gelled, but the final  $G'$  was lower than 100 Pa, significantly lower  
257 than the values obtained for the EWP-containing gels ( $>3000$  Pa) (Table 1).  
258 Regardless of pH, mixtures containing more than 50% EWP exhibited a two-step  
259 increase in  $G'$  around 60 and 85 °C, previously attributed to the  
260 denaturation/aggregation of OVT and OVA, respectively (Kuang et al., 2023b). When  
261 EWP represented less than 50% of the mixture, a one-step increase was observed  
262 between 80 and 90 °C, consistent with the denaturation temperature of PPI globulins  
263 (Kuang et al., 2023b).

264 The final  $G'$  value of gelled systems significantly increased with an increased content  
265 of EWP, except for the 75/25 PPI-EWP mixture at pH 9 (Table 1). Surprisingly, this  
266 last sample presented a final  $G'$  value comparable to the 25/75 PPI-EWP sample at  
267 the same pH with a very high standard error. Moreover, the  $G'$  of this sample  
268 primarily increased during cooling (about 90-fold) compared to other samples (about  
269 3-4-fold). These observations indicated a very unstable behavior of this mixture,  
270 having a final heterogeneous gel structure (Fig. 4 & 5) that could not be specified as a  
271 self-supporting gel network. While  $G'$  values of the 50% EWP-based gels seemed  
272 independent of pH,  $\tan(\delta)$  was significantly lower at pH 9.0 compared to pH 7.5,  
273 indicating a more viscous contribution at lower pH (Table 1).  $\tan(\delta)$  value also  
274 decreased with an increase in EWP content and reached 0.13 and 0.11 for the 100%  
275 EWP sample at pH 7.5 and 9.0, respectively. These values were characteristic of weak

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

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

293 With the increased proportion of PPI in the mixture, the YP first increased up to 50%  
294 PPI (the 50/50 weight ratio sample presented a maximum at both pHs) and then  
295 decreased. This behavior could be explained by the variable structure of the gels at a  
296 microscopic level depending on the percentage of each protein type in the mixture and  
297 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 &



320 McClements (2022) for RuBisCo gels. The color of PPI-EWP gels at pH 9.0 was  
321 darker than the those at pH 7.5, in agreement with the observations of Zhang et al.  
322 (2023) for gellan gum gels in presence of tea polyphenols.

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

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

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

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

347

### 348 **3.3 Microstructure of PPI-EWP gels**

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

355 For the pure EWP system (Fig. 4A/a), the microstructural organization of the gel  
356 constituted of fine aggregates appeared quite different between pH 7.5 and 9.0. At pH  
357 9.0, the EWP gel presented a denser and more homogeneous protein network than at  
358 pH 7.5, where the protein network was more porous and loosely packed. This result is  
359 similar to previously published SEM and cryo-TEM data showing granular (pH 7) vs.  
360 smooth (pH 9) EWP gel microstructure (Nyemb, et al., 2016; Clark, Kavanagh, &  
361 Ross-Murphy, 2001), and CLSM observations showing a more homogeneous  
362 structure of EWP gels at pH 9 than at pH 5 (Somaratne et al., 2020a). The different  
363 gel structures observed at both pHs may be attributed to the different behavior of

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

375 Fig. 4 E/e shows the microstructure of heated PPI at pH 7.5 and 9.0, respectively. At  
376 pH 9.0, protein particles and small aggregates were poorly interconnected (indicating  
377 no gel formation as previously mentioned), whereas at pH 7.5, a denser protein  
378 network with gel-like properties was observed. Additionally, larger particles were  
379 observed at pH 7.5 (Fig. 4E), whereas only spaced small particles were apparent at pH  
380 9.0 (Fig. 4e). The higher repulsive force between protein particles at high pH, as  
381 indicated previously, could explain the formation of smaller aggregates with  
382 insufficient interconnections to form a solid network.

