Effects of High Hydrostatic Pressure on the conformational structure and the functional properties of Bovine Serum Albumin

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Abstract

Non-thermal technologies, such as High Hydrostatic Pressure (HHP), are able to induce extensive changes in the structure of biological macromolecules, namely proteins. HHP treatments disrupt the electrostatic interactions, which stabilize the quaternary and the tertiary structure of the proteins, and activate the reactions of sulfhydryl-disulfide bond exchange. These structural changes result in the dissociation and refolding of proteins during HHP treatments, and consequently in the modification of protein functional properties, namely physicochemical properties (solubility, binding and surfactant properties, water and oil absorption capacity, emulsifying and foaming properties). The technological behavior of the proteins in food preparation, processing, storage, as well as their contribution to determine quality perception of foods mainly depends on these functional properties.

This work aims at investigating the effects of HHP treatments on the conformational (quaternary, tertiary and secondary) structure and the functional properties of a globular water soluble protein, the Bovine Serum Albumin (BSA). BSA (50-100 mg/mL) solutions in Sodium Phosphate Buffer were processed at different pressure levels (100-500 MPa) and treatment times (15, 25 min). BSA unfolding and refolding were analyzed in terms of free sulfhydryl (SH) groups, changes of secondary structure, foaming and emulsifying properties.

Analyzing the experimental data it can be concluded that the unfolding of BSA samples with a concentration of 50 mg/mL occurred in the pressure range between 100 and 400 MPa. In fact an increased number of the free SH groups as well as an improved foaming and emulsifying ability were detected in the treated samples. Pressure levels above 400 MPa promoted the interactions between adjacent polypeptide chains and the formation of soluble high molecular mass aggregates. The concentration of the protein in the samples, also, controlled the occurrence of unfolding and aggregation. Extensive

changes in BSA secondary structure were observed at pressure level above 300 MPa, for longer processing times and higher protein concentrations. In these processing conditions β-sheet aggregates were likely to replace the initial α-helixes.

Keywords: *High Hydrostatic Pressure, unfolding, aggregation, BSA*

1. Introduction

In the last years proteins, the major structural and functional components of several foodstuffs, such as dairy products, meat, bakery products, have been widely investigated, with the aim of formulating novel foods or innovate the texture of traditional foods.

The functional properties of proteins, namely physicochemical properties (solubility, binding and surfactant properties, water and oil absorption capacity, emulsifying and foaming properties) influence food preparation, processing and storage, and contribute to the quality perception during food consumption. The exposure of hydrophobic groups on protein surface controls the interactions with oils (emulsions), air (foam) or other proteins (gels and coagula) (Li-Chan & Nakai, 1989). The hydrophobic amino acids are usually buried inside the globular proteins but, as a consequence of the unfolding of the native structure, these hydrophobic groups can be involved in the intermolecular interactions (Kato et al., 1989, Monahan ET AL., 1995).

Food processing can affect the functional properties of proteins since it can induce unfolding and aggregation, depending on the technology used, the processing conditions applied and the type of product. The knowledge of the mechanism underlying the structural arrangement of protein molecules may be useful to predict and control protein functionality in food formulations (McClements, 1999). Consequently, processing methods, able to modify the functional properties of proteins, are of interest to the food, chemical and pharmaceutical industries utilizing proteins as functional ingredients in their products.

Non-thermal technologies, namely High Hydrostatic Pressure (HHP), Pulsed Light (PL) and Pulsed Electric Fields (PEF), are able to induce changes in functional properties of food protein similarly to the traditional thermal treatments (pasteurization, sterilization). HHP technology is widely applied to inactivate spoiling microorganism and increase the shelf life of foods, preserving their nutritional and sensorial properties. HHP treatments, which have an irrelevant effect on small nutrients, such as vitamins, amino acids and flavour compounds, are instead able to induce changes in macromolecules, mainly proteins and carbohydrates, whose extent is dependent on the treatment temperature, the applied pressure level and the treatment time (Galaska, Dickinson & Ledward, 2000; Barba et al., 2012).

HHP processes are able to modify the protein quaternary structure by destroying the hydrophobic interactions, the tertiary structure by reversible unfolding and the secondary structure via irreversible unfolding (Tauscher, 1995). The quaternary structure dissociates at moderate pressures (150–200 MPa), the tertiary structure is also significantly affected at pressure level above 200 MPa and secondary structure changes take place at very high pressure (300–700 MPa) (Ahmed et al. 2010). Protein unfolding, induced by HHP treatments in the pressure range between 100 and 500 MPa, allows the inaccessible SH groups to be exposed. Consequently, the number of the free sulfhydryl (SH) groups and disulfide (SS) bonds also undergo changes during HHP treatments. Under severe processing conditions (above 500-600 MPa, depending on the protein and the temperature applied), the number of free SH group, instead, decreases, probably due to the formation of disulfide bonds by oxidation, especially at alkaline pH where the thiolate anions are more reactive. For instance, the SH-SS interchange reactions induced by HHP treatments were observed for β-lactoglobulin by Funtenberg (1995). The high degree of exposure of sulfhydryl groups, and the subsequent oxidation and sulfhydryl-disulfide bond exchange reactions result in insoluble and/or soluble aggregates and gel formation. Tang & Ma (2009), who investigated the occurrence of aggregates formation in soy protein isolate (SPI) treated by HHP processes at 200–600 MPa, observed combined insoluble (IA) and soluble (SA) aggregates. The relative ratio of IA and SA aggregates depended on the pressure levels. At pressure level above 200 MPa, more soluble aggregate were formed at the expense of insoluble aggregates (Tang & Ma, 2009). Soluble aggregates were also observed for β-conglycinin and glycinin treated at pressure levels between 300-600 MPa (Speroni et al., 2009). Similarly HHP promoted aggregation and significantly increased the surface hydrophobicity of Rapeseed Protein Isolates (RPI) (Rong et al., 2013).

The number of the disulfide bonds, moreover, affects the texture of the HHP induced gels, being the storage modulus, related to the elasticity of a solid, proportional to the cross-linking density in the gel network (Qin et al., 2012).

HHP treatments affect also the proteins functional properties: the viscosity and the surface tension are increased, as observed for egg white (Yang et al., 2009) and the foaming capability is increased, as demonstrated for ovalbumin (Denda & Hayashi, 1992) and egg white (Knorr et al., 1992; Yang et al., 2009). Yang et al. (2009) observed a direct correlation between the egg white foaming ability and the number of free SH

groups. At the lowest hydrophobicity level of egg white foaming capacity and foam stability of egg white reached the maximum value (Yang et al., 2009).

The changes of functional properties as well as the variation of the conformational structure may be particularly relevant in case of allergens, which are mainly specific glycoproteins able to activate the reaction of the human immune systems (Gross, M. & Jaenicke, 1994; Penãs, et al., 2006; Shriver et al., 2010). Bovine Serum Albumin (BSA), a globular protein soluble in water, represents a very interesting case of investigation, being a protein component of whey and blood and a common allergen of bovine meat and whey proteins. Moreover BSA physicochemical and structural properties have been investigated and measured, making it a desirable model for the research on food protein. BSA is a protein responsible for several allergic cross-reactions: most children with beef allergy are also allergic to cow's milk and should avoid the consumption of dairy products. Sensitization to bovine serum albumin is a marker of cow's milk allergy in children with beef allergy (Martelli et al., 2002). Furthermore, cooking or other processing methods do not necessarily eliminate the allergenic epitopes from milk and meat products. Nevertheless few papers, which investigated the effects of HPP processing on BSA, demonstrated that the solution properties of the protein can be affected by pressure (Buckow et al., 2011).

According to these findings, this paper proposed an extensive characterization of a HHP treated allergenic protein, Bovine Serum Albumin (BSA), with the aim of individuating the mechanism of action of HHP technology on the structure as well as on the functional and biological properties of this compound and determining to which extent the physical and structural changes occurring to the proteins previously described can be observed for this particular case. An extended experimental campaign was carried out to assess the effects of HHP process parameters, namely pressure level and time, and protein concentration on the structural characteristic of the allergens.

The effect of processing conditions, namely pressure level and treatment time, on the conformational structure and the functional properties of BSA solubilized in a buffer solutions at different concentration in buffer solution was analyzed. BSA unfolding and refolding were analyzed in terms of free sulfhydryl (SH) groups, changes of secondary structure, foaming and emulsifying properties. The experimental data were analyzed in order to identify the role of the process parameters in the transition from reversible unfolding to irreversible unfolding/aggregation of this protein. A deeper understanding

of the mechanisms governing the conformational changes will be very useful to design novel processes addressed to the reduction and/or elimination of food allergenicity.

