

# $\beta$ -lactoglobulin denaturation and aggregation reactions and fouling deposit formation: a DSC study

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## Summary

The thermal stability of  $\beta$ -lactoglobulin was studied using differential scanning calorimetry (DSC) as part of a larger investigation into milk and whey fouling. Measurement of kinetics using DSC is very difficult as the resultant trace is the sum of simultaneous denaturation and aggregational enthalpies; the two processes were demonstrated by measurements at different heating rates.  $\beta$ -lactoglobulin denaturation proceeds via an intramolecular disulphide stabilized intermediate and is irreversible even before the denaturation temperature is reached. Denaturation and aggregational processes can be partially separated by measurements over a range of pH. The literature shows that the rate of fouling decreases as solution pH increases. However, DSC shows that the thermal stability of  $\beta$ -lactoglobulin decreases as pH increases, particularly above pH 6. It is hypothesised that aggregation rather than denaturation might be the critical step in the formation of fouling deposit.

## Keywords

Calorimetry, heat denaturation of milk protein, pH effects, reaction kinetics.

## Introduction

The formation of fouling deposit is a severe problem in food processing plant operation. Fouling deposit from milk fluids contains both proteins and minerals in different combinations. Two types of deposit were described by Burton (1968): (1) a soft white voluminous spongy deposit occurring near the fluid inlet to heat exchangers, at temperatures up to 100°C, and containing 50–60% proteins, 30–35% minerals, and 4–8% fat, and (2) a grey brittle structure formed at temperatures above 100°C containing 70% minerals, 15–20% proteins, and 4–8% fat.

A more detailed chemical analysis of deposits in pasteurization and ultra-high temperature (UHT) plant, reported by Tissier *et al.* (1984), confirms the above. The pasteurizer contained a deposit similar to Type A, whose protein composition was 50%  $\beta$ -lactoglobulin, 23% immunoglobulins, and 18%  $\alpha$  s-1 casein. The sterilizer contained two types of deposit, one similar to Type A and the other to Type B. The

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protein composition of Type A sterilizer deposit was 62%  $\beta$ -lactoglobulin, 9%  $\alpha$ -lactalbumin, 9% immuno-globulins, and 9%  $\kappa$ -casein, while the protein composition of type B sterilizer deposit was 27%  $\alpha$ s-1-casein and 50%  $\beta$ -casein. The composition of type A deposit is significantly different from that of milk, of which  $\beta$ -lactoglobulin makes up about 9.8% of the protein content (Walstra & Jenness, 1984).

Deposit analysis also showed the mineral composition of fouling deposits to be different from milk. Lyster (1965) reported that calcium and phosphate typically accounted for 90% of the total mineral fraction in fouling deposits, while they represent only about 30% of the mineral composition in milk (Walstra & Jenness, 1984). Burton (1968) suggested that mineral salt deposition was caused by the lower solubility of calcium phosphate as temperature increases. These conclusions were supported by Visser (1988), who reported that in milk soluble calcium phosphate precipitates on heating via an amorphous intermediate before crystallizing as hydroxyapatite. Proteinaceous and mineral components in the deposit are thus formed by different mechanisms and deposit at different times during the process; Belmar-Beiny & Fryer (1991) demonstrate that the first layer of deposit from heated skimmed milk is protein, and that minerals are not deposited for several minutes. The precise interactions between mineral and protein during deposition are unknown; therefore, it is useful to study the thermal behaviour of individual components of milk in an attempt to elucidate the mechanisms of fouling.

A number of studies have linked the thermal stability of  $\beta$ -lactoglobulin and the formation of fouling deposit. Lalande *et al.* (1985) modelled pasteurizer fouling using  $\beta$ -lactoglobulin denaturation kinetics. Skudder *et al.* (1981) showed deposition could be significantly reduced by adding potassium iodate to prevent  $\beta$ -lactoglobulin aggregation before pasteurization. The thermal behaviour of  $\beta$ -lactoglobulin is complex, involving both molecular unfolding (denaturation) and subsequent intermolecular polymerization (aggregation) (Gotham, 1990). Unfolding during denaturation changes the protein conformation to expose groups that were buried in the native protein structure, thus resulting in the increased activity of such groups and enabling disulphide interchange reactions to occur as part of aggregation.

The overall thermal stability of  $\beta$ -lactoglobulin has been extensively investigated, by, *inter alia* Sawyer (1968), Lyster (1970), McKenzie (1971), Harwalker (1980), and Paulsson *et al.* (1985). Differential scanning calorimetry (DSC) has been used to study both the mechanism (de Wit & Klarenbeek, 1981) and kinetics (de Wit & Swinkels, 1980; Park & Lund, 1984; Relkin & Launay, 1990) of the process. Proposed rate orders vary (Dannenberg, 1986); de Wit & Swinkels (1980) suggest the process is first-order at pH 6.75 between 65 and 70°C, and higher orders above, whilst Park & Lund (1984) and Relkin & Launay (1990) suggest that the process is second order at that pH.

In DSC of solutions, a reference (solvent) and a test sample (solution) are both heated at a constant rate (commonly 5–10°C min<sup>-1</sup>). The difference between the heat needed to heat the two samples is measured as a function of temperature; this difference arises because of reactions in the test sample which result in the evolution or absorption of heat. An idealized DSC 'thermogram' is shown in Fig. 1; the two main parameters by which the thermogram is characterized are the area under the curve, the denaturation enthalpy, and the temperature at which the peak heat load occurs, the denaturation temperature. The error in the enthalpy arises because of uncertainties in the position of the baseline AB between the onset and end temperatures  $T_1$  and  $T_F$ ; the sharper the peak, the less error there will be.

