Differential scanning calorimetry study of pressure/temperature processed β-lactoglobulin: effect of dextran sulphate

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Abstract

β-lactoglobulin at pH 7 in the presence or absence of dextran sulphate (1:1 weight ratio) was subjected to a combined pressure and temperature treatment (pressures up to 280 MPa, temperature up to 60°C and time up to 30 minutes). The effect of the treatment was assessed post-processing using differential scanning calorimetry. The thermograms in absence of dextran sulphate were very broad with possibly a shoulder on the low temperature side and showed a decrease in calorimetric enthalpy as the intensity of treatment increased. In the presence of dextran sulphate the thermograms had a much sharper shape. The calorimetric enthalpy was reduced further than in the absence of dextran sulphate. This demonstrated that the polysaccharide sensitised the protein to the combined treatment. Treatment with pressure and temperature alone demonstrated that dextran sulphate sensitised the protein structure to pressure. The species created by the combined process could be in the molten globule state and possess improved functional properties. Also the operating parameters applied could potentially be conditions for improved functionality.

Keywords: pressure, temperature, protein, polysaccharide, molten globule, functionality, food processing.

1. Introduction

In recent years, alternative ways for ensuring food preservation with minimal product quality loss and less chemical additives have been sough because of an increased consumer demand for less processed products (Mertens & Knorr, 1992; Butz & Tauscher, 2002). High pressure processing is the most promising non-thermal technology and extensive work has been done for food preservation since the Japanese renewed the interest in the technology (Hayashi, 1989). Indeed it has been demonstrated that high pressure (usually 400-800 MPa) could inactivate micro-organisms and extend products shelf life. In addition to the preservation side of the technology, interest has incorporated the possibility of creating new products because of the effect of pressure on the different food constituents (Knorr, 2002). High pressure acts in a different way to temperature as it does not disrupt covalent bonds but weakens non-covalent bonds (Balny, Mozhaev & Lange, 1997) leading to the possibility of creating new foods. In the case of proteins, for example, pressure affects the tertiary and quaternary structure but there is little effect at the secondary structural level (Heremans, 1982; Silva & Weber, 1993). This modifies the balance of intramolecular and protein-solvent bonds hence leading to conformational changes and functional properties modifications (Pittia, Wilde, Husband & Clark, 1996; Messens, VanCamp & Huyghebaert, 1997). Different intensities of treatments could lead to structures with different molecular structures having distinct functional properties. Hence a single protein could give the

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opportunity to obtain different degrees of a given functionality (Farrell Jr. et al., 2002; Qi, Brown & Farrel Jr, 2001). These different structures are thought to be intermediates in the denaturation pathway and are often referred to as molten globules (Hirose, 1993; Mine, 1995; Yang, Dunker, Powers, Clark & Swanson, 2001).

This paper will present some differential scanning calorimetry results on the effect of a combined pressure/temperature at moderate pressures (up to 280 MPa) and temperatures (up to 60°C) on β-lactoglobulin at concentrations close to those found in milk (3 mg/ml) and neutral pH. The effect of adding a polysaccharide will also be investigated. The use of moderate temperature, in addition to pressure, enables the same level of processing at lower pressures and hence could minimise the investment cost. Indeed, while pressurising itself is not costly, it must be remembered that the equipment costs are very high and found to be proportional to the operating pressure (Mertens & Deplace, 1993).

Bovine β-lactoglobulin is the major protein in whey and it is though that it is mainly responsible for the functional properties of whey. This globular protein is quite sensitive to pressure when processed at relatively high concentration as aggregation occurs. For example it was demonstrated that solutions at 25 mg/ml pressurised at 450 MPa presented soluble aggregates (Dumay, Kalichevsky & Cheftel, 1994). However, no significant effects have been recorded for the tertiary structure and surface hydrophobicity at 900 MPa (Iametti et al, 1997). Identically, no irreversible effects were observed at the secondary level at 800 MPa (Galazka & Ledward, 1996) and 900 MPa (Pittia, Wilde, Husband & Clark, 1996). Hence, it is believed that the proteins apparent higher resistance to pressure at low concentration is because of it's higher flexibility and due to some refolding occurring post-processing when the protein is not implicated in aggregates (Galazka, Sumner & Ledward, 1996). It has been shown, using circular dichroism, that a combination of pressure and temperature (maximum of 294 MPa and 62°C) could alter, in an important way, the secondary and tertiary structure of β-lactoglobulin (Aouzelleg et al., 2004) even if the pressures used were lower than those required to modify irreversibly the protein structure at low concentration.