2. Materials and Methods

2.1 Preparation of the samples

Different amount of BSA protein (Sigma-Aldrich, Italy) to reach the concentration of 50 and 100 mg/mL was dissolved, at a temperature of 25 °C, in Sodium Phosphate Buffer (50 mM, pH=8), according to the protocols reported by Penãs et al. (2006). After a gentle mixing, a homogenous solution was obtained. The pH of the protein solution was measured with a pH-meter (S400 SevenExcellence, Mettler Toledo International Inc.). The protein solutions were stored under refrigerated conditions before HHP treatments.

2.2 Experimental Apparatus

The HHP system U22 (Institute of High Pressure Physics, Polish Academy of Science, Unipress Equipment Division, Poland), which is a laboratory scale unit provided with a vessel which has a maximum processing volume of 50 mL, was used during the experiments.

The system can be operated in a wide pressure range (0 - 1400 MPa) under thermal controlled conditions (25 - 120 °C).

Operating pressure, ramp rate and processing time are set up on a control panel, which, in turn, allows the opening and the closure of the HHP vessel. A portable Temperature Power and control Unit (TCU) connected to the main unit with electrical cables and thermocouples (K-type) permits the set up and control of the operative temperature in the HHP vessel. The vessel is heated with electrical heaters and cooled withcompressed air. The pressurizing medium is Plexol (Bis (2-ethylhexyl) sebacate from Sigma-Aldrich, Italy) and the estimated temperature increase due to pressure build-up is 2-3 °C/ 100 MPa.

2.3 Experimental plan

2.3.1 Experimental protocols

For the experimental campaign 5 mL of BSA solution were sealed in flexible pouches made of a multilayer film (Polyethylene-Aluminium-Polypropylene). The pouches were introduced into the U22 vessel and the pressure cycle set at the desired processing

conditions. All tests were carried out at ambient temperature (25 °C), while the operating pressure was varied in the range between 100 and 600 MPa, and the operating time between 15 and 25 min. After the pressure release, the pouches were collected from the vessel, dried with blotting paper and stored at 4 °C before undergoing chemical characterization. All experiments were conducted in triplicate.

SH free groups, foaming properties, FT-IR spectra, and emulsifying properties were determined on unprocessed and processed samples according to the protocols reported in the following paragraphs.

2.3.2 Determination of free SH groups

Free sulfhydryl (SH) groups of BSA samples were evaluated according to the protocols reported by Beveridge et al. (1974) and by Hardham (1981). Ellman's reagent was prepared by dissolving 4 mg of 5,5-dithio-bis 2-nitrobenzoic acid (DTNB) (Sigma-Aldrich, Italy) in 1 mL of Tris–Glycine buffer (0.1 M Tris-(hydroxymethyl)-aminomethane (Tris), 0.1 M glycine, and 4 mM ethylenediamine-tetraacetic acid disodium salt, pH=8.0; Sigma-Aldrich, Italy)). BSA (10 mg), dissolved in 5 mL of 8M urea in Tris–Glycine buffer, was added to the Ellman's reagent (40 μL) and incubated for 30 min at room temperature (25 °C). The absorbance was measured at 412 nm with a UV/VIS spectrophotometer (V-650, Jasco Europe Srl, Italy), using Tris–Glycine buffer as blank. Free sulfhydryl groups were evaluated according to Equ.1:

$$SH(\mu M/g) = 73.53 * A_{412} * D/C \quad (1)$$

being A_{412} the absorbance at 412 nm, C the sample concentration in mg solid/mL and D the dilution factor. Results were expressed as the mean of three measurements.

2.3.3 Determination of FT-IR spectra

The secondary structure of the BSA samples was determined with a FT-IR spectrophotometer (FT-IR-4000, Jasco Europe Srl, Italy) at ambient temperature (25 °C). BSA samples (50-100 mg/mL) were dissolved in Sodium Phosphate Buffer (pH= 8) and treated under HHP (pressure level=100-600 MPa; treatment time=15, 25 min).

For each test the transitions among different vibrational energetic levels were recorded in terms of absorbance spectra as a function of the measurement time.

The Fourier Transform was applied to the recorded spectra in order to observe the behavior of the absorbance values as a function of the frequency (wavelength factor, cm^{-1}). The second derivative and Fourier self-deconvolution were applied to the infrared spectra.

2.3.4 Determination of the foaming properties

The foaming properties of BSA solutions untreated and treated under HHP (P=100-600 MPa; t=15, 25 min) were determined according to the protocol of Hammershoj and Larsen (1999) reported by Lomakina et al. (2006). A volume of 5 mL of the sample was placed in a glass beaker of 100 mL and stirred at 18000 rpm for 5 minutes at ambient temperature (25 °C). The produced foam was gently transferred into a graduated glass cylinder (diameter= 2 cm, height=16 cm, graduated volume= 25 mL) to determine the foam volume (V_f). The Foaming Capacity (FC) was calculated as the ratio between the measured foam volume (V_f) and the initial volume of liquid solutions (V_0), according to Equ. 2:

$$FC = V_f/V_0\% \quad (2)$$

The results were expressed as the mean value of three independent measurements.

2.3.5 Determination of turbidity, emulsifying activity and emulsion stability

Turbidity, Emulsifying Activity Index (EAI) and Emulsion Stability Index (ESI) of the protein solutions were determined according to the turbidimetric method developed by Pearce and Kinsella (1978), and modified by Tang et al. (2005) and Yang et al. (2010).

6 mL of BSA protein solution (50-100 mg/mL in Sodium Phosphate Buffer (50 mM, pH=8)) and 2 mL of peanut oil were homogenized in a high-speed homogenizer (T-25 ULTRA-TURRAX Digital Homogenizer, IKA®-Werke GmbH & Co. KG) for 1 min at 10.000 rpm. A 50-µL emulsion was taken at different times from the bottom of the homogenized emulsion, and diluted (1:100) in a sodium dodecyl sulfate (SDS) 0.1% (w/v) solution.

After the diluted emulsions were shaken in a vortex mixer for a moment, the absorbance was read at 500 nm against a blank (dilution solution) in a UV/VIS spectrophotometer (V-650, Jasco Europe Srl, Italy).

The EAI was calculated using Equ. (3), proposed by Cameron et al. (1991) and adopted by Silva et al. (2003) and Tang et al. (2005):

$$EAI\ \left(\frac{m^2}{g}\right) = 2 * \frac{T}{[(1-\theta)*C*1000]} \quad (3)$$

where T is the turbidity, θ is the fraction of oil used to form the emulsion (0.25) and C is the initial concentration of protein (5-10 g/100 mL). The turbidity was calculated using Equ. (4):

$$T = 2.303 * \frac{A*DF}{OP} \quad (4)$$

where A is the absorbance of the sample at 500 nm, DF is the dilution factor (100) and OP is the optical path (0.01 m).

The ESI was measured according to the method described by Chobert et al. (1988) and adopted by Silva et al. (2003), with a minor modification. The ΔEAI% was calculated according the Equ. (5):

$$\Delta EAI\% = \frac{(EAI_{MAX} - EAI_{MIN})}{EAI_{MAX}} * 100 \quad (5)$$

where EAI_{MAX} is the highest value of EAI obtained for the diluted emulsions right after their formation, and EAI_{MIN} is the value obtained from the emulsion after storage for 10 min.

The values of ESI were calculated using Eq. (6):

$$ESI = 1/\Delta EAI\% \quad (6)$$

The results were expressed as the mean value of three independent measurements.

2.4 Statistical analysis

The experiments were carried out in triplicate (3 independent runs) and the standard deviations of the results were calculated and reported.

The analysis of variance test for significant effects of treatments and assay samples were determined using the SPSS Statistics software. Experimental data were statistically

analyzed performing an analysis of variance (two-way ANOVA). Main effect differences were considered significant at the $p<0.05$ level.

The Pearson product-moment correlation coefficient was used to measure the strength of the linear relationship between two variables.

3. Results and discussion

3.1 Effect of HHP treatments on free SH groups

Most of the free SH groups in the native globular proteins are masked to the attack by Ellman's reagents, due to their location in poorly accessible regions of the polypeptide chain. The application of an external agent, such as temperature and/or pressure, modifies the protein conformational structure with the consequent unmasking and activation of SH groups, which can be detected according to the protocol of the Ellman's reaction (Beveridge et al., 1974; Hardham, 1981). Therefore the analysis of the free SH-groups may be considered as an indication of protein unfolding and/or denaturation.

The concentration of free SH groups in the BSA samples (50 and 100 mg/mL) treated at pressure levels from 100 to 600 MPa for 15 and 25 min was estimated. In Figure 1 the experimental data obtained according to the protocol based on Ellman's reagents are reported. The concentration of free SH groups in the native protein solutions is around 0.27 μmol SH/g.

