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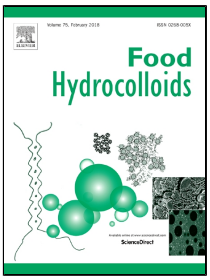

Emulsification performance and interfacial properties of enzymically hydrolyzed peanut protein isolate pretreated by extrusion cooking

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Highlights

"Emulsification performance and interfacial properties of enzymically hydrolyzed peanut protein isolate pretreated by extrusion cooking" by Chen *et al*.

Food Hydrocolloids.

- Extrusion pretreatment increased the protease accessibility of peanut protein isolate.
- The insoluble protein particles in peanut protein isolates tended to induce bridging flocculation of emulsion droplets during homogenization.
- Hydrolysates of extruded peanut protein isolate showed a high protein solubility of ~90%.
- The production of surface active peptides during enzymic proteolysis of peanut protein isolate was promoted after extrusion pretreatment.
- Extrusion pretreatment produced noticeable benefits in improving emulsification performances of protein hydrolysates.

Emulsification performance and interfacial properties of enzymically hydrolyzed peanut protein isolate pretreated by extrusion cooking

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Abstract

In this study, peanut protein isolate (PPI) was modified with extrusion pretreatment and papain-induced proteolysis. SDS-polyacrylamide gel electrophoresis showed that extrusion pretreatment conducted at 130 °C substantially increased the protease accessibilities of the major constitutive proteins (conarachin and arachin) in EPPI (extrudates of PPI), resulting in a remarkable increase in the degree of hydrolysis (DH) and protein solubility for the hydrolysates. Analysis of droplet size distributions and microstructures of oil-in-water model emulsions formed by PPIH (PPI hydrolysates) and EPPIH (EPPI hydrolysates) with different DH showed that extrusion pretreatment led to a marked enhancement in the emulsification performance for the hydrolysates. EPPIH (6.2% DH) was capable of producing a stable emulsion (20 vol.% sunflower seed oil) with fine droplets (d_{32}=0.4 μm, d_{43}=1.6 μm) at 2.5% (w/v) sample content, whilst the equivalent emulsions made with control PPI and PPIH (0.9% DH) required 6.5% and 5.5% (w/v) level of sample, respectively. Based on investigations of surface pressure versus sample concentration profiles and saturation surface Loads (Γ_{sat}) for some selected PPIH and EPPIH, it was found that with most insoluble protein particles in EPPIH being enzymically hydrolyzed and becoming soluble, the production of surface active peptides with low Γ_{sat} was substantially promoted during enzymic proteolysis, which was responsible for the efficient use of EPPIH (6.2% DH) on generating and stabilizing small emulsion droplets against bridging flocculation during homogenization. These results indicated that hydrolyzed PPI could be used an efficient food emulsifying agent with extrusion pretreatment substantially increasing its protease accessibility.

Keywords: peanut protein isolate; extrusion pretreatment; protease accessibility; emulsification performance; saturation surface load; surface pressure

1. Introduction

Many legume protein isolates have been investigated as possible emulsifying agents in emulsion-based food (Benjamin, Stilcock, Beaucham, Buettner, & Everett, 2014; Ghatak & Sen, 2013; Karaca, Low, & Nickerson, 2011; Ma, Boye & Simpson, 2016). Among them, peanut protein isolate (PPI) is usually preferred due to the good surface active properties of its major constitutive proteins arachin and conarachin, which have shown to be able to substantially lower the oil/water interfacial tension (Benjamin et al., 2014; Karaca et al., 2011). In addition, PPI has been considered as a good nutritional source due to its high content of essential amino acids, low risk of allergic reactions and steady supply, which has gained preference among both consumers and producers (Ghatak et al, 2013). However, due to the rigid globular structures of native peanut proteins, PPI is less capable as an emulsifying agent when compared to milk proteins with flexible molecular structures (McClements & Gumus, 2016). Moreover, extensive denaturation and aggregation of peanut proteins usually occur during the production of commercial PPI, resulting in the loss of much of its soluble proteins and emulsification capability (Taherian et al., 2011).

Modification of proteins based on enzymolysis has been considered to be safe and of great potential to improve their emulsification performances. This is attributed to 3 distinct structural changes caused by enzymic proteolysis: a decrease in average molecular mass, the exposure of hydrophobic groups and the liberation of ionizable groups (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). However, peanut proteins or protein aggregates in PPI were generally resistant to enzymolysis due to their highly compact structures, leading to a limited improvement on functionalities for the resulting hydrolysates (Perrot, Quillien, & Guéguen, 1999; Zhao, Liu, Zhao, Ren, & Yang, 2011). With this regards, attempts should be made to enhance the protease accessibility

of PPI, which has been proved to be a key influencing factor in achieving desirable functionalities of final products (Jung, Murphy, & Johnson, 2005; Surówka, Żmudziński, Fik, Macura, & Łaocha, 2004; Zeeb, McClements, & Weiss, 2017; Zheng et al., 2006).

Extrusion cooking is a high-temperature-short-time physical treatment during which feed materials are subjected to high temperature (90–200 ºC), high pressure (1.5–30.0 MPa) and mechanical shear simultaneously in the extruder (Day & Swanson, 2013). According to the literature, these effects could cause unfolding, denaturation and realignment of protein molecules (Alam, Kaur, Khaira, & Gupta, 2016). Enzymic proteolysis of plant-protein products is usually enhanced by extrusion cooking, resulting in significant changes in physicochemical and functional properties for the resulting hydrolysates. For instance, Alonso, Aguirre, and Marzo (2000a) compared extrusion cooking with several other thermal processing methods on trypsin-induced hydrolysis of legume proteins, and showed that extrusion is the most effective pretreatment method in promoting enzymic proteolysis. Zhen et al. (2006) reported that extrusion pretreatment substantially increased the protease accessibilities of the major components of corn gluten, resulting in a strong increase in protein solubility for corn gluten hydrolysates. Moreover, the enhancement of enzymic proteolysis of peanut extrudates has also been reported by several other studies (Abd El-Hady & Habiba, 2003; Alonso, Grant, Dewey, & Marzo, 2000b; Chen & Phillips, 2005).

Noticeably, emulsifying properties of hydrolysates of extruded soy proteins have been investigated, but the experimental results reported in the literature appear ambiguous. As Surówka et al. (2004) reported that extrusion pretreatment followed by limited enzymic proteolysis using Neutrase caused a marked increase in emulsifying activity index (EAI) but a decrease in emulsifying stability index (ESI) for soy flour. On the contrary, Jung et al. (2005) found that such a treatment

could increase the ESI for soy flour. Such contradictory could be caused by the misleading EAI and ESI results based only on the turbidity measurements, which has been proved to be not most reliable for the characterization of micron-size droplets (McClements, 2007).

Commercial laser diffraction particle size analysis (LD-PSA) instruments are capable of determining particle size distributions and average particle diameters within the range of about 100 nm to 1000 μm and have been used as a standard technique for emulsion characterization (McClements, 2007). Laser scanning confocal microscopy (LSCM), on the other hand, is a powerful technique which can deliver high-resolution microimages on fine emulsion in real space, and has a variety of novel contrast mechanisms that enable us to monitor the size and spatial distributions of droplets in emulsions, and even to examine the structures of emulsion interfacial layers (Kwok & Ngai, 2016). Therefore, a combination of these two complementary techniques could not only give information for assessment of emulsification performances among different emulsifying agents, but also provide new insights into the underpinning mechanisms of emulsion formation and stabilization (Hu, Ting, Hu, & Hsieh, 2017). However, to our knowledge, no such work has been done on the emulsification performance of hydrolyzed PPI.

Hence, this study was designed to improve the emulsification performance of PPI using extrusion pretreatment and controlled enzymic proteolysis. Emulsification performances of protein samples were investigated by analyzing droplet sizes and microstructures of freshly formed oil-water emulsions as characterized using LD-PSA and LSCM. Some key interfacial properties including surface pressure (π) versus sample concentration profile and saturation surface Load (Γ_{sat}) were also measured accordingly, in order to explore the underpinning mechanisms of improved emulsification performances for hydrolyzed products caused by extrusion pretreatment.

2. Materials and methods

2.1. Materials

Commercial PPI was kindly provided by Tianshen Bioprotein Co. (Linyi, China). This PPI was produced using alkaline extraction followed by isoelectric pH precipitation; the precipitated proteins were then re-suspended in alkaline solution and underwent intensively thermal treatments including high temperature sterilization and spray-drying. The protein content of the PPI product was 88.1 % (w/w, dry basis), determined using Kjeldahl method (N × 5.46); the moisture content was 5.2% (w/w), determined according to AOCS Official Method Ba 2a-38; based on the information provided by the manufacturer, this PPI contains <2.0% fat, <4.0% ash. Food grade papain (EC 3.4.22.2) with the nominal activity of 114 460 U/g was obtained from Baiao Biochemistry Co. (Jiangmen, China), and was applied to induce the proteolysis of protein samples without activation. Nile blue, Nile red, and phenylmethanesulfonyl fluoride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Laemmli sample buffer, Tris-HCl precast gel (4–15 %), β-mercaptoethanol, and Coomassie Brilliant Blue R-250 were purchase from Bio-Rad Laboratories (Hercules, CA, USA). Sunflower seed oil was purchased from a local grocery store. Sunflower seed oil purified with Florisil (PR grade, 60–80 mesh, Sigma-Aldrich) was used as the oil phase in the interfacial tension measurements, and that without further purification was used in the preparation of emulsions. All chemicals were of reagent grade. Deionized water prepared with a Milli-Q apparatus (Millipore, USA) was used throughout. The pH was adjusted using 0.1–1.0 M HCl solutions and 0.1–1.0 M NaOH solutions.

