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0308-8146(95)00239-1

*Food Chemistry*, Vol. 57, No. 3, pp. 393–398, 1996

0308-8146/96 $15.00+0.00

# Changes in protein–protein and protein–polysaccharide interactions induced by high pressure

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(Received 8 September 1995; revised version received 20 October 1995; accepted 20 October 1995)

β-Lactoglobulin and bovine serum albumin (BSA) protein solutions (0.1%, 0.2% and 2.5%), when subjected to high pressure treatment (800 MPa for 20 min) at neutral pH, were denatured and some aggregates formed. The total calorimetric enthalpy of 2.5% solutions of the pressure-treated proteins decreased to virtually zero for both β-lactoglobulin and BSA following pressure treatment. Isoelectric focussing patterns (IEF) indicated that aggregation occurred in both proteins and there was a concomitant loss of sulphydryl groups (42% for β-lactoglobulin and 55% for BSA), suggesting that protein aggregation after high pressure processing was caused, at least in part, by the formation of –S–S– bridges. The surface hydrophobicity of the two proteins was modified, increasing (40%) with β-lactoglobulin and decreasing (41%) with BSA. Pressure treatment of 1:1 mixtures of BSA and dextran sulphate (DS) yielded structures with a significant enthalpy. However, addition of DS to β-lactoglobulin had little effect on the thermograms, suggesting that the DS either protects the protein against pressure induced unfolding or enables the pressure-denatured protein to regain some secondary structure. Copyright © 1996 Elsevier Science Ltd

## INTRODUCTION

In recent years high pressure technology has received considerable attention as a method of food processing and preservation (Galazka & Ledward, 1995; Ledward, 1995). Bridgman (1914) reported that egg-white proteins could be coagulated under certain conditions, demonstrating that high pressure, apart from having a lethal affect on certain microorganisms, could affect protein reactivity. It is now well established (Heremans, 1982; Balny *et al.*, 1989) that high pressures (up to 800 MPa) will cause most water-soluble proteins to undergo denaturation. This phenomenon appears to be dependent upon protein structure, temperature, magnitude of the applied pressure and nature of the solvent (Masson, 1992). Several studies (Zipp & Kauzmann, 1973; Balny & Masson, 1993; Defaye *et al.*, 1995) have shown that most proteins undergo denaturation and, on pressure release, may reform into a modified conformation. There is some evidence (Denda & Hayashi, 1992; Knorr *et al.*, 1992; Galazka *et al.*, 1995*a,b*) that this modifies the functional properties of the reformed protein.

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β-Lactoglobulin and bovine serum albumin (BSA) account for approximately 55% and 12% of the total whey proteins, respectively (Morr, 1992). It has recently been found (Dumay *et al.*, 1994; Hayakawa *et al.*, 1994; Galazka *et al.*, 1995*a*) that native β-lactoglobulin undergoes some limited aggregation and change in structure following pressure treatment. The solution properties of BSA are also influenced by pressure (Hayakawa *et al.*, 1992, 1994); the loss of secondary structure is dependent on treatment time and applied pressure.

Previous research (Dickinson & Galazka, 1991, 1992) has shown that dextran sulphate (DS) forms an electrostatic complex at neutral pH with BSA, although there is no evidence to suggest that β-lactoglobulin also forms such a complex.

The effect of pressure on such mixtures is unknown. During pressurization all carboxyl groups on a protein or polysaccharide will ionize, irrespective of the initial pH. In an unbuffered system, the pH thus decreases and increased numbers of potential ionic binding sites are formed. When pressure is released, the degree of ionization decreases, and the conformation of the protein may be altered, thus modifying any protein–polysaccharide interactions. If no specific interaction occurs,

then the increased hydration of the polymers (due to the increased charge on the proteins) may change their behaviour.

Our aims were to determine the effects of high pressure on the solution properties of the two water-soluble globular proteins, β-lactoglobulin and BSA, and to see how the change in conformation modifies the possible electrostatic protein–polysaccharide interactions.

