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# Kinetics of Denaturation and Aggregation of Whey Proteins in Skim Milk Heated in an Ultra-high Temperature (UHT) Pilot Plant

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## ABSTRACT

Skim milk was subjected to various temperature–time (70–130°C for 3–1800 s) treatments in a pilot-scale ultra-high temperature (UHT) plant using a direct steam injection (DSI) system. The rates of denaturation and aggregation of β-lactoglobulin (β-lg) and α-lactalbumin (α-la) were determined by quantitative polyacrylamide gel electrophoresis while that of immunoglobulin G (IgG) was determined by affinity chromatography. The order of reaction, the rate constant and the activation energy were determined for the denaturation of β-lg, α-la and IgG using non-linear regression methods. The kinetic parameters obtained for β-lg and α-la showed a marked temperature dependence. Aggregation of β-lg mainly involved the formation of intermolecular disulphide linkages at all temperatures studied, while the aggregation of α-la, particularly below 80°C, appeared to also involve hydrophobic interactions. The kinetic parameters obtained for β-lg aggregation, due to disulphide bond formation, were similar to those observed for β-lg denaturation. © 1998 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

Heat treatment of milk during commercial processing operations results in a number of physicochemical changes in the milk constituents, in particular denaturation of the whey proteins, that subsequently influence the functional properties of the milk products. For example, in the manufacture of yoghurt, milk must be preheated to cause high levels of whey protein denaturation, in order to develop desirable textural properties. Excessive heating of milk, on the other hand, can lead to increased rennet clotting times and low gel strength which are detrimental in the manufacture of cheese.

Denaturation of whey proteins is generally assumed to be a process consisting of at least two steps (Mulvihill and Donovan, 1987; de Wit, 1990). Initially, the native protein unfolds cooperatively to expose side chain groups originally buried within the native structure. The second step involves aggregation of unfolded protein molecules through thiol-disulphide interchange reactions, hydrophobic interactions and ionic linkages. Denaturation of whey proteins has been measured by numerous authors in a number of model and milk systems, using a wide range of methods, including DSC (Park and Lund, 1984), serological activity (Baer *et al.*, 1976), solubility at pH 4.5, specific optical rotation (Harwalkar, 1980), electrophoretic techniques (Hillier and Lyster, 1979; Dannenberg and Kessler, 1988) and FPLC (Manji and Kakuda, 1986). The denaturation of α-lactalbumin (α-la) appears to follow first-order kinetics, but the denaturation of β-lactoglobulin (β-lg) has been described as following first-order (Gough and Jenness, 1962; de Wit and Swinkels, 1980; Luf, 1988), second-order (Hillier and Lyster, 1979; Manji and Kakuda, 1986) or 1.5 order (Dannenberg and Kessler, 1988; Roefs and de Kruif, 1994). Despite these differences, there is general agreement that the denaturation reaction shows a non-linear Arrhenius plot, although activation energy ($E_a$) has been found to vary between 260 and 310 kJ mol$^{-1}$ for the 70–90°C range, and 32–50 kJ mol$^{-1}$ for the 90–150°C range. The differences between the results obtained by various workers could be attributed to a number of factors: the concentration and composition of the protein solution, the medium of the protein solution (skim milk, whey or isolated protein in buffer solutions), the assay methods used to detect residual protein levels and a lack of sufficient data to permit the accurate determination of the kinetic parameters.

In addition, various heating methods have been used, including heating samples in glass tubes, capillary tubes immersed in water/oil baths and laboratory-scale heat exchangers. It is likely that whey proteins respond differently depending on how the sample is heated, i.e., on the time required to reach the desired temperature, flow conditions and cooling times and rates. Hence, the kinetics of denaturation of whey proteins on heating milk under industrial conditions is likely to be different from that observed in milks heated in the laboratory. In most of the previous studies, the kinetics of denaturation were determined in milks heated by indirect heating methods, and no studies are available on the kinetics of denaturation of whey proteins in milk heated by

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direct steam injection using typical commercial processing conditions.

Under the conditions that exist in milk, the denaturation of whey proteins is a very complex process; unfolding of protein molecules is followed immediately by irreversible aggregation reactions. The denatured $\beta$-lg polymerises via disulphide bonds and forms a complex with $\alpha$-la and/or $\kappa$-casein in the micelles (Singh, 1995). Little information is available on the kinetics of aggregation of whey proteins involving intermolecular disulphide bonds in milk systems. Because of the complexity of the reaction pathways, the denaturation and aggregation of $\beta$-lg and $\alpha$-la have been studied in model systems rather than in milk itself.