2.2. Extrusion cooking of PPI

Extrusion experiments were carried out using a laboratory-type twin-screw extruder (SYSLG30-IV, Saibainuo Technology Co. Ltd, Jinan, China) with four individual barrel zones from the feeder

133 to the die, each with separate temperature control. **Fig. 1** shows the schematic diagram of this
134 extruder. The temperature profile in the extruder from the first zone to the third zone was constantly
135 set at 25, 50, and 90 ºC, respectively. The fourth zone was set at the desired cooking temperature
136 (100, 130, and 160 ºC). The diameter of the screw was 30 mm, and the extruder had a barrel length-
137 to-diameter ratio of 23: 1, with a cooling die attached at the end of the extruder. The screw elements
138 included kneading blocks and reverse paddles, and the screw speed was set at 325 rpm. Prior to
139 extrusion, the moisture content of PPI was adjusted to 15 % (w/w). Moisturized PPI was fed into the
140 extruder with a loss-in-weight feeder at a rate of 12.8 kg/h. The extrudates of PPI (EPPI) were allowed
141 to cool to room temperature and then ground to pass through a screen of 40 mesh. Ground samples
142 were dried in a convection oven at 40 ºC for about 18 h to reach a moisture content of ca. 5.2 % (w/w)
143 similar to that of raw PPI. The resulting EPPI powder was sealed and stored at 4 ºC for further use.

144 **2.3. Preparation of PPIH and EPPIH and determination of degree of hydrolysis (DH)**

145 Papain, a protease having a broad specificity to peptide bonds, was used to induce the
146 proteolysis. PPI or EPPI powder was fully dispersed into deionized water (powder: water = 1:10, w/v)
147 by stirring at room temperature for 2 h using magnetic stirrers, with pH being adjusted to 7.0. Papain
148 was then added into the resulting sample suspensions, and the enzymic proteolysis was carried out at
149 50 ºC and pH 7.0 in a temperature-controlled shaking water bath operated at 120 rpm rate. An auto-
150 titrator (848 Titrino plus, Metrohm, Switzerland) loaded with 0.1–1.0 M NaOH solutions was used
151 to maintained the pH of suspensions constantly at pH 7.0 during proteolysis. On the basis of
152 preliminary experiments, different enzyme-to-substrate ratios (E:S, 0.05–0.5%, w/w) were used to
153 prepare PPIH and EPPIH with desirable DH values. The DH of protein hydrolysates refers to the ratio
154 of cleaved peptide bonds against the total peptide bonds before proteolysis. In this study, the pH-stat

155 method described by Adler-Nissen, Eriksen & Olsen (1983) was used to control and determine the
156 DH (%) of different samples, which was calculated as Eq. (1):

157 $$\mathrm{DH}\,(\%) = \frac{V_{\mathrm{NaOH}} \times C_{\mathrm{NaOH}}}{\alpha \times M_{\mathrm{P}} \times h_{\mathrm{tot}}} \times 100\% \qquad (1)$$

158 where α is the average degree of dissociation of α-amino groups; M_P is the mass of protein (N × 5.46,
159 g); h_{tot} is the total number of peptide bonds in the substrate (meqv/g protein); C_{NaOH} is the
160 concentration of NaOH solution (M), and V_{NaoH} is the consumption of NaOH solution (mL).
161 According to the literature, α is taken as 0.44 at 50 ºC and pH 7.0 and h_{tot} value is 7.52 meq/g for PPI
162 (Adler-Nissen et al., 1983). The hydrolysis time was set at 120 min, wherein a plateau in DH over
163 time can be achieved for each E:S combinations. At the end of the reaction time, the protease inhibitor
164 phenylmethanesulfonyl fluoride was added into the sample suspensions to a concentration of 1 mM
165 so as to terminate the papain-induced proteolysis (Luo et al., 2010). The amount of NaOH solution
166 consumed was recorded for determining the DH. Finally, the hydrolysates were lyophilized, finely
167 milled, and sealed in plastic bags. As controls, suspensions of PPI and EPPI were treated with the
168 same incubation conditions and enzyme inactivation treatment as described above, but without papain
169 added and also required minimal NaOH addition. Throughout this article, samples were designated
170 according to the extrusion profiles used and the DH obtained. For example, EPPIH-6.2% refers to the
171 hydrolysate of PPI being pretreated with extrusion and having a DH of 6.2%.

172 **2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

173 The polypeptide profiles of different protein samples were determined by SDS-PAGE under
174 reducing conditions using the Laemmli method (Laemmli, 1970). Briefly, 100 μL of protein sample
175 suspension (2.0 %, w/v) was mixed with 100 μL of Laemmli sample buffer (containing 5 % v/v β-
176 mercaptoethanol). The mixtures were heated to 95 °C for 10 min and were centrifuged for 5 min at

177 10,000 *g*. An aliquot (15 μL) of the resulting sample supernatant was loaded onto the Tris-HCl precast
178 gel (4–15 %) for electrophoresis running in a Mini-protean Tetra system (Bio-Rad Laboratories).
179 Electrophoresis was conducted at 200V until the indicator dye reached the gel bottom. After
180 separation, proteins were fixed and stained using Coomassie Brilliant Blue R-250.

181 **2.5. Determination of protein solubility (PS)**

182 The PS values of samples were determined according to the method of Karaca et al. (2011), with
183 some modifications. Sample suspensions (1.0 %, w/v) were magnetically stirred for 2 h, with pH
184 being adjusted to 7.0. The resulting suspensions were centrifuged (10,000 *g*, 30 min) to collect
185 supernatants. The soluble nitrogen content of the supernatant was determined using Kjeldahl method
186 and PS was expressed as the ratio of soluble nitrogen in the supernatant to total nitrogen in the sample.

187 **2.6. Emulsion formulation and characterization**

188 Protein samples were dispersed into deionized water to reach different concentrations (1.5–
189 7.0 %, w/v), followed by magnetically stirring for 2 h. The resulting suspensions were adjusted to pH
190 7.0. Oil-in-water emulsions were prepared by homogenizing 20 % (v/v) sunflower seed oil and 80 %
191 (v/v) sample suspensions. Initially, a coarse emulsion was formed by blending the oil and sample
192 suspensions in a high shear blender (Shanghai Specimen Model Co., China) at 20,000 rpm for 2 min.
193 The coarse emulsion was then passed through a high-pressure valve homogenizer (APV-2000 Gaulin,
194 Abvertslund, Denmark) twice at 30 MPa.

195 Droplet size distributions (DSD) of emulsions were measured using a Mastersizer 3000 laser
196 diffraction particle size analyzer (Malvern Instruments, Malvern, UK). The refractive index and
197 adsorption of the sunflower seed oil were taken as 1.462 and 0.001, respectively; and the refractive
198 index of water was taken as 1.330. Average droplet sizes were characterized as Sauter mean diameter

199 $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ and volume mean diameter $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of droplets
200 of diameter d_i. The d_{43} diameter was used to examine differences in the droplet size distributions for
201 different sample emulsions, since this parameter was particularly sensitive to the appearance of large
202 droplets or droplet aggregates in a size distribution due to, for example, flocculation (Hu et al., 2017).

203 The microstructures of fresh emulsions were visualized using a LSM 710 Laser scanning
204 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) operating in fluorescence
205 mode. Nile Red dye was used to stain the emulsion oil phases, with fluorescence excited at 488 nm.
206 The Nile Red staining solution was prepared by dispersing 0.001g of Nile Red dye in 10 mL of 1,
207 2-propanediol. Nile Blue dye was used to stain the proteins in emulsion, with fluorescence excited at
208 633 nm. The Nile Blue staining solution was prepared by dispersing 0.001g of Nile Blue dye in 10
209 mL deionized water. An aliquot (20 μL) of the Nile Red or Nile Blue solution was thoroughly mixed
210 with 5 mL of emulsion. The samples were scanned at 25 ºC, using 20× (NA 0.1) and 63× (NA 1.2)
211 water-immersion objective lens. As the Nile Red stained the oily substance in emulsion, the oil phase
212 appeared as greenish colour, whilst the water/protein phase appeared dark in the microimages; as the
213 Nile Blue stained the proteinaceous substance, the protein phase appeared as reddish colour, whilst
214 the oil/water phase appeared dark.

215 **2.7. Determinations of surface pressure (π)**

216 Interfacial tension measurements of protein samples at oil-water interface were determined
217 based on the sessile drop method using an OCA-50 contact angle and drop contour analysis system
218 (Dataphysics Instruments GmbH, Germany). Suspensions of PPIH and EPPIH in 50 mM phosphate
219 buffer solution (pH 7.0) were serially diluted to a final concentration of 0.001–5.0 % (w/v). After
220 centrifugation (10,000 *g*, 20 min), the resulting supernatants were collected. The interfacial tension

measurements were carried out at 25 °C. A drop (15 μL) of the sample supernatants was delivered into an optical glass cuvette containing purified sunflower seed oil by the automatic sampling system, and allowed to stand for 180 min to achieve protein adsorption at the oil-water interface. Charged coupled device camera started to photograph the contour of the drop immediately after its formation, from which the interfacial tension were automatically calculated based on Young-Laplace equation by the instrument. The surface pressure was calculated from the interfacial tension measurements: $\pi = \gamma_0 - \gamma$, where γ_0 is the interfacial tension of the phosphate buffer–sunflower seed oil interface (30.2 ± 0.3 mN/m) and γ is the interfacial tension in the presence of protein samples.

