



Rheological characterization and modelling of high pressure processed Bovine Serum Albumin



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ABSTRACT

In the last years the utilization of novel mild technologies to induce structural modifications of food allergens and, consequently, to inhibit the allergic reaction was extensively investigated. Among these technologies High Hydrostatic Pressure (HHP) was proved to be very promising being able to induce significant modification of protein secondary, tertiary and quaternary structure, causing reversible or irreversible denaturation, aggregation and gelation.

In this work the rheological characterization of a selected allergenic protein after HHP processing was carried out and the effect of the processing parameters was described through the modelling of the rheological behavior of processed samples. The Bovine Serum Albumin (BSA), a globular protein, solubilized in sodium phosphate buffers (50 mM) was selected and the effect of different protein concentrations (12, 25, 50 and 100 mg/mL), pressure levels (600, 700 and 800 MPa) and treatment times (15, 25 min) was studied during the experimental trials.

The experimental results demonstrate that the protein solution processed at pressure levels above 600 MPa show the characteristic behavior of a pseudoplastic fluid and that the processing variables, namely pressure level and treatment time, as well as protein concentration, play an important role on the rheological properties of the system. The values of the viscosity and the shape of the curves of the viscosity as a function of the shear stress change with increasing the pressure level, prolonging the treatment time and/or increasing the protein concentration. Indeed, HHP treatments at pressure levels below 600 MPa are able to induce changes of the protein structure at the microscopic scale, as demonstrated by the reduction of the denaturation temperature. HHP processes may be successfully applied to modify and/or control the rheological behavior of the allergenic proteins.

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1. Introduction

In recent years the use of novel technologies, such as PEF (Pulsed Electrical Field), HILP (High Intensity Light Pulses), γ -radiations and HHP (High Hydrostatic Pressure) has been intensively investigated as innovative suitable methods to induce protein modifications without affecting the quality of the food. Among the food proteins, which may be affected by these technologies, the allergens represent a very interesting target. The challenge is to design new processes able to selectively modify the epitopes (linear or conformational) and spatial conformation of allergens and to preserve at the same time the overall quality of foodstuffs (Alessandri et al., 2010). If the allergen folding is

changed, its digestion in the gastrointestinal tract may be modified and the allergic reaction mechanism inactivated.

A few recent studies (Shriver and Yang, 2011) have demonstrated the potential applicability of novel technologies, namely PEF, HILP and HHP, to modify the allergen structure and, consequently, reduce their allergenic power (Vallons et al., 2010; Renzetti et al., 2008, 2010). It is well known that HHP process is able to induce functional and structural modifications of food biomolecules. As far as proteins are concerned, the structural changes induced by HHP treatments depend on several factors such as protein species, pH, ionic strength, type of buffer, pressure and temperature levels, and processing time (Messens et al., 1997). HHP process is able to act on the quaternary structure, which is stabilized by hydrophobic interactions, the tertiary structure causing the reversible unfolding, and secondary structure causing the irreversible unfolding. For globular proteins, such as albumin,

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HHP process, which affects both the tertiary and quaternary structures (Gross and Jaenicke, 1994), promotes the dissociation and the subsequent assembling of the systems with higher complexity on the one hand, and the unfolding and disassembling on the other hand (Jaenicke, 1987).

The mechanism of HHP induced unfolding is yet not well understood for different proteins, even though it has been hypothesized that it consists of several reversible steps, in which the intermediates play an important role during aggregation occurring after pressure release. Pressure induced conformations are more prone to aggregation since the hydrogen bridges can be formed at lower temperatures under pressure (Heremans, 2001). Unlike the thermally induced denaturation, high pressure induced denaturation can be reversible (Mozhaev et al., 1996; Rastogi, 2007), as well as irreversible, due to the unfolding of monomers and the aggregation/formation of gel structures (Cheftel et al., 1992). This was observed for ovalbumin (Okamoto et al., 1990), β -lactoglobulin (Galazka et al., 1996a,b), fish protein (Pérez-Mateos and Montero, 1997), meat protein (Okamoto et al., 1990 and Jimenez-Colmenero et al., 1998), whey proteins (Van Camp and Huyghebaert, 1995). Protein unfolding is much less intense during processing at moderate pressure than in thermal processing (Heremans et al., 1992), but protein denaturation at pressures as high as 1000 MPa has been found to be more severe than thermal denaturation for different proteins (Hayakawa et al., 1996). For instance, with respect to thermally induced gels, HHP induced gels of whey proteins show a lower hardness and a higher fraction of non-incorporated liquid. Moreover the denaturation of single-chain proteins occurs in the pressure range between 300 and 500 MPa (Hayakawa et al., 1992; Heremans et al., 1992; Silva and Welber, 1993).