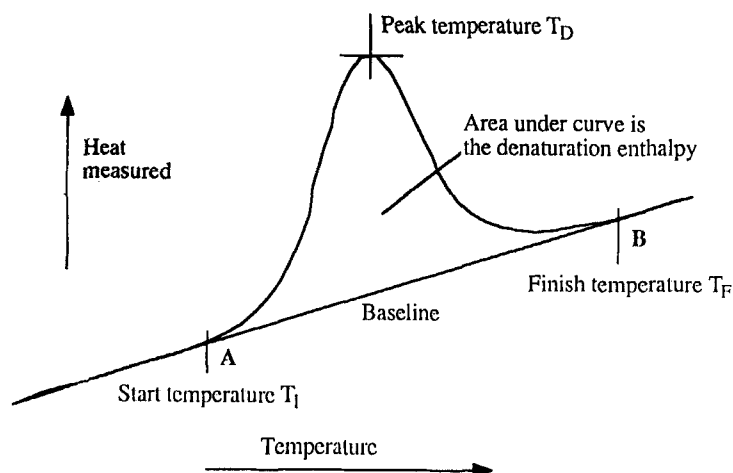


Figure 1. Schematic diagram of a DSC thermogram showing the parameters measured in this study.

DSC is well suited to the analysis of kinetic situations where only one reaction is occurring. However, sequential reactions such as denaturation and aggregation of proteins are more difficult to interpret. Since denaturation is an endothermic process, whilst aggregation is exothermic, the two processes may give rise to a thermogram which, although a single peak, is composed of data from both processes and is thus difficult to analyse.

DSC was initially thought to be particularly suited to studies of protein thermal stability because aggregational enthalpies were small and would not affect the result (de Wit & Swinkels, 1980). If this is correct, DSC has a major advantage over other methods of studying protein denaturation. However, for  $\beta$ -lactoglobulin, some questions, particularly the denaturation reaction order, remain unanswered. In addition, the precise nature of the reaction which leads to fouling is unclear, perhaps because the differences between the inter- and intramolecular processes of denaturation and aggregation are often not clear in the biochemical literature. This study attempts to distinguish the two processes of denaturation and aggregation of  $\beta$ -lactoglobulin on heating, and discusses the possible influence of these processes on fouling from milk products in industrial plant.

## Materials and methods

### Materials and solutions

Bovine serum albumin (product no. A3912), and bovine  $\beta$ -lactoglobulin (product no. L2506) were obtained from Sigma Chemicals Ltd. (St Louis, MO, USA) and used without further purification. All other chemicals were analytical grade reagents and only distilled deionised water was used throughout these experiments. The purity of the  $\beta$ -lactoglobulin can be seen in Fig. 2 which shows the result of SDS polyacrylamide gel electrophoresis.  $\beta$ -lactoglobulin was dissolved in 10 mM Tris/HCl, 1 mM EDTA at a concentration of  $1 \text{ mg ml}^{-1}$ . Protein was then desalted using a Pharmacia PD-10 Sephadex G-25M column which had previously been equilibrated

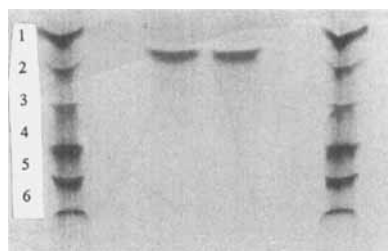


**Figure 2.** SDS gel of the  $\beta$ -lactoglobulin used in this study (two centre tracks). The markers on the outside tracks are (1)  $\alpha$ -lactalbumin (mol wt. 14 400); (2) trypsin inhibitor (20 100); (3) carbonic anhydrase (30 000); (4) ovalbumin (43 000); (5) bovine serum albumin (67 000); (6) phosphylase B (94 000).

with the electrophoresis solution. Electrophoresis was carried out using a Pharmacia Phast-System horizontal unit (Pharmacia, Uppsala, Sweden), with a PhastGel gradient 8–25 gel together with SDS buffer strips [0.55% (w/v)] SDS. Protein samples (1  $\mu$ l) were loaded and the gels stained with Coomassie Blue dye. Although the chloride concentration of the  $\beta$ -lactoglobulin product is substantial, this does not affect the thermal behaviour of the protein; the calcium content was claimed to be very small by Sigma. The gel shows that the protein consists of a single component.

The buffers used were; 0.2 M Na acetate (pH 4.0–5.6), 0.2 M K phosphate (pH 5.6–8.0), adjusted to the required pH with 0.1 M HCl or KOH. Preliminary work, based on the proposal that published variations in  $\beta$ -lactoglobulin denaturation temperatures and enthalpies may be due to the use of different buffer systems (Park & Lund, 1984) shows no difference between the thermal stability of  $\beta$ -lactoglobulin in simulated milk ultrafiltrate (SMUF) (Jenness & Knoop, 1962) and 0.2 M phosphate at pH 6.6 or 0.2 M phosphate and 0.2 M acetate at pH 5.6 (Gotham, 1990), suggesting that this is not the reason for variations in the literature. The experiments with potassium iodate were done using SMUF as sole buffer at pH 2.5 and 6.6.