2.8. Determinations of saturation surface load (Γ_{sat})

The Γ_{sat} values of protein samples were determined according to the method of Zhao et al. (2014), with some modifications. Freshly prepared emulsions were diluted with 1 × deionized water, and were centrifuged for 2 h at 12,000 g to separate oil droplets from serum layer. The oil droplet phase was carefully removed using a syringe. The amount of non-adsorbed protein remained in the serum phase and precipitates was determined using Kjeldahl method (N × 5.46). The mass adsorbed protein per unit volume of emulsion (C_a) is equal to the initial concentration of protein minus that of non-adsorbed protein after homogenization. The Γ_{sat} (mg m^{-2}) is then calculated from Eq. (2):

$$\Gamma_{sat} = \frac{C_a \times d_{32}}{6 \times \emptyset_{oil}} \qquad (2)$$

where d_{32} diameters of emulsions are measured using Mastersizer 3000; $\emptyset_{oil}$ is the volume fraction of the oil in emulsions (0.2).

2.9. Statistical analysis

All measurements were repeated at least 3 times using duplicate samples. The averages and the standard deviations were calculated from all measurements using Excel Software (Microsoft

243 Corporation, USA). Statistically significant differences between samples were assessed by a one-way
244 ANOVA and Tukey's test with a α-level of 0.05 using Minitab 17.0 software (Minitab Inc., USA).

245 **3. Results and discussion**

246 **3.1. Influences of extrusion pretreatment on the enzymic proteolysis of PPI**

247 For analyzing the influences of extrusion pretreatment on the enzymic proteolysis of PPI, the
248 DH, PS (pH 7.0), and SDS-PAGE profiles of hydrolysates prepared with and without extrusion
249 pretreatment were studied. **Fig. 2A** shows digital images of EPPI prepared at different extrusion
250 temperatures (100, 130, 160 °C). It is seen that at relatively low extrusion temperature of 100 °C, the
251 extrudate showed a low expansion extent. With increasing extrusion temperature to 130 °C, the
252 expansion extent of the extrudate increased remarkably. Besides, from the cross section of the
253 extrudate of EPPI-130 °C, we can see that it presented characteristic vacuoles and laminated
254 structures of extruded products. However, with a further increase of extrusion temperature to 160 °C,
255 the expansion extent of the extrudate decreased, and the product became brown in colour and hard in
256 texture. **Fig. 2B** shows effects of extrusion temperature on the DH of hydrolysates. Compared to
257 PPIH, all tested EPPIH (100, 130, 160 °C) obtained significantly (p<0.05) higher DH under the same
258 enzymolysis conditions, suggesting that extrusion pretreatment was able to effectively enhance the
259 enzymic proteolysis of PPI. In addition, EPPIH-130 °C obtained a significantly (p<0.05) higher DH
260 than EPPIH-100 °C and EPPIH-160 °C. From these results, we can see that extrusion temperature
261 was an important factor that posed a striking impact on both morphology and enzymic proteolysis of
262 EPPI, which agreed with those reported in the literature (Abd EI-Hady et al., 2003; Alam et al., 2016).
263 Seeing that among all the EPPI prepared in this study, EPPI-130 °C showed the highest expansion
264 extent and obtained the highest DH after enzymic proteolysis, it seems that the DH of EPPIH was

positively correlated to the expansion extent of EPPI. Since extrusion pretreatment conducted at 130 °C showed better effect in enhancing the enzymic proteolysis of PPI in terms of DH, this extrusion temperature was selected to prepare EPPI for the following studies.

To characterize the protein degradation in samples caused by different treatments, SDS-PAGE experiment was performed under reducing condition. **Fig. 3** shows the electrophoretic patterns of control PPI, control EPPI and their hydrolysates prepared with papain-induced proteolysis at different E:S ratios (0.05–0.5%, w/w). It is seen that the electrophoretic patterns of control PPI and control EPPI were similar, both displayed five major bands, S66, S41, S40, S38, and S26, named by their molecular weights (MW). Among them, Band S66 was identified as the subunit of conarachin, bands S41, S40, and S38 were identified as the acidic subunits (AS) of arachin, and band S26 was identified as the basic subunit (BS) of arachin (Ghatak et al., 2013; Zhao et al., 2011). According to the literature, arachin and conarachin are the major constitutive proteins in PPI, which account for approximately 90% of the total proteins (Ghatak et al., 2013). After papain-induced proteolysis, the protein components in PPI degraded gradually with increasing E:S ratios, but showed different protease accessibilities: conarachin (S66) disappeared at E:S = 0.1% (w/w); AS-arachin (S41, S40, and S38) disappeared at E:S = 0.5% (w/w); BS-arachin (S26) appeared highly resistant to papain-induced proteolysis, and was still identifiable at E:S = 0.5% (w/w). By contrast, it appeared that conarachin, AS-arachin, and BS-arachin in EPPI were hydrolyzed more readily by papain, because they degraded completely at lower E:S ratios of 0.05%, 0.1%, and 0.5% (w/w), respectively. From these observations, it is clear that the accessibilities of the major protein components to papain in PPI were substantially increased after extrusion pretreatment. This finding was similar with previous studies and confirmed that extrusion pretreatment was a highly effective way to improve the protease

accessibilities of plant proteins (Chen et al., 2005; Jung et al., 2005; Surówka et al., 2004; Zheng et al., 2006). According to the literature, the denaturing action of high temperature and high pressure on proteins in the extruder, which, together with shearing forces, led to the formation of laminated structures easily accessible to proteases (Day et al., 2013; Surówka et al., 2004). On the other hand, on the lower part of the gel, some new polydisperse bands were detected for both PPIH and EPPIH, which probably arised from the production of peptides during papain-induced hydrolysis of peanut proteins. It is seen that since almost all of the subunits in EPPI had been degraded completely, EPPIH with DH between 6.2% and 8.3% mainly consisted of peptides with MW < 25 kDa.

Adequate protein solubility is a key factor for a protein to be as an efficient emulsifying agent. Poor emulsification performance usually goes together with poor protein solubility (McClements et al, 2016). From **Table 1**, it is seen that the PS of control PPI was very poor (27.6%). This may be because the harsh processes used for manufacturing commercial PPI usually cause extensive denaturation and aggregation of peanut proteins, resulting in the loss of much of its soluble proteins and the formation of a large amount of insoluble protein particles (Ghatak et al., 2013; Taherian et al., 2011). In contrast, the control EPPI showed a lower PS of 22.5%. This finding was similar with previous studies that extrusion cooking was ineffective in improving the PS of peanut protein products (Alonso, Orúe, Zabalza, Grant, & Marzo, 2000c). After enzymic proteolysis, the DH and PS of both PPIH and EPPIH increased steadily as E:S ratios increased. It is well known that the breakdowns of peptide bonds induced by enzymolysis caused an increase in ionizable amino and carboxyl groups and a decrease in molecular mass for hydrolyzed proteins, leading to an improvement on their PS (Wouters et al., 2016). Moreover, enzymolysis probably induced the unfolding of protein molecules and/or protein aggregates, whereby more hydrophilic groups could be exposed and showed

309 increased intramolecular hydration (Zhao et al., 2011). Therefore, limited enzymic proteolysis could
310 cause a strong increase in the PS of food proteins, on the premise that the protease is accessible to the
311 protein being hydrolyzed (Wouters et al., 2016; Zeeb et al., 2017; Zhao et al., 2011). From **Table 1**,
312 we can see that at the same E:S ratios, EPPIH always got significantly (p<0.05) higher DH and PS
313 than PPIH did. For instance, at E:S = 0.2% (w/w), the DH and PS of EPPIH were 6.2% and 89.3%,
314 much higher than those of PPIH (DH = 1.3%, PS = 42.7%). This may be because the protease
315 accessibilities of the major enzymolysis-resistant protein components (conarachin and arachin) in PPI
316 were substantially increased after extrusion pretreatment, more peanut proteins in EPPI could then be
317 readily hydrolyzed and became soluble. It is noteworthy that EPPIH with DH between 6.2% and 8.3%
318 showed a high PS of ~90%, which meant that most of insoluble proteins and protein aggregates in
319 EPPIH had been hydrolyzed and become soluble peptide fragments, as shown in SDS-PAGE analysis.

320 **3.2. Emulsification performances of PPIH and EPPIH**

321 To be effective as emulsifying agents, proteins should possess some surface activity, thereby
322 facilitating the production of fine emulsion droplets by lowering the oil/water interfacial tension.
323 Once adsorbed onto droplets, proteins should spread out quickly on the interface, thereby stabilizing
324 the freshly formed droplets against immediate aggregation by forming a protective interfacial layer
325 around the droplets. Therefore, the emulsification performance refers to how effective an emulsifying
326 agent is at forming emulsions with small droplets during emulsification (McClements, 2007). Effects
327 of DH on average droplet sizes d_{43} and microstructures of fresh oil-in-water emulsions formed by
328 PPIH and EPPIH are demonstrated in **Fig. 4** and **Fig. 5**, respectively. In **Fig. 5**, LSCM images with
329 oil stained and DSD superimposed were used to monitor the spatial distributions of droplets in
330 emulsions, and those with protein stained were used to examine the structures of emulsion interfacial

layers. Emulsions formed by control PPI and control EPPI both showed a high d_{43} values of 29.4 μm and 33.6 μm, respectively. Based on LSCM observations, it was found that the control PPI emulsion contained many big droplets and droplet flocs, suggesting that re-coalescence and flocculation of emulsion droplets occurred during homogenization (**Fig. 5a**). In this experiments, in order to amplify differences in emulsification performance between different protein samples, a relatively low protein/oil ratio (sample concentration = 2.0 % w/v, oil fraction = 20 vol.%) was used to make emulsions. It is clear that control PPI could not make a stable emulsion with fine droplet size under this emulsification condition. Additionally, from **Fig. 5b**, it is seen in the control PPI emulsion that a lot of protein particles were attached on the droplet surfaces, with emulsion droplets being connected by them. The reason for this may be due to the fact that under the turbulent flow conditions of high-pressure homogenization, it is mainly by convection that emulsifying agents are transported to the freshly created oil/water interfaces, so that insoluble protein particles could also adsorb fast onto droplet surfaces (Dickinson, 2017). However, compared to soluble proteins, protein particles are less efficient in coating oil droplets at relatively low concentration, because they have a low diffusive mobility and could not unfold on the droplet surface after adsorption, and are prone to induce coalescence and bridging flocculation (sharing of the adsorbed layer amongst adjacent droplets, see **Fig. 6**) of emulsion droplets during homogenization (Dickinson, 2017; Tcholakova, Denkov, & Lips, 2008; Tcholakova, Denkov, Sidzhakova, Ivanov, & Campbell, 2003).