According to these findings, the rheological characterization of HHP treated allergens can be suggested as a useful indirect method to detect the structural modification of the allergens and to select the processing condition to be used to design novel processing cycles to obtain low-allergenic foods.

A globular protein, the Bovine Serum Albumin (BSA), an allergen of bovine meat and whey proteins and responsible for several allergic cross-reactions, was selected for the experiments. 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 experimental data obtained in flow measurement tests and temperature ramp tests on untreated and processed samples were used to set up the model describing the rheological characteristics of the HHP processed BSA samples as a function of the processing and product variables.

The aim of this work was to assess the mechanism of action of HHP on BSA protein and to clarify the role of process and product parameters on the modification of this allergenic protein in view of designing processing cycles to produce foodstuff with reduced allergenic power.

2. Materials and methods

2.1. Preparation of the samples

BSA (Sigma–Aldrich, Italy) was dissolved at a temperature of 25 °C in Sodium Phosphate Buffer (50 mM, pH = 8), according to the protocols reported by Penās et al. (2006), at different concentrations (12, 25, 50 and 100 mg/mL) and gently mixed until 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 experimental campaign was carried out utilizing the HHP system U22 (Institute of High Pressure Physics, Polish Academy of Science, Unipress Equipment Division, Poland), a laboratory scale unit with a vessel having a maximum processing volume of 50 mL. The system can be operated in a wide pressure range (0–1400 MPa) under thermal controlled conditions (25–120 °C). A control panel allows the operative pressure, the ramp rate and the processing time to be set up, as well as to control the opening and the closure of the HHP vessel. The operating temperature, in the HHP vessel, is set and controlled by means of a portable Temperature Power and control Unit (TCU) connected to the main unit with supplying and thermocouples cables (K-type). The heating of the vessel is provided by means of an electrical heater while to cool the system compressed air is utilized. 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 each experiment BSA samples (5 mL) 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 experimental conditions. All tests were carried out at ambient temperature (25 °C), while the operating pressure was varied in the range between 500 and 800 MPa, and the operating time between 15 and 25 min. At the end of the pressure release, the pouches were collected from the vessel, dried with blotting paper and stored at 4 °C before the rheological characterization.

2.3.2. Rheological characterization

The rheological characterization of processed samples was carried out in stationary regime using the rheometer TA Instruments AR 2000 (TA Instruments, New Castle, DE). A plate–cone geometry was used (40 mm diameter, 2°) with a fixed gap width of 1 mm. 1 mL of the sample was loaded on the rheometer plate and left undisturbed for 10 min to equilibrate at the measurement temperature of 25 °C. Different types of test were used to determine the rheological behavior of BSA samples, as described in details in the following sections.

2.3.2.1. Flow measurement tests. Flow measurement tests were carried out on BSA samples with two protein concentrations, namely 50 and 100 mg/mL, processed in HHP treatments, at pressure level of 600 MPa, 700 MPa and 800 MPa, and processing time of 15 and 25 min. The apparent viscosity and shear stress were measured at a fixed angular velocity of 6.3 rad/s in the range of shear rates between 0.01 and 100 Hz.

2.3.2.2. Temperature ramp tests. Stationary temperature ramp tests were carried out BSA samples of different protein concentration, namely 12, 25, 50 and 100 mg/mL, treated with HHP at pressure level of 600, 700 and 800 MPa. During each test the temperature was changed from 25 °C to 95 °C with a ramp rate of 1.5 °C/min, constant strain of 0.1% and angular velocity of 6.3 rad/s. The trend of the apparent viscosity was recorded as a function of temperature.