The objective of this study was to investigate the kinetics of irreverible denaturation of $\beta$-lg, $\alpha$-la and IgG, using native-PAGE, in skim milk heated under typical commercial processing conditions over a wide range of temperatures. The kinetics of aggregation of $\beta$-lg, due to disulphide bond formation, were also determined using SDS-PAGE.

## MATERIALS AND METHODS

### Milk supply

On six occasions, raw whole milk was obtained from the University Dairy Farm or Tui Dairy Company, Longburn, New Zealand. Milk was skimmed without pasteurization and stored at 5°C.

### UHT processing

Skim milk was processed on a pilot-scale UHT plant (Type D, Alfa-Laval, Sweden) at the New Zealand Dairy Research Institute, Palmerston North (Fig. 1). For each run, skim milk was first heated to ~65°C by a plate heat exchanger and then direct steam injection (DSI) was used to give an instantaneous step increase to the desired temperature. The heated milk then flowed through the holding tube and into the flash vessel, which provided an instantaneous reduction in milk temperature to ~65°C. A range of temperatures (70–130°C) and holding times (3–160 s) were used. When longer holding times were required, heated milks were collected in a screw cap glass test tube (125 mm length, 20 mm diameter, 1 mm wall thickness) just after the DSI unit and placed in a water bath at the appropriate temperature for a set time. It took ~10 s to transfer a 100 ml sample to the water bath. The temperature of the sample dropped by ~2°C, and ~1 min was required for samples to come up to temperature. At the end of the heat treatments, skim milks were cooled to room temperature in an ice bucket. The dilution of milk caused by the injection of steam by the DSI unit varied between 2 and 10%, depending upon the temperature required. In order to investigate the effects of dilution on the extent of whey protein denaturation, samples of skim milk were deliberately diluted by up to 20% with water and heated in a UHT-plant; the extent of denaturation in the diluted samples was comparable with that of normal heated milk, indicating that dilution of milk due to the injection of steam had negligible effect on the denaturation of whey proteins.

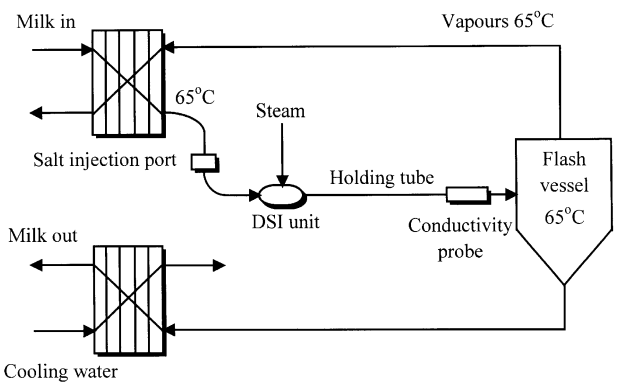


**Fig. 1.** Schematic diagram of the UHT plant set-up.

The residence time distribution (RTD) of skim milk in the holding tube was measured using a cross-correlation technique, as described by Janssen (1994). Reynolds number ranged from 4800 to 7080, indicating that the flow conditions in the tubes were in the transitional region. A salt tracer was injected into the 90° bend just prior to the DSI unit. The conductivity of skim milk at the end of the holding tube was measured by a conductivity cell and a RTD curve was plotted. The mean residence time was calculated from the RTD curve and used in the kinetic calculations. The mean residence times were close to the average holding time; for example, milk flowing through a holding tube with a nominal holding time of 30 s at 115 kg h$^{-1}$ had a mean residence time of 31.6 s with a standard deviation of 1.87 s.

### Milk protein analysis

Skim milk samples were centrifuged at 50,000 rpm (average 175,000 ***g***) for 1 h at 20°C in a Beckman L8-80M (Beckman, Palo Alto, California) and supernatants were removed carefully. These supernatants and some of the pellets were analysed for $\beta$-lg and $\alpha$-la by polyacrylamide gel electrophoresis (PAGE), which was performed on a mini-gel electrophoresis unit (Biorad Laboratories, Richmond, CA, USA), under non-dissociating (native-PAGE), dissociating but non-reducing (SDSNR-PAGE) and dissociating and reducing (SDSR-PAGE) conditions as described by McSwiney *et al.* (1994). The gels were scanned on a Computing Densitometer (Molecular Dynamics, Sunnyvale, CA 94086) and the integrated intensities of the $\beta$-lg or $\alpha$-la bands were calculated by a software program, ImageQuant (Molecular Dynamics). Standard curves prepared from purified $\beta$-lg and $\alpha$-la (Sigma Corp, St. Louis, MO, USA) were used to determine the concentration (g kg$^{-1}$) of individual whey proteins. This procedure gave good repeatability (error ~6%) in quantification of the protein bands.