The present paper reports on the differential scanning calorimetry characterisation of these molecular species that could correspond to molten globules. β-lactoglobulin molten globule could offer potentials for the food industry as a food ingredient with controlled and increased functionality. The thermal denaturation is a complex phenomenon with a transition at approx. 70°C and a second one at 130°C (Dewitt & Klarenbeek, 1981).

The last element of the study is the consequence of the presence of a polysaccharide, dextran sulphate, on the effect of the combined process on the protein. Polysaccharides are the other important biopolymers in food systems. Polysaccharides are mainly responsible for controlling rheological characteristics while proteins are more known for their functional properties (Galazka, Smith, Ledward & Dickinson, 1999a). The presence of polysaccharides could greatly influence the thermal and functional behaviour of proteins (Baeza & Pilosof, 2002). Due to it's higher charge density, dextran sulphate has been found to interact strongly with different proteins depending on the conditions (Galazka, Smith, Ledward & Dickinson, 1999a; Galazka, Smith, Ledward & Dickinson, 1999b; Galazka, Dickinson & Ledward, 2001a).

There have been no evidences suggested so far for β-lactoglobulin (Galazka, Sumner & Ledward, 1996). In the case of bovine serum albumin, this phenomenon protected the protein against pressure unfolding.

The present paper investigates the effect of combined pressure and temperature on the thermograms of β-lactoglobulin and solutions of β-lactoglobulin and dextran sulphate using differential scanning calorimetry analysis.

2. Materials and methods

β-lactoglobulin derived from bovine milk (3 times crystallised and lyophilised, approx. 90%) was purchased from Sigma Chemical Co. (St. Louis, MO). Dextran sulphate (~5.10^5 Da, containing 0.5-2.0% phosphate buffer salts pH 6-8) was also obtained from Sigma.

The protein was prepared by dissolving in 50mM Tris-HCl buffer at pH7 to provide a protein concentration of approximately 3 mg/ml. The concentration was measured spectrophotometrically before and after processing using an absorbance coefficient of 0.96 $l.g^{-1}.cm^{-1}$ (Yang, Dunker, Powers, Clark & Swanson, 2001). Solutions of protein and polysaccharide mixtures were prepared in the same way with a ratio 1:1, w/w, as was done by other authors (Galazka, Sumner & Ledward, 1996).

Experimental design methodology was used to assess the effects of the three independent variables. A full factorial design was used (Box & Wilson, 1951). Hence, 8 experiments were performed (a full central composite design with 15 experiments was actually carried out, but in this paper, only the 8 results of the factorial design are shown as no added value came from the other experiments). The combinations of factors at different levels are presented in table 1. The design used allows carrying out a minimum number of experiments to assess the effects of the variables.

Experiments performed with pressure or temperature alone were done at 70°C and 294 MPa for 30 minutes. This temperature was chosen because it could be the one attained in the combined processing conditions for the highest temperature studied taking into account the temperature rise due to pressurisation. This is a worse case temperature. Indeed, the adiabatic heat increase should be limited as the pressure rise is slow (less than 100 MPa/min) and the pressure vessel is located in a water bath. The pressure of 294 MPa was chosen as it is the maximum pressure used in the full experimental design used.