The effect of HHP treatments on the concentration of free SH groups depends on the processing conditions (pressure level and holding time) as well as on the BSA concentration in the processed protein solutions.

In BSA samples with a concentration of 50 mg/mL the number of free thiol groups increases with the pressure level up to a threshold value, which depends on treatment time and protein concentration in the solution. According to the experimental data plotted in Figure 1A, the value of the threshold pressure is 400 MPa for a treatment time of 15 minutes and 300 MPa about for a treatment time of 25 minutes. The number of SH groups estimated for the samples treated at the same pressure level decreases with prolonging the processing times, which, similarly to pressure level, contribute to anticipate aggregation phenomena in protein solutions.

Above the defined value of the threshold pressure, the dependence of the number of free SH groups on pressure level follows an opposite trend. Experimental data demonstrate

that, similarly to temperature, high hydrostatic pressure is able to induce protein unfolding, which allows the masked SH groups to be exposed and consequently detected. However, under pressure the unmasked SH groups are more reactive and above the threshold pressure level the decrease in total SH content could be due to the sulfhydryl-disulfide bond exchange reactions, which are especially promoted at alkaline pH. Similarly Qin et al. (2012) detected a higher number of SH groups in the pressure range between 300-400 MPa and their significant decrease in the pressure range of 400-500 MPa probably due to the formation of aggregates (Quin et al., 2012). Van der Plancken et al. (2005), who studied the effect of heating and high pressure treatments on egg white proteins, observed the exposure of buried SH groups and the formation of aggregates at pressure levels of 500-600 MPa with a consequent decrease in free SH content due to the oxidation of the thiols and the formation of disulphide bonds.

The experimental data reported in Figure 5.2 demonstrate the relevant role of protein concentration in determining the effect of HHP treatment on the conformational structure of BSA. As already observed at lower BSA concentration, the number of free SH groups in the HHP treated samples is higher than in the native protein solution. However, if the data of BSA samples with protein concentrations of 50 mg/mL and 100 mg/mL are compared, higher protein concentrations reduce the number of free SH groups detectable.

This result can be explained by hypothesizing a different mechanism governing the protein behavior under pressure rather than a reduced efficacy of the HHP treatment in unmasking the buried SH groups. The availability of free SH groups unmasked under pressure and of more contact points due the high protein concentration in solution enhance the protein-protein interaction and the formation of both short and long terms bindings between neighboring polypeptide side chains (Cheftel et al., 1985). In fact, samples with protein concentration of 100 mg/mL have a lower number of free SH groups, due to the aggregation phenomena which already occur at pressure level of 300 MPa. In this case prolonged holding time do not have any significant effect on this parameter ($p<0.05$), while pressure levels above 300 MPa determine a slight increase of the number of free SH groups, thus demonstrating a progressive modification of the conformational structure of the pressure-induced aggregates.

3.2 Effect of HPP on secondary structure of BSA

3.2.1 FTIR spectrum of BSA at ambient temperature

FT-IR spectroscopy, unlike X-ray crystallography and NMR spectroscopy, provides information about the secondary structure content of proteins in water solutions as well as in deuterated forms and dried states (Susi et al., 1986).

FT-IR spectrum of native BSA (50 mg/mL) in water solution at ambient temperature is shown in Figure 2A, in which the absorbance of IR radiation as a function of wavenumber (in cm^{-1}) is reported. The protein structural units are able to absorb specific wavelengths of radiation in the infrared region, giving rise to a characteristic set of nine absorption bands, namely Amide A, B and Amide I-VII. Among them the amide I and II represent the vibrational bands of the protein backbone (Krimm et al., 1986). In particular, the absorption associated with the Amide I band takes into account the stretching vibrations of the C=O bond (approximately 80%) of the amide groups, while the Amide II band leads primarily to bending vibrations of the N—H bond (40−60% of the potential energy) and CN stretching vibration (18−40%). Both the C=O and the N—H bonds are involved in the hydrogen bonding between the different units of secondary structure, and, accordingly, the locations of the Amide I and Amide II bands are sensitive to the secondary structure content of a protein. Generally the analysis of FT-IR spectra is restricted to the region of the Amide I bands, because, according to several studies reported in the literature, Amide II band is not as good a predictor for quantifying the secondary structure of proteins (Fabian et al. 2002; Kong &Yu, 2007; Krimm et al., 1986; Murayama and Tomida, 2004; Surewicz & Mantsch, 1988). According to the FT-IR spectrum shown in Figure 2A, the Amide I region is located in the range between 1700 and 1600 cm^{-1} in agreement with the data reported in the literature (Kong & Yu, 2007). The database, available in the literature and containing the association between FT-IR peaks and secondary structure types, allows the secondary structure of BSA to be defined through the analysis of the FT-IR spectra of native protein and HHP treated samples. In order to narrow and separate the intrinsically broad bands of the BSA spectrum, the second derivative analysis was selected among the mathematical data analysis methods suitable to enhance the resolution of the FT-IR spectrum.

The second derivative spectrum of BSA (50 mg/mL) at ambient pressure, shown in Figure 2B, is dominated by four peaks at 1654, 1674, 1630, and 1610 cm^{-1} in the Amide I region, in agreement with the studies of Murayama and Tomida (2004). The structure of BSA protein is composed of 76% helix, 10% turns and 23% extended chain and no

beta (ß) sheets (Fabian et al. 2002). The peak at 1654 cm^{-1} corresponds to α–helical structures while the peak at 1674 cm^{-1} is associated with turn structures. Furthermore the band at 1630 cm^{-1} is assigned to short-segment chains connecting to α–helix segments of BSA and the one at 1610 cm^{-1} is due to the vibrations of glutaminyl (Gln) residues and the aromatic side chain, as already observed by Fabian et al. (2002).

3.2.2 FT-IR spectra of HHP treated BSA

Figure 3A and 3B and Figure 4A and 4B show the second derivative of the FT-IR spectra of untreated BSA samples (50 and 100 mg/mL respectively) and treated at different pressure levels (100-600 MPa) and treatment times (15-25 min) in the region of the Amide I (1600-1700 cm^{-1}). As already observed for the samples of the native BSA, the second derivative analysis clearly resolves the peaks of FT-IR spectra, which can be associated to the secondary structure types, namely α-helix, β-sheets, random coils and turns.

The second derivative spectrum of BSA samples (50 mg/mL) treated at high pressure (200-600 MPa) for 15 minutes (Figure 5.4A) changes with the pressure level applied and the holding time under pressure. BSA samples treated at 200 and 400 MPa show two higher and broader peaks at 1645 and 1630 cm^{-1}, thus clearly indicating that high pressure treatments are able to modify the secondary structure. In particular, in the case of protein in water solution, the band between 1642 and 1624 cm^{-1} are assigned to α-helix structure. At higher pressure levels, the changes in the secondary structure of BSA become more relevant. Accordingly, the spectrum of BSA treated at 600 MPa for 15 minutes is characterized by a new peak at 1685 cm^{-1}, which can be associated with the formation of intermolecular β-sheet aggregates.

Prolonged treatment times (25 min) enhance the effect of pressure, thus determining higher differences among the spectra of the samples treated at different pressure level (200, 400 and 600 MPa), as shown in Figure 3B. Furthermore, a more evident occurrence of the peak at 1685 cm^{-1} is observed together with the occurrence of a new peak at 1615 cm^{-1} associated with the formation of β-sheet intermolecular aggregates (Lefevre et al., 2000). In particular, the 1685 cm^{-1} band correspond to antiparallel β-sheet while the 1615 cm^{-1} band is due to the intermolecular β-sheets resulting from the formation of molecular aggregates.

In more concentrated samples (Figure 4A and 4B), the spectra result more amplified in the region between 1660-1630 cm^{-1} and a new peak at 1654 cm^{-1} associated with α -helix structure is formed. At longer treatment times (Figure 4B) the α-helix structures (1645 cm^{-1}) unfold into the disordered structure and turns, as demonstrated by the peak at 1654 cm^{-1}. Also in this set of experiments, prolonged processing times enhance the extent of molecular aggregation in protein secondary structure.

3.3 Foaming properties

Foaming capacity (FC%) of BSA samples with different concentration (50-100 mg/mL) processed in high pressure treatments (100-600 MPa, 15-25 min) was evaluated and compared to the foaming ability of the untreated samples. The experimental data are shown in Figure 5A and 5B for the BSA sample with the concentration of 50 mg/mL and 100 mg/mL, respectively.