2.3.3. Mathematical modelling

The Ostwald de Waele model (Eq. (1)), which assumes a power-law dependence between shear stress, τ , and shear rate, $\dot{\gamma}$, was used to model the rheological behavior of BSA samples:

$$\tau = K(\dot{\gamma})^{n-1}\dot{\gamma} \quad (1)$$

where K , the consistency index (Pa s^n), is the shear stress at a shear rate of 1.0 s^{-1} and the exponent n , the flow behavior index, is a dimensionless number indicating the closeness to Newtonian flow. For the special case of a Newtonian fluid ($n = 1$), the consistency index K is coincident to the viscosity of the fluid, η .

The model parameters, K and n , were estimated by applying the least squares method. The values of the statistical parameters, R^2 and the standard deviation of the curves from the experimental data, were also estimated in order to evaluate the correctness of the fitting equations.

3. Results and discussions

3.1. Flow measurement tests

The effect of HHP treatments on structural modifications of BSA was assessed by flow measurements in stationary regime. According to previous results (De Maria et al., 2013), HHP induced gelation of BSA occurs at pressure levels above 600 MPa and this threshold value is strictly related to the pH and concentration of the protein solution. Therefore, in this study HHP treatments were carried out on BSA samples with protein concentrations of 50 and 100 mg/mL at pressure levels above the threshold value, namely 600, 700 and 800 MPa applied for two treatment times of 15 and 25 min. The data obtained are reported in Figs. 1 and 2.

According to the experimental results, HHP treated samples behave as shear thinning fluid, for which the apparent viscosity decreases with increasing the shear rate (Fig. 1). This rheological behavior is typical of disperse materials and polymeric systems, which are characterized by tridimensional aggregates. At lower values of the shear rate, the Newtonian behavior (constant value of the viscosity, η_0 , namely the zero viscosity) observed is due to the Brownian motion of the molecules, which ensure their isotropic distribution, even though there are some initial effects of orientation to the flow. With an increase in the shear rate up to a certain critical point, the molecular orientation induced by the shear forces prevails on the casual orientation due to Brownian motions and, accordingly, the values of the viscosity drastically decrease until reaching a constant value, η_∞ .

According to the results shown in Fig. 1, the extension of the Newtonian region depends on the applied pressure and treatment time, reducing with increasing the pressure level or prolonging the

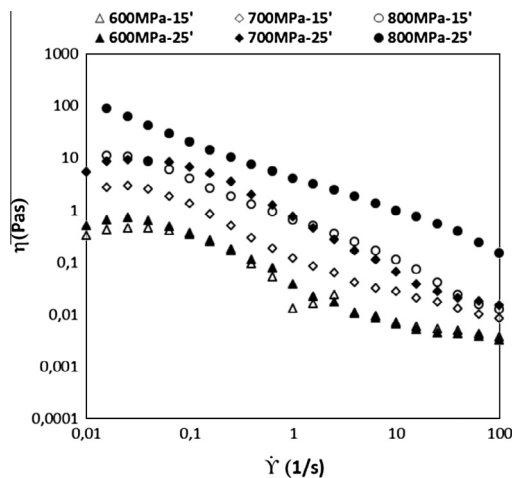


Fig. 1. Viscosity of BSA samples (50 mg/mL in sodium phosphate buffer) processed under different pressures levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min), as a function of the shear rate.

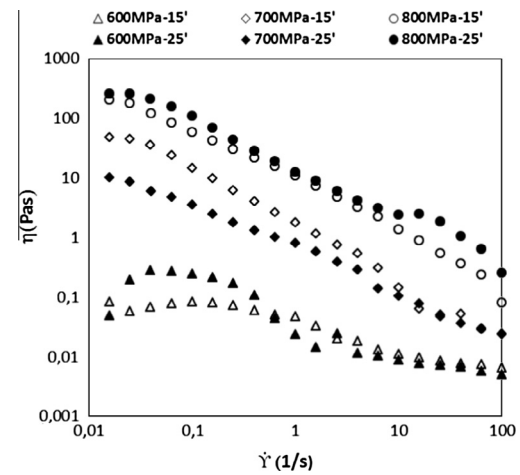


Fig. 2. Viscosity of BSA samples (100 mg/mL in sodium phosphate buffer) processed under different pressures levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min).