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

386 protein network. It was assumed that this homogeneous network was formed by egg  
387 protein since egg white was predominant in the mixture, and the appearance of this  
388 domain resembled that observed for 100% EWP gels. With 50% of PPI in the  
389 mixtures (Fig. 4C), more spherical aggregates with black holes were present, and the  
390 surrounding network area decreased. With a higher concentration of PPI in PPI-EWP  
391 mixtures, the gel structure appeared more heterogeneous, forming random protein  
392 clusters of smaller size and irregular shape (Fig. 4D). In contrast, PPI-EWP gels at pH  
393 9.0 (Fig. 4b-d) exhibited some differences. When EWP was the dominant component  
394 (Fig. 4B-C), the gel showed some large aggregates (~10-20  $\mu\text{m}$ ) resembling brain-like  
395 structures, surrounded by a continuous network similar to pure EW gel. When PPI  
396 comprised 50% of the mixtures, the gel contained numerous protein clusters of  
397 smaller size. When PPI was the dominant component, the irregular clusters were  
398 dispersed in a less well-defined continuous phase. Similar observations regarding  
399 mixed gels were previously reported by Kornet et al. (2020), who found that whey  
400 protein-PPI gels contained large clusters with a high pea protein content. Silva,  
401 Cochereau, Schmitt, Chassenieux, & Nicolai (2019) demonstrated that mixtures of  
402 micellar caseins and PPI at pH 5.8 formed gels with protein clusters, whereas more  
403 homogeneous gels were obtained for individual proteins. McCann et al. (2018) and  
404 Roesch & Corredig (2005) observed a discontinuous network in soy protein-whey  
405 protein gels at a total protein concentration of around 6%, indicating phase separation,  
406 while Gómez-Mascaraque & Pinho (2021) found a microgel structure between soy  
407 and whey protein gels.

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

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

442

### 443 **3.4 Intermolecular interactions involved in PPI-EWP gels**

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

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

### 453 **3.4.1 Effect of dissociating agents on 100% PPI- and 100% EWP- gels**

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

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

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

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

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



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

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

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

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

527

#### 528 **3.4.2 Effect of dissociating agents on PPI-EW mixed gels**

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

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

538 On the contrary, when mixed gels were rich in PPI (PPI-EWP 75/25), urea was the  
539 most efficient dissociating agent ( $47.8\% \pm 0.9$ ), followed by guanidinium-HCl ( $29\% \pm$   
540  $0.5$ ) at pH 7.5 (Fig. 6A). This result suggests a combination of non-specific and lower  
541 energy interactions, similar to the case of 100% PPI gels, with a dominance of  
542 hydrogen and hydrophobic bonds.

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

558

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

560 **mixtures**

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

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

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602 Competing interests' statement:

603 The authors have no competing interests to declare

604

605 Figure captions:

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 <sup>aA</sup>	14446 ± 413 <sup>aA</sup>	0.135 ± 0.002 <sup>aA</sup>	0.115±0.001 <sup>aB</sup>
25/75	7284 ± 192 <sup>bA</sup>	7204 ± 281 <sup>bA</sup>	0.138 ± 0.001 <sup>aA</sup>	0.118±0.003 <sup>aB</sup>
50/50	4725 ± 324 <sup>cA</sup>	4182 ± 440 <sup>cA</sup>	0.151 ± 0.002 <sup>bA</sup>	0.134±0.004 <sup>bB</sup>
75/25	3446 ± 331 <sup>cA</sup>	9237 ± 3249 <sup>bB</sup>	0.157 ± 0.003 <sup>bA</sup>	0.158±0.008 <sup>cA</sup>
100/0	97 ± 4 <sup>d</sup>	no gel	0.227 ± 0.005 <sup>c</sup>	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 <sup>a</sup>	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 <sup>a</sup>	0.73±0.02 <sup>a</sup>	0.95±0.04 <sup>a</sup>	3.90±0.08 <sup>a</sup>	0.75±0.01 <sup>a</sup>	0.93±0.02 <sup>a</sup>
25/75	2.78±0.17 <sup>a</sup>	0.67±0.02 <sup>a</sup>	0.92±0.04 <sup>ab</sup>	3.45±0.16 <sup>a</sup>	0.74±0.01 <sup>ab</sup>	0.91±0.03 <sup>ab</sup>
50/50	1.69±0.03 <sup>b</sup>	0.58±0.02 <sup>b</sup>	0.84±0.00 <sup>ab</sup>	1.84±0.08 <sup>b</sup>	0.71±0.00 <sup>bc</sup>	0.89±0.01 <sup>ab</sup>
75/25	0.79±0.00 <sup>c</sup>	0.59±0.02 <sup>b</sup>	0.80±0.01 <sup>b</sup>	0.51±0.01 <sup>c</sup>	0.67±0.01 <sup>c</sup>	0.81±0.01 <sup>b</sup>

