Influence of High Pressure on Bovine Serum Albumin and Its Complex with Dextran Sulfate

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High-pressure processing of bovine serum albumin (BSA) solutions (0.1−1 wt % protein, pH 7) has shown decreasing protein surface hydrophobicity with increasing pressure, which is further reduced in the presence of dextran sulfate (DS) (BSA:DS weight ratio of 2:1 and 4:1). The total calorimetric enthalpy ΔH for pure BSA is substantially reduced after treatment at 600 MPa, and both the endothermic peak temperature and the value of ΔH for BSA + DS is reduced under the same treatment conditions. Size exclusion chromatography indicates extensive pressure-induced protein unfolding and aggregation during BSA treatment at 400 MPa. Complexation with polysaccharide at low ionic strength protects the globular protein against pressure-induced aggregation. The loss of the protective effect of DS on addition of electrolyte (0.1 M NaCl) is consistent with the predominantly electrostatic character of the protein−polysaccharide interaction.

Keywords: *Protein−polysaccharide interaction; high-pressure processing; surface hydrophobicity; protein aggregation; differential scanning calorimetry; size exclusion chromatography*

INTRODUCTION

Proteins and polysaccharides are present together in most food products of commercial significance. Both types of macromolecules contribute to the structure, texture, and stability of food colloids through their gelation and aggregation behavior (Dickinson and Stainsby, 1982; Mitchell and Ledward, 1986; Dickinson and McClements, 1995). The colloidal properties are dependent, not only on the functionality of the individual food biopolymers, but also on the nature and strength of the protein−polysaccharide interactions (Dickinson, 1993, 1995a,b).

Macromolecular interactions may be weak or strong, specific or nonspecific, attractive or repulsive. In solution, protein and polysaccharide molecules may be joined together in two main ways (Dickinson, 1993, 1995a): (a) through a covalent linkage arising from chemical reaction to form a permanent protein−polysaccharide supermolecule; or (b) through noncovalent association (e.g., attractive electrostatic interaction) to form a (potentially) reversible protein−polysaccharide complex. Strong covalent linkages between proteins and polysaccharides are less susceptible to disruption—for example, by pH change or heating—than are electrostatic complexes. The latter can be formed from binary mixtures of biopolymers having either the opposite or the same net charge (Ledward, 1979, 1996; Tolstoguzov, 1986). The nature and strength of electrostatic protein−polysaccharide interactions are sensitive to environmental conditions (pH, ionic strength, temperature) as well as to the detailed protein structure. For instance, at pH 7 and low ionic strength, it has been shown that the anionic polysaccharide dextran sulfate (DS) forms an interfacial electrostatic complex with adsorbed bovine serum albumin (BSA) in both emulsions (Dickinson and Galazka, 1992) and foams (Izgi and Dickinson, 1995), but no such complex with adsorbed β-lactoglobulin (Dickinson and Galazka, 1991).

Growing interest from the food industry in high-pressure processing technology has led to increased research on the effects of high pressures (0−1000 MPa) on the structures of globular proteins in aqueous solution (Heremans, 1982, 1995; Hayakawa *et al.*, 1992, 1994, 1996; Masson, 1992; Dumay *et al.*, 1994; Defaye *et al.*, 1995; Johnston and Murphy, 1995; Galazka *et al.*, 1996a). Since formation of ion pairs and hydrophobic bonds is accompanied by a large positive volume change (Masson, 1992), the application of high isostatic pressures to protein molecules in solution can have a strongly disruptive effect on intramolecular electrostatic and hydrophobic interactions. Conversely, hydrogen bond formation is associated with smaller volume changes (+1 to −3 mL mol^{-1} per interacting group), and so it is less pressure sensitive. This means that, while high-pressure treatment readily disrupts the tertiary and quaternary structure of globular proteins, it has less influence on the secondary structure. As with thermal processing, the pressure-induced denaturation of globular proteins can rapidly lead to aggregation (Dumay *et al.*, 1994; Galazka *et al.*, 1996a,b), and ultimately, at high enough concentrations under the appropriate conditions, to precipitation or gelation (Johnston, 1992). In particular, changes in the structure and state of aggregation of BSA are dependent on the magnitude of the applied pressure and on the treatment time (Hayakawa *et al.*, 1992, 1994, 1996; Galazka *et al.*, 1996a). During pressure treatment of BSA in solution at (pre-pressure) pH 7, the strong protein aggregation is considered to be mainly due to formation of intermolecular disulfide bridges via −SH/−SS− interchange (Galazka *et al.*, 1996a).