The lyophilized protein powders were dissolved in buffers, filtered (Millipore 22  $\mu$ m filters, Harrow, UK) to remove undissolved material and the protein concentration measured spectrophotometrically using the following extinction coefficients ( $E_{cm}^{1\%}$ ) and molecular weights; bovine serum albumin: 6.67 at 279 nm (Aoki *et al.* 1973) and 66000 (Spahr & Edsall 1964),  $\beta$ -lactoglobulin: 9.3 at 278 nm (Ruegg *et al.* 1977) and 18422 (McKenzie 1971).

### Calorimetry

A Mettler Instruments power compensated DSC consisting of a TA20 measuring cell containing high sensitivity sensors was linked to a TC 10A microprocessor with an IBM based data storage system; an online program calculates reaction parameters assuming only a single reaction. Protein solution (30  $\mu$ l) and reference sample (buffer) were sealed into DSC pans and heated from 20 to 120°C at 7.5°C min<sup>-1</sup> (unless otherwise stated) to give maximum pre- and post-peak baseline stability and calculate enthalpies as accurately as possible. Heating rates varied from 0.3 to 20°C min<sup>-1</sup>. The reversibility of  $\beta$ -lactoglobulin denaturation was measured by heating samples to a preset temperature (between 60–80°C), cooling to 25°C, and reheating to 120°C. The weights of protein and reference samples were recorded before and after each run and results were discarded if either weight changed by more than 0.3 mg. Examination of samples after heating showed them to have aggregated.

## Results and discussion

### Accuracy of results

As all curves showed only a single maximum, results were expressed in conventional DSC terms of denaturation enthalpy and temperature. Data quoted are all averages of 5 experiments unless otherwise stated. Standard deviations of denaturation temperatures were  $\pm 0.4^\circ\text{C}$  or less, and denaturation enthalpies  $\pm 20\text{ kJ mol}^{-1}$ , results similar to others in the literature (Ruegg *et al.*, 1977; de Wit & Swinkels, 1980; Sochava *et al.*, 1985).

### Effect of $\beta$ -lactoglobulin aggregation on DSC results

A series of experiments were conducted to study the heating of  $\beta$ -lactoglobulin in the DSC at pH 6.6 for various protein concentrations and heating rates.

As  $\beta$ -lactoglobulin concentrations increased from 3.28 to 7.10 mM (60 and 130  $\text{mg ml}^{-1}$ ) at pH 6.6, denaturation temperatures increased by about  $1.0^\circ\text{C}$  (Fig. 3a), while denaturation enthalpies increased from 280 to about 300  $\text{kJ mol}^{-1}$  (Fig. 3b).

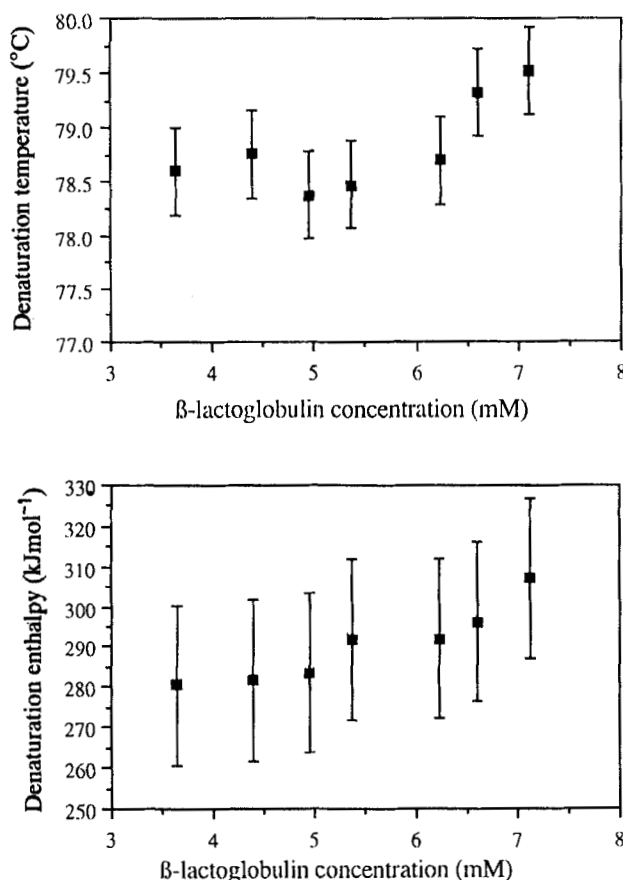


**Figure 3.** (a) The effect of  $\beta$ -lactoglobulin concentration on denaturation temperature, pH 6.6,  $7.5^\circ\text{C min}^{-1}$ , phosphate buffer. (b) The effect of  $\beta$ -lactoglobulin concentration on denaturation enthalpy, pH 6.6,  $7.5^\circ\text{C min}^{-1}$ , phosphate buffer. All data quoted are means of 5 replicates, 30  $\mu\text{l}$  sample.

The enthalpy data confirms those of Park & Lund (1984) and de Wit & B Swinkels (1980). Comparison of the variation in the measured parameters with the accuracy of the experiment suggests that neither change is significant. A second series of experiments were then carried out to measure the effect of changing the heating rate from 3.0 to 20°C. The denaturation temperature increased from 72 to 81°C, (Fig. 4a) whilst denaturation enthalpy increased by about 20 kJ mol<sup>-1</sup> (Fig. 4b). Again, the enthalpy changes are probably not significant. However, the change in denaturation enthalpy is much greater than experimental error.

