



Structural behaviour in condensed bovine serum albumin systems following application of high pressure



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ABSTRACT

The present study shows that application of high hydrostatic pressure of 600 MPa for 15 min at ambient temperature on condensed bovine serum albumin systems (BSA) with up to 80% w/w solids content has a limited effect on the conformational structure of the protein, as compared to thermal treatment. This was demonstrated throughout the experimental concentration range using small-deformation dynamic oscillation, differential scanning calorimetry and infrared spectroscopy. BSA possesses seventeen disulfide linkages per molecule, which constitutes a stable arrangement with high energy requirements for substantial structure alteration. Upon cooling, pressurised materials undergo vitrification and networks exhibit comparative mechanical strength to that of thermally treated counterparts. The mechanical manifestation of the glass transition region and glassy state for atmospheric and pressurised samples was examined by the method of reduced variables and the combined framework of WLF/free volume theory producing disparate predictions of the glass transition temperature for the two types of polymeric network.

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1. Introduction

In the last ten years, there has been a marked increase in the application of high pressure processing (HPP) in the food industry worldwide to create “fresh” fruit juices with enhanced natural flavour retention and higher content of natural vitamins, compared to conventional thermally treated products. In terms of protein functionality, which is the subject of interest in this work, pressure processing can lead to denaturation and different states of viscosity, aggregation or gelation, depending on the protein system, treatment temperature, physicochemical environment of the solution and magnitude or duration of the applied pressure (Patel, Singh, Havea, Considine, & Creamer, 2005).

The effect of high hydrostatic pressure on the structure of proteins in aqueous solution has received considerable attention over the last few years (Dumay, Kalichevsky, & Cheftel, 1994; Hayakawa, Kajihara, Morikawa, Oda, & Fujio, 1992). These studies have confirmed the view that, since formation of ion pairs or hydrophobic bonds is accompanied by a substantial positive volume change, high pressure treatment has a disruptive effect on intermolecular electrostatic and hydrophobic interactions. On the other hand, hydrogen bond formation is associated with a small (usually negative) volume change, and so hydrogen bonding is relatively insen-

sitive to pressure. This means that high pressure may be able to disrupt the secondary, tertiary and quaternary structure of globular proteins but has no effect on the primary structure (Dumay et al., 1994).

Bovine serum albumin (BSA) is a protein component of whey and blood. Its physicochemical and structural properties have been well-characterised, making it a desirable model system for food related research (Leibman, Tiktupulo, & Privalov, 1975). In terms of thermal processing, the formation of a gel network takes place at moderate operating temperatures (80 °C for 30 min). Pressure-induced unfolding of BSA can rapidly lead to aggregation at pressures above 400 MPa, depending on protein concentration and treatment time, but there is no subsequent gelation in samples subjected to high pressure of 400 MPa for 30 min. Using spectrofluorometry, it was demonstrated that the conformation of BSA remains fairly stable at pressures of 400 MPa and a low protein concentration. This pressure stability of BSA is positively correlated with the high number of disulphide bonds stabilizing its three-dimensional structure (Michnik, 2003; Hayakawa et al., 1992).

The application of high pressures affects the secondary structure of BSA, with the changes mainly consisting of a decrease in the α -helical structure, accompanied by a slight increase in β -sheet structure (Hosseini-Nia, Ismail, & Kubow, 2002). Most studies report that pressures between 800 and 1000 MPa are required to bring upon a considerable alteration to the secondary structure of the protein. Hayakawa, Linko, and Linko (1996) reported a

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decrease in the BSA α -helix content of up to 50% at 1000 MPa at 30 °C after 10 min. These structural modifications of the protein under pressure can be irreversible depending on pH, protein concentration and accompanying thermal treatment or shear.

Literature thus far has reported on relatively dilute aqueous systems, i.e. up to 10% or 20% (w/w) solids content, and demonstrated the effect of the level and time of pressurisation on reversible or irreversible conformational change in the protein structure (Hayakawa et al., 1992; Hosseini-Nia et al., 2002; Patel et al., 2005). The present investigation employs several physicochemical techniques and theoretical modelling for the characterisation of the structural properties of atmospheric and pressurised condensed BSA systems, reaching a solids content of up to 80% (w/w) in preparations. In these high-density systems, protein macromolecules are unable to rearrange themselves into regularly ordered forms and instead exhibit an amorphous rubbery or glassy consistency (Kasapis & Mitchell, 2001).

The passage from the rubbery to glassy state is in the nature of a second-order thermodynamic transition, describing changes in state but not in phase (Kasapis, 2008). Headway in this type of research is achieved by adapting theoretical frameworks from the “sophisticated synthetic polymer science”, which allows for the determination of viscoelastic functions in relation to time or temperature of observation. Molecular and macroscopic properties of concentrated BSA matrices have been thus examined in this work using FTIR, microDSC and dynamic oscillatory rheometry in shear, where very little, if anything, has been reported in the literature in terms of the pressure effects.

2. Materials and methods

2.1. Materials, sample preparation and treatment

Bovine serum albumin (BSA) was a lyophilized powder from Sigma–Aldrich, Castle Hill, NSW, Australia. It had purity of more than 98% based on agarose gel electrophoresis, the molecular weight was about 66 kDa and the pH was between 6.8 and 7.2 in a 1% (w/w) solution of 0.15 M NaCl.

Dispersions of 10–30% (w/w) solids were prepared by mixing the powder in deionized water at neutral pH and ambient temperature. These were stirred for approximately 2 h using an IEC magnetic stirring at 510 rpm, to ensure proper dissolution. They were stored overnight at 4 °C to achieve thorough hydration and the removal of air bubbles. A series of concentrations for the BSA protein samples (40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% and 80%, w/w) were prepared by dehydrating the original material using a rotary evaporator at 40 °C (± 1 °C) and all samples were made in triplicate to allow for experimental replication.