The concentration of immunoglobulin G (IgG) in the supernatant was determined by an affinity column (Hi-Trap affinity column. Protein G, Pharmacia, Uppsala, Sweden) according to the method of Kinghorn *et al.* (1995).

### Concentration and dilution effects

Using the method of van Boekel and Walstra (1989), a correction factor of 0.917 was determined and used to

convert whey protein concentration in the supernatant to that in the heated skim milk. This factor accounts for the decrease in water volume caused by water associated with the pellet and water unavailable as solvent due to steric exclusion at the micelle surface.

The changes in protein concentration in heated milk, due to steam injection and water removal in the flash vessel, were determined by comparing the protein nitrogen content of the heated milk sample with that of raw milk. This was used to convert the concentration of whey protein in the heated milk to that in the original skim milk. The nitrogen content was determined by the Kjeldhal method using a Kjel-Foss automatic 16200 (Foss Electric, Hillerød, Denmark).

### Statistical analysis of kinetic data

The kinetics of whey protein reactions were determined using a non-linear regression (NLR) method, as described by Oldfield *et al.* (1996). The rate of protein denaturation can be described by an integrated form of the general rate equation (1) and the Arrhenius equation (2),

$$\left(\frac{C_t}{C_0}\right)^{1-n} = 1 + (n-1)k_n C_0^{n-1} t, \tag{1}$$

where, $n$ is the reaction order, $k_n$ is the rate constant $(\mathrm{g\,kg^{-1}})^{(1-n)}\,\mathrm{s^{-1}}$, $t$ is the time (s) and $C_t$ and $C_0$ are the concentrations ($\mathrm{g\,kg^{-1}}$) of native protein at time $t$ and time 0, respectively,

$$k_n = k_0\, \mathrm{e}^{-(E_a/RT)} \tag{2}$$

where, $k_0$ is the pre-exponential term $(\mathrm{g\,kg^{-1}})^{(1-n)}\,\mathrm{s^{-1}}$, $R$ is the universal gas constant ($8.314\ \mathrm{J\,mol^{-1}\,K^{-1}}$), $T$ is temperature (K) and $E_a$ is the activation energy ($\mathrm{kJ\,mol^{-1}}$).

A disadvantage of the Arrhenius equation is the high correlation between the kinetic parameters $E_a$ and $k_0$. This problem can be overcome by subtracting a reference temperature ($T_{ref}$) from the heating temperature (Oldfield *et al.*, 1996; van Boekel, 1996),

$$k_n = k_{ref} \exp\left(-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right), \tag{3}$$

where, $k_{ref}$ is the reference pre-exponential term $(\mathrm{g\,kg^{-1}})^{(1-n)}\,\mathrm{s^{-1}}$ and $T_{ref}$ is the reference temperature (K).

Substitution of eqn (3) into eqn (1), followed by rearrangement, gives

$$C_t = C_0\left[1 + (n-1)\,k_{ref}\,C_0^{n-1}\,t \times \exp\left(-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right)\right]^{(1/(1-n))} \tag{4}$$

The concentration/time data were fitted directly into eqn (4) and $E_a$, $k_{ref}$, $n$ and $C_0$ were determined simultaneously by NLR. The overall fit of the model to the experimental data was given by the adjusted $R^2$,

$$R^2 = 1 - \frac{\mathrm{MSSE}}{\mathrm{MSST}}, \tag{5}$$

where MSSE is the mean sum of squares residuals and MSST is the mean sum of squares total.

### Thermodynamics of denaturation

The effect of temperature on denaturation rates can be studied using the activated complex theory. The basis of this theory is that reactants ($A$ and $B$) form an unstable intermediate (activated complex, $X^{\ddagger}$), which then immediately decomposes to form products ($P$ and $Q$) [eqn (6)] (Levenspiel, 1972). A quasi-equilibrium is assumed to exist between the reactants and the activated complex. The thermodynamic properties of the activated complex can then be determined by use of the Eyring equations (7)–(9) (Moore, 1972),

$$A + B \leftrightarrow X^{\ddagger} \rightarrow P + Q, \tag{6}$$

$$k_f = \frac{k_b T}{h}\, \mathrm{e}^{-\Delta H^{\ddagger}/RT}\, \mathrm{e}^{\Delta S^{\ddagger}/R}, \tag{7}$$

