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Influence of High-Pressure Processing and Microbial Transglutaminase on the Properties of Pea Protein Isolates

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Abstract: This study investigated the effects of high-pressure processing (HPP; 600 MPa/15 min) and microbial transglutaminase-catalyzed (MTG; 30 U·g of protein^{-1}) crosslinking on the concentration of dissolved proteins (SOL), free sulfhydryl groups (SH), surface hydrophobicity (H_0), and viscosity of pea protein isolates (PPI) at different concentrations (1–13%; w/v). The SOL increased by increasing protein concentration (max. 29%). MTG slightly affected SOL. HPP decreased SOL with increasing protein concentration, and the combination MTG + HPP resulted in a lower SOL than HPP alone. The concentration of SH in untreated PPI increased with increasing protein concentration, reaching a maximum of 8.3 µmol·mg prot^{-1}. MTG increased SH at higher protein concentrations. HPP lowered SH, but its concentration increased by increasing protein concentration. HPP + MTG offset the effect of MTG, yielding lower SH. MTG did not affect H_0 at 1% concentration but increased it for concentrations from 3–5%, and there was a decrease with 7–9%. HPP increased H_0 up to 37% for intermediate protein concentrations but did not affect it at higher concentrations. MTG + HPP decreased H_0 at all protein concentrations. The viscosity of the dispersions increased with protein concentration. HPP increased the viscosity of the dispersions for concentrations above 7%, while MTG only caused changes above 9%. Combined MTG + HPP resulted in viscosity increase. The results underscore the opportunity for innovative development of high-protein products with improved properties or textures for industrial application.

Keywords: high-pressure processing; transglutaminase; pea protein isolates

Citation: Queirós, R.P.; Moreira, N.; Pinto, C.A.; Fidalgo, L.G.; Saraiva, J.A.; Lopes-da-Silva, J.A. Influence of High-Pressure Processing and Microbial Transglutaminase on the Properties of Pea Protein Isolates. *Macromol* **2024**, *4*, 213–226. https://doi.org/10.3390/macromol4020011

Academic Editor: Dimitrios Bikiaris

Received: 24 February 2024
Revised: 11 March 2024
Accepted: 28 March 2024
Published: 8 April 2024

1. Introduction

The demand for protein-rich foods is increasing, and plant-based proteins are becoming a more viable substitute for animal-based proteins due to the high cost and environmental impact of animal husbandry. Legumes have been identified as a more sustainable source of protein, as their cultivation requires fewer resources than animal agriculture [1]. Peas are an important source of plant-based protein, containing 20–30% protein by weight, and are used for various purposes such as emulsification, gelation, and enhancing texture [2,3]. However, the utilization of plant proteins as food ingredients is limited by certain functional restrictions, necessitating the implementation of strategies to improve their techno-functional properties. Modifications to these properties can generally be achieved through physical or enzymatic techniques, thus broadening the potential of proteins as

structural ingredients [4]. Research has demonstrated that high-pressure processing (HPP) and microbial transglutaminase (MTG) treatments, which are conducted at low temperatures and without additives, can significantly alter the structural and techno-functional properties of food proteins. These treatments have been found to improve the emulsifying, foaming, and gelation abilities of the proteins [5,6].

HPP is a technology that applies pressure in the range of 400–600 MPa for a few minutes to inactivate microorganisms without the use of heat. HPP is versatile, as it can be applied to a wide range of foods regardless of size or shape. Additionally, HPP does not significantly alter the nutritional and physicochemical properties of food, such as color or flavor, and has minimal impact on bioactive compounds [7]. HPP has primarily been applied in the food industry to inactivate pathogenic and spoilage microorganisms, ensuring product safety and extending shelf-life, with several products commercially available nowadays [8]. However, recent research has explored other potential food applications of HPP, such as modifying the structural and functional properties of proteins [5,9] and enhancement of enzymes [10,11].

MTG (EC 2.3.2.13) is an extracellular enzyme belonging to the transferase class with a molecular weight of 38 kDa. It is commercially produced through traditional fermentation by the bacterium *Streptoverticillium moboarense* [12,13]. It is generally recognized as safe (GRAS) by the U.S. Food and Drugs Administration (FDA) and it has been widely used in the food industry in recent years [6]. It can modify the techno-functional properties of proteins through the incorporation of amines, promoting intra- and intermolecular crosslinks, or through deamination. MTG catalyzes the acyl transfer reaction between the γ-carboxamide group of protein-bound glutamine residues and primary amines, preferably the ε-amino group of lysine residues. In the absence of lysine or primary amines, water may act as the acyl acceptor and promote hydrolytic deamidation of the glutamine and asparagine residues, converting them into glutamic and aspartic acid [12,14].

MTG has been shown to be relatively resistant to pressure up to 600 MPa [15], thus the combination of HPP and MTG seems to have promising synergistic effects on protein crosslinking and the improvement of protein functionality. This technology has been demonstrated to work well with protein isolates, such as bovine serum albumin, γ-globulin, and β-lactoglobulin, as well as high-protein foods, such as meat reconstruction, surimi gelation, and yogurt setting. HPP can alter the conformation of proteins, exposing the target residues for MTG and enhancing the enzyme's activity, which leads to improved protein crosslinking and functionality [16].

The enhanced functionality of proteins could potentially eliminate or reduce the need for additives, such as emulsifiers or thickeners, or even be utilized to create new products or even establish new categories. Therefore, the objective of this work is to evaluate the individual and combined effects of MTG and HPP on the soluble protein, the content of free sulfhydryl groups (SH), surface hydrophobicity (H_0), and viscosity of pea protein isolates (PPI) with various dispersion concentrations.

2. Materials and Methods

2.1. Materials

A readily dispersible pea protein isolate (Pisane® M9, Cosucra) was obtained from Induxtra (Induxtra de Suministros Llorella Portuguesa—Industria Alimentar, Lda., Moita, Portugal). The protein content was determined to be 80.9 ± 0.2% by elemental analysis (N × 6.25). The protein isolates had a water content of approximately 10%. According to the supplier, the ash and fat contents were less than 6% and 4%, respectively. All reagents used were of analytical grade. Activa® transglutaminase (100 U·g^{-1}; 1 U is defined as the amount of enzyme that catalyzes the formation of 1 µmol hydroxamate from hydroxylamine and carbobenzoxy-L-glutaminylglycine per minute at pH 6.0, 37 °C) was kindly provided by Ajinomoto Foods Europe SAS (Hamburg, Germany).