After enzymic proteolysis, with the increase of DH, d_{43} of emulsions formed by PPIH and EPPIH both decreased gradually, and then increased rapidly at high DH values. Emulsions formed by PPIH displayed a minimum d_{43} of 20.6 μm at DH 0.9%. Microscopic analyses showed that compared to the control PPI emulsion, the PPIH-0.9% emulsion showed an increase in small droplets distributing from

353 0.1 to 1 μm (**Fig. 5c**). However, strong droplet flocculation was still evident in the PPIH-0.9%
354 emulsion, and its emulsion interfacial layers still contained a lot of protein particles (**Fig. 5d**). It
355 appears that some soluble proteins/peptides with high surface activity were produced during papain-
356 induced proteolysis of PPI, which enabled PPIH-0.9% could generate and stabilize some fine droplets
357 during homogenization. However, due to their compact structures, peanut proteins or protein particles
358 in PPI were resistant to enzymolysis, which limited the production of surface active proteins/peptides
359 during enzymic proteolysis. As a result, bridging flocculation caused by adsorbed protein particles
360 occurred in the PPIH-0.9% emulsion, because still there were insufficient emulsifying agents
361 available to fully coat all newly created droplets during homogenization. These findings suggest that
362 modification of PPI using papain-induced proteolysis alone could only induced a limited
363 improvement on its emulsification performance.

364 By contrast, emulsions formed by EPPIH showed a minimum d_{43} of ~4.5 μm at DH = 4.6−6.2%.
365 Microscopic analyses showed that the emulsion formed by EPPIH-6.2% appeared homogeneous,
366 with most of droplets distributing from 0.1 to 10 μm (**Fig. 5e**). These results clearly showed that
367 modification of PPI using extrusion pretreatment and controlled enzymic proteolysis was more
368 effective in improving the emulsification performance of PPI than that using enzymic proteolysis
369 alone. As shown before, the protease accessibilities of the major protein components (conarachin and
370 arachin) in PPI were substantially increased after extrusion pretreatment such that most of insoluble
371 proteins and protein aggregates in EPPI could be readily hydrolyzed and became soluble. Therefore,
372 it is reasonable to infer that a lot of more surface active peptides were produced during papain-induced
373 proteolysis of EPPI as compared with that of PPI, leading to a marked improvement on the
374 emulsification performance for EPPIH.

375 However, after reaching their minimum d_{43}, emulsions formed by PPIH and EPPIH both showed
376 a rapid increase in d_{43} with a further increase in DH, which suggested that excessive enzymic
377 proteolysis caused a decrease in the emulsification performances for both PPIH and EPPIH. Another
378 interesting finding was that although the emulsion formed by EPPIH-8.3% contained a lot of big
379 droplets and had a large d_{43} of 12.7 μm, there was no sign of droplet flocculation observed in this
380 emulsion (**Fig. 5g**). In addition, it is observed that the droplet surfaces were covered by a thin layer
381 of peptides, with little particulates attached (**Fig. 5h**). Considering that EPPIH-8.3% mainly consisted
382 of soluble peptides with small molecular weight (PS = 87.2%, MW < 25 kDa) and control PPI
383 contained a lot of protein particles, it seems that when insufficient emulsifying agents were available
384 during homogenization, bridging flocculation of droplets probably occurred in emulsions formed by
385 protein particles (e.g., control PPI emulsion), but not occurred in those formed by small peptides (e.g.,
386 EPPIH-8.3% emulsion). The reason for this finding is not fully understood, but it may be that protein
387 particles were of large size and might have several different regions available for anchoring to the
388 droplet surfaces, and were therefore apt to cause the bridging of protein particles between droplets at
389 relatively low concentrations during homogenization (see **Fig. 6**) (Dickinson, 2010), which posed a
390 negative effect on the emulsification performance for PPI.

391 The d_{43} values as a function of sample concentration for emulsions (20 vol.% oil) formed by
392 control PPI, PPIH-0.9% and EPPIH-6.2% are shown in **Fig. 7**. For all tested protein samples, d_{43} of
393 emulsions formed by them decreased gradually with increasing their concentration until a minimum
394 value was reached, which indicated that sufficient emulsifying agents were available during
395 homogenization to saturate all of newly created droplets. For control PPI, PPIH-0.9% and EPPIH-
396 6.2%, the minimum concentration required to form stable emulsions was 6.5 %, 5.5 %, and 2.5 %

(w/v), respectively; the measured average droplet sizes of these emulsions were small and without too much difference (d_{32} = 0.4 μm, d_{43} = 1.2–1.6 μm, see **Table 2**). LSCM observations showed that these emulsions were homogeneous without droplet flocculation (images not shown). From these results, we can see that compared with control PPI and PPIH-0.9%, EPPIH-6.2% was capable of forming a stable emulsion with comparable fine droplet size at a relatively lower concentration, and therefore possessed a better emulsification performance.

3.3. Interfacial properties of PPIH and EPPIH

In an attempt to explore the underpinning mechanisms of improved emulsification performances for PPIH and EPPIH, the surface pressure (π) versus sample concentration profiles and saturation surface loads (Γ_{sat}) of some selected PPIH and EPPIH were determined. Plots of the dependence of surface pressure on sample concentration for control PPI, PPIH-0.9% and EPPIH-6.2% are shown in **Fig. 8**. For all tested protein samples, as sample concentration increased, π continued to increase until it reached a relatively constant level at a certain protein concentration where the interface had been saturated with proteins, i.e. saturation surface pressure (π_{sat}). The value of π_{sat} gives an indication of how effectively an emulsifying agent is capable of reducing the oil/water interfacial tension at saturation, which is closely related to how easily droplets are disrupted during homogenization (McClements et al., 2017). The measured π_{sat} were 15.5, 14.7, 14.3, and 9.4 mN m^{-1} for PPIH-0.9%, control PPI, EPPIH-6.2%, and EPPIH-8.3%, respectively. The π_{sat} values of PPIH-0.9%, control PPI and EPPIH-6.2% were comparable to those of protein emulsifiers (around 11–18 mN m^{-1}) commonly used in emulsion-based food (McClements et al., 2016), suggesting that they had adequate surface activity that enabled the generation of fine droplets during homogenization. However, it should be noted that the minimum concentration required to reach π_{sat} for different samples were in the sequence

of EPPIH-6.2% (0.01 %, w/v) < PPIH-0.9% (0.1 %, w/v) < control PPI (0.5 %, w/v). These results indicated that compared to control PPI and PPIH-0.9%, EPPIH-6.2% was capable of lowering oil/water interfacial tension to a comparable extent at saturation, but at a much lower bulk concentration. Indeed, this finding was consistent with that of emulsification performance measurements, and provided further evidence that EPPIH-6.2% probably contained a lot of more surface active peptides than control PPI and PPIH-0.9%. On the other hand, compared to EPPIH-6.2%, EPPIH-8.3% showed a much lower π_{sat} at a higher concentration (0.5 %, w/v), suggesting that excessive proteolysis caused the loss of surface activity for protein hydrolysates, and therefore the decrease of their emulsification performances.

The effectiveness of a protein as an emulsifying agent depends on how much is present at the oil/water interface when droplets are completely saturated, i.e. Γ_{sat} (McClements, Bai, & Chung, 2017). The emulsions stabilized by 6.5 % (w/v) control PPI, 5.5 % (w/v) PPIH-0.9%, and 2.5 % (w/v) PPIH-6.2% were chosen for Γ_{sat} measurements, because these sample concentrations were the minimum amount required for them to emulsify all the oil phase without bridging flocculation. From **Table 2**, we can see that the measured Γ_{sat} for different samples were in the sequence of control PPI (11.3 mg m^{-2}) > PPIH-0.9% (9.6 mg m^{-2}) > EPPIH-6.2% (2.7 mg m^{-2}), suggesting that the emulsifying agents in EPPIH-6.2% were capable of saturating per unit area of droplet surface with less amount when compared with those in control PPI and PPIH-0.9%. This finding may be explained by the fact that EPPIH-6.2% mainly consisted of soluble peptides, which probably had better molecular flexibility than globular peanut proteins in PPI. According to the literature, compared with proteins with rigid structures, proteins with flexible structures tended to have a lower Γ_{sat}, because they could rapidly alter their conformation and spread out after adsorption onto droplet surface (Dickinson,

441 2017). On the other hand, it is seen that the Γ_{sat} of control PPI and PPIH-0.9% were on the high end
442 of values typically reported for food proteins (ca. 1–10 mg m^{-2}) (McClements et al., 2017). This may
443 be because the emulsion interfacial layers formed by control PPI and PPIH-0.9% were a mixture of
444 soluble proteins and protein particles as shown in LSCM observations. Extraordinary high Γ_{sat} values
445 were also reported by several studies on emulsifying properties of heat-denatured soy proteins and
446 whey proteins, which suggested that with particulates adsorbing to the droplet surface, the amount of
447 emulsifying agent required to saturate per unit area of droplet surface can be much higher than that
448 for soluble proteins, because protein particles were of much larger size and could not unfold at droplet
449 surface as compared to soluble proteins (see **Fig. 6**) (Cui, Chen, Kong, Zhang, & Hua, 2014; Keerati-
450 u-rai & Corredig, 2009; Tcholakova et al., 2003).