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), Tanford (1968), Léger & Arntfield (1993), Sun & Arntfield (2012)
Guanidinium-HCl (GuHCl)	Disrupt	Weaken	Disrupt		Tanford (1962), Ustunol et al. (1992), Utsumi & Kinsella (1985)
Propylene glycol (PG)	Promote	Disrupt	Promote		Gordon & Jencks (1963), Uruakpa & Arntfield (2006), Ustunol et al. (1992), Nozaki & Tanford (1963)
Urea		Disrupt	Disrupt		

645

646

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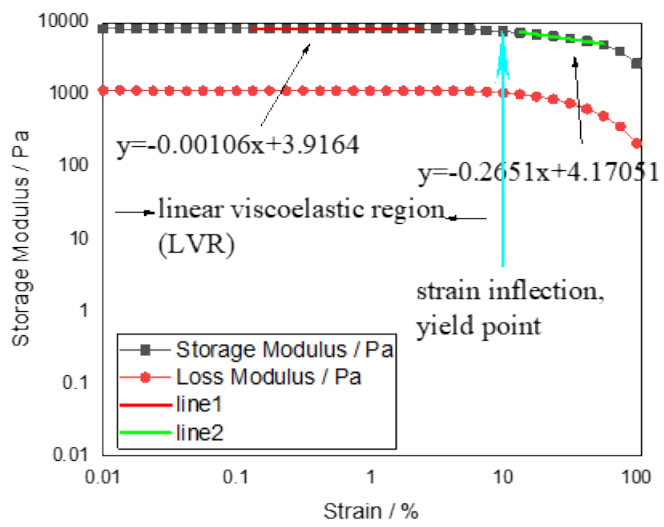


Figure 1.

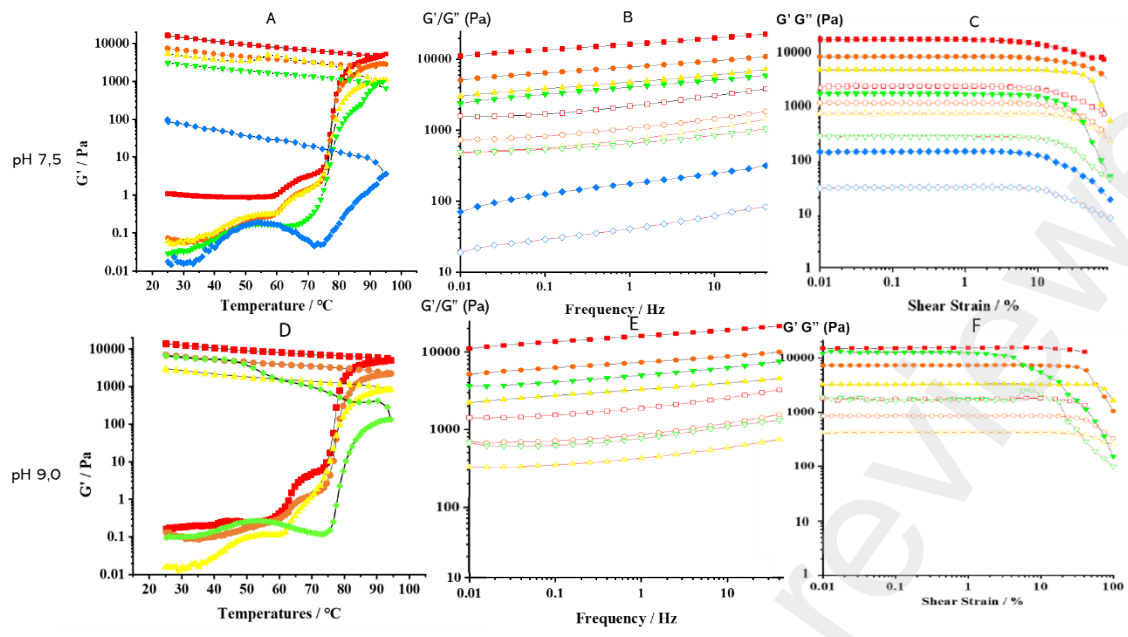


Figure 2.