## MATERIALS AND METHODS

### Materials

BSA (99% purity) and β-lactoglobulin (3× crystallized and lyophilized) was obtained from Sigma Chemical Co. (St Louis, MO, USA). DS ($\approx 5\times10^5$ Da, containing 0.5–2.0% phosphate buffer salts pH 6–8), made by treatment of dextran T-500 with chlorosulphonic acid in pyridine, was also purchased from Sigma. DL-Dithiothreitol (DTT), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and buffer salts were obtained from Sigma. Isoelectric focussing (IEF) gels and buffers were purchased from NOVEX™ (San Diego, CA, USA).

Sequence analyses of the proteins were from the PIR database and University of Wisconsin genetics computer group software (Pir1:lgbo and Pir1:ABBOS) (George *et al.*, 1986; Devereux *et al.*, 1984).

### Preparation of protein and protein + polysaccharide solutions

Solutions of protein (0.1, 0.2 and 2.5%) and mixtures of protein + DS (1:1, w/w) were prepared with HPLC grade water (Rathburn Chemicals Ltd., Walkerburn, UK). pH adjustment was by addition of 0.05 M HCl or NaOH.

The buffer in this system at neutral pH is primarily due to the histidines and α-amino groups of the protein, since ionization of these groups does not lead to a volume change (Funtenberger *et al.*, 1995); it is likely that the pH remains relatively constant on the application of high pressure.

### High pressure treatment

Samples of protein and mixtures of protein + polysaccharide solutions were sealed in cryovac bags and subjected to pressures of 800 MPa for 20 min at 23 ± 2°C (Galazka *et al.*, 1995*b*) using a prototype Stansted 'Food-Lab' high pressure 'rig' (Stansted Fluid Power Ltd., Stansted, Essex, UK). Solutions were kept on ice for 1–2 h before analysis.

### Analytical procedures

*Differential scanning calorimetry (DSC)*
Calorimetric measurements were performed on a Microcal MA2 scanning calorimeter (MicroCal Inc., Northampton, MA, USA) at a scan rate of 1°C $min^{-1}$ under nitrogen at 2 bar. Data were analysed using the Microcal Origin data analysis package. Proteins at 2.5% were heated through the range 10–90°C with $T_m$ and total calorimetric enthalpies being recorded.

*Spectrofluorometry*
The surface hydrophobicity of BSA and β-lactoglobulin was determined using 1 ml of 0.1% protein solution mixed with 1 ml of $4\times10^{-5}$ M 1-anilinonaphthalene-8-sulphonate (ANS) at ambient temperature. The fluorescence intensity was recorded on a Perkin-Elmer LS50 spectrofluorimeter (excitation at 365 nm, emission at 470 nm), and the relative intensity measured over the range 400–500 nm.

*Isoelectric focussing*
IEF was run under native conditions using pH 3–10 NOVEX IEF gels containing 5% polyacrylamide. Protein solutions (unfiltered and filtered, 0.2 μm Millipore) were diluted with IEF sample buffer (pH 3–10) to give a sample concentration of 0.1%, and 20 ml of the solution loaded into each well. The gels were run for 2.5 h (100 V constant for 1 h, 200 V constant for 1 h and 500 V for 30 min). Fixation of the protein was by 12% trichloroacetic acid for 30 min, and Coomassie Brilliant Blue G250 was used to stain the protein. To give an indication on the size of aggregate formation samples were filtered (2 μm Millipore) and compared with unfiltered samples.

*Gel permeation chromatography*
A Superose 12 (preparative grade, Pharmacia, Uppsala, Sweden) column, calibrated within the range 200 000–14 000 daltons, was used to measure molecular mass. The column buffer was 10 mM Tris–HCl supplemented with 100 mM NaCl pH 8.0. Protein was eluted from the column (1 cm×30 cm) at a flow rate of 0.8 ml $min^{-1}$. The column was run with and without 5 mM DTT to determine the extent of disulphide mediated aggregation.

*Determination of protein thiols*
The content of free cysteine groups on the proteins was determined using DTNB (Ellman, 1959; Janatova *et al.*, 1968) with $\epsilon_{412\,nm} = 13600\ M^{-1}\ cm^{-1}$.

