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High pressure homogenization to boost the technological functionality of native pea proteins

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ABSTRACT

Pea proteins are being increasingly used for the formulation of plant-based products, but their globular structure and the presence of aggregates can affect their technological properties. In this study, the effect of high pressure homogenization (HPH) at different intensities (60 and 100 MPa) was investigated as a pre-treatment to modulate the techno-functional properties of a pea protein isolate (IP) extracted through an alkaline extraction/isoelectric precipitation process. SDS-PAGE, circular dichroism, thermal properties, total free sulfhydryl groups, antioxidant capacity and reducing properties were evaluated along with technological indices as solubility, WHC and OHC, interfacial tension and emulsifying capacity. HPH treatments were able to unfold and modify proteins structure, leading also to a change of the relative abundance of pea protein globulins (SDS-PAGE) and of the vicilin to legumin ratio. Solubility, WHC and OHC were improved, while interfacial tension and emulsifying capacity were weakly affected. However, an enhanced physical stability over time of the emulsions prepared with the 60 MPa-treated protein was found, likely as an effect of the decreased ratio between vicilin and legumin after treatment.

Results of this study will contribute to deepen the effect of the HPH technology used as pre-treatment, adding useful results and expanding knowledge about the structure and techno-functional properties of native and modified pea proteins.

1. Introduction

In recent years pea proteins have found a growing interest in the food industry as alternative protein source and this is driving an increasing research work on their characterization. They are extracted from the seeds of *Pisum sativum* L., a plant that grows in moderate climates, and it is worldwide cultivated at relatively low costs (Lu et al., 2020); they have high nutritional values and in particular a high lysine content (Burger and Zhang, 2019; Tömösközi et al., 2001). Pea protein structure is mostly globular, as the main storage proteins in the seed are globulins, namely legumin (a hexamer), vicilin (a trimer) and convicilin (a trimer with high homology with vicilin), strictly linked together (Barać et al., 2015). Thanks to some of these characteristics, pea proteins have been increasingly used in the food industry for the formulation of plant-based, vegan and *meat-analogues* products. However, due to their compact globular structure, pea proteins are unable to express to their best their technological properties (emulsifying, foaming, stabilizing and/or gelling) (Lam et al., 2018; McClements, 2004). To overcome such issues, in the formulation of innovative plant-based products based on pea proteins it is quite common to add many other ingredients to guarantee, depending on the type of product, the expected softness, juiciness, stability and so on. Hence, various technologies, based on physical, chemical or enzymatic modification, have spread to modify plant proteins structure and modulate their technological properties.

High pressure homogenization (HPH) is an innovative mild technology that is primarily used to stabilize multiphasic products, to homogenize and emulsify immiscible phases and also to decrease the microbial load (Levy et al., 2021). During the process, the fluid is forced to pass through a narrow gap in the homogenizer valve, where it is submitted to a rapid acceleration. Consequently, cavitation, shear stress, and turbulence simultaneously occur allowing the induction of phenomena like mechanical disruption and membrane alteration.

Recently, other interesting applications of this technology have also spread in which HPH has been successfully applied for the encapsulation

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of bioactive molecules for delivery purposes, for the extraction of added value compounds (polyphenols, proteins and pigments) from industrial by-products and for the structural modification of food biopolymers like starch and proteins (Fernandez-Avila et al., 2019; Jurić et al., 2019; Nikbakht Nasrabadi et al., 2021). Regarding this last application, HPH is indeed increasingly being used as a pre-treatment for the structural modification of different plant proteins with the aim of tailoring targeted technological proteins. In pea proteins, HPH was shown to have the ability to increase solubility and oil holding capacity (Melchior et al., 2022), while on lentil proteins, HPH improved the solubility, foaming and emulsifying capacity when applied at pressures up to 100 MPa (Saricaoglu, 2020). Also, faba bean proteins improved their solubility and interfacial properties upon HPH treatment, but the emulsifying capacity was not affected (Yang et al., 2018).

Commercial plant protein isolates can however be obtained by applying rather different extraction procedures and technologies, leading to finished products with uneven technological properties depending on the variability in the composition and in the native/denatured state of the proteins (D'Alessio et al., 2022). Therefore, the effects a preliminary treatment can achieve may be strictly related to the structural properties of the raw/untreated protein before modification (Corredig et al., 2020; Geerts et al., 2017; Stone et al., 2015).

The aim of the present work was thus to investigate the effect of HPH treatment carried out at two different intensities on the technological and functional properties of native pea proteins, including solubility, WHC, OHC, surface properties, emulsifying capacity and antioxidant activity. To deepen the structure-functionality relationship, the effect of HPH treatments on the pea proteins structural properties was also studied by means of SDS-PAGE, circular dichroism, differential scanning calorimetry and total free sulfhydryl group quantification.

2. Materials and methods

2.1. Materials

Commercial pea protein isolate (CP) was donated by VICTA Food SRL (Mogliano Veneto, Italy) on behalf of Cosucra (Warcoing, Belgium) and obtained through different steps such as alkaline extraction, decantation, pasteurization, purification and spray-drying, as reported in the technical data sheet. The mean composition is 86% of protein, 2.4% of fibers and 0.8% of carbohydrates; the technological functionality was investigated and previously reported (D'Alessio et al., 2022).

A pea protein isolate (IP) was obtained from frozen raw peas following the method described by Kornet et al. (2020), based on isoelectric precipitation, with slight modifications; frozen peas were mixed, dried at 70 °C for ca 6 h, grinded and sieved (100 μm). The flour was mixed in water at pH 8 for 2 h in a 1:10 ratio and centrifuged (6000 rpm for 15 min); the supernatant was collected, adjusted at pH 4, mixed for 1 h and centrifuged under the same conditions as before. The obtained pellet was recovered, re-dispersed in water at pH 7 and mixed for other 2 h. At last, this suspension was freeze dried and collected for use. The protein content of the freeze-dried protein, as determined by Kjeldahl nitrogen method (N x 5.7), was comparable to Kornet et al. (2020), and was of 87% ± 2%. Frozen peas and sunflower oil used for emulsions were purchased in a local supermarket (Teramo, Italy). All other reagents and chemicals were of analytical grade.

2.1.1. Preparation of solutions and HPH pre-treatments

The IP solutions (1.0% w/v) were prepared in a Phosphate Buffer Solution (PBS 50 mM, pH 6.55), stirred for 2 h and then pretreated by means of a high-pressure homogenizer (Panda Plus 2000; GEA Niro Soavi, Parma, Italy), at two different pressure intensities (60 MPa -IP60 and 100 MPa -IP100) for 5 cycles. The homogenizer was equipped with a heat exchanger to control suspensions temperature and keep it lower than 30 °C during all the cycles of homogenization.