Samples were treated with high hydrostatic pressure at ambient (initial) temperature (22 °C) for 15 min using the cylindrical chamber of a high pressure unit, with a 35 litre volume and 70 mm plunger diameter (Quintus Press – QFP 35L, Avure Technologies, Kent, WA, USA). The pressure medium was demineralised water, the pressure build up was performed at about 100 MPa per 20 s and the accessible pressure range extended to 600 MPa, which was sufficient to induce considerable structural effects on the materials. The recorded temperature profile during HPP showed that during compression to 600 MPa the temperature increased by about 20 °C. The maximum temperature (about 42 °C) was not high enough to induce thermal denaturation of BSA during pressurisation. Atmospheric samples, and pressurised counterparts (following HPP treatment), were subjected to physicochemical experimentation over a broad temperature range of –70 to 90 °C (Dissanayake et al., 2012; Kasapis & Mitchell, 2001).

2.2. Methods of analysis

2.2.1. Small-deformation dynamic oscillation

Small-deformation dynamic oscillation in shear was used to analyse the BSA samples, being pressure or thermally treated, with a controlled strain AR-G2 rheometer (TA Instruments, New Castle, DE, USA). Standard steel parallel plate geometries of 10, 20 and 40 mm were needed to obtain measurements of samples containing 10%, 20%, 30%, 40%, 50%, 60%, 70% and 80% (w/w) total solids. Thermal gelation of the BSA samples was carried out by applying small amplitude oscillatory measurements at a constant strain of 0.1–0.001% and an angular frequency of 1 rad/s. About 3.1 ml of sample was introduced onto the Peltier plate of the instrument and a thin layer of silicon oil was placed on the gap between the two measuring plates to minimise evaporation. Prior to analysis, samples were equilibrated at 25 °C for 2 min. Samples were then heated from 25 to 85 °C at a rate of 2 °C/min, kept at that temperature for 20 min under observation and further monitored with an increasing frequency from 0.1 to 100 rad/s at 85 °C to examine the nature of formed gels. Thus, the low-amplitude oscillatory measurements of the real (G' ; storage modulus) and imaginary (G'' ; loss modulus) parts of the complex shear modulus ($G^* = G' + iG''$), and $\tan \delta$ (G''/G') were recorded at atmospheric pressure (Dissanayake et al., 2012; Kasapis, 2012). A similar experimental protocol was adopted for materials at this solid content that were previously subjected to high pressure. All experiments were performed in triplicate and effectively overlapping traces were reported.

The investigation of vitrification phenomena using small amplitude oscillatory measurements of 80% (w/w) BSA samples at atmospheric pressure or pressurised at 600 MPa for 15 min was obtained at a constant strain of 0.001% and angular frequency of 1 rad/s. In doing so, the 80% BSA samples at atmospheric pressure were thermally denatured by heating from 25 to 85 °C at a rate of 2 °C/min, kept at that temperature for 20 min and then cooled down at 2 °C/min to subzero temperatures. Frequency sweeps were also carried out from 0.1 to 100 rad/s and strain of 0.001% at the low temperature end of the experimental routine, at constant temperature intervals of 4 °C to facilitate theoretical modelling of the molecular processes responsible for the observed viscoelastic behaviour in the glass transition region and glassy state. Pressurised samples of 80% BSA were cooled from 25 °C to subzero temperatures at the above controlled scan rate followed by frequency sweeps to match the analysis implemented for the atmospheric pressure counterparts. In the high solid (80%) regime, there was no ice formation at subzero temperatures, which allowed for treatment of the results with the glass transition theory.

2.2.2. Micro differential scanning calorimetry

Thermal analysis of the BSA samples was measured using a micro differential scanning calorimeter VII (Setaram, France). The calorimetric furnace of Micro DSC VII was made of a double aluminium wall, with the experimental and reference vessels being inserted into the furnace via two cylindrical cavities. The minimum and maximum temperatures that could be reached using a Peltier device were –20 and 120 °C, respectively. The purging rate of nitrogen gas was 50 ml/min. Samples of around 850 mg were accurately weighed into aluminium pans and hermetically sealed. A pan with water of equal weight was used as the reference. The 10%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% and 80% (w/w) BSA samples at atmospheric pressure were analysed by equilibrating at 25 °C, ramping the temperature to 100 °C at a rate of 1 °C/min and then cooling to 20 °C at 1 °C/min. Similar experimental settings were utilised for the pressure treated samples at the same concentrations. The enthalpy of denaturation (ΔH) and denaturation temperature (T_d) were determined using the instrument software after manually setting the start and end

points of the peak. An excellent reproducibility was recorded amongst the various replicates of each experimental concentration. All experiments were performed in triplicate with data statistical testing using ANOVA (one way, $p > 0.05$).

2.2.3. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) for the pressurised, heat-treated and atmospheric samples of BSA was carried out using a Perkin Elmer Spectrum 100 FT-IR spectrometer combined with Spectrum software, Version 6.0.2 (Springvale Road, Glen Waverley, Melbourne, Vic, Australia). Sample spectra were obtained in the absorbance mode. For each spectrum, an average of four scans was recorded at 4 cm^{-1} resolution in the range of $1400\text{--}1800\text{ cm}^{-1}$ after atmospheric and background subtraction. The spectra of the amide I and amide II peaks were deconvoluted, using the proprietary software Spectrum Version 6.0.2, to obtain the characteristic signals that represented the secondary conformation of the protein. All experiments were performed in triplicate with data statistical testing using ANOVA (one way, $p > 0.05$).