## RESULTS AND DISCUSSION

### β-Lactoglobulin

*Denaturation*
Figure 1 shows DSC thermograms for solutions of native and pressure-treated β-lactoglobulin (2.5%) and a pressurized mixture of β-lactoglobulin + DS (1:1, w/w). Native β-lactoglobulin shows a predominant endothermal peak with a $T_m$ of 73.32°C, and the $T_m$ for pressure-treated protein shifts towards a lower temperature region (38.26°C). The calorimetric enthalpy for the pressure-treated sample falls to virtually zero. This and the decreased $T_m$ indicates that there is a major loss of structure which is not recovered on pressure release.

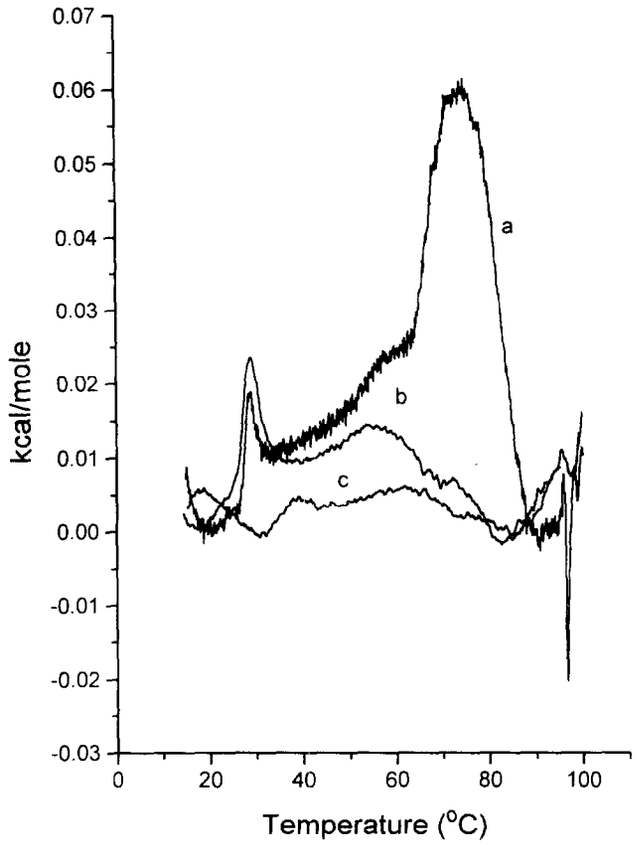


**Fig. 1.** DSC thermograms of β-lactoglobulin solutions (pH 7.0) containing 2.5% protein: (a) native (unpressurized) β-lactoglobulin solution; (b) β-lactoglobulin + DS (1:1, w/w) solution processed at 800 MPa for 20 min; (c) pressurized β-lactoglobulin solution at 800 MPa for 20 min. Heating rate 1°C $min^{-1}$.

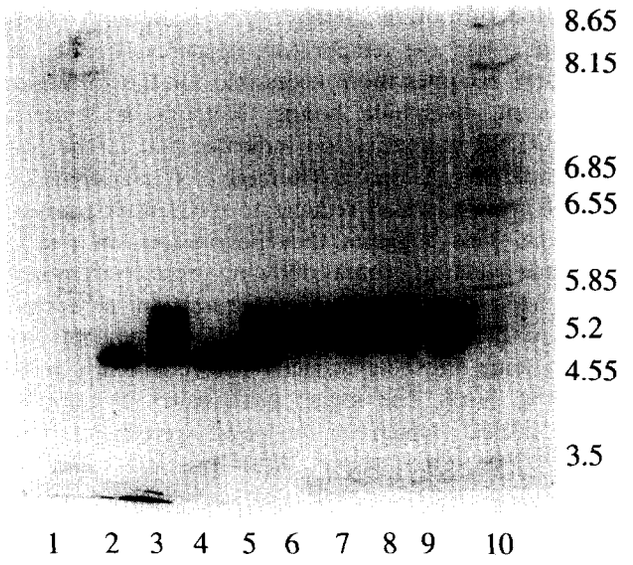


**Fig. 2.** Effect of high pressure treatment (800 MPa for 20 min) on the aggregation properties of bovine serum albumin (BSA) and β-lactoglobulin. Isoelectric focussing of filtered (0.2 μm Millipore) and unfiltered native and pressure-treated protein solutions: (1) standards; (2) filtered native BSA; (3) filtered pressure-treated BSA; (4) not filtered control BSA; (5) not filtered pressure-treated BSA; (6) filtered unpressurized β-lactoglobulin; (7) filtered pressure-treated β-lactoglobulin; (8) unfiltered native β-lactoglobulin; (9) unfiltered pressurized β-lactoglobuin; (10) standards.