duration of the treatment. The samples treated at 600 MPa show a constant viscosity up to shear rates of 0.1 Hz . At higher pressure levels or longer processing times the Newtonian region is narrow, while, at a pressure level of 800 MPa and processing time of 25 min, the Newtonian region is not present any more and the viscosity decreases linearly with the shear rate.

Moreover, it can be observed that as the processing pressure increases, the values of the viscosity, measured with the flow tests, increases. The same trend was observed with processing time, the longer the time the higher the viscosity measured. Therefore, analyzing the results of the rheological characterization of processed samples, namely the shape of the curve and the values of the viscosity, it is possible to assess the effects of HHP treatment on protein rheology. The effects of the HHP processing on protein structure. The effects of the HHP processing on protein rheology may be correlated to the modification of the molecular interactions, which determine the conformation of proteins—ionic and hydrophobic interactions, as well as of the hydrogen bonds (Mozhaev et al., 1996). The HHP treatments of proteins, generally, cause the rupture of the hydrogen bonds and the formation of stretch-conformation (Wu et al., 2009). According to the observations reported by Galazka et al. (1996a), high pressure treatment affects the tertiary and quaternary structure of globular proteins, thus leading to protein aggregation and, under drastic processing conditions, to precipitation or gelation. Moreover, the aggregation of BSA in solution is mainly due to the formation of intermolecular disulfide bridges via SH/SS interchange. The changes of the conformational structure of the proteins and the networking of protein molecules affect the texture of the gel obtained (Kinsella and Whitehead, 1989). At higher pressure and longer holding time, the irreversible unfolding and the consequent aggregation of the BSA protein are enhanced, thus inducing a higher resistance to the shear stress and, consequently, an increase of the viscosity.

Fig. 2 shows the apparent viscosity of BSA samples at a concentration of 100 mg/mL and treated with the same processing conditions (600–800 MPa, 15–25 min). In this case, significantly different values of the viscosity were measured. The zero viscosity range was wider with respect to the previous case, namely between 0.1 Pa s for the samples processed at 600 and 1000 Pa s for the samples processed at 800 MPa.

According to these results, the protein concentration emphasizes the effects of HHP processing parameters on the apparent viscosity. Indeed, with higher protein concentration protein aggregation is promoted and the interactions among the peptide of adjacent chains are more likely to occur.

These findings are in agreement with the previous work done by De Maria et al. (2013), who demonstrated that BSA protein gelation occurred at a pressure level above 600 MPa and the texture of the gels obtained are controlled by the protein concentration and pH. Similarly Galazka et al. (1997), who investigated the effects of HHP treatments (300–800 MPa, 20 min) on BSA in solution (pH = 7), have demonstrated the formation of protein oligomers at a pressure level of 400 MPa, an extensive polymerization of BSA protein in dimers, trimers and higher oligomers held together by disulfide bonds as well as a relevant loss of the tertiary structure for the samples processed at 800 MPa. Higher protein concentration enhanced the extent of protein–protein interactions (Galazka et al., 1997). Similar conclusions were reported by Vèlez-Ruiz et al. (1998) for concentrated milk, Shibauchi et al. (1992) for casein, and Wu et al. (2009) for soy-proteins. Wu et al. (2009) investigated the effects of HHP treatments on WPI (Whey Protein Isolates) and observed that the HHP treatment caused the loss of the protein tertiary structure and the increase of its volume, due to the formation of high molecular weight aggregates and to swelling, typical of HHP treated globular proteins. As a consequence, the authors observed an increase of protein hydrodynamic radius and resistance to flow (in terms of viscosity).