3. Results and discussion

3.1. Structure formation in BSA samples

Small deformation dynamic oscillation in shear was employed to provide an insight into the mechanical properties of BSA preparations ranging in solids content from 10% to 80% (w/w). The development of a three-dimensional structure for the atmospheric materials is illustrated in Fig. 1(a), where the logarithm of the storage modulus (G') was plotted against a linear temperature–time equivalence that extended to $85\text{ }^{\circ}\text{C}$ over a 50 min period of observation. Samples of up to 50% solids demonstrated liquid-like behaviour during the initial period of treatment, but transformed to gels with increased heating. As discussed in the literature, heating unfolds the interior pocket of the molecule to expose a single cysteine molecule at position 34 and provides easy access to the creation of disulphide bridges for further aggregate formation (Leibman et al., 1975; Oakes, 1976). Raising the BSA content further, from 60% to 80%, exhibited an immediate solid-like behaviour, where the values of the storage modulus stayed well above 10^3 Pa throughout the experimental routine.

In contrast to the above discussion, pressurised BSA samples recorded from the beginning of the heating scan strong structures at low concentrations, for example, in the storage modulus values of 20% (w/w) solids that were approximately $10^{2.5}\text{ Pa}$ at $25\text{ }^{\circ}\text{C}$ (Fig. 1(b)). Packed polymeric matrices achieved with HHP treatment were partially loosened during thermal treatment, but the samples remained very rigid, with G' values remaining close to 10^7 Pa throughout the combined temperature/time run for the 80% solids formulation. In both atmospheric and pressurised systems, there was a gradual drop in the G' values from 45 to $75\text{ }^{\circ}\text{C}$ with increasing temperature, owing to the destabilisation of hydrogen bonding and electrostatic interactions in the protein molecule. This was followed by protein denaturation, the build-up of the thiol/disulphide interchange reactions and hydrophobic interactions leading to an increase in the values of G' at the end of the heating and isothermal runs at $85\text{ }^{\circ}\text{C}$ (Clark & Lee-Tuffnell, 1986; Considine, Patel, Anema, Singh, & Creamer, 2007).

Rheological results in Fig. 1(a,b) argued that high pressure processing had an effect on BSA, which was shown by the early structure formation upon commencing the heating run. Samples, however, further denature at higher temperatures, according to experience with the thermally treated systems, and create cohesive three-dimensional structures at the end of the experimental test. The micromolecular nature of the two types of associations

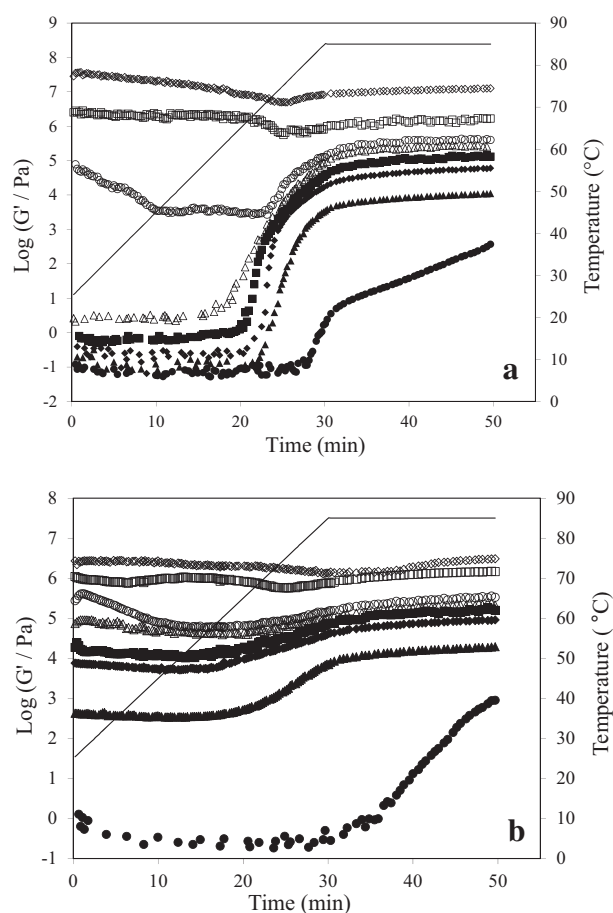


Fig. 1. Changes in storage modulus (G') of 10 (●), 20 (▲), 30 (◆), 40 (■), 50 (△), 60 (○), 70 (□) and 80% (◇) (w/w) BSA samples during heating from 25 to $85\text{ }^{\circ}\text{C}$ at a rate of $2\text{ }^{\circ}\text{C}/\text{min}$ and holding at $85\text{ }^{\circ}\text{C}$ for 20 min at a frequency of 1 rad/s and strain from 0.1% to 0.001% for (a) atmospheric pressure and (b) pressurisation at 600 MPa for 15 min.

produced by the atmospheric and pressurised samples will be further examined below.

3.2. Micromolecular aspects of the thermal behaviour in BSA systems

The extent of molecular rearrangements, as a function of thermal and high pressure treatments in BSA, was monitored by measuring the denaturation temperature of the protein systems and the change in enthalpy (ΔH) in endothermic events using micro differential scanning calorimetry. Fig. 2(a,b) depict the thermograms of our samples containing up to 80% solids during heating from 25 to $100\text{ }^{\circ}\text{C}$ at a scan rate of $1\text{ }^{\circ}\text{C}/\text{min}$. As before, the systems were tested either at atmospheric pressure or after pressurising at 600 MPa for 15 min. In both cases, there were well-defined troughs, but with some variation in size and range of temperature bands, which reflect the main characteristics of the endothermic event. The emphasis here was to pinpoint the temperature range of first-order thermodynamic transitions and to calculate the change in enthalpy (ΔH) in the endothermic processes from the area under the peak of the thermal event.