Two-ml plastic capsules were filled without headspace with the protein or protein/dextran sulphate solution and were placed in a 70 ml pressure vessel (Staffordshire Hydraulic Ltd, UK). The pressure reactor was located in a Grant water bath and was allowed to equilibrate at the desired experiment temperature before inserting the samples. The pressurisation media was water and pressure was achieved using a manual pump. The vessel and its contents were pressurised and held under pressure for the required length of time before the pressure was released. The times to close or open the reactor were of approximately 1 min and the depressurisation was almost instantaneous. The times required to reach pressure were between 1 and 3.5min depending on the final pressure. Pressure and heat treatments when studied on their own were carried out in the same vessel.

All samples appeared clear after processing. After treatment, the samples were stored at -4°C before analysis. In the same way as has been reported by others at higher concentrations (Kolakowski, Dumay & Cheftel, 2001), the single freezing and thawing cycle did not affect the β-lactoglobulin thermogram.

The samples were analysed on a Microcal MC-2 microcalorimeter (Northampton, USA). The samples were thawed, degassed under vacuum and the 1.2249 ml sample cell was filled with the solution. Tris HCl 50 mM pH 7 buffer with and without dextran sulphate (3 mg/ml) was used in the reference cell when analysing the protein solution alone and with dextran sulphate, respectively. The solutions were heated from 20 to 100°C at 1°C/min. In addition to a base line correction (subtraction of buffer-buffer thermogram to the buffer-sample trace) the thermograms were normalised for concentration. The temperature of the transition and calorimetric enthalpies were determined after smoothing of the thermograms.

3. Results

3.1 *β-lactoglobulin solution*

Figure 1 shows the thermograms obtained and table 2 a summary of the data. The native β-lactoglobulin thermogram presented is similar to those obtained with similar conditions by other authors (Holt et al, 1998). For β-lactoglobulin the maximum heat capacity reached and transition temperature found were close to those obtained for similar conditions (Holt et al, 1998). However, they are higher than the ones normally reported for higher concentrations. For example, the maximum heat capacity reached at low concentrations, this study and Holt et al. (Holt et al, 1998), were 8-10 kcal.mole^{-1}°C^{-1}. For the transition temperature they were 83°C and 76-80.6°C respectively.

Differential scanning calorimetry is a useful technique used to characterise the thermodynamics of protein denaturation. β-lactoglobulin has been extensively studied using that technique and the transition was found to be irreversible due to aggregation occurring. The present thermogram of the native protein is very wide with a slow heat capacity increase starting at 40°C up to a maximum at 83°C and a steep dip on the high temperature side probably due to additional exothermic aggregation occurring. As can be observed, the size of the denaturation peak is greatly reduced when the processing intensity is increased. This is in agreement with what is observed for studies at higher protein concentration (25, 50 mg/ml) with high pressure alone (Dumay, Kalichevsky & Cheftel, 1994). The transition denaturation process is endothermic but it represents a combination of exothermic reactions (e.g. disruption of hydrophobic interactions, aggregation) and of endothermic contribution (e.g. disruption of hydrogen bonds) (Murray, Arntfield & Ismond, 1985). Although this makes the differential scanning calorimetry analysis more difficult it is still possible to assess qualitatively the amount of residual structure present in the analysed protein. The calorimetric enthalpy does not have any real significance as the process is controlled kinetically due to aggregation but it still represents the enthalpy of the transition and can be used to assess the amount of structure in the protein. The endothermic thermogram is thought to result mainly from intramolecular hydrogen bonds disruption (Dumay, Kalichevsky & Cheftel, 1994) and can be used as a measure of the protein loss of structure and denaturation. Hence, in terms of calorimetric enthalpy, the native sample and conditions constituted by the less processed ones (1 and 2) show a gradual decrease in enthalpy with processing. Then, all other processing conditions,

except from condition 8, present similar levels of calorimetric enthalpy and show a distorted thermogram with a shoulder appearing (although this can already be guessed for lower processing intensities) on the low temperature side. The shoulder at c.a. 60-65°C is getting more and more pronounced with the intensity of processing while not observed for the native protein. The peak becomes broader the more the protein is denatured by the treatment.

The more severe processing condition 8 shows even more reduced enthalpy. The transition occurring at relatively high temperature, unpredictable exothermic aggregation affects the thermogram on the high temperature side making it dip in a faster way resulting in an inaccurate calorimetric assessment.