The energy density, generally described as the energy input per unit volume (E_v, MJ/m^3) and that transferred from the valve of the homogenizer to the protein suspensions, was evaluated for each treatment following the equation proposed by Stang et al. (2001):

$$E_v = \Delta P$$

where ΔP is the pressure difference that operates at the nozzle. In a treatment with multiple passes under high pressures as in this study, the energy density is considered as the ΔP multiplied by the numbers of cycles.

2.2. Methods

2.2.1. Circular dichroism

The circular dichroism spectra were recorded by a Jasco J-810 Spectropolarimeter (Jasco Corporation, Tokyo, Japan) at 25 °C, using a 5 mm quartz cell in the spectral range of 190–300 nm, and at 1 mg/mL of protein concentration. Analyses were carried out on the pea protein obtained from the purification process and on treated pea protein isolates. The secondary structure components of the samples were obtained by the elaboration of the spectra by means of CDNN software.

2.2.2. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method described by Battista et al. (2013) with slight modifications by using the Mini PROTEAN ® Tetra Cell system (Biorad). The concentrations of resolving and stacking gels were 12.5% and 5%, respectively. The amount of protein was determined by Bradford assay using Bovine Serum Albumin (1 μg/μL, Sigma-Aldrich) as protein standard. Then, equivalent amounts of protein (25 μg) were loaded and each gel was run at 90 V for 15 min and 120 V for 1 h and 15 min under reducing and non-reducing conditions. Samples run on reducing conditions were treated with 2-β-mercaptoethanol (1%) and heated at 70 °C for 10 min. Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (Biorad) were run with samples for molecular weight estimation (10–250 kDa). The gels were stained with Coomassie Blue G-250 and scanned by Azure c400 Gel Imaging System (Azure Biosystems).

Image analysis of gels was carried out using the ImageJ software, in order to estimate the relative abundance of legumin, vicilin and convicilin in IP and treated samples (IP60 and IP100). Relative abundance indicates the presence of a certain protein residue after the treatment compared to its initial amount and was calculated as the band intensity of a specific protein residue divided by the total sample band intensity of the electrophoretic gel under investigation; then, the band intensity of each residue of the treated samples was normalized with respect to their abundance in the control sample (IP), in order to evaluate the effect of the treatment when compared to the control. Consequently, the vicilin/legumin ratio was calculated.

2.2.3. Total free sulfhydryl groups

The determination of total free sulfhydryl group was carried out following the method described by Peng et al. (2016) by using Ellman's reagent (50, 5-dithiobis (2-nitrobenzoic acid)); 2.5 mL of sodium phosphate buffer (10 mM, pH 7) added with 6 M urea was used to dilute the samples at a concentration of 2 mg/mL; then 80 μL of Ellman's reagent was added and the samples were stored for 30 min in the dark before reading the absorbance at 412 nm. Phosphate buffer added with 6 M urea and 80 μL of Ellman's reagent was used as blank solution. The following equation was used to calculate the total free sulfhydryl groups:

$$\mu M\ SH\ /\ g\ protein = [(A_{412}\text{-}A_{412\ B})\ /\ \varepsilon C]\ x\ 100000$$

where A_{412} is the absorbance of the sample, $A_{412\ B}$ is the blank absorbance, ϵ is the extinction coefficient (chosen as 136000 M^{-1} cm^{-1} from the works by Peng et al., 2016; Shi et al., 2020) and C is the concentration of the sample.

2.2.4. Thermal properties

Thermal properties, in particular denaturation temperature, were investigated using a DSC (Pyris 8500, PerkinElmer, Shelton, USA); 5 μL of 10% (w/v) IP, IP60 and IP100 solutions, dispersed in PBS (50 mM, pH 6.55), were placed in a 50 μL closed pan while an empty stainless-steel pan was used as reference. Samples were heated from 10 °C to 105 °C at 10 °C/min and nitrogen was used as carrier gas.

2.2.5. Antioxidant activity and reducing properties

The antioxidant activity and reducing properties of the protein samples were determined by means of the ABTS assay, and the Folin-Ciocalteu method, respectively. The antiradical activity of the samples was determined according to the radical scavenging method as described by Re et al. (1999), with some modifications. The 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+•) radical stock solution was first diluted to obtain an absorbance of 0.70 ± 0.02 at 734 nm ($Abs_{initial}$), then, 15 μL of sample, or dilution of it, were mixed with 1.485 mL of the ABTS+• solution and the antiradical activity was read after 7 min (Abs_{final}) with a Lambda Bio 20 spectrophotometer (Perkin-Elmer, Waltham, MA, USA). For each dilution the percentage of inhibition was calculated by the following equation and plotted against sample's concentration:

$$[(Abs_{initial} - Abs_{final})/ Abs_{final}] \times 100 \quad (1)$$

TEAC (Trolox Equivalent Antioxidant Capacity) was calculated as the ratio of the linear regression coefficient of samples and that of Trolox, used as standard. Results were expressed as μmol TE on dry mass of sample (μmol TE/g).

The reducing properties (RP) of pea proteins were evaluated by using the Folin-Ciocalteu reagent, following the method described by Georgé et al. (2005) with slight modifications. Each sample (120 μL) was added to 600 μL of Folin-Ciocalteu reagent (Sigma-Aldrich, Darmstadt, Germany) diluted 1:10 and then incubated for 2 min in the dark before adding 960 μL of a 7.5% (w/v) Na_2CO_3 solution. The reaction mix was then kept in a water bath at 50 °C for 5 min and total phenolic content was determined by reading the absorbance at 765 nm. Gallic acid solutions were used as standard for the calibration curve and results were expressed as mg of gallic acid equivalents/g of dry weight of sample (mg GAE/g).

2.2.6. Solubility

Solubility was evaluated by using the method described by Kornet et al. (2020), with slight modifications: 1.0% (w/v) of protein samples (IP, IP60, IP100) were dissolved in deionized water; the pH was adjusted to get values of 2-4-7-10 with NaOH and HCl 1 M and let to stir for 1 h. Samples were centrifuged at 9000 rpm for 30 min, the supernatants were collected and freeze-dried. The solubility percentage was obtained by the ratio between the initial dry weight and the supernatant residue dry weight.