While there has been little work reported so far on the high-pressure processing of mixed biopolymer sys-

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tems, one might expect non-covalent protein−polysaccharide interactions to be susceptible to considerable modification as a result of pressure-induced changes in electrostatic interactions and protein conformational structure. During high-pressure treatment, all carboxyl groups on proteins and polysaccharides will become ionized, irrespective of the initial pH. So, in a weakly buffered system, the pH may fall and more potential ionic binding sites may be produced. On pressure release, the degree of ionization will decrease again, but the conformation of the protein may in part be irreversibly changed, which in turn may modify any interactions. Even in a system where no specific protein−polysaccharide interaction is present, the increased hydration of the macromolecules at high pressure, which is associated with the increased net charge, may affect the mixed biopolymer solution behavior.

The model system chosen for investigation here, BSA + DS, forms an electrostatic complex at neutral pH (Dickinson and Galazka, 1992). In a recent study of oil-in-water emulsions containing these two biopolymers (Dickinson and Pawlowsky, 1996a), it was found that high-pressure treatment (≥400 MPa) of the protein prior to emulsification affects the flocculation and rheology of the resulting BSA-stabilized emulsions containing added polysaccharide. A preliminary study of BSA + DS in solution has suggested (Galazka *et al.*, 1996a) that the presence of DS (1:1 by weight) affects the extent of BSA unfolding determined after pressurization (at 800 MPa). This new paper reports on a more detailed investigation of the influence of high pressures from 300 to 800 MPa on the unfolding and aggregation of BSA in rather more dilute solution at neutral pH in the absence and presence of this anionic polysaccharide at BSA:DS weight ratios 2:1 and 4:1.

MATERIALS AND METHODS

Bovine serum albumin (Fraction V powder, product A-2934, lot 93H0291) and dextran sulfate (5×10^5 Da, containing 0.5−2.0% phosphate buffer salts pH 6−8, made by treatment of dextran T-500 with chlorosulfonic acid in pyridine, product D-6001, lot no. 112H0372) were purchased from Sigma Chemical Co. (St. Louis, MO). The protein alone (1 wt %) or a mixture of BSA (1 wt %) + DS (0.25 or 0.5 wt %) was dissolved in HPLC grade water, and pH adjustments were made by addition of 0.05 M HCl or 0.05 M NaOH. Buffer solutions for use in the chromatography were prepared from analytical grade reagents and HPLC grade water. 1-Anilinonaphthalene-8-sulfonate (ANS) ammonium salt (316.4 Da) was obtained from SERVA (Feinbiochemica, Heidelberg).

Approximately 5 mL samples of protein solution (1 wt %) and solutions of BSA + DS (containing 1 wt % BSA) at pH 7.0 were hermetically sealed in Cryovac bags (Cryovac−W. R. Grace Ltd., London, U.K.). These were then subjected to high-pressure treatment for 20 min at a pressure in the range 300−800 MPa (i.e., 3−8 kbar) at ambient temperature (Galazka *et al.*, 1996b) using a prototype Stansted Food-Lab high-pressure rig (Stansted Fluid Power, Essex, U.K.). Maximum pressure (800 MPa) was reached in 3 min, held for 20 min, and then released to atmospheric pressure in 1 min. The maximum temperature attained was 37 °C, and on pressure release the temperature decreased to 11 °C. The pH of the pressure-treated solutions *before* and *after* treatment was 7.0 ± 0.2, but we do not know the precise values of pH *during* pressure treatment. [We suppose here, however, that the pH during pressurization was reasonably constant (within 0.5 unit) since protein buffering in water at neutral pH is mainly influenced by histidine and α-amino groups whose dissociation is accompanied by relatively little volume change (Funtenberger *et al.*, 1995).] Pressure-treated solutions were kept on ice for 1−2 h before analysis.