As a first step in analysis, the data in Fig. 4a can be extrapolated to estimate a denaturation temperature at an infinitely small heating rate. An apparent denaturation temperature of 72°C is obtained, a value between those of 70°C and 72.8°C obtained by de Wit & Swinkels (1980) and Ruegg *et al.* (1977).

Some mathematical treatment of single reactions is possible. The DSC signal of a single n<sup>th</sup> order reaction in which native protein N is transformed into denatured protein D

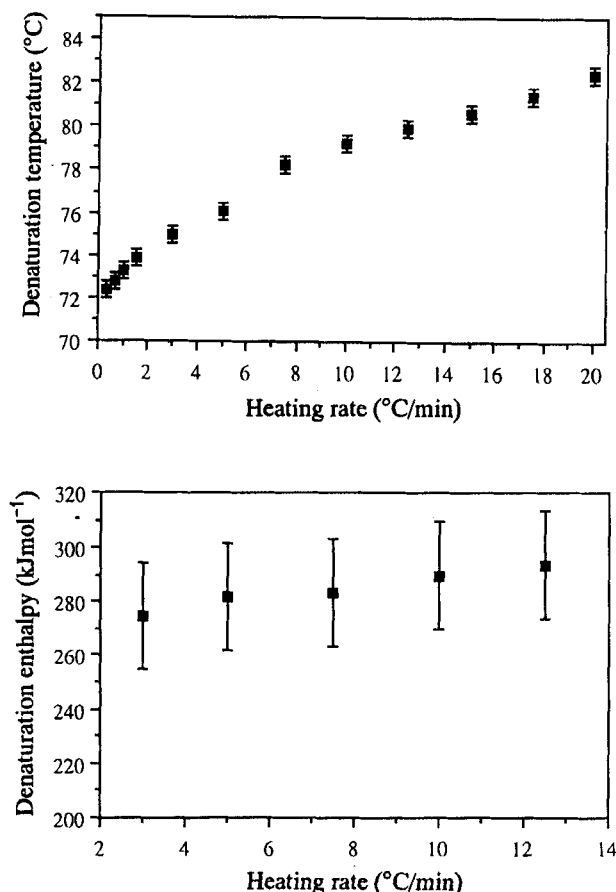


**Figure 4.** (a) The effect of heating rate on denaturation temperature,  $\beta$ -lactoglobulin concentration 3.28 mM, pH 6.6, phosphate buffer. (b) The effect of heating rate on denaturation enthalpy,  $\beta$ -lactoglobulin concentration 3.28 mM, pH 6.6, phosphate buffer. All data quoted are means of 5 replicates, 30  $\mu$ l sample.



with a reaction rate constant

$$\frac{dc}{dt} = -k_d c^n \text{ where } k_d = A_d \exp \frac{-E_d}{RT} \quad (2)$$

and enthalpy of reaction  $\Delta H_d$ , can be calculated from the heat given off or adsorbed  $W$ , temperature  $T$ ;

$$W = V \Delta H_d \frac{dc}{dt} \quad (3)$$

$$W = V \Delta H_d A_d \exp \left[ \frac{-E_d}{RT} \right] c_o^n (1-x)^n \quad (4)$$

$V$  is the volume of the sample,  $c_o$  the initial protein concentration, and  $x$  the fractional extent of reaction at time  $t$ . The total heat change measured  $Q$ , i.e. the area between the curve and the baseline, is given by

$$Q = \int_{T_i}^{T_f} W \cdot dt = V \Delta H_d c_o \quad (5)$$

so that the measured enthalpy of reaction should be a constant providing reaction is complete between the initial and final temperatures  $T_i$  and  $T_f$ .

The temperature at which  $W$  is a maximum is the denaturation temperature,  $T_d$ . Differentiation of equation (4) followed by considerable manipulation gives the Kissinger relationship between the denaturation temperature, protein concentration, and heating rate  $\alpha^\circ\text{C min}^{-1}$ ;

$$\ln \left( \frac{\alpha}{T_d^2} \right) = \ln \left( \frac{A_d R}{E_d} c_o^{(n-1)} \right) - \frac{E_d}{RT_d} \quad (6)$$

(Kissinger, 1957). For a single reaction of any order a graph of  $\ln (\alpha/T_d^2)$  vs.  $1/T_d$  should be a straight line from which the activation energy  $E$ , of the reaction can be found. Such a plot is given as Fig. 5. It can be seen that the line is not straight, suggesting that rather than a single reaction, there are multiple reactions occurring. The slopes of the lines on Fig. 4, fitted to the first and last four points on the plot, give activation energies of 260 and 820 kJ mol<sup>-1</sup> respectively. This shows clearly that the assumption of a single reaction is not tenable. These straight lines have correlation coefficients of 0.968 and 0.993 respectively. A straight line fit to the whole curve, with a correlation coefficient of 0.942, gave an activation energy of 420 kJ mol<sup>-1</sup>. However, the curve was better fitted by a parabola, which has a correlation coefficient of 0.990. Relkin & Launay (1990) present Kissinger plots for  $\beta$ -lactoglobulin, fit straight lines through all the data, and find activation energies in the region of 500 kJ mol<sup>-1</sup>, intermediate between the values obtained here. However, the denaturation temperatures in their work were all above 82°C, whilst the majority of denaturation temperatures in this work were lower.