As the β-lactoglobulin is more intensively processed, the temperature of denaturation increases. The general trend is an increase from 81-83°C for the less processed protein to 84-85°C for the more processed samples (table 2). This however is quite difficult to assess as the maximum is quite flat and, as already mentioned, the signal on the high temperature side is more variable because of aggregation.

The aim of the study was to identify the relative importance of the different parameters (pressure, temperature and time) composing the combined process on the calorimetric enthalpy quantitatively and quantify linear and interactive effects using a multiple linear regression. This was done successfully for near-UV circular dichroism results (Aouzelleg et al., 2004) and it was hoped that parallels could be drawn between the two techniques. However, in this case the thermograms were very close together and many of them were not significantly different.

Hence, experiments were carried out with pressure and temperature alone to visualise the effect of each of them separately on the protein structure. As can be seen on figure 3, both pressure and temperature have a very similar and limited effect on the thermograms. The effect of each of them separately is much lower than their combination (figure 1). The most severe combined treatment 8 is shown for comparison on figure 3. This shows that the combined treatment is much more efficient to destroy the protein structure than when the parameters are applied separately.

3.2. *β-lactoglobulin/dextran sulphate mixtures*

Figure 2 shows the thermograms of solutions of β-lactoglobulin in the presence of dextran sulphate. The native protein thermogram in the presence of dextran sulphate differs fundamentally from the one without dextran sulphate. The transition temperature is approximately the same for the two thermograms (c.a. 83°C) and the maximum heat capacity reached is similar (~9 kcal/mol/K). However, the thermogram in the presence of dextran sulphate is much sharper and the slow heat capacity increase between 40 and 60°C observed for the globular protein alone does not occur. This results in a calorimetric enthalpy for the protein-polysaccharide mixture, which is less than for the protein alone. After smoothing it was calculated as 90 kcal/mole and 160 kcal/mole for the native protein in presence and absence of dextran sulphate, respectively.

The calorimetric enthalpy is reduced as the intensity of treatment intensity increases gradually. Table 2 compares the calorimetric enthalpies with conditions without dextran sulphate and shows the sensitising effect of dextran sulphate. Treatments 4 and 5 still present a recognisable endotherm but for more intense treatment the thermogram is very weak and becomes nearly flat for treatment 8. The transition temperature

does not significantly change with processing, but again, this is difficult to assess on the weak thermograms. The small transition temperature for condition 8 is due to the fact that the thermogram is very flattened and wide with no real maximum transition temperature. As for the case when dextran sulphate was absent it was not possible to do a linear regression as many of the thermograms are close to the base line. When comparing the thermograms it seems that pressure is the most important parameter to bring about structural destruction of the protein as all combinations at 280 MPa show very flattened thermograms.

In order to check this, pressure and temperature where applied alone (figure 4). As can be observed, similarly to what happens with the combined process, the calorimetric enthalpies in the presence of dextran sulphate are smaller than for the protein alone. In addition, a very strong sensitising effect of the protein to pressure is induced by the presence of dextran sulphate. This would entail that pressure was the main parameter responsible for the protein denaturation in the combined process in the presence of dextran sulphate. Temperature may however have a role in the combined process at the lower pressures studied and facilitate the denaturing effect of pressure. Indeed, it can be seen that time and temperature also have important roles in the combined process at lower pressures as can be assessed by comparing treatments 1 and 2 or 1 and 3 for example (figure 2).

4. Discussion

Several hypotheses can explain the wide shape of the thermogram for the globular protein alone at low concentration. These are exposed in the following.

One possibility is that the β-lactoglobulin globular protein has two different domains unfolding during the programmed temperature increase of the differential scanning calorimetry analysis as hypothesised by other authors (Fessas, Iametti, Schiraldi & Bonomi, 2001). The first one with a maximum transition temperature of c.a. 65°C and the second one 82-83°C. The low temperature domain could be more resistant and explain why the thermogram appears distorted as the processing intensity increases.

