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Improvement of the foaming properties of pea protein concentrate suspensions by physical or enzymatic treatments

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22 Abstract:

23 The ability of proteins to foam is an important functionality in aerated food products. The
24 functionalities of animal proteins over plant proteins are undeniable. However, plant-based ingredients
25 have now become common substitutes. Among the available plant protein sources, yellow peas have
26 been widely studied. This paper focuses on the functionalities of a pea protein concentrate (PPC)
27 obtained after simpler and less damaging processes than those for isolates.

28 Pea protein concentrate dispersions underwent ultrasounds, heating, or enzymatic hydrolysis. Physical
29 treatments did not affect pea protein's structure, unlike enzymatic hydrolysis, which significantly
30 altered the molecular size distribution. Enzymatic hydrolysis emerged as the most effective pathway
31 for enhancing PPC foamability. Foams produced from concentrated hydrolyzed PPC (HYD PPC)
32 dispersions were compared to whole egg foams. Interestingly, HYD PPC foams exhibited solid-like
33 behavior, while whole egg foams displayed flowing behavior and lower stability over time. The solid
34 fraction and the presence of polysaccharides in HYD PPC dispersions prevented drainage and
35 coalescence in HYD PPC foams, ensuring excellent stability. Interfacial behavior differed in terms of
36 kinetics of adsorption at the air/water interface between HYD PPC and whole egg proteins, yet both
37 formed interfacial films with high viscoelastic properties.

38

39 Keywords: Pea protein concentrates, Foaming, Air-liquid interface, Enzymatic hydrolysis, Ultrasounds,
40 Thermal treatment

41

Abbreviations: **DH:** Degree of hydrolysis, **FC:** Foaming Capacity, **FS:** Foaming Stability, **HYD PPC:** Hydrolyzed Pea Protein Concentrate, **HYD PPI:** Hydrolyzed Pea Protein Isolate, **PPC:** Pea Protein Concentrate, **PPI:** Pea Protein Isolate, **TH PPC:** Heated PPC, **US PPC:** Sonicated PPC

42 1. INTRODUCTION

43 Dietary proteins, primarily derived from animals and plants, exhibit significant variations in molecular
44 structure, amino acid composition, digestibility, and technical functionalities in foods, such as gelation,
45 emulsification, foaming, and water binding [1].

46 A growing interest in plant-based ingredients as substitutes for animal constituents is undeniable,
47 driven by sustainability considerations to meet the rising global population demand. Plant-based
48 protein production boasts a lower carbon footprint than animal proteins [2,3]. Increasing health
49 awareness and ethical concerns have further propelled consumer demand for plant proteins,
50 prompting food companies to introduce plant-based alternatives.

51 Despite the advantageous functionalities of animal proteins such as egg proteins that exhibit excellent
52 foaming, gelling or thickening properties [4], the shift towards plant-based proteins, particularly from
53 sources like yellow peas (*Pisum sativum L.*), has gained traction. More than twelve million tons were
54 produced worldwide in 2021 [5]. Yellow pea seeds contain approximately 20-30% proteins and 65%
55 carbohydrates, mainly starch [6]. Pea proteins, available as flour (20-30% proteins), protein
56 concentrate (50% minimum), or protein isolate (80% minimum), are increasingly incorporated into
57 plant-based substitutes for meat or yogurt [3].

58 Plant proteins can be categorized into four primary classes [6] : albumins, globulins, prolamins, and
59 glutelins, based on their solubility and extractability in various solvents. Regarding pea proteins,
60 globulins are the primary component (60 – 80%). They exhibit poor water solubility contrary to
61 albumins that account for the second major protein fraction (15 – 25%) [7]. In contrast to animal
62 proteins, which generally exhibit low molecular weight and a relatively simple globular structure [8],
63 plant proteins display intricate quaternary structures with monomers of high molecular weight.

64 Although pea proteins exhibit functional properties such as emulsification and foaming, these
65 functionalities fall short compared to animal proteins [9–11]. The limited physicochemical
66 functionalities of plant proteins often constrain their utilization in food products; for instance, low

67 solubility hinders their incorporation into aqueous-based products, and their tendency to be compact,
68 aggregated, and inflexible restricts their capacity to adsorb at interfaces. Unlike animal proteins that
69 are directly available, plant proteins need to be extracted from the seeds or the grains. The processes
70 of extraction that are used comprise several steps such as milling followed by air fractionation or
71 alkaline extraction/acid precipitation depending on the desired level of purity. For a wet extraction, a
72 final drying step is added. These various extraction treatments tend to leave the proteins in a
73 denatured and aggregated state with the majority of the functional groups buried inside the structure
74 [8,12]. In order to improve their functionality, proteins modification can be implemented. The
75 fundamental principles of protein modification approaches, based on physical and biochemical
76 treatments, have been extensively reviewed elsewhere [9,13]

77 Physical treatments encompass heating [14,15], static high-pressure treatment [16], ultrasonication
78 [17,18], pulsed electric fields [19], or other shear treatments [20]. These physical treatments denature
79 proteins, inducing aggregation or unfolding by exposing hydrophobic residues previously buried within
80 the protein's interior. Denatured albumins and globulins exhibit enhanced adsorption at interfaces
81 compared to their native state, forming gels *via* disulfide bonds and hydrophobic interactions.

82 Among the deliberate biochemical modifications applied to proteins, enzymatic crosslinking or
83 hydrolysis is common [21–23]. Proteases used for enzymatic protein hydrolysis are commonly
84 obtained from either traditional or genetically modified microorganisms, and certain proteases are
85 extracted from fruit sources. Hydrolysis has demonstrated its ability to improve solubility, gelling, and
86 emulsifying characteristics of plant proteins [24].

87 Even though previous studies have already demonstrated the enhancement of pea protein foamability
88 through various physicochemical treatments [17,25–27], they relied on protein isolates with high
89 degree of protein purity. A specific nomenclature is used depending on the purity of the protein
90 sources. Protein concentrates, typically contain 50-70% protein, with the remainder consisting of
91 carbohydrates, fats, and other nutrients. They are produced by removing some of the non-protein

92 components (e.g. fats, fibers and carbohydrates) through basic processing methods like defatting,
93 dispersion, filtration or centrifugation. Protein Isolates usually contain 80% or more protein, making
94 them highly pure. They undergo more extensive processing to remove almost all non-protein
95 components. Techniques include isoelectric precipitation, ultrafiltration, ion exchange. In this study,
96 we examine how different treatments – namely sonication, heat treatment, or enzymatic proteolysis
97 – affect the foaming properties of yellow pea protein concentrates. Parameters such as solubility, foam
98 overrun, stability, and dynamic tension at the water-air interface were analyzed. The results obtained
99 for pea proteins were compared to a reference system based on whole eggs, which are commonly
100 used for the production of aerated structures in foods.

101

102 2. MATERIALS AND METHODS

103 2.1. Materials

104 Pea protein concentrate (PPC, Pea Protein 55 – Deflavored) was obtained from AGT Foods Ingredients
105 (Canada), and pea protein isolate (PPI, Vitessence Pulse 1803) from Ingredion (USA).

106 Whole eggs with a dry matter content of 23% (MetroChef) were procured from Metro (France). Sodium
107 hydroxide (NaOH 32% w/v solution) and Endoprotease Sumizyme BNP were acquired from Sigma-
108 Aldrich (France) and Takabio (France), respectively. Deionized water was employed in all experiments.