Addition of DS to the native β-lactoglobulin (not shown) leads to no significant change in $T_m$ (73.61°C). The sample denatured by high pressure treatment (Fig. 1) shows a $T_m$ of 59.27°C, and a very low calorimetric enthalpy. The small peak at 29°C could be due to an artifact or impurities present within the native β-lactoglobulin sample. Overall, these results suggest that if any complexes are formed between β-lactoglobulin and DS during high pressure treatment the binding is likely to be weak and reversible.

*Aggregation*

Fluorometry studies of ANS bound to the native and refolded β-lactoglobulin at pH 7.0 showed an increase (40%) in the fluorescence intensity in the pressure-treated sample (Table 1), which indicates a significant increase in protein surface hydrophobicity. IEF (Fig. 2) patterns provide some evidence for low molecular weight aggregation in the pressurized sample. Our results are consistent with Nakamura *et al.* (1993) who have shown that high pressure processing (200–600 MPa) of whey protein concentrate induces extensive aggregation of β-lactoglobulin, and Dumay *et al.* (1994) have recently demonstrated that pressure treatment (450 MPa for 15 min) causes extensive aggregation and unfolding of β-lactoglobulin. Recent circular dichroism measurements at neutral pH (Ledward *et al.*, 1995) have shown a reduction in the α-helical content (3%) following high pressure treatment at 800 MPa for 20 min. The modest loss of α-helical content suggests that there is some refolding of the protein at low concentrations; possibly, at higher concentrations (2.5%), aggregation prevents the reformation of secondary structure. β-Lactoglobulin has a high degree of flexibility and compressibility (Gekko, 1991) and because of this it is thought that a large proportion of the unfolding is reversible, even though the exact mechanism is unknown. It is suggested that the extent of unfolding seems to be enhanced by increasing the protein concentration, presumably due to reduced reversibility caused by aggregation (Galazka *et al.*, 1995*a*).

β-Lactoglobulin contains five cysteine groups of which four are associated as disulphides (Pir1:lgbo) leaving a single free residue. Under normal conditions this free group is unavailable for intermolecular

**Table 1. Surface hydrophobicity (mean ± SD) of native and denatured bovine serum albumin and β-lactoglobulin solutions (0.1% protein)**

| Protein | pH | *I* (native) | *I* (treated) | Change |
|---|---|---|---|---|
| β-Lactoglobulin | 7.0 | 36.2 ± 5.7 | 60.1 ± 8.1 | 40% increase |
| Bovine serum albumin | 7.0 | 628.9 ± 20 | 371.2 ± 41.5 | 41% decrease |

The measurements were made in the presence of $4 \times 10^{-5}$ M 1-anilinonaphthalene-8-sulfonate, and values of the relative fluoresence intensity (*I*) before and after treatments were recorded at pH 7.0. Fluoresence intensity was excited at 365 nm, and emission monitored at 470 nm. Measurements were performed four times.

association with other sulphydryl or disulphide groups. However, when the temperature is increased, the free sulphydryl becomes more accessible for the formation of covalent disulphide bonds (Katsuta & Kinsella, 1990). DTNB quantification indicates that in the control sample there is only 0.036 mole of free cysteine per mole of protein, which reduces to 0.021 after pressure treatment. This suggests that polymerization due to disulphide bonding occurs following pressure treatment at 800 MPa for 20 min. Gel permeation chromatography of the naturally occurring material indicates that there is some size heterogeneity before pressure treatment [Fig. 3(a)]. The major peak elutes at approximately 20000, which is in good agreement with the 18200 reported weight. After pressure treatment (Fig. 3(b)) the apparent molecular size of the main peak has increased to approximately 40000, indicating the formation of dimers. It is seen that new species are produced on high pressure treatment and their presence corresponds to the aggregation reported by Dumay *et al.* (1994). Treatment of the pressure-treated sample with 5 mM DTT (Fig. 3(c)) reduces the level of dimerization, considerably supporting the view that a disulphide mediated polymerization has occurred during or after pressure treatment. These findings are in agreement with those of Johnston & Murphy (1995), who have shown that the number of -SH groups found in milk are reduced following high pressure treatment.

