LWT - Food Science and Technology 137 (2021) 110436

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Changes of structural and techno-functional properties of high hydrostatic pressure (HHP) treated whey protein isolate over refrigerated storage

D. Carullo [a], G.V. Barbosa-Cánovas [b], G. Ferrari [a,c,*]

[a] Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano, SA, Italy
[b] Center for Nonthermal Processing of Food, Biological Systems Engineering, Washington State University, Pullman, WA, 99164-6120, USA
[c] ProdAl Scarl – University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano, SA, Italy

ARTICLE INFO

Keywords:
High hydrostatic pressure (HHP)
Whey protein isolate (WPI)
Refolding
Techno-functional properties
Denaturing gel electrophoresis (SDS-PAGE)

ABSTRACT

This work assessed the effect of storage time on the structure and functionality of HHP-treated whey protein isolate (WPI). Different pressures (100–600 MPa) and treatment times (15–30 min) were applied to aqueous WPI dispersions (5% w/v). The induced degree of unfolding was evaluated to select optimal HHP treatment conditions of WPI before storage at 4 °C. Conformational and techno-functional properties of untreated and optimally HHP-treated WPI samples were determined by UV–Vis and IR spectroscopy, foaming capacity, and interfacial tension measurements, respectively. Further tests of HHP-assisted hydrolysis of WPI were performed by α-chymotrypsin, bromelain, or their mixture (1:1 w/w), with the degree of hydrolysis (DH%) and electrophoretic patterns analyzed.

The maximum unfolding degree was detected after a treatment of 400 MPa and 15 min and, at these processing conditions, no aggregation occurred. However, the structural changes achieved upon HHP were gradually lost during storage through a first-order refolding process ($k_{REF.} = 0.031\ h^{-1}$), with restoring of native functionality.

Hydrolysis performances of selected enzymes towards WPI were significantly promoted by high-pressure. Interestingly, a clear synergistic effect of α-chymotrypsin and bromelain combination on the WPI hydrolysis yield was detected, which resulted in the highest protein rupture (DH = 17%).

1. Introduction

In the last decades, whey proteins have been gaining increasing attention at the industrial level, since they are used for the formulation/development of functional foods or as ingredients for cosmetics and pharmaceuticals, due to their well-recognized techno-functional properties such as solubility, foamability, thickening and emulsifying capacity (Ambrosi, Polenta, Gonzales, Ferrari, & Maresca, 2016; De Maria, Ferrari, & Maresca, 2016; Rodiles-Lopez et al., 2008).

However, several authors have highlighted that the change in protein functional properties, being structure-dependent parameters, could occur under a physical/chemical stress induced to the protein system (Queirós, Saraiva, & Lopes da Silva, 2018). Consequently, significant arrangements at structural level via polypeptide chain breakage or unfolding/aggregation phenomena could be produced (Bouaouina, Desrumaux, Loisel, & Legrand, 2006).

Recently, innovative approaches for structural modifications of food proteins, in replacement to conventional thermal processing methods, were proposed, such as those based on the use of pulsed electric fields, high-intensity pulsed light, high hydrostatic pressure, and high-pressure homogenization treatments (Carullo, Donsì, & Ferrari, 2020; De Maria et al., 2016; Siddique, Maresca, Pataro, & Ferrari, 2016, 2017; Xiang, Ngadi, Ochoa-Martinez, & Simpson, 2011).

High hydrostatic pressure (HHP) is the most utilized nonthermal technology at the industrial scale, especially for microbial inactivation processes due to its minimal impact on organoleptic, nutritional, and sensorial properties of foods (De Maria et al., 2016; Larrea-Wachtendorff, Tabilo-Munizaga, & Ferrari, 2019). However, HHP treatments may also trigger the conformational disordering of food proteins, as well as affect their main techno-functional properties, depending on pressure, time and temperature applied (Ambrosi, Polenta, Gonzalez, Ferrari, & Maresca, 2016; Rodiles-Lopez et al., 2008).

According to Le Chatelier's principle, increasing hydrostatic pressure shifts the equilibrium towards a minimization of protein specific volume

* Corresponding author. Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano, SA, Italy.
E-mail address: gferrari@unisa.it (G. Ferrari).

https://doi.org/10.1016/j.lwt.2020.110436
Received 9 July 2020; Received in revised form 6 October 2020; Accepted 21 October 2020
Available online 23 October 2020

(Espinosa, Caffarena, & Grigera, 2019). This is due to the penetration of water molecules surrounding proteins into the polypeptide core, which then gradually fill and disassemble cavities initially solvent-free, thus destabilizing the tertiary/quaternary structures and, eventually, leading to unfolding (de Oliveira & Silva, 2015, 2017). Moreover, under these conditions, a greater exposure of free sulfhydryl groups occurs, with subsequent increase in hydrophobic interactions (De Maria et al., 2016; Khan, Mu, Sun, Zhang, & Chen, 2015).

As far as whey proteins are concerned, several works showed that HHP-assisted unfolding could enhance both their techno-functional properties (Lim, Swanson, & Clark, 2007; Lopez-Fandino, 2006; Padiernos, Lim, Swanson, Ross, & Clark, 2009) and the yields of enzymatic hydrolysis, with the latter generally performed to obtain peptides with higher bioactivity and lower allergenicity than native proteins (Ambrosi et al., 2016; Blayo, Vidcoq, Lazennec, & Dumay, 2016).

For instance, Lim et al. (2007) demonstrated that the application of HHP treatments at 300 MPa for 15 min significantly promoted the foam stability of whey proteins concentrate (WPC) with respect to untreated samples, thus suggesting the potential utilization of WPC as fat-replacers in the formulation of ice creams and whipping creams. Ambrosi et al. (2016) concluded that the great unfolding degree of WPC achieved at 400 MPa caused the unmasking of previously inaccessible hydrolytic sites, which promoted both rates and yields of reaction over samples hydrolyzed at ambient pressure, independently of the utilized proteolytic enzyme (α-chymotrypsin, bromelain). However, the authors did not investigate the effect of a combination of enzymes on the performances of HHP-assisted hydrolysis of whey proteins, whose concomitant action may potentially lead to an increase in the degree of protein breakage over single enzymes. This aspect is of utmost importance in order to furtherly widen their range of application, thus expanding market opportunities (Segat et al., 2014).

To the best of our knowledge, only a few works studied the reversibility of HHP-induced unfolding of proteins derived from whey (e.g. β-Lactoglobulin) as a function of temperature and pH during short storage time (t < 2 days) (Belloque, Chicon, & Lopez-Fandino, 2007; Ikeuchi et al., 2001; Møller, Stapelfeldt, & Skibsted, 1998). Moreover, none of them were addressed to demonstrate whether the potential occurrence of refolding after pressure release would have an influence on whey proteins functionality, which might have allowed to define the proper storage conditions for their potential industrial exploitation.

Therefore, this work aimed to investigate the impact of storage time on structural and techno-functional properties of whey proteins previously modified by HHP treatments, as well as to assess the effectiveness of using a combination of enzymes to further enhance the hydrolysis yields under pressure. In particular, the objectives of this study included: (i) the analysis of the effect of pressure level and processing time on the extent of proteins unfolding; (ii) the evaluation of the stability over time of structural and techno-functional properties of untreated and HHP-treated whey proteins; and (iii) the yield determination of the hydrolysis reaction, carried out at high pressure (HHP, 400 MPa) or ambient (0.1 MPa) pressure, with single proteolytic enzymes or their combination.

2. Materials and methods

2.1. Sample preparation and chemicals

Lyophilized whey protein isolate (WPI, UltraWhey 90 instant), derived from sweet cheese whey and purchased from Volac International Ltd. (Orwell, UK), was used in this work and stored under refrigerated conditions (T = 4 °C) until processing. The weight composition of WPI was as follows: 90% proteins, 1.0% fat, 2.5% lactose, 2.0% ash, and 4.5% moisture. The protein fraction included β-Lactoglobulin (β-Lg, 50–60%), Glycomacropeptide (GMP, 15–20%), α-Lactalbumin (α-La, 15–20%), Bovine Serum Albumin (BSA, 1–2%), Immunoglobulin G (IgG, 1–2%), Immunoglobulin A (IgG, < 1%), and Lactoferrin (Lf, < 1%). All chemicals and enzymes used in this study were purchased from Sigma Aldrich (Milan, Italy) unless otherwise specified. MilliQ water was used to dilute samples and prepare all reagents and buffers. WPI samples were obtained by dissolving the protein at a constant concentration (5% w/v) in a sodium phosphate buffer (50 mmol/L, pH = 7.5) and kept under gentle stirring in an ice-water bath until complete solubilization. The pH of the dispersions, which were stored under refrigerated conditions before use, was determined by pH-meter (S400 Seven Excellence, Mettler Toledo International Inc., Milan, Italy). Enzymatic solutions for hydrolysis were prepared by dissolving α-chymotrypsin, bromelain or their mixture (1:1 w/w) in a sodium phosphate buffer (50 mmol/L, pH = 7.5) at a constant concentration (2% w/v) and stored under refrigerated conditions (T = 4 °C) before usage.

2.2. High hydrostatic pressure processing

HHP treatments of WPI dispersions were performed in a U111 high-pressure multivessel system (UNIPRESS-Polish Academy of Sciences, Warsaw, Poland), as previously described in detail in the work of Larrea-Wachtendorff et al. (2019), coupled to a thermostatic bath for temperature control (Huber CC245 wl, Offenburg, Germany). The HHP unit can be operated at pressures up to 700 MPa in the temperature range of −40 to 100 °C. A silicon oil (Huber thermofluid M40.165/220.10, Offenburg, Germany) was used as pressurizing medium and as the main fluid in the thermostatic bath.