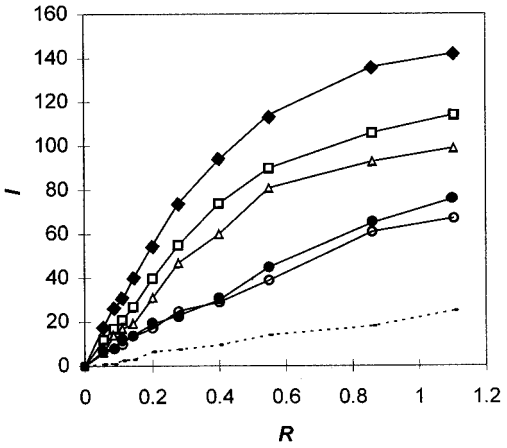

Figure 1. Probe spectrofluorometry for ANS binding to BSA at pH 7. Measurements were made for solutions of native and pressure-treated BSA (0.13−12 μM) at a constant ANS concentration of 2.40 μM. Relative fluorescence intensity *I* (monitored at 470 nm from excitation at 350 nm) is plotted against the BSA−ANS molar ratio *R* for various high-pressure treatments of 20 min duration: ◆, untreated; □, 300 MPa; △, 400 MPa; ●, 600 MPa; ○, 800 MPa; ---, untreated BSA + DS mixture. Plotted values are means based on triplicate experiments.

Probe spectrofluorometry with ANS ammonium salt was used to determine protein surface hydrophobicity before and after pressure treatment (Nakai *et al.*, 1996). The binding of ANS to BSA in the presence and absence of DS was determined as a function of the magnitude of the applied pressure. Aliquots of solutions of BSA or BSA + DS (2:1 by weight) were mixed with a solution of ANS and the fluorescent intensity after pressure treatment was recorded on a Perkin-Elmer LS50 spectrofluorimeter (excitation 350 nm, slit 2.5 nm; emission 470 nm, slit 2.5 nm) at ambient temperature. The ANS concentration was kept constant at 2.40 μM and the BSA concentration was varied in the range 0.13−12.0 μM. Relative intensity was measured at 470 nm. Quoted values are the mean ± sd from three determinations.

Differential scanning calorimetry (DSC) measurements were carried out on a MicroCal MA2 scanning microcalorimeter (MicroCal Inc., Northampton, MA) at a scan rate of 1 °C min^{-1}. The samples were kept under nitrogen gas at 2 bar to prevent oxidation and to maintain a positive pressure in the vessel. The total (integrated) calorimetric enthalpy ΔH and the endothermic peak temperature T_m were calculated using the MicroCal Origin data analysis package. The solutions of BSA (1 wt %) or BSA + DS (1 wt % BSA; [BSA]/[DS] = 2 or 4) were heated through the temperature range 20−85 °C.

Size exclusion chromatography was carried out using a Sephacryl S-300 column (100 × 1.6 cm) calibrated with thyroglobin (6.7×10^5 Da), apoferitin (4.4×10^5 Da), β-amylase (2.0×10^5 Da), alcohol dehydrogenase (1.5×10^5 Da), bovine serum albumin (6.6×10^4 Da), carbonic anhydrase (2.9×10^4 Da), and tryptophan (2.0×10^2 Da). The column buffer was 20 mM Tris-HCl at pH 7. In some cases, 0.1 M NaCl was added to the buffer in order to assess the importance of the electrostatic contributions to the protein−protein and protein−polysaccharide interactions. Biopolymers were eluted at 280 nm from the column at a flow rate of 0.75 mL min^{-1}. All experiments with the exception of the spectrofluorometry studies were carried out using 1 wt % protein.

RESULTS AND DISCUSSION

The probe spectrofluorimetry experiments indicated a substantial reduction in the surface hydrophobicity of pure BSA following the high-pressure treatment. Figure 1 shows a plot of fluorescence intensity *I* against the BSA−ANS molar ratio *R* for treatments at various pressures (for 20 min) carried out on a 0.1 wt % protein solution initially at pH 7. There is a consistent trend

Table 1. Effect of High-Pressure Processing on the Protein Hydrophobicity (S_0) of Native and Denatured BSA (0.13−12.0 μM) and Mixtures of BSA (0.13−12.0 μM) + DS (2:1 by Weight) in Aqueous Solution (pH 7.0, 2.40 μM ANS)[a]

BSA:DS ratio (by weight)	treatment pressure (MPa)	S_0 (absorbance units/mol of ANS)
0	0	266
0	300	198
0	400	169
0	600	82
0	800	71
0.5	0	27
0.5	300	20

[a] Quoted values are the averages of triplicate measurements. Estimated error ± 10%.

of reduction in fluorescent intensity with increasing pressure. Compared with the native protein, there is a reduction in I by 20% arising from treatment at 300 MPa and a reduction in I by 53% arising from treatment at 800 MPa at a BSA−ANS molar ratio of $R = 1.1$. The S_o values determined from the initial slopes (Nakai *et al.*, 1996) are shown in Table 1. The value of the untreated BSA is about three times that of the sample treated at 800 MPa. Assuming equal binding strengths, this implies that only around one-third of the binding sites remain available to the hydrophobic probe following treatment at 800 MPa.