2.2. Sample Preparation and Processing

A dispersion of 15% (*w*/*v*) of the protein isolate was prepared in distilled water and stirred for 20 h at room temperature (20–22 °C) for hydration. The dispersions were then diluted in distilled water to obtain dispersions of 1, 3, 5, 7, 9, 11, and 13% (*w*/*v*) that were stirred for an additional 4 h. The resulting dispersions (40 mL) were placed in flasks (Thermo Scientific™ Nalgene™ Wide-Mouth Lab Quality HDPE Bottles, Waltham, MA, USA). The dispersions with concentration of 11% and 13% were only used for viscosity analysis as they gelled under certain conditions.

To perform the MTG reaction, a solution of MTG was prepared in distilled water and then diluted to have a final concentration of 30 U·g of protein^{-1} when added to the dispersions. After adding the MTG solution to the protein dispersions, the samples were kept at 4 °C overnight. To evaluate the combined effects of HPP and MTG, the enzyme was added to the dispersions immediately before processing. Protein isolates, with and without added MTG, were treated at 600 MPa for 15 min at room temperature (approximately 20 °C), using a hydrostatic press (Hiperbaric 55, Burgos, Spain). This HPP equipment has a pressure vessel with a 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 the pressurizing fluid.

Protein isolates without MTG or HPP were used as control samples. All samples were kept at 4 °C overnight and then centrifuged at 6000 rpm for 20 min at 4 °C before analysis, except for samples intended for viscosity analysis.

2.3. Soluble Protein

The protein concentration in the supernatant was determined using the Bradford method [17] with some modifications. To an aliquot of 50 µL of the protein dispersion, 250 µL of Bradford reagent was added, 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 with BSA standards (0.05–0.70 mg·ml^{-1}). The results were expressed as:

$$\text{soluble protein } (\%) = \frac{\text{protein in the supernatant } \left(\text{mg·mL}^{-1}\right)}{\text{initial protein } \left(\text{mg·mL}^{-1}\right)} \times 100 \quad (1)$$

2.4. Sulfhydryl Groups

The concentration of free sulfhydryl groups was determined using the method of Beveridge et al. (1974) [18] with some modifications. The centrifuged samples were diluted in 0.086 mol·L^{-1} Tris buffer (pH 8.0). To 500 µL of the sample, 500 µL of Tris buffer and 50 µL of Ellman's reagent were added, and the mixture was kept for 60 min at room temperature (~20 °C). The absorbance of the mixture was then measured at 412 nm using a spectrophotometer (Shimadzu UV-1280, Kyoto, Japan). The concentration of SH was determined by dividing the absorbance value by the molar extinction coefficient of 13,600.

2.5. Surface Hydrophobicity

The H_0 of the protein dispersions was determined using the fluorescent probe 1-aniline-8-naphthalene-sulfonate (ANS) according to the method of Kato et al. (1980) [19]. The protein dispersions (0.05–0.25 mg·mL^{-1}) were prepared in 0.01 mol·L^{-1} phosphate buffer pH 7 by diluting the original centrifuged dispersion. An aliquot of 20 µL of ANS (0.008 mol·L^{-1} in 0.01 mol·L^{-1} phosphate buffer) was added to 4 mL of each protein solution, and the fluorescence intensity was measured at 390 nm (excitation) and 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 the fluorescence intensity vs. protein concentration (mg·mL^{-1}) plot, which was determined by linear regression analysis.

2.6. Shear Viscosity

Shear flow measurements were performed in an AR-1000 controlled stress rheometer (TA Instruments, Wilmslow, UK) equipped with a cone and plate measuring system (6 cm diameter acrylic cone, 2° angle). The flow curves were obtained after equilibrating the sample on the rheometer geometry, at 20 °C, for 10 min, by an up–down step program applying a different shear stress range to each sample. Apparent viscosity measured at a shear rate of 50 s^{-1} was considered to compare among different samples. Deviation from the Newtonian behavior was quantified by applying a power law model (Equation (2)) to the shear-thinning region of the flow curves and considering the flow index:

$$\eta_a = K\,\dot{\gamma}^{n-1} \quad (2)$$

where η_a is the apparent viscosity (Pa·s), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the consistency coefficient (Pa·s^n), and n is the flow behavior index (dimensionless).

2.7. Statistical Analysis

Results of soluble protein, sulfhydryl groups, and surface hydrophobicity are expressed as the average of three replicates ± standard deviation, whereas one replicate was used for shear flow measurements. ANOVA followed by Tukey's honestly significant difference test with a significance level of 5% was used to determine any significant differences between the samples. All statistical analyses were performed with Minitab v19 (State College, PA, USA) and Microsoft Excel 2013 (Microsoft Office System, Redmond, WA, USA).

3. Results and Discussion

3.1. Effects of HPP and MTG on the Concentration of Dissolved Proteins

The concentration of dissolved proteins depends on the hydrophilicity and hydrophobicity of their surface and is closely related to their technological properties, such as emulsification and gelation capabilities. The concentration of the dissolved proteins in the dispersion was determined to be 14.7 ± 0.5% at a concentration of 1% (w/v) and a pH of 7. As the total protein concentration of the dispersion increased, there was a corresponding increase in the concentration of the dissolved proteins, reaching a maximum of 29.2 ± 1.0% in protein isolates at 9%.

Figure 1 displays the outcomes obtained for PPI dispersions regarding the concentration of the dissolved proteins. The results indicate that the presence of MTG had a minimal impact on the concentration of the dissolved proteins, as observed in the protein concentration range analyzed. Overall, there was a trend of decreasing the concentration of the dissolved proteins; however, it was not statistically significant in most cases ($p > 0.05$). The inability of MTG to influence the solubility of plant-based protein dispersions at low concentrations has already been reported in the literature [20]. Most likely, higher protein concentrations correspond to a greater availability and amount of substrate for the catalytic action of MTG, which promotes the formation of a larger number and/or larger size of protein aggregates [21]. This consequently leads to a reduction in the concentration of the dissolved proteins.