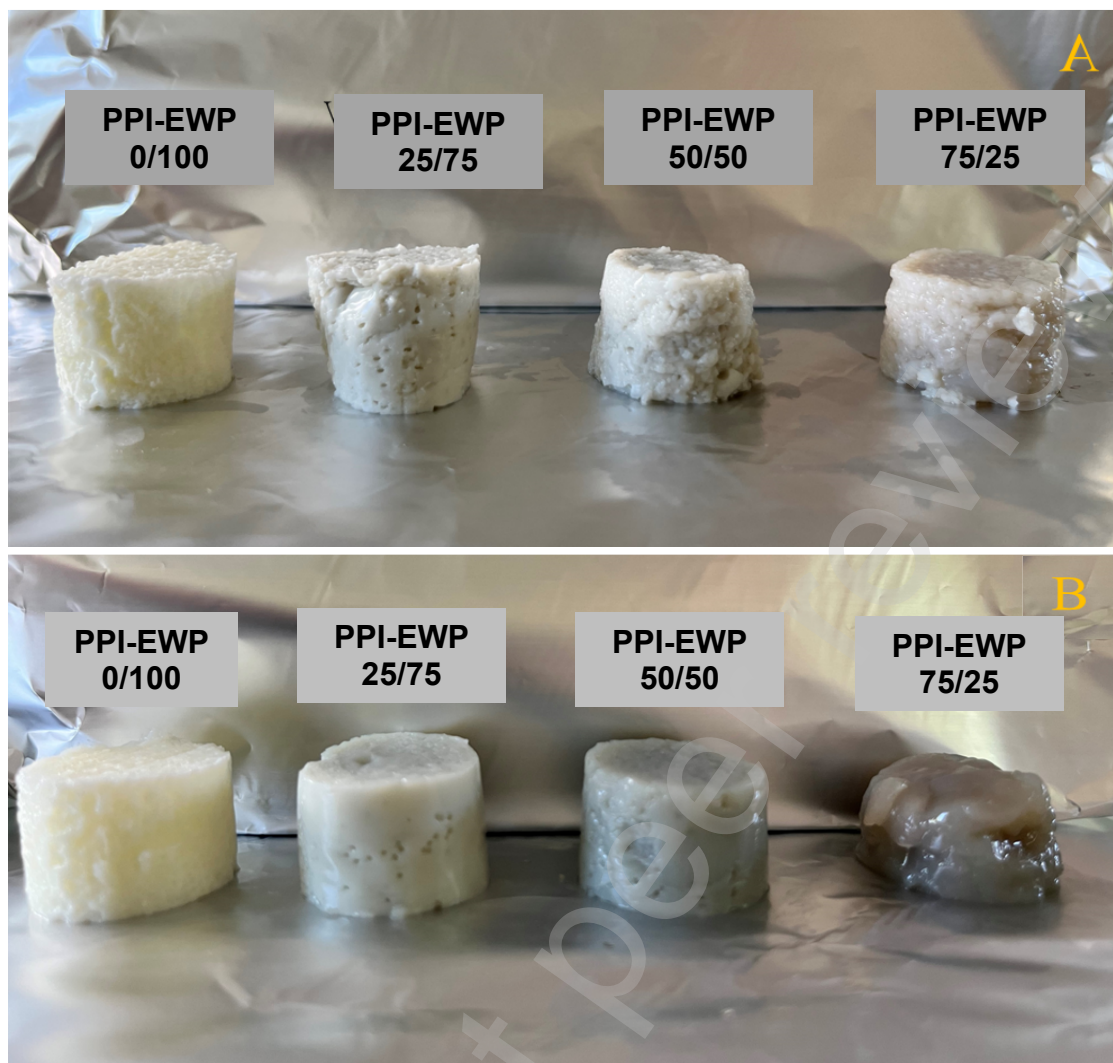