The reduction in surface hydrophobicity of pure BSA on pressure treatment shown by the data in Figure 1 and Table 1 is in agreement with previous results of Galazka *et al.* (1996a) and Hayakawa *et al.* (1992, 1996). The loss of surface hydrophobicity in the pressure-treated BSA could be due to intermolecular associations which reduce the accessibility of fluorescent probe binding sites, or possibly also to the burying of some of the binding sites in the refolded, partly denatured protein following pressure release. It is noteworthy that, under similar experimental conditions, the globular milk protein β-lactoglobulin exhibits a substantial *increase* in surface hydrophobicity (Galazka *et al.*, 1996a; Hayakawa *et al.*, 1996).

In the presence of anionic dextran sulfate (BSA:DS weight ratio = 2:1), the BSA surface hydrophobicity is greatly reduced, both for the native and pressure-treated protein. We see in Table 1 that the protein hydrophobicity (S_0) for the untreated mixture is 27 AU/mol ANS whereas for the pressure-treated mixture we find $S_0 = 20$ AU/mol ANS. This change is greater than the estimated error, and, in all experiments, the ANS fluorescence measurements for the pressurized mixtures were always lower than for the untreated mixtures at all R values. Because ANS has been shown not to bind at all to the polysaccharide alone, it must be assumed that the large reduction in measured surface hydrophobicity on addition of DS is due to the extensive blocking of fluorescent probe binding sites arising from BSA−DS complexation and/or electrostatic repulsion between the two negatively charged molecules. The further slight reduction in the surface hydrophobicity for BSA + DS detected on application of the high pressure suggests that some pressure-induced modification of the protein occurs also in the complexed state.

Table 2 compares DSC data at pH 7 for solutions of native and pressure-treated BSA (1 wt %) and mixtures of BSA (1 wt %) + DS (0.25 or 0.5 wt %). We can see that the endothermic peak temperature T_m and the total (integrated) calorimetric enthalpy ΔH are the same (within experimental error) for the native BSA and the BSA treated at 300 MPa for 20 min. However, treatment at 600 MPa leads to a 50% reduction in ΔH, and treatment at 800 MPa induces a decrease in T_m by several degrees, as well as a large drop in ΔH, which suggests a very substantial loss of tertiary structure as a result of the high-pressure processing. While these results broadly agree with earlier preliminary DSC results determined at a higher BSA concentration of 2.5 wt % ($T_m = 51.5$ °C and $\Delta H < 25$ MJ mol^{-1} for samples treated at 800 MPa) (Galazka *et al.*, 1996a), we note that the decrease in ΔH on pressure treatment is even greater in the more concentrated BSA solution. This is probably due to greater pressure-induced aggregation at the higher protein concentration. Dumay *et al.* (1994) reported similar trends with β-lactoglobulin at pH 7.

It is shown in Figure 2 that the addition of DS (0.5 wt %) to BSA leads to a reduction in T_m and ΔH, both for pressure-treated and untreated samples. Dextran sulfate alone (not shown) does not deviate from the baseline. The experiments carried out here on 1 wt % BSA systems provide confirmation of the findings presented previously for 2.5 wt % BSA systems (Galazka *et al.*, 1996a). Table 2 shows that mixtures containing 0.25 wt % polysaccharide (BSA:DS weight ratio of 4:1)

Table 2. Influence of High-Pressure Treatment on the DSC Endothermic Peak Temperature T_m and Total Calorimetric Enthalpy ΔH of Solutions of Pure BSA (1 wt %) and Mixtures of BSA (1 wt %) + DS (0.25 or 0.5 wt %) at pH 7[a]

BSA:DS ratio (by weight)	treatment pressure (MPa)	T_m[b] (°C)	ΔH[c] (MJ mol^{-1})
0	0	62.4	635
0	300	62.2	655
0	400	62.4	ND[d]
0	600	62.4	335
0	800	56.7	215
0.25	0	51.8	180
0.25	600	51.3	120
0.5	0	52.6	90
0.5	300	53.2	120
0.5	400	51.6	ND
0.5	600	46.9	105
0.5	800	44.6	70

[a] All experiments were performed in duplicate. [b] Experimental error ± 0.5 °C. [c] All values fell within ± 25 MJ mol^{-1} of the mean quoted (expressed in MJ per mole of monomeric BSA). [d] ND, not determined.