Increasing the polymer concentration up to 55% in preparations produced bimodal thermograms with increasing temperature. Thus, the spectrum of denaturation temperatures for the atmospheric samples was from 61 to $77\text{ }^{\circ}\text{C}$ and from 76 to $91\text{ }^{\circ}\text{C}$ for the first and second unfolding transitions, respectively (Table 1). In pressurised materials, the corresponding values for the two

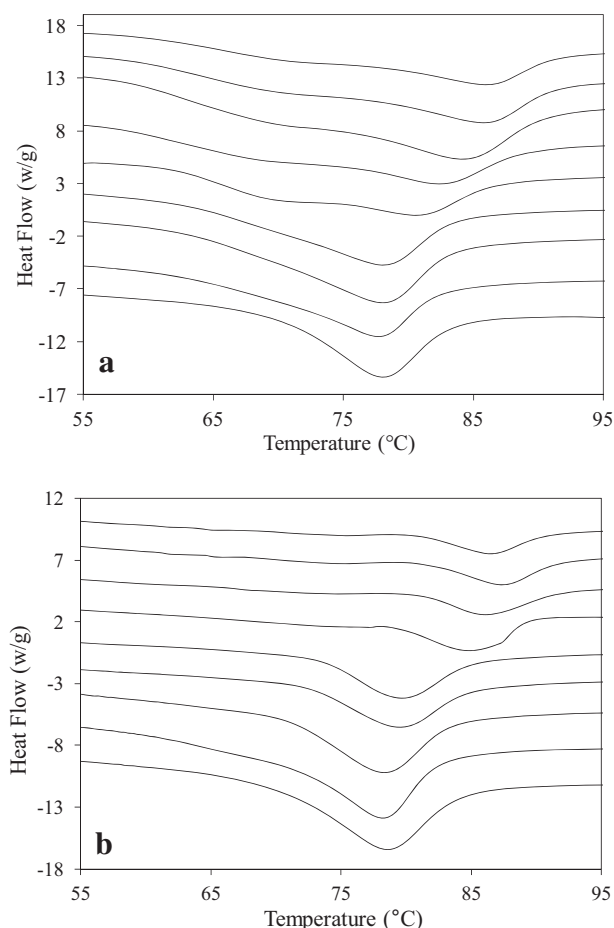


Fig. 2. Microcalorimetry thermograms of 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75% and 80% (w/w) BSA samples during heating from 25 to 100 °C at a rate of 1 °C/min for (a) atmospheric pressure and (b) pressurisation at 600 MPa for 15 min arranged successfully downwards (complete set of data for all experimental concentrations is shown in Table 1).

endothermic peaks were 65–81 °C and 80–92 °C, respectively. In general, there was an increase in the values of various denaturation temperatures (onset, peak and endset) of 4–5 °C with treatment, which should be attributed to the high density effect of HHP on protein matrices. As expected, the enthalpy requirement (ΔH) for denaturation raised with the protein content from 10% to 80% w/w.

Literature has reported the deconvolution of DSC thermograms for aqueous BSA preparations, at a relatively dilute regime (e.g. 10% solids), into two independent phase transitions, which depend on pH and ionic strength (Kang & Singh, 2003). It has been suggested that the two peaks appear when the native conformation of the protein is broken leading to the formation of a crevice surrounded by two thermodynamically independent domains (Barone et al., 1995; Yamazaki, Yano, & Aoki, 1990). The low temperature DSC peak corresponded to a BSA domain in the vicinity of tryptophan at position 212. The high temperature DSC peak is associated with the cooperative collapse of two homologous BSA structures (second domain), which together assembled to form a heart shaped molecule in the native conformation (Barone, Del Vecchio, Fessas, Giancola, & Graziano, 1993; Giancola, De Sena, Fessas, Graziano, & Barone, 1997).

This deconvolution pattern was reflected in our results, but at the higher BSA concentrations (>60%, w/w) a single peak developed, Fig. 2(a,b). In this regime, the molecular domains of the BSA structure appear to collapse together *via* a broad endothermic

event due to the scarcity of water molecules needed for the formation of thermodynamically distinct morphologies in the protein (Michnik, 2003). Overall, application of high pressure processing had a partial effect on the native morphology of BSA, which undergoes further denaturation during heating congruent with the thermograms of the atmospheric counterparts.

3.3. Further probing into the micromolecular aspects of BSA matrices

In this part of the present discussion, we have taken advantage of the specific absorbance of protein molecules under infrared spectroscopy that is characteristic of the native conformation and structure. In doing so, atmospheric, temperature and high pressure treated samples were examined using infrared spectroscopy (FTIR) to observe transformations in molecular architecture. The infrared active amide vibrations characteristic of BSA were observed in FTIR spectra as amide I and amide II bands in the 1700–1480 cm^{-1} region. The amide I band presented in the range of 1700–1600 cm^{-1} , was primarily attributed to an out-of-phase combination of C=O and C–N stretching of amide groups and relates directly to the secondary structure (Doesseau & Pezolet, 1990; Surewicz, Mantsch, & Chapman, 1992). The amide II band occurred in the region of 1575–1480 cm^{-1} and was attributed to an out-of-phase combination of in-plane C–N stretching and N–H bending of amide groups (Byler & Susi, 1986; Dong, Huang, & Caughey, 1990).