$$E_a = \Delta H^{\ddagger} + RT, \tag{8}$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}, \tag{9}$$

where $k_f$ is the reaction rate constant for the breakdown of the activated complex to product ($\mathrm{s^{-1}}$), $k_b$ is the Boltzmann's constant ($1.38062 \times 10^{-23}\ \mathrm{J\,K^{-1}}$), $h$ is the Planck's constant ($6.6262 \times 10^{-34}\ \mathrm{J\,s}$) and $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta G^{\ddagger}$ are the enthalpy, entropy and free energy of formation of the activated complex, respectively. The activation energy determined by NLR was used in eqn (8) to calculate $\Delta H^{\ddagger}$ and eqn (7) was then used to calculate $\Delta S^{\ddagger}$; $\Delta G^{\ddagger}$ was determined from the reaction rate constant and $\Delta H^{\ddagger}$ using eqn (9).

## RESULTS AND DISCUSSION

### Loss of native whey proteins

Changes in the concentrations of native $\beta$-lg A, $\beta$-lg B and $\alpha$-la as a function of heating time were determined at temperatures in the range 70–130°C by native-PAGE; the results obtained for $\beta$-lg A, $\beta$-lg B and $\alpha$-la at 100 and 120°C are shown in Fig. 2. As expected, the concentration of native whey proteins decreased with an increase in heating time at all temperatures studied. The rate of loss of different whey proteins was in the order; IgG (determined using affinity chromatography) > $\beta$-lg B > $\beta$-lg A > $\alpha$-la, which is in agreement with the results of previous workers (Dannenberg and Kessler, 1988; Singh and Creamer, 1991).

Denaturation has been described as a two-step process, unfolding of unimolecular native protein followed by intermolecular aggregation to form polymeric products (Mulvihill and Donovan, 1987; deWit, 1990). The native-PAGE method used in this study differentiates between monomeric and aggregated proteins but does not distinguish between unfolded and native conformations of $\beta$-lg and $\alpha$-la. Therefore, the loss of protein based on native-PAGE reflects the extent of irreversible denaturation (i.e. aggregation involving both non-covalent interactions and disulphide bond formation).

### Kinetics of denaturation of whey proteins

The concentration versus time data for the loss of native proteins, obtained at each temperature, were used to determine the kinetics of whey protein denaturation. The kinetic parameters derived from NLR analysis

[eqn (4)] are shown in Table 1. Analysis of the experimental data for β-lg and α-la showed that there were two temperature regions in which the $E_a$ values were markedly different (Fig. 3). Both β-lg A and B showed a break in the Arrhenius plot at approximately 90°C, while for α-la, this break occurred at 80°C (Fig. 3). On the other hand, IgG showed no break in the Arrhenius plot over the temperature range 70–85°C.

The calculated activation energies, along with their 95% confidence intervals (Table 1), can be compared with those reported previously. In the temperature range 70–90°C, the $E_a$ values for β-lg A and B were slightly higher than those reported by Dannenberg and Kessler (1988) (265 and 280 kJ mol$^{-1}$). However, in the higher temperature range, 95–130°C, the values were similar to those of Dannenberg and Kessler (1988) (54 and 45 kJ mol$^{-1}$). For α-la, the $E_a$ values in both the lower (70–80°C) and higher temperature (85–130°C) ranges were somewhat lower than the values reported by Dannenberg and Kessler's (1988) i.e., 269 and 69 kJ mol$^{-1}$, respectively. The activation energy for IgG in the range 70–85°C was slightly lower than the values reported by Resmini *et al.* (1989) (345 kJ mol$^{-1}$) and Li-Chan *et al.* (1995) (298.5 kJ mol$^{-1}$), but differed considerably from Luf *et al.* (1993) (70–75°C; 674.42 kJ mol$^{-1}$, 76–79°C; 89.22 kJ mol$^{-1}$) who found a break in the Arrhenius plot at 76°C.

These differences in the level of denaturation could be caused by different heating methods, and/or analytical techniques used. Dannenberg and Kessler (1988) used indirect heating (tubular heat exchanger), and a thermostatically controlled stainless-steel holding tube section, in which the milk was held at the desired temperature and time by control valves. The DSI and flash vessel used in the present study operates as an instantaneous step up and step down in temperature, respectively. Thus, the differences in heating up and cooling down times between these two investigations may have affected the denaturation kinetics. Both studies used gel electrophoresis to determine the amount of native, undenatured whey proteins, although Dannenberg and Kessler (1988) acidified the heated milk samples to pH 4.6 to precipitate caseins and denatured whey proteins.

