Article

High-Pressure Effects on Selected Properties of Pea and Soy Protein Isolates

Rui Queirós [1], Rita Ferreira [2], Jorge A. Saraiva [2,*] and José A. Lopes-da-Silva [2,*]

[1] Department of Applications and Food Processing, Hiperbaric S.A., Calle Condado de Treviño, 6, 09001 Burgos, Spain
[2] LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal
* Correspondence: jorgesaraiva@ua.pt (J.A.S.); jals@ua.pt (J.A.L.-d.-S.)

Abstract: The use of vegetable proteins has been the focus of research efforts to develop new products and/or to replace other sources of protein. Ergo, there is a need to assess the effects of new processing technologies on this type of protein. This work evaluated the influence of high-pressure processing (HPP) (pressure: 200, 400 and 600 MPa; holding time: 5, 10 and 15 min) on selected properties of pea (PPI) and soy (SPI) protein isolates at three pH values (6, 7 and 8). SPI presented a higher percentage of soluble proteins than PPI, still, HPP increased protein solubility of both isolates. This effect was more pronounced on SPI, particularly at pH 7 and 8, where the percentage of soluble proteins almost tripled under some HPP conditions. Similarly, the surface hydrophobicity also increased with HPP for proteins from both sources, increasing, in general, with increasing pressure and holding time. On the contrary, the content of free sulfhydryl groups generally decreased with HPP for proteins from both sources, suggesting a complex balance between protein unfolding and further aggregation under certain conditions. The effects of HPP on the emulsifying properties of the protein isolates were dependent on pH, pressure, holding time and whether the soluble or total fraction of the protein isolates were used.

Keywords: protein isolates; pea; soy; high-pressure processing; surface changes; emulsifying activity

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1. Introduction

In recent decades, there has been an increase in the pursuit of technologies and products that allow us to tailor the technological properties of macromolecules present in foods to improve nutritional and sensorial quality of food products. In this way, the use of vegetable proteins has been the focus of many research efforts to develop new products and to replace other sources of protein [1]. One of the most cultivated crops in the world is soybean, due to the global growing demand for its oil and protein content (ca. 35%). Soy proteins are among the most studied legume proteins and have several applications in food production, mainly due to their nutritional and functional properties. Similar to soy, pea has been increasingly exploited as a source of protein, largely due to its ability to grow worldwide. Peas contain approximately 25% of proteins that have several applications in the food industry, for instance, emulsification, gelation and texture improvement, which is increasing this pulse popularity [1,2].

The widespread applications of these proteins, particularly in food formulations processed by newer technologies, is restricted due to the limited knowledge concerning adequate processing parameters [3]. Since the structure, conformation and physicochemical properties of proteins can be affected by pressure, high-pressure processing (HPP) can be a useful tool to tailor the techno-functional properties of food proteins [4]. Under HPP, changes in protein conformation are expectable, related to a change from a high-volume conformation to a low-volume conformation, which usually follows a non-linear response to pressure and occurs predominantly at higher pressures, i.e., above 400 MPa. The collapse of molecular cavities due to water penetration, accompanied by their hydration, and the

shortening of the hydrogen bonds are the most relevant consequences of pressure [5]. These effects promote intermolecular interactions that destabilize the tertiary structure and the eventual unfolding of the protein [6]. After the release of pressure, the structure of proteins will frequently be different from the native structures, leading to altered properties such as foaming, emulsification or gelation [7].

Several studies using vegetable proteins have shown the advantages of HPP to modify the supramolecular structures of proteins, creating new structured systems and textures and improving their functionality [8,9]. However, there is still a lack of information about the effect of HPP on proteins with different structures and macromolecular organization, namely for commercial protein isolates, which are often composed of proteins that are far from their original native state, and under different environment conditions and HPP processing. Therefore, the objectives of the present study were (i) to evaluate the effects of different HPP parameters, i.e., pressure and holding time, on selected structural and technological protein characteristics, e.g., solubility, content of free sulfhydryl groups, surface hydrophobicity and emulsifying properties; (ii) to assess these effects for commercial pea and soy protein isolates, which typically correspond to proteins with a high degree of denaturation; and (iii) to evaluate these effects for proteins with a different charge density, achieved by studying a relatively short pH range (6–8), slightly above the isoelectric point for most pea and soy proteins and with applied interest.

2. Materials and Methods

2.1. Materials

Readily dispersible pea protein isolate (PPI, Pisane® M9, Cosucra, Warcoing, Belgium) and soy protein isolate (SPI, Induxtra W, Induxtra, Girona, Spain) were obtained from Induxtra (Induxtra de Suministros Llorella Portuguesa–Indústria Alimentar, Lda., Moita, Portugal). Protein content was determined by elemental analysis (N $\times$ 6.25): PPI = 81.1 $\pm$ 0.1%; SPI = 86.7 $\pm$ 0.03%. The water content of both protein isolates was less than 3%. According to the supplier, ash and fat content were less than 6% and 4%, respectively. All reagents used were of analytical grade.

2.2. Sample Preparation

The protein isolates were dispersed in distilled water (1% (*w*/*v*)) and stirred for 4 h at room temperature for hydration. The pH was adjusted to 6, 7 or 8 with 0.1 mol·L^{-1} citric acid or 0.01 mol·L^{-1} NaOH and stirred for 40 min at room temperature. The dispersions (approximately 40 mL) were placed in flasks (Thermo Scientific™ Nalgene™ Wide-Mouth Lab Quality HDPE Bottles, Waltham, MA, USA) for processing.

2.3. Pressure Treatments

The samples were treated at 200, 400 or 600 MPa for 5, 10 or 15 min at room temperature (approximately 20 °C) using a hydrostatic press (Hiperbaric 55, Burgos, Spain). This HPP equipment has a pressure vessel of 200 mm inner diameter and 2000 mm length and a maximum operating pressure of 600 MPa. It is connected to a refrigeration unit (RMA KH 40 LT, Ferroli, San Bonifacio, Italy) that allows controlling the temperature of the input water used as pressurizing fluid. For those tests where the soluble protein fraction was to be studied, the dispersions were centrifuged at 6000 rpm for 20 min at 4 °C.

2.4. Electrophoresis

Each sample solution was diluted (1:2) in loading buffer (0.5 M Tris-HCl pH 6.8, 4% (*w*/*v*) SDS, 15% (*v*/*v*) glycerol, 1 mg·mL^{-1} bromophenol blue and 2% (*v*/*v*) β-mercaptoethanol) and incubated at 100 °C for 5 min. SDS-PAGE electrophoresis was performed as previously described [10].