2.2.7. Water and oil holding capacity

Water and oil holding capacity were assessed following the method described by Fuentes-Alventosa et al. (2009), with slight modifications. Pea proteins samples (IP, IP60, IP100) were solubilized in PBS (50 mM, pH 6.55) to reach the concentration of 10.0 g/L and stirred at room temperature for 1 h. After standing at ambient temperature for 30 min, solutions were centrifuged at 5000 rpm for 20 min. The supernatant was thrown away and the tubes with the sediment were weighed, frozen at −40 °C and then freeze-dried at −10 °C for 24 h. Water holding capacity (WHC) was expressed as g of water retained per g of sample. Oil holding capacity (OHC) was determined under the same condition of WHC by using sunflower oil instead of water; after the centrifuge, the oil was allowed to drain until complete removal. The OHC was expressed as g of oil retained per g of sample.

2.2.8. Interfacial tension

Interfacial tension between the protein sample solutions IP, IP60 and IP100 at increasing concentrations (0.001% - 0.005% - 0.01% w/v) and sunflower oil, was measured after 30 min of equilibration time at 20 °C with an Attension Sigma 700/701 tensiometer (Biolin Scientific Oy, Espoo, Finland), equipped with a Du Noüy platinum ring (diameter: 120.39 mm).

2.2.9. Emulsifying capacity

Oil-in-water (O/W) emulsions were prepared by dispersing different concentration of IP, IP60, and IP100 (concentration range: 0.05% - 1.0% w/v) in PBS (50 mM, pH 6.5); 10% of sunflower oil was used as lipid phase. Solutions were pre-homogenized with a rotor-stator device (YellowLine DI 25 Basic, IKA Werke GmbH & Co, Germany) at 13500 rpm for 1 min and then emulsified at 15 MPa, for 10 cycles using high-pressure homogenizer (Panda Plus 2000; GEA Niro Soavi, Parma, Italy). Emulsifying capacity was evaluated by measuring particle size and distribution of oil-in-water model emulsions using a laser diffraction particle size analyzer (Mastersizer 3000; Malvern, Worcestershire, UK). 0.001 and 1.474 were chosen as the absorption and refractive index for sunflower oil, respectively. The stability of the emulsions was also evaluated after 7 days of storage at 4 °C.

2.3. Statistical analysis

All the experiments were carried out in triplicate and a one-way analysis of variance (ANOVA) and Tukey's test were used to establish the significance of differences among the mean values at the 0.05 significance level; principal component analysis (PCA), data analysis and modelling were carried out by using OriginPro 2016 software (OriginLab Corporation, Northampton, MA, USA).

3. Results and dicussions

3.1. Structural and molecular characterization as affected by HPH

During a first step of the work, preliminary analyses were carried out using a commercial pea protein isolate (CP); upon investigation, several critical issues in terms of technological functionality were observed, especially when the pea proteins were submitted to the HPH treatment, showing a behavior which is typical of denatured proteins. To get more insight on the structural properties of CP, circular dichroism was then used; the spectrum (Fig. 1) was quite flat and did not show the profile expected from a native protein, in which peaks at specific wavelengths

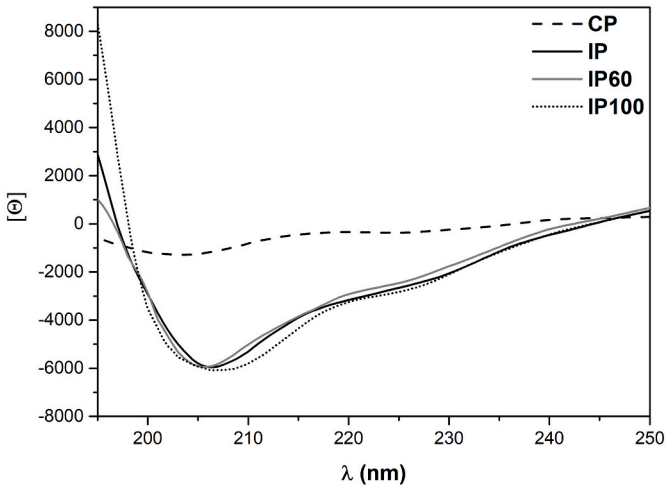

Fig. 1. Circular dichroism spectra of commercial, native and modified pea proteins.

(range between 195 and 230 nm) give information about the secondary structure (Hou et al., 2017; Zhang et al., 2022). In fact, it is known that commercial pea protein isolates can be in an unfolded state due to aggressive conditions adopted during the extraction procedures and purification technologies, which did not allow the preservation of the native state (Tanger et al., 2020). For this reason, the experimentation was carried out with a lab-extracted pea protein isolate from frozen peas by using a mild extraction protocol able to ensure the preservation of proteins secondary structure (Kornet et al., 2020). However, for comparison, some results on CP were still included in the results section.

Fig. 1 reports also the spectra of IP, IP60 and IP100 samples that, contrarily to CP, showed a typical "boat profile", corresponding to proteins with an intact secondary structure. Compared to IP, an increase in the intensity of the peak at 198 nm, which is typically associated with α-helix conformation, was highlighted for the protein treated at 100 MPa (IP100), while for IP60 sample there was a decrease in the intensity of the negative peak at about 216 nm, corresponding to a decrease in the β-sheet conformation. These results demonstrates that HPH pre-treatments modified the protein secondary structure. To provide a quantitative evaluation of the impact induced by the pre-treatments, the percentage of β-sheet and α-helix of the native and modified proteins were calculated together with those of the commercial protein for comparison (Table 1). The random coils in CP resulted high, confirming the occurrence of a very disordered and therefore denatured structure (Kelly et al., 2005). IP also had a quite high percentage of random coils, albeit lower than CP, but associated to a higher percentage of α-helices: this could be due to the fact that the dehydration process may have led to a partial modification and/or rearrangement of the protein structure, which, however, did not lead to its denaturation, as can also be seen from its spectrum. Moreover, IP showed a higher amount of α-helices than that found by Zhi et al. (2022) (19.36 ± 0.01% for IP vs. 7.9 ± 0.4% for PPI), and of antiparallel β-sheets compared to the values reported by Shevkani et al. (2015), while the values of general β-sheets (parallel and antiparallel), β-turn and random coils appear to be similar to those identified by Zhu et al. (2021). A high degree of variability of the data related to the secondary structure of plant proteins is frequently experienced in literature and is usually associated to factors like cultivar and/or extraction method (Shevkani et al., 2019); however, a greater proportion of β-conformations than α-ones is generally reported (Shevkani et al., 2015), as also confirmed by the results obtained in this study.