Fig. 3(a) illustrates the original FTIR spectra for selected BSA preparations obtained at atmospheric pressure without any treatment, after pressurisation and finally following thermal processing. Fig. 3(b) summarises all the infrared results in amide I throughout the experimental concentration range. Clearly, pressurisation had an effect on the secondary structure of the molecule, which is further altered by thermal treatment, seen in some detail in the intensity of amide I peaks. Quantitative estimation of the secondary structure is based on the assumption that the protein conformation is the linear sum of certain fundamental secondary elements. Thus, deconvolution of a specific band (in particular amide I) generated a series of secondary peaks that represented different components within the secondary structure of the protein. The approach helps considerably with determination of the proportion of secondary elements in the molecule, as a result of processing or physicochemical environment (Dong et al., 1990).

Fig. 3(c,d) explored this approach by displaying changes in the secondary structure recorded at ambient temperature for native preparations, samples that have been previously pressurised at 600 MPa for 15 min, and heat treated at 85 °C for 20 min by assigning band frequencies to conformational characteristics of the protein. The secondary conformation of 30% (w/w) BSA in Fig. 3(c) was in agreement with the reported percentages of α -helix and β -sheets structures that are about 40–60% and 15–20% in the native form, respectively (Carter & Ho, 1994; Clark, Saunderson, & Suggett, 1981). When the protein was in the denatured form, the intensity of the former signal decreased, suggesting partial loss of the α -helix content. Moreover, this decrease was accompanied by the appearance of intermolecular β -sheet strands, which are an important conformational component of a partially aggregated secondary structure (Stokkum et al., 1995).

The FTIR work was repeated for all experimental concentrations, with preparations of 80% solids also shown (Fig. 3d), to produce similar patterns of changing conformation with the low-solid counterparts. Results argue that BSA largely retained its native conformation following pressurisation, which should also maintain bioactivity alongside structural functionality. Network formation in high-solid systems, in relation to the glass transition phenomena, merits further investigation using the rheological techniques described below.

Table 1

Thermal properties of 10–80% (w/w) BSA systems detected by micro differential scanning calorimetry.

Sample	Onset temperature (°C)	Peak temperature (°C)	Endset temperature (°C)	ΔH (J/g)
A – 10%	64.0 ± 1.8	67.0 ± 1.0	73.7 ± 0.4	0.126 ± 0.026
	75.7 ± 1.5	85.3 ± 0.4	87.6 ± 0.8	0.390 ± 0.110
A – 20%	67.3 ± 1.2	70.7 ± 1.0	76.7 ± 0.8	0.143 ± 0.050
	80.0 ± 1.2	86.3 ± 1.8	90.0 ± 0.4	1.039 ± 0.172
A – 30%	61.7 ± 0.8	68.7 ± 1.1	76.3 ± 1.0	0.151 ± 0.052
	79.0 ± 0.8	86.6 ± 0.8	90.6 ± 0.6	1.209 ± 0.367
A – 35%	60.7 ± 0.8	68.3 ± 0.4	75.3 ± 1.0	0.147 ± 0.031
	79.6 ± 0.5	86.6 ± 0.8	90.6 ± 1.5	1.254 ± 0.074
A – 40%	61.0 ± 0.4	68.3 ± 0.3	75.3 ± 1.0	0.165 ± 0.381
	79.0 ± 1.2	86.6 ± 0.4	91.0 ± 0.4	1.304 ± 0.097
A – 45%	61.3 ± 1.7	69.0 ± 1.0	75.0 ± 0.4	0.179 ± 0.031
	78.3 ± 1.6	85.6 ± 0.5	90.0 ± 1.7	1.553 ± 0.103
A – 50%	63.3 ± 1.1	68.3 ± 0.4	74.3 ± 0.9	0.239 ± 0.059
	81.1 ± 1.1	85.1 ± 0.2	90.0 ± 0.4	1.711 ± 0.237
A – 55%	60.0 ± 1.7	67.7 ± 1.0	73.7 ± 0.4	0.357 ± 0.036
	77.3 ± 0.5	83.3 ± 0.4	87.6 ± 0.8	2.085 ± 0.144
A – 60%	64.0 ± 0.9	69.3 ± 0.8	74.0 ± 0.7	0.407 ± 0.016
	76.3 ± 0.1	82.0 ± 1.3	85.6 ± 0.2	3.203 ± 0.347
A – 65%	65.7 ± 0.1	78.3 ± 1.8	83.3 ± 1.1	3.754 ± 0.247
A – 70%	73.3 ± 1.1	78.3 ± 0.6	81.6 ± 0.9	3.815 ± 0.227
A – 75%	67.7 ± 0.8	77.7 ± 1.1	82.3 ± 1.0	4.011 ± 0.251
A – 80%	70.7 ± 1.8	78.0 ± 0.1	83.3 ± 0.9	4.230 ± 0.130
P – 10%	65.3 ± 1.0	71.7 ± 0.4	77.0 ± 1.5	0.057 ± 0.018
	80.0 ± 1.3	85.3 ± 0.9	89.3 ± 0.4	0.306 ± 0.094
P – 20%	66.5 ± 0.7	72.6 ± 0.6	81.2 ± 1.6	0.098 ± 0.014
	82.3 ± 0.9	85.7 ± 0.4	89.7 ± 0.4	0.776 ± 0.076
P – 30%	70.0 ± 1.7	75.0 ± 1.5	80.7 ± 0.5	0.117 ± 0.008
	82.0 ± 1.3	86.1 ± 0.1	90.7 ± 0.4	0.960 ± 0.131
P – 35%	70.0 ± 1.8	76.3 ± 1.0	79.3 ± 1.5	0.110 ± 0.012
	81.7 ± 0.4	87.0 ± 0.5	91.2 ± 1.2	1.043 ± 0.065
P – 40%	68.4 ± 0.8	74.7 ± 1.1	78.6 ± 1.3	0.160 ± 0.036
	81.5 ± 0.7	87.3 ± 0.3	91.9 ± 0.1	1.108 ± 0.236
P – 45%	67.7 ± 0.4	74.7 ± 0.4	78.7 ± 0.4	0.159 ± 0.039
	81.5 ± 0.8	86.6 ± 0.5	91.3 ± 1.6	1.342 ± 0.029
P – 50%	68.6 ± 0.5	73.7 ± 0.4	77.9 ± 0.3	0.162 ± 0.024
	81.1 ± 1.3	86.0 ± 0.4	92.0 ± 0.5	1.531 ± 0.148
P – 55%	70.4 ± 0.8	74.7 ± 0.7	77.9 ± 0.3	0.167 ± 0.001
	80.3 ± 1.0	85.0 ± 1.2	89.4 ± 1.3	2.011 ± 0.205
P – 60%	73.5 ± 0.7	79.7 ± 0.4	85.2 ± 1.5	3.270 ± 0.100
P – 65%	72.7 ± 0.4	79.6 ± 0.7	84.6 ± 1.1	3.432 ± 0.052
P – 70%	71.7 ± 0.4	78.3 ± 0.9	83.0 ± 0.6	3.550 ± 0.185
P – 75%	71.0 ± 0.6	78.3 ± 1.3	82.6 ± 0.7	3.789 ± 0.248
P – 80%	71.3 ± 0.9	78.7 ± 0.6	83.7 ± 0.6	4.023 ± 0.162