2.5. Solubility

Pressurized and non-pressurized (control) protein dispersions were centrifuged at 6000 rpm for 20 min at 4 °C. Soluble protein concentration was determined in the supernatant using the method of [11] with a few modifications. A total of 250 µL of Bradford reagent were added to an aliquot of 50 µL of protein solution (supernatant), mixed for 30 s and then incubated for 20 min at room temperature. The absorbance was measured at 595 nm using a spectrophotometer (Microplate Spectrophotometer Multiskan Go, Thermo Scientific, Waltham, MA, USA) and the protein concentration was determined using a calibration curve using BSA standards. Protein solubility was expressed as the ratio of soluble to initial total protein, in percentage.

2.6. Sulfhydryl Groups

The content of free sulfhydryl groups (SH) was determined according to the method of [12] with some modifications. After centrifugation (6000 rpm for 20 min at 4 °C), 500 µL of the control or each pressurized protein solution were added to 500 µL 0.086 $mol \cdot L^{-1}$ Tris buffer (pH 8.0) and 50 µL Ellman's reagent and kept for 60 min at room temperature (~20 °C). Absorbance was measured at 412 nm using a spectrophotometer (Shimadzu UV-1280, Kyoto, Japan). The content of SH was determined by dividing the absorbance value by the molar extinction coefficient of 13,600.

2.7. Surface Hydrophobicity

Protein surface hydrophobicity (H_0) was determined using the fluorescent probe 1-anilino-8-naphthalene-sulfonate (ANS) according to the method of [13]. Control and pressurized protein solutions, after centrifuging, were diluted to 0.05–0.25 $mg \cdot mL^{-1}$ with 0.01 $mol \cdot L^{-1}$ phosphate buffer pH 7. An aliquot of 20 µL of ANS (0.008 $mol \cdot L^{-1}$ in 0.01 $mol \cdot L^{-1}$ phosphate buffer) was added to 4 mL of each protein solution and then the fluorescence intensity was measured (390 nm—excitation; 470 nm—emission) using a fluorescence spectrometer (Hitachi F2000 fluorescence spectrophotometer, Tokyo, Japan). The index of H_0 was calculated using the initial slope of fluorescence intensity vs. protein concentration ($mg \cdot mL^{-1}$) plot (calculated by linear regression analysis).

2.8. Emulsifying Properties

Emulsifying properties were evaluated for both the whole isolate protein dispersions and for the soluble protein fractions obtained after centrifugation (6000 rpm for 20 min at 4 °C). Oil-in-water emulsions were prepared by mixing 3 mL of 8 $mg \cdot mL^{-1}$ protein solution (centrifuged or not) with 1 mL of sunflower oil, and then homogenizing using a T25 Ultra-turrax homogenizer (IKA-Werke, Königswinter, Germany) at 10,000 rpm for 30 s. The emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined according to the method of [14]. An aliquot (50 µL) of the emulsion was retrieved from the bottom of the emulsion, immediately (0 min) and after 10 min and diluted (1:500, *v*/*v*) in 0.1% (*w*/*v*) SDS solution. The absorbance was measured at 500 nm using a spectrophotometer (Perkin-Elmer Instruments Lambda 35, Perkin-Elmer Instruments, Waltham, MA, USA). The indexes were calculated according to Equations (1) and (2).

$$\mathrm{EAI}\left(\mathrm{m^2 \cdot g^{-1}}\right) = \frac{2 \times 2.303 \times A_0 \times DF}{c \times \varphi \times (1-\theta)} \quad (1)$$

$$\mathrm{ESI}\ (\mathrm{min}) = \frac{A_0}{A_0 - A_{10}} \times \Delta t \quad (2)$$

where A_0 and A_{10} are the absorbance (500 nm) of the diluted emulsion at 0 and 10 min, respectively, DF is the dilution factor (500), c is the initial protein concentration ($g \cdot 100\ mL^{-1}$), φ is the optical path (0.01 m), θ is the oil volumetric fraction (0.25) and Δt is 10 min.

To help understand the emulsifying behavior of the unprocessed and HPP PP and SP samples, measurements of surface tension (air–water interface, γ) were carried out for

centrifuged 0.01 g·mL^{-1} protein solutions at pH 7. The surface tension was determined by analyzing the shape of a pendant drop using a Dataphysics contact angle system OCA-20. Drop volumes of 5 ± 1 µL were obtained using a Hamilton DS 500/GT syringe connected to a Teflon-coated needle, with a diameter of 0.52 µm, placed inside an aluminum air chamber. The temperature inside the aluminum chamber was measured with a Pt100 within ±0.1 °C, placed at approximately 2 cm to the liquid drop. The drop was formed, and the measurements were carried out after 10 min stabilization at 20 °C. The analysis of the drop shape was performed using the SCA 20 software module, with basis on the Young–Laplace equation.

2.9. Statistical Analysis

ANOVA and Tukey's honestly significant difference test were used to determine significant differences between samples with a 5% level of significance. All the performed analyses were done in duplicate, from triplicated samples, except if stated otherwise.

3. Results

3.1. Protein Composition and Solubility

Electrophoretic analysis was performed for the whole protein isolate dispersions under reducing conditions, for unprocessed samples and for those submitted to the more pronounced HPP conditions, i.e., at 400 and 600 MPa for 15 min. There were no major changes in the relative volume of the bands for the identified protein fractions, for both pea (PP) and soy proteins (SP), at the different pH values and HPP conditions analyzed (results are shown as supplementary material—Figures S1 and S2). The similar polypeptide composition observed for unprocessed and HPP-treated samples indicates that HPP did not induce any relevant changes in the relative amount and molecular weight of the main proteins and their subunits under the electrophoretic conditions analyzed.

The solubility of proteins strongly affects their techno-functional properties, including gelation and interfacial activity, and, consequently, their ability to help in the formation/stabilization of disperse systems. Non-pressurized PPI showed soluble protein values of 25.5 ± 0.6, 25.4 ± 0.3 and 28.0 ± 0.3% at pH 6, 7 and 8, respectively. In general, plant proteins show lower solubility in the pH range of 4 to 6, in the vicinity of the isoelectric point, with expected increases above and below this range.