Both HPH pre-treatments led to a significant increase in α-helix conformations and a significant decrease in the quantity of parallel β-sheets and disordered structures (random coils). This trend was also highlighted on rapeseed proteins treated at 40 MPa with dynamic high pressure microfluidization (Zhang et al., 2022), as well as on pea proteins heat-treated at pH 12 (Zhi et al., 2022). The pressure developed in the homogenization valve, as well as the phenomena of cavitation, turbulence and shear stress, can influence both covalent and non-covalent bonds, leading to depolymerization and rearrangement of the secondary structure, transforming β-sheets and random coils into α-helices (Fan et al., 2020; Zhi et al., 2022). According to literature, β-sheets are ordered structures in which many hydrogen bonds between the sheets stabilize the protein structure, making their digestion more difficult (Shevkani et al., 2019; Withana-Gamage et al., 2011); moreover, a negative correlation between the amount of β-sheets present in different protein sources and their digestibility was reported, while the higher the proportion of α-helices, the higher the *in vitro* digestibility and solubility (Bai et al., 2015). The results obtained in the present work can be considered promising for the formulation of new food products, as the application of HPH on pea proteins may improve their digestibility and, in turn, also enhance their nutritional value.

Table 1
Effect of HPH pre-treatment on the pea protein secondary structure contents. Values in the same columns with different letters showed significant differences ($p < 0.05$).

	α-helix (%)	β-sheets antiparallel (%)	β-sheets parallel (%)	β-turn (%)	Random coils (%)
CP	10.94 ± 0.07^{d}	13.86 ± 0.03^{a}	14.79 ± 0.04^{c}	17.63 ± 0.03^{a}	42.79 ± 0.02^{c}
IP	19.36 ± 0.01^{c}	14.35 ± 0.05^{a}	11.62 ± 0.01^{a}	17.96 ± 0.01^{a}	36.64 ± 0.03^{a}
IP60	34.18 ± 0.04^{b}	11.20 ± 0.05^{a}	8.26 ± 0.01^{b}	18.11 ± 0.01^{a}	28.28 ± 0.02^{b}
IP100	41.33 ± 0.04^{a}	8.55 ± 0.02^{b}	7.13 ± 0.01^{b}	17.50 ± 0.01^{a}	25.46 ± 0.03^{b}

SDS-PAGE was carried out to understand the structure of both the native and HPH-modified proteins, by focusing on the relative abundance of the protein portions of interest, i.e. legumin, vicilin and convicilin. SDS-PAGE profiles of IP, IP60 and IP100 under non-reducing and reducing conditions (Fig. 2 a, b) showed the three major globular proteins: convicilin with a Mw band of about 75 kDa, legumin which reported a ≈60 kDa band and vicilin, for which the three typical subunits have been identified, whose molecular weights fall within the ranges also reported in the literature: ≈50 kDa, ≈35 kDa and about 15 kDa (Chang et al., 2022; Mession et al., 2013).

On the other hand, in reducing conditions, due to the breaking of the SS bonds, legumin was split into legumin α (whose band has been identified at ≈40 kDa) and legumin β, which has a band corresponding to Mw of ≈20 kDa. For vicilin, as expected, no significant differences were highlighted between the two electrophoretic runs as also reported by Chang et al. (2022). The SDS-PAGE were then performed on IP60 and IP100; to understand how the HPH pre-treatments affected the different fractions, the band intensities of legumin, vicilin and convicilin were quantified and normalized with respect to the intensity of the corresponding bands in IP (Fig. 3, abcd). With regard to the smallest subunit of vicilin at about 15 kDa, it seems to be no longer detectable by SDS-PAGE, as it was not possible to identify the intensity of the corresponding band on the electrophoretic gel. This may be due to the fact that the HPH treatments were able to break the weak bonds (hydrophobic interactions, hydrogen bonds and electrostatic forces) that hold vicilin subunits together (Lam et al., 2018), in residues smaller than 10 kDa, not detectable with the experimental conditions used. Furthermore, the SDS-PAGE revealed that the HPH treatments led to a decrease in the amount of convicilin with a subsequent significant increase in the relative abundance of the vicilin subunit of ≈50 kDa and, above all, of legumin. A possible explanation of this result may be related to their structure: in fact, convicilin has an important homology with vicilin and differs from it only for the N-terminus (Tzitzikas et al., 2006). Based on this, it could be hypothesized that HPH treatments detached the N-terminus from convicilin, leaving only the homologous part with vicilin, thus increasing the relative quantity of the latter and decreasing that of convicilin. Although the aspect of legumin increase is still not very clear, a possible explanation could be attributed to the random association of its dissociated subunits after HPH treatment, due to the formation of non-covalent bonds and reaggregation through non-specific interactions, as also demonstrated by Mession et al. (2013, 2015) on heat-treated legumin, with the subsequent formation of multiple structures homologous to the legumin. The variation in the relative abundance of these proteins certainly demonstrate that HPH pre-treatments changed the proteins structure with possible implications on their technological properties as previously reported by several studies (Chang et al., 2022; Cui et al., 2020; Boye et al., 2010).

The structural changes induced by the HPH pre-treatments were evaluated also by DSC analysis. The thermograms (Fig. 4) clearly highlighted the denaturation peak of the native IP and of the treated IP60 and IP100, while, on the contrary, no denaturation phenomena were observed in the CP sample, confirming the denatured state of the commercial protein isolate. As far as the IP, IP60 and IP100 proteins are concerned, being made up of several protein fractions, it would be expected to detect different denaturation peaks; however, only one larger peak is visible and it derives from the overlapping of the denaturation

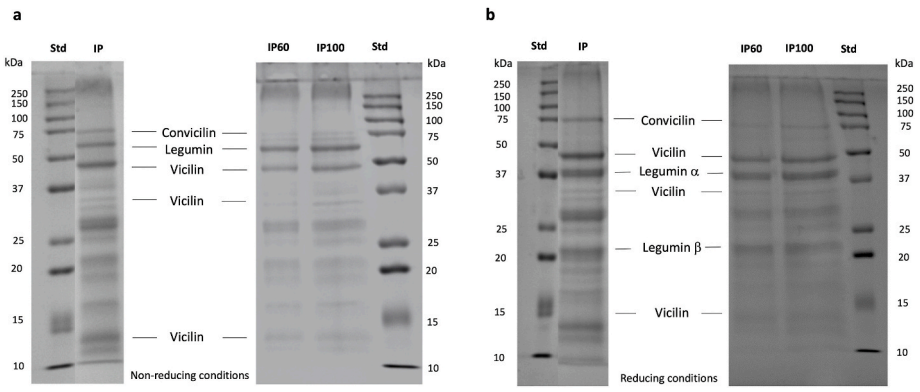

Fig. 2. Pea protein IP-IP60-IP100 SDS-PSGE profiles of convicilin, legumin and vicilin fractions, under non-reducing (a) and reducing conditions (b).