109 2.2. Compositional analysis

110 PPC and PPI powders were characterized according to AOAC methods to determine moisture content
111 (14.004, 1999), fat content (2003.06, 2006) and ashes (942.05, 1942). The total nitrogen content of the
112 PPC and PPI powders was determined using Kjeldahl method (AOAC method 991.20, 1995). A nitrogen-
113 to-protein conversion factor of 5.4 was adopted [28]. The carbohydrates content was estimated as the
114 remainder to 100%.

115 2.3. Preparation of PPC and PPI dispersions

116 The protein powder was dispersed in demineralized water at ambient temperature by using a magnetic
117 stirrer for around 30 min to allow powder hydration. Dispersions were prepared at 8 wt% or 23 wt%
118 for PPC and 16 wt% for PPI. The weight percentages are given in relation to the total mass of the phase
119 under consideration.

120 **2.4. Physical treatments of PPC dispersions**

121 2.4.1. Ultrasounds treatment

122 Sonication was applied to 8 wt% PPC dispersions using a SFX 550 (Branson-Emerson) sonicator.
123 Ultrasound (US) treatment was delivered with cycles (pulse duration of on-time 5 s and off-time 3 s)
124 for a total of 20 min at 10 % full power output (550 W, 20 kHz). To control the temperature, an iced
125 bath was used during US process and the temperature was monitored.

126 2.4.2. Thermal treatment

127 A two-step heat treatment was applied to the 8 wt% PPC dispersions: 90 min at 55°C, followed by
128 20 min at 80°C using water baths and under magnetic stirring. The second phase of the heat treatment
129 at 80°C was adopted to replicate the enzyme inactivation step described in the next paragraph, and
130 thus to compare systems identically processed. Immediately after the whole thermal treatment, PPC
131 dispersions were chilled in an iced bath.

132 **2.5. Enzymatic hydrolysis**

133 2.5.1. Enzymatic proteolysis of 8 wt% PPC dispersions

134 Enzymatic proteolysis of PPC dispersions was performed for 90 min at 55°C using the Sumizyme BNP
135 neutral protease extracted from *Bacillus subtilis* ($2.25 \cdot 10^4$ AU/g, enzymatic activity determined with
136 Anson method [29], equivalent to $3.75 \cdot 10^{-4}$ kat) with an enzyme-to-substrate ratio (E/S) of 1000 AU/g.
137 A volume of 57.6 mL of demineralized water was used to disperse 5.6 g of PPC powder and heated.
138 Once the dispersion attained 55°C, the enzyme solution (10 g/L) was added to reach a total volume of
139 70 mL and a dispersion concentration of 8 wt%.

140 Immediately after the hydrolysis step, the enzyme was inactivated through a thermal treatment of the
141 hydrolysate at 80°C, for 20 min, and finally cooled down with an iced bath.

142 2.5.2. Scale up of enzymatic proteolysis of concentrated PPC or PPI dispersions

143 Enzymatic proteolyses of concentrated dispersions of PPC (23 wt%) and PPI (16 wt%) were performed
144 under the same conditions as for 8 wt% PPC dispersions, at higher volume (500 mL) using 100 g/L
145 enzyme solution. Stirring was achieved by a mechanical stirrer set at 400 rpm (RW 20 digital, IKA)
146 equipped with a helix (5 cm diameter).

147 In the following sections, hydrolyzed PPC and PPI dispersions will be denoted as HYD PPC and HYD PPI,
148 respectively.

149 2.6. Dispersions and hydrolysates characterizations

150 2.6.1. Soluble protein content

151 The soluble protein content of supernatants from PPC and PPI dispersions, obtained after
152 centrifugation (4600 g, 15 min), was determined using bicinchoninic acid (BCA) spectrophotometric
153 method with bovine serum albumin (BSA) as standard (Pierce rapid gold protein assay, Thermo Fisher
154 Scientific, Massachusetts, USA). The absorption wavelength was 480 nm.

155 2.6.2. Degree of hydrolysis (DH)

156 The degree of hydrolysis allows to quantify the enzymatic proteolysis extent. It represents the
157 percentage of peptides bonds cleaved compared to the initial number of peptide bonds of the protein.

158 DH was calculated according to pH-Stat method [30] using Eq. [1]:

$$DH(\%) = \frac{B \times N_b}{M_p \times h_{tot} \times \alpha} \times 100 \quad [1]$$

159 B: base volume (mL), N_b : concentration of the base (NaOH, 0.5 mol/L), α : average dissociation degree,
160 M_p : mass (g) of proteins ($5.4 \times N$: nitrogen protein powder content), h_{tot} : theoretical total number of
161 peptide bonds in the protein substrate (meqv.g^{-1} of protein). h_{tot} was calculated from the theoretical

162 amino acid composition of the pea protein substrate by summing the concentrations of each amino
163 acid, and was equal to 7.63 meqv.g⁻¹ of protein.

164 α was calculated via Eq. [2] and found equal to 0.168, within the experimental conditions at pH = 6.37,
165 and by adopting a pK value of 7.06.

$$\alpha = \frac{10^{(pH-pK)}}{1 + 10^{(pH-pK)}} \quad [2]$$

166 2.6.3. Size-exclusion chromatography (SEC)

167 The molecular weight distribution of the native proteins and of the hydrolysates was determined by
168 size-exclusion chromatography on a Superdex Peptide 10/300 gl column (GE Healthcare, USA) using a
169 Nexera Series HPLC apparatus (Shimadzu, Japan). The sample was filtered (0.2 μ m) and 10 μ L were
170 injected. The column was equilibrated and eluted with water/acetonitrile/trifluoroacetic acid
171 (60/40/0.1, v/v/v), at a flow rate of 0.6mL/min, at room temperature. The peptides were detected at
172 214 nm. Proteins and PEG (polyethylene glycol) standards ranging from 160 Da to 38 kDa (Sigma-
173 Aldrich) were used for calibration. The relative size distribution of the peptides and the proteins was
174 estimated by integration of the area of the relevant peaks. SEC elution profiles were divided into 7
175 fractions determined to properly report the differences between the molecular weights of
176 unhydrolyzed and hydrolyzed soluble pea proteins.

177 2.7. Foams preparation

178 Two devices were employed to prepare protein-based foams. The shear device was adapted to the
179 sample volume. Indeed, the stirring system must preferably be of a size comparable to the container
180 to ensure that the entire volume is continuously mixed. The initial series of experiments were
181 conducted on limited volumes. The shear module used was a turbulent agitator, with the stirring rod
182 being of a size comparable to the container's diameter. In the second series of trials, we aimed to scale
183 up and compare the foaming capacity of protein suspensions with that of whole eggs, at comparable
184 dry extract levels. In this case, a planetary mixer was used.

185 2.7.1. Preparation of foams from 8 wt% PPC dispersions

186 Foams were made using a high-speed shearing method. Briefly, 30 mL of dispersion were placed in a
187 100 mL graduated cylinder immersed in an iced bath. The dispersion was processed at 12,000 rpm for
188 1 min using a homogenizer (T25 digital ULTRA-TURRAX, IKA).

189 2.7.2. Preparation of foams from whole eggs or 16 wt% and 23 wt% pea protein dispersions

190 Foams were prepared using a planetary mixer equipped with a whisk (Cooking chef KCL95, Kenwood).
191 Plant protein dispersions or eggs (approximately 300 mL) were placed in the mixer bowl and whipped
192 for 15 min at the maximum speed (190 rpm).