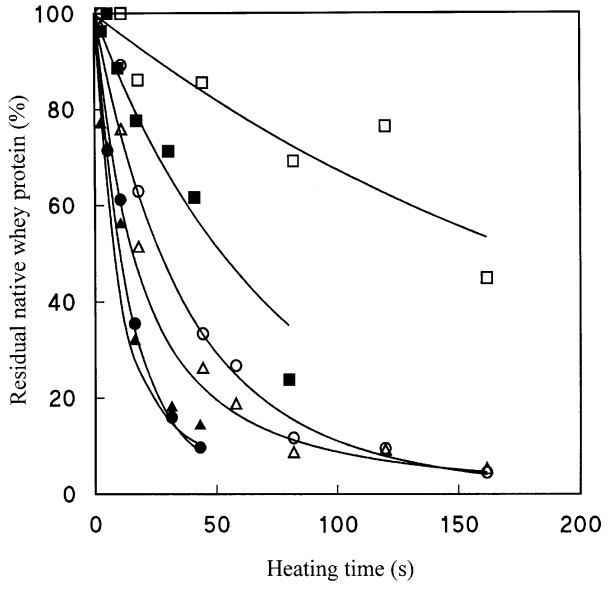


**Fig. 2.** Loss of β-lg A (○, ●), β-lg B (△, ▲) and α-la (□, ■) based on native-PAGE gels of ultracentrifugal supernatants obtained from skim milk heated at 100 (open symbols) or 120°C (closed symbols). Lines represent the predicted denaturation from the model.

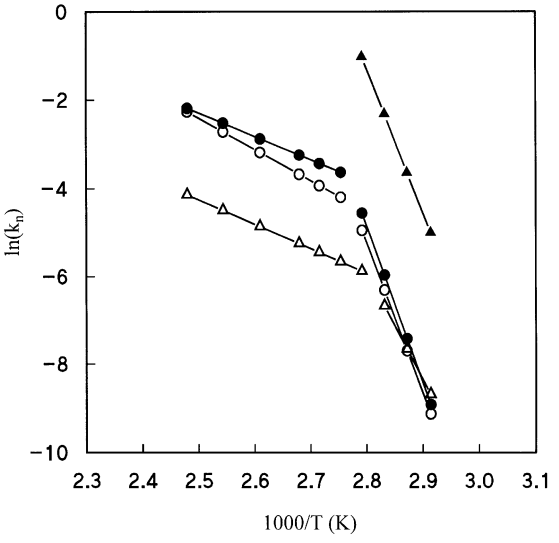


**Fig. 3.** Arrhenius plot for the denaturation of β-lg A (○), β-lg B (●), α-la (△) and IgG (▲) in skim milk. $k_n$ has units of [(g kg$^{-1}$)$^{(1-n)}$ s$^{-1}$].

**Table 1.** Kinetic Parameters for Denaturation of Individual Whey Proteins in Skim Milk

| Whey protein | Temperature range (°C) | Parameters from NLR[a] with 95% confidence intervals | | | | | |
|---|---|---|---|---|---|---|---|
| | | $n$ | $E_a$(kJ mol$^{-1}$) | ln($k_{ref}$)[b] | ln($k_0$) | $C_0$(g kg$^{-1}$) | $R^2$[c] |
| β-lg A | 95–130[d] | 1.2 ± 0.2 | 58.8 ± 5.7 | −3.06 ± 0.11 | 15.3 ± 1.8 | 1.56 ± 0.08 | 0.971 |
| | 70–90[e] | 1.0 ± 0.3 | 285.5 ± 30.8 | −6.29 ± 0.10 | 91.0 ± 10.5 | 1.79 ± 0.08 | 0.985 |
| β-lg B | 95–130[d] | 1.6 ± 0.2 | 44.0 ± 5.8 | −2.78 ± 0.11 | 10.9 ± 1.8 | 1.76 ± 0.12 | 0.980 |
| | 70–90[e] | 1.4 ± 0.4 | 296.7 ± 42.2 | −5.26 ± 0.25 | 95.1 ± 14.4 | 1.94 ± 0.25 | 0.994 |
| α-la | 85–130[f] | 1.1 ± 0.3 | 52.9 ± 5.6 | −4.79 ± 0.27 | 11.9 ± 1.8 | 0.73 ± 0.02 | 0.952 |
| | 70–80[g] | 0.9 ± 0.4 | 203.3 ± 33.2 | −7.66 ± 0.37 | 62.6 ± 11.5 | 0.74 ± 0.05 | 0.985 |
| IgG | 70–85[h] | 2.0 ± 0.4 | 269.3 ± 37.6 | −2.91 ± 0.99 | 89.5 ± 12.9 | 0.45 ± 0.04 | 0.940 |

[a] Non-linear regression.
[b] $T_{ref}$ is the average of the temperature range quoted in the table.
[c] Adjusted $R^2$ [eqn (5)].
The number of data points in each temperature range, [d] 43, [e] 25, [f] 61, [g] 15 and [h] 28.