A – atmospheric, P – pressure at 600 MPa for 15 min, ΔH – denaturation enthalpy.

3.4. Vitrification phenomena in condensed BSA samples

In the last part of this work, we examined the molecular packing of condensed BSA matrices, with reference to possible applications in high-solid systems for the food and pharmaceutical industries. Fig. 4(a) reproduces small deformation oscillatory profiles of 80% (w/w) atmospheric and pressurised BSA samples. The former needed thermal activation at 85 °C to form a three dimensional network. Upon cooling of both materials, at a rate of 1 °C/min, constant frequency of 1 rad/s and a small amplitude of oscillatory strain at 0.001%, the values of the storage modulus increased considerably to represent distinct consistencies in the viscoelastic master curve.

In further experimentation, materials were heated to reproduce the outcome of the cooling protocol in Fig. 4(a), but the effective timescale of observation was extended from 0.1 to 100 rad/s. The frequency dependence of storage and loss modulus was plotted in double logarithmic plots at regular temperature intervals of 4 °C for both the atmospheric systems and their counterparts that have been subjected to prior pressurisation (data not shown). Relatively low values were observed at the high temperature end (e.g. 16 °C for atmospheric and –39 °C for the pressurised samples), but there was a substantial build-up of viscoelasticity at lower temperatures (e.g. –20 °C for atmospheric and –75 °C for pressurised systems).

Following this, we employed a method of reduced variables to construct master curves of viscoelasticity as a function of time of observation (Prolongo, Salom, & Masegosa, 2002). Data were processed by choosing arbitrarily a point as the reference temperature (T_0), and shifting the remaining spectra along the log frequency axis until a uniform curve was obtained. Reference temperatures within the glass transition region were 12 and –55 °C for the atmospheric and pressurised BSA samples, respectively. Fig. 4(b and c) reproduce the master curves of viscoelasticity for the two types of BSA at 80% (w/w) solids, with the reduced variables of shear modulus (G'_p and G''_p) being plotted logarithmically as a function of reduced frequency of oscillation (ωa_T). Data reduction unveiled an extensive range of oscillatory frequencies of up to fourteen orders of magnitude, for example, from 10^{-4} to 10^{11} rad/s, and there was also a combined increase in the values of G'_p and G''_p , for example, from 10^4 to 10^8 Pa (Fig. 4c).

Data reduction yielded two sets of shift factors (a_T), which possessed a fundamental value in describing the patterns of molecular relaxation of the system within vitrification (Maltini & Anese, 1995). Plotting the shift factors, as a function of experimental temperature in Fig. 5, adapted the approach used extensively by material scientists in the development of mechanistic understanding of glassy phenomena in combination with the theory of free volume (Ferry, 1980). According to Ferry, holes between the packing irregularities of long chain segments or the space required for their