Regarding the effect of HPP alone (600 MPa, 15 min), there was an increase in the concentration of the dissolved proteins observed for low protein concentrations. Still, the concentration of the dissolved proteins seems to be highly dependent on the protein concentration, with a significant decrease in the concentration of the dissolved proteins as the protein concentration increases. At the highest concentration analyzed, the concentration of the dissolved proteins for the pressurized samples was even lower than for the non-pressurized samples. The possible increase in the concentration of the dissolved proteins promoted by HPP is likely due to the dissociation of protein aggregates, as well as some

protein unfolding, which enhances their interactions with the solvent (water) [22–25]. On the other hand, pressure might expose hydrophobic residues and increase intermolecular interactions, resulting in the formation of insoluble aggregates, especially at higher protein concentrations. The data reported here suggest that high protein concentration can promote the formation of these insoluble aggregates, as previously reported for amaranth protein isolates [26].

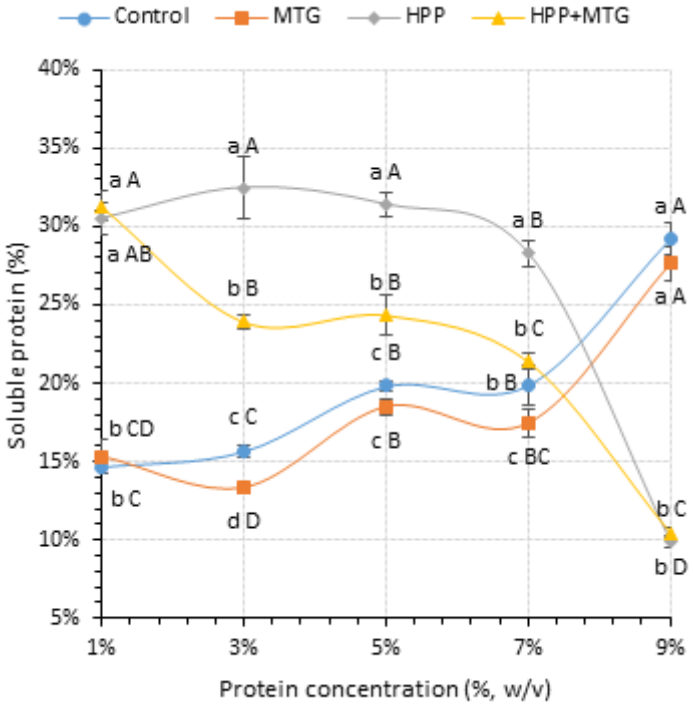

Figure 1. Variation of the concentration of the dissolved proteins as a function of protein concentration for protein dispersions subjected to various processing conditions: (●) control; (■) in the presence of 30 U/g protein MTG; (⧫) treated by HPP at 600 MPa, 15 min; (▲) processed by a combination of both treatments (MTG + HPP). Different capital letters indicate significant differences ($p < 0.05$) between the same treatments at different protein concentrations. Different lowercase letters indicate significant differences ($p < 0.05$) between different treatments at the same protein concentration.

The effect of the combined treatments, MTG + HPP, on the concentration of the dissolved proteins of pea proteins (Figure 1) showed a similar trend as that observed for the HPP-treated samples, which is not surprising given the minimal effect observed for MTG. Overall, the combined treatments resulted in protein samples with lower concentration of the dissolved proteins than those treated with HPP alone, supporting the hypothesis that the presence of MTG and HPP had a synergistic effect on intermolecular interactions and aggregation. The exposure of glutamine and lysine residues caused by HPP, which are typically buried within the protein tertiary structure, may have contributed to this effect by making them more accessible to the action of MTG [27]. Previous studies have demonstrated HPP's ability to facilitate protein crosslinking catalyzed by MTG for several animal-derived proteins such as β-lactoglobulin, casein, bovine serum albumin, and ovalbumin [16,28]. Therefore, it is likely that HPP may promote protein aggregate dissociation and induce conformational changes that increase the concentration of the dissolved proteins and facilitate access by MTG. As discussed earlier, MTG can catalyze crosslinking reactions that produce high-molecular-weight compounds, thereby reducing the concentration of the dissolved proteins.

3.2. Effects of HPP and MTG on the Amount of Free Sulfhydryl Groups

Sulfhydryl and disulfide interchange and the formation of new disulfide bonds play a crucial role in the tertiary structure of proteins. Therefore, manipulating these processes is essential to control the technological properties of proteins [29]. The concentration of free SH groups in untreated protein isolates at 1% was 3.00 ± 0.50 μmol·mg prot^{-1}. This concentration increased linearly (μmol·mg prot^{-1} = 62.57·protein concentration (%) + 2.85; R^2 = 0.92) with an increased protein concentration of the dispersion, reaching a maximum of 8.26 ± 0.16 μmol·mg prot^{-1}.

The addition of MTG to PPI at a 1% protein concentration had no statistically significant effects ($p > 0.05$) compared to control samples, in agreement with previous reports [20]. Conversely, a significant increase in free SH groups was observed for higher protein concentrations in the presence of MTG (Figure 2).