Preliminary tests allowed to assess the effect of HHP treatments on structural modifications induced on WPI, with the aim to identify the optimal processing conditions, as those granting the highest unfolding degree, which were then applied on samples for stability evaluation. To this purpose, samples of 2.5 mL of whey protein dispersions were packed into flexible pouches made of a polymer/aluminum/polymer film (OPP30-A19-LDPE70), avoiding headspace, and immediately processed at different pressures (P = 100, 200, 300, 400, 500, and 600 MPa) and treatment time (t = 15, and 30 min). All the experiments were executed at a temperature of 25 °C, while the compression and decompression rates were set at 8 MPa/s. Although the depressurization rate is known to affect the structural features of proteins (Fertsch, Müller, & Hinrichs, 2003), the U-111 system could not be set at variable pressure release rate, due to equipment limitations. At the end of each treatment, samples were collected in plastic tubes and immediately placed in an ice-water bath before analyses. Untreated samples (controls) were also collected and used as a reference for further characterization.

For the stability tests, the structural and techno-functional properties of untreated and optimally HHP treated WPI dispersions were observed by performing analyses immediately after treatment and after 1, 2, 3, 4, 7, and 10 days of refrigerated storage (T = 4 °C). Longer storage times were not considered due to microbial proliferation in both untreated and HHP pre-treated samples (data not shown).

HHP-assisted hydrolysis experiments were carried out adding the proteolytic enzymes (α-chymotrypsin, bromelain, or their mixture) to WPI samples at a constant enzyme/substrate ratio (1:10, w/w), and then subjecting the dispersions to HHP treatments ($P_{OPT.}$, $t_{OPT.}$) at the optimal activation temperature of the proteolytic enzyme utilized (37 °C for α-chymotrypsin; 45 °C for bromelain and enzymatic mixture). For the sake of comparison, the hydrolysis of untreated samples was performed also at ambient pressure (0.1 MPa). The hydrolysis reaction was stopped via enzymatic inactivation, carried out by heating all the samples at 100 °C for at least 5 min. The hydrolysates were stored in an ice-water bath for further analyses.

2.3. Stability tests: analysis of WPI dispersions

2.3.1. Free sulfhydryl (-SH) groups

The estimation of free sulfhydryl groups (-SH) has been successfully used to study conformation variations induced upon HHP treatments of proteins from different sources, such as milk whey (Ambrosi et al.,

2016), egg white (Quirós, Chichón, Recio, & López-Fandiño, 2007), soybean (Li, Zhu, Zhou, & Peng, 2012) and squid (Jin et al., 2015).

In this work, the content of free -SH groups of both untreated and HHP treated WPI dispersions was analyzed according to the method of Ellman (1959), with slight modifications. The protein dispersions were diluted to a final concentration of 2 g/L with a 50 mmol/L Tris-HCl buffer (pH = 7.0) in 15 mL plastic tubes. Subsequently, 2.75 mL of diluted protein were mixed with 0.25 mL of a 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) solution (1 g/L) in 50 mmol/L Tris-HCl buffer. A spectrophotometer V-650 (Jasco Inc. Easton, MD, USA) was used to measure the absorbance at 412 nm against a blank (2.75 mL of 50 mmol/L Tris-HCl buffer + 0.25 mL of DTNB reactant), after an incubation time of 30 min. The concentration of free -SH groups was calculated as reported by Siddique, Maresca, Pataro, and Ferrari (2016), and the results were expressed in μmol free -SH groups/g protein.

2.3.2. FTIR measurements

The evolution of secondary structure components of untreated and HHP treated WPI dispersions was monitored utilizing an IR spectrophotometer (FTIR-4100, Jasco Europe Srl, Italy), with the spectra collected at a constant resolution (2 cm^{-1}) and a fixed number of scans (64) in the Amide I region (1700 - 1600 cm^{-1}). Background spectra were also collected and used to remove spectral signals that originated from air, moisture, and coating materials on reflecting mirrors along the IR radiation path. The resulting averaged spectra, each deriving from 9 repetitions per sample, were smoothed with an eleven-point under adaptive-smoothing function to remove any eventual noise. Finally, the baseline modification was applied for each spectrum.

2.3.3. Foamability (FA) and foam stability (FS)

The foaming capacity of WPI samples during refrigerated storage was determined according to the method of Segat et al. (2014), with some slight modifications. 10 mL of WPI dispersion were placed in a 50 mL graduated tube and homogenized at 10,000 rpm for 2 min using an Ultra Turrax T25 disperser (IKA Werke GmbH & Co., DE). At the end of each homogenization cycle, the rotor-stator head was gently lifted to avoid affecting the volume of the formed foam. Foamability (FA) and foam stability (FS) were calculated according to Eq. (1) and Eq. (2):

$$FA\ (\%) = \frac{V_{F,\,0}}{V_L} \cdot 100 \tag{1}$$

$$FS\ (\%) = \frac{V_{F,\,30\ min}}{V_{F,\,0}} \cdot 100 \tag{2}$$

where V_L, $V_{F,0}$, and $V_{F,30min}$ represent the initial volume of WPI dispersion and the volumes of the foam formed immediately after whipping (time 0) or after standing at room temperature for 30 min, respectively.

2.3.4. Air-water interfacial tension

Interfacial tension at the air-water interface was measured by using the method of the pendant drop, as thoroughly described by Donsì, Sessa, and Ferrari (2012). Drops (5–10 μL in volume) of WPI dispersions, prepared at a constant concentration (1 g/L), were produced using a micrometric syringe (D = 0.71 mm), with the needle put in an empty transparent cuvette. Data of air-water interfacial tension were collected via CAM200 tensiometer (KSV Instruments, Finland), consisting of an experimental cell, an illuminating and viewing system, and a data acquisition system. After the digitalization of captured drop frames, the interfacial tension was calculated as a function of the curvature radius at the apex. The shape of the captured drop images was fitted to the Young-Laplace equation (Eq. (3)) which correlates the interfacial tension to the shape of the drop:

$$\frac{d\varnothing}{ds} = 2 + \beta Z - \frac{\sin\varnothing}{X} \tag{3}$$

$$\beta = -\frac{g\sigma q^2}{\rho_L} \tag{4}$$

where φ is the angle of the tangent at the point (X, Z), s is the linear distance along the drop profile, dφ/ds corresponds to the radius of curvature at the point (X, Z) and β is the shape parameter, given by Eq. (4), where g is the gravitational constant, ρ_L is the effective density of the liquid drop, σ is the surface or interfacial tension, expressed in mN/m, and q is the radius of curvature at the origin. For all the samples, the interfacial tension was monitored up to 1.5 10^3 s from the instant of drop formation by sequential acquisition of drop frames at specified time intervals (5 s). Higher observation times were not tested due to the detachment of the pendant drop from the syringe needle.

2.4. Degree of hydrolysis

The degree of enzymatic hydrolysis (DH, %) of untreated and HHP treated samples was evaluated by measuring the o-phthaldialdehyde (OPA) reaction (Nielsen, Petersen, & Dambmann, 2001). OPA reagent was prepared by dissolving sodium tetraborate decahydrate, sodium dodecyl sulfate (SDS), o-phthaldialdehyde 97% (OPA), and dithiothreitol 99% (DTT) in a deionized water solution. A serine solution (0.1 g/L) in deionized water was used as standard. For each measurement, 3 mL of OPA reagent were added to 400 μL of deionized water (blank), serine solution (standard), or WPI samples (untreated, HHP treated). The degree of hydrolysis was spectrophotometrically measured at 340 nm after 2 min of reaction, as previously described by Hardt, van der Goot, and Boom (2013).

2.5. Reducing SDS-PAGE

SDS-PAGE electrophoresis was carried out under reducing conditions as described by O'Loughlin, Murray, Kelly, Fitz Gerald, and Brodkorb (2012), with slight modifications. To this purpose, A TV100Y twin-plate mini-gel unit equipped with an Apelex power supply (APELEX-Massy, France) was used. Briefly, untreated and hydrolyzed at ambient and high pressure WPI samples were diluted in a Tris-HCl buffer (0.125 mol/L, pH = 6.8) containing SDS (2% w/w), glycerol (10% w/w), bromophenol blue (0.02% w/w) and β-mercaptoethanol (5% w/w) as a reducing agent. Separating (12%) and stacking (6%) polyacrylamide gels were added with 50 μL of ammonium persulfate solution (10% w/v) and 5 μL of N, N, N', N'-tetra methylethylenediamine (TEMED), right before being poured in the electrophoretic system. 5 μL of all samples were loaded into the prepared gels, together with a pre-stained Protein Marker (peqGOLD, 10–260 kDa), and run at constant voltage (100 V) for 1 h. Afterward, gels were recovered from the equipment and stained with a staining solution (0.1% Coomassie Brilliant Blue R 250, 10% acetic acid, 20% isopropanol) overnight. A de-staining solution (30% methanol, 10% acetic acid) was used until the background became clear.

2.6. Statistical analysis

All treatments and analyses were repeated three times unless otherwise specified. The mean values and standard deviations (SD) of the experimental data were calculated. Statistically significant differences ($p \leq 0.05$) among the averages were evaluated using a one-way analysis of variance (ANOVA) and Tukey's test ($p \leq 0.05$). Statistical analysis was carried out using IBM SPSS Statistics 20 software (IBM Corp., Armonk, New York, USA). The Pearson product-moment correlation coefficient was used to measure the strength of the linear relationship between each couple of the investigated variables.