451 **4. Conclusions**

452 In this work, we investigated effects of extrusion pretreatment on the enzymic proteolysis of PPI
453 and on the emulsification performances and interfacial properties of its hydrolysates. Results showed
454 that extrusion pretreatment effectively enhanced the papain-induced proteolysis of PPI in terms of
455 DH, with a preferable extrusion temperature at 130 °C. After extrusion pretreatment, the protease
456 accessibilities of the major enzymolysis-resistant protein components (conarachin and arachin) in PPI
457 were substantially increased, resulting in a remarkable increase in PS for the resulting hydrolysates.
458 EPPIH with DH between 6.2% and 8.3% mainly consisted of peptides with MW < 25 kDa, and
459 showed a PS of ~90%, much higher than those of control PPI (27.6%) and PPIH.

460 Based on the analysis of emulsion microstructures visualized using LSCM, it was found that the
461 commercial PPI used in this study contained a lot of protein particles, which could adsorb onto
462 emulsion droplet surfaces during high-pressure homogenization. Protein particles had a very high

463 saturation surface load and tended to induce bridging flocculation of emulsion droplets during
464 homogenization at relatively low concentration, which posed a negative effect on the emulsification
465 performance for PPI. It is encouraging to find that modification of PPI using extrusion pretreatment
466 and controlled papain-induced proteolysis caused a marked improvement on its emulsification
467 performance. The improved emulsification performance of EPPIH was arisen from the increased
468 protease accessibility of EPPI after extrusion pretreatment, which caused two important changes for
469 the resulting hydrolysates: (1) most of insoluble protein particles in EPPIH were enzymically
470 hydrolyzed and become soluble, which effectively eliminated the negative effects of protein particles
471 on emulsification performance; (2) the production of surface active peptides with low saturation
472 surface Load was substantially promoted in EPPIH during enzymic proteolysis.

473 In conclusion, hydrolyzed PPI showed potential as a valuable new source of emulsifying agent
474 for emulsion-based food products. Extrusion pretreatment was proved to be a highly effective
475 technique to enhance the enzymic proteolysis of globular plant proteins, and produced noticeable
476 benefits for the functionality improvement of protein hydrolysates. However, what structural changes
477 actually occurred to PPI during extrusion cooking, which made the resulting extrudates become easily
478 accessible to protease, was still not very clear. Future detailed investigations on this topic will be
479 helpful to understand the relationship between protein structure and protease accessibility and to
480 establish industrial applications of extrusion pretreatment.

481 **Acknowledgement**

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References

Abd EI-Hady, E. A., & Habiba, R. A. (2003). Effect of soaking and extrusion conditions on antinutrients and protein digestibility of legume seeds. *LWT–Food Science and Technology, 36*(3), 285–293.

Adler-Nissen, J., Eriksen, S., & Olsen, H. S. (1983). Improvement of the functionality of vegetable proteins by controlled enzymatic hydrolysis. *Plant Foods for Human Nutrition, 32*(3–4), 411–423.

Alam, M. S., Kaur, J., Khaira, H., & Gupta, K. (2016). Extrusion and extruded products: changes in quality attributes as affected by extrusion process parameters: a review. *Critical Reviews in Food Science and Nutrition, 56*(3), 445–473.

Alonso, R., Aguirre, A., & Marzo, F. (2000a). Effects of extrusion and traditional processing methods on antinutrients and in vitro digestibility of protein and starch in faba and kidney beans. *Food Chemistry, 68*(2), 159–165.

Alonso, R., Grant, G., Dewey, P., & Marzo, F. (2000b). Nutritional assessment in vitro and in vivo of raw and extruded pea (*Pisum sativum* L.). *Journal of Agricultural and Food Chemistry, 48*(6), 2286–2290.

Alonso, P., Orúe, E., Zabalza, M. J., Grant, G., & Marzo, F. (2000c). Effect of extrusion cooking on structure and functional properties of pea and kidney bean proteins. *Journal of the Science of Food and Agriculture, 80*(3), 397–403.

Benjamin, O., Stilcock, P., Beaucham, A., Buettner, A., & Everett, D. W. (2014). Emulsifying properties of legume proteins compared to beta-lactoglobulin and tween 20 and the volatile release from oil-in-water emulsions. *Journal of Food Science, 79*(10), E2014–E2022.

Chen, L., & Phillips, R. D. (2005). Effects of twin-screw extrusion of peanut flour on in vitro digestion of potentially allergenic peanut proteins. *Journal of Food Protection, 68*(8), 1712–1719.

Cui, Z. M., Chen, Y. M., Kong, X. Z., Zhang, C. M., & Hua, Y. F. (2014). Emulsifying properties and oil/water (O/W) interface adsorption behavior of heated soy proteins: effects of heating concentration, homogenizer rotating speed, and salt addition level. *Journal of Agricultural and Food Chemistry, 62*(7), 1634–1642.

Day, L., & Swanson, B. G. (2013). Functionality of protein-fortified extrudates. *Comprehensive Reviews in Food Science and Food Safety, 12*(5), 546–564.

Dickinson, E. (2010). Food emulsions and foams: Stabilization by particles. *Current Opinion in Colloid & Interface Science, 15*(1–2), 40–49.

Dickinson, E. (2017). Biopolymer-based particles as stabilizing agents for emulsions and foams. *Food Hydrocolloids*, *68*, 219–231.

Ettelaie, R., Zengin, A., & Lee, H. (2014). Fragment proteins as food emulsion stabilizers: A theoretical study. *Biopolymers, 101*(9), 945–958.

Ghatak, S. K., & Sen, K. (2013). Peanut proteins: Application, aliments and possible remediation. *Journal of Industrial and Engineering Chemistry, 19*(2), 369–374.

Hu, Y. T., Ting, Y. W., Hu, J. Y., & Hsieh, S. C. (2017). Techniques and methods to study functional characteristics of emulsion systems. *Journal of Food and Drug Analysis, 25*(1), 16–26.

Jung, S., Murphy, P. A., & Johnson, L. A. (2005). Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science, 70*(2), C180–C187.

529 Karaca, A. C., Low, N., & Nickerson, M. (2011). Emulsifying properties of chickpea, faba bean,
530 lentil and pea proteins produced by isoelectric precipitation and salt extraction. *Food Research*
531 *International, 44*(9), 2742–2750.
532 Keerati-u-rai, M., & Corredig, M. (2009). Heat-induced changes in oil-in-water emulsions stabilized
533 with soy protein isolate. *Food Hydrocolloids*, *23*(8), 2141–2148.
534 Kwok, M. H., & Ngai, T. (2016). A confocal microscopy study of micron-sized poly (N-
535 isopropylacrylamide) microgel particles at the oil-water interface and anisotopic flattening of
536 highly swollen microgel. *Journal of Colloid and Interface Science*, *461*, 409–418.
537 Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of head of bacteriophage.
538 *Nature*, *227*(5259), 680–685.
539 Luo, D. H., Zhao, Q. Z., Zhao, M. M., Yang, B., Long, X. T., & Ren, J. Y., et al. (2010). Effects of
540 limited proteolysis and high-pressure homogenization on structural and functional characteristics
541 of glycinin. *Food Chemistry, 122*(1), 25–30.
542 Ma, Z., Boye, J. I., & Simpson, B. K. (2016). Preparation of salad dressing emulsions using lentil,
543 chickpea and pea protein isolates: A response surface methodology study. *Journal of Food*
544 *Quality, 39*(4), 274–291.
545 McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of
546 emulsion stability. *Critical Reviews in Food Science and Nutrition*, *47*(7), 611–649.
547 McClements, D. J., Bai, L., & Chung, C. (2017). Recent advances in the utilization of natural
548 emulsifiers to form and stabilize emulsions. *Annual Review of Food Science and Technology, 8*,
549 205–236.

McClements, D. J., & Gumus, C. E. (2016). Natural emulsifiers-Biosurfactants, phospholipids, biopolymers, and colloidal particles: Molecular and physicochemical basis of functional performance. *Advances in Colloid and Interface Science, 234*, 3–26.

Perrot, C., Quillien, L., & Guéguen, J. (1999). Identification by immunoblotting of pea (*Pisum sativum* L.) proteins resistant to in vitro enzymatic hydrolysis. *Sciences Des Aliments, 19*(3), 377–390.

Surówka, K., Żmudziński, D., Fik, M., Macura, R., & Łaocha, W. (2004). New protein preparations from soy flour obtained by limited enzymic hydrolysis of extrudates. *Innovative Food Science and Emerging Technologies, 5*(2), 225–234.

Taherian, A. R., Mondor, M., Labranche, J., Drolet, H., Ippersiel, D., & Lamarche, F. (2011). Comparative study of functional properties of commercial and membrane processed yellow pea protein isolate. Food Research International, *44*(8), 2505–2514.

Tcholakova, S., Denkov, N. D., & Lips, A. (2008). Comparison of solid particles, globular proteins and surfactants as emulsifier. *Physical Chemistry Chemical Physics, 10*(12), 1608−1627.