The apparent order of denaturation reactions varied among the whey proteins ($n = 0.9$–$2.0$, Table 1). The break in the Arrhenius plot did not cause the reaction orders of the whey proteins to change markedly between the two temperature regions (Table 1), although in the lower temperature region ($<95°C$) the reaction orders were slightly lower. The reaction order for $\beta$-lg B (1.6–1.4) was found to be higher than that for $\beta$-lg A (1.2–1.0) (Table 1). The denaturation of $\alpha$-la could be described by a reaction order of $1.0 \pm 0.3$ while a reaction order of 2.0 was found for IgG (Table 1). The results obtained for $\beta$-lg B are essentially in agreement with those of Dannenberg and Kessler (1988) who observed 1.5 order kinetics. Other workers, however, have shown that denaturation of $\beta$-lg follows first- or second-order kinetics (Baer *et al*., 1976; Hillier and Lyster, 1979; Manji and Kakuda, 1986). The reaction order found for IgG in this study is in agreement with Resmini *et al*. (1989). However, Luf *et al*. (1993) reported a best fit of 1.16, and Li-Chan *et al*. (1995) assumed a first-order reaction. Because of the complexity of the reactions occurring in milk, the reaction order for denaturation may not be that significant in defining a reaction mechanism and thus should be treated as an empirical constant, fitting the experimental data to a relationship between the reaction rate and a number of the reactants.

## Thermodynamics of denaturation of whey proteins

The enthalpy ($\Delta H^{\ddagger}$), entropy ($\Delta S^{\ddagger}$) and free energy of formation ($\Delta G^{\ddagger}$) of the activated complex for $\beta$-lg A and B and $\alpha$-la are shown in Table 2. In the lower temperature range, i.e below 90°C, the $\Delta H^{\ddagger}$ values varied from 280 to 290 kJ mol$^{-1}$ for $\beta$-lg A and B, and were about 200 kJ mol$^{-1}$ for $\alpha$-la. These values are typical for heat-induced protein denaturation reactions (Labuza, 1980). Above 90°C, $\Delta H^{\ddagger}$ values were similar to those of chemical reactions (40–56 kJ mol$^{-1}$). At temperatures below 90°C, these values were higher than those reported by Dannenberg and Kessler (1988) for $\beta$-lg A and B (260–280 kJ mol$^{-1}$) and lower for $\alpha$-la (260 kJ mol$^{-1}$), although above 90°C, the $\Delta H^{\ddagger}$ values were similar (45–65 kJ mol$^{-1}$).

Below 90°C, $\Delta S^{\ddagger}$ was positive (0.27–0.53 kJ mol$^{-1}$ K$^{-1}$), indicating a gain in translational and rotational entropy, which could be interpreted as the protein changing into a more open conformation. At higher temperatures, $\Delta S^{\ddagger}$ was negative ($-0.17$ to $-0.13$ kJ mol$^{-1}$ K$^{-1}$), indicating a decrease in disorder which suggests that association reactions were becoming the rate limiting step (Dannenberg and Kessler, 1988).

**Table 2.** Average Enthalpy ($\Delta H^{\ddagger}$), Entropy ($\Delta S^{\ddagger}$) and Free Energy ($\Delta G^{\ddagger}$) Values Obtained for Denaturation of Whey Protein in Heated Skim Milk

| Whey protein | Temperature (°C) | $\Delta H^{\ddagger}$ (kJ mol$^{-1}$) | $\Delta S^{\ddagger}$ (kJ mol$^{-1}$ K$^{-1}$) | $\Delta G^{\ddagger}$ (kJ mol$^{-1}$) |
|---|---|---|---|---|
| $\beta$-lg A | 100–130 | 55.5 | −0.128 | 105.3 |
| | 70–90 | 282.6 | 0.501 | 105.5 |
| $\beta$-lg B | 100–130 | 40.7 | −0.162 | 103.6 |
| | 70–90 | 293.8 | 0.536 | 103.7 |
| $\alpha$-la | 90–130 | 49.7 | −0.158 | 110.1 |
| | 70–80 | 200.4 | 0.266 | 107.8 |