193 **2.8. Foams characterizations**

194 2.8.1. Foaming capacity, FC, and foam stability, FS

195 FC corresponds to the air-to-liquid volume ratio (Eq. [3]) measured right after whipping. FS is defined
196 as the ratio of the total foam volumes (Eq. [4]) measured after a time t equal to 1 or 2 hours (V_t) and
197 after 5 min storage (V_5 , the delay necessary to establish a discernable interface between the foam and
198 the underlying liquid phase).

$$FC (\%) = \frac{V_{air}}{V_{liq}} \times 100 = \frac{V_{foam} - V_{liq}}{V_{liq}} \times 100 \quad [3]$$

$$FS (\%) = \frac{V_t}{V_5} \times 100 \quad [4]$$

199 2.8.2. Visco-elastic properties

200 Visco-elastic properties in the linear domain were measured with a controlled strain rheometer (AR
201 G2, TA Instruments, USA) equipped with a parallel plate geometry (40 mm diameter) and 1 mm gap at
202 20°C. Around 2 g of foam were carefully placed in the bottom plate and the gap was slowly narrowed.
203 A cap was used to cover the geometry in order to prevent sample drying during the test.

204 The viscoelastic linear domain was determined by performing a strain sweep from 0.01 to 1000 %, at a
205 frequency of 1 Hz. The storage modulus G' (Pa) and loss modulus G'' (Pa) were measured in the linear
206 domain, at a strain of 0.5 % after a 5 min equilibration step allowing sample relaxation after the gap
207 was narrowed.

208 2.8.3. Microscopic images of foams

209 Foam samples were placed onto a hollow microscope slide under a phase contrast microscope
210 (Olympus BX53) to observe the foam microstructure directly after whipping.

211 2.9. Air-water interfacial properties

212 Since foaming properties are, to a large extent, determined by protein adsorption, two techniques were
213 implemented to characterize the kinetics of adsorption and the rheological properties of the protein
214 layer at the water-air interface.

215 2.9.1. Dynamic surface pressure

216 Surface tension at the air/solution interface was measured as a function of time by the pendant drop
217 technique (Tracker drop tensiometer, Teclis, France). The supernatants from HYD PPC as well as from
218 eggs (4600 g, 15 min) were diluted with distilled water to adjust the soluble proteins concentration to
219 0.1 g/L. A 10- μ L drop of sample was then generated and the evolution of the surface tension, $\sigma(t)$, was
220 recorded over time for a 1200 to 5400 s period. Data were converted to a time-dependent surface
221 pressure $\Pi(t) = \sigma_0 - \sigma(t)$, where σ_0 (72 mN/m) is the surface tension at the air/ pure water interface.

222 2.9.2. Dilatational interfacial elasticity

223 Interfacial dilatational properties of soluble egg proteins and soluble HYD PPC were characterized by
224 the oscillating drop technique using the drop tensiometer (Tracker drop tensiometer, Teclis, France). A
225 sinusoidal volume deformation of 5% amplitude was applied to a 10 μ L drop with a period of 1 s during
226 1200 s. The interfacial viscoelastic modulus, E (mN/m), is a complex number, comprising an in-phase

227 component (real part), and an out-of-phase component (imaginary part). The two components were
228 deduced from a Fourier transform using the apparatus software (Eq. [5]),

$$E = \frac{d\sigma}{dA/A} = \frac{-d\Pi}{d\ln A} = E_d + iE_v \quad [5]$$

229 where E_d is the surface dilatational elastic modulus (mN/m), and E_v is the surface dilatational viscous
230 modulus (mN/m).

231 **2.10. Statistical analysis**

232 All the results are presented as mean \pm standard deviation from at least 3 replicates ($n = 3$). A one-way
233 analysis of variance (ANOVA) was performed to identify significant differences ($p < 0.05$). Least
234 significant difference (LSD) multi comparison tests were used to identify sample means that were
235 significantly different. All the statistical analyses were performed with R software version 4.2.1 [31]
236 using *agricolae* package for LSD test.

237

238 **3. RESULTS AND DISCUSSION**

239 **3.1. Biochemical characterization of the protein powders**

240 The composition of the studied commercial plant protein samples is presented in Table 1. The main
241 component is protein, accounting for 50% in PPC and for 71% in PPI. PPC displays a significantly
242 elevated carbohydrate content, nearly 36% of its composition, in accordance with reported starch
243 content of approximately 40% [7]. The lipids content is lower for PPI than for PPC, 0.4 versus 2.1%
244 respectively.

245 **3.2. Effects of physical treatments and enzymatic hydrolysis on dilute PPC dispersions**

246 **3.2.1. Impacts on the soluble protein fraction**

247 Dilute PPC dispersions at 8 wt% were first examined. The soluble protein content was quantified
248 following physical and enzymatic treatments to evaluate their impact on protein solubilization

249 (Fig. 1 (A)). Regarding the enzymatic treatment, only one enzyme was used in this study. It was selected
250 for its efficiency in terms of enzymatic activity across a wide range of conditions and its non-GMO
251 nature. The operating conditions were optimized by evaluating the degree of hydrolysis, solubility,
252 foaming capacity, and molecular weight distribution measured by size-exclusion chromatography (see
253 Section 2.6.3). This optimization was conducted through an experimental design in which the selected
254 control factors were temperature (35°C to 55°C), enzyme/substrate ratio (100 to 1000), dispersion dry
255 matter content (2% to 8%), and duration (15 to 90 minutes).

256 Sonication resulted in a slight improvement of the soluble protein concentration compared to the
257 untreated PPC dispersion (25.6 ± 1.8 g/L *versus* 21.9 ± 1.5 g/L, respectively). This increase can be
258 attributed to structural changes in the proteins induced by ultrasound, exposing hydrophilic groups and
259 thereby enhancing protein water solubility. However, the solubility increase is less remarkable than
260 reported in the literature [17], likely due to the lower intensity of the ultrasound treatment used here
261 (55 W, 20 min compared to 200 W, 5 min). Indeed, we chose to use a low power level to ensure that
262 the samples did not experience heating.

263 Despite the high temperature adopted (80°C for 20 minutes), the thermal treatment did not
264 significantly alter solubility. This finding aligns with the work of Chao et al. [32] reporting that heating
265 did not modify pea protein solubility when thermal treatments between 55°C and 90°C were applied
266 to PPI dispersions.

267 In the conditions of enzymatic hydrolysis studied (55°C for 90 min, E/S = 1000 AU/g), the degree of
268 hydrolysis (DH) of pea proteins was determined to be $13.7 \pm 1.9\%$. The soluble protein content of HYD
269 PPC was unexpectedly the lowest among all the conditions studied (18.9 ± 1.5 g/L). This outcome was
270 surprising, considering that hydrolysis is generally known to enhance protein solubility. For instance,
271 Shahbal et al. [26] previously reported that enzymatic hydrolysis of various plant proteins using Alcalase
272 improved solubility for all sources. Additionally, in the study by Shuai et al. [33], hydrolysis of PPI by

273 different proteases increased protein solubility by at least 5%, even at low degrees of hydrolysis (DH =
274 2%). The increase in solubility was attributed to the reduction in the molecular size of proteins.

275 The unexpected result obtained in our case may be due to the limitation of the soluble protein assay
276 (BCA assay), which may not accurately quantify very small peptides. Cortés-Rios et al. [34] indicated
277 that the BCA assay could quantify tri-peptides as the minimum length. To verify this hypothesis, the
278 dispersions were characterized for their soluble protein size profile using size exclusion
279 chromatography (SEC). The SEC elution profiles of both initial and treated PPC dispersions were
280 segmented into seven size fractions (Fig. 1 (B)). The results concerning native PPC align with the
281 expected composition, as the dispersion comprised more than 40% of proteins over 13.5 kDa. Notably,
282 pea albumins, the water-soluble protein fraction, are larger than 10 kDa [7].