It is clear from the analysis of the Kissinger plot that the assumption of a single reaction is not a good one. More work is necessary to determine how much kinetic information can be obtained from DSC curves for proteins undergoing multiple reactions. Protein denaturation and aggregation may be represented by a pair of consecutive reactions;

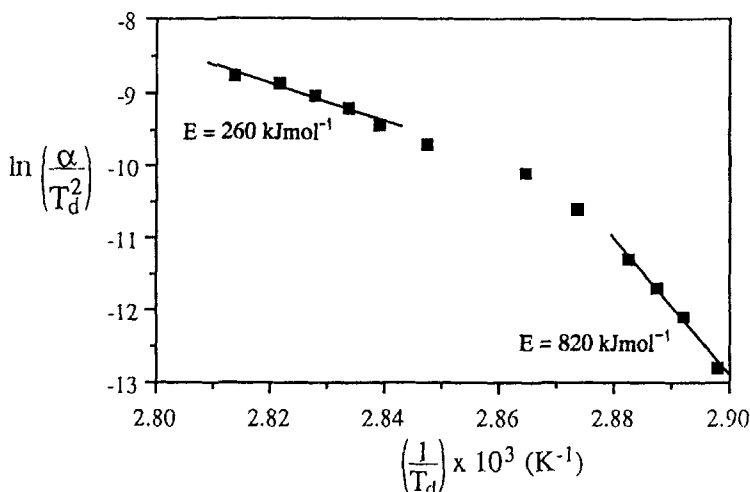


**Figure 5.** Plote of equation (6) demonstrating that the heating rate data can be fitted to two straight lines rather than one.



in which native protein N of concentration  $c$ , and initial concentration  $c_0$ , is successively transformed into denatured (D) and aggregated (A) protein of concentrations  $c_d$  and  $c_a$  respectively by  $n^{\text{th}}$  and  $m^{\text{th}}$  order reactions. Each reaction will have its own rate constant and activation energy:

$$\frac{dc}{dt} = -k_d c^n; \quad \frac{dc_a}{dt} = k_a c^m \quad (8)$$

$$k_d = A_d \exp\left[\frac{-E_d}{RT}\right]; \quad k_a = A_a \exp\left[\frac{-E_a}{RT}\right] \quad (9)$$

and its own enthalpy of reaction  $\Delta H_d$  and  $\Delta H_a$ .

Thus, the signal measured by the DSC will be

$$W = V [\Delta H_d k_d c^n + \Delta H_a k_a c_d^m] \quad (10)$$

which can be rewritten at a given temperature  $T$  as;

$$W = V \left[ \Delta H_d A_d \exp\left[\frac{-E_d}{RT}\right] c^n + \Delta H_a A_a \exp\left[\frac{-E_a}{RT}\right] c_d^m \right] \quad (11)$$

The change in  $W$  as a function of temperature constitutes the DSC thermogram. As before, equation (10) can be integrated to give the peak area;

$$Q = \int_{T_i}^{T_f} W \cdot dt = V (\Delta H_d + \Delta H_a) c_0 \quad (12)$$

i.e. that the heat measured is the sum of the two processes. Equations (5) and (12) show that for both single and multiple reactions the measured enthalpy will be independent of concentration and heating rate, demonstrating that the non-significant increase of the denaturation enthalpy in Figs 3b and 4b is fully consistent with a two-stage reaction.

The data plotted in Fig. 5 suggest that a single reaction cannot be assumed, i.e. that DSC detects exothermic aggregational enthalpies. Analysis of the DSC results assuming a single reaction [equation (6)] gave variable reaction orders of between 1 and 2, suggesting that aggregation makes it impossible to use the approximation in the range studied. To obtain accurate kinetics it will be necessary to deconvolute the DSC signal to separate denaturation and aggregation processes. Analytical treatments such as that of Sandu & Singh (1989) tend to consider only those conditions where the two processes are effectively distinct, where two peaks can be seen on the thermogram. The analysis of single peaks is more difficult; differentiation of equation (11) to obtain a simple equation analogous to equation (6) is not possible. Development of a numerical scheme to fit parameters to the equation is currently underway. The results indicate that kinetic data for  $\beta$ -lactoglobulin obtained from DSC should be treated with caution.

#### *Disulphide bonding in $\beta$ -lactoglobulin denaturation and aggregation*

The above section shows that denaturation and aggregation reactions cannot be easily separated by DSC. McKenzie (1971) proposed the initial step in  $\beta$ -lactoglobulin aggregation is the formation of intermolecular disulphide bonds, which can be blocked by chemical oxidation reactions. Experiments were conducted in which potassium iodate, the same oxidizing agent as that used by Skudder *et al.* (1981) was added to  $\beta$ -lactoglobulin before testing by DSC; it was hoped that this would suppress aggregation and allow the effects of denaturation alone to be measured.