For $\beta$-lg A, $\beta$-lg B and $\alpha$-la, the value of $\Delta G^{\ddagger}$ remained relatively constant at approximately 100 kJ mol$^{-1}$, over the temperature range studied (70–130°C). Similar values for $\Delta G^{\ddagger}$ have been reported for the denaturation $\beta$-lg A and B, and $\alpha$-la denaturation in milks and reconstituted milks (Gough and Jenness, 1962; Dannenberg and Kessler, 1988; Anema and McKenna, 1996).

The downward concave shape of the Arrhenius plot (high slope at low temperature and *vice versa*) (Fig. 3) is consistent with two reactions in series with different temperature-dependent rate constants (Bunnett, 1974). At low temperatures ($<90°C$), the high activation energy of denaturation caused this to be the rate-limiting step. As the temperature was increased, the rate of denaturation increased until it became faster than that of the aggregation step and consequently aggregation became the rate limiting step.

## Loss of SDS-monomeric whey protein

Quantitative SDSNR-PAGE analysis was used to follow the aggregation of $\beta$-lg and $\alpha$-la in the supernatant of heated skim milks. In this PAGE system, SDS solubilized and dispersed most of the non-covalently linked aggregates into monomeric proteins whereas the covalently-linked protein aggregates remained intact. Thus, the protein that was resolved by SDSNR-PAGE is referred to as SDS-monomeric protein. The amounts of SDS-monomeric $\beta$-lg ($\beta$-lg A + $\beta$-lg B) and $\alpha$-la decreased with an increase in heating time at temperatures in the range 75–130°C; the results obtained after heating at 100 and 120°C are shown in Fig. 4. The loss of SDS-monomeric protein from the supernatant was apparently caused by the formation of disulphide-linked aggregates in the serum and/or aggregates that associated with the casein micelles and subsequently sedimented during ultracentrifugation.

The quantities of $\beta$-lg and $\alpha$-la in the ultracentrifugal pellets of skim milks heated at 75–90°C were determined by SDSNR- and SDSR-PAGE. $\beta$-Lg in the pellet was present almost entirely in the form of disulphide-linked

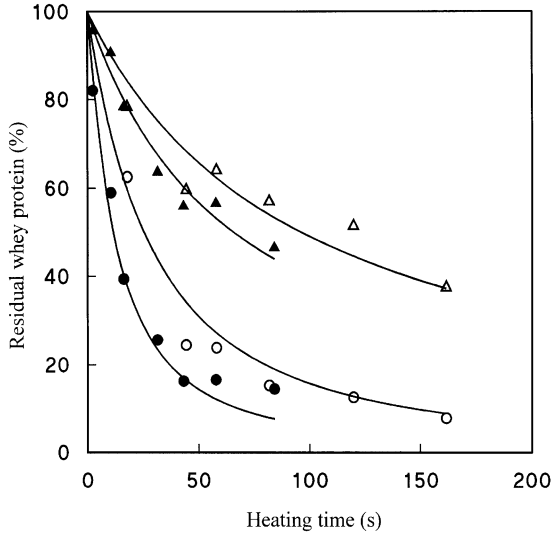


**Fig. 4.** Loss of SDS-monomeric $\beta$-lg (○, ●) and $\alpha$-la (△, ▲) from SDSNR-PAGE gels of ultracentrifugal supernatants obtained from skim milk heated at 100 (open symbols) or 120°C (closed symbols).

**Table 3.** Kinetic Parameters for Aggregation of β-Lg via Disulphide Bond Formation in Skim Milk

| Temperature range (°C) | Parameters from NLR[a] with 95% confidence intervals | | | | | |
|---|---|---|---|---|---|---|
| | $n$ | $E_a$ (kJ mol$^{-1}$) | ln($k_{ref}$)[b] | ln($k_0$) | $C_0$ (g kg$^{-1}$) | $R^{2c}$ |
| 95–130 | 1.6 ± 0.3 | 47.12 ± 10.41 | −3.61 ± 0.24 | 11.09 ± 10.41 | 3.28 | 0.940 |
| 75–90 | 1.7 ± 0.4 | 298.46 ± 52.16 | −6.02 ± 0.22 | 94.92 ± 52.16 | 3.84 | 0.984 |

[a] Non-linear regression.
[b] $T_{ref}$ is the average of the temperature range quoted in the table.
[c] Adjusted $R^2$ [eqn (5)].

aggregates (results not shown); presumably, these aggregates were β-lg/κ-casein and β-lg/β-lg complexes. In contrast, only about 60% of total α-la in the pellet was in the form of disulphide-linked aggregates; the remaining α-la was presumably aggregated via non-covalent (hydrophobic) interactions (results not shown).