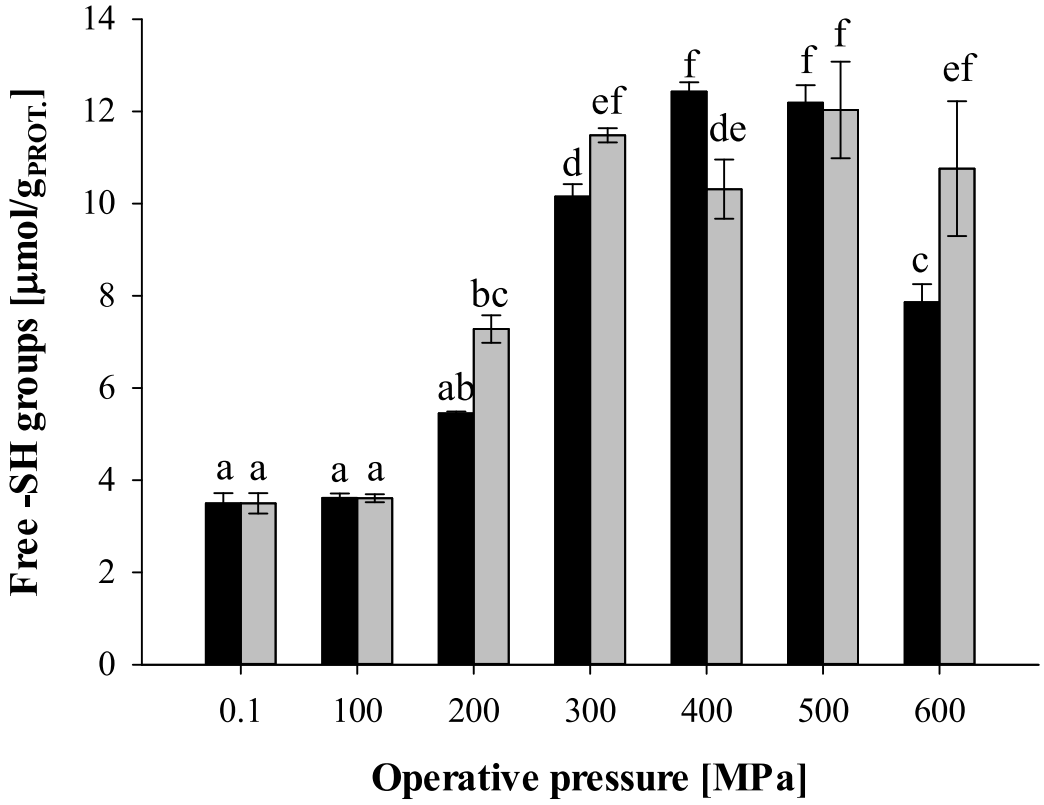

Fig. 1. Concentration of free sulfhydryl groups (µmol/$g_{protein}$) from untreated and HHP treated WPI dispersions, as a function of pressure level (100–600 MPa) and treatment time (black bar: 15 min, light grey bar: 30 min). Different letters above the bars indicate significant differences among the mean values ($p \leq 0.05$).

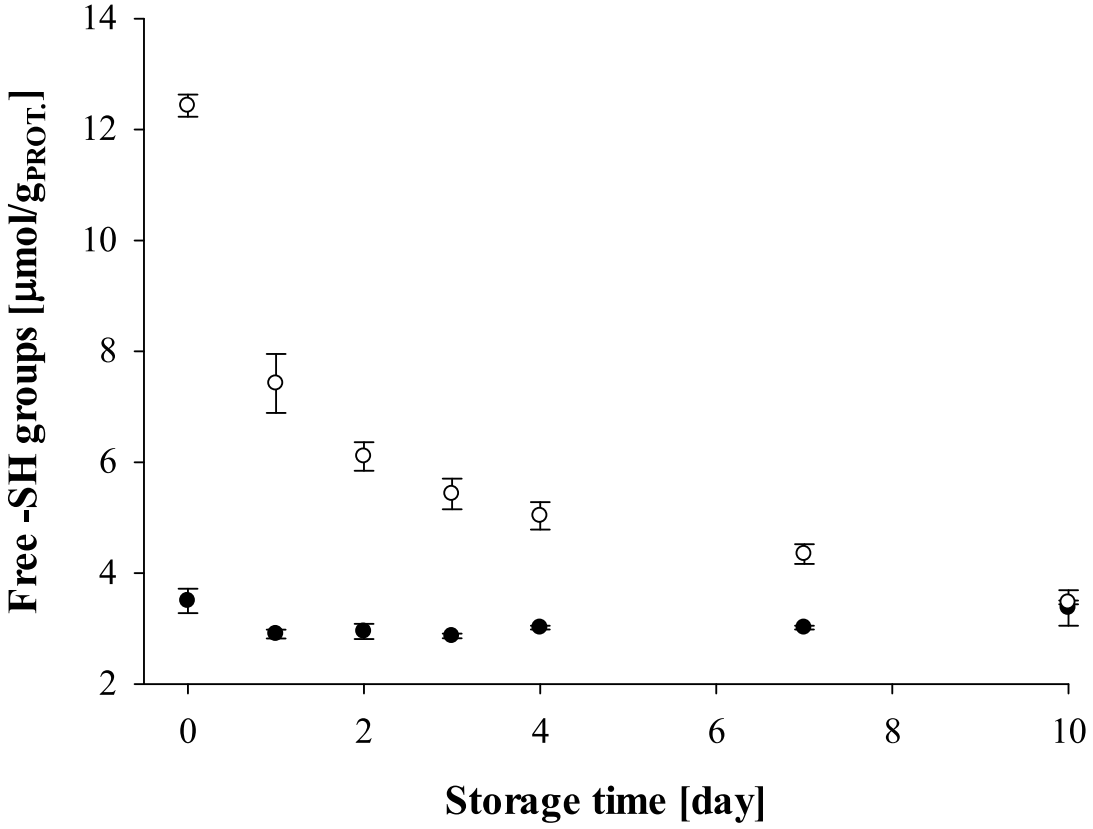

Fig. 2. Changes of free sulfhydryl groups concentration (µmol/$g_{protein}$) from untreated (black circle) and HHP (white circle) treated (400 MPa, 15 min) WPI dispersions, as a function of the storage time (0–10 days). Standard deviations were used as error bars ($p \leq 0.05$).

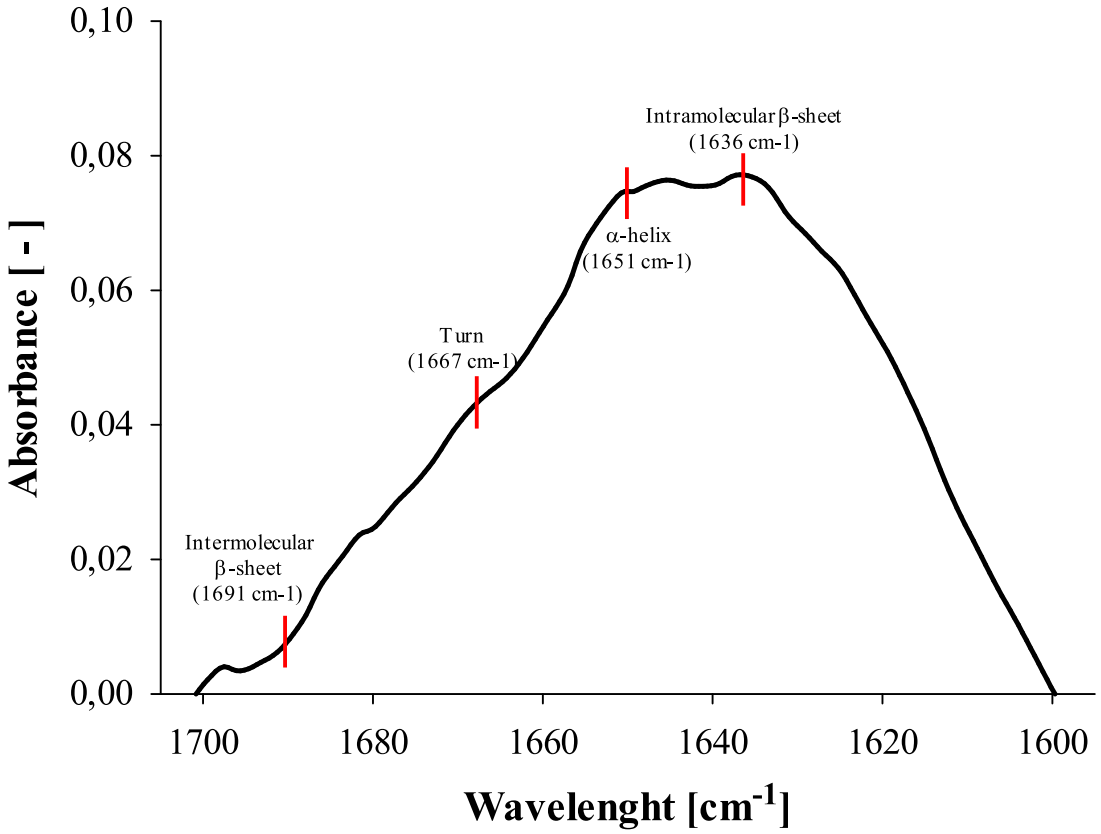

Fig. 3. FTIR spectrum of untreated WPI sample plotted as a function of the wavenumber (cm^{-1}).