Table 1 shows that addition of 5 mM potassium iodate to 3.28 mM  $\beta$ -lactoglobulin at pH 6.6 in SMUF greatly decreased the denaturation temperature by 7°C and the enthalpy by 137 kJ mol<sup>-1</sup>, but in similar experiments at pH 2.5 the denaturation temperature decreased by only 2.4°C and the enthalpy by only 55 kJ mol<sup>-1</sup>. Minimal aggregation occurs at pH 2.5 (Harwalker, 1980) and, as observed, little change in the enthalpy should result when iodate is added. If iodate blocked aggregation at pH 6.6 and left denaturation unaffected the results should show only a small increase in the measured endothermic denaturation enthalpy due to the absence of the exothermic aggregation enthalpy. It thus seems impossible to measure aggregational enthalpies by this method; no other experiments were conducted.

Potassium iodate had no significant effect on bovine serum albumin (BSA) denaturation at pH 6.6 (Table 1) and no detectable thermal activity of its own in the

**Table 1.** The effect of the addition of 5 mM potassium iodate on protein denaturation temperature and enthalpy.

| Protein     | Treatment | pH  | Denaturation temperature (°C)<br>±SD 5 replicates | Enthalpy (kJ mol <sup>-1</sup> )<br>±SD |
|-------------|-----------|-----|---------------------------------------------------|-----------------------------------------|
| $\beta$ -lg | none      | 6.6 | 78.2 ± 0.2                                        | 268 ± 25                                |
| $\beta$ -lg | iodate    | 6.6 | 71.2 ± 0.3                                        | 131 ± 11                                |
| $\beta$ -lg | none      | 2.5 | 83.0 ± 0.4                                        | 304 ± 25                                |
| $\beta$ -lg | iodate    | 2.5 | 80.6 ± 0.7                                        | 249 ± 14                                |
| BSA         | none      | 6.6 | 68.3 ± 0.7                                        | 1129 ± 63                               |
| BSA         | iodate    | 6.6 | 67.1 ± 0.4                                        | 1090 ± 49                               |
| none        | iodate    | 6.6 | NMP                                               | NMP                                     |

NMP. No measurable peak.

temperature range 20–120°C. This confirms the results of Marshall (1986), who used gel electrophoresis to study the heating of raw milk in the presence of potassium iodate and concluded (1) that iodate addition increased  $\beta$ -lactoglobulin denaturation at both 75°C and 95°C, and (2) that BSA denaturation was unaffected by addition of potassium iodate at either 75°C or 95°C. Both BSA and  $\beta$ -lactoglobulin contain disulphide bridges and sulphhydryl groups. Thus, the effect of potassium iodate is not due simply to chemical oxidation of sulphur-containing amino acids; it must alter the denaturation process. A possible explanation is offered by de Wit & Klarenbeek (1981), who suggest that, during denaturation, intramolecular disulphide bonds take part in the formation of a partially unfolded denatured intermediate at pH 6.6. Addition of potassium iodate would block the formation of this intermediate. The reaction scheme followed on heating under these conditions will thus not be that followed by the molecule under normal circumstances.

### Reversibility

Once intermolecular aggregation begins, it is likely that the changes induced by heating will be irreversible. De Wit & Swinkels (1980) suggest  $\beta$ -lactoglobulin denaturation is reversible up to the DSC denaturation temperature, and cite biochemical analysis by Mills (1976) and Pantaloni (1964) in support. However, Arnebrant *et al.* (1987) suggest that denaturation is irreversible.

The reversibility of the thermal behaviour of  $\beta$ -lactoglobulin was examined by heating protein to temperatures up to, and just above, the DSC denaturation temperature, followed by cooling to room temperature and reheating in the DSC. Any reduction in the denaturation enthalpy or change in the denaturation temperature for the reheated sample will be due to irreversible changes during the initial heating. Results are given in Table 2. Comparison with results for  $\beta$ -lactoglobulin heated only once ( $T_d = 78.4^\circ\text{C}$ ,  $\Delta H = 284 \text{ kJ mol}^{-1}$ ) shows some irreversible reaction occurs before the denaturation temperature is reached. The denaturation temperatures of reheated  $\beta$ -lactoglobulin starts to increase for samples initially heated above 72.5°C. This trend is similar to that observed by Arnebrant *et al.* (1987), and may be due to the increasing thermal stability of the partially denatured intermediate on reheating. The results emphasise that the DSC denaturation temperature is not a fundamental

**Table 2.** Increasing irreversibility of  $\beta$ -lactoglobulin denaturation at pH 6.6: DSC results on reheating  $\beta$ -lactoglobulin.

| First heating maximum temperature (°C) | Denaturation temperature on reheating (°C)<br>±SD, 5 replicates | Enthalpy (kJ mol <sup>-1</sup> )<br>±SD |
|----------------------------------------|-----------------------------------------------------------------|-----------------------------------------|
| Control (room temperature)             | 78.4 ± 0.4                                                      | 284 ± 20                                |
| 60.0                                   | 78.8 ± 0.2                                                      | 236 ± 10                                |
| 65.0                                   | 78.9 ± 0.1                                                      | 194 ± 10                                |
| 67.0                                   | 77.8 ± 0.3                                                      | 164 ± 5                                 |
| 70.0                                   | 78.4 ± 0.4                                                      | 179 ± 11                                |
| 72.5                                   | 78.7 ± 0.2                                                      | 167 ± 19                                |
| 75.0                                   | 81.0 ± 1.5                                                      | 137 ± 20                                |
| 77.5                                   | 83.0 ± 0.5                                                      | NMP                                     |
| 80.0                                   | NMP                                                             | NMP                                     |

NMP. No measurable peak.

quantity, and certainly does not correspond to the onset either of denaturation or aggregation. In these experiments the start temperature  $T_i$  was in the region of 70°C. It is difficult to measure this quantity accurately. The effect of preheating is more marked in the enthalpy of denaturation; all the enthalpies are statistically different from the control sample. The results show that denaturation and aggregation are intimately linked, and that the overall process is not reversible even after heating to quite low temperatures.