283 Non-hydrolyzed dispersions, including those treated with ultrasound (US PPC) and heat (TH PPC),
284 exhibited a protein molecular weight distribution very similar to that of native PPC, with over 84% of
285 the protein sizes exceeding 1.8 kDa. Sonication slightly raised the proportion of proteins over 13.5 kDa
286 (49% *versus* 42%). It is likely that ultrasounds facilitate the solubilization of higher molecular weight
287 proteins and form soluble protein complexes simultaneously [17]. TH PPC exhibited a lower proportion
288 of proteins with a molecular weight greater than 13.5 kDa and an increased proportion of the 13.5–5
289 kDa size fraction. This change could be attributed to the aggregation of high molecular weight soluble
290 proteins that were retained during sample filtration before SEC analysis. This hypothesis is supported
291 by the lower signal intensity observed for TH PPC compared to native PPC (Fig. SI 1, Supplementary
292 information). However, these proteins remained soluble and were quantified by spectrophotometric
293 soluble protein assays, leading to similar soluble concentrations in TH PPC and native PPC (20.8 and
294 21.9 g/L, respectively). Elution profiles of native PPC, US PPC, and TH PPC presented the same pattern,
295 suggesting that these physical treatments did not damage the ternary or quaternary structures of
296 proteins.

297 As expected, enzymatic hydrolysis resulted in a clear shift in the molecular weight distribution of pea
298 proteins: 64% of the peptides had molecular weights lower than 0.9 kDa for HYD PPC, compared to
299 only 10% for native PPC. This aligns with our hypothesis regarding the BCA assay limitations to quantify
300 small peptides, as the SEC profile shows that HYD PPC primarily comprised peptides lower than
301 tripeptides that were not detected by the assay. Previous studies on the effects of the commonly used
302 Alcalase endoprotease showed a similar trend, with the production of peptides with low molecular
303 weights below 10 kDa. SDS-PAGE profiles demonstrated the disappearance of bands with molecular
304 sizes between 17 and 100 kDa after proteolysis, leaving a thick area with indistinguishable bands below
305 10 kDa [26].

306 3.2.2. Impact on the foaming properties

307 The 8 wt% dispersions submitted to the different treatments were evaluated for their foaming capacity
308 (FC) and foam stability (FS) after 1h storage. The high-speed shearing method described in section 2.7.1
309 was employed to generate foams from the diluted PPC dispersions. In Fig. 2, the results are compared
310 to those obtained from the untreated dispersion (PPC).

311 In the realm of physical treatments, heat treatment demonstrated an enhancement of the foaming
312 capacity (FC) when compared to the native PPC dispersion ($69 \pm 4\%$ versus $54 \pm 2\%$). Conversely,
313 sonication did not exhibit any significant improvement ($61 \pm 10\%$). The impact of enzymatic proteolysis
314 was markedly more pronounced than that of the physical treatments, resulting in an FC as high as
315 $114 \pm 7\%$, which is more than twice the FC value of the native PPC dispersion. Foaming stability (FS)
316 surpassed 57% for all examined conditions, indicating that more than half of the foam volume was
317 retained one hour after foam production. Enzymatic hydrolysis emerged as the sole treatment that
318 significantly enhanced foam stability compared to the native PPC dispersion, with values of $84 \pm 3\%$
319 and $57 \pm 2\%$, respectively. A control test was performed with PPC and the enzyme thermally inactivated,
320 which did not result in any improvement in FC nor FS parameter. Therefore, the improvement in
321 foaming properties is clearly attributable to the use of the enzyme.

322 In the present study, sonication did not alter significantly the foaming properties of pea proteins, even
323 though previous research has shown that this treatment can have an influence on it [17,27]. The
324 enhancement of pea proteins' foaming properties after sonication is attributed to structural
325 modifications of the proteins. Ultrasound induces partial unfolding of the proteins, leading to a quicker
326 adsorption of unfolded proteins at the air/water interface, thereby improving foam formation.

327 The marginal improvement in foaming capacity observed for the TH PPC in this study is in line with
328 observations made by Chao et al. [32]. These researchers noted an enhancement in foam stability but
329 observed varying effects on the foaming capacity of PPI dispersions based on the applied thermal
330 treatment: the foaming capacity remained unchanged after 30 min at 80°C, while it increased following
331 30 min at 50°C, suggesting that heating at high temperature reduces proteins foamability. In our study,
332 results revealed that a treatment of 55°C for 1h30, followed by 20 min at 80°C, led to a 15% increase
333 in both foaming capacity and foam stability. Here, the time/temperature pattern used for the thermal
334 treatment did not affect proteins foamability. Similarly to sonication, it can be hypothesized that
335 protein unfolding and enhanced polypeptide chain flexibility induced by the thermal treatment
336 facilitated adsorption at the air/water interface, thereby promoting foam formation.

337 Improvements in pea protein foaming capacity through enzymatic hydrolysis have been observed in
338 various studies on PPI. For instance, there were increases from 100% to 243% with pepsin hydrolysis
339 (76 g/L protein solution, DH = 9-12%) as reported by Tang et al. [35], and from 45% to 168% with trypsin
340 hydrolysis (67 g/L protein solution, DH = 4%) as reported by Shuai et al. [33]. However, in those studies,
341 foam stability did not show significant differences between the untreated and the hydrolyzed pea
342 proteins.

343 In contrast, our results demonstrated a positive effect of enzymatic proteolysis on both foaming
344 capacity and foam stability. The enhanced foaming capacity may be attributed to the reduction in
345 molecular weight of the proteins, facilitating a faster migration rate at the air-water interface [35]. It is

346 likely that due to their enhanced flexibility, these peptides develop stronger lateral interactions,
347 creating a rigid layer that contributes to foam stability, as suggested by Wouters et al. [24].

348 Our findings unequivocally demonstrate that enzymatic proteolysis, involving peptide bond cleavage
349 and molecular weight reduction, effectively functionalized pea proteins. In comparison to the two
350 physical treatments, proteolysis resulted in a significant enhancement of foaming properties. This
351 investigation, conducted on dilute PPC dispersions, led to the conclusion that enzymatic hydrolysis
352 stands out as the optimal approach for augmenting their foaming properties.

353 3.3. Scale-up and functionalities of hydrolyzed concentrated dispersions

354 With the goal of using PPC dispersions as a food ingredient that could substitute whole eggs, they were
355 formulated with the same dry extract (23 wt%). Their foaming characteristics were examined and
356 compared to those of eggs. It is noteworthy that the foaming attributes of PPC dispersions can be
357 affected by the presence of endogenous starch revealed by Maltese crosses in polarized micrographs
358 (Fig. SI 2 (A), Supplementary Information). However, these crosses were no longer visible after thermal
359 treatment (20 minutes at 80°C), indicating that starch underwent gelatinization during enzyme
360 inactivation (Fig. SI 2 (C), Supplementary Information). To assess the role of gelatinized starch, PPI
361 dispersions containing around 70% protein (Table 1) and no starch were also examined. Their dry
362 extract was fixed at 16% to achieve the same protein content as whole eggs [36] and HYD PPC 23 wt%
363 dispersions, which is 11.5%. Foams were produced from the above-mentioned hydrolyzed
364 concentrated protein dispersions and from whole eggs.

365 3.3.1. Foam characteristics

366 The foaming capacity (FC) and the foam viscoelastic moduli G' and G'' were measured and the values
367 are reported in Table 2.