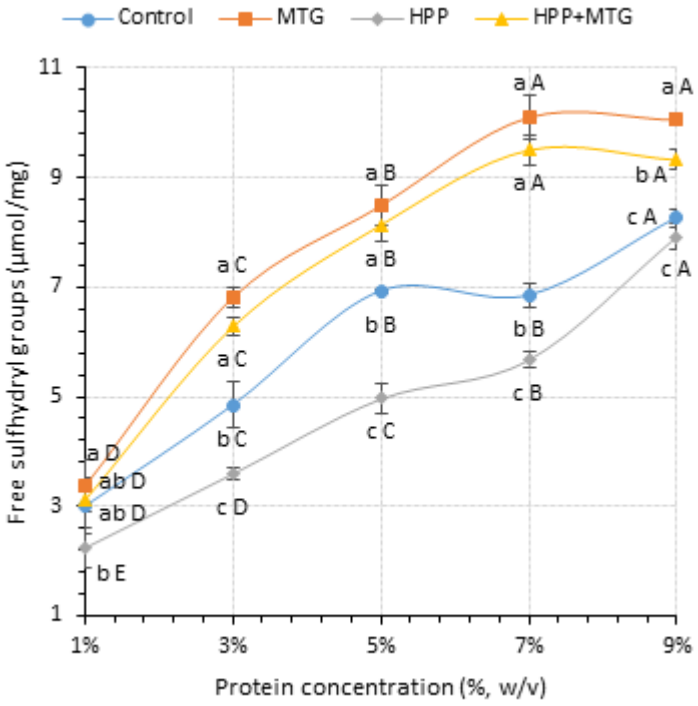

Figure 2. Variation of the concentration of free sulfhydryl groups as a function of protein concentration for protein dispersions subjected to different processing conditions: (●) control; (■) in the presence of 30 U/g protein MTG; (♦) treated by HPP at 600 MPa, 15 min; (▲) processed by a combination of both treatments (MTG + HPP). Different capital letters indicate significant differences ($p < 0.05$) in the same treatment at different protein concentrations. Different lowercase letters indicate significant differences ($p < 0.05$) between different treatments at the same protein concentration.

On the other hand, for more concentrated PPI, the addition of MTG increased the concentration of free SH compared to the controls. It is worth noticing that up to a concentration of 7% of total protein, the concentration of free SH increased linearly (μmol·mg prot^{-1} = 109.50·protein concentration (%) + 2.81; R^2 = 0.96) with increasing protein concentration. Further increasing the concentration of protein to 9% did not increase the content of free SH. Still, no further statistically significant alteration in free SH groups was observed upon increasing protein concentration above 3% (Figure 2). An increase in free SH of peanut protein was also reported as a result of MTG enzymatic activity (Hu et al., 2011). The authors suggested that MTG, by catalyzing crosslinking, may induce conformational changes (unfolding) of the proteins, thus exposing free SH. This explanation may also help to understand the observed results in the current study. The more pronounced effect observed at higher protein concentrations may be due to a higher degree of crosslinking resulting from the greater amount of substrate available for the enzymatic reaction.

As expected from the previous results [22], when HPP was applied individually to PPI, the concentration of free SH was lower than in the controls (Figure 2), contrary to MTG. Nonetheless, like the controls, the concentration of free SH in HPP samples increased linearly (μmol·mg prot^{-1} = 67.11·protein concentration (%) + 1.53; R^2 = 0.98) with an increased protein concentration of the dispersion. However, as the protein concentration increased, the effect of the decrease was less pronounced, showing similar levels of free SH groups for the unprocessed sample and the processed one, for a protein concentration of 9%.

As previously discussed, HPP can decrease the content of free SH by promoting hydrophobic interactions that lead to S-S exchange and/or the formation of new disulfide bonds [30,31]. On the other hand, pressure can also lead to an increase in free SH by dissociation/unfolding of the proteins, consequently exposing buried SH groups. [32].

The simultaneous application of HPP and MTG on PPI appears to offset the effect of MTG alone, yielding lower free SH. Similar to individually applied MTG, the concentration of free SH increased linearly (μmol·mg prot^{-1} = 104.77·protein concentration (%) + 2.58; R^2 = 0.96) with increasing protein concentration up to 7%, where it reached 9.33 ± 0.20 μmol·mg prot^{-1}. An additional increase in the protein concentration to 9% did not increase the content of free SH (Figure 2). Nonetheless, the combination of HPP and MTG resulted in higher free SH than the controls in all protein concentrations used, except for 1%, where no differences were observed. Overall, the combined HPP and MTG treatment increased the content of exposed SH compared to the control or only HPP-treated samples. An increase in the content of SH was also reported for sweet potato protein submitted to HPP with the subsequent addition of MTG compared to only HPP and controls [33]. As previously discussed, pressure-induced unfolding allowed buried SH groups to be exposed and most likely contributed to the crosslinking of protein catalyzed by MTG. The reason why the combined treatments led to an increase in free SH groups intermediate to that observed for treatment by HPP or MTG individually cannot be clearly explained based on the available data. However, speculating, we can consider the hypothesis that the catalytic activity of MTG on proteins that have undergone greater conformational expansion due to pressure, leading to a greater degree of crosslinking, contributes to a reduction in the number of free or reactively available SH groups in relation to the effect caused by the enzyme activity when not under treatment by HPP.

3.3. Effects of HPP and MTG on the Proteins' Surface Hydrophobicity

Changes in surface hydrophobicity are associated with the exposure or enclosure of the side chains of aromatic amino acids, which can affect the technological properties of proteins. The surface hydrophobicity of proteins isolates at 1% (H_0 = 2489 ± 69) was not significantly affected by the increase in protein concentration up to 5%, although it increased at higher concentrations (7% and 9%). Figure 3 shows the variation in protein H_0 for pea samples subjected to the different processing treatments under study, relative to the results obtained for the non-processed protein samples.

The effect of MTG without HPP treatment on H_0 was not significant ($p > 0.05$) for PPI at a protein concentration of 1% (Figure 3), which was expected based on previously reported results [20]. However, for protein concentrations above 1%, the effect of MTG became more pronounced, with an initial increase in H_0 for protein concentrations of 3–5%, followed by a significant decrease for protein concentrations of 7–9%, with H_0 reaching significantly lower values than for the control samples. It is possible that crosslinking promotes conformational changes that result in protein unfolding and exposure of hydrophobic regions, thereby increasing H_0, as previously described for lower protein concentrations [34]. However, in most cases, the crosslink catalyzed by MTG may close off hydrophobic residues inside the structure of the higher-molecular-weight polymers formed [35], consequently decreasing H_0, as reported here for higher concentrations of PPI [21]. A higher degree of crosslinking, as likely occurs with increasing protein concentration, probably promotes the occurrence of this second mechanism.