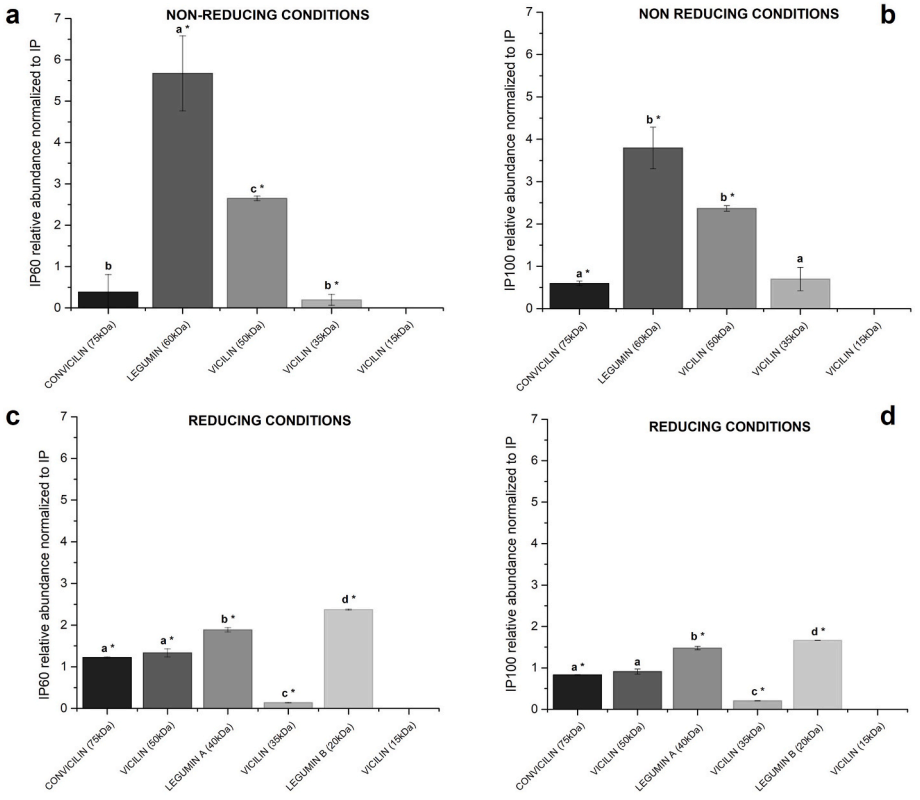

Fig. 3. Relative abundance of pea protein globulin fractions of IP60 (**a** non-reducing conditions, **c** reducing conditions) and IP100 (**b** non-reducing conditions, **d** reducing conditions), normalized to untreated protein (IP). Columns with different letters show significant differences between them and columns with asterisk shows significant difference from IP ($p < 0.05$).

peaks of legumin and vicilin, as stated also in other works (Chang et al., 2022; Withana-Gamage et al., 2011; Shand et al., 2007). What is clearly visible is a shift of the denaturation peaks towards lower temperatures as the treatment pressure increased, as a consequence of the progressive unfolding of the protein structure. The trend is confirmed by the IP value of T_{ONSET} (Table 2) at around 91.49 ± 0.55 °C that is in line with the one reported in literature (Sun and Arntfield, 2011). The significant decrease of T_{ONSET} of the treated samples (IP60 and IP100) compared to the control (IP) is a consequence of the HPH treatments that caused the unfolding of the structure, due to the breakage of bonds and interactions within the protein, making it more prone to unfold. Same trends were observed by other authors in whey and chickpea proteins subjected to HPH treatment (Huang et al., 2022; Wang et al., 2020).

Protein structure modification has been also confirmed by the analysis of the total free sulfhydryl groups (Table 2), a parameter that can represent a macroscopic indicator of modification in the tertiary and quaternary structure of proteins, which were significantly reduced upon HPH process. The decrease of thiol groups can possibly indicate the formation of disulfide bridges and/or a refolding of the proteins. Indeed, stressing treatments on plant proteins, as HPH, can induce hydrophobic interactions leading to S–S exchange or to the formation of new disulphide bonds due to the oxidation of –SH groups (Peng et al., 2016; Queirós et al., 2018; Wang et al., 2017). Another contribution to the decrease of thiol groups may derive from the reduction upon HPH of convicilin, which is rich of sulfur-containing amino acids (Boye et al., 2010 and Reinkensmeier et al., 2015).

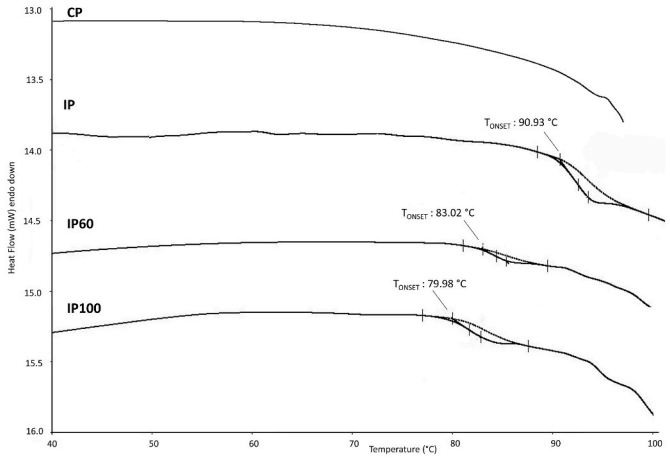

Fig. 4. Thermograms obtained by DSC analysis, showing the denaturation peaks of the pea protein samples. Dashed lines represent integrations performed.

Table 2
Onset temperature and total free –SH groups obtained for IP, IP60 and IP100. Values in the same columns with different letters showed significant differences ($p < 0.05$).