368 Whole eggs and the hydrolyzed dispersions exhibited the capability to trap substantial volumes of air,
369 although with notable differences. HYD PPI 16 wt% showed the highest FC value (1111%), followed by
370 whole eggs (646%), and finally HYD PPC 23 wt% (485%). The foaming capacity of whole eggs is higher

371 than the value (350%) reported by Li et al. [37], possibly due to a difference in whipping duration (5 min
372 *versus* 15 min in our study).

373 The viscoelastic properties of all studied foams exhibited a consistent trend, with the storage modulus
374 (G') surpassing the loss modulus (G''), indicating a dominance of elastic behavior in the foams. For eggs
375 and HYD PPI 16 wt% foams, the G' and G'' values were relatively low ($G' = 15$ Pa, $G'' = 10$ Pa for eggs;
376 $G' = 99$ Pa, $G'' = 15$ Pa for HYD PPI 16 wt%) compared to HYD PPC 23 wt% foams ($G' = 459$ Pa,
377 $G'' = 132$ Pa). In comparison to eggs and HYD PPI 16 wt%, HYD PPC 23 wt% foams exhibited a solid-like
378 behavior: they did not show flowing behavior and supported their own weight. Microscope
379 observations indicated that HYD PPC 23 wt% foams had smaller and more uniform bubble sizes
380 compared to egg foams (Fig. 3 (A) and (B)). The bubbles in egg foams exceeded 200 μm in diameter,
381 whereas most bubbles in HYD PPC 23 wt% foams measured around 100 μm .

382 The observed differences can be attributed to the presence of gelatinized starch in HYD PPC
383 dispersions. A similar correlation was noted in the study by Martin Torrejon et al. [38], which involved
384 foams made from gelatin suspensions with and without pregelatinized tapioca starch. Substantial
385 differences were observed in terms of the incorporated air volume: in presence of gelatinized starch,
386 the highly viscous suspension could not incorporate as much air as the suspension without. A further
387 comparison between foams obtained from HYD PPC 23 wt% and HYD PPI 16 wt% highlights the
388 disparities between a pea protein concentrate and a pea protein isolate. Despite nearly identical total
389 protein contents (11.5%), foam characteristics differed significantly in terms of air volume fraction,
390 primarily due to the elevated carbohydrate content of PPC dispersions. Concomitantly, the higher
391 viscosity of the HYD PPC 23% dispersions generated higher viscous stresses during the whipping
392 process and thus smaller bubbles than the HYD PPI 16% dispersions, as evidenced by microscope
393 observations (Fig. 3 (B) and (C)).

394 Foam stability was monitored for up to 2 hours (Fig. 4). During the initial hour, the egg foam remained
395 relatively stable, with a volume loss close to 30% ($FS = 69 \pm 2\%$). However, a significant decline in egg

396 foam stability occurred after 1.5 hours, resulting in almost complete collapse. In contrast, foams
397 generated from HYD PPC and HYD PPI exhibited consistent stability throughout the entire observation
398 period.

399 The destabilization of aqueous foams is attributed to three primary mechanisms: drainage,
400 disproportionation (Ostwald ripening), and coalescence [39]. Coalescence, the merging of air bubbles,
401 typically occurs as a result of liquid drainage. As films between bubbles and Plateau borders (interstitial
402 zones in between 3 adjacent bubbles) thin out, the interfaces approach to each other, leading to
403 eventual film disruption. In our investigation, we noted both drainage and coalescence contributing to
404 the destabilization of whole egg foam. Indeed, over the observation time, we could observe an increase
405 of the drained liquid as well as a swifter raise of the air bubbles sizes than the average, revealing the
406 occurrence of coalescence phenomena [40] (Fig. SI 3, Supplementary Information). In their study
407 involving microscopic observation of whole egg foam, Spencer et al. [41] revealed a destabilization
408 mechanism via disproportionation. Over time, bubble size increased as gas diffused from smaller
409 bubbles to larger ones. Previous research has explored the destabilizing impact of egg yolk on egg white
410 foams. Li et al. [37] found that adding 30% egg yolk to egg white increased foam drainage by 45% within
411 30 minutes. Wang et al. [42] determined that even a low concentration of 0.5% egg yolk decreased egg
412 white foam stability by 10% and increased drainage by 40% within the same timeframe. Therefore,
413 given the use of whole eggs in our study, the observed limited foam stability is in agreement with these
414 authors' findings. Egg white has a favorable effect on both foaming and stability, while egg yolk
415 predominantly exerts a destabilizing effect.

416 For HYD PPC and HYD PPI foams, no destabilization similar to that observed in whole eggs was identified
417 and the total foam volume remained unaffected. Indeed, no drainage was observed over the 2-hour
418 observation period, even though HYD PPC and HYD PPI foams evidenced significantly bigger bubbles
419 sizes as the experiment was going on (Fig. SI 3, Supplementary Information).

420 The lifespan of foams is generally influenced by drainage through the liquid films separating air bubbles
421 and the Plateau borders [39]. In HYD PPC 23 wt% foams, the continuous phase is thickened due to the
422 dissolved polysaccharides and the gelatinization of starch, resulting in a measured viscosity of 65 mPa.s
423 for HYD PPC 23 wt% *versus* 10 mPa.s for whole eggs at 500 s⁻¹. Furthermore, aggregates from the
424 insoluble fraction form an interconnected three-dimensional network which slows down drainage.
425 Interestingly, in Fig. 3 (B), dark spots seem to indicate the presence of particles at the air-water
426 interfaces. It is likely that particles or clusters from the insoluble fraction adsorb at the air-water
427 interfaces or intercalate into the films, thus preventing them from thinning.

428 3.3.2. Interfacial properties of hydrolyzed pea proteins soluble fraction and whole egg proteins

429 A critical factor influencing the formation and stability of protein foams is the ability of proteins to
430 function as surface-active species, thereby reducing the interfacial tension between air and water and
431 generating interfacial stiffness.

432 Drop tensiometry measurements were conducted to compare the performance of egg proteins and
433 hydrolyzed pea proteins at the air-water interface. For HYD PPC 23 wt%, the measurements were
434 conducted using only the water-soluble fraction.

435 The surface pressure (Π) was monitored over time for eggs and HYD PPC at 0.1 g/L (Fig. 5 (A)). The
436 observations were continued until the surface pressure value approached a nearly asymptotic value.
437 The process of protein adsorption typically involves three distinct steps: diffusion, adsorption, and
438 rearrangement. The diffusion phase is characterized by a lag period in the evolution of surface pressure
439 as proteins move towards the interface. Subsequently, the surface pressure increases rapidly as
440 proteins adsorb at the interface. Finally, during the rearrangement phase, only minimal alterations in
441 surface pressure are observed as proteins undergo rearrangements [8].

442 For HYD PPC proteins, no lag phase was observed. The surface pressure exhibited a rapid increase
443 within the initial 100 seconds, rising from 1 to 8 mN/m, indicating a swift diffusion of peptides to the
444 interface. Similar immediate surface pressure increases have been reported in studies on the interfacial

445 properties of pea or legume proteins. In a study by Kornet *et al.* [43], unhydrolyzed soluble pea proteins
446 (1 g/L) raised the surface pressure by 10 mN/m in 100 s. However, our achieved surface pressure value
447 after 1000 s was slightly lower (17 mN/m compared to 21 mN/m).