Another explanation could be that, in a given protein solution, although all molecules are in the same physico-chemical conditions, a distribution of conformations is observed. Indeed, the majority of proteins are in the conformation that has a minimal free energy for these conditions but a number of molecules are in a slightly higher energetic state, i.e. misfolded forms. These states are partially or incorrectly folded conformers presenting similarities with the native structure but with non-native regions (Cooper, 1999). The heat capacity increase between 40 and 60°C could be mainly due to these misfolded forms beginning to unfold.

Another element potentially explaining what happens on the thermogram between 40 and 70°C is the heat of complex dissociation of the dimer which is present at neutral pH (Qi, Brownlow, Holt & Sellers, 1995). The thermograms have been shown to be concentration dependent (Qi, Brownlow, Holt & Sellers, 1995). At low concentration (4 mg/ml), the thermogram was wider, showing no return to a base line and the presence of shoulders. Heat induced dissociation of the β-lactoglobulin dimer was believed to be mainly the cause for this spread thermogram on the low temperature side. The temperature at which this dissociation appears on the thermogram increases with concentration. Above 25 mg/ml the denaturation transition occurs when the protein is mainly dimeric and the thermogram obtained is a symmetrical sharp peak. At low

concentration, the endothermic dissociation, denaturation and exothermic aggregation are separated and the peak is wider.

This study, like others before (Qi, Brownlow, Holt & Sellers, 1995; Holt et al., 1998), shows how complex the thermal denaturation of β-lactoglobulin is at neutral pH and low concentration. Therefore making the analysis more difficult.

The present results show that the moderate combined process had irreversible effects on the protein structure. However, it seems that a plateau of denaturation, with denatured states having similar enthalpies, occurs from sample number 2, a relatively non-heavy treatment (150 MPa, 40°C, 30 min.). To have further significant denaturation, a higher level of treatment is required (treatments 8, i.e. 280 MPa, 60°C, 30 min). Both pressure and temperature on their own have limited effects while their combined effect is much more important.

When dextran sulphate is present in addition to the protein, the resulting thermograms are greatly modified. The peak is sharper and the calorimetric enthalpy is smaller. This would entail that an interaction between the two biopolymers occurs. It is known, for example, that on complexing with dextran sulphate the secondary structure of the protein is modified (Tolstoguzov, 1986). In addition, the calorimetric enthalpy of the thermograms of β-lactoglobulin treated with dextran sulphate, with a given treatment, is always smaller than the one without dextran sulphate. This entails that the presence of dextran sulphate in the solution made β-lactoglobulin more sensitive to the combined process. The results with pressure treatment alone show that dextran sulphate has a strong sensitising effect on the protein to pressure.

The results presented in this paper show that an interaction occurs between the polysaccharide and the protein. Even if both polysaccharides have a net negative charge at pH 7, attractive electrostatic interactions can occur between the negatively charged polysaccharide and positively charged patches on the protein (Dickinson, 2003). These interactions could be particularly strong with sulphated polysaccharides as the positively charged groups on the protein, $-NH_3^+$, interact strongly with $–OSO_3^-$ on the polysaccharide even at neutral or alkaline pH (Dickinson, 2003). The electrostatic nature of the interactions has been demonstrated with dextran sulphate and bovine serum albumin (Galazka, Smith, Ledward & Dickinson, 1999a).

In an earlier study, some of these authors (Galazka, Sumner & Ledward, 1996) found that bovine serum albumin at 2.5% had a higher calorimetric enthalpy post-processing (800 MPa, 20 min, pH7) in the presence of dextran sulphate than in its absence. The author's hypothesis was as follow (Galazka, Sumner & Ledward, 1996; Galazka, Dickinson & Ledward, 2001b), the complex present before pressurisation is dissociated under pressure and the protein would unfold, exposing more charges. A stronger bovine serum albumin-dextran sulphate would be formed on pressure release. The large negative charge of the complex would prevent protein-protein interaction allowing reformation of secondary structure post-processing explaining the higher calorimetric enthalpy in the presence of dextran sulphate. In the same study with β-lactoglobulin the effect of dextran sulphate was not considered significant and it was concluded that the electrostatic binding between the two biopolymers was probably very weak.