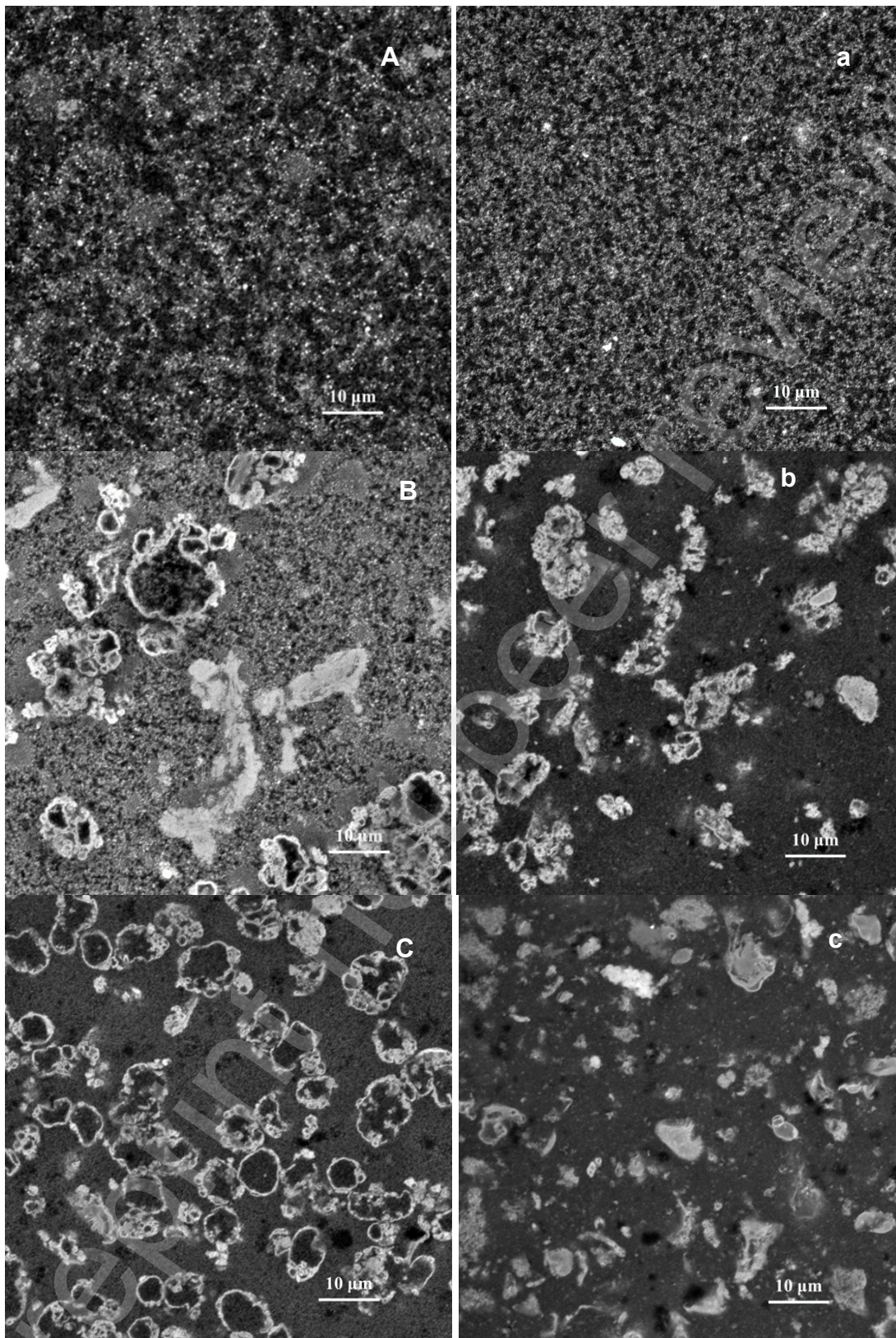