#### *The effect of pH on $\beta$ -lactoglobulin denaturation and protein fouling*

This study has shown that it is difficult to use the DSC to obtain clear kinetics of a type which could be used in fouling studies. However, it is possible to use DSC evidence, together with that for fouling, to suggest the controlling mechanism for deposit formation. Fouling in pasteurization and UHT plants increases rapidly below pH 6.6 (Hege & Kessler, 1986; Skudder *et al.*, 1986). Hege & Kessler (1986) found deposit formation to be a maximum at pH 5.1. However, the thermal stability of  $\beta$ -lactoglobulin, measured between pH 4.0–8.0, shows that denaturation temperatures decrease with increasing pH (Fig. 5a), particularly between pH 6.25 and 7.0. Over the pH range 4.0–8.0, (Fig. 5b) the denaturation enthalpy decreases steadily from 330 kJ mol<sup>-1</sup> to 243 kJ mol<sup>-1</sup>. Effects of this type were also noted by Ruegg *et al.* (1977). Park & Lund (1984) and De Wit & Swinkels (1980). Thus, the decreasing thermal stability of  $\beta$ -lactoglobulin with pH increase does not coincide with increased deposit formation in fouling, but the reverse is true. The rate of deposit formation increases below pH 6, rather than above. It is important to explain this anomaly.

De Wit & Klarenbeek (1981) found the thermal behaviour of  $\beta$ -lactoglobulin to be two-stage, with DSC peaks at 80°C and 140°C. At the lower temperature only partial unfolding occurred, so the process was only complete at 140°C when all residual structure, mainly constrained by disulphide bonds, was disrupted. De Wit & Klarenbeek (1981) also noted that as the pH increased the enthalpy change associated with the low-temperature peak decreased, as seen here in Fig. 4a, whilst that of the high-temperature peak increased. They proposed that, as pH increased, low temperature denaturation was increasingly limited by constraining disulphide bonds which reduce the amount of molecular unfolding possible at 80°C. Watanabe & Klostermeyer (1976) showed that the number of sulphhydryl groups available for intermolecular disulphide bond formation is pH dependent, increasing significantly below pH 7.0. Sulphydryl groups buried within the molecule are sterically unable to take part in disulphide exchange reactions until exposed by initial unfolding.

Hegg *et al.* (1985) and de Wit (1981) both show that  $\beta$ -lactoglobulin aggregate formation increases significantly as pH decreases from 7.0 to 6.0, reflecting increased molecular unfolding during the denaturation phase. The trend in the pH dependence of fouling deposit formation found by Hege & Kessler (1986) was thus similar to that of the dependence of  $\beta$ -lactoglobulin aggregation on pH (de Wit, 1981). This suggests that  $\beta$ -lactoglobulin aggregation, rather than denaturation, may be the controlling factor in deposit formation. Although above pH 6.5 the rate of  $\beta$ -lactoglobulin denaturation will increase, the amount of molecular unfolding associated with denaturation will be reduced, allowing fewer intermolecular disulphide exchange reactions, and thus decreased aggregation. The importance of aggregation is further demonstrated by Skudder *et al.* (1986) who found that the extent of denaturation was similar at pH 5.0 and 7.0. However, fouling amounts are significantly different at the two pH values, as is the extent of aggregation.