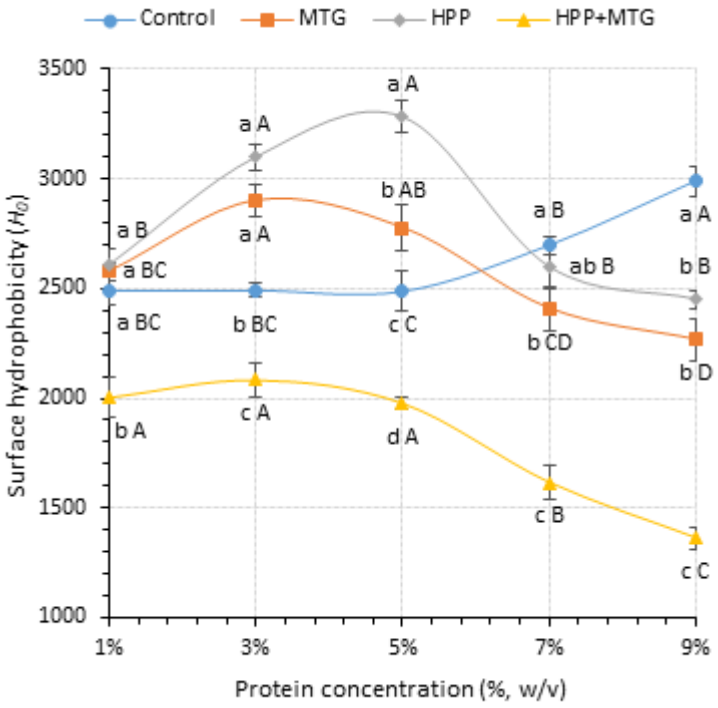

Figure 3. Variation of protein surface hydrophobicity as a function of protein concentration, for protein dispersions subjected to different processing conditions: (●) control; (■) in the presence of 30 U/g protein MTG; (♦) treated by HPP at 600 MPa, 15 min; (▲) processed by a combination of both treatments (MTG + HPP). Different capital letters indicate significant differences ($p < 0.05$) in the same treatment at different protein concentrations. Different lowercase letters indicate significant differences ($p < 0.05$) between different treatments at the same protein concentration.

Similarly to MTG, HPP did not significantly affect the H_0 of protein dispersions at 1%. It increased H_0 by up to 37% in the intermediate protein concentration range of 3–5%. However, for higher protein concentrations of 7% and 9%, HPP did not alter H_0 (Figure 3). It is worth recalling that similar pressure and time conditions for HPP of protein solutions increased protein H_0, as previously discussed [22]. Pressure may unfold proteins, exposing hydrophobic groups on the surface due to resulting conformational changes. This explains the higher H_0 observed in the lower protein concentration range. However, at higher concentrations, pressure may promote interactions between proteins, changing the equilibrium between aggregation and dissociation processes, leading to a decrease in H_0 [36,37].

The combined MTG + HPP treatments resulted in a significant decrease in H_0 for the pea protein samples compared to the non-processed samples, across the entire protein concentration range, especially for higher concentrations (Figure 3). As discussed earlier, the combination of HPP and MTG may alter the equilibrium between aggregation and dissociation processes, leading to a decrease or increase in H_0. For PPI, the results indicate that increased crosslinking and/or aggregation processes seem to be more prominent as there is a decrease in H_0.

3.4. Effects of HPP and MTG on the Viscosity of Pea Protein Dispersions

Figure 4 shows representative flow curves obtained for non-processed pea protein dispersions at different protein concentrations. Within the range of protein concentrations between 1 and 7% the measured values of viscosity were very low, and the measurable shear rate range was limited due to the resolution limit of the rheometer.

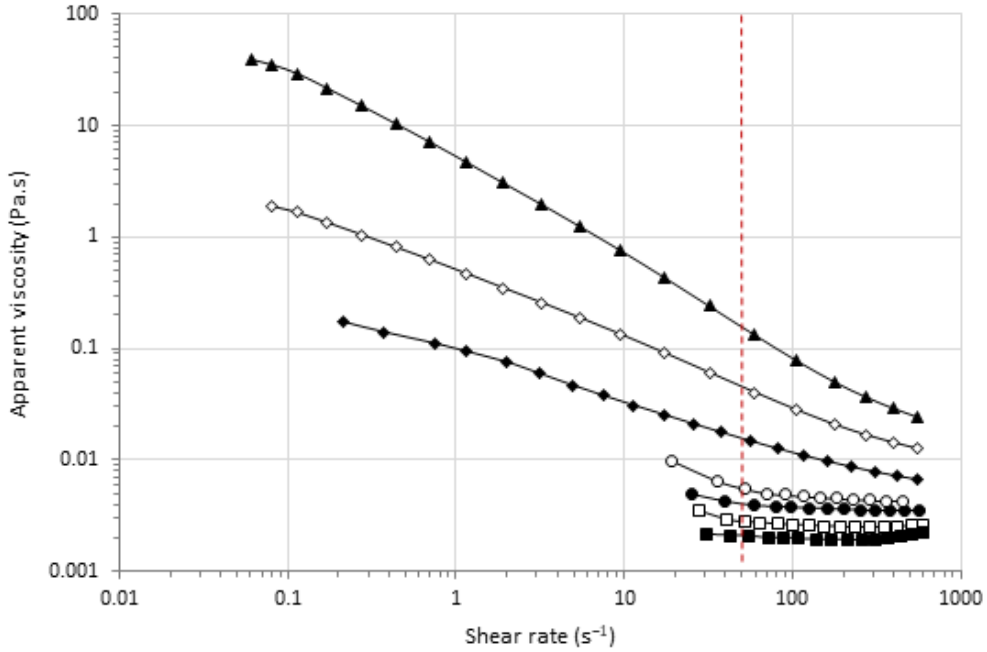

Figure 4. Representative flow curves obtained from the applied up stress ramp (increasing shear stresses), at 20 °C, for unprocessed protein dispersions at different concentrations: (■) 1%, (□) 3%, (●) 5%, (○) 7%, (⧫) 9%, (◇) 11%, and (▲) 13% (*w*/*v*). The dotted line marks a shear rate of 50 s^{-1}.