	T_{ONSET} (°C)	μM SH g^{-1}
IP	91.49 ± 0.55 [a]	1.47 ± 0.08 [a]
IP60	82.76 ± 1.07 [b]	0.32 ± 0.06 [b]
IP100	80.93 ± 2.12 [b]	0.39 ± 0.02 [b]

3.2. Effect of HPH on pea proteins technological properties

3.2.1. Solubility, WHC and OHC

Solubility is one of the technological properties most influenced by high pressure treatments, as reported in many works in the literature dealing with plant proteins (Cheng et al., 2022; Primozic et al., 2018; Yang et al., 2018). This property is very important as it precludes the use of such proteins for the formulation of different food products such as emulsified dressings or beverages, which are normally formulated at acidic or neutral pH. As shown in Fig. 5, the solubility of the commercial protein isolate (CP) was extremely lower than IP, presumably as a consequence of the extraction and purification processes carried out at industrial level (Tanger et al., 2020), which caused the partial or complete unfolding of the proteins or a potential aggregation during spray-drying (Lu et al., 2020; Shand et al., 2007), leading to low solubility. Furthermore, based on the previous technological characterization of CP used in this study (D'Alessio et al., 2022), the high particle size reported ($D_{[4;3]}$ 92.08 ± 5.23 μm), which can be ascribable to the presence of aggregates, caused a reduction of solubility. The solubility of IP, on the other hand, was much higher than CP, likely because the extraction and purification methods were carried out under mild conditions and did not impair the protein structure as also evidenced by the circular dichroism spectra previously discussed (Fig. 1). It is interesting to note that both the HPH pre-treatments significantly increased the solubility within the pH range studied and this was more evident at acid pH, in particular at the isoelectric point of pea proteins (4.3–4.5). The increase in the solubility of IP60 and IP100 is likely due to the disruption of aggregates and a decreased particle size upon high pressure processing, which allowed a greater dispersion and hydration of the protein, as widely reported in literature on different HPH-treated plant proteins, like pea, faba bean and lupin proteins (Melchior et al., 2022; Yang et al., 2018; Bader et al., 2011) as well as on animal proteins (Chen et al., 2016; Yu et al., 2018). In such works, solubility resulted enhanced by the application of HPH because of the decrease in the particle size of the proteins, which in turn led to an increase in the surface area of the proteins and therefore to an improvement in the interactions between water molecules and the protein itself. The slight decrease in solubility that was noted for IP100 compared to IP60, could be due to the formation of small aggregates or a reaggregation of the protein, usually observed at high pressures (Melchior et al., 2022; Saricaoglu, 2020).

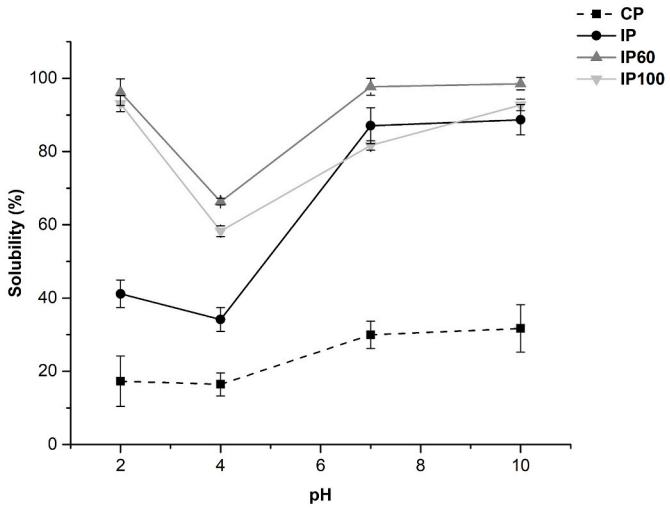

Fig. 5. Solubility (%) of commercial (CP), untreated (IP) and treated pea protein isolates (IP60 and IP100) as a function of pH.

It is interesting to highlight that, if the energy density applied from the valve to the flowing sample during the HPH treatment is taken into consideration (Calligaris et al., 2018), different effects were achieved on pea proteins solubility, energy density being equal. Indeed, in terms of energy density, the two treatments used in this work (60 MPa and 100 MPa for 5 cycles) were comparable to those applied by Melchior et al. (2022) (100 MPa and 150 MPa for 3 cycles, respectively) on commercial pea proteins. In this study, therefore, the solubility increased to a higher extent compared to the results obtained by Melchior et al. (2022): 34.03 ± 1.19% (100 MPa for 3 passes) vs. 97.69 ± 2.30% (60 MPa for 5 passes) and 19.94 ± 1.00% (150 MPa for 3 cycles) vs. 81.68 ± 1.27% (100 MPa for 5 cycles). It can be hypothesized that such variability may be ascribable, among other factors, to differences in the initial state of the proteins (native/denatured). It seems thus that, to improve pea proteins solubility and enhance their hydrophilic character, the native state of the protein is a pre-requisite to HPH pre-treatments. Further investigations are needed to verify whether the effects achieved with the HPH process may be dependent on the native/denatured state of the proteins.

Also, WHC was positively affected by HPH pre-treatments (Fig. 6), leading to a significant increase of this functionality at 100 MPa, a result which is similar to what reported for other protein treated with HPH (Lu et al., 2020; Dong et al., 2011). In fact, HPHs were able to reduce the size of protein residues, increase the surface in contact with water and the number of hydrophilic amino acid residues capable to interact and hold water (Lu et al., 2020). Furthermore, as observed in other works, the increase in WHC was also linked to a greater exposure of polar groups and side chains, that can facilitate the formation of hydrogen bonds (Stone et al., 2015).

The oil holding capacity (OHC), on the other hand, was significantly affected by HPH and a higher capacity to bind oil was found with the pre-treatment at higher intensity (100 MPa), likely for a change of arrangement that favored the exposure of hydrophobic groups previously hidden inside the structure. Such results seems contradictory as both WHC and OHC were positively improved upon the HPH treatment carried out at 100 MPa; however, different phenomena could have occurred contextually at different levels: the disruption of aggregates and decrease in particles size, which led to an increase in hydration and

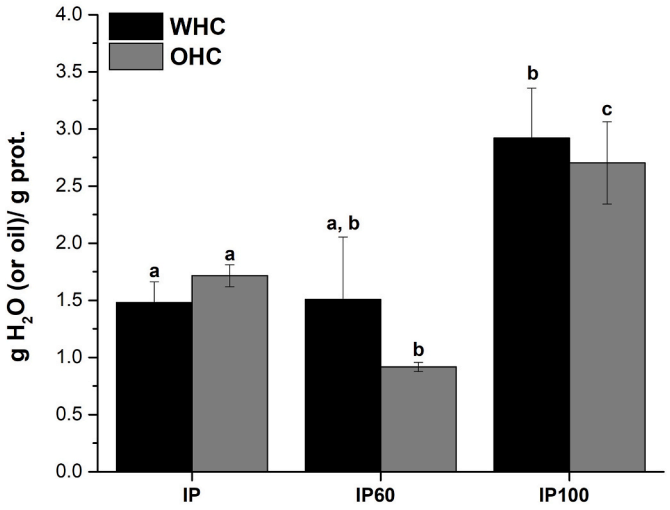

Fig. 6. WHC and OHC of the three samples analyzed. Columns with different letters means significant differences ($p < 0.05$).

therefore in solubility; the decrease in particles size which simultaneously led also to an increase in the exposed surface area and likely in the exposed hydrophilic groups, with an improvement of the WHC; finally, the structural modification that led to a partial unfolding of the proteins, as also demonstrated by DSC analyses, and consequent exposure of hydrophobic groups and an increase in OHC. Finally, another aspect to consider is that both WHC and OHC include a part of water/oil which is bound and a part which is physically entrapped within structures. To this regard, the possibility that HPH may have caused the formation of loops that can physically entrap the oil or water phase cannot be excluded.