448 The surface pressure curve for eggs exhibited a prolonged induction time of approximately 500 seconds
449 before a sharp increase. Between 300 and 2000 seconds, the surface pressure increased by 10 mN/m
450 and reached a value of 15 mN/m after 3000 seconds. In a study by Jin *et al.* [44], the surface pressure
451 in binary systems of egg white proteins (ovalbumin/ovomuroid 5:1) reached a value exceeding
452 20 mN/m, which is higher than the measurement obtained here for whole eggs. In the case of whole
453 eggs, the three steps described for protein adsorption are clearly observable, contrary to the
454 observations of Jin *et al.* [44] on egg white proteins. The extended lag phase at the beginning may
455 indicate a slower diffusion of proteins to the interface because of the presence of proteins of higher
456 molecular weight (e.g., ovomucin, ovotransferrin). Li *et al.* [45] demonstrated that contamination of
457 egg white with egg yolk reduces its ability to adsorb at the air-water interface and, consequently, its
458 foaming properties.

459 For both HYD PPC and eggs, the surface pressure did not truly reach a plateau regime at the end of the
460 observation, but its evolution slowed down. The continuous increase of surface pressure over time
461 suggests that protein adsorption is a dynamic phenomenon with constant reorganizations at the
462 interface.

463

464 Dilatational interfacial properties may contribute to understanding the phenomena involved in foam
465 formation and the differences in foaming properties between whole eggs and HYD PPC. Foam
466 formation relies on the ability of proteins to quickly adsorb at the interface when the system undergoes
467 perturbations that introduce air or stretch the interfaces.

468 The viscoelastic modulus (E) curves exhibited different shapes (Fig. 5 (B1)). For eggs, an induction
469 period occurred within the first 200 s, after which the viscoelastic modulus increased continuously

470 before stabilizing at around 55 mN/m after 1000 s. In contrast, for HYD PPC, there was no induction
471 phase. The viscoelastic modulus increased rapidly between the beginning of the observation and 200 s,
472 reaching a plateau value of 40 mN/m.

473 The viscoelastic moduli curves closely resembled those obtained for the elastic modulus, E_d (Fig. SI 4,
474 Supplementary Information), with E_d being higher than the viscosity modulus, E_v , indicating the
475 dominance of elastic behavior at the interface. The absence of an induction period for HYD PPC
476 suggests rapid protein adsorption at the interface. In a study by Shen et al. [46], the viscoelastic
477 modulus of native PPIs also exhibited an early increase. However, the value reached upon protein
478 adsorption at the interface was much lower than that observed for our pea protein hydrolysate
479 (10 mN/m *versus* 40 mN/m). The higher viscoelastic modulus values for HYD PPC suggest that
480 hydrolyzed pea proteins establish stronger interactions at the interface compared to native pea
481 proteins.

482 We observed significant variability in the duration of the induction phase for eggs. This is illustrated in
483 Fig. 5 (B2), which presents two separate measurements. As depicted in Fig. 5 (B1) representing the
484 mean of the replicates, the large error bars in the viscoelastic modulus mainly result from the difference
485 in lag time that could extend up to 300 seconds. Nevertheless, the trend in the evolution of interfacial
486 viscoelasticity remains consistent across both replicates. A competition for adsorption at the interface
487 may occur between proteins from egg white and egg yolk, resulting in an induction phase of variable
488 duration at the beginning of the observation. This is in contrast to the study by Jin et al. [44], where
489 the viscoelastic modulus of egg white proteins exhibited a sharp increase without any induction phase.
490 Despite this difference, our results align with those presented in the literature regarding the reached
491 viscoelastic modulus values: at long times of observation, they were comparable for egg white proteins
492 (around 50 mN/m in the study of Jin et al. [44]) and whole eggs (55 mN/m). This suggests that the long
493 term- interfacial properties of whole eggs are primarily influenced by egg white proteins.

494 The trend in the evolution of viscoelastic moduli for HYD PPC and whole eggs shown in Fig. 5 hints at a
495 potentially faster strengthening of the interfaces covered by HYD PPC proteins. This phenomenon can
496 be attributed to the lower molecular weight resulting in higher flexibility of the hydrolyzed pea proteins
497 compared to globular and rigid egg proteins. In both systems, high values of viscoelastic modulus
498 (> 40 mN/m) were obtained, indicating the solid-like nature of the interfacial films formed.

499 It is worth noting that the apparatus employed for investigating the air/water interface may have
500 limitations in elucidating further distinctions between the two systems. Particularly in the context of
501 surface viscoelasticity measurement, the applied deformations may not mirror the real conditions of
502 foam formation and evolution [47]. Furthermore, these findings regarding interfacial behavior solely
503 reflect the influence of the soluble fractions.

504 In summary, under the examined conditions, both systems demonstrated similar surface pressure and
505 viscoelastic modulus values, indicating their capability to foam through the formation of rigid films with
506 in-plane (lateral) protein interactions. Contrary to systems where enhanced stability against
507 coalescence was associated to an increase in surface viscoelasticity [48], our observations did not
508 support this correlation. This suggests that in our systems other factors are more influential. Again, it
509 can be argued that in foams based on HYD PPC the presence of both polysaccharides dissolved in the
510 aqueous phase and aggregates formed by the insoluble fraction lead to increased viscosity and even
511 gelation, which slows down the draining process and thus extends the lifespan of the foams.

512

513

514 4. CONCLUSION

515 In this study, our primary objective was to identify protein modification methods that could enhance
516 the foamability of pea protein concentrates. We explored both physical and enzymatic treatments on
517 low dry matter pea protein dispersions, with a focus on characterizing the soluble fraction before

518 conducting foaming experiments. Sonication increased the soluble protein content and resulted in the
519 formation of soluble protein complexes. Thermal treatment did not modify the soluble protein
520 concentration. Analysis of protein size distribution revealed significant alterations in protein structures
521 with enzymatic proteolysis, leading to the formation of low molecular weight peptides.

522 Size exclusion chromatography did not show any modification of the primary protein structure with
523 physical treatments. However, the enhancement of foaming capacity indicated that physical
524 treatments induced structural changes in the polypeptide chains, promoting protein unfolding and
525 exposure of hydrophobic regions that facilitated the formation of aqueous foams and stabilized them.
526 Following enzymatic modification, the foaming properties of proteins were significantly higher
527 compared to the native protein dispersion. The comparison of different systems highlighted the synergy
528 of soluble and insoluble components in hydrolyzed protein concentrates. The reduced molecular
529 weight of the hydrolyzed species facilitated their diffusion towards the interface and resulted in the
530 formation of firm interfaces. Our study was focused on protein modification, but other components
531 may contribute to the improvements in foaming properties through sonication, heat treatment, or
532 enzymatic proteolysis. For instance, the presence of insoluble particles and polysaccharides
533 contributed to bolstering physical stability.

534 Using plant protein concentrates in place of isolates could be a way to develop stable foams by taking
535 advantage of the texturing properties of the carbohydrate content without resorting to food additives.