However, in the present study, dextran sulphate induced a strong sensitising effect over the β-lactoglobulin protein structure to pressure. The difference in results with Galazka et al. (Galazka, Sumner & Ledward, 1996) could possibly due to the higher protein concentration (25 mg/ml instead of 3 mg/ml) and/or to the higher operating pressure (800 MPa instead of 280 MPa) used in their study. These conditions could result in near-complete unfolding of the protein alone hence potentially preventing visualising the sensitising effect of dextran sulphate on the β-lactoglobulin structure to pressure. In the present case, it could be thought that the more the protein is processed, the greater the level of unfolding and the exposure of positive patches rises which in turn increases the protein-polysaccharides interactions. When the protein is associated in the complex with dextran sulphate during or after pressurisation the close interaction might prevent the protein molecule from regaining some structure post-processing. In a similar way, it was found that the presence of another polysaccharide (κ-carrageenan or ι-carrageenan (2 mg/ml)) with bovine serum albumin (5 mg/ml) at pH 7 sensitised the protein structure to a 20 min treatment at 600 MPa treatment (Galazka, Smith, Ledward & Dickinson, 1999a). This was evidenced by a lower enthalpy post-processing in presence of polysaccharide. In the same study it was demonstrated that complexation between the protein and κ-carrageenan, ι-carrageenan or dextran sulphate protected the protein against aggregation through disulphide bridging.

These results suggest that more unfolding of the β-lactoglobulin protein could occur in the presence of dextran sulphate. The combined process without dextran sulphate has been shown to induce the formation of species that could be trapped in the molten globule state as they have less tertiary structure but still conserve some secondary structure (Aouzelleg et al., 2004). The so called molten globules created have been shown to be stable for at least three months (Yang, Dunker, Powers, Clark & Swanson, 2001) and have been postulated to have higher functionality (Hirose, 1993; Dickinson & Matsumura, 1994; Yang, Dunker, Powers, Clark & Swanson, 2001). The presence of dextran sulphate could result in additional functional properties (e.g. due to higher surface hydrophobicity of the partly unfolded protein) or this could allow reducing he operating pressure even further. For example, in the present study, 150 MPa in the presence of dextran sulphate at various temperatures are sufficient to give a range of denatured structures. However, dextran sulphate could have detrimental effects on the functionality. For example, it was found that while high pressure up to 800 MPa increased the surface hydrophobicity of ovalbumin, treatment in the presence of dextran sulphate reduced it. This was probably due to dextran sulphate blocking the hydrophobic binding sites on the surface of the protein (Galazka, Smith, Ledward & Dickinson, 1999b; Galazka, Dickinson & Ledward, 2001b). This should be assessed with β-lactoglobulin as proteins differ greatly in their responses to denaturation conditions (Hayakawa, Linko & Linko, 1996). In addition, actual functional properties should be tested. Interfacial properties such as foaming properties are relevant, as they require low concentrations in protein, and could demonstrate if partially unfolded structure have higher foaming properties. Pressure has been shown to affect the foaming properties of whey protein isolate (Ibanoglu and Karatas, 2003). The different structures obtained in the present study, combined with other structural information (Aouzelleg et al., 2004), could allow correlating the processing conditions to the protein structure and foaming properties.

5. Conclusions

Although moderate and insufficient on their own to induce significant denaturation, the combination treatments with moderate pressures and temperatures can result in distinct structures. The results show that even operating in moderate processing condition it is possible to optimise the processing parameters so as to obtain significant structural changes potentially leading to functional benefits. In the presence of dextran sulphate the globular protein was sensitised to the combined process studied. This would seem to be due principally to a sensitisation to pressure. Thus, dextran sulphate could enable a wider range of structures with more advanced unfolding to be obtained for given moderate combined processes and with possible added functional benefits. This could allow potential savings as a given functionality could be obtained with less protein material or with lower operating pressure.