3. Results and discussion

3.1. Impact of HHP treatments on WPI unfolding

Fig. 1 shows the content of free -SH groups in untreated and HHP treated WPI dispersions, as a function of pressure intensity (100–600 MPa) and processing time (15–30 min). Regardless of the duration of the treatment, the application of a pressure level of 100 MPa was insufficient to significantly ($p > 0.05$) modify the conformational structure of WPI, being the free -SH groups content similar to that detected in untreated samples. Instead, more severe HHP treatment conditions (P = 200–400 MPa) contributed to trigger partial unfolding of WPI samples due to unmasking of previously buried thiol groups which linearly increased ($p \leq 0.05$) with the pressure intensity. The highest concentration of thiol groups (12.43 ± 0.21 μmol -SH/$g_{PROT.}$) was detected at 400 MPa for 15 min. In these conditions, neither aggregation nor polypeptides size-reduction was detected through particle size distribution (PSD) measurements, being the PSD curves of untreated and HHP treated samples almost overlapped (data not shown). Further increases of pressure up to 600 MPa did not significantly ($p > 0.05$) intensify the WPI unfolding, independently of treatment time. The observed behavior could be attributed to the likely concomitant occurrence of protein aggregation through inter-/intramolecular SH/SS exchange reactions, promoted at P > 400 MPa, as already reported by De Maria et al. (2016). Similar trends were observed by Li et al. (2012) in a study on the influence of HHP treatments on structural properties of soy protein isolate (SPI). The authors found that a significant increase in free -SH content of SPI was induced in the pressure range of 200–300 MPa while, at higher pressure, it progressively decreased due to the predominance of aggregation over unfolding.

Based on the results shown so far, additional investigations on the influence of HHP pre-treatments on structure and functionality of WPI during refrigerated storage, in comparison with untreated samples, were performed setting HHP parameters at 400 MPa and 15 min.

3.2. Effect of storage time on WPI structural properties

The concentration of free -SH groups in untreated and HHP pre-treated WPI dispersions was monitored over time to gain insight on the kinetics of polypeptide chains rearrangement after pressure release (Fig. 2). Untreated WPI showed no appreciable changes in thiol reactivity with storage time, thus indicating a certain degree of conformational stability under refrigerated conditions, as previously observed by Ambrosi et al. (2016) and Siddique et al. (2017) within the range 60–120 min. Conversely, pressurized WPI samples exhibited a significant ($p \leq 0.05$) reduction in free -SH content during storage, until an equilibrium condition was reached. In particular, as shown in Fig. 2, almost 80% of free thiol groups of WPI, previously made accessible by HHP processing, was lost approximately during the first 3 days of storage. Then, the free -SH groups slowly reduced with time to attain concentrations similar to those of untreated samples ($p > 0.05$).

In agreement with previous studies (Ambrosi et al., 2016; Belloque et al., 2007; Møller et al., 1998), the gradual loss in thiol reactivity detected in HHP pre-treated WPI could be ascribed to the occurrence of refolding, taking place during storage, through which proteins regain the original structure, reaching a thermodynamically stable configuration. Møller et al. (1998) found that the exposure of thiol groups in β-Lg (0.2–0.5% w/v in water) processed at 100–250 MPa for 30 min decreased over time after pressure release, by following an exponential decay kinetics with a refolding rate ($k_{REF.}$) of 0.104 h^{-1} (T = 5 °C). Additionally, based on the transition state theory, the authors reported a negative activation entropy ($\Delta S = -247$ kJ/kmol·K), corroborating the hypothesis of proteins refolding due to the evolution of the system towards a more ordered state.

It is worth mentioning that β-Lg, the most abundant protein in whey, might be responsible for the overall functionality of WPI (Bouaouina

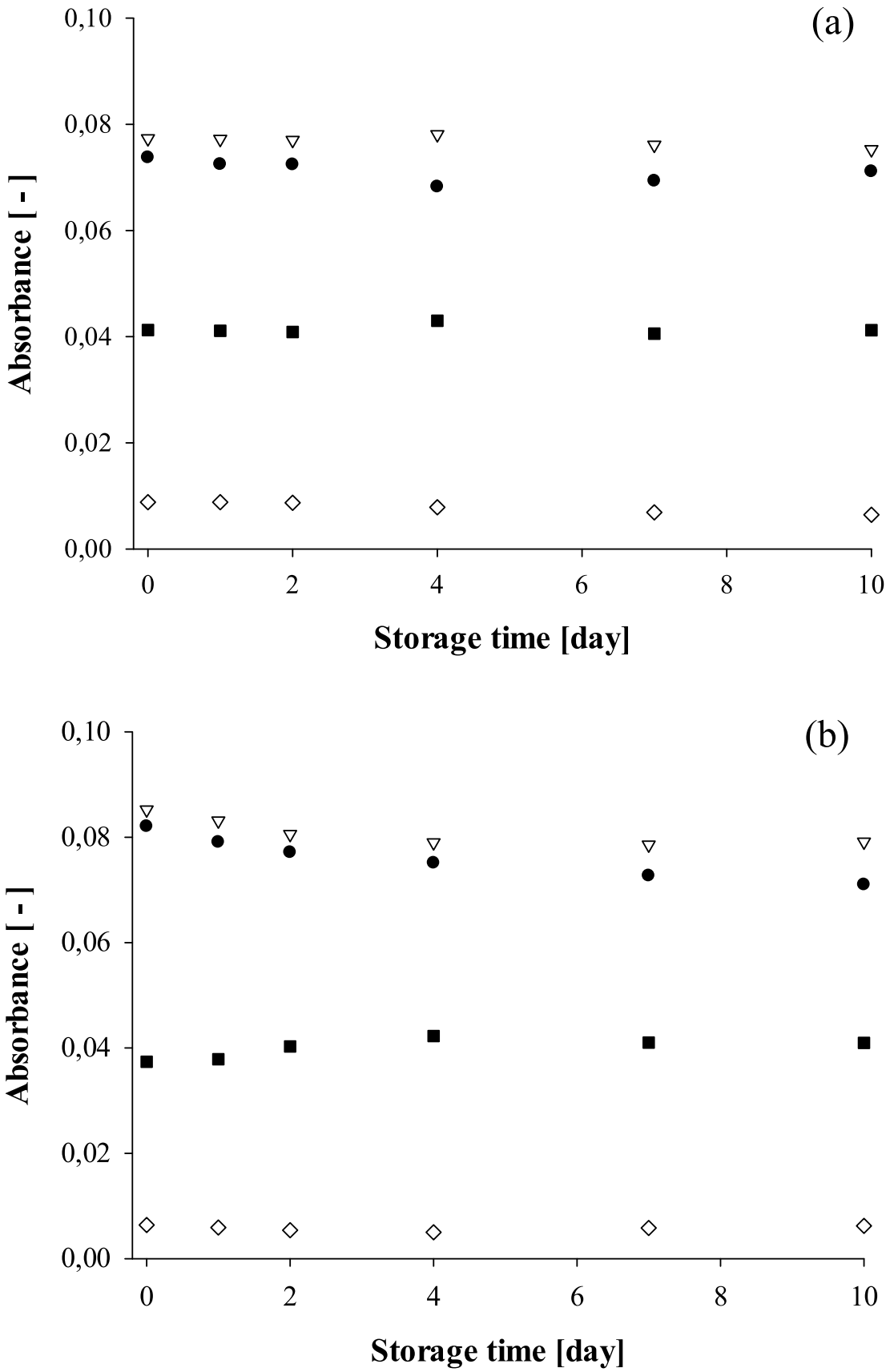

Fig. 4. Changes of the secondary structure main components, namely α-helix (black circle), intramolecular β-sheet (white triangle), turn (black square) and intermolecular β-sheet (white rhombus), of untreated (a) and HHP treated at 400 MPa for 15 min (b) WPI dispersions, as a function of the storage time (0–10 days).