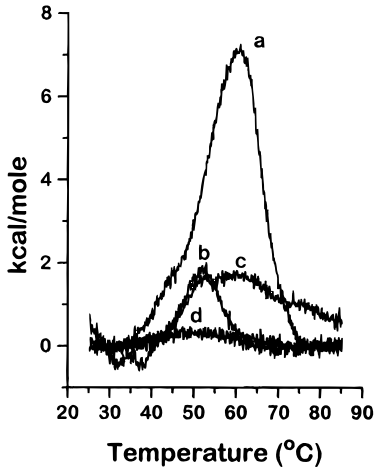

Figure 2. DSC thermograms of bovine serum albumin solutions (pH 7.0) containing 1.0% protein: (a) unpressurized BSA solution; (b) native BSA + DS (2:1 weight ratio) solution; (c) pressurized BSA solution at 800 MPa for 20 min; (d) pressure-treated BSA + DS (2:1 weight ratio) mixture at 800 MPa for 20 min.

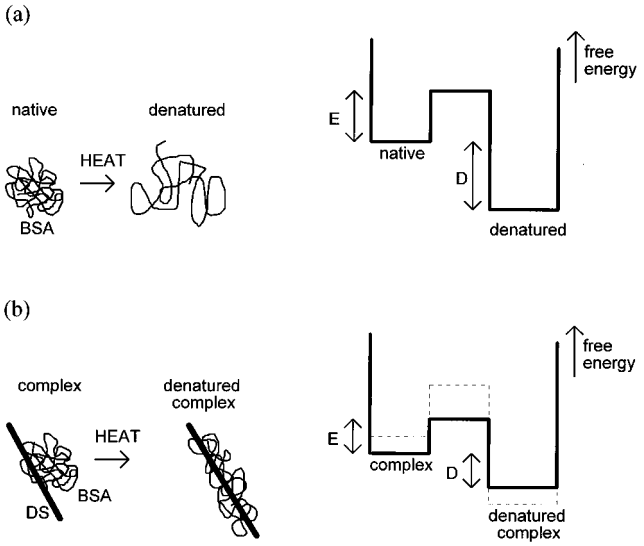

Figure 3. Schematic representation of the effect of complexation with dextran sulfate on the free energy diagram for conversion of BSA by heating from the native state to the denatured state: (a) in absence of polysaccharide; (b) in presence of polysaccharide. The two free energy diagrams show the energy of activation (E) and the free energy difference between native and denatured states (D). The lowering of the values of E and D due to polysaccharide complexation are equivalent to the lowering of the experimental values of T_m and ΔH, respectively, in the DSC experiments (see Table 2).

have rather higher values of ΔH than those containing 0.5 wt % polysaccharide (BSA:DS weight ratio of 2:1). In the latter case, there was such a large reduction in the value of ΔH due to the presence of the polysaccharide that the pressure treatment had little additional effect. These results suggest that complexation with DS reduces the extent of BSA unfolding due to heating and that high-pressure treatment of the mixed BSA + DS can reduce this even further.

The influence of protein−polysaccharide complexation on the quantities T_m and ΔH derived from the DSC experiments (see Table 2) can be understood in qualitative terms as illustrated in Figure 3. Interaction between BSA and DS leads to a lowering of the activation (free) energy, E, for the heat-induced conversion of the native protein into the denatured form via the partially unfolded transition state. The postulated reduction in E is consistent with the reduction in the endothermic peak temperature T_m by more than 10 °C. This means that complexation with polysaccharide allows the protein more easily to adopt the configuration of the transition state along the unfolding pathway accompanying thermal denaturation. BSA−DS complexation also leads to reduction in the ("equilibrium") free energy difference, D, between the denatured and native forms of the protein. The postulated reduction in D is consistent with the large reduction in the total calorimetric enthalpy in Table 2. This means that the complexation with polysaccharide during and after thermal treatment does not allow the protein to unfold to the same highly disordered state as is normally the case with BSA alone. As high-pressure treatment (800 MPa) also leads to a reduction in both T_m and ΔH, we can speculate that the thermally induced conversion of pressure-treated BSA to denatured BSA is also accompanied by lower values of the parameters E and D.