SPI showed higher protein solubility than PPI, namely 33.6 ± 1.3, 43.8 ± 1.7 and 34.3 ± 0.3% at pH 6, 7 and 8, respectively. The decrease in solubility observed when the pH increased from 7 to 8 was somewhat surprising. Probably, the relatively high isoelectric point of the glycinin's basic subunits (8.0–8.5; [15]) contributes to the overall decrease of SPI solubility at pH 8.

The relative effects on protein solubility of the different HPP treatments within the pH 6–8 range are shown in Figure 1.

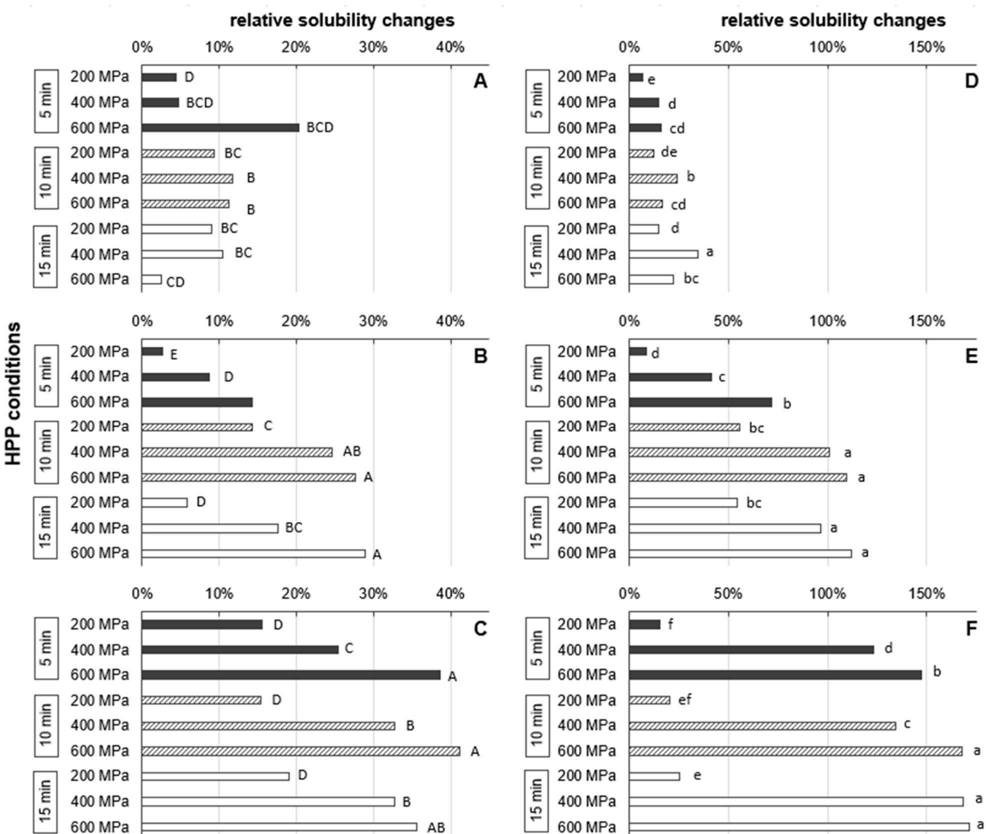

Figure 1. Relative variation of protein solubility (%) for the high-pressure processed samples, having as reference the solubility measured for non-pressurized samples. Results are shown for PPI ((**A**)—pH 6, (**B**)—pH 7, (**C**)—pH 8) and SPI ((**D**)—pH 6, (**E**)—pH 7, (**F**)—pH 8) samples. Different letters indicate significant differences ($p < 0.05$) between samples represented in each figure.

Overall, the HPP treatments lead to higher protein solubility than that observed for the unprocessed samples. The increase in protein solubility was considerably higher for SPI than for PPI samples; for SPI at pH 7 and 8, more intensive conditions (i.e., higher pressure or holding time) lead to protein solubilities above 90%. At pH 6, the increase in solubility was less pronounced than at higher pH for both protein isolates and HPP conditions. At this pH (Figure 1A,D), where we expect lower electrostatic repulsions among protein chains if compared to higher pH, increasing pressure for longer processing times leads to lower increases in solubility, probably related to pressure-induced protein aggregation.

In general, a more pronounced solubility increase was observed at pH 8 regardless of the protein isolate (Figure 1C,F). In addition, the solubility increase was more pronounced as the pressure increased, with a lower effect of increasing the processing time.

3.2. *Sulfhydryl Groups*

Figure 2 shows the effects of HPP (200–600 MPa/5–15 min) on the free SH of PPI and SPI at different pH values. For PPI, some general trends were observed among the significant effects of the HPP conditions on the content of free SH groups: (1) increasing the holding time of the HPP at 200, 400 and 600 Pa, at the studied pHs (Figure 2A–C), leads to a general decrease in free SH; and (2) the effects of increasing pressure were dependent on the holding time and pH—at pH 6, increasing pressure during 5 min treatments increased the content of free SH, reaching values even higher ($p < 0.05$) than that for the control sample. No significant changes ($p > 0.05$) comparatively to the control were observed for a holding time of 10 min, though a decrease in SHs was already observed for a longer pressurization time. At pH 7, and mainly at pH 8, the general observed trend was a decrease in the content of free SH as the pressure increased.