According to the data shown in Figure 5A, HHP treatment is able to enhance the foaming capacity of BSA, independently of the processing conditions. The values of this functional properties increases with increasing the pressure level in the range between 100 and 400 MPa, while a decreasing trend is detected at pressure level above 400 MPa. The dependence of the foaming ability on the pressure level, therefore, follows the same trend already observed for the free SH groups, thus confirming BSA partial unfolding in the pressure range between 100 and 400 MPa and the progressive loss of the tertiary structure and the consequent polymerization of BSA protein in dimers, trimers and higher oligomers held together by disulfide bonds.

The foaming properties, in fact, are mainly related to the protein capability to form film at the air-water interface. The interfacial behavior of the protein, therefore, is strictly controlled by the electrostatic and hydrophobic interactions as well as by the stability of the disulphide bonds, which stabilize its secondary and tertiary structures. High pressure disrupts hydrophobic interactions and ionic bonds resulting in protein molecules being more suitable to adsorb air bubbles at air-water interface (Dickinson, 1989). This effect is enhanced for the albumin proteins, which are amphiphilic molecules and are easy to extend on the water-air interface (Li & Zhang, 2005; Yang et al., 2009). Similar results are shown by Yang et al. (2009), who observed an increased foaming capability and stability of egg white processed at pressure levels of 100-400 MPa. The authors explained that HHP treatment reduced the hydrophobicity and solubility of the protein

and observed a positive correlation between the foaming capability and the surface sulfhydryl content. The egg white samples exhibited greater foaming properties after a HHP treatment at 350 MPa for 10 min.

The reduced foaming capacity observed for the protein samples treated with pressure level above 400 MPa may be related to the irreversible unfolding, as demonstrated by the reduction of the number of free SH-groups and FTIR spectra. Similarly Galaska et al. (1997) observed a reduction in surface hydrophobicity of pure BSA in solution (pH=7) processed with pressure level between 400 and 800 MPa. The authors attributed the loss of surface hydrophobicity to the partial unfolding and the intermolecular association of BSA protein after pressure release. Moreover, as the increase of the pressure levels enhances the formation of aggregates the foaming ability of the samples decreases.

Similarly, Krešić et al. (2006) observed that high-pressure treatments (300 MPa and 600 MPa, for 5 and 10 min) significantly improved the foaming capacity of WPI and WPC due to the protein unfolding. A decrease of the foaming volume (FV) was detected if processing pressure was higher than 600 MPa because of the loss in protein solubility occurring at very high pressures. A significant reduction of the solubility of whey proteins after pressurization was also observed by Pittia et al. (1996). Van der Plancken et al. (2006) investigated the effect of a combined process of high pressure (400-700 MPa) and heating (50-85 °C) on foaming properties of egg white solutions. The authors observed an increase in foaming ability of those samples with high extent of unfolding and residual solubility with the best stability and volume foam reached at pH 8.8 above 500 MPa. The authors reported the association between foaming ability and protein flexibility/solubility and noticed an increase in foaming ability at higher exposed SH content (Van der Plancken et al., 2006).

If the effect of the processing time is considered, foaming ability was clearly higher in samples treated for a shorter processing time (15 min). Prolonged processing times (25 min) promote a larger extent of aggregation which prevent the formation of protein foams. A similar mechanism can explain the effect of protein concentration in the processed samples. If the results shown in Figure 5A and 5B are compared, the foaming ability decreases with the protein concentration in the processed samples. Protein concentration in solution controls the extent of protein-protein interaction, this allowing the formation of small aggregates at lower pressure level.

3.4 Emulsifying properties

The emulsifying properties are generally recognized as the most important parameters among the functional properties and used to determine the degree of protein denaturation and aggregation.

Figure 6 shows the values of the EAI (m^2/g) of BSA samples with different concentration (50-100 mg/mL) processed in high pressure treatments (100-600 MPa, 15-25 min).

The EAI is a measure of the interfacial area stabilized per unit weight of protein and represents the protein capacity to stay at the water/oil interface after the emulsion formation (Boye et al., 2010). The dependence of EAI values on the processing pressure varies with protein concentration, as already observed for the free-SH groups and the foaming capacity. In BSA sample with a concentration of 50 mg/mL, the emulsifying activity index increases with the processing pressure in the range between 100 and 400 MPa, while at higher pressure level a slight decrease in the EAI values can be observed. Prolonged processing times (25 min) still contribute to enhance the emulsifying capacity of the HHP treated protein solutions. In BSA sample with a concentration of 100 mg/mL, the EAI values follow a different trend. For shorter processing times (15 min), the EAI values are higher than the one measured for the control samples and almost independent on the processing pressure. The BSA samples processed for 25 min, instead, show a significant decrease in the EAI values at pressure level above 300 MPa ($p<0.05$). When the pressure levels is above 400 MPa, the BSA samples show significantly lower ($p<0.05$) EAI values than that of untreated one. As already observed for the foaming capacity, the observed variation of the emulsifying property could be due to the unfolding of proteins and subsequent loss of protein solubility. Thus, the proteins could not adsorb at the water/oil interface when they were insolubilized and aggregated.

Figure 7 shows the values of the ESI (%) of BSA samples with different concentration (50-100 mg/mL) processed in high pressure treatments (100-600 MPa, 15-25 min. ESI provides a measure of the ability of protein to stabilize the structure of the emulsion over a defined period of time (Boye et al., 2010). According to the experimental data shown in Figure 7A, the HHP treatments enhance the stability of the protein/oil emulsion in the pressure range between 100 and 400 MPa for the sample processed for 15 min and in the pressure range between 100 and 300 MPa for the sample processed

for 25 min. Pressure levels above the mentioned range determine a significant decrease in the ESI values ($p<0.05$), which are, in any case, higher than the ones measured for the untreated samples. If the protein concentration increases (Figure 7B), the ESI values estimated for the samples processed for 15 min are almost stable and not-significantly different ($p>0.05$) from the values of the untreated samples, if the processing pressure is lower than 400 MPa. At pressure level above 400 MPa, the HHP treatments enhance the stability of the protein/oil emulsion. An opposite trend can be observed for the samples treated for 25 min, showing ESI values decreasing with the processing pressure above 400 MPa. This effect may result from a decline in molecular flexibility of the proteins because of protein aggregation, since molecular flexibility is an important factor influencing emulsion stability (Tang et al., 2005). According to the mechanism of the emulsification process proposed by Tang et al. (2005), it consists of at least three steps: the adsorption of protein on the O/W interface; the changes of quaternary and tertiary conformations; the formation of a stable viscoelastic film. The first step is clearly affected by protein solubility and highly dependent on the flexibility in quaternary conformation. The occurrence of protein unfolding under pressure and the consequent modification of the tertiary structure affect the emulsion stability, clearly influencing the second and the third step of the emulsification process.

Moreover protein concentration influences the stability of the protein/oil emulsions, thus determining lower values of the ESI at higher protein concentration and confirming the formation of small aggregates.

3.5 Correlation analysis

Correlations between the functional parameters and the content of free SH-groups of BSA samples as affected by HHP treatments were summarized in Table 1. The data still confirm the observation reported in the previous paragraphs. For the BSA samples with a concentration of 50 mg/mL processed for 15 min, the whole analyzed parameters show a strong positive correlation, while at prolonged processing times, only the number of free SH-groups has a strong positive correlation with the foaming capacity, the ESA and ESI values. Moreover the correlation between ESI values and ESA or FC value becomes moderate. For the samples with a concentration of 100 mg/mL for 15 min, instead, a strong positive correlation exists between the number of free SH-groups and the ESA values, as well as between the foaming capacity and the ESA; positive or

negative moderate correlations are detected for the other analyzed parameters. At longer processing times, the positive strong correlations are replaced by very weak or weak correlations (negative and positive), thus demonstrating that different mechanisms control the effect of HHP treatment on the analyzed parameters.

4. Conclusions

High Hydrostatic Pressure resulted in protein unfolding and/or aggregation of proteins showing the potential to induce changes in BSA structure as well as in its functional properties. Protein unfolding occurs in the pressure range, which depends on protein concentration and treatments time. In the unfolded state, BSA proteins show improved foaming properties, expose a higher number of SH free groups and are characterized by negligible modifications of the secondary structure. Pressure levels above the threshold valued, prolonged processing times and/or high protein concentration allow the polymerization of BSA protein in dimers, trimers and higher oligomers held together by disulfide bonds, as demonstrated by the reduction of foaming and emulsifying properties and the significant changes in secondary structure detected.

The experimental results demonstrated so far that the effect of HHP treatments on protein structure may be predicted through a proper selection of both protein concentration and process conditions. The availability and reactivity of free SH groups represent the key factor controlling the mechanism of HHP action on proteins and determining the functional properties as well as the stability of the proteins in solution, in a certain range of protein concentration. Protein concentration, indeed, in solution controls the extent of protein-protein interaction, as demonstrated by the variation of the functional properties and the secondary structure of the HHP treated proteins.