3.2.2. *Surface properties and emulsifying capacity*

The effect of the HPH process on the technological functionality of pea proteins in emulsified systems was investigated by studying the adsorption behavior at the oil/water interface and the capacity to form and stabilize o/w emulsions under standardized conditions and results are reported in Figs. 7 and 8, respectively. A decrease in interfacial tension was observed for all the samples with the increase of protein concentration (Fig. 7), but no significant differences were found among IP, IP60 and IP100. A limited effect of the HPH treatment was observed also in the emulsification properties evaluated after systems preparation

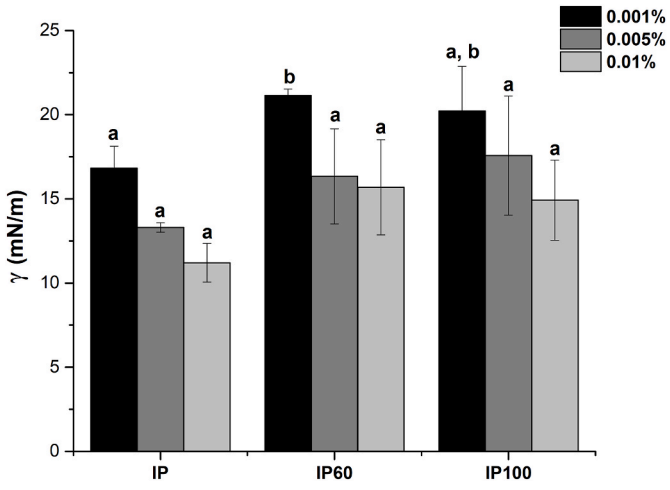

Fig. 7. Interfacial tension of pea protein solution (native and treated) at different concentrations and sunflower oil, after 30 min of equilibrium. Columns with different letters means significant differences ($p < 0.05$).

(Fig. 8a) as all emulsions exhibited a bimodal distribution, with a minor population of smaller droplets centred around 1 μm and a main population of larger particles centred on 8–9 μm. However, although the distributions were very similar, by taking into account the $D_{[4;3]}$, significant differences ($p < 0.05$) were highlighted among the oil droplets size of the three emulsion systems with values of 5.77 ± 0.02 μm for IP, 4.96 ± 0.02 μm for IP60 and 6.61 ± 0.04 μm for IP100, being IP60 the system with the lowest particle size. Moreover, it is interesting to underline that upon storage (Fig. 8b), the IP60-emulsions remained quite unchanged with a slight increase of $D_{[4;3]}$ (6.57 ± 0.02 μm), compared to emulsions formulated with IP and IP100, whose droplet size distribution moved towards larger diameters (10.88 ± 0.05 μm for IP and 18.38 ± 0.37 μm for IP100) and wider distributions centred mostly on 100 μm. The surface properties were thus not affected by the modifications induced by HPH to the proteins structure as observed also on lentil proteins treated with HPH at different intensities by Primozic et al. (2018). Regarding the role of pea globulins ratio and of the individual proteins legumin and vicilin on surface properties, it is still a widely debated topic in literature and controversial results can be found. Indeed, some studies reported that a lower legumin/vicilin ratio is associated with a greater emulsifying capacity and stability over time (Dagorn-Scaviner et al., 1987), while other studies affirmed that a lower vicilin/legumin ratio resulted in a greater emulsifying stability (Barać et al., 2015; Pedrosa et al., 2020; Sathe, 2002). The present work seems to confirm this last hypothesis, since the HPH pre-treatments lead to a lower vicilin to legumin ratio, particularly evident in the IP60-systems.

3.2.3. *Antioxidant activity*

The TEAC assay and the Folin-Ciocalteu method were applied to respectively investigate, with two different mechanisms, the antiradical activity and reducing properties (RP) of the treated proteins as affected by the HPH process, with respect to the untreated IP sample (Fig. 9). It is well established that the antioxidant properties of proteins are closely linked to the molecular structure, molecular weight, amino acid sequence of the protein and the presence of specific amino acids like cysteine and aromatic ones (Žilić et al., 2012). Results obtained for the native IP are similar to those found in literature (Wang et al., 2017). HPH treatments significantly affect both TEAC and RP values, to a higher extent in sample IP60 than IP100 with respect to the control (IP). Some treatments such as heat, enzymatic and electron beam irradiation, were proven to increase the antioxidant activity of proteins (Chang et al., 2021; Wang et al., 2017, Žilić et al., 2012), however, to the best of the authors' knowledge, the effect of high-pressure treatments on the antiradical activity and reducing properties of pea proteins has not been investigated yet and thus no data for comparison are available. Even though the antioxidant activity in proteins and peptides and the mechanism behind it are far from being fully understood, the integrity of the molecular structure (Medina-Navarro et al., 2010), together with the interactions between side chains of different amino acids, have been recognized to be key factors for the exploitation of such functionality (Elias et al., 2008, Žilić et al., 2012), indeed, amino acids act as antioxidants both with a reducing mechanism, exerted by the –SH groups, and as proton donors to electron-lacking radicals from the aromatic residues of amino-acids like tryptophan, phenylalanine and tyrosine (Žilić et al., 2012). This behavior may explain the decrease of both the TEAC and RP values due to the HPH pre-treatments, related to the loss of the reducing centers of the proteins as indicated by the decrease of the total free –SH groups (Table 2). Regarding the TEAC and RP values observed at 100 MPa, the higher values compared to 60 MPa could be explained by the progressive unfolding of the protein structure due to the more intense treatment, which enabled the exposure of some highly reactive amino acids, like tyrosine and tryptophan, usually hidden within the protein core (Žilić et al., 2012).