We have used size exclusion chromatography to assess the molecular sizes of protein aggregates and protein−polysaccharide complexes. Figure 4 shows the

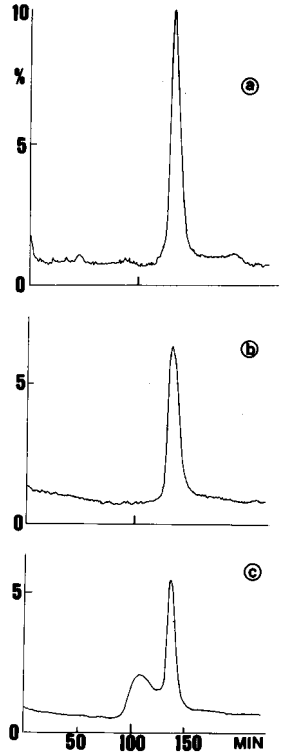

Figure 4. Effect of high-pressure treatment on size exclusion chromatograms of BSA (1 wt %) at pH 7 in absence of salt. The absorbance at 280 nm is plotted against the elution time: (a) untreated; (b) 300 MPa for 20 min; (c) 400 MPa for 20 min.

effect of high-pressure treatment on the elution profile of a 1 wt % solution of pure BSA at pH 7 in the absence of added NaCl. Chromatogram a for the untreated BSA shows a single narrow peak at *ca.* 7×10^4 Da corresponding to the native monomeric protein. Chromatogram b for the BSA treated at 300 MPa for 20 min shows a slightly broader single peak which is suggestive of some small change in protein structure without significant aggregation. Increasing the treatment pressure to 400 MPa leads to chromatogram c with a second broader peak at shorter elution times clearly corresponding to aggregated protein oligomers in the relative molecular weight range $2-5 \times 10^5$ Da. It seems likely that these pressure-induced BSA aggregates are stabilized by −SS− linkages, since BSA (like β-lactoglobulin) has a free cysteine residue which, under appropriate conditions in the partly unfolded molecule, can become available for intermolecular association with other sulfhydryl or disulfide groups. Earlier work has demonstrated (Galazka *et al.*, 1996a) that there is a loss of more than half of the free sulfhydryl groups during treatment at 800 MPa for 20 min, and that dithiothreitol (DTT) addition reduces these units to monomer status in the pressure-treated samples.

We turn now to the size exclusion chromatography of the mixed system of BSA (1 wt %) + DS (0.5 wt %). Figure 5 shows the effect of the high-pressure treatment on the elution profile in the absence of added NaCl. Chromatogram a for the untreated mixture shows a strong narrow peak corresponding to distinct protein−polysaccharide complex(es) of molecular weight *ca.* 5×10^5 Da (i.e., of similar order to that of the DS alone) together with a very small peak probably corresponding to residual monomeric BSA. Chromatogram b for the pressure-treated mixture (400 MPa for 20 min) shows no significant change in the complex molecular weight; the increase in optical density following high-pressure

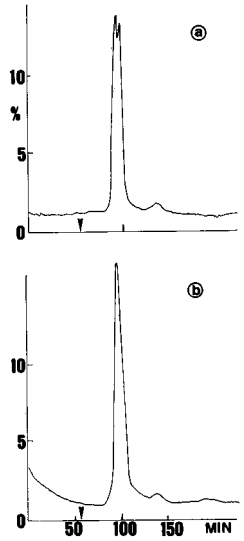

Figure 5. Effect of high-pressure treatment on size exclusion chromatograms of mixture of BSA (1 wt %) + DS (0.5 wt %) at pH 7 in absence of salt. The absorbance at 280 nm is plotted against the elution time: (a) untreated; (b) 400 MPa for 20 min. Arrow indicates position of the void volume.