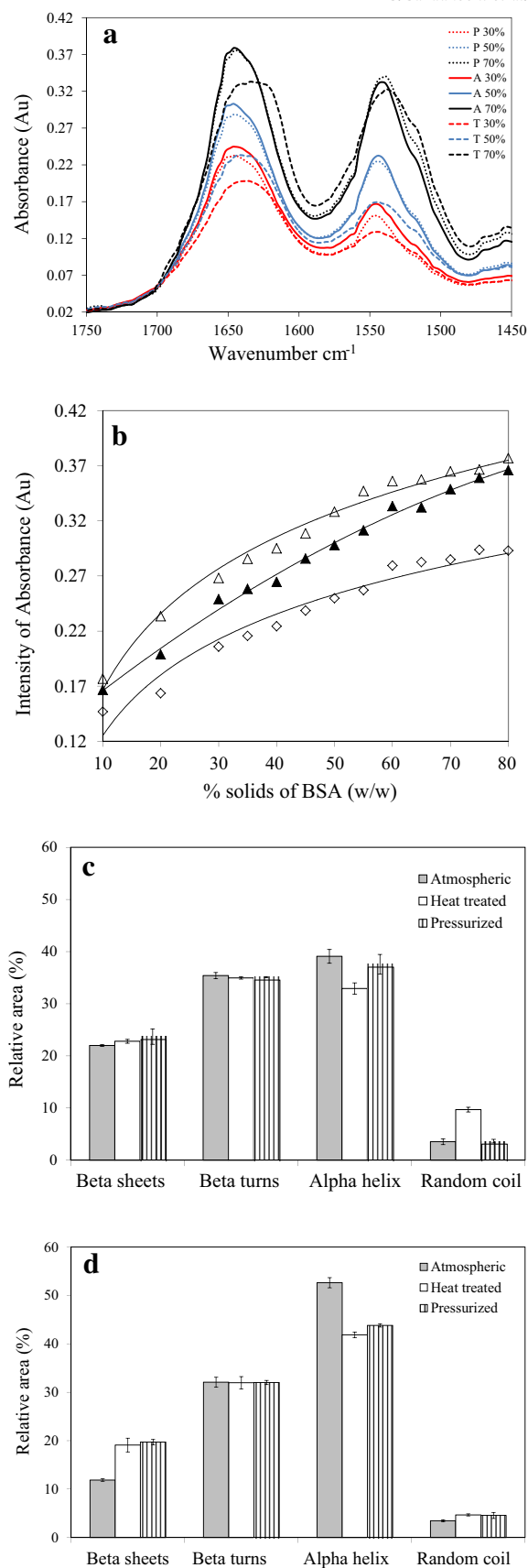


Fig. 3. (a) FTIR spectra of 30%, 50% and 70% (w/w) BSA samples at atmospheric pressure, after pressurization and with heat treatment at 85 °C for 20 min; (b) absorbance variation as a function of concentration for the samples tested at atmospheric pressure (Δ), after pressurization at 600 MPa for 15 min ($\blacktriangle$) and with heat treatment at 85 °C for 20 min ($\diamond$) observed by amide I infrared spectroscopy; secondary conformation of BSA at 30 (c) and 80% (d) total solids (w/w).

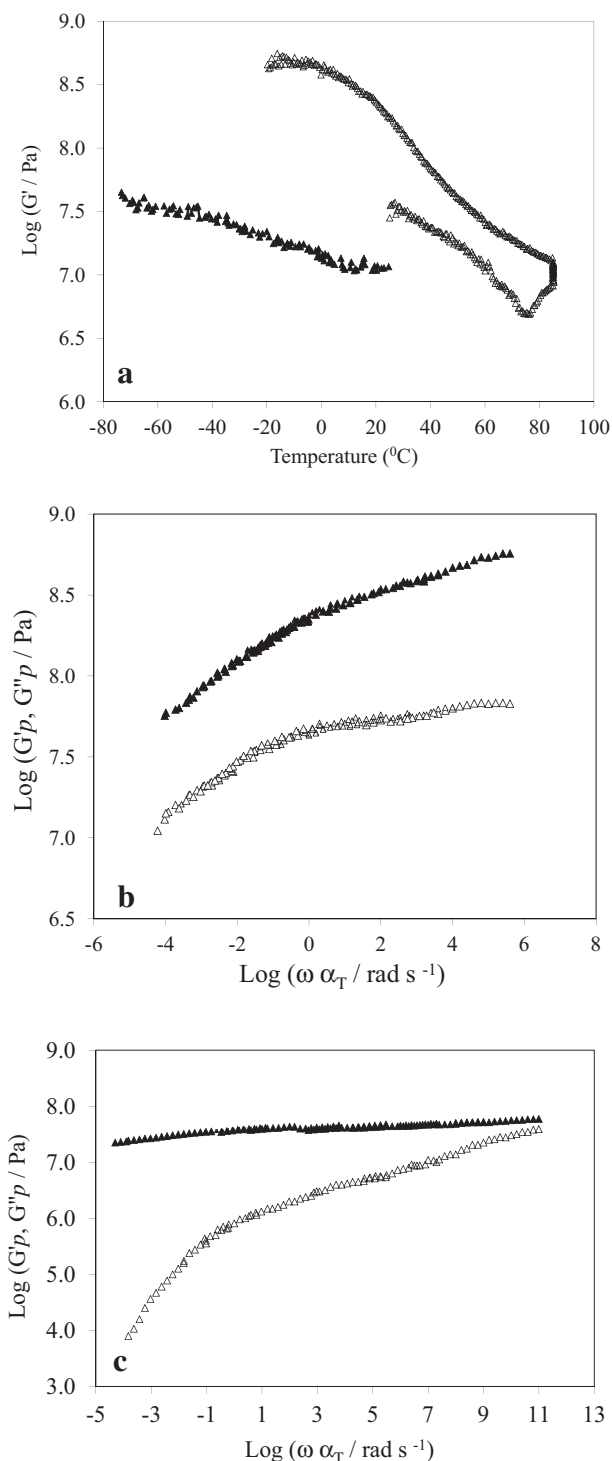


Fig. 4. (a) Variation of storage modulus (G') for 80% (w/w) BSA at constant frequency of 1 rad/s and strain of 0.001%, with atmospheric pressure samples being heated from 25 to 85 °C, held at 85 °C for 20 min and cooled down to –20 °C (Δ) and pressurized samples at 600 MPa for 15 min being cooled from 25 to –80 °C ($\blacktriangle$); master curves of reduced shear modulus [G'_p (Δ) and G''_p (Δ)] as a function of reduced frequency of oscillation (ωa_T) for 80% (w/w) BSA samples at atmospheric (b) and pressurized (c) conditions.

string-like movements account for the free volume. Adding to that, the space occupied by the van der Waals radii of polymeric contours and the thermal vibrations of individual residues, i.e. the occupied volume, we come up with the total volume per unit mass of a macromolecule.