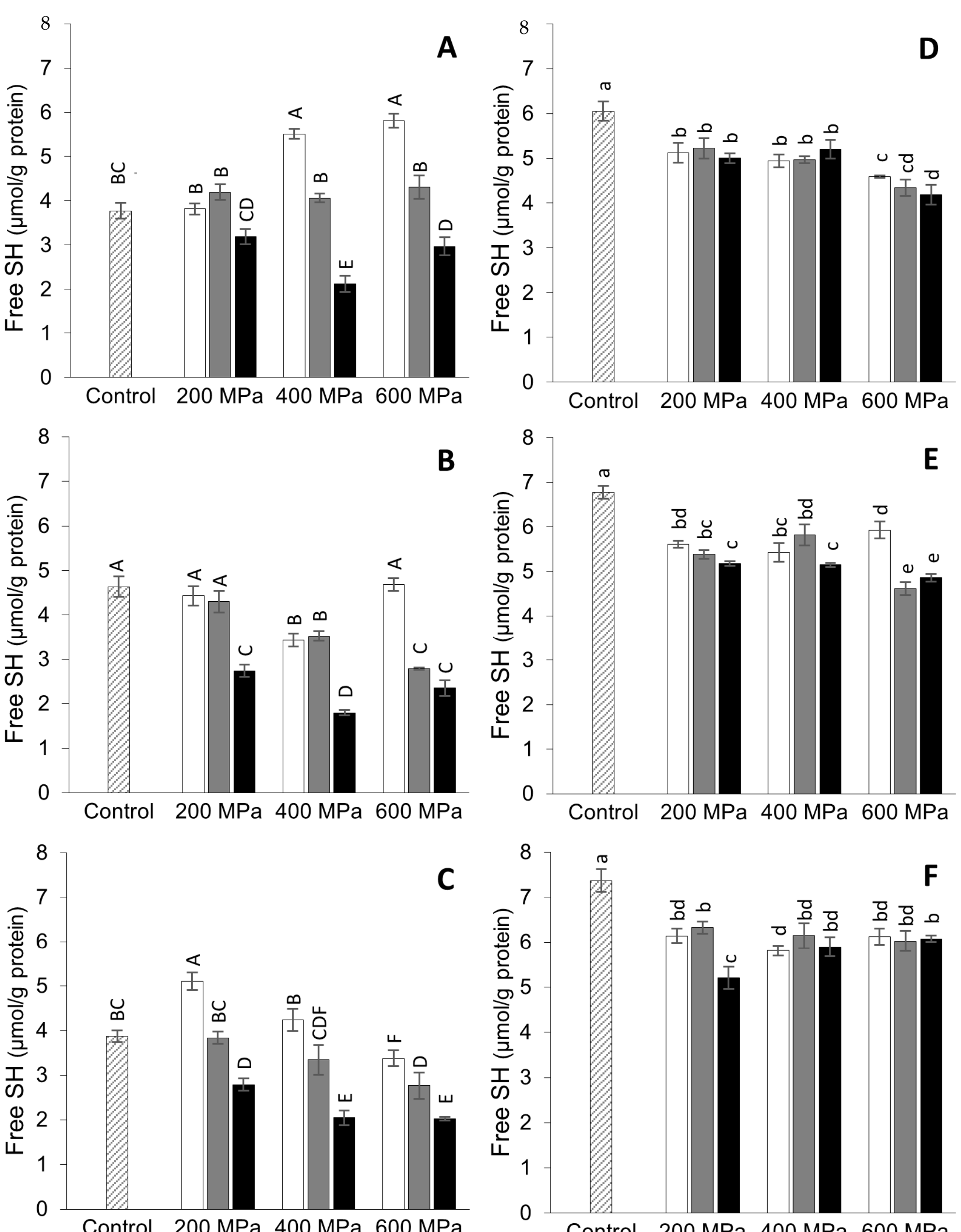

Figure 2. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 min on the content of free sulfhydryl groups of pea ((**A**)—pH 6, (**B**)—pH 7, (**C**)—pH 8) and soy ((**D**)—pH 6, (**E**)—pH 7, (**F**)—pH 8) protein isolates at different pH values. The columns with diagonal stripes represent control samples (0.1 MPa). Blank, gray and black filled columns denote 5-, 10- and 15-min processing times, respectively. Different letters indicate significant differences ($p < 0.05$) between samples represented in each figure.

Regarding SPI, overall, all processing conditions lead to a lower content of free SH groups (between 15 and 30%) than those observed for the unprocessed sample for the three pHs analyzed (Figure 2D–F). Increasing pressure or processing times either did not significantly ($p > 0.05$) affect the content of SH or decreased it. In general, pressure level and holding time had a lower effect than that observed for the PPI samples.

3.3. Surface Hydrophobicity

Figure 3 presents the relative effects of HPP (200–600 MPa for 5–15 min) at different pH values (6–8) on the surface hydrophobicity (H_0) of pea and soy solubilized proteins. For

unprocessed PP, the highest value of H_0 was found at pH 7 (H_0 = 1735), followed by pH 8 and 6. It is worth to note that a higher amount of free SH groups was also observed at pH 7, probably related to a net balance favoring more expanded protein conformations at this pH, thus exposing more hydrophobic zones and free SH groups. Unprocessed SP samples showed higher H_0 values (H_0 = 2103–2209) than for PP, but a much lower pH effect.