The apparent shear-thickening behavior observed for the lower concentrations at high shear rates is an artifact due to the usual turbulence observed for cone–plate geometry. Anyway, the observed flow behavior was essentially Newtonian, with the apparent viscosity showing a very low dependence on the shear rate. Increasing the protein concentration clearly increased the apparent viscosity and the degree of shear-thinning flow behavior, with the apparent viscosity showing a much more pronounced dependence on the shear rate.

The viscosity of protein dispersions processed only by HPP did not vary significantly for concentrations equal to or less than 5%, presenting a flow behavior very close to Newtonian, like unprocessed dispersions. Figure 5 shows the flow curves obtained for 7% PPI dispersions. At this protein concentration, there was already a significant increase in viscosity with HPP treatments, with the flow curves also showing a more pronounced shear-thinning behavior and a greater structural hysteresis between the up and down curves.

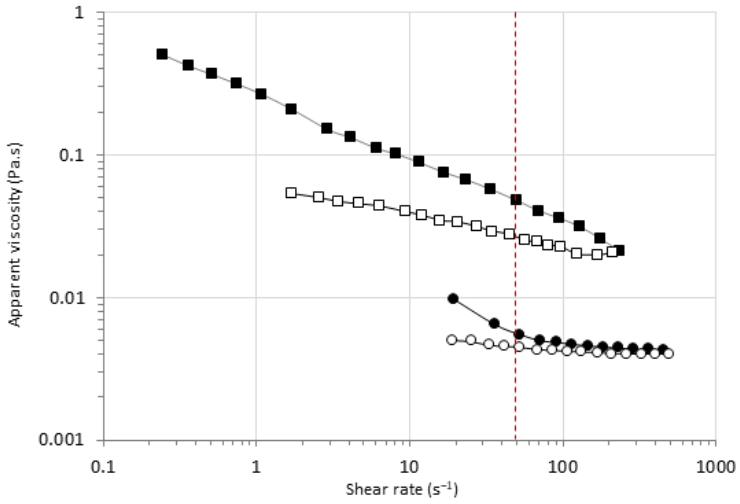

Figure 5. Representative flow curves obtained from the applied up (filled) and down (open symbols) stress ramps, at 20 °C, for 7% protein dispersions subjected to different treatments: unprocessed samples (circles); and HPP 600 MPa, 15 min (squares). The dotted line marks a shear rate of 50 s^{-1}.

The influence of HPP on the flow characteristics of the PPI dispersions is further compared in Figure 6.

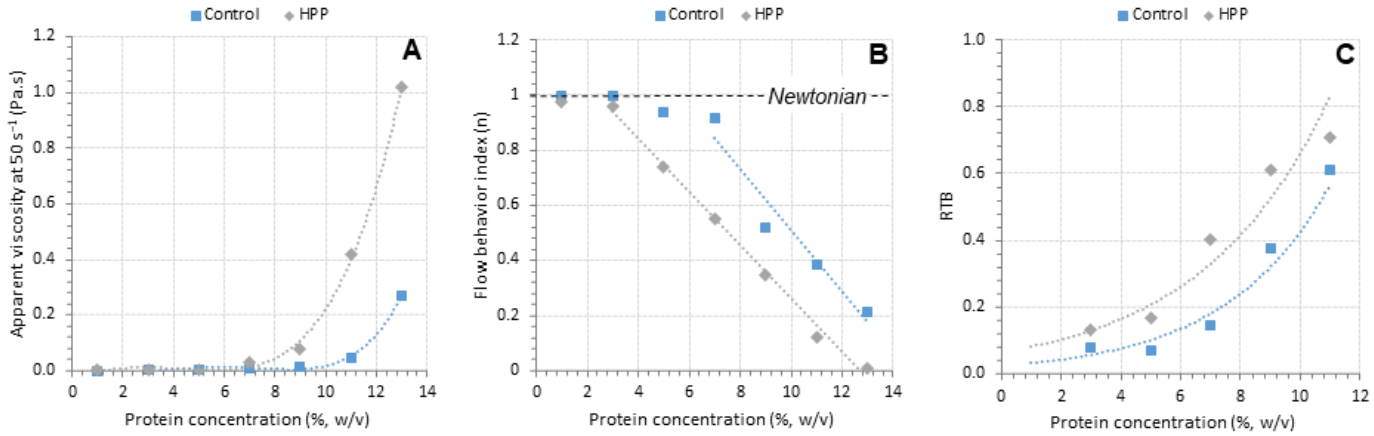

Figure 6. Comparison between flow behavior characteristics obtained for (■) unprocessed and (⧫) HPP-treated protein dispersions: (**A**) Dependence of apparent viscosity (50 s^{-1}) on pea protein concentration; (**B**) Dependence of the flow behavior index *n* (Equation (2)) on pea protein concentration; (**C**) Dependence of RTB, the relative thixotropic structural breakdown, on pea protein concentration. Dotted lines have no physical meaning and are only a guide for the eyes.

The apparent viscosity for the PPI dispersions treated by HPP was not significantly different from that of unprocessed dispersions for protein concentrations up to 5%.

However, for concentrations equal to or greater than 7%, HPP clearly leads to higher apparent viscosities (Figure 6A). There is not much information regarding the effects of HPP on the viscosity of protein solutions, however, an increase in apparent viscosity with pressure, particularly at higher protein concentrations, was also reported for HPP-treated soymilk [38] and amaranth protein isolates [26]. Like the results obtained here, the apparent viscosity of low-concentration amaranth protein dispersions (1%) was not affected by HPP. Still, for protein concentrations $\geq$ 5%, increasing pressure increased the apparent viscosity of the dispersions. This increase was larger for a protein concentration of 10% than 5% [26]. Furthermore, pressure alone can directly induce the formation of pea protein gels when the protein concentration exceeds 10% [39]. The increase in apparent viscosity may be associated with the formation of aggregates promoted by HPP, as previously discussed, which is favored at higher protein concentrations. The deviation from the Newtonian flow behavior was also markedly increased by HPP as shown by the more pronounced decrease in the flow behavior index *n* (Figure 6B).