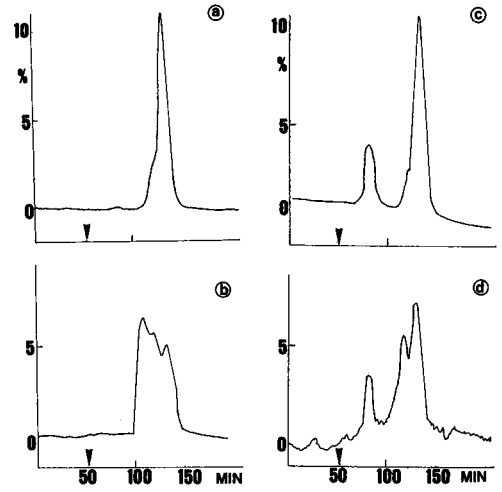

Figure 6. Size exclusion chromatography in presence of 0.1 M NaCl for BSA (1 wt %) and BSA (1 wt %) + DS (0.5 wt %) at pH 7. The absorbance at 280 nm is plotted against the elution time: (a) untreated native BSA; (b) BSA treated at 800 MPa for 20 min; (c) untreated BSA + DS; (d) BSA + DS treated at 800 MPa for 20 min. Arrow indicates position of the void volume.

treatment may be simply due to recovering the complex(es) in a slightly smaller volume, the double peak apparently disappears on pressure treatment.

To investigate the extent of the electrostatic character of the protein−protein and protein−polysaccharide interactions, the chromatography was repeated in the presence of 0.1 M NaCl. The elution profiles are given in Figure 6. Chromatogram a for the native BSA shows the monomeric peak at *ca.* 7×10^4 Da, but now with some indication of larger oligomers. Chromatogram b for the high-pressure treated BSA (800 MPa for 20 min) is indicative of extensive polymerization of the protein into dimers, trimers, and higher oligomers presumably held together by intermolecular disulfide bonds (Galazka *et al.*, 1996a). Chromatogram c for the untreated mixture of BSA + DS in the presence of salt shows two distinct main peaks at *ca.* 7×10^4 Da and $> 5 \times 10^5$ Da corresponding to the monomeric protein and some residual high molecular weight BSA−DS complex, respectively. Comparison of Figure 6c with Figure 5a indicates that the addition of 0.1 M NaCl induces reversible dissociation of most of the complexed BSA to monomeric form. This is consistent with the protein−polysaccharide complex being held together predominantly by electrostatic interactions. Chromatogram d for the high-pressure-treated mixture of BSA + DS (800 MPa for 20 min) in the presence of salt shows a complex system of peaks that is indicative of the presence of both monomeric and oligomeric proteins, as well as some residual BSA−DS complex. Comparison of Figure 6d with Figure 6b shows that, at the higher ionic strength, where the BSA−DS electrostatic attractive interaction is substantially screened, the high-pressure-induced protein aggregation occurs both in the presence and absence of the polysaccharide.

Taking the chromatography results of Figures 4−6 as a whole, we can infer that electrostatic interaction is an important factor influencing protein−polysaccharide complexation in these systems, with or without high-pressure treatment. On the other hand, protein−protein complexation induced by high-pressure treatment is more influenced by covalent −SS− bridging. The most important finding (*cf.* Figures 4c and 5b) is that complexation with DS at low ionic strength protects the globular protein against aggregation caused by intermolecular −SS− bonding during high-pressure processing. This protective effect may be due, in part at least, to the blocking of the hydrophobic surface binding sites on the protein by the bulky polysaccharide moieties.

Whether the BSA−DS interaction is entirely electrostatic in character is not entirely clear. It has been found separately from circular dichroism (CD) measurements (V. B. Galazka, unpublished results) that addition of DS (1:1 by weight) to unpressurized BSA at pH 7 reduces the inferred α-helical content by 15% as compared with the pure protein. Pressurization of the BSA + DS mixture (800 MPa for 20 min) showed only a further loss of 10% α-helical content, as compared with a loss of α-helical content of 35% for pressurization at 800 MPa in the absence of polysaccharide (Galazka *et al.*, 1997) and a loss of 50% for pressurization at 1000 MPa (Hayakawa *et al.*, 1996). These inferences based on CD measurements are consistent with the chromatography results at low ionic strength insofar that they confirm that the interacting polysaccharide has a protective effect on the protein, reducing its sensitivity to pressure-induced modification and aggregation.