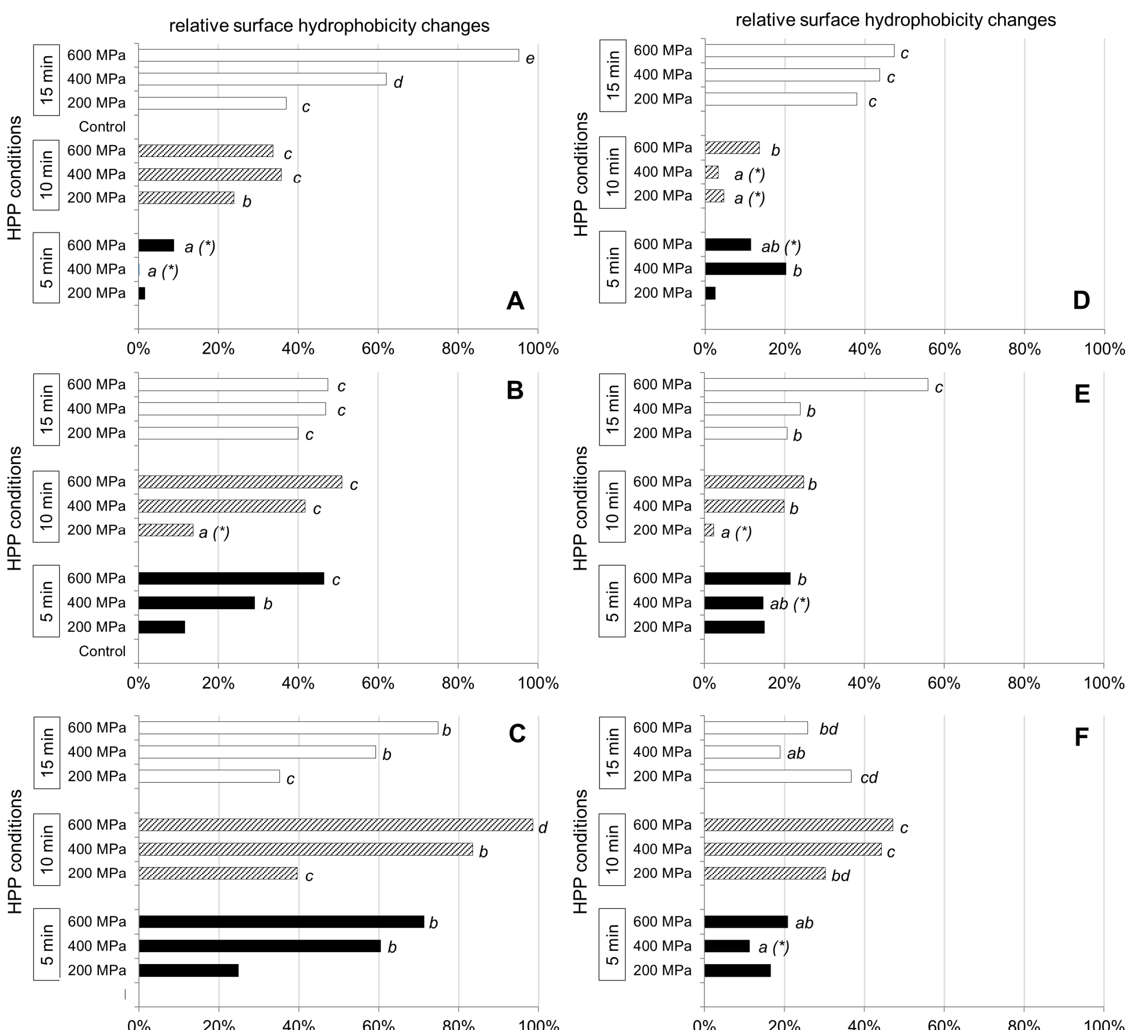

Figure 3. Relative variation (%) of protein surface hydrophobicity for the HPP-treated samples, having as reference the surface hydrophobicity measured for unprocessed samples. Results are shown for pea ((**A**)—pH 6, (**B**)—pH 7, (**C**)—pH 8) and soy ((**D**)—pH 6, (**E**)—pH 7, (**F**)—pH 8) protein samples. Different letters indicate significant hydrophobicity differences ($p < 0.05$) between samples at the same pH. Significant differences ($p < 0.05$) in relation to the unprocessed samples, at the same pH, were observed for all tested samples except those indicated by an asterisk (* $p > 0.05$).

In general, all HPP conditions tested led to an increase in proteins' surface hydrophobicity, most probably related to protein partial unfolding and exposure of hydrophobic groups previously hindered inside the protein folded structures, or, in a few cases, to non-significant changes ($p > 0.05$) in this parameter [16,17]. HPP effects were more pronounced for PP than for SP samples, similar to what has already been observed for the HPP effects on free SH groups (Section 3.2). Higher pressures and/or higher processing times seem to originate more pronounced increases in protein surface hydrophobicity. As observed for the HPP effects on free SH groups, also, in this case, the influence of pH on the magnitude of the effects of HPP on protein hydrophobicity was much more pronounced for PP (Figure 3A–C) than for SP (Figure 3D–F). It is interesting to note the effects observed for

short pressurization times (5 min): for SP there were no significant differences compared to non-pressurized samples, for pH 6–8, while, for PP there was a clear dependence on pH, with samples at pH 6 (Figure 3A) not showing significant differences ($p > 0.05$) comparatively to the control sample or among them, there was already a tendency towards an increase in H_0 at pH 7 (Figure 3B) and greater differences were observed at pH 8, with H_0 increasing significantly ($p < 0.05$) comparatively to the control sample and with increasing pressure.

3.4. Emulsifying Properties

Since HPP can alter protein solubility, conformation and hydrophobicity, it is expected to affect proteins' influence on emulsion formation and stability, and, thus, the EAI and ESI values. However, the effect of HPP on the surface tension of PP and SP solutions was minor and, in most cases, not statistically significant, either in relation to the unprocessed control sample or in relation to the different processing conditions (results not shown; see Section S2 and Figure S3 as Supplementary Data). Among all HPP conditions studied, for both the PP and SP samples and the three different pH, the more pronounced effects of HPP (and those that lead to significant differences ($p < 0.05$) in relation to the unprocessed samples) were observed mainly at pH 8, as shown in Figure 4 (the whole set of results is shown in Figures S4 and S5 as Supplementary Data).

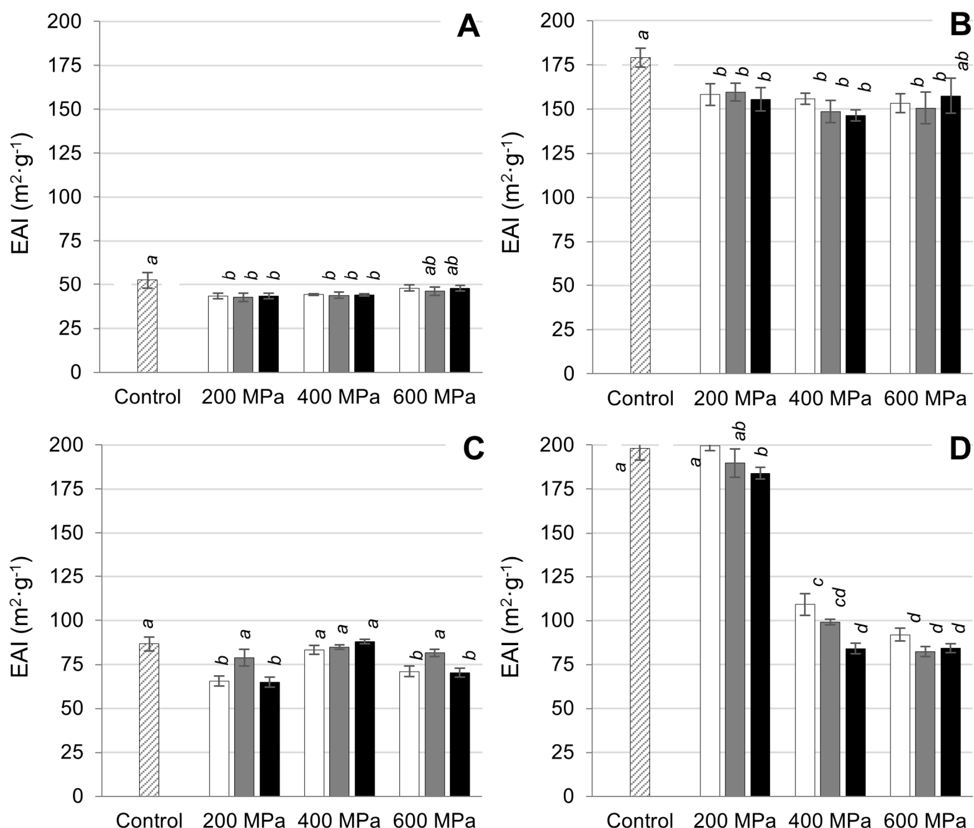

Figure 4. Emulsifying activity index (EAI) measured for O/W emulsions prepared with pea ((**A**)—total fraction, (**B**)—soluble fraction) and soy ((**C**)—total fraction, (**D**)—soluble fraction) protein dispersions (pH 8), previously subjected to different high-pressure processing treatments (200, 400 and 600 MPa for 5, 10 and 15 min). The columns with diagonal stripes represent unprocessed (0.1 MPa) control samples. Blank, gray and black filled columns denote 5-, 10- and 15-min processing times, respectively. Different letters indicate significant differences ($p < 0.05$) between samples represented in each figure.