A significant departure from the Newtonian behavior for the unprocessed dispersions was verified for protein concentrations above 7%, whereas for HPP-treated dispersions this departure was already observed for concentrations above 3%. A decline in the *n* value after HPP was also observed in soymilk, being more noticeable when the protein concentration was higher [38]. The relative difference between the viscosities obtained from the up and down curves, at the same shear rate of 50 s^{-1}, was used to approximately quantify the relative thixotropic structural breakdown (RTB) (Figure 6C). Clearly, HPP originated more structured dispersions and consequently higher RTB values within the whole range of protein concentrations analyzed.

The enzymatic treatments with MTG alone only caused significant changes in the flow profile and viscosity of the PPI dispersions for protein concentrations greater than 9%, increasing the apparent viscosity and the degree of the shear-thinning behavior, as illustrated in Figure 7.

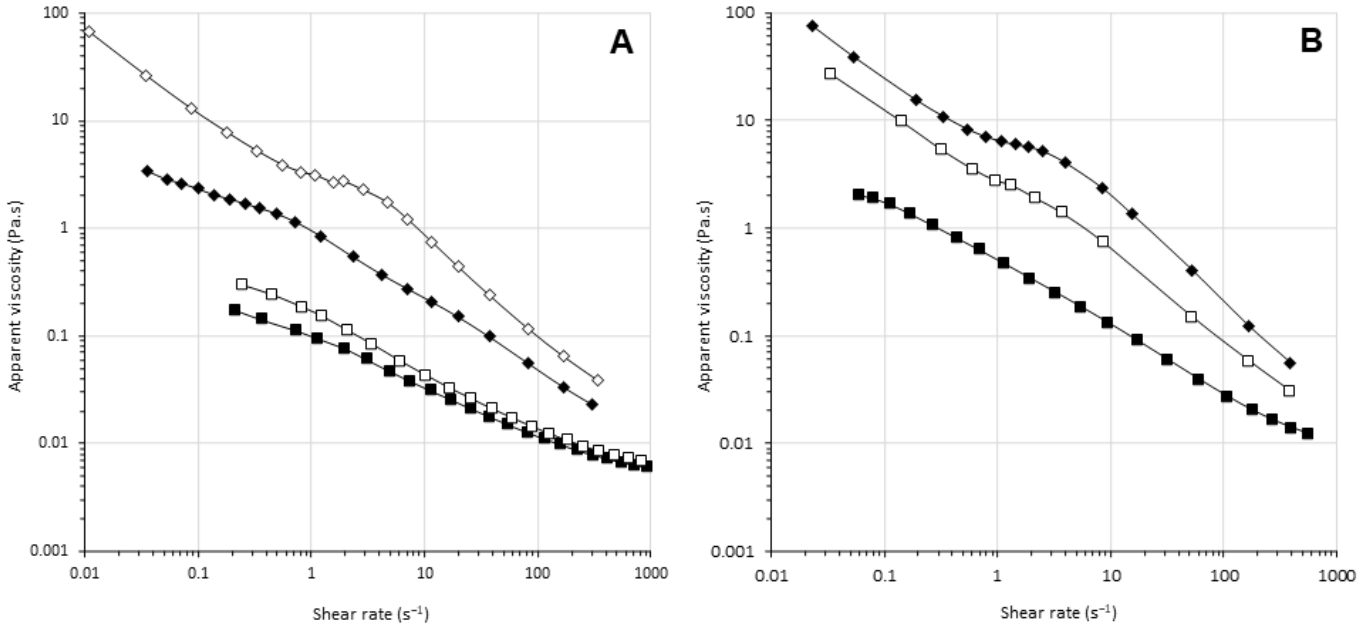

Figure 7. Flow curves (rising stress ramp) obtained for dispersions at (**A**) 9% and (**B**) 11%, at 20 °C, subjected to different treatments: (■) unprocessed samples; (□) MTG 30 U/(g protein); (♦) HPP 600 MPa, 15 min; (◇) MTG 30 U/(g protein) + HPP 600 MPa, 15 min. In (**B**), the 11% protein dispersion subjected to combined treatment by MTG and HPP gave rise to a gel.

Equally to HPP, there is very little information on the effects of MTG on the viscosity of protein dispersions, particularly plant-based proteins. Still, the increase in the apparent viscosity observed was expected, as the high-molecular-weight polymers, promoted by the crosslinking catalyzed by MTG, can reduce the mobility of the water in the protein network, increasing the flow resistance [6].

It is worth noting the flow profile observed for the 9% PPI dispersion subjected to the MTG + HPP combined treatment (Figure 7A) (like what is observed at 11% protein for each treatment alone, Figure 7B). Besides the pronounced shear-thinning behavior observed, these systems showed a tendency for a viscosity plateau around 1 s^{-1} but a further (asymptotic-like) increase in viscosity as the shear rate decreased, suggesting the existence of significant yield stress, probably associated with the dispersed nature of these systems. The combined MTG and HPP treatments seem to originate a synergistic effect, at least for pea protein concentrations above 7%, leading to a more pronounced increase in viscosity and on the shear-thinning flow profile. For 11% protein, the combined treatment even led to the formation of a gel.

Similar to the analysis previously performed for the other parameters under study (concentration of the dissolved proteins, free SH, H_0), the relative variation (Equation (2)) of the measured apparent viscosity at a shear rate of 50 s^{-1} caused by HPP, MTG, or the combined MTG + HPP treatments, by comparison to that measured for the control solutions (PPI dispersions without any treatment), was also investigated, as a function of the protein concentration (Figure 8).