On the basis of the combined experimental information presented in this paper, we may assume that the influence of pressure treatment on the BSA−DS complex is to induce an unfolding of the protein molecule in close proximity to the polysaccharide, thereby inhibiting protein aggregation during and after pressure application. While before pressure treatment and afterward, the two biopolymers are complexed together electrostatically, this may not be the case *during* the pressure treatment since it is known that ionic bonds are disrupted under high pressure. Hence, we may speculate that the application of high pressure leads to dissociation of the electrostatic complex, whereupon the protein becomes partially denatured in a manner analogous to the pressure-induced denaturation of the BSA alone. Finally, on pressure release the attractive electrostatic BSA−DS interaction is restored, leading to rapid recomplexation of the unfolded protein with the polysaccharide on return to ambient pressure, and protecting the denatured BSA against protein−protein aggregation. The alternative mechanisms involving no

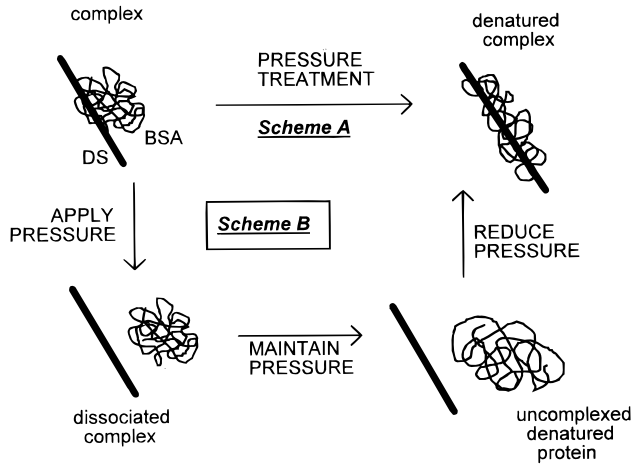

Figure 7. Schematic representation of the effect of high pressure treatment on the protein−polysaccharide complexation. Two possible mechanisms exist: scheme A, where the biopolymers remain complexed during pressure treatment; and scheme B, where the biopolymers dissociate following pressure application, the protein unfolds during maintenance of high pressure, and the complex of DS with the pressure-unfolded BSA reforms during pressure release.

complex dissociation (scheme A) and complex dissociation during pressure treatment (scheme B) are illustrated diagrammatically in Figure 7. A definitive confirmation of scheme B would require *in situ* measurements of protein structure in the mixed biopolymer solutions at the high pressure.

Finally, we note that the findings from the BSA + DS solution experiments reported here are of use in interpreting recent rheological data (Dickinson and Pawlowsky, 1996a,b) obtained for BSA-stabilized oil-in-water emulsions at pH 7 containing various amounts of the same polysaccharide (added after emulsification). Interaction at low ionic strength between the anionic polysaccharide in the aqueous phase and the adsorbed protein at the oil−water interface leads to a large increase in the small-deformation elastic modulus due to flocculation of droplets by bridging polysaccharide molecules at concentrations well below surface saturation coverage. Increasing the ionic strength from 5 to 70 mM was found to reduce substantially the strength of the bridging flocs (Dickinson and Pawlowsky, 1996b). This can be readily understood in terms a weakening of the attractive electrostatic protein−polysaccharide interaction due to much greater screening of electrical charge in the presence of the additional electrolyte. High-pressure treatment (≥400 MPa) of BSA before emulsification was found to lead to large changes in flocculation behavior and rheology of the emulsion after DS addition (Dickinson and Pawlowsky, 1996a). This implies a substantially different type of polysaccharide interaction with the pressure-denatured protein than with the untreated native protein.

CONCLUSIONS

Using a combination of spectrofluorometry, calorimetry, and size exclusion chromatography, we have demonstrated that high-pressure treatment (>300 MPa) has a considerable effect on the conformational and aggregation behavior of BSA in dilute solution under neutral pH conditions. The loss of surface hydrophobicity following pressure treatment can be attributed to intermolecular association which reduces the accessibility of fluorescent probe binding sites, or to the burying of some of the binding sites in the refolded partly denatured protein following pressure release. The pressure-induced BSA aggregates are probably mainly stabilized by −SS− linkages, with polymerization involving the free cysteine residue of the BSA monomer.

Performing the same experiments in the presence of polysaccharide at BSA:DS ratios of 2:1 and 4:1 has demonstrated the formation of small soluble ionic protein−polysaccharide complexes which are largely dissociated in 0.1 M NaCl. The large reduction in surface hydrophobicity on complexation with DS can be attributed to extensive blocking of fluorescent probe binding sites. Complexation with anionic polysaccharide at low ionic strength then has the effect of protecting the protein against extensive aggregation following the high-pressure treatment. This result may have practical implications for the control of protein functionality in food products subjected to high-pressure processing.

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