Tcholakova, S., Denkov, N. D., Sidzhakova, D., Ivanov, I. B., & Campbell, B. (2003). Interrelation between drop size and protein adsorption at various emulsification conditions. *Langmuir, 19*(14), 5640–5649.

Wouters, A. G. B., Rombouts, I., Fierens, E., Brijs, K., & Delcour, J. A. (2016). Relevance of the functional properties of enzymatic plant protein hydrolysates in food systems. *Comprehensive Reviews in Food Science and Food Safety, 15*(4), 786–800.

Zeeb, B., McClements, D. J., & Weiss, J. (2017). Enzyme-based strategies for structuring foods for improved functionality. *Annual Review of Food Science and Technology, 8*, 21–34.

572 Zhao, G. L., Liu, Y., Zhao, M. M., Ren, J. Y., & Yang, B. (2011). Enzymatic hydrolysis and their
573 effects on conformational and functional properties of peanut protein isolate. *Food Chemistry,*
574 *127*(4), 1438–1443.
575 Zhao, Q. Z., Liu, D. L., Long, Z., Yang, B., Fang, M., Kuang, W. M., et al. (2014). Effect of sucrose
576 ester concentration on the interfacial characteristics and physical properties of sodium caseinate-
577 stabilized oil-in-water emulsions. *Food Chemistry, 151*, 506–513.
578 Zheng, X. Q., Li, L. T., Liu, X. L., Wang, X. J., Lin, J., & Li, D. (2006). Production of hydrolysate
579 with antioxidative activity by enzymatic hydrolysis of extruded corn gluten. *Applied*
580 *Microbiology and Biotechnology, 73*(4), 763–770.

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582

583 **Legends**

584 **Table 1.** DH and PS (pH 7.0) of PPIH and EPPIH hydrolyzed with papain at different E/S ratios (0–
585 0.5%, w/w)*

586 **Table 2.** Properties of emulsions (20 vol.% oil) formed by control PPI, PPIH-0.9% and EPPIH-6.2%*

587 **Fig. 1.** Schematic diagram of the twin-screw extruder employed in this study.

588 **Fig. 2.** Effects of extrusion pretreatment conducted at different temperatures (100, 130, 160 °C) on
589 the morphology of EPPI (A) and on the DH of EPPIH hydrolyzed with papain at E:S = 0.2% (w/w)
590 (B).

591 **Fig. 3.** SDS-PAGE patterns of PPIH and EPPIH hydrolyzed at different E:S ratios (0–0.5%, w/w).
592 S66: conarachin; S41, S40, and S38: acidic subunits of arachin; S26: basic subunits of arachin; M,
593 molecular weight markers.

594 **Fig. 4.** Effects of DH on the initial average droplet size (d_{43}) of emulsions (20 vol.% oil, 2.0% w/v
595 samples) formed by PPIH and EPPIH.

596 **Fig. 5.** LSCM images of fresh emulsions (20 vol.% oil, 2.0 % w/v samples) formed by some selected
597 PPIH and EPPIH: (a and b) control PPI; (c and d) PPIH-0.9%; (e and f) EPPIH-6.2%; (g and h)
598 EPPIH-8.3%. DSD is superimposed on oil stained microimages, with horizontal scale showing
599 particle size (μm); arrows in protein stained microimages highlight the presence of protein particles
600 at droplet surfaces.

601 **Fig. 6.** Schematic representation of effects of protein particles on the formation and stabilization of
602 emulsions during homogenization. Collision of droplets with insufficient coverage of emulsifying
603 agents leads to coalescence and/or bridging flocculation (bridging of protein particles between
604 droplets).

605 **Fig. 7.** Effects of concentration of protein samples on the initial average droplet size (d_{43}) of emulsions
606 (20 vol.% oil) formed by control PPI, PPIH-0.9% and EPPIH-6.2%, respectively.

607 **Fig. 8.** Changes in surface pressure at sunflower seed oil/water interface as a function of sample
608 concentration for some selected PPIH and EPPIH.

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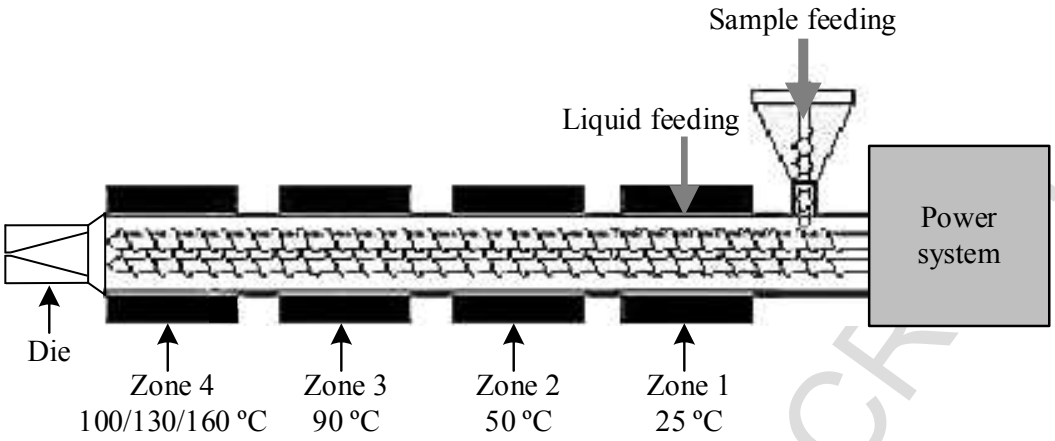

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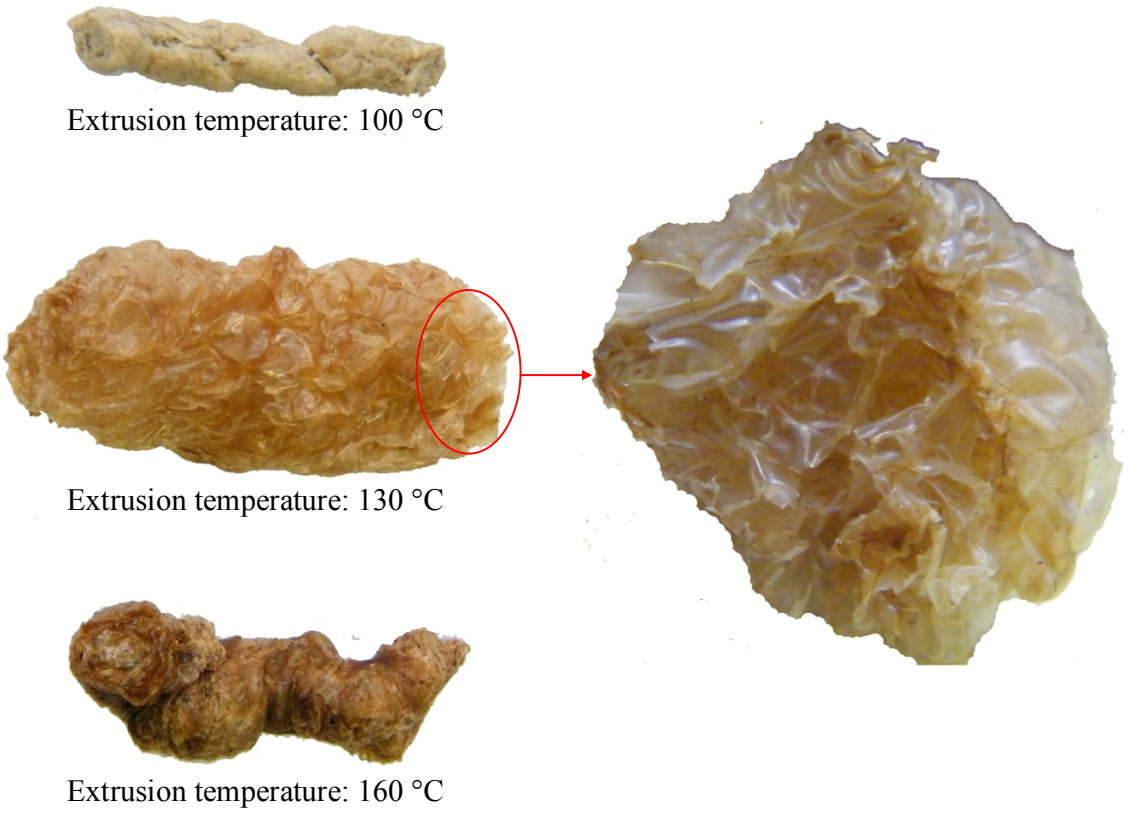
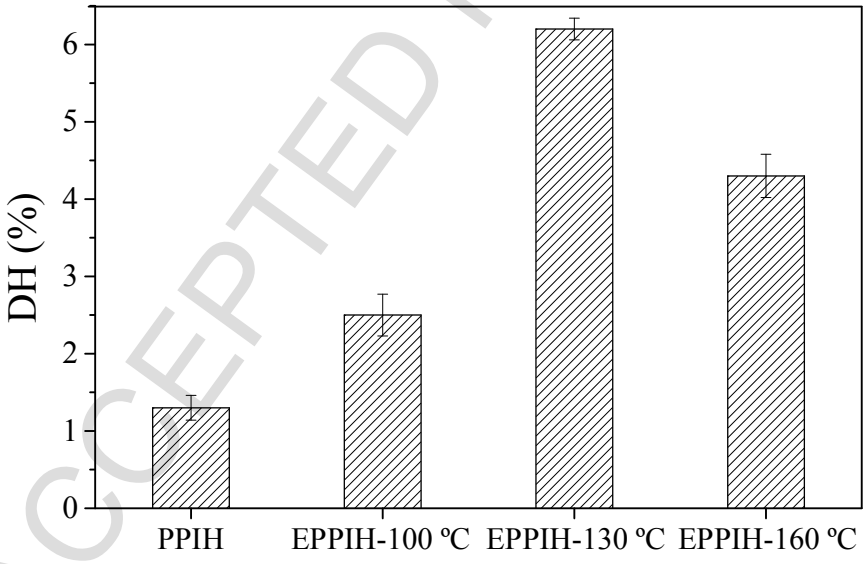


A. Digital images of EPPI prepared at different extrusion temperatures



B. DH of PPIH and EPPIH

Fig. 2.