Similarly, Marjanović and Jovanović (2011) applied HHP cycles (0.1–600 MPa) on β -lactoglobulin and noticed that the treatments with pressure below 100 MPa did not affected the viscosity of β -lactoglobulin solution, suggesting that structural changes in the protein did not occur. HHP treatments in the pressure range between 100 and 300 MPa, instead, promoted the dissociation of protein dimers into monomers, the penetration of water inside the protein structure and the exposure of hydrophobic zones to the aqueous solvent, causing an increase of the viscosity and hydration of β -lactoglobulin molecules. Moreover, the authors reported a partial unfolding only at pressure levels above 300 MPa, this inducing the formation of intermolecular disulfide bonds through sulphhydryl/disulfide interchange reactions, followed by the formation of β -lactoglobulin aggregates.

3.2. Temperature-ramp tests

The rheological characterization of HHP treated BSA samples was also made by means of the temperature ramp tests in stationary regime. This kind of measurements is suitable to detect if high pressure processing, even if carried out at pressure levels unable to induce BSA gelation, can cause structural modifications of the protein, which cannot be detected with flow measurement tests. If a partial irreversible unfolding occurs, the unfolded protein should be more sensible to other stresses such as a temperature increase. Temperature ramp tests were carried out on the BSA samples with a concentration of 50 mg/mL treated at pressure levels between 500 and 700 MPa for 15 min. The values of the viscosity were measured in the temperature range between 25 and 95 °C, and data are reported in Fig. 3.

Independently of the processing conditions considered, the curves of the apparent viscosity are clearly characterized by the same trend: the values of the viscosity are constant up to temperatures around 75–80 °C and suddenly increase with increasing the temperature above this value (see Fig. 3).

Therefore, for each curve the threshold temperature, above which the dependence of the viscosity on temperature clearly appears, can be estimated. With increasing the temperature above the threshold value, the viscosity of the BSA protein increases due to the thermal unfolding and to the interaction among the polypeptide chains, resulting in a larger hydrodynamic radius of the particles. Thus, the threshold temperature has the physical meaning and corresponds to the denaturation temperature of the protein. The estimated values of this parameter are listed in Table 1.

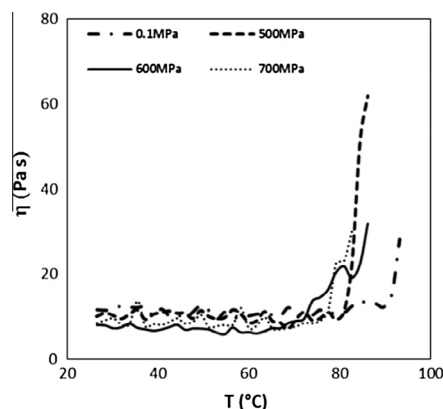


Fig. 3. Temperature ramp tests in stationary regime of BSA samples (50 mg/mL in sodium phosphate buffer) processed under different pressures levels (0.1 MPa, 500 MPa, 600 MPa, 700 MPa) for 15 min.

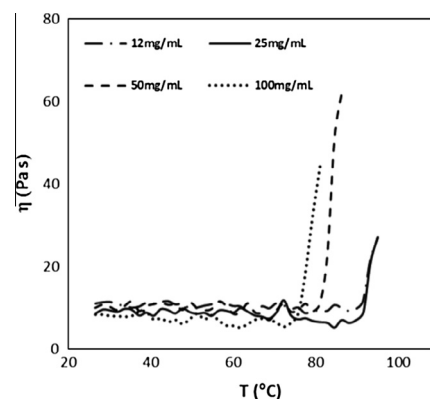


Fig. 4. Temperature ramp tests in stationary regime of BSA samples at different concentration (12, 25, 50 and 100 mg/mL in sodium phosphate buffer) processed at 500 MPa for 15 min.

Table 1

Values of the denaturation temperature estimated for BSA samples (50 mg/mL in sodium phosphate buffer) processed under different pressures levels (0.1 MPa, 500 MPa, 600 MPa, 700 MPa) for 15 min.

P (MPa)	T (°C)
0.1	81.0 ± 3.65
500	77.5 ± 3.02
600	74.0 ± 2.77
700	73.9 ± 2.62

Table 2

Values of the denaturation temperature estimated for BSA samples at different concentration (12, 25, 50 and 100 mg/mL in sodium phosphate buffer) processed at 500 MPa for 15 min.