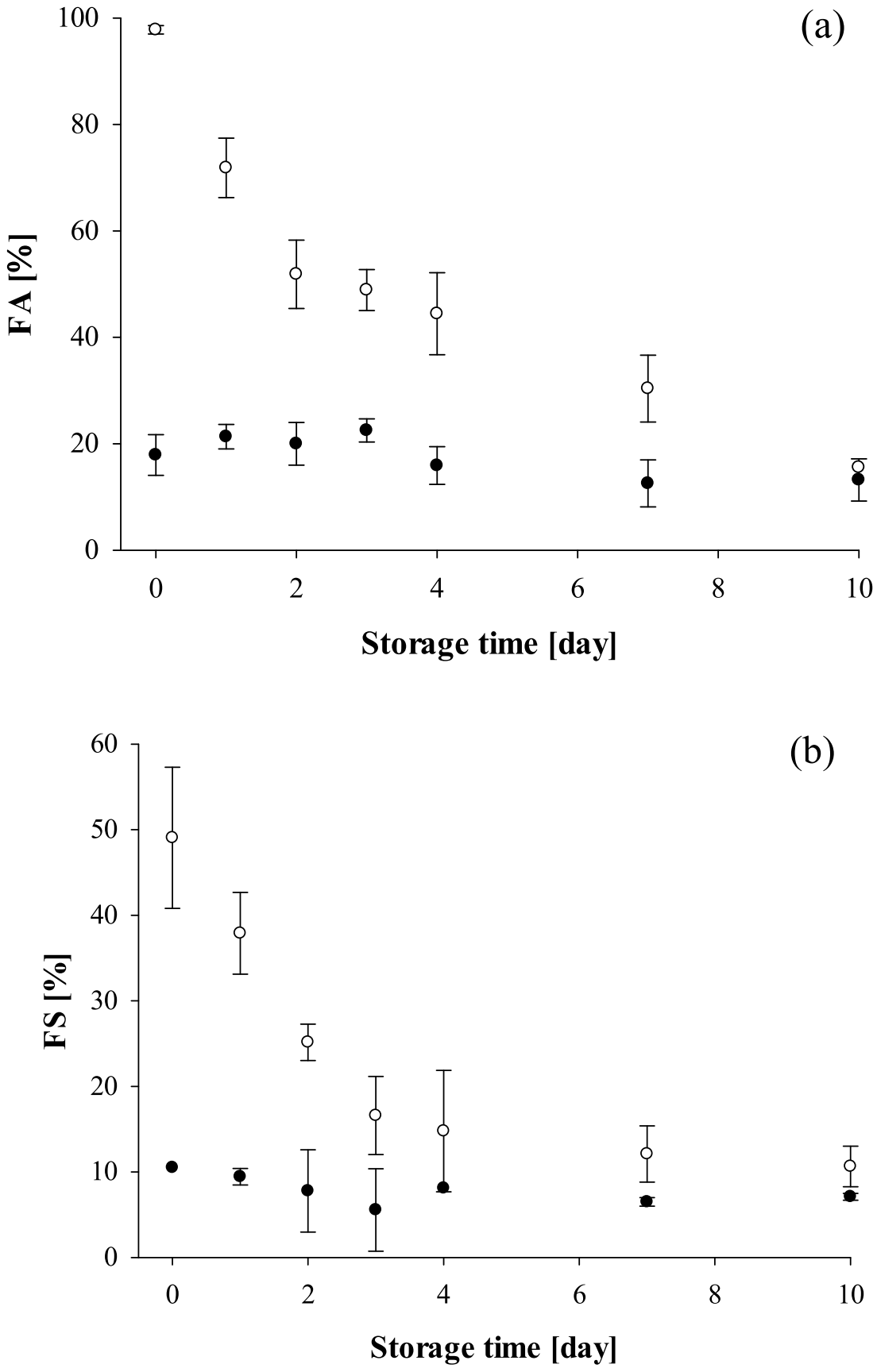

Fig. 5. Changes of foamability (a) and foam stability (b) from untreated (black circle) and HHP (white circle) treated (400 MPa, 15 min) WPI dispersions, as a function of the storage time (0–10 days). Standard deviations were used as error bars ($p \leq 0.05$).

et al., 2006). Hence, taking this into account, the predictive exponential-decay model of Møller et al. (1998) could be used to fit the data of Fig. 2. The model described quite well ($R^2 = 0.976$) the data, with a refolding rate ($k_{REF.}$) of 0.031 h^{-1} at 4 °C. The significantly lower refolding rate observed in this work could be due to the processing conditions and protein concentration utilized. More severe HHP treatment intensities applied to WPI samples (P = 400 MPa) caused greater structural modifications, thus longer storage times were required for protein refolding and recovering of the initial state. Moreover, protein-protein interactions generally depend on their concentration in the solubilizing medium (Wang & Roberts, 2018). Therefore, the more intense "crowding" effect achieved at the WPI concentration tested in this work might have decreased the average inter-protein distance, as well as reduced the fluctuation of hydrophobic residues, thus significantly slowing down the refolding rate. Interestingly, data not shown revealed that the acidity of untreated and HHP treated WPI dispersions, evaluated by means of pH measurements, did not appreciably change over the investigated duration of the storage phase.

The reversible nature of pressure-induced unfolding of WPI at 400 MPa for 15 min was confirmed by the results of FTIR measurements. Fig. 3 shows a typical FTIR spectrum of untreated whey proteins in the Amide I region (1700–1600 cm^{-1}). The identification of peaks corresponding to different secondary structure components of WPI was performed based on the assignments by Barth (2007). The secondary structure of whey proteins showed two major peaks at 1636 cm^{-1} and 1651 cm^{-1}, due to the greater contribution of intramolecular β-sheet and α-helix components, and two smaller peaks characteristics of turn (1667 cm^{-1}) and intermolecular β-sheet (1691 cm^{-1}) structures. In this work, the changes in WPI conformational structure during refrigerated storage were highlighted by plotting the absorbance of these peaks over time (Fig. 4), for both untreated (a) and optimally HHP pre-treated (b) WPI samples. In agreement with the results of Fig. 2, untreated WPI samples did not show any variation in terms of secondary structure components, which then remained constant during the entire storage period. Pressure treatments at 400 MPa for 15 min induced an evident alteration of WPI conformation, with increments in both α-helix and intermolecular β-sheet structures over untreated samples, counterbalanced by a reduction in intramolecular β-sheet and turn components. However, such induced conformational modifications by HHP were completely restored already after 2 days of refrigerated storage, above which any difference in characteristic absorbances of untreated and HHP treated WPI samples could be hardly evidenced. This trend is probably due to the elastic behavior of polypeptide chains under pressure, in good agreement with the previous findings of Larrea-Wachtendorff, Tabilo-Munizaga, Moreno-Osorio, Villalobos-Carvajal, and Perez-Won (2015) who detected a full reversibility of pressure-induced secondary structure variation in palm ruff muscles during a refrigerated storage of 35 days.

3.3. Effect of storage time on WPI functionality

The foaming capacity of untreated and HHP pre-treated (P = 400 MPa, t = 15 min) WPI dispersions was assessed measuring foamability (FA, %) and foam stability (FS, %), whose time dependence during refrigerated storage is reported in Fig. 5a and Fig. 5b, respectively.

Untreated WPI samples were characterized by poor foaming properties (FA = 19%; FS = 10%), due to their compact structures which prevented the exposure of hydrophobic groups towards the aqueous medium. However, in the absence of any physical stress, FA and FS values did not appreciably change during storage, thereby confirming the stability of untreated WPI at 4 °C.

HHP treatments drastically enhanced the foaming capacity of whey proteins, which yielded significantly ($p \leq 0.05$) higher values of foamability (98%) and foam stability (49%), compared to untreated samples. Remarkably, a strong positive correlation was observed between the concentration of free -SH groups and the foaming capacity values, as reported in Table 1. This can be explained by the increased amount of hydrophobic residues upon unfolding which improved the protein capacity to absorb air bubbles at the air/water interface, in agreement with previous literature findings (De Maria et al., 2016; Rodiles-Lopez et al., 2008).

Table 1
Correlation coefficient among structural and techno-functional properties, namely free -SH groups concentration (μmol/$g_{prot.}$), foamability (%), foam stability (%) and air-water superficial tension (mN/m), of untreated (control) and HHP treated (P = 400 MPa, t = 15 min) WPI dispersions, observed along the whole refrigerated storage phase (0–10 days).

Properties	Free -SH groups	Foamability	Foam stability	Air-water superficial tension
Free -SH groups	–	0.959**	0.952**	−0.983**
Foamability	0.959**	–	0.956**	−0.946**
Foam stability	0.952**	0.956**	–	−0.951**
Air-water superficial tension	−0.983**	−0.946**	−0.951**	–

ns = not significant for $p > 0.05$; * = significant for $p \leq 0.05$; ** = significant for $p \leq 0.01$.
*** = significant for $p \leq 0.001$.

Fig. 5 also highlights that the foaming capacity of HHP treated samples was not maintained after pressure release since its values linearly decreased with storage time in the first 4 days until reaching similar values observed in untreated WPI samples. These data are consistent with the previously detected proteins refolding over storage (Fig. 2).

Additional information on WPI surface properties and, hence, on their ability to produce/stabilize foams were obtained from air/water interfacial tension measurements. Fig. 6 shows the dynamics of interfacial tension of untreated and HHP treated WPI dispersions (measured immediately after treatment (a) and after 1 (b), 4 (c) and 7 (d) days of refrigerated storage). Regardless of the storage time, the interfacial tension of the air/WPI dispersion system was about 52 mN/m at t = 0 s. Moreover, the curves of surface tension from both untreated and HHP treated samples decreased with the time elapsing from the pendant drop formation (0 s) and the last acquired frame (1500 s). This was likely due to the dynamic nature of proteins adsorption step at the gas/liquid interface, being driven by the concentration gradient between the bulk and boundary layer (Donsì et al., 2012). However, the observed trend was more pronounced for pressurized samples due to a greater surface hydrophobicity and enhanced mobility of polypeptide chains in the bulk phase upon partial unfolding. Similarly, Bouaouina et al. (2006) observed a more rapid surface tension reduction at the air/water interface for dynamic high-pressure (DHP) treated (P = 300 MPa) WPI dispersion with respect to control samples, caused by the improved unmasking of hydrophobic groups which enhanced the adsorption kinetics.

A significant correlation among time-averaged interfacial tension (Fig. 6), foam capacity (Fig. 5), and free -SH groups content (Fig. 2) of HHP treated WPI dispersions was found (Table 1). This confirmed the dependence of physicochemical properties governing the overall WPI functionality on the structural rearrangement occurring upon processing (unfolding) and storage (refolding).