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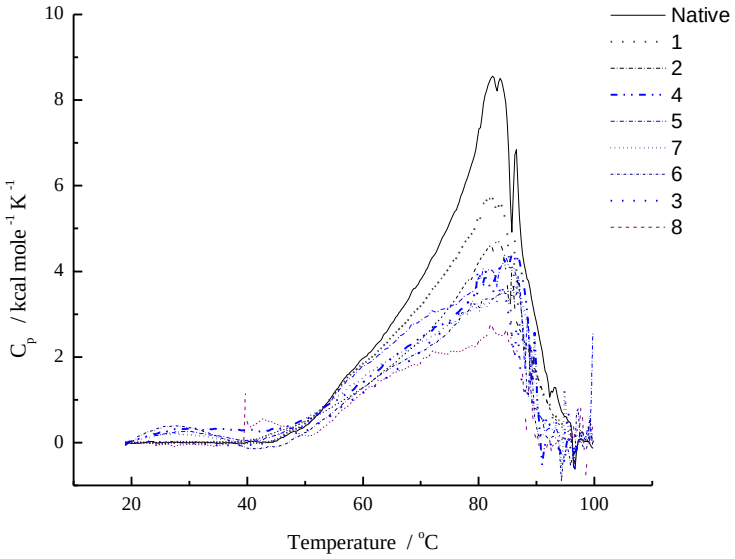

Figure 1: Differential scanning calorimetry results of β-lactoglobulin solution in Tris HCl pH 7 after the indicated combined heat/pressure treatment. The order of the graphs at the peak temperature 83°C is the same as the one given in legend.

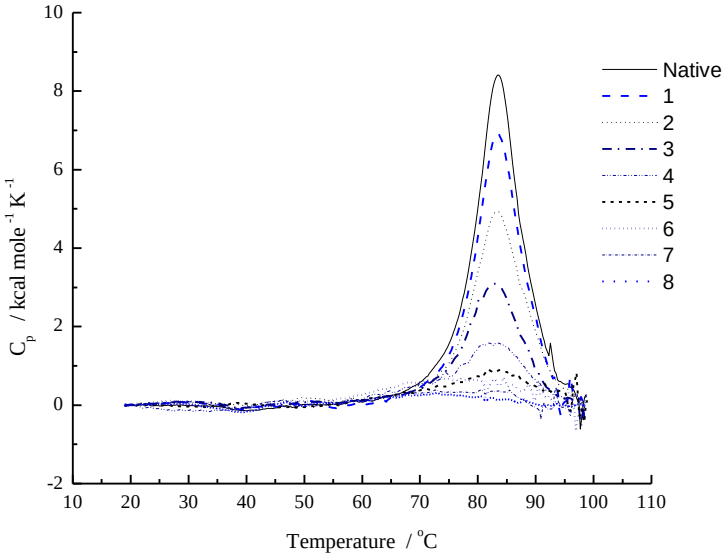

Figure 2: Differential scanning calorimetry results of β-lactoglobulin and dextran sulphate (1/1) solution in Tris HCl pH 7 after the indicated combined heat/pressure treatment. The order of the graphs at the peak temperature 83°C is the same one as given in legend.