Since the EAI is calculated in relation to the protein concentration, and considering the relatively low solubility of the commercial protein isolates, one can expect that the amount of protein in the total PPI and SPI fractions that effectively will contribute to the interfacial activity will be relatively low. Therefore, not unexpected, the soluble protein fractions showed higher EAI values (Figures 4, S4 and S5).

HPP seems to decrease the interfacial area coated by the protein [14], at least at pH 8 and for certain HPP conditions, as suggested by the reduced EAI values observed for the HPP-processed samples at this pH value. For the PP total fraction (Figure 4A), EAI was 52 ± 4 $m^2 \cdot g^{-1}$ for the unprocessed samples and decreased by approximately 16–19% with HPP, whereas, for the emulsions prepared with the PP soluble fraction (Figure 4B), EAI was 179 ± 5 $m^2 \cdot g^{-1}$ for the unprocessed samples, also decreasing by a similar order with HPP treatments. In both cases, there were negligible effects of pressure level or pressurizing times. There was also a reduced effect of HPP on EAI of emulsions prepared with the total SP fraction (Figure 4C), with a reduction of EAI up to 25% for certain HPP conditions (200 and 600 MPa/5 and 15 min). Emulsions prepared with HPP-treated SP soluble fraction showed a considerable reduction of EAI higher pressures (Figure 4D).

Considering PP dispersions, significant effects ($p < 0.05$) on ESI were only observed at pH 7, for certain HPP conditions, as shown in Figure 5. For greater clarity, only the results obtained for 15 min of processing by HPP are shown, although, qualitatively, the effect observed for other times was similar. In this case, there was a general tendency for ESI to increase with the HPP treatments, especially for the soluble protein fraction.

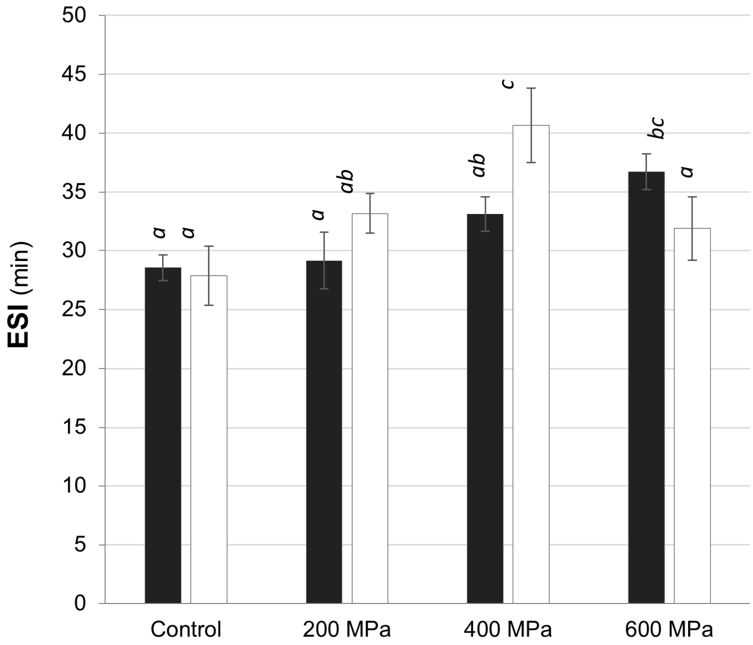

Figure 5. Emulsifying stability index (ESI) measured for O/W emulsions prepared with pea (total fraction—filled columns; soluble fraction—blank columns) protein dispersions (pH 7) previously subjected to different HPP treatments (200, 400 and 600 MPa for 15 min). Different letters indicate significant differences ($p < 0.05$) between samples; vertical bars denote the standard deviation from three replicates.

For SP dispersions, a different behavior was observed for the total and soluble protein samples. Regarding the total SP, the HPP treatment resulted in a significant decline in ESI at pH 6 ($p < 0.05$) (Figure 6A), while significant increases, though less pronounced, were observed at pH 8 for certain HPP conditions, and an intermediate behavior at pH 7. Regarding the soluble SP, significant effects ($p < 0.05$) on ESI were observed at pH 6, although only for the shortest pressurization time tested (5 min) (Figure 6B) at pH 8, in this

case, mainly for longer pressurization times (10 and 15 min) (Figure 6D). In both cases, the noticed effect was an increase in ESI with pressure treatment.