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684

Figure Captions

Figure 1 Free SH-groups in BSA samples processed in HHP treatments at different pressure levels (100-600 MPa) and processing times (15 min, 25 min). **A**: protein concentration of 50 mg/mL; **B**: protein concentration of 100 mg/mL.

Figure 2 FT-IR Spectra of BSA (50 mg/mL) native protein at 25 °C. **A**: FT-IR spectrum; **B:** Second derivative spectrum of the Amide I.

Figure 3 FT-IR Spectra of BSA (50 mg/mL) samples processed in HHP treatments at different pressure levels (100-600 MPa). **A**: treatment time=15 min; **B**: treatment time=25 min.

Figure 4 FT-IR Spectrum of BSA (100 mg/mL) samples processed in HHP treatments at different pressure levels (100-600 MPa). **A**: treatment time=15 min; **B**: treatment time=25 min.

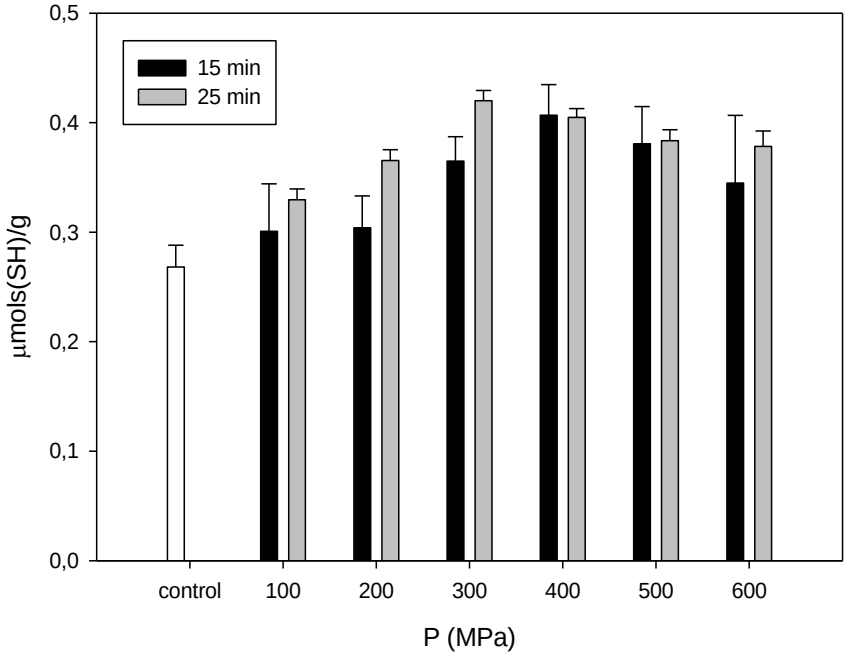
Figure 5 Foaming ability of BSA samples processed in HHP treatments at different pressure levels (100-600 MPa) and processing times (15 min, 25 min). **A**: protein concentration of 50 mg/mL; **B**: protein concentration of 100 mg/mL.

Figure 6 Emulsion Ability Index (EAI) of BSA samples processed in HHP treatments at different pressure levels (100-600 MPa) and processing times (15 min, 25 min). **A**: protein concentration of 50 mg/mL; **B**: protein concentration of 100 mg/mL.

Figure 7 Emulsion Stability Index (ESI) of BSA samples processed in HHP treatments at different pressure levels (100-600 MPa) and processing times (15 min, 25 min). **A**: protein concentration of 50 mg/mL; **B**: protein concentration of 100 mg/mL.

Figure 1

A


0,5
0,4
0,3
0,2
0,1
0,0
µmols(SH)/g
15 min
25 min
control
100
200
300
400
500
600
P (MPa)

B

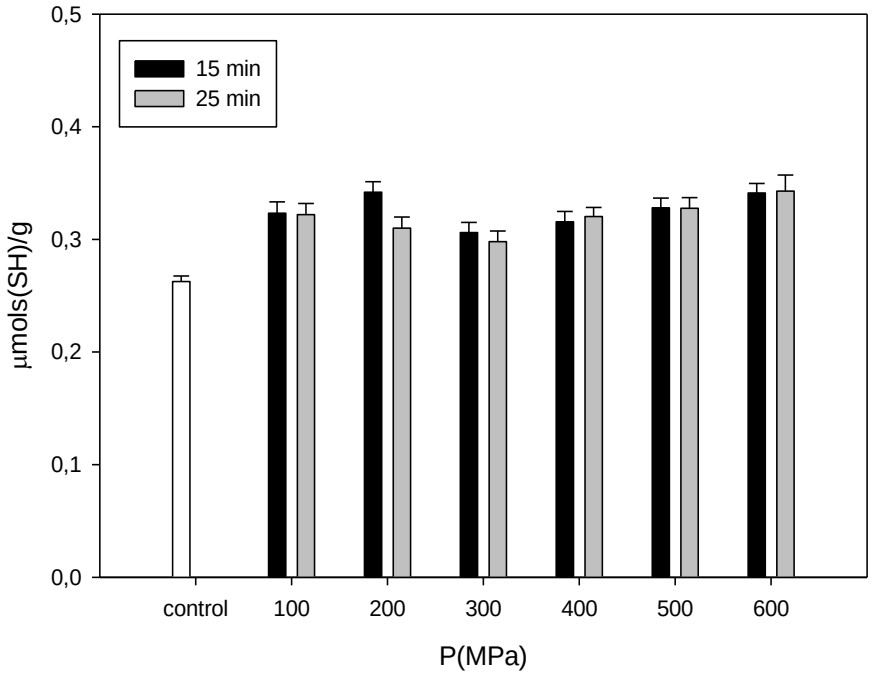
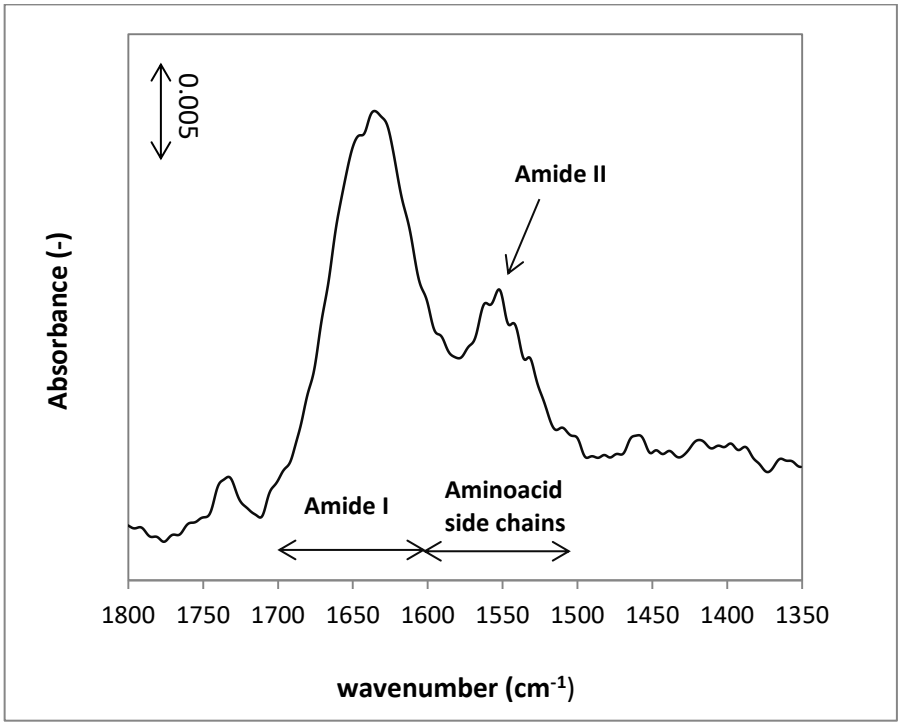

0,5
0,4
0,3
0,2
0,1
0,0
µmols(SH)/g
15 min
25 min
control
100
200
300
400
500
600
P(MPa)

Figure 2

A


0.005
Amide II
Absorbance (-)
Amide I
Aminoacid
side chains
1800 1750 1700 1650 1600 1550 1500 1450 1400 1350
wavenumber (cm-1)