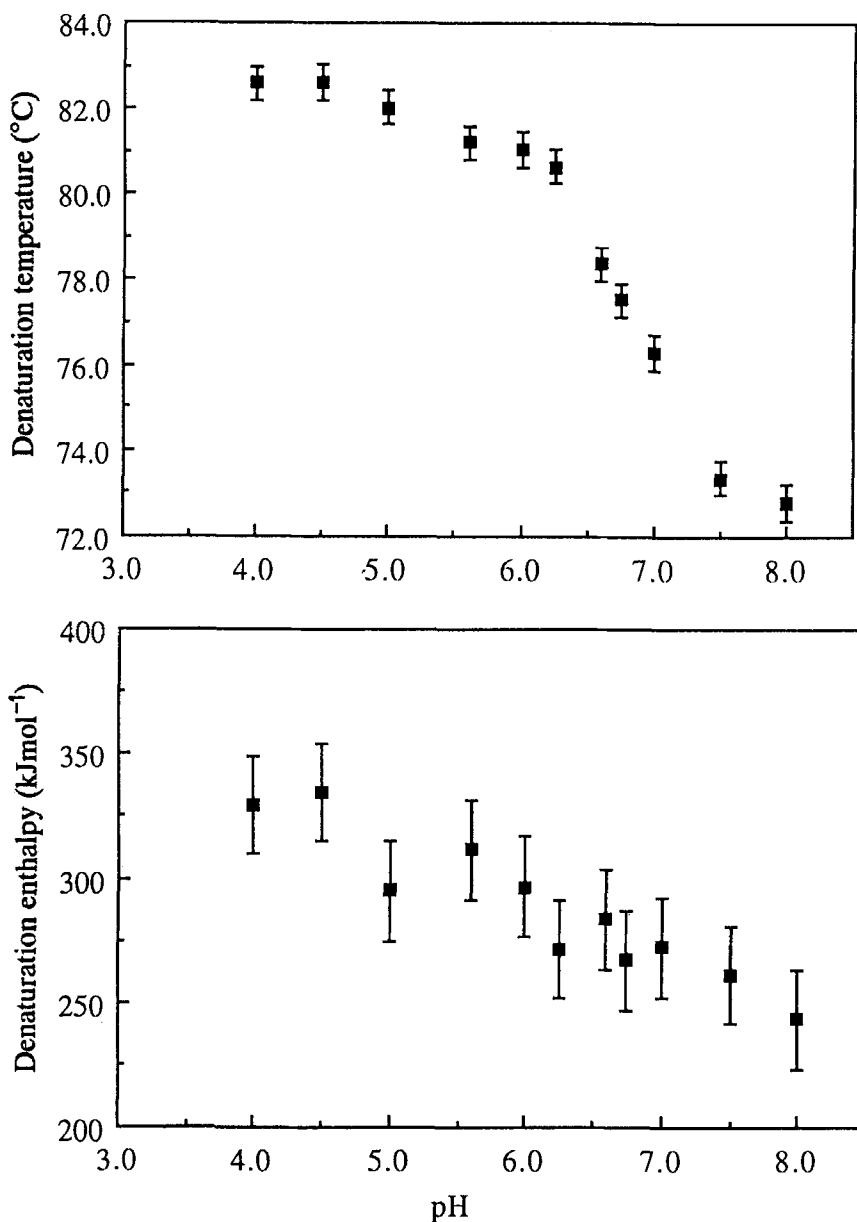


**Figure 6.** (a) The effect of  $\beta$ -lactoglobulin denaturation temperature, concentration 60 mg ml<sup>-1</sup>, acetate buffer pH 4.0–5.6, phosphate buffer pH 5.6–8.0, heating rate 7.5°C min<sup>-1</sup>. (b) The effect of  $\beta$ -lactoglobulin denaturation enthalpy, concentration 60 mg ml<sup>-1</sup>, acetate buffer pH 4.0–5.6, phosphate buffer pH 5.6–8.0, heating rate 7.5°C min<sup>-1</sup>. All data quoted are means of 5 replicates, 30  $\mu$ l sample.

Further work will be required to confirm this hypothesis. For example, it would be useful to measure aggregation and denaturation as a function of pH for the same sample of  $\beta$ -lactoglobulin, to determine the precise pH dependence of the two processes. However, this work suggests that DSC is not well suited to studies which attempt to separate the processes of denaturation and aggregation. It should be

noted that the kinetic scheme proposed in equation (7) is likely to be a considerable simplification of the actual processes which  $\beta$ -lactoglobulin undergoes during heating. Relkin & Launay (1990) propose a four-stage reaction. The use of DSC to analyse such multistage processes is unlikely to produce a clear reaction order.

## Conclusions

A DSC study of the thermal stability of  $\beta$ -lactoglobulin has been carried out. It is not easy to obtain kinetics from the DSC trace, because DSC measures thermal changes associated with both denaturation and aggregation. Analysis of data taken at a variety of heating rates demonstrates that the assumption of a single reaction is not possible. Addition of oxidizing agents such as potassium iodate to suppress aggregation also affects the denaturation step. The overall process is irreversible even before the denaturation temperature is reached. The amount of unfolding associated with denaturation at ca. 80°C reduces with increasing pH, reducing the amount of aggregation possible. The amount of fouling deposit formed in process plant also decreases with increasing pH, suggesting that aggregation, i.e. inter-molecular polymerisation is the rate-controlling step in deposit formation.

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## Appendix: Nomenclature

|                |                                         |                         |
|----------------|-----------------------------------------|-------------------------|
| A              | pre-exponential factor                  |                         |
| c              | Concentration                           | (mol m <sup>-3</sup> )  |
| c <sub>0</sub> | Initial concentration of native protein | (mol m <sup>-3</sup> )  |
| D              | Denatured protein                       |                         |
| E              | Activation energy                       | (kJ mol <sup>-1</sup> ) |
| k <sub>d</sub> | Rate constant for denaturation reaction | (ms <sup>-1</sup> )     |
| m              | Order of reaction                       |                         |

|                |                            |                                         |
|----------------|----------------------------|-----------------------------------------|
| N              | Native protein             |                                         |
| n              | Order of reaction          |                                         |
| Q              | Total heat measured by DSC | (J)                                     |
| R              | Gas constant               | (kJ mol <sup>-1</sup> K <sup>-1</sup> ) |
| T              | Temperature                | (K)                                     |
| T <sub>d</sub> | Denaturation temperature   | (K)                                     |
| V              | Volume of DSC cell         | (m <sup>3</sup> )                       |
| W              | Heat output                | (W)                                     |
| x              | Percentage reaction        |                                         |
| α              | Heating rate               | (°C min <sup>-1</sup> )                 |
| ΔH             | Enthalpy                   | (kJ mol <sup>-1</sup> )                 |

## Subscripts and superscripts

|   |              |
|---|--------------|
| a | Aggregation  |
| d | Denaturation |
| I | Initial      |
| F | Final        |