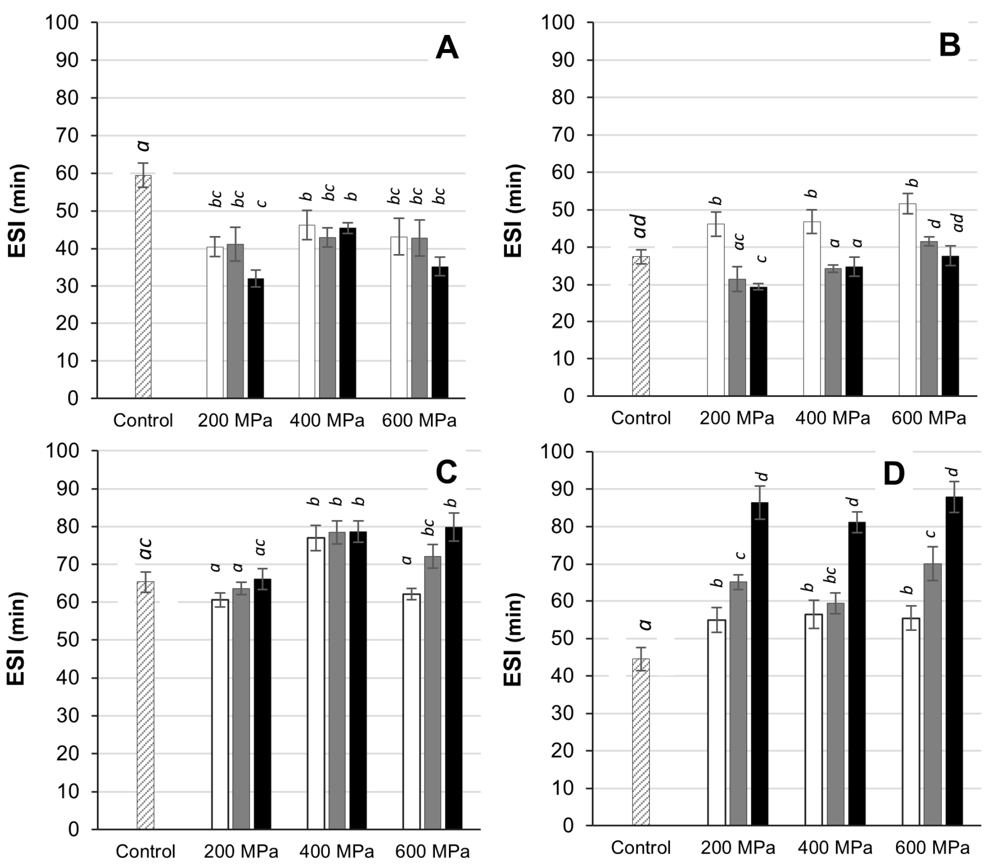

Figure 6. Emulsifying stability index (ESI) measured for O/W emulsions prepared with soy protein dispersions previously subjected to different high-pressure processing treatments (200, 400 and 600 MPa). The columns with diagonal stripes represent control samples (0.1 MPa); blank, gray and black filled columns denote 5-, 10- and 15-min processing times, respectively. Selected data shown correspond to (**A**)—total soy protein at pH 6; (**B**)—soluble soy protein at pH 6; (**C**)—total soy protein at pH 8; and (**D**)—soluble soy protein at pH 8. Different letters indicate significant differences ($p < 0.05$) between samples represented in each figure; vertical bars denote the standard deviation from three replicates.

4. Discussion

4.1. Protein Composition and Solubility

Eight bands were identified for the PPI samples (Figure S2A,B), being most likely legumin's acid (α = ~39 kDa) and basic (β = ~20 kDa) subunits, the three vicilin's subunits (~46 kDa; ~32 kDa; ~29 kDa), convicilin's globular fraction (~68 kDa), lipoxygenase (LOX = ~93 kDa) and albumin's major subunit (PA2 = ~23 kDa) [18]. For SPI samples, eleven bands were identified (Figure S2C,D) and attributed to β-conglycinin subunits (α′ = ~74 kDa; α = ~70 kDa; β = ~52 kDa), glycinin's acid ($A_{1–4}$ = ~31–45 kDa) and basic (B = ~20 kDa) subunits, glycinin's acid-basic subunits pair (AB = ~62 kDa), lipoxygenase (LOX = ~93 kDa) and agglutinin (~28 kDa) [19].

The observed solubility increases for PPI at pH 8 can be attributed to electrostatic repulsion brought on by negative net charges on the protein surface, as it was also reported for other commercial pea protein isolates [20,21]. A decrease in the solubility of SPI with the increase of the pH, from 6.5 to 7.5, particularly for the glycinin fraction, was previously reported [22].

Overall, both protein isolates showed a relatively low protein solubility, what seems to be a general characteristic of commercial protein isolates, related to a high degree of protein denaturation and the presence of varying amounts of soluble and insoluble proteins (aggregates) [16,23].

The observed increase in protein solubility with pressure is probably related to protein unfolding and dissociation of preformed aggregates [16,24], an effect that will be even more noticeable due to more significant electrostatic repulsions occurring at higher pHs. The observed trend of increased solubility can be attributed to the increase in interactions between proteins and solvent, which may be caused by the dissociation of aggregates and the unfolding of proteins promoted by pressure [16,24].

4.2. Sulfhydryl Groups and Surface Hydrophobicity

Although likely dependent on a complex set of factors, including pH and intrinsic factors related to the structure of the studied proteins, the increase in the content of free SH groups is probably related to the dissociation/unfolding of the proteins exposing SH groups, while those conditions that led to a decrease in SH content, which predominated, are probably related to the promotion of new intermolecular interactions and partial aggregation of proteins, or even to the occurrence of sulfhydryl/disulphide interchange and/or formation of new disulfide bonds [16,25,26], masking these groups.

Previous studies on vegetable proteins (including soybean, pea, red kidney bean, lentil and faba) have shown similar results to those here reported, i.e., an increase in protein surface hydrophobicity with HPP [16,17,27,28], while others reported an opposite effect [29], which, again, reveals the complexity of the factors involved in conformational changes caused by HPP and the expected complex balance between protein unfolding and aggregation under different pressure conditions and pH values.

4.3. Emulsifying Properties

The general trend observed in previous studies regarding the effects of HPP on emulsifying properties of plant-based proteins was the increase of EAI with pressure [8,30]. These effects were explained mainly by protein unfolding caused by the HPP and consequent exposure of hydrophobic groups, thus improving emulsifying properties [31], although, the contrary was also already reported [28]. It is expected that a complex set of factors interacting with each other influence the interfacial activity of these proteins, including protein charge, solubility, surface hydrophobicity and the overall distribution of hydrophilic and hydrophobic regions on the protein chains. In fact, and contrarily to what was previously reported [32], in the present study, no significant correlations were observed between the changes of H_0 and EAI with pressure, both in the total and soluble fractions at the different pHs. However, in general, for both proteins under the three pHs analyzed, a strong negative correlation was observed between the effect of HPP on solubility and EAI: for example, under pH 7, r = −0.702 ($p < 0.001$) for PPI and r = −0.953 ($p < 0.001$) for SPI. For these samples and under the conditions analyzed, a possible positive effect of HPP on protein solubility and in obtaining more open and extended protein conformations, which, at first, would be advantageous to obtain an increase in the emulsifying capacity of these proteins, may be compromised by an increase in intermolecular protein interactions, even though mostly forming soluble aggregates that will have lower diffusion rates and molecular flexibility, thus compromising their interfacial activity and emulsifying activity.

Regarding the effects of HPP on the proteins' ability to stabilize emulsions, the treatment by HPP has been shown to have varied and complex effects, not only dependent on HPP conditions but also on the pH, making difficult a reasoned interpretation of these effects. However, also in this case, for many conditions of HPP and for certain pH values, the observed effects were not statistically significant (the whole set of results is shown in Figures S6 and S7 as Supplementary Data).