B

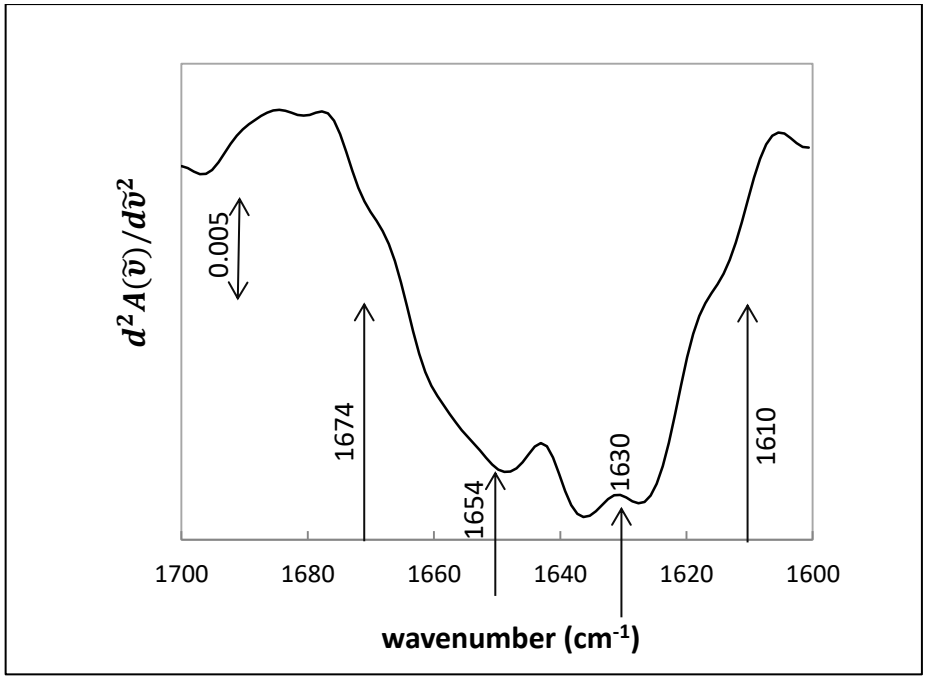
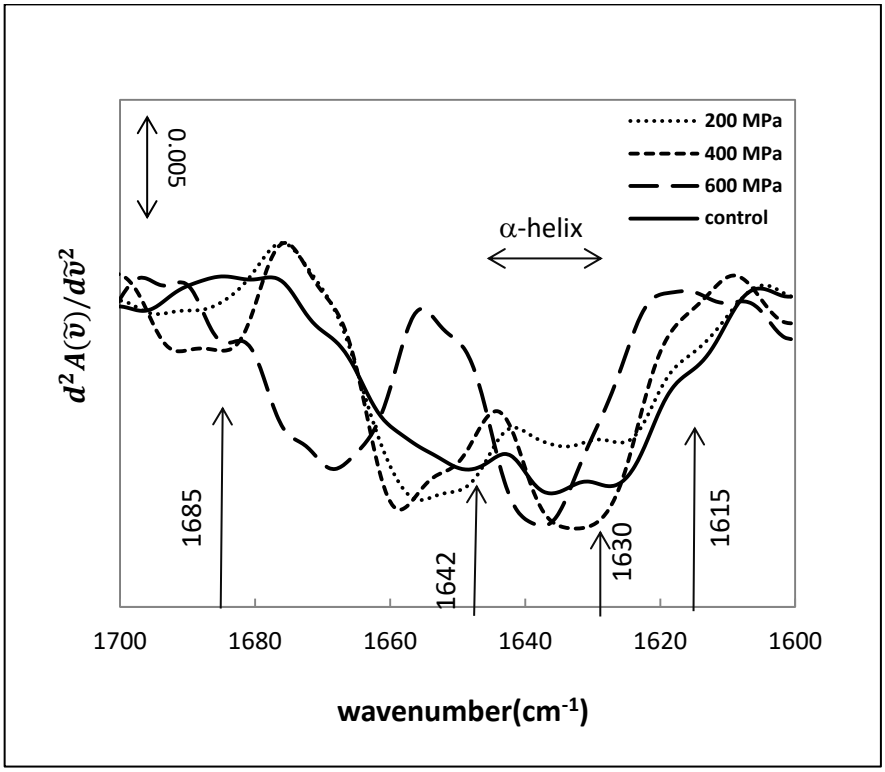

$d^2A(\tilde{\nu})/d\tilde{\nu}^2$
0.005
1674
1654
1630
1610
1700 1680 1660 1640 1620 1600
wavenumber (cm-1)

Figure 3

A


200 MPa
400 MPa
600 MPa
control
0.005
α-helix
$d^2A(\tilde{v})/d\tilde{v}^2$
1685
1642
1630
1615
1700
1680
1660
1640
1620
1600
wavenumber(cm-1)

B

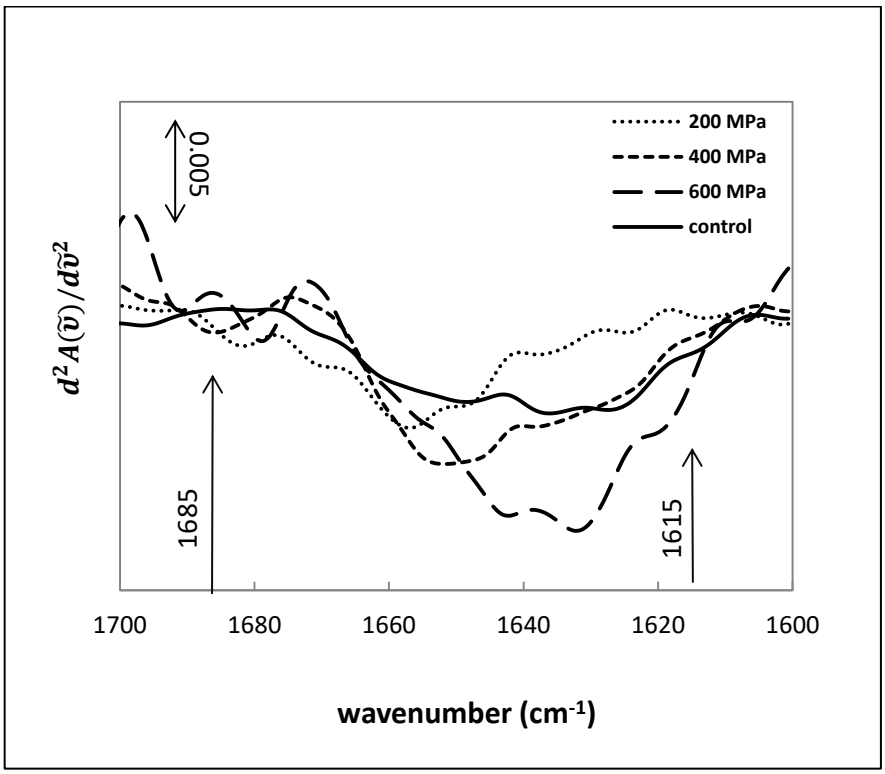
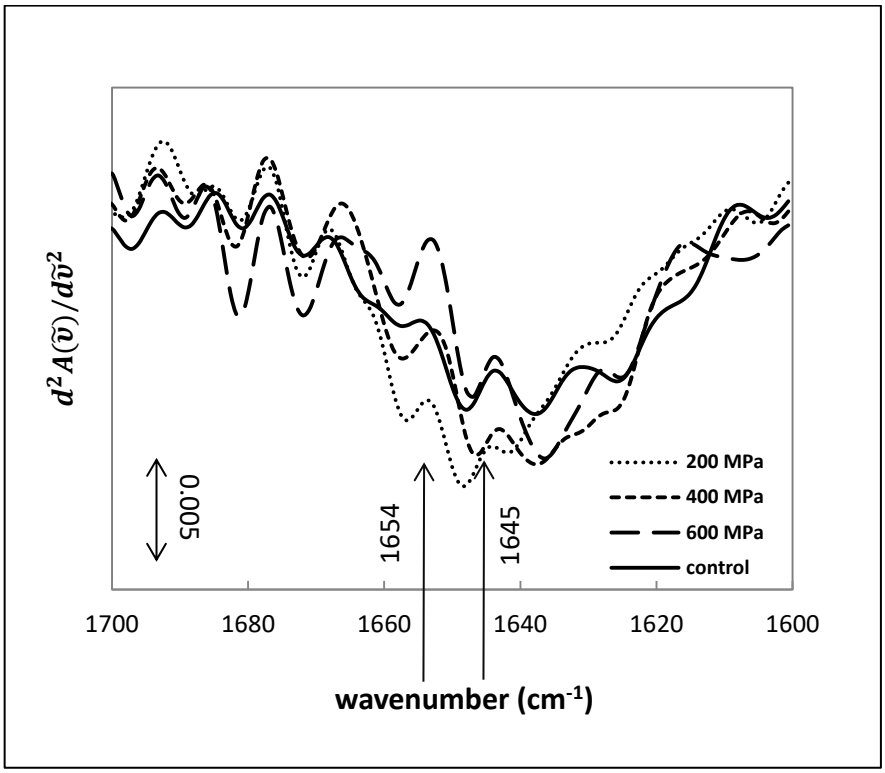