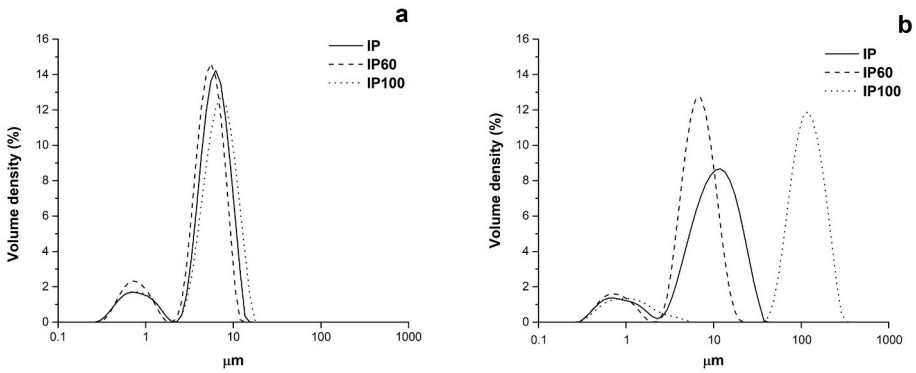

Fig. 8. Droplet size distributions of emulsions formulated with 1.0% (w/v) of IP, IP60 and IP100, 1 min after formulation (a) and after 7 days of storage at 4 °C.

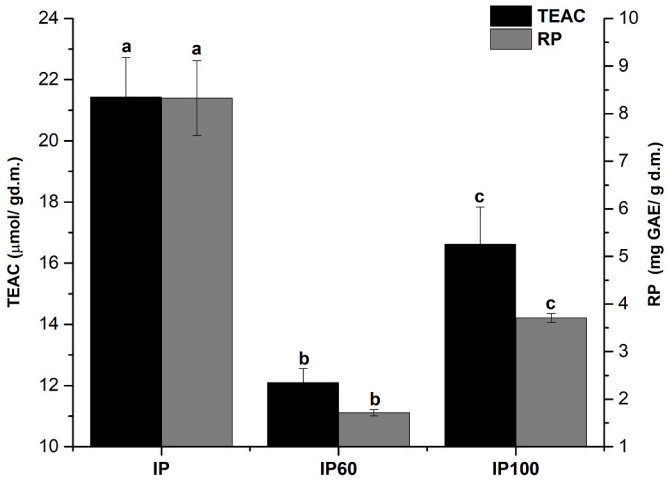

Fig. 9. Total antioxidant activity of native and modified pea proteins: TEAC to evaluate antiradical capacity and RP to assess reducing properties. Columns with different letters means significant difference for the same spectrophotometric assay ($p < 0.05$).

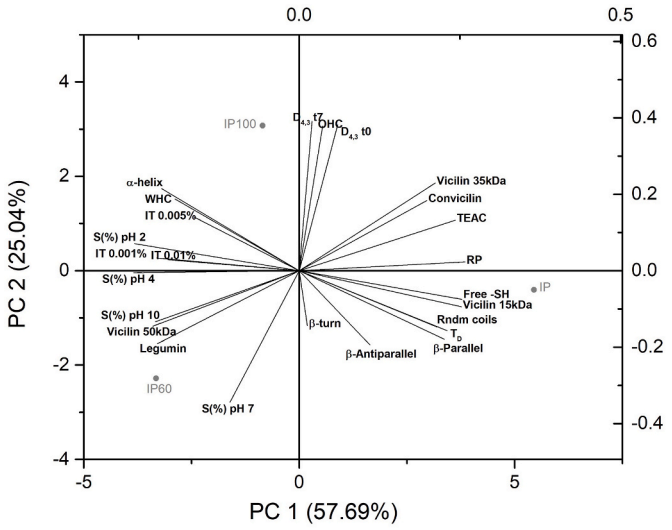

Fig. 10. Biplot of structural and techno-functional properties of pea proteins as affected by HPH pre-treatments.

3.3. Principal component analysis

PCA was performed to have an overview of the impact of HPH pre-treatments on the structure and, consequently, on the techno-functional properties of pea proteins. Therefore, these changes have been analyzed taking into account the secondary structure's conformations percentages, the quantity of total free sulfhydryl groups, the relative abundance of convicilin, legumin and vicilin, the denaturation temperature, TEAC and RC, solubility, interfacial tension, WHC and OHC and $D_{[4;3]}$ at time zero and after 7 days of storage.

In Fig. 10 is reported the biplot obtained from the first two principal components (PC), which accounted for 57.69% and 25.04% of the total variance respectively, and for a cumulative contribution of 82.73%.

The sample scores (i.e. IP, IP60 and IP100) resulted clearly separated in three different quadrants, meaning that the applied pressures exerted significant changes on the native protein. IP was more characterized by β- and disordered conformations, mostly described along PC1, while, in the opposite position, α-helix structures better characterized the IP100 scores. These results confirmed those obtained from the deconvolutions carried out on circular dichroism's spectra (section 3.1). The biplot also highlighted how the treatment at 60 MPa deeply affected the relative abundance of vicilin 50 kDa and legumin that resulted positively correlated to IP60. Conversely, the content of vicilin 35 kDa and convicilin were negatively correlated to IP60, since a decrease of their content was observed after the HPH treatment. The antioxidant activity and reducing properties of pea proteins, well described by PC1, showed a clear separation of IP sample, in agreement with the results obtained and the decrease of functionality experienced after the application of HPH treatments. In contrast, the technological functionalities as solubility, WHC and interfacial tension, characterized IP60 and IP100 since an increase of these values has been observed after the HPH treatment. As regards PC2, IP100 was strongly separated considering $D_{[4;3]}$ at time zero ($D_{[4;3]}$ t0) and after storage ($D_{[4;3]}$ t7), as well as the OHC.

4. Conclusions

In this study, the use of HPH at different intensities as a pre-treatment resulted in structural changes (secondary structure, relative abundance of pea protein globulins and total free sulfhydryl groups) which were reflected on pea proteins techno-functionality. HPHs were able to unfold and modify protein structure, as demonstrated by the decrease of the denaturation temperature and of the total free sulfhydryl groups, as well as by the variation in the relative abundance of vicilin and legumin and by the changes occurred in the secondary structure. From a technological functionality perspective, HPH pre-treatments significantly enhanced solubility, WHC and OHC, especially at the highest intensity whilst did not influence both the surface properties and the emulsifying capacity; however, in the emulsions formulated with IP60, characterized by the lowest vicilin/legumin ratio, an improvement of the physical stability was observed. The insights from this study may contribute to the exploitation of HPH as a useful technology to tailor pea proteins technological functionality; to this regard, further

investigations are needed to verify whether the effects achieved with HPH may be dependent on the native/denatured state of the proteins before processing.

CRediT authorship contribution statement

Giulia D'Alessio: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, preparation. **Federica Flamminii:** Conceptualization, Methodology, Data curation, Writing – review & editing. **Marco Faieta:** Formal analysis, Data curation. **Roberta Prete:** Formal analysis, Data curation. **Alessandro Di Michele:** Formal analysis, Data curation. **Paola Pittia:** Resources, Writing – review & editing, Project administration, Funding acquisition. **Carla Daniela Di Mattia:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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