C (mg/mL)	T (°C)
12	91.4 ± 3.5
25	89.7 ± 2.83
50	77.5 ± 2.92
100	74.0 ± 2.45

It can be observed that with increasing the pressure level, the values of the denaturation temperature reduce. The denaturation temperature of the native protein (0.1 MPa) is about 81 °C. The

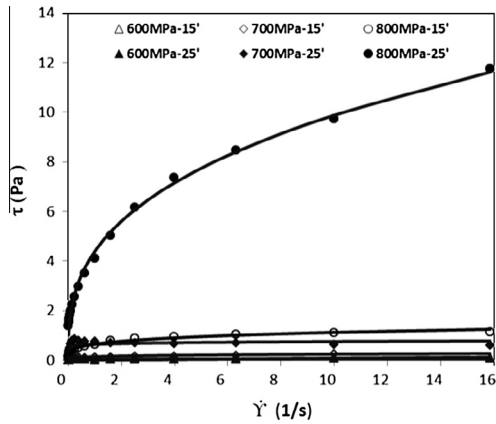


Fig. 5. Rheograms of BSA samples (50 mg/mL in sodium phosphate buffer) processed under different pressures levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min). Points represent the experimental data and lines the fitting equations.

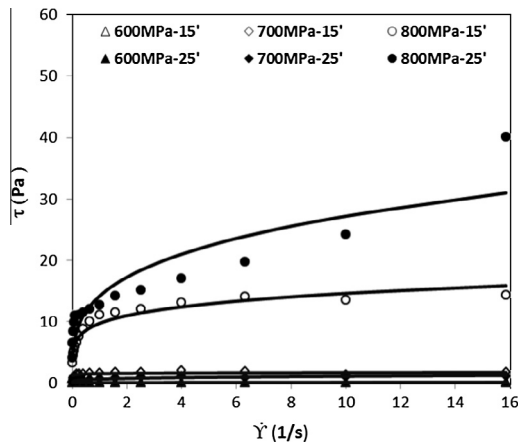


Fig. 6. Rheograms of BSA samples (100 mg/mL in sodium phosphate buffer) processed under different pressures levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min). Points represent the experimental data and lines the fitting equations.

denaturation temperature of the HHP treated samples decreases with an increasing pressure level up to 600 MPa. Further increases of the pressure level do not affect the value of the denaturation temperature (about 74.0 °C), confirming that microscopic changes in the BSA protein structure occur at pressure levels below the critical value of 600 MPa. As already observed, the HHP-induced gelation occurs at pressure level above 600 MPa, where macroscopic changes in the protein structure are detectable. Similar results were reported for egg yolk dispersions by Aguilar et al. (2007). The authors, using differential scanning calorimeter (DSC), detected a progressive decrease of the thermal denaturation enthalpy as the processing pressure level was raised. An increase of

the pressure applied produces significant changes in the linear viscoelastic behavior of the sample which typically takes place in sol–gel transitions. Our results are also in agreement with those reported by Speroni et al. (2005), who analyzed the influence of high-pressure treatments on diluted LDL (Low-Density Lipoproteins) from hen egg yolk.

Temperature ramp tests were also carried out on BSA samples with different concentrations (12, 25, 50 and 100 mg/mL) treated at 500 MPa for 15 min to analyze the influence of protein concentration (see Fig. 4). Also in this case, the curves of the viscosity as a function of the temperature were used to determine the denaturation temperature. The estimated values of the latter parameter are listed in Table 2. As observed for samples treated at different pressure levels, protein concentration may influence the mechanism of denaturation. In more diluted samples the denaturation occurs at higher temperatures, while higher concentrations enhance the interaction among the polypeptide chains and the formation of bonds among adjacent chains, which are at the base of the formation of a gel with a higher capability of water retention (Van Camp and Huyghebaert, 1995).