In general, the effect of HPP on the surface tension of PP and SP solutions was small and, in most cases, not statistically significant, either in relation to the control sample or in

relation to the different processing conditions. For PP, the most significant influence of HPP that is worth highlighting is the decrease in surface tension with increasing pressure for samples processed for a reduced holding time (5 min, $p < 0.05$) (Figure S3A). The decrease of γ with pressure was also observed for 15 min holding time, but, apparently, higher values of γ were obtained by increasing the holding time. For soy proteins (Figure S3B), the treatment by HPP seems to originate a more pronounced increase of γ in relation to the control. For these samples, it is noteworthy to mention the significant decrease ($p < 0.05$) of γ with the increase of the holding time for those samples treated at 600 MPa.

Thus, the influence of HPP upon the ability of the proteins to decrease the air-water surface tension is dependent on the processing conditions (pressure and holding time), but also on the type of protein, which can help to better understand the diversity of results regarding the emulsifying properties of the pea and soy proteins, as affected by HPP.

Previous studies have already shown different effects of HPP on ESI for emulsions prepared with different vegetable proteins, with both increases and decreases in the stability of emulsions being reported [16,33,34]. Since pressure affects different proteins in different ways and affects the exposure of hydrophobic groups and/or changes in the molecular flexibility [6], it can either increase or decrease the interactions between the proteins within the interfacial viscoelastic film on the oil droplets, altering the ESI in different ways. It is worth to note that relatively small effects of HPP on the surface tension of PP and SP solutions were observed, but, even so, without any well-defined tendency for an increase or decrease, this also demonstrates a complex dependence of the surface tension of these protein solutions on HPP treatments (Figure S3, available as Supplementary Material). The results obtained here suggest that certain pressure/time combinations may alter protein conformation in a way that increases the availability of hydrophobic groups, ergo decreasing the free energy at the air–water interface. On the other hand, the possible changes in the molecular organization of proteins due to HPP may also promote greater interaction between proteins and formation of aggregates that will hinder the migration of the protein to the interface and subsequent adsorption, resulting in higher values of surface tension, compared to non-pressurized samples, as was observed for soy proteins.

5. Conclusions

HPP can lead to substantial changes in the structure of pea and soy proteins that may improve some of their techno-functional properties. Therefore, HPP increases, for instance, the solubility and surface hydrophobicity of protein isolates from both pea and soy, although the consequence on interfacial and emulsifying protein properties was dependent on the selected processing conditions, and often with a compromised positive effect due to further protein aggregation. In addition, similar HPP conditions can also lead to a decrease in free SH groups, which would point to protein folding and establishment of intermolecular interactions, corroborating again the complexity of the HPP effects on proteins' conformation and their propensity to interact. The effects of a different pH during the HPP treatment were previously studied, but mainly comparing the behavior above and below the proteins' isoelectric point. Here, we clearly showed that, even within a relatively short pH range, above p*I*, the degree of protein ionization does have a significant influence on the conformational changes undergone by proteins under pressure. In particular, the application of HPP to both PPI and SPI revealed an increase in the solubility of proteins in both isolates. Specifically, the solubility of SPI was significantly more pronounced at pH 7 and 8 and pressure above 400 MPa, with almost three times the percentage of soluble proteins in comparison to without HPP. Furthermore, the surface hydrophobicity of proteins from both sources increased with HPP, with the increase correlating to increasing pressure and holding time. Unlike the hydrophobicity, the content of free sulfhydryl groups in proteins from both sources decreased with HPP, particularly for PPI at pressures above 400 MPa, suggesting a balance between protein unfolding and further aggregation under certain conditions. Finally, the emulsifying properties of the protein isolates were found to be dependent on pH, pressure, holding time and the fraction of the protein isolates used.

Overall, findings from this study reveal that HHP can be effectively used to improve and tailor pea or soy proteins to be used as technological ingredients in food formulations, however, additional studies are needed to fully understand, from a fundamental point of view, the digestibility issues related to the consumption of HPP-treated proteins and their effects on the human body.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13042359/s1, Figure S1. SDS-PAGE analysis of high pressure-treated (A) pea and (B) soy protein isolates at 0.1, 400 and 600 MPa for 15 min at pH 6 (lanes 2–4), pH 7 (lanes 5–7) and pH 8 (lanes 8–10). Lane 1—protein molecular weight markers. To the right are the most likely protein fractions based on the standards' molecular masses—LOX denotes for lipoxygenase, and PA2 for the major component of the albumin fraction; in (B) α', α and β denote for the β-conglycinin subunits, A1, A2, A3, and A4 for the glycinin's acid subunits, B for the glycinin's basic subunit, and AB for acid-basic subunits pair. Figure S2. Relative amount of the main proteins and their subunits obtained from the SDS-PAGE electrophoretic analysis for the PPI (A,B) and SPI (C,D) samples. Results are shown for two different pH (pH 6, A and C; pH 8, B and D). Black filled columns denote for non-pressurized samples, dashed columns for samples treated at 400 MPa, 15 min, and blank filled columns denote for samples treated at 600 MPa for 15 min. In A and B, LOX denotes for lipoxygenase, and PA2 for the major component of the albumin fraction; in C and D, α', α and β denote for the β-conglycinin subunits, A1, A2, A3, and A4 for the glycinin's acid subunits, B for the glycinin's basic subunit, and AB for acid-basic subunits pair. Figure S3. Effects of high-pressure processing on the surface tension of (A) pea and (B) soy proteins (soluble fractions at 0.01 $g \cdot mL^{-1}$, pH 7), for different pressure and holding time values. The non-pressurised control sample is indicated by the blank column. Different letters indicate significant differences ($p < 0.05$) among the different samples. Figure S4. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 min on the emulsifying activity index (EAI) of the total fraction of pea and soy protein isolates at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal stripes represent pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein samples. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein samples. Figure S5. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 min on the emulsifying activity index (EAI) of the soluble fraction of pea and soy protein at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal stripes represent pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein samples. Different lowercase letters indicate significant differences ($p < 0.05$) between soy samples. Figure S6. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 min on the emulsifying stability index of the total fraction of pea and soy protein isolates at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal stripes represent pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein isolates. Figure S7. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 min on the emulsifying stability index of the soluble fraction of pea and soy protein at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal strips represent pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein isolates.

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