3.4. Impact of HHP treatments and enzyme composition on WPI hydrolysis

The nature of proteolytic enzyme and its interaction with the substrate under pressure may strongly influence the extent of polypeptide chains rupture, as well as the molecular weights distribution of the generated peptides. Therefore, we also investigated the effect of an enzymatic cocktail, used in combination with HHP treatments, on the achieved degree of WPI hydrolysis, as compared with the utilization of

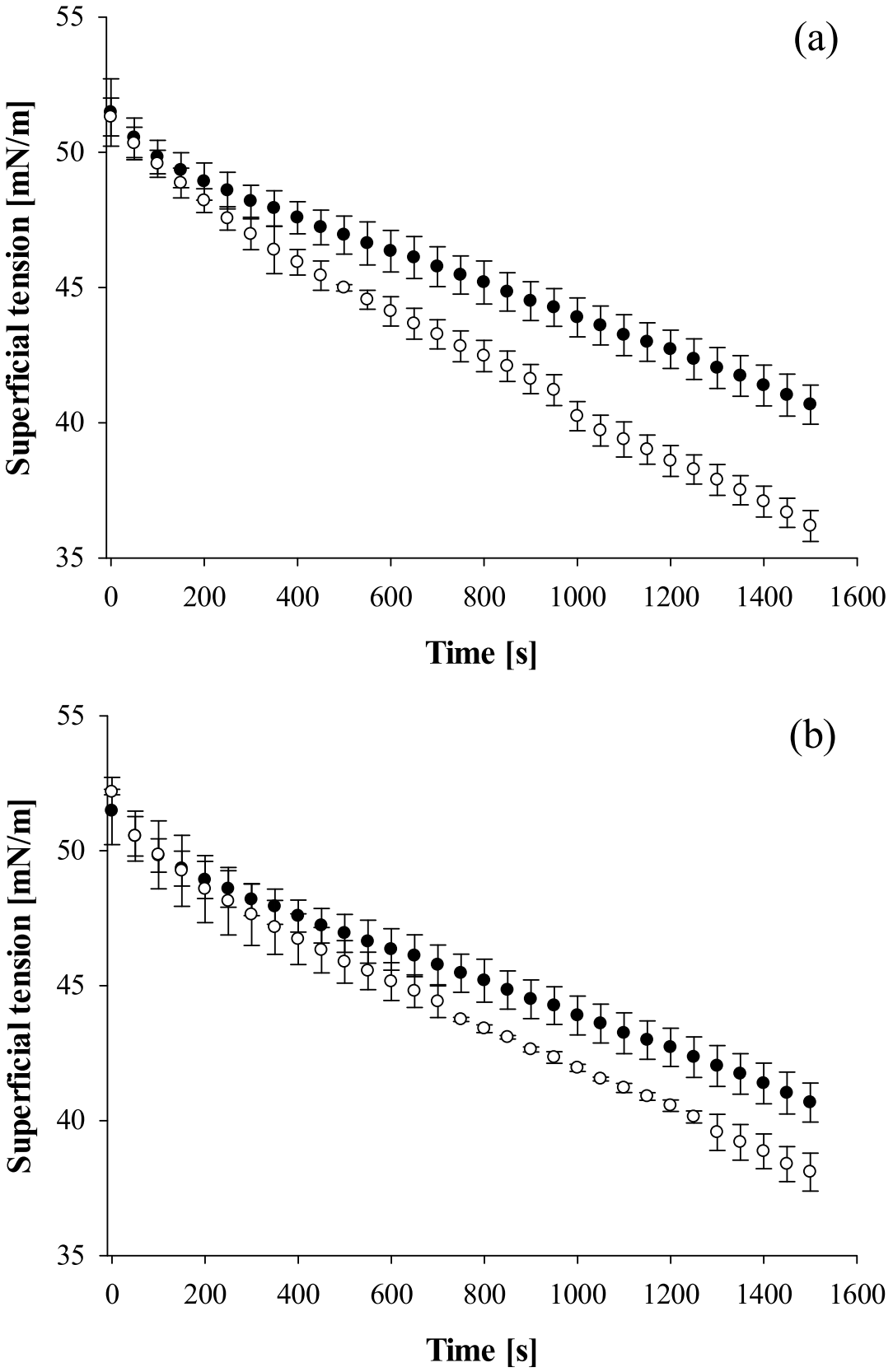

Fig. 6. Air-water superficial tension as a function of time from untreated (black circle) and HHP (white circle) treated (400 MPa, 15 min) WPI dispersions, observed at day 0 (a), day 1 (b), day 4 (c) and day 7 (d) of storage. Standard deviations were used as error bars ($p \leq 0.05$).

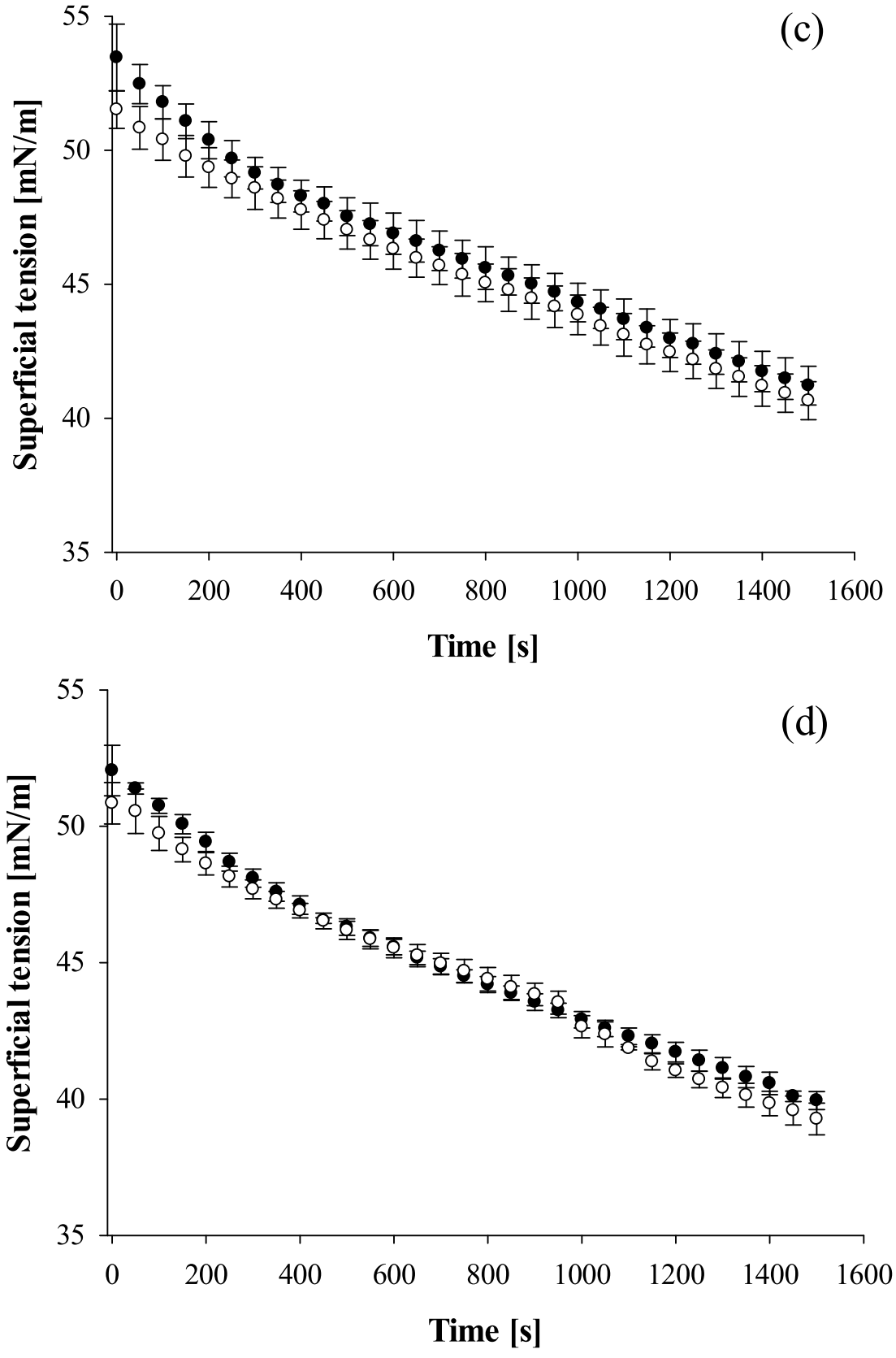

Fig. 6. (*continued*).

single enzymes. Fig. 7 shows the hydrolysis degree of HHP-assisted (P = 400 MPa, t = 15 min) enzymatic reaction of WPI with α-chymotrypsin, bromelain, or their mixture (1:1 w/w). For the sake of comparison, the results obtained by hydrolyzing with the same enzymes at ambient pressure (t = 15 min) untreated and HHP pre-treated WPI (P = 400 MPa) are reported.