3.3. Modelling of the rheological data

The experimental data of the shear stress (τ) as a function of the shear rate ($\dot{\gamma}$) measured for the BSA samples with concentration between 50 and 100 mg/mL treated with HHP processes at pressure level of 600–800 MPa and processing time between 15 and 25 min were fitted by Ostwald de Waele model equation (Eq. (1)). As already described, this model assumes a power-law dependence of the shear stress on the shear rate. Figs. 5 and 6 show the model curves and the experimental data for the BSA samples with the two concentration of 50 mg/mL and 100 mg/mL, respectively, while in Table 3 the values of the model parameters, K and n , as well as the parameters of the statistical analysis, R^2 and the standard deviation, for the two samples are listed.

According to these estimated values, the flow consistency index (K) of BSA samples increases with increasing pressure levels, treatment times and protein concentration. The experimental data demonstrate that the consistency index increases with increasing protein concentration and pressure level applied. For instance, the value of the parameter K , estimated for the samples treated under pressure for 15 min, varies from 0.15 to 0.66 Pa s ^{n} when processing pressure is increased from 700 to 800 MPa. Accordingly, when applying the same shear rate, higher values of the shear stress are measured, thus demonstrating a higher resistance to flow and an increase of the compactness and hardness of the HHP gels. The parameter K , therefore, accounts for the changes of textural and rheological properties of HHP gels. Thus, the model equation correctly describes the mechanism of action of the processing and product parameters, as already discussed in the previous sections.

According to the values of the parameter n , independently of processing conditions and protein concentration, BSA samples is clearly a non-Newtonian fluid, behaving as a pseudoplastic fluid.

Table 3

Values of the model parameters (K and n) and statistical parameters (correlation coefficient and standard deviation) estimated for the BSA samples (50 mg/mL and 100 mg/mL in sodium phosphate buffer) treated at different pressure levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min).

Pressure–time	C = 50 mg/mL				C = 100 mg/mL			
	K (Pa s ^{n})	n (–)	R^2	SD	K (Pa s ^{n})	n (–)	R^2	SD
600 MPa–15 min	0.01	0.83	0.86	0.01	0.03	0.56	0.99	0.00
700 MPa–15 min	0.15	0.22	0.88	0.03	1.55	0.05	0.82	0.30
800 MPa–15 min	0.66	0.24	0.99	0.05	9.72	0.18	0.97	0.34
600 MPa–25 min	0.04	0.17	0.88	0.01	0.04	0.39	0.92	0.01
700 MPa–25 min	0.65	0.07	0.72	0.19	0.65	0.24	0.95	0.12
800 MPa–25 min	4.39	0.35	1.00	0.21	14.19	0.28	0.92	0.51

With more drastic processing conditions or higher protein concentration, the values of the parameter n change, thus demonstrating its clear dependence on process and product variables.

Further studies are necessary to set up a model equation able to describe and account for the dependence of both model parameters on the pressure level, processing time as well as protein concentration. However, the *Ostwald de Waele* model equation utilized in this work is able to correctly represent the mechanism of action of the HHP and the role played by the variables analyzed.

4. Conclusions

The experimental work performed so far has demonstrated that the irreversible unfolding and the consequent gelation of BSA protein solutions occur at pressure level above 600 MPa. These results are consistent with the observation that HHP treatments above 600 MPa induce a relevant loss of the tertiary structure of BSA protein, thus leading to the formation of intermolecular disulfide bridges and the polymerization of the protein, in accordance with the findings of a previous work (De Maria et al., 2013). Moreover, the texture and the rheological behavior of the processed BSA samples depended on pressure level, processing time and/or protein concentration.

However, even when the HHP process is carried out at pressure level that do not induce the gelation of BSA protein, namely pressure levels below the threshold value of 600 MPa, the treatments cause the partial modification of the protein structure, thus allowing the interaction between the unfolded chains and the formation of small aggregates, as demonstrated by the reduction of the denaturation temperature.

The *Ostwald de Waele* model is able to describe the pseudoplastic behavior of the HHP processed BSA samples. Further work is planned to set up a generalized model equation, which will take into account the dependence of the model parameters on processing and product variables on the one hand, and to extend results to different proteins, on the other hand.

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