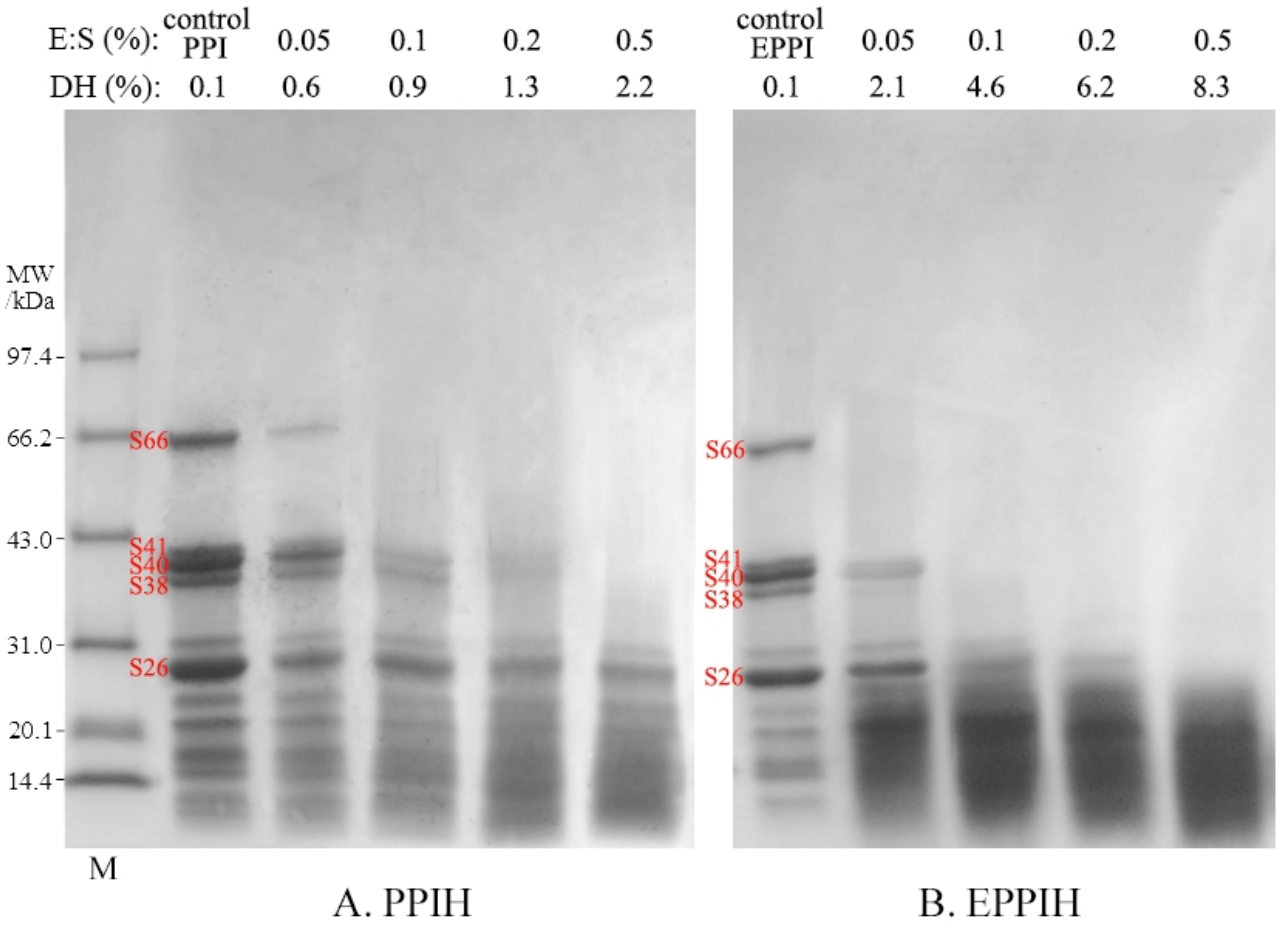


Fig. 3

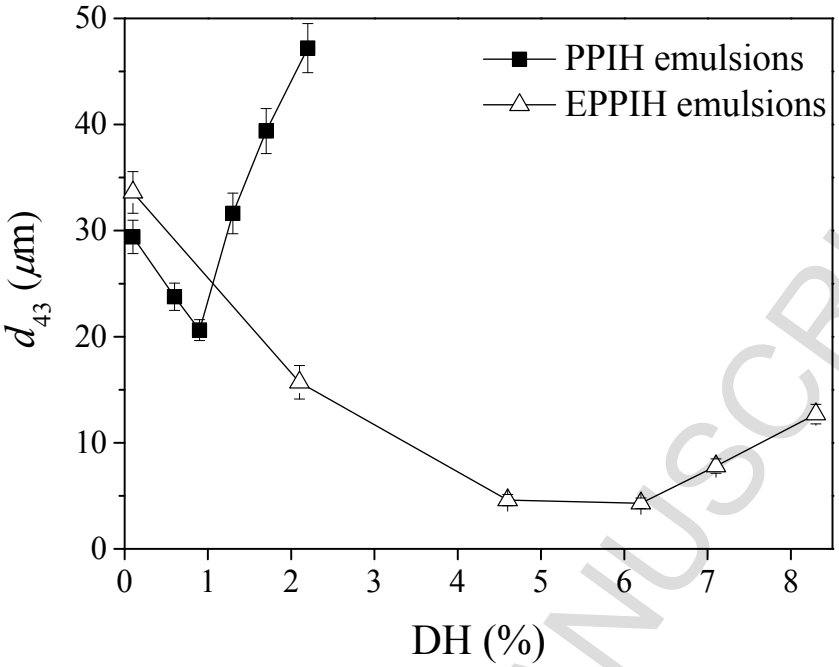


Fig. 4.

Fig. 5.

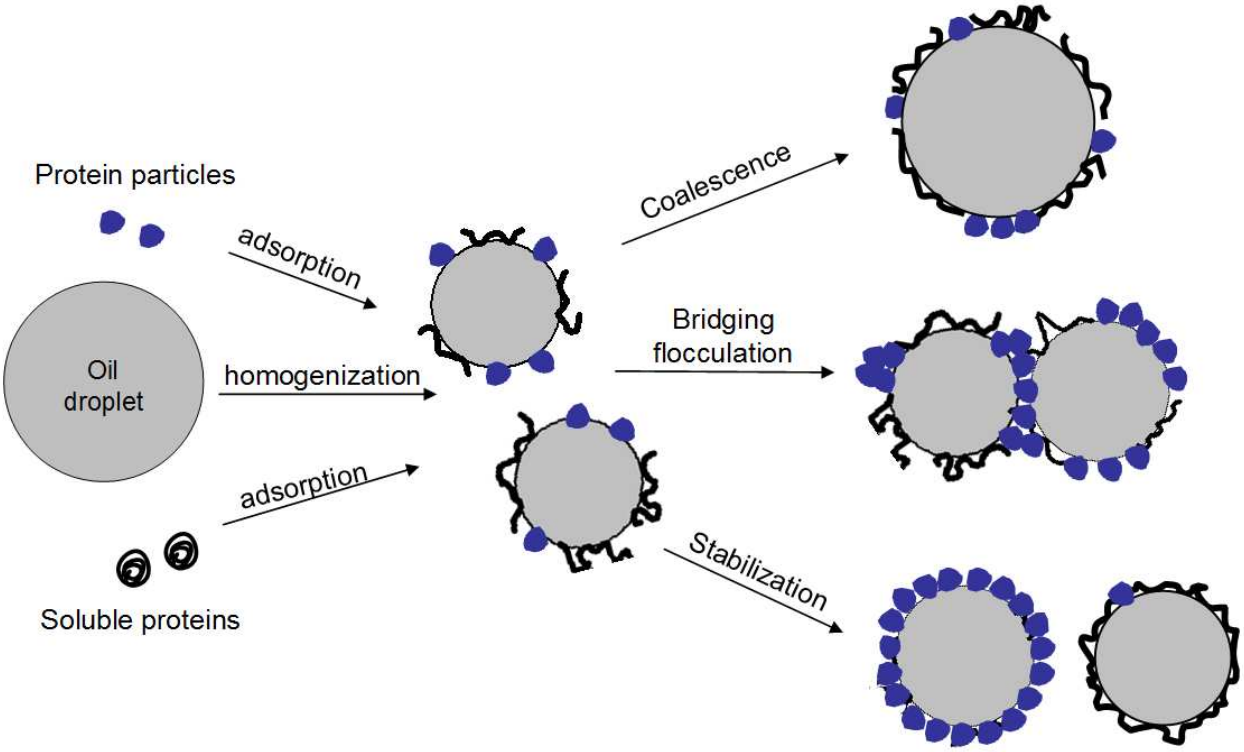


Fig. 6.