200 MPa
400 MPa
600 MPa
control
0.005
$d^2A(\tilde{v})/d\tilde{v}^2$
1685
1615
1700
1680
1660
1640
1620
1600
wavenumber (cm-1)

Figure 4

A


$d^2A(\tilde{\nu})/d\tilde{\nu}^2$
0.005
1654
1645
200 MPa
400 MPa
600 MPa
control
1700
1680
1660
1640
1620
1600
wavenumber (cm-1)

B

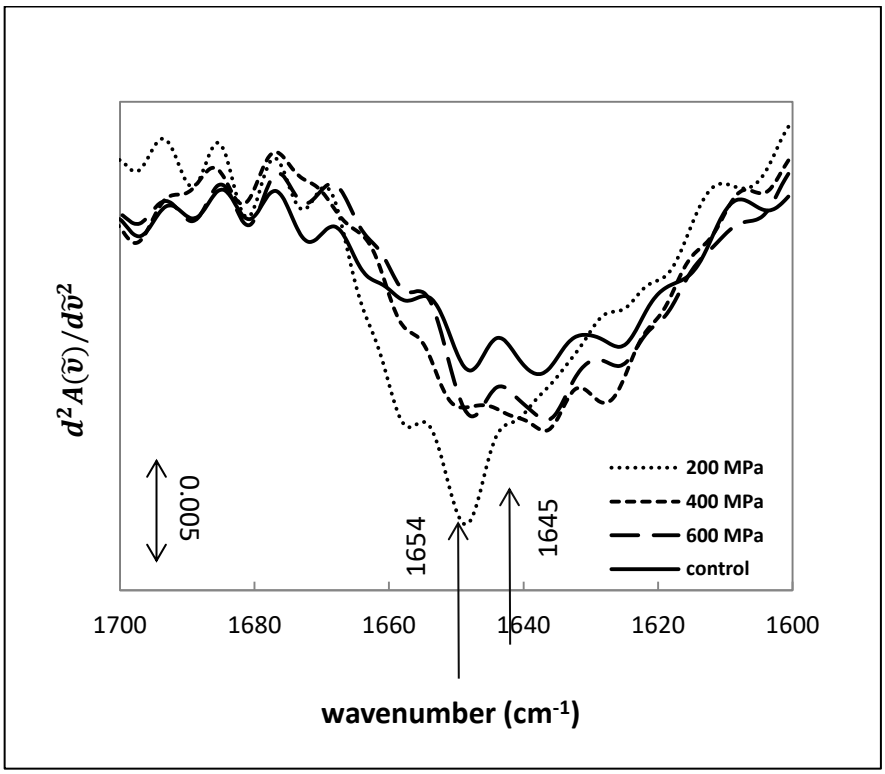
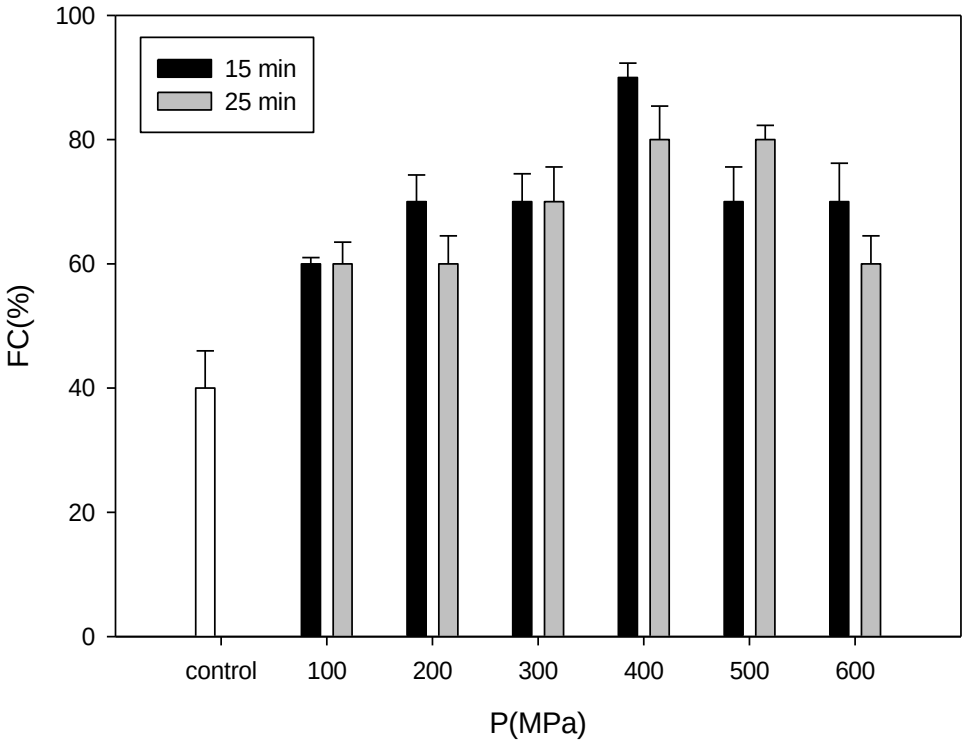

$d^2A(\tilde{\nu})/d\tilde{\nu}^2$
0.005
1654
1645
200 MPa
400 MPa
600 MPa
control
1700
1680
1660
1640
1620
1600
wavenumber (cm-1)

Figure 5

A


100
80
60
40
20
0
FC(%)
15 min
25 min
control
100
200
300
400
500
600
P(MPa)

B

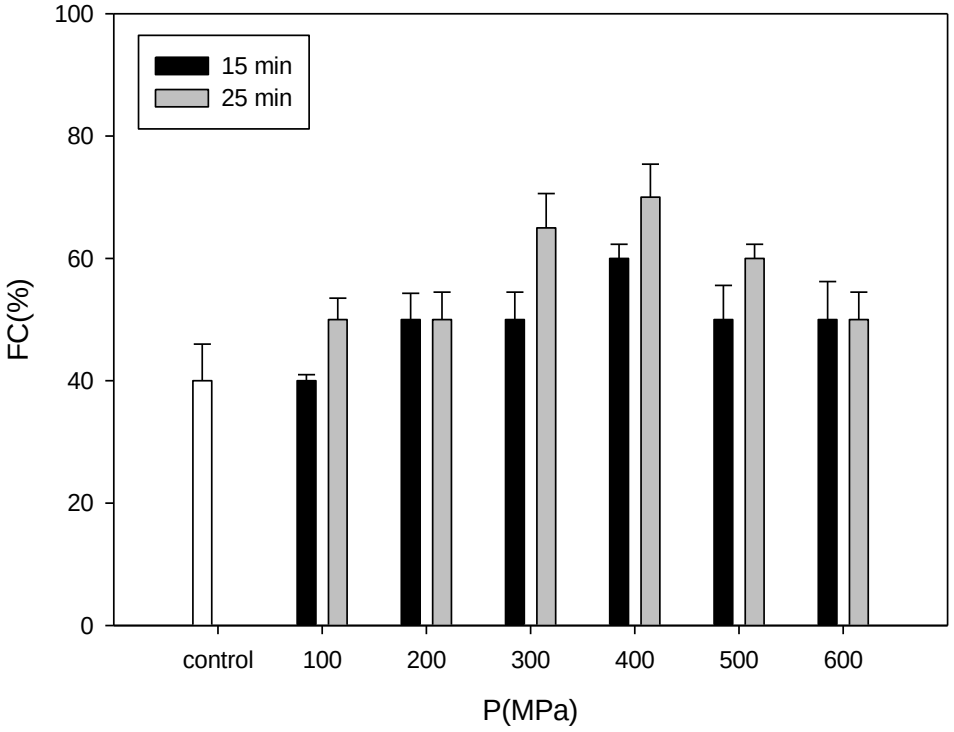
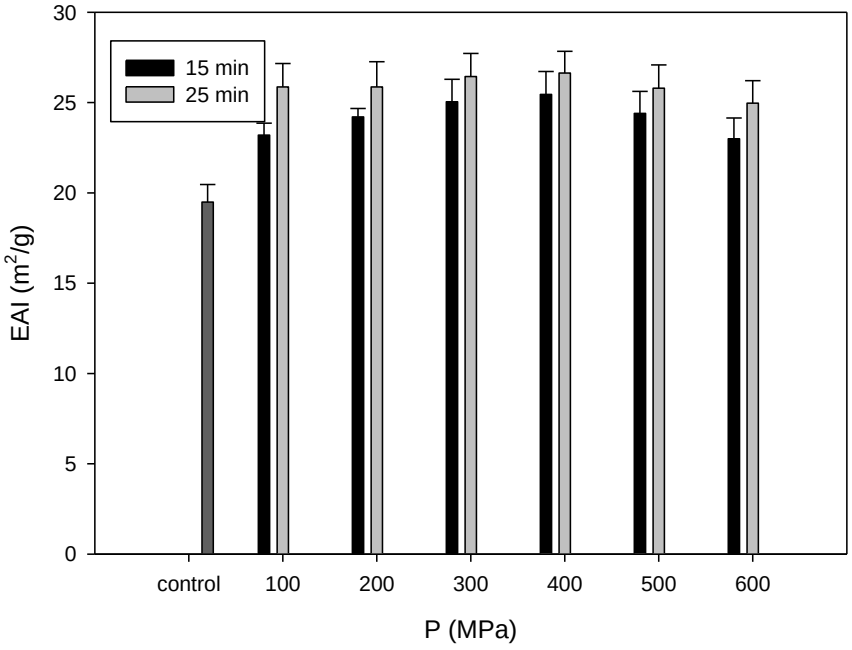