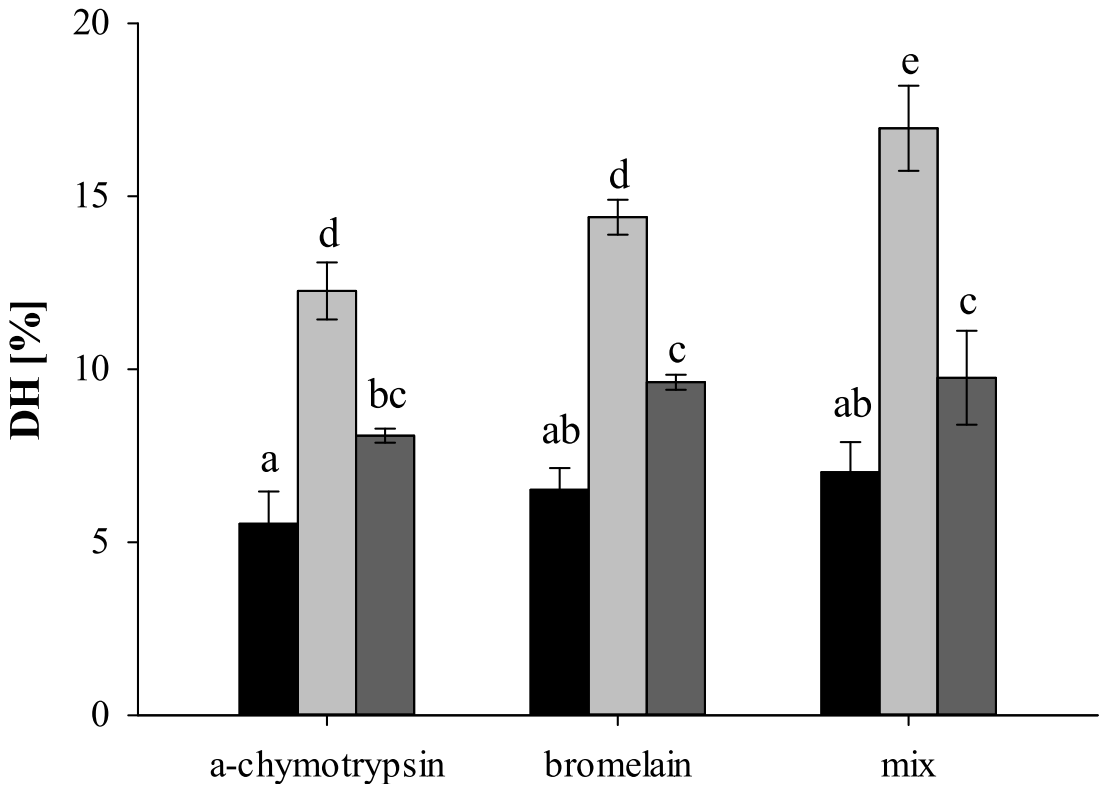

Fig. 7. Hydrolysis degree (%) of untreated (black bar) and HHP (light grey bar) treated (400 MPa) WPI samples in the presence of α-chymotrypsin, bromelain, and their mixture (1:1 w/w). The reaction time was set at 15 min. For comparison purposes, reaction yields of samples hydrolyzed at 0.1 MPa after HHP (dark grey bar) treatment (400 MPa, 15 min) are also reported. Different letters above the bars indicate statistical differences among the samples ($p \leq 0.05$).

Results demonstrated that the hydrolysis of control samples is almost independent on the type of enzyme utilized, being the values of DH% statistically similar ($p > 0.05$) among them. This can be explained by considering that untreated whey proteins are characterized by a highly folded structure with a reduced accessibility to active sites for the enzymatic attack (Ambrosi et al., 2016). Instead, WPI unfolding occurring during HHP treatments positively affected the extent of proteins rupture, due to the significant ($p \leq 0.05$) increase in DH% values over control samples, independently on the type of enzyme. Nonetheless, higher hydrolysis yields were observed when the enzymatic reaction was performed under high pressure rather than at ambient conditions (Fig. 7). The continuous conformational change of WPI upon HHP treatments likely increased the number of sites susceptible to enzymatic attack and the enzyme/substrate interaction, in good agreement with previous findings (Ambrosi et al., 2016; Belloque et al., 2007). It could be also hypothesized that the catalytic activity of α-chymotrypsin, bromelain, and their mixture (1:1 w/w) improved at 400 MPa, thus reducing the reaction time to achieve a given degree of protein rupture, in comparison with the hydrolysis performed at atmospheric pressure. The increase in α-chymotrypsin activity due to HHP treatments is supported by literature (Mozhaev, Kudryashova, & Bec, 1996). However, additional research is required to better elucidate the effect of pressure and time on the activity of both bromelain and the enzymatic mixture used in this work.

Interestingly, a clear synergistic effect by combining the two enzymes was detected when WPI hydrolysis was assisted by HHP treatments (Fig. 7), leading to a significant ($p \leq 0.05$) higher yield (DH = 17%) with respect to those obtained using α-chymotrypsin (DH = 12.3%) or bromelain (DH = 14.3%) alone. These results could be due to the different substrate specificity of the investigated proteolytic enzymes. In particular, α-chymotrypsin cleaves preferably peptide bonds on the C-terminal side of phenylalanine, tyrosine, tryptophan, and leucine (De Maria, Ferrari, & Maresca, 2017). Instead, bromelain catalyzes the selective rupture of protein sites with arginine, lysine, glutamic acid, glycine, ornithine, methionine sulfoxide, and alanine (Ee, Khoo, Ng, Wong, & Chai, 2019). Therefore, it could be speculated that the transient unfolding phenomena occurring upon HHP treatments caused a cumulative effect between both enzymes' specificity, thus further enhancing the hydrolysis performance.

The reducing SDS-PAGE profile of hydrolysates from untreated and high-pressure treated (400 MPa) WPI dispersions (Fig. 8), corroborated the data of Fig. 7. Untreated WPI was dominated by two major bands, being β-Lg (MW ≈ 17 kDa) and α-La (10 kDa < MW < 17 kDa), together with a lighter band, attributable to BSA, detected between 52 kDa and 72 kDa. A clear reduction in the intensity of the β-Lg band and the complete disappearance of α-La were observed in samples hydrolyzed by α-chymotrypsin at ambient pressure. These results are in good agreement with the findings of Kim et al. (2007), who highlighted the lower susceptibility of β-Lg to tryptic hydrolysis. Despite the slight but insignificant ($p > 0.05$) increase in DH% observed over α-chymotrypsin (Fig. 7), the different substrate specificity of bromelain or enzymatic mixture yielded different electrophoretic patterns of WPI (Fig. 8), which showed only a low-intensity band between 10 kDa and 17 kDa. Conversely, due to the increased effectiveness of HHP-assisted hydrolysis (Fig. 7), all WPI characteristic bands completely vanished on hydrolysates obtained under pressure, whatever was the utilized enzyme. The complete hydrolysis of WPI determined the formation of very small peptides that were not visible in the electrophoretogram, being presumably characterized by MW < 10 kDa. Overall, these data are consistent with the results previously obtained by other scientists, who demonstrated the key role played by high-pressure in generating structural modification of proteins, thus intensifying the enzymatic hydrolysis process (Ambrosi et al., 2016; De Maria et al., 2017; Jin et al., 2015; Quirós et al., 2007; Rodiles-Lopez et al., 2008).

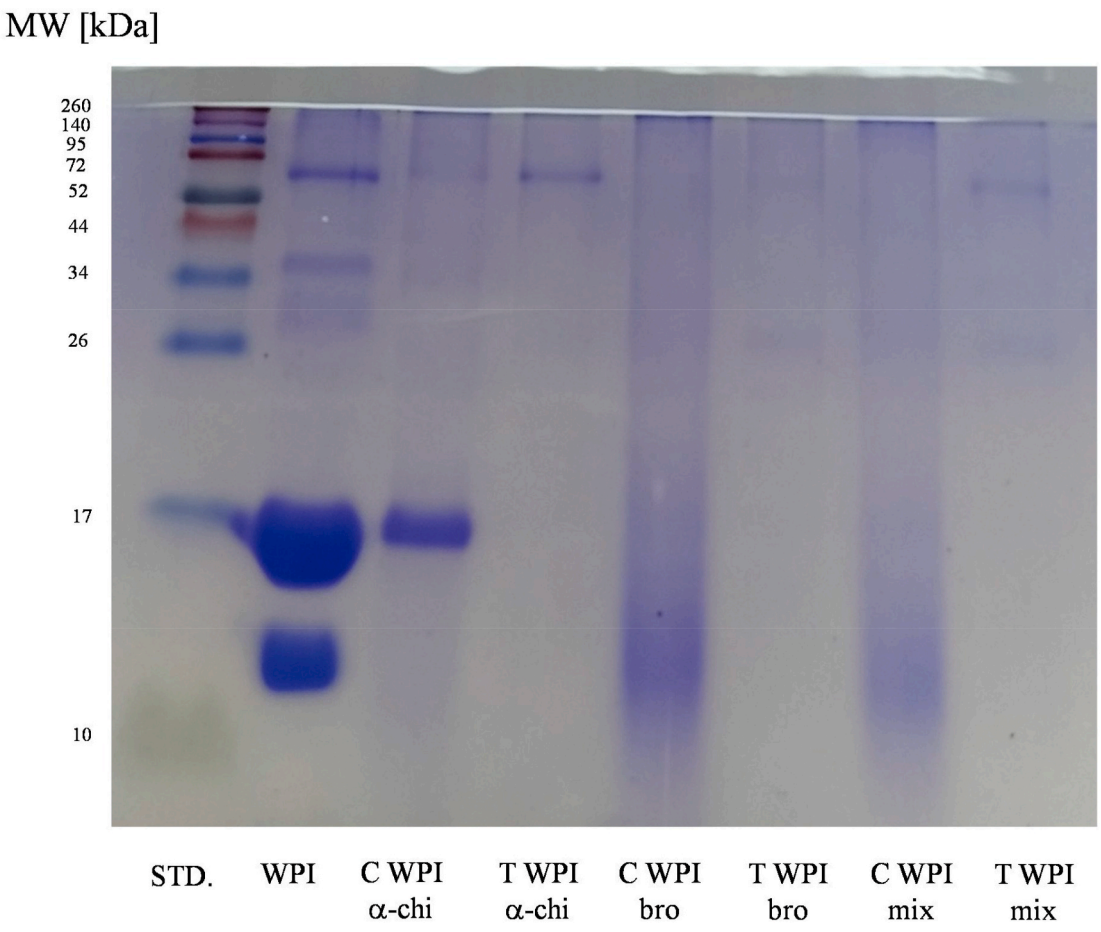

Fig. 8. Reducing SDS-PAGE patterns of untreated and HHP treated (400 MPa) WPI samples in the presence of α-chymotrypsin, bromelain, and their mixture (1:1 w/w). The reaction time was set at 15 min. STD – protein marker, WPI – untreated sample, C - sample hydrolyzed at ambient pressure (control), T – sample hydrolyzed during HHP processing.

4. Conclusions

This work investigated the impact of storage, in refrigerated conditions, on the structural and techno-functional properties of WPI, previously subjected to HHP treatments. Measurements of free -SH groups revealed that the changes in WPI conformation depended on both pressurization intensity and time, with the highest extent of unfolding detected at 400 MPa for 15 min. Under these conditions, a strong positive correlation between the conformational changes and the improved interfacial properties of WPI was found. However, after pressure release, proteins underwent a first-order refolding process which restored the secondary, tertiary, and quaternary structure of untreated samples, as well as their original functionality, mostly within three days of storage. As far as HHP-assisted enzymatic hydrolysis is concerned, the α-chymotrypsin/bromelain mixture remarkably improved the degree of protein breakage as compared to single enzymes, showing a clear synergistic effect due to an increase in the overall substrate specificity.