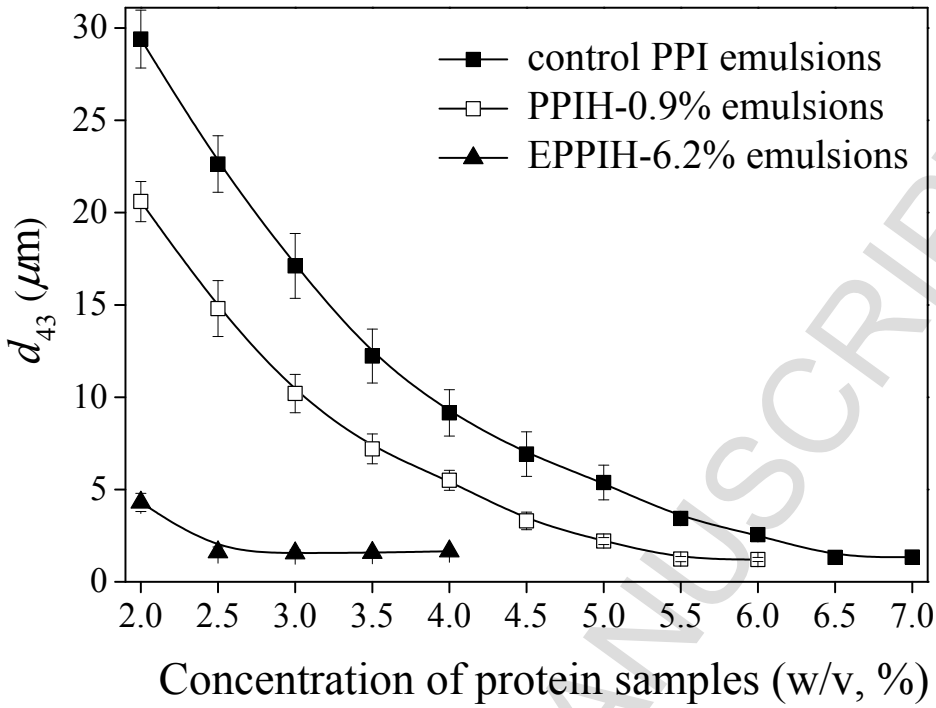


Fig. 7.

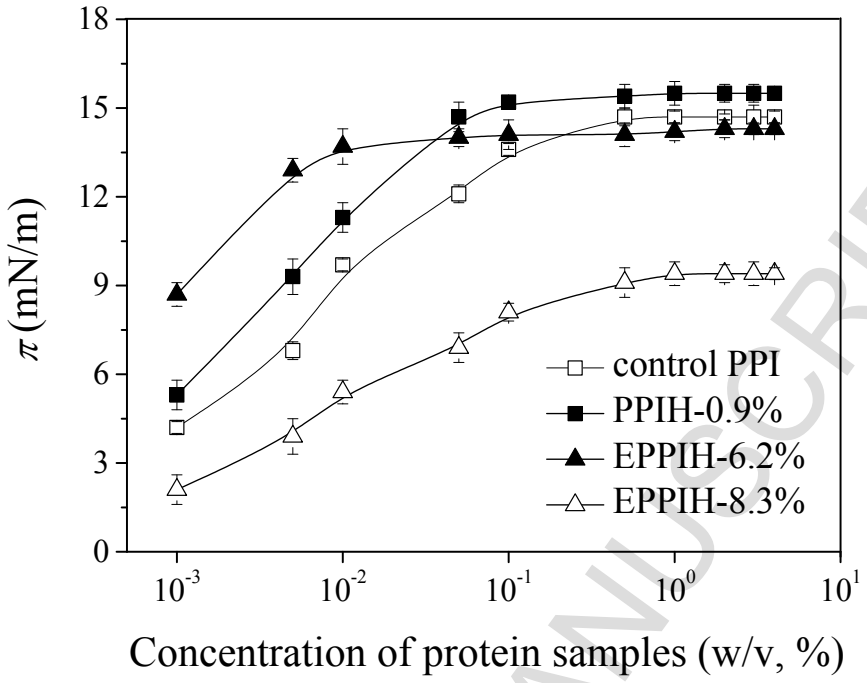


Fig. 8.

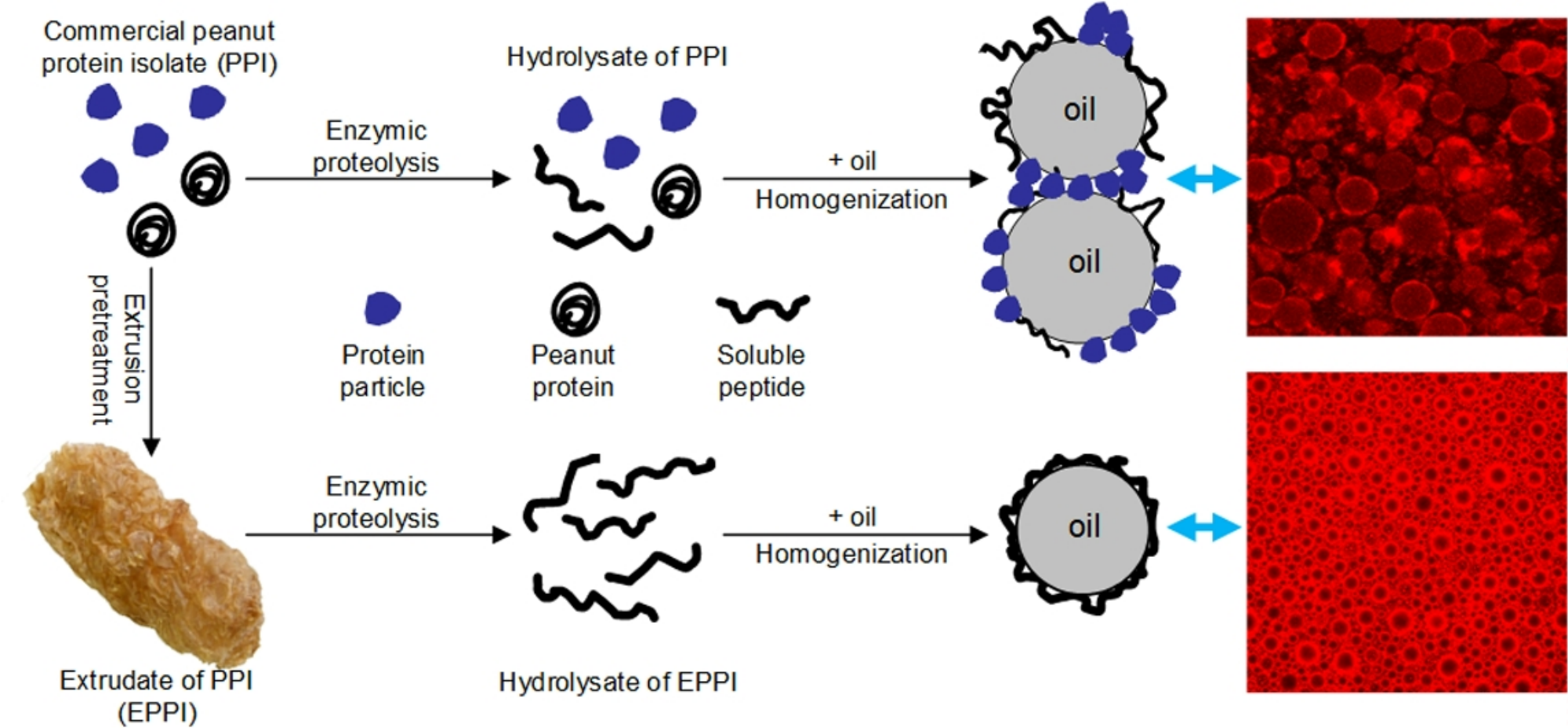

Commercial peanut protein isolate (PPI)
Enzymic proteolysis
Hydrolysate of PPI
+ oil
Homogenization
oil
oil
Extrusion pretreatment
Protein particle
Peanut protein
Soluble peptide
Enzymic proteolysis
+ oil
Homogenization
oil
Extrudate of PPI (EPPI)
Hydrolysate of EPPI

Table 1. DH and PS (pH 7.0) of PPIH and EPPIH hydrolyzed with papain at different E/S ratios (0–0.5%, w/w) *

E/S ratios	DH (%)		PS (%)	
(%, w/w)	PPIH	EPPIH	PPIH	EPPIH
0	0.1 ^{j}	0.1 ^{j}	27.6 ^{j}	22.5 ^{k}
0.05	0.6 ^{i}	2.1 ^{e}	34.3 ^{i}	57.4 ^{d}
0.1	0.9 ^{h}	4.6 ^{d}	39.5 ^{h}	83.5 ^{c}
0.2	1.3 ^{g}	6.2 ^{c}	42.7 ^{g}	89.3 ^{a}
0.3	1.7 ^{f}	7.1 ^{b}	46.4 ^{f}	90.6 ^{a}
0.5	2.2 ^{e}	8.3 ^{a}	51.6 ^{e}	87.2 ^{b}

* In the comparison of the same type of index, results having different letters are significantly different (p<0.05).

Table 2. Properties of emulsions (20 vol.% oil) formed by control PPI, PPIH-0.9% and EPPIH-6.2%*

Sample	C_0 (w/v, %)	d_{32} (μm)	d_{43} (μm)	Γ_{sat} (mg m^{-2})
control PPI	6.5	0.4 [a]	1.3 [b]	11.3 [a]
PPIH-0.9%	5.5	0.4 [a]	1.2 [c]	9.6 [b]
EPPIH-6.2%	2.5	0.4 [a]	1.6 [a]	2.7 [c]

* In the comparison of the same type of index, results having different letters are significantly different (p<0.05); C_0: sample concentration in the aqueous phase before emulsification.