**Bovine serum albumin**

*Denaturation*

DSC thermograms for solutions of BSA (2.5%) and mixtures of BSA+DS (1:1, w/w), before and after

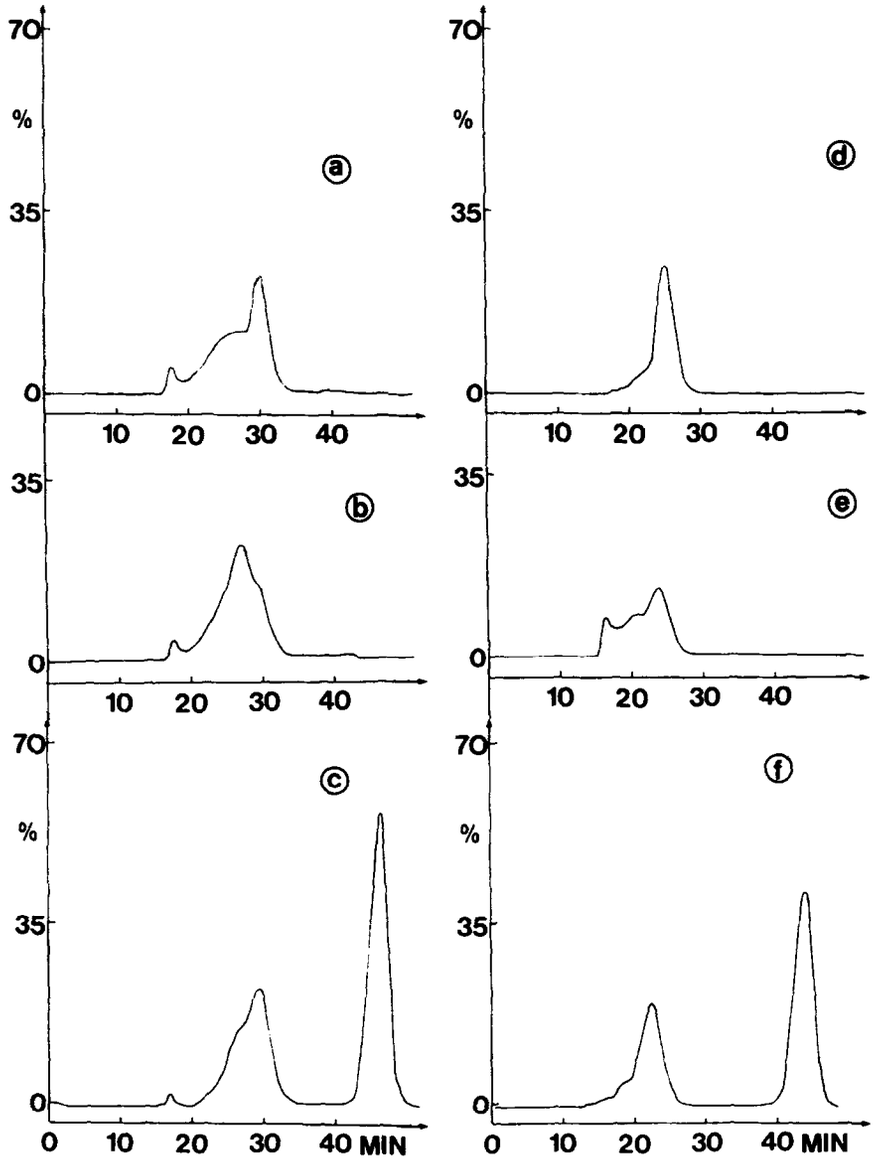


**Fig. 3.** Pressure-induced crosslinking of β-lactoglobulin and bovine serum albumin (BSA) containing 0.2% protein. Gel permeation chromatography (Superose 12 column at pH 8.0) of protein constituents from native and pressure-treated (800 MPa for 20 min) solutions: (a) native β-lactoglobulin; (b) pressure-treated β-lactoglobulin; (c) pressure-processed β-lactoglobulin with 5 mM dithiothreitol (DTT); (d) control BSA; (e) high pressure-treated BSA; (f) treatment of pressurized BSA with 5 mM DTT. Absorbance was measured at 280 nm. The time axis is frequently referred to as elution time. Note that, in the presence of DTT, the pressure-treated profile returns to that of the native material with both proteins.

pressure treatment are presented in Fig. 4. We see that native BSA has a $T_m$ of 59.24°C and BSA, which had been subjected to pressure treatment (800 MPa for 20 min), leads to a large reduction in the calorimetric enthalpy with a shift of $T_m$ to 51.47°C, indicating a significant loss of native structure. The effect of a 2.5% DS addition to the BSA solution (Fig. 4) leads to a decrease in $T_m$ (53.94°C) and calorimetric enthalpy. High pressure treatment in the presence of DS (Fig. 4) decreases the calorimetric enthalpy with no significant change in $T_m$ (54.18°C) when compared with the untreated mixture. The $T_m$ and calorimetric enthalpy for the pressure-treated BSA + DS mixture are higher than for pressure-treated BSA in the absence of DS. The presence of DS during treatment indicates that some mechanism acts at around neutral pH, possibly involving complex formation between the protein and polysaccharide, which helps secondary structure formation in the denatured form or inhibits its loss during pressure treatment.