Figure 3.





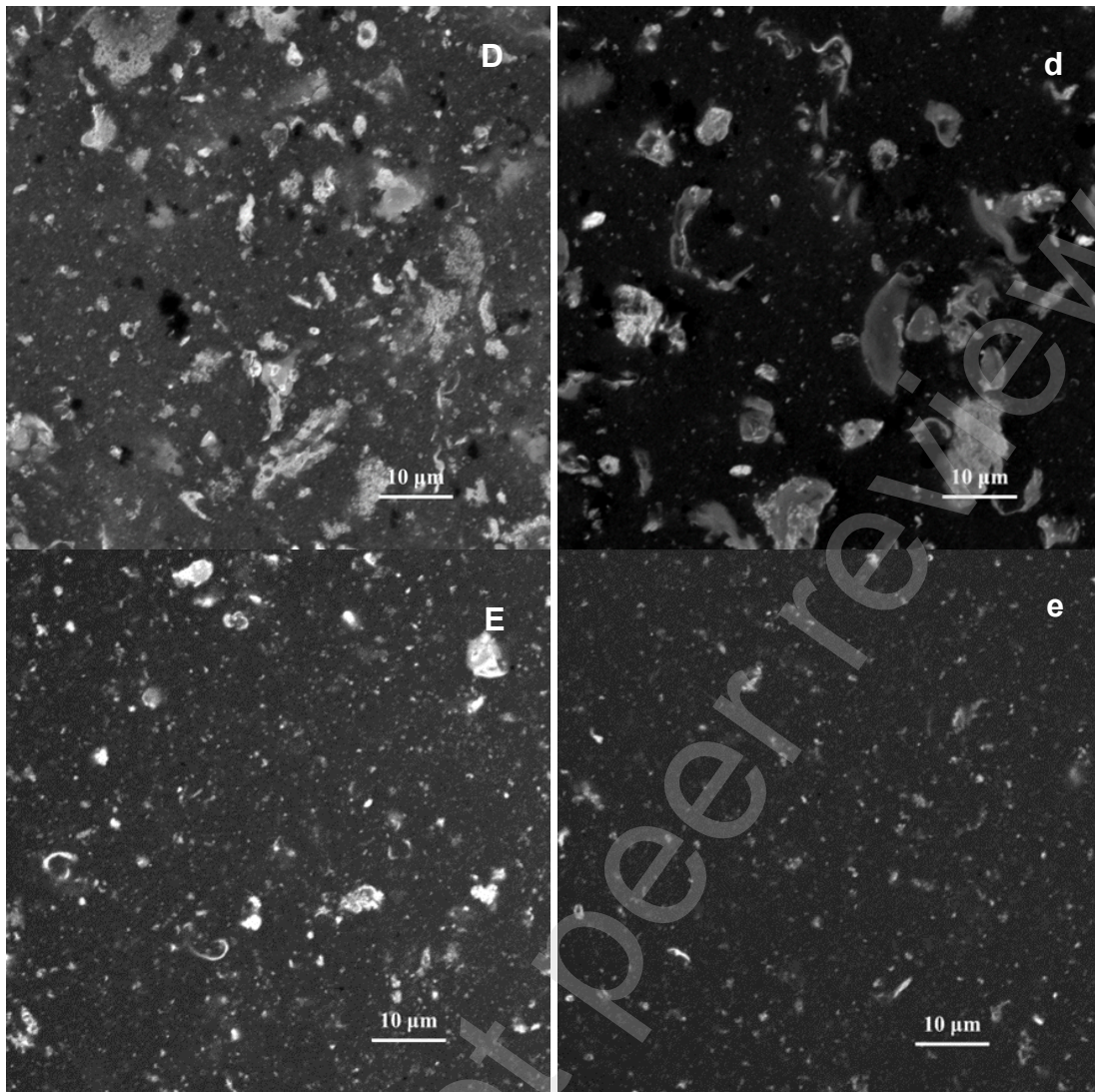


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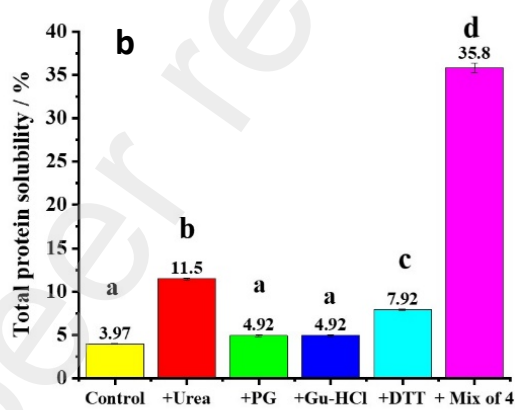
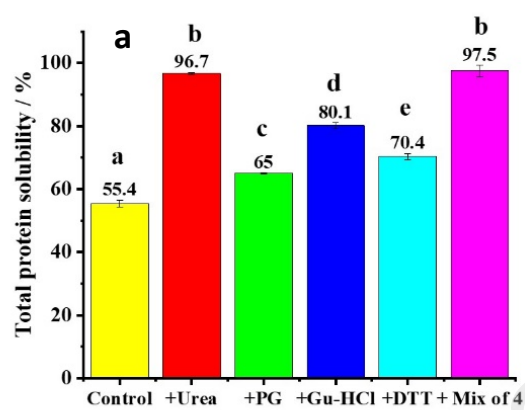