536 *CRedit authorship contribution statement*

537 **Lucie Périé:** Investigation, Methodology, Writing – Original draft, Visualization, **Raphaëlle Savoie:**

538 Conceptualization, Methodology, Writing – review and editing, **Christelle Harscoat-Schiavo:**

539 Conceptualization, Methodology, Writing – review and editing, **Mathieu Delample:** Project

540 administration, **Mathilde Roze:** Writing – review and editing, **Cécile Lebrun:** Funding acquisition,

541 Project administration, **Martine Crepin:** Project administration, **Fernando Leal-Calderon:**

542 Conceptualization, Methodology, Writing – review and editing.

543

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681 Table 1: Biochemical composition of pea protein concentrate (PPC) and pea protein isolate (PPI)
 682 (Values are presented as mean \pm standard deviation)

| Composition (g/100 g) | PPC | PPI |
|--|------------------|-----------------|
| Protein | 49.97 \pm 0.01 | 71.2 \pm 0.3 |
| Lipids | 2.1 \pm 0.4 | 0.44 \pm 0.02 |
| Carbohydrates* | 36.2* | 16.0* |
| Moisture | 5.3 \pm 1.2 | 7.1 \pm 0.1 |
| Ash | 6.4 \pm 0.2 | 5.27 \pm 0.01 |
| *Assumed to be equal to the complement to 100% relative to all other measured components | | |

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684 Table 2 : Foaming properties of whole eggs, hydrolyzed pea protein concentrate dispersions at 23 wt% (HYD PPC
 685 23 wt%) and hydrolyzed pea protein isolate dispersion at 16 wt% (HYD PPI 16 wt%) (Means with different letters
 686 within the same column are significantly different, $p < 0.05$).

| | FC (%) | Foam viscoelasticity (0.5% strain) | |
|-----------------------|----------------------------|------------------------------------|------------------------------|
| | | G' (Pa) | G'' (Pa) |
| Eggs | 646 \pm 7 ^b | 15.3 \pm 1.3 ^c | 9.5 \pm 0.3 ^c |
| HYD PPC 23 wt% | 485 \pm 36 ^c | 458.6 \pm 20.1 ^a | 132.3 \pm 4.6 ^a |
| HYD PPI 16 wt% | 1111 \pm 37 ^a | 98.9 \pm 8.1 ^b | 15.3 \pm 2.2 ^b |

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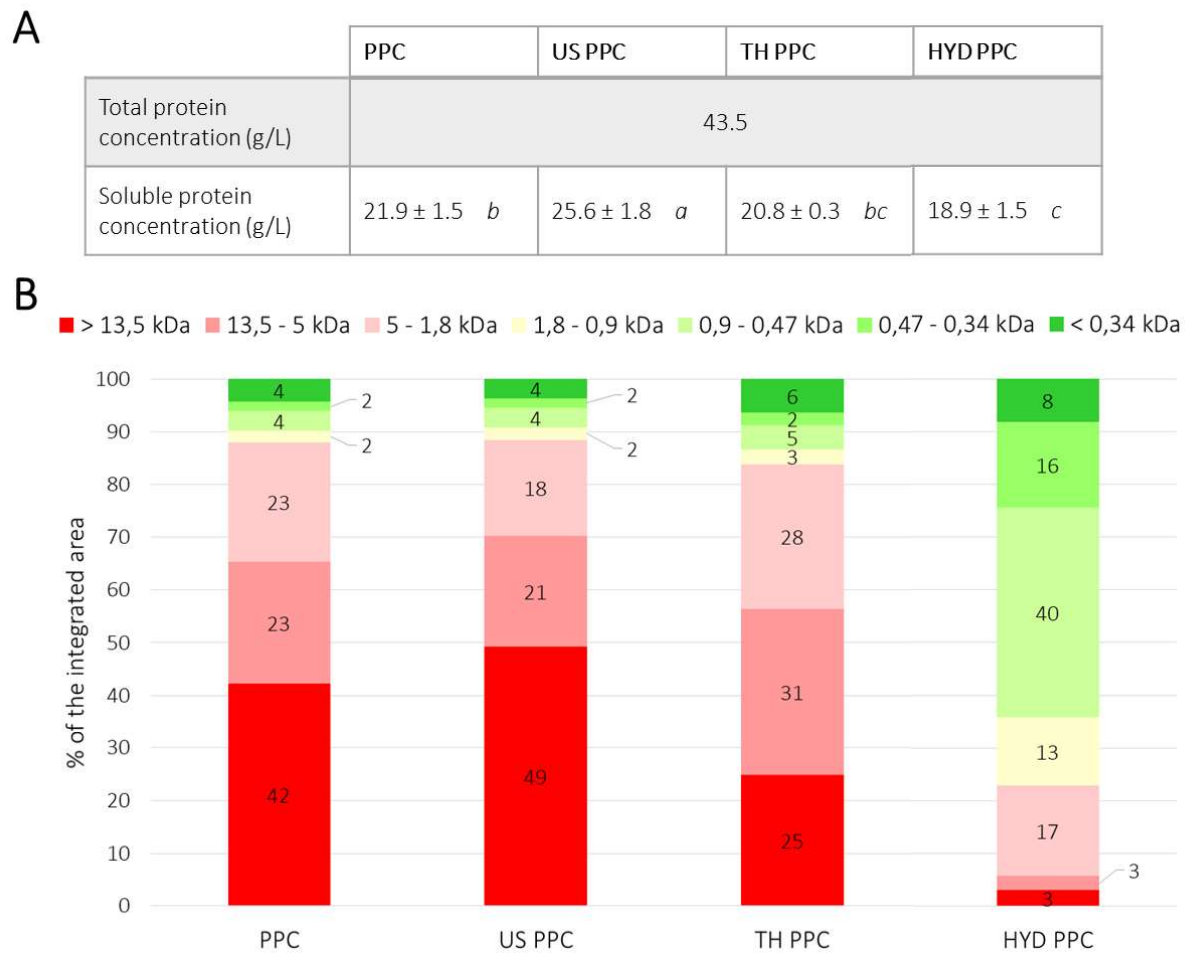


Figure 1: (A) Total and soluble protein contents and (B) Size distribution from SEC-HPLC for native (PPC), sonicated (US PPC), heated (TH PPC) and enzymatically hydrolyzed (HYD PPC) pea protein concentrate dispersions at 8 wt%. (Values are presented as mean ± standard deviation; means with the same letter are not significantly different ($p < 0.05$))

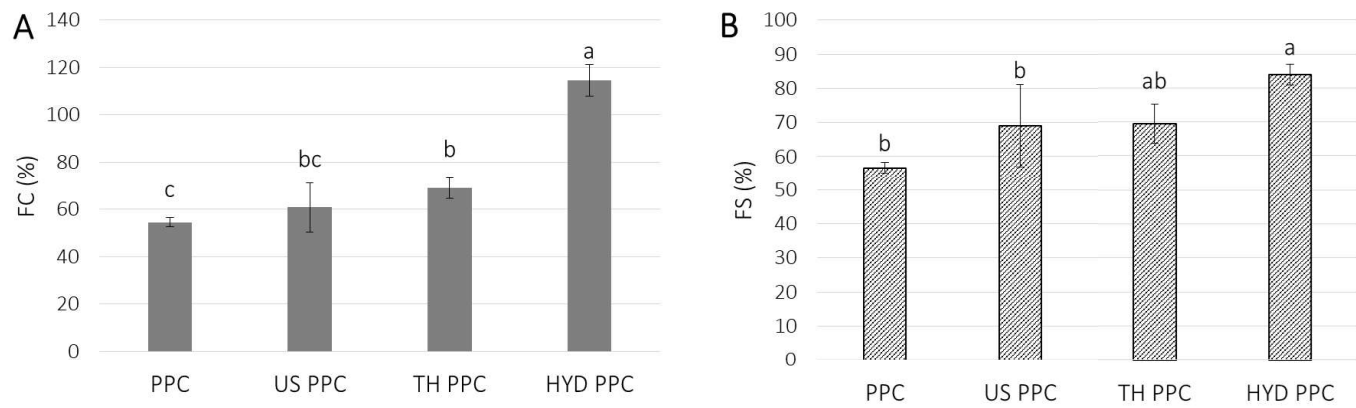


Figure 2: (A) Foaming capacity (FC) and (B) foam stability (FS) of 8 wt% pea protein concentrate (PPC) dispersions after sonication (US PPC), thermal treatment (TH PPC) and enzymatic hydrolysis (HYD PPC) (significant differences between conditions are indicated by different letters, $p < 0.05$).