100
80
60
40
20
0
FC(%)
15 min
25 min
control
100
200
300
400
500
600
P(MPa)

Figure 6

A


15 min
25 min
EAI (m²/g)
0
5
10
15
20
25
30
control
100
200
300
400
500
600
P (MPa)

B

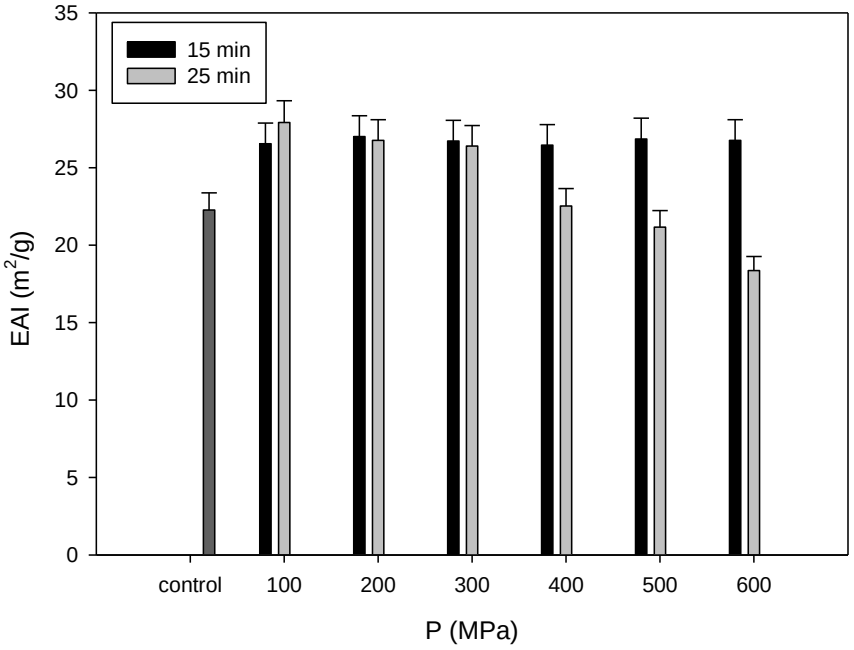
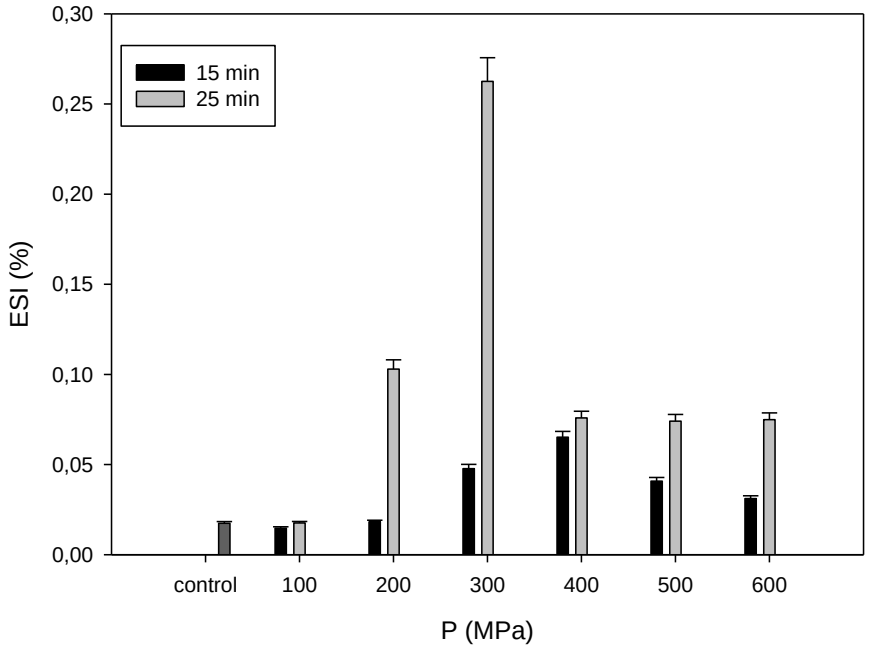

15 min
25 min
EAI (m²/g)
0
5
10
15
20
25
30
35
control
100
200
300
400
500
600
P (MPa)

Figure 7

A


0,30
0,25
0,20
0,15
0,10
0,05
0,00
ESI (%)
15 min
25 min
control
100
200
300
400
500
600
P (MPa)

B

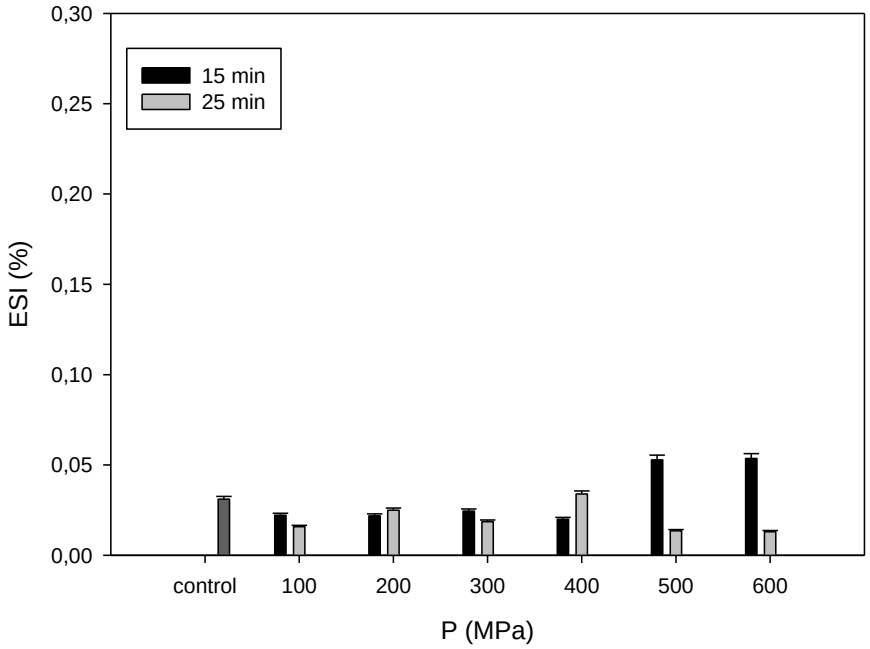

0,30
0,25
0,20
0,15
0,10
0,05
0,00
ESI (%)
15 min
25 min
control
100
200
300
400
500
600
P (MPa)

Table captions

Table 1 Correlations among functional properties and the content of frre SH- groups of BSA samples processed in HHP treatments at different pressure levels (100-600 MPa) and processing times (15 min, 25 min). A: protein concentration of 50 mg/mL; B: protein concentration of 100 mg/mL.

Table 1

A

	Processing time: 15 min			
	SH	**FC**	**ESA**	**ESI**
SH		0,866	0,805	0,921
FC	0,866		0,910	0,723
ESA	0,805	0,910		0,613
ESI	0,921	0,723	0,613	
	Processing time: 25 min			
	SH	**FC**	**ESA**	**ESI**
SH		0,855	0,634	0,708
FC	0,855		0,547	0,373
ESA	0,634	0,547		0,348
ESI	0,704	0,374	0,348	

B

	Processing time: 15 min			
	SH	**FC**	**ESA**	**ESI**
SH		0,273	0,906	0,244
FC	0,273		0,593	-0,158
ESA	0,906	0,593		0,074
ESI	0,244	-0,158	0,074	
	Processing time: 25 min			
	SH	**FC**	**ESA**	**ESI**
SH		0,444	-0,270	-0,591
FC	0,444		-0,2793	0,245
ESA	-0,270	-0,280		0,089
ESI	-0,591	0,245	0,089	