Even for those protein concentrations where MTG alone did not cause any significant changes in viscosity and flow profile of the pea protein dispersions, the simultaneous MTG and HPP treatments lead to a much more significant increase in viscosity than HPP alone, probably related to the fact that the effect of the conformational alteration of proteins caused by HPP promotes the crosslinking action of the enzyme, leading to the formation of a larger number of aggregates and/or of a larger size, leading to a more noticeable increase in viscosity and the shear-thinning character when compared to unprocessed dispersions or dispersions treated separately by each process.

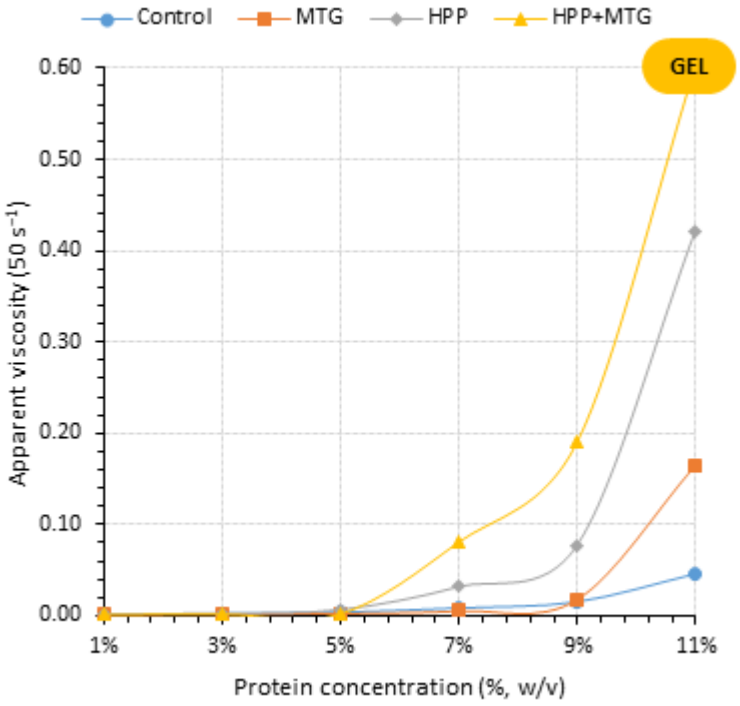

Figure 8. Variation of apparent viscosity measured at a shear rate of 50 s^{-1}, as a function of protein concentration under different processing conditions: (●) control; (■) in the presence of 30 U/g protein MTG; (♦) treated by HPP at 600 MPa, 15 min; (▲) processed by a combination of both treatments (MTG + HPP).

4. Conclusions

The combination of HPP and MTG shows promise as a tool for modifying food proteins and their techno-functional properties, enabling the development of new products and applications. However, the effects of these treatments depend on the selected processing conditions and the protein concentration. Throughout the study, synergistic and antagonistic effects were observed between HPP and MTG, particularly at higher protein concentrations. For example, the addition of MTG counteracted the increase in the concentration of the dissolved proteins promoted by HPP. On the other hand, for higher pea protein concentrations, the combined application of HPP and MTG resulted in a higher apparent viscosity than when the treatments were applied individually. HPP may promote the dissociation of aggregates and protein conformational expansion, making the proteins more accessible to MTG, which further modified the protein properties. These findings provide valuable insight into how combined HPP and MTG treatments can modify protein structure and tailor their techno-functional properties and hold significant implications for the food industry, both in theory and practice. The results indicate a considerable potential for innovation in the development process, either through the reduction of additive requirements or the creation of new textural properties. This could entail the invention of entirely new products or the enhancement of existing ones by introducing unique features. Such innovations could be facilitated using HPP and MTG, either individually or in combination. Future work could focus on expanding the range of protein types and concentrations studied to further investigate the synergistic and antagonistic effects of HPP and MTG on protein structure and techno-functional properties. Additionally, it would be interesting to explore the effects of different processing conditions, such as varying pressure and time for HPP and different concentrations of MTG, on the same protein samples to better understand the mechanisms underlying these effects. Furthermore, future studies could investigate the impact of the combined HPP and MTG treatments on other properties of proteins, such as emulsifying or gelling properties, and assess their potential for developing novel food products with tailored properties.

Author Contributions: Conceptualization, J.A.S. and J.A.L.-d.-S.; methodology, J.A.S. and J.A.L.-d.-S.; validation, R.P.Q. and L.G.F.; formal analysis, R.P.Q., N.M. and C.A.P.; investigation, R.P.Q., C.A.P. and L.G.F.; resources, J.A.S.; writing—original draft preparation, R.P.Q., N.M., C.A.P. and L.G.F.; writing—review and editing, L.G.F., J.A.L.-d.-S. and J.A.S.; supervision, J.A.L.-d.-S. and J.A.S.; project administration, J.A.S.; funding acquisition, J.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work received financial support from FCT/MCTES (LA/P/0008/2020 DOI 10.54499/LA/P/0008/2020, UIDP/50006/2020 DOI 10.54499/UIDP/50006/2020, and UIDB/50006/2020 DOI 10.54499/UIDB/50006/2020) through national funds. Carlos A. Pinto thanks Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior (FCT/MCTES) and European Social Fund (ESF) through NORTE 2020 (Programa Operacional Região Norte) for his PhD grant refs. SFRH/BD/137036/2018 and COVID/BD/153220/2023. This work was also supported by FCT/MCTES via the doctoral fellowship (SFRH/BD/111002/2015) of Rui P. Queirós.

Data Availability Statement: Data will be available upon request to the corresponding author.

Acknowledgments: This work received support and help from FCT/MCTES (LA/P/0008/2020 DOI 10.54499/LA/P/0008/2020, UIDP/50006/2020 DOI 10.54499/UIDP/50006/2020, and UIDB/50006/2020 DOI 10.54499/UIDB/50006/2020) through national funds. Carlos A. Pinto thanks Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior (FCT/MCTES) and European Social Fund (ESF) through NORTE 2020 (Programa Operacional Região Norte) for his PhD grant refs. SFRH/BD/137036/2018 and COVID/BD/153220/2023. This work was also supported by FCT/MCTES via the doctoral fellowship (SFRH/BD/111002/2015) of Rui P. Queirós.

Conflicts of Interest: The authors have no conflicts of interest to declare.

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