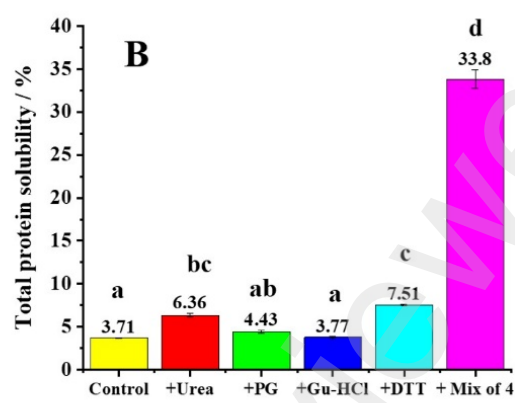
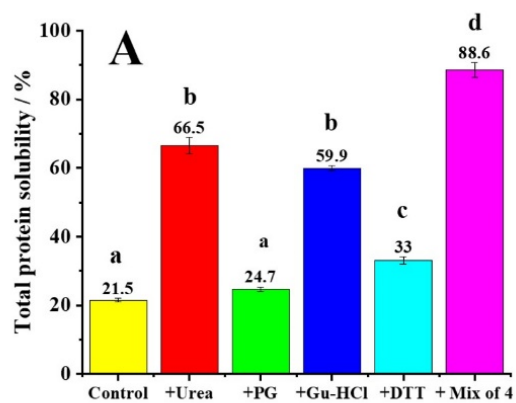


Figure 5.

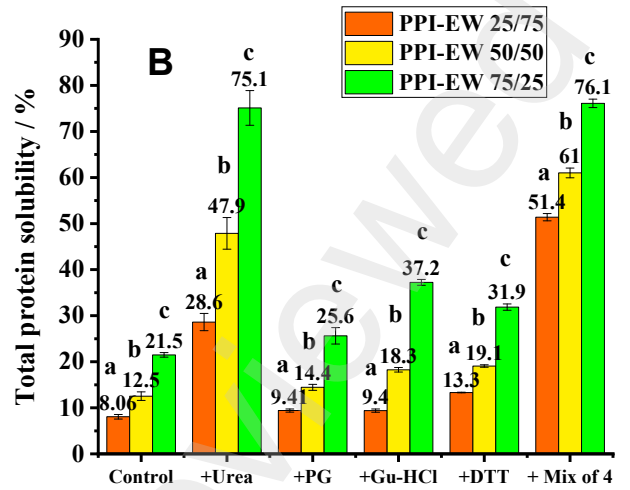
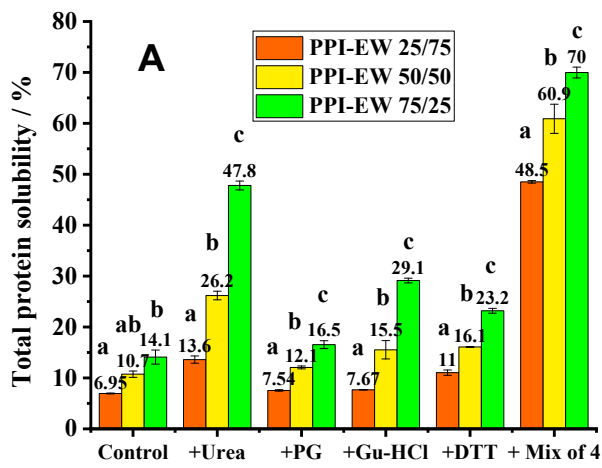


Figure 6.