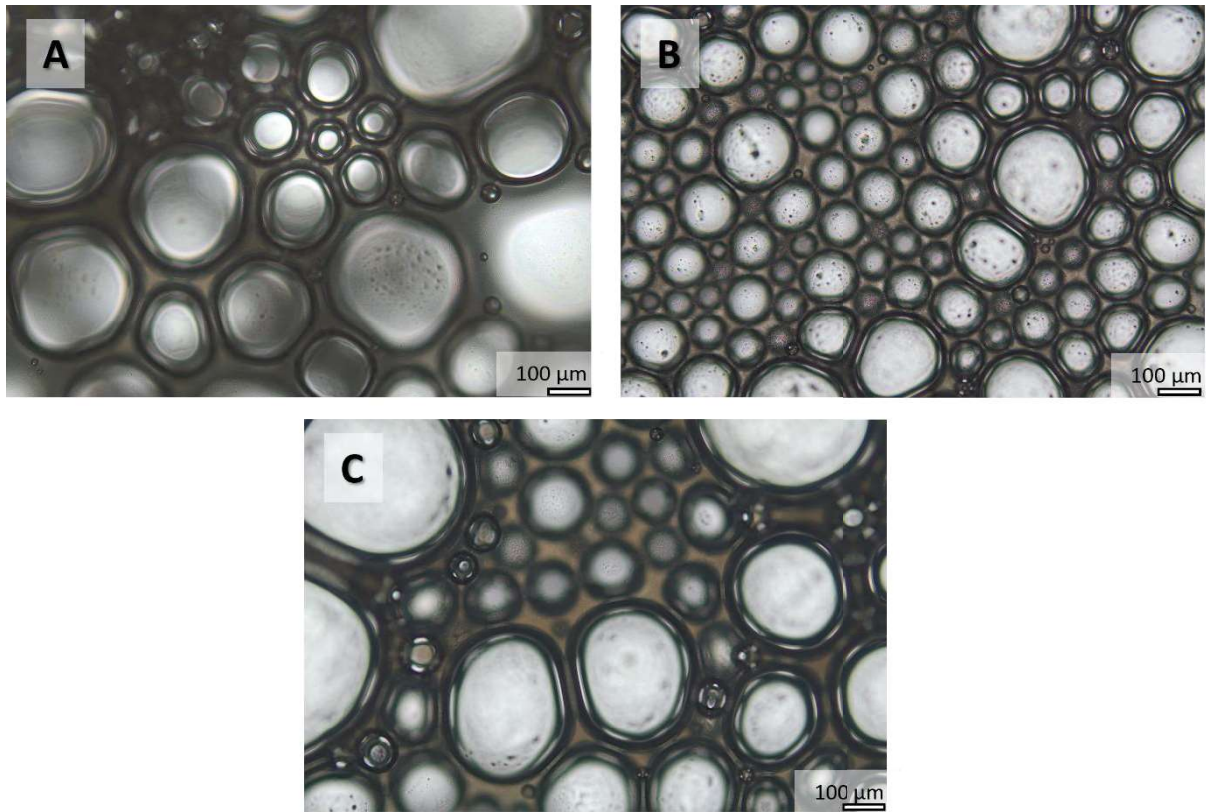


Figure 3: Micrographs of whole eggs (A), HYD PPC 23 wt% (B) and HYD PPI 16 wt% (C) foams

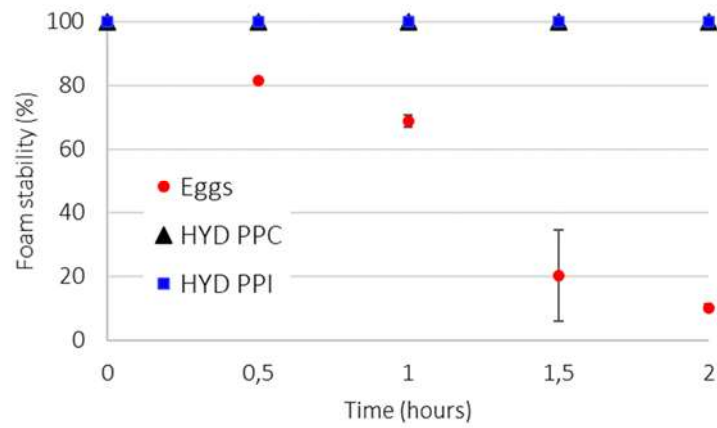


Figure 4: Foam stability (FS) over time of whipped wholes eggs, hydrolyzed pea protein concentrate dispersion (HYD PPC 23 wt%) and hydrolyzed pea protein isolate dispersion (HYD PPI 16 wt%). Results are presented as means; standard deviations are shown as error bars.

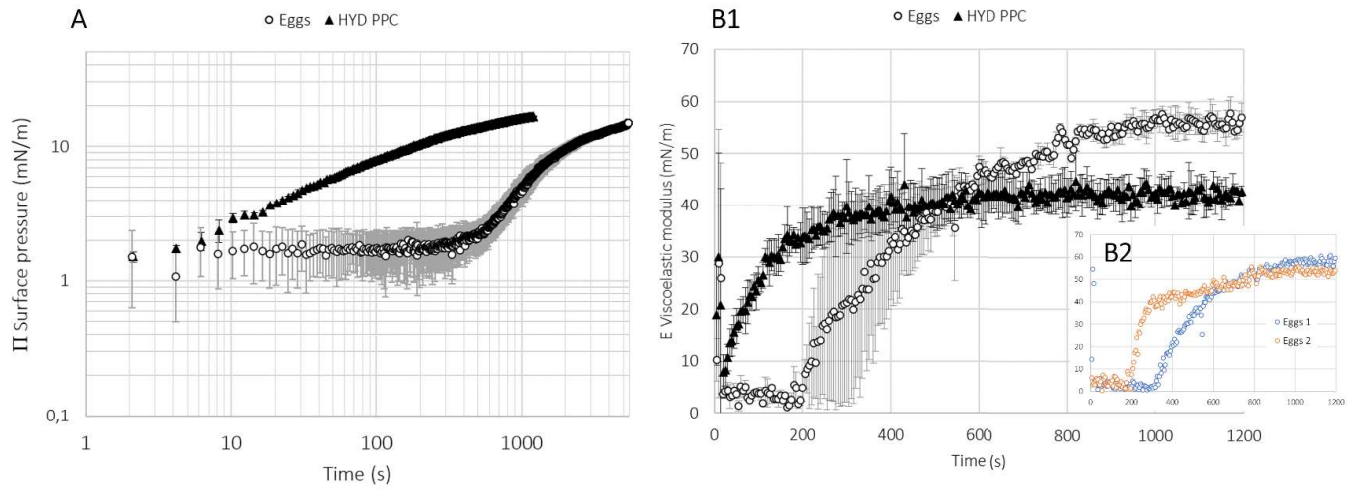


Figure 5: Time-dependent changes in (A) surface pressure (Π) and (B1) viscoelastic modulus, E , at the air-water interface of proteins (0.1 g/L) from whole eggs and hydrolyzed pea protein concentrate (HYD PPC, soluble fraction). (B2) Detail of the two curves for whole eggs represented as a mean in (B1).

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