*Aggregation*

Spectrofluorometry data (Table 1) based on ANS binding to BSA in aqueous solution indicates a decrease in the protein surface hydrophobicity (41%) after pressure-processing at 800 MPa for 20 min. The loss of surface hydrophobicity could be due to the lower number of hydrophobic groups binding to the ANS because of intermolecular interactions (Hayakawa *et al.*, 1992), or the pressure-treated sample has refolded into a slighly different conformation thus burying some of the hydrophobic groups. Some evidence for low molecular weight aggregation of BSA following high pressure treatment is provided by native IEF (Fig. 2) patterns. The addition of bands seen on the IEF data after high pressure treatment is due to the presence of high molecular weight aggregates observed in the gel permeation chromatography scans (Fig. 3). Recent studies (Hayakawa *et al.*, 1994; Ledward *et al.*, 1995) have shown that high pressures have a substantial effect on the secondary structure of BSA. Our (Ledward *et al.*, 1995) circular dichroism data suggest a reduction in the α-helix content from 69% in the native protein to 44% following high pressure treatment (800 MPa for 20 min) at pH 7.0. This compares well with the study of Hayakawa *et al.* (1994) who found a 50% decrease in the α-helical content of pressure-treated BSA (1000 MPa for 10 min). At lower pressures (600 MPa for 9 min) there was no significant change (Hayakawa *et al.*, 1992).