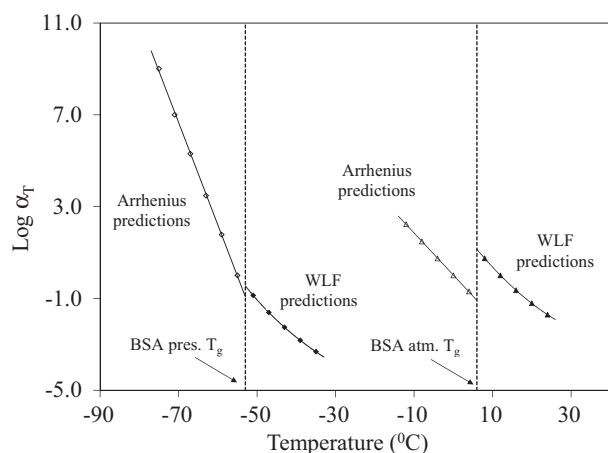


Fig. 5. Temperature variation of factor a_T within the glass transition region (closed symbols) and the glassy state (open symbols) for 80% (w/w) BSA samples at atmospheric conditions (triangles) and pressurised at 600 MPa for 15 min (diamonds); solid lines reflect the WLF and modified Arrhenius fits of the shift factors during the vitrification process, with the dashed lines indicating the predictions of the mechanical glass transition temperature.

The mathematical format of the theory of free volume for dynamic oscillatory data is given by the Williams, Landel and Ferry (WLF) equation, as follows (Ferry, 1980):

$$\log a_T = - \frac{(B/2.303f_o)(T - T_o)}{(f_o/\alpha_f) + T - T_o} \quad (1)$$

where, f_o is the fractional increase in the free volume at T_o (or f_g at T_g), α_f is the thermal expansion coefficient, and the value of B is set to be about one.

An alternative approach to probe mechanical data in the viscoelastic master curve is based on the predictions of the classical reaction-rate theory. For our purposes, this can be formulated in terms of the modified Arrhenius equation (Gunning, Parker, & Ring, 2000):

$$\log a_T = \frac{Ea}{2.303R} \left(\frac{1}{T} - \frac{1}{T_o} \right) \quad (2)$$

where, R is the gas constant. This mathematical expression follows a progress in shift factors by integrating two sets of temperature data. It returns a constant activation energy (E_a), which argues that the relaxation processes in the temperature range of interest are heavily controlled by specific chemical features.

As shown in Fig. 5, the WLF equation follows the progress in viscoelasticity at the upper range of experimental temperatures thus making free volume the molecular mechanism dictating diffusional mobility. Application of the combined WLF/free volume theoretical framework yielded f_g , α_f and T_g estimates of 0.040, 8×10^{-4} deg and 6 °C for the atmospheric samples, with the corresponding parameters for the pressurised counterparts being 0.040, 6×10^{-4} deg and -53 °C, respectively. The values of fractional free volume at the glass transition temperature and thermal expansion coefficient are congruent with those reported in the literature for synthetic amorphous polymers (Kasapis, 2012; Shrinivas & Kasapis, 2010), hence arguing that BSA undergoes glassy transformation within the present experimental settings.

In addition, there was an apparent change in the pattern of the shift factor at the lower temperature range in Fig. 5 that cannot be followed by the WLF equation. Instead, progress in viscoelasticity at these temperatures is better described by Eq. (2), which yields the concept of activation energy for an elementary flow process that is independent of temperature (Kasapis, Al-Marhoobi, Deszczynski, Mitchell, & Abeysekera, 2003). Based on the

assumption that the free volume decreases linearly with temperature, leading to a discontinuity in the linear trend of factor a_T in Fig. 5, the glass transition temperature is defined empirically as the point below which thermal motions become extremely slow. Thus, the experimental T_g should be located at the end of the glass transition region (or onset of the glassy state), where the free volume declines to about 3–4% of the total volume of the material (Ferry, 1980). According to Fig. 5, the experimental T_g observations were in close agreement with those predicted in the preceding paragraph based on WLF/free volume modelling.

4. Conclusions

The present work extends the application of high pressure processing to globular proteins in relation to their techno- and bio-functionality. Previously, we reported on whey protein preparations, with the work indicating protein unfolding and irreversible denaturation with pressurization (Dissanayake et al., 2012). The high-pressure labile whey protein in the native state was attributed to the presence of a hydrophobic core in β -lactoglobulin, which is disturbed irreversibly to allow for the transfer of water molecules to nonpolar residues in the corpuscular interior. Current results demonstrate the opposite effect of HHP on the structural properties of a globular protein. It appears that the BSA molecule, with a sequence of seventeen disulphide bridges and accompanying secondary bonds, can largely resist pressurisation in formulations made of low or high levels of solids.

Cooling of high-solid BSA structures subjected to prior high pressure produced thermomechanical profiles of coherent structure formation, as for the atmospheric counterparts. It appeared that the limited denaturation of the protein under high pressure supports vitrification at subzero temperatures. This network type in largely undenatured and “partially activated” BSA molecules is known as a molten globular state. The characteristics of this state, which have been reported for a number of globular proteins in the literature, include large retention of native secondary structure, compact volume which is 10–20% larger than the native state, and propensity towards aggregation. This type of network is, of course, distinct from the conventional “string of beads” structure obtained from thermally treated BSA molecules and is reflected in the distinct predictions of the glass transition temperature for the two systems.

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