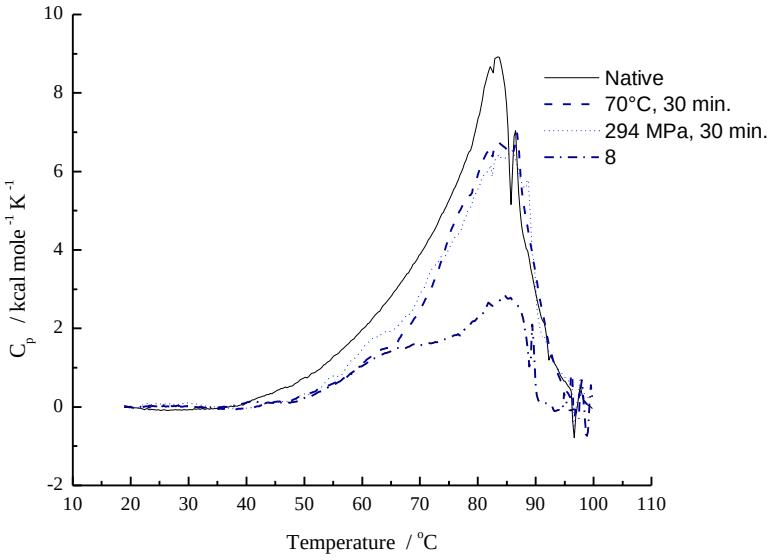

Figure 3: Differential scanning calorimetry results of β-lactoglobulin solution treated with pressure or temperature alone or with combined heat/pressure treatment.

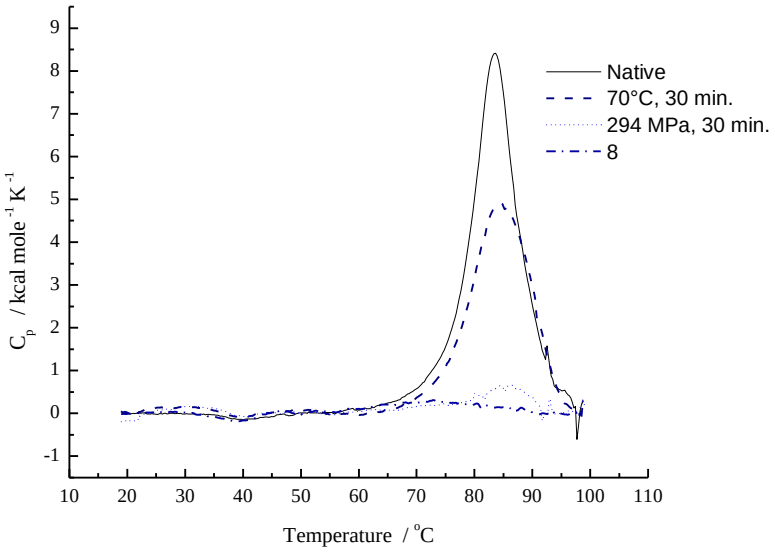

Figure 4: Differential scanning calorimetry results of β-lactoglobulin/dextran sulphate solutions treated with pressure or temperature alone or with combined heat/pressure treatment.

Figure 1: Differential scanning calorimetry results of β-lactoglobulin solution in Tris HCl pH 7 after the indicated combined heat/pressure treatment. The order of the graphs at the peak temperature 83°C is the same as the one given in legend.

Figure 2: Differential scanning calorimetry results of β-lactoglobulin and dextran sulphate (1/1) solution in Tris HCl pH 7 after the indicated combined heat/pressure treatment. The order of the graphs at the peak temperature 83°C is the same one as given in legend.

Figure 3: Differential scanning calorimetry results of β-lactoglobulin solution treated with pressure or temperature alone or with combined heat/pressure treatment.

Figure 4: Differential scanning calorimetry results of β-lactoglobulin/dextran sulphate solutions treated with pressure or temperature alone or with combined heat/pressure treatment.

Table 1: Central composite experimental design. Factor levels for each experiment.

Experiment	Pressure (MPa)	Temperature (°C)	Time (min.)
1	150	40	10
2	150	40	30
3	150	60	10
4	150	60	30
5	280	40	10
6	280	40	30
7	280	60	10
8	280	60	30

Table 2: Differential scanning calorimetry data summary results for the experimental design conditions of β-lactoglobulin with and without dextran sulphate (thermograms in Figure 1 and 2).

Condition	Without dextran sulphate		With dextran sulphate	
	Calorimetric enthalpy (cal/mol)	Transition temperature (°C)	Calorimetric enthalpy (cal/mol)	Transition temperature (°C)
Native	160544	82.9	91779	83.7
1	119415	81.8	74335	83.3
2	82427	81.3	60481	83.3
3	73170	81.3	44116	83
4	89937	85.2	16956	82.1
5	90200	83.6	27749	83.2
6	85767	83.9	7982	84.4
7	79735	84.9	8770	83.1
8	58272	82.8	6190	72