In conclusion, the results of this work provided useful additional insight into the utilization of HHP technology at mild conditions to modify the structural features of food proteins, whose time-dependent behavior may allow defining their processability at the industrial scale.

CRediT authorship contribution statement

D. Carullo: Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing. **G.V. Barbosa-Cánovas:** Conceptualization, Writing - review & editing. **G. Ferrari:** Conceptualization, Formal analysis, Methodology, Resources, Supervision, Writing - review & editing.

Acknowledgments

Daniele Carullo is indebted to the University of Salerno (Dept. of Industrial Engineering) for having provided funds for his post-doctoral scholarship.

References

Ambrosi, V., Polenta, G., Gonzalez, C., Ferrari, G., & Maresca, P. (2016). High hydrostatic pressure assisted enzymatic hydrolysis of whey proteins. *Innovative Food Science & Emerging Technologies, 38*, 294–301.

Barth, A. (2007). Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta, 1767*, 1073–1101.

Belloque, J., Chicon, R., & Lopez-Fandino, R. (2007). Unfolding and refolding of α-lactoglobulin subjected to high hydrostatic pressure at different pH values and temperatures and its influence on proteolysis. *Journal of Agricultural and Food Chemistry, 55*, 5282–5288.

Blayo, C., Vidcoq, O., Lazennec, F., & Dumay, E. (2016). Effects of high pressure processing (hydrostatic high pressure and ultra-high pressure homogenisation) on whey protein native state and susceptibility to tryptic hydrolysis at atmospheric pressure. *Food Research International, 79*, 40–53.

Bouaouina, H., Desrumaux, A., Loisel, C., & Legrand, J. (2006). Functional properties of whey proteins as affected by dynamic high-pressure treatment. *International Dairy Journal, 16*, 275–284.

Carullo, D., Donsì, F., & Ferrari, G. (2020). Influence of high-pressure homogenization on structural properties and enzymatic hydrolysis of milk proteins. *LWT – Food Science & Technology, 130*, 109657.

De Maria, S., Ferrari, G., & Maresca, P. (2016). Effects of high hydrostatic pressure on the conformational structure and the functional properties of bovine serum albumin. *Innovative Food Science & Emerging Technologies, 33*, 67–75.

De Maria, S., Ferrari, G., & Maresca, P. (2017). Effect of high hydrostatic pressure on the enzymatic hydrolysis of bovine serum albumin. *Journal of the Science of Food and Agriculture, 97*, 3151–3158.

Donsì, F., Sessa, M., & Ferrari, G. (2012). Effect of emulsifier type and disruption chamber geometry on the fabrication of food nanoemulsions by high pressure homogenization. *Industrial & Engineering Chemistry Research, 51*, 7606–7618.

Ee, K.-Y., Khoo, L.-Y., Ng, W.-J., Wong, F.-C., & Chai, T.-T. (2019). Effects of bromelain and trypsin hydrolysis on the phytochemical content, antioxidant activity, and antibacterial activity of roasted butterfly pea seeds. *Processes, 7*, 534.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics, 82*, 70–77.

Espinosa, Y. R., Caffarena, E. R., & Grigera, J. R. (2019). The role of hydrophobicity in the cold denaturation of proteins under high pressure: A study on apomyoglobin. *The Journal of Chemical Physics, 150*, Article 075102.

Fertsch, B., Müller, M., & Hinrichs, J. (2003). Firmness of pressure-induced casein and whey protein gels modulated by holding time and rate of pressure release. *Innovative Food Science & Emerging Technologies, 4*, 143–150.

Hardt, N. A., van der Goot, A. J., & Boom, R. M. (2013). Influence of high solid concentrations on enzymatic wheat gluten hydrolysis and resulting functional properties. *Cereal Science, 57*, 531–536.

Ikeuchi, Y., Nakagawa, K., Endo, T., Suzuki, A., Hayashi, T., & Ito, T. (2001). Pressure-induced denaturation of monomer beta-lactoglobulin is partially irreversible: Comparison of monomer form (highly acidic pH) with dimer form (neutral pH). *Journal of Agricultural and Food Chemistry, 49*, 4052–4059.

Jin, Y., Deng, Y., Qian, B., Zhang, Y., Liu, Z., & Zhao, Y. (2015). Allergenic response to squid (Todarodes pacificus) tropomyosin Tod p1 structure modifications induced by high hydrostatic pressure. *Food and Chemical Toxicology, 76*, 86–93.

Khan, N. M., Mu, T.-H., Sun, H.-N., Zhang, M., & Chen, J.-W. (2015). Effects of high hydrostatic pressure on secondary structure and emulsifying behavior of sweet potato protein. *High Pressure Research*. https://doi.org/10.1080/08957959.2015.1005013

Kim, S. B., Ki, K. S., Khan, M. A., Lee, W. S., Lee, H. J., Ahn, B. S., et al. (2007). Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity. *Journal of Dairy Science, 90*, 4043–4050.

Larrea-Wachtendorff, D., Tabilo-Munizaga, G., & Ferrari, G. (2019). Potato starch hydrogels produced by high hydrostatic pressure (HHP): A first approach. *Polymers, 11*, 1673.

Larrea-Wachtendorff, D., Tabilo-Munizaga, G., Moreno-Osorio, L., Villalobos-Carvajal, R., & Perez-Won, M. (2015). Protein changes caused by high hydrostatic pressure (HHP): A study using differential scanning calorimetry (DSC) and fourier transform infrared (FTIR) spectroscopy. *Food Engineering Reviews, 7*, 222–230.

Lim, S.-Y., Swanson, B. G., & Clark, S. (2007). High hydrostatic pressure modification of whey protein concentrate for improved functional properties. *Journal of Dairy Science, 91*, 1299–1307.

Li, H., Zhu, K., Zhou, H., & Peng, W. (2012). Effects of high hydrostatic pressure treatment on allergenicity and structural properties of soybean protein isolate for infant formula. *Food Chemistry, 132*, 808–814.

Lopez-Fandino, R. (2006). Functional improvement of milk whey proteins induced by high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition, 46*, 351–363.

Møller, R. E., Stapelfeldt, H., & Skibsted, R. H. (1998). Thiol reactivity in pressure-unfolded beta-lactoglobulin. Antioxidative properties and thermal refolding. *Journal of Agricultural and Food Chemistry, 46*, 425–430.

Mozhaev, V. V., Kudryashova, E. V., & Bec, N. (1996). Modulation of enzyme activity and stability by high pressure: α-Chymotrypsin. *Progress in Biotechnology, 13*, 221–226.

Nielsen, P. M., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Food and Chemical Toxicology, 66*, 642–646.

de Oliveira, G. A. P., & Silva, J. L. (2015). A hypothesis to reconcile the physical and chemical unfolding of proteins. *Proceedings of the National Academy of Sciences, 112*, E2775–E2784.

de Oliveira, G. A. P., & Silva, J. L. (2017). The push-and-pull hypothesis in protein unfolding, misfolding and aggregation. *Biophysical Chemistry, 231*, 20–26.

O'Loughlin, I. B., Murray, B. A., Kelly, P. M., Fitz Gerald, R. J., & Brodkorb, A. (2012). Enzymatic hydrolysis of heat-induced aggregates of whey protein isolate. *Journal of Agricultural and Food Chemistry, 60*, 4895–4904.

Padiernos, C. A., Lim, S.-Y., Swanson, B. G., Ross, C. F., & Clark, S. (2009). High hydrostatic pressure modification of whey protein concentrate for use in low-fat whipping cream improves foaming properties. *Journal of Dairy Science, 92*, 3049–3056.

Queirós, R. P., Saraiva, J. A., & Lopes da Silva, J. A. (2018). Tailoring structure and technological properties of plant proteins using high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition, 58*, 1538–1556.

Quirós, A., Chichón, R., Recio, I., & López-Fandiño, R. (2007). The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chemistry, 104*, 1734–1739.

Rodiles-Lopez, J. O., Jaramillo-Flores, M. E., Gutierrez-Lopez, G. F., Hernandez-Arana, A., Fosado-Quiroz, R. E., Barbosa-Canovas, G. V., et al. (2008). Effect of high hydrostatic pressure on bovine a-lactalbumin functional properties. *Journal of Food Engineering, 87*, 363–370.

Segat, A., Misra, N. N., Fabbro, A., Buchini, F., Lippe, G., Cullen, P. J., et al. (2014). Effects of ozone processing on chemical, structural and functional properties of whey protein isolate. *Food Research International, 66*, 365–372.

Siddique, M. A. B., Maresca, P., Pataro, G., & Ferrari, G. (2016). Effect of pulsed light treatment on structural and functional properties of whey protein isolate. *Food Research International, 87*, 189–196.

Siddique, M. A. B., Maresca, P., Pataro, G., & Ferrari, G. (2017). Influence of pulsed light treatment on the aggregation of whey protein isolate. *Food Research International, 99*, 419–425.

Wang, W., & Roberts, C. J. (2018). Protein aggregation – mechanisms, detection, and control. *International Journal of Pharmaceutics, 550*, 251–268.

Xiang, B. Y., Ngadi, M. O., Ochoa-Martinez, L. A., & Simpson, M. V. (2011). Pulsed electric field-induced structural modification of whey protein isolate. *Food and Bioprocess Technology, 4*, 1341–1348.