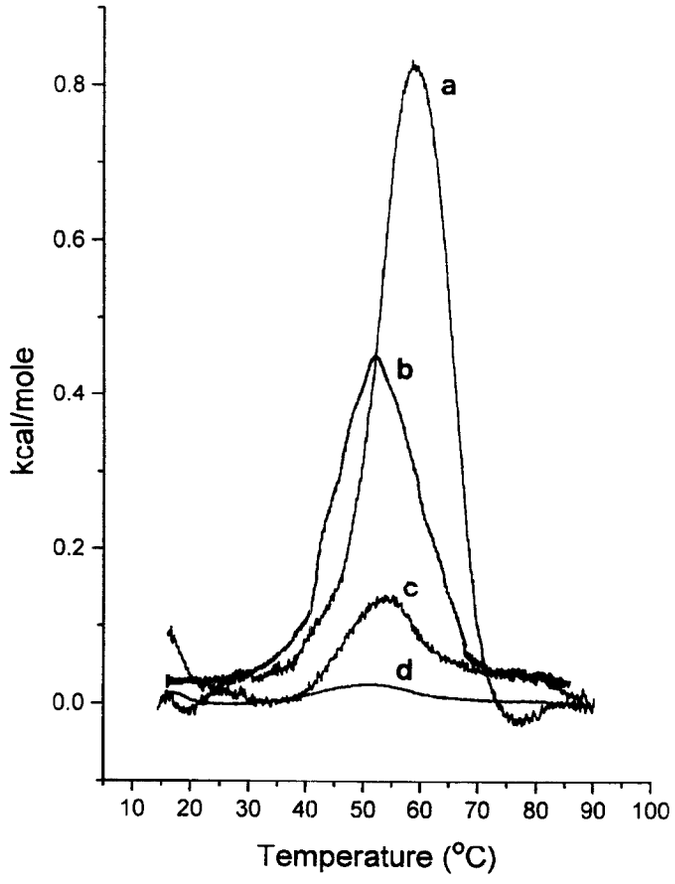


**Fig. 4.** Pressure-induced unfolding of bovine serum albumin (BSA) solutions (pH 7.0) containing 2.5% protein: (a) control (unpressurized) BSA solution; (b) native BSA + dextrana sulphate (DS) (1:1, w/w) mixture; (c) pressure-treated BSA + DS

Sequence data (Pir1:ABBOS) for BSA indicate that for the mature protein there are 35 cysteine residues of which 34 are present as disulphides, leaving a single free cysteine which is well shielded and thus unavailable for reaction with other sulphydryl or disulphide groups under 60°C (Katchalski *et al.*, 1957). However, as the temperature is increased, the group becomes progressively more available for the formation of intermolecular disulphide bridges, leading to dimerization, unfolding and eventual aggregation (Wetzel *et al.*, 1980; Peters, 1985). Our DTNB quantification data suggest a mole percentage of 0.12 (free cysteine per molecule) for the control sample which is reduced to 0.054 free cysteine per molecule after pressure treatment. This would suggest that the free cysteine is partially hidden in the protein and that, after pressure treatment, there is a subtle reorganization that leads to some of the free cysteine being irreversibly oxidized or becoming associated as disulphides. The gel permeation chromatography data (Fig. 3) indicate that BSA behaves similarly to β-lactogloblin. The unpressurized sample shows a molecular weight of approximately 70 000 with some indications of larger units (Fig. 3(d)). Pressurization induces extensive dimer, trimer and higher aggregate formation (Fig. 3(e)). These units appear to be stabilized by disulphide bonding, as demonstrated by the return to monomer status after DTT treatment (Fig. 3(f)). The presence of a single free cysteine in BSA coupled with this multimerization suggests that, during pressurization, some level of disulphide interchange occurs.

Several studies in our laboratory (Defaye & Ledward, 1995; Ledward *et al.*, 1995) have shown that metmyoglobin forms dimers after pressure treatment at pH 7.0. These dimers are dissociated in the presence of sodium dodecyl sulphate, which indicates that association is primarily due to hydrophobic interactions and not –S–S– bridging as in BSA and β-lactoglobulin.

## CONCLUSION

High pressure treatment of β-lactoglobulin and BSA causes the proteins to undergo unfolding and aggregation as shown by DSC, spectrofluorometry, IEF and measurement of sulphydryl groups. Protein aggregation, after pressure treatment, is presumably due to sulphydryl groups and perhaps exposed hydrophobic regions on the protein reacting to form oligomers. The presence of DS was shown to apparently reduce the degree of BSA unfolding induced by pressure but not that associated with β-lactoglobulin. At neutral pH, BSA, DS and β-lactoglobulin all carry a net negative charge, but electrostatic interaction may take place between negative charges on the anionic polysaccharide and positively charged regions on the protein to form soluble ionic complexes (Tolstoguzov, 1986). Stronger ionic complexes are formed between BSA and DS than between β-lactoglobulin and DS. It is noteworthy that, although DS decreases the thermal stability of BSA by 5–6°C, the pressure-treated samples have some secondary structure, even at 2.5% concentration (Fig. 4). It may well be that the large negative charge on the complexes inhibits protein–protein interactions, thus allowing some reformation of secondary structure after pressure treatment.

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