### Kinetics of formation of β-lg aggregates

Because of the complex nature of α-la aggregation, the kinetics of disulphide-linked aggregate formation were attempted for β-lg only. The concentration versus time data for the loss of SDS-monomeric β-lg were used to determine the kinetics of β-lg aggregation in milk (Table 3). A break in the Arrhenius plot was observed at 90°C, which was the same break-point observed for the denaturation of β-lg. The reaction order was found to be 1.6–1.7 which was higher than the average denaturation order of 1.3 (Table 1). The numerical values of the parameters, $E_a$, ln($k_{ref}$) and ln($k_0$) (Table 3) were similar to those for β-lg denaturation (Table 1). The similar values for the kinetic parameters for denaturation and disulphide aggregation suggest that all of the denatured β-lg aggregates rapidly either with itself or with κ-casein in the micelles through thiol-disulphide interactions.

### Relationship between denaturation and the formation of disulphide-linked aggregates

Figure 5a shows the quantity of disulphide-linked β-lg aggregates formed in relation to denatured β-lg in the supernatant. Over the temperature range 75–130°C, the increase in denatured β-lg was mirrored by a similar increase in disulphide-linked β-lg aggregates. A corresponding plot for α-la (Fig. 5b) was more scattered, but showed a similar trend to that for β-lg at temperatures >85°C. However, at temperatures <85°C considerable denaturation of α-la was observed with little effect on the formation of disulphide-linked aggregates. Dalgleish (1990) observed a close correlation between the amount of disulphide-linked aggregates formed and the amount of serum protein lost in heated milk, although he did not report the behaviour of β-lg and α-la separately.

A number of mechanisms and models have been suggested previously for β-lg denaturation and aggregation in model solutions (e.g. Griffin *et al.*, 1993; McSwiney *et al.*, 1994; Roefs and deKruif, 1994; Qi *et al.*, 1995). The key features of these models could be used to explain the overall denaturation and aggregation behaviour of β-lg in heated milk systems. Based on the results of this study and the model proposed by Roefs and deKruif (1994), the following reactions could be taking place during heat

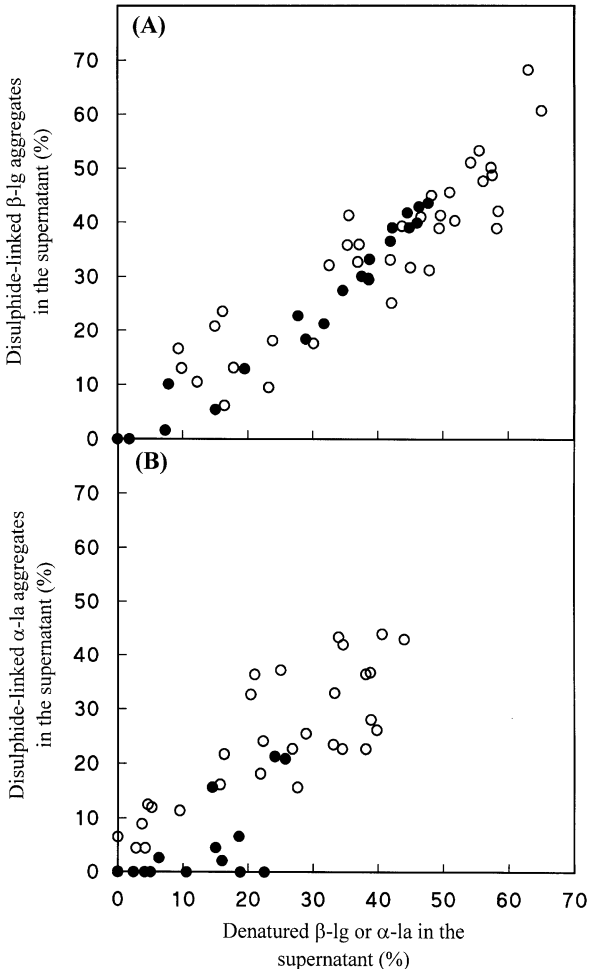


**Fig. 5.** Formation of disulphide-linked β-lg (A) or α-la (B) aggregates in relation to denatured β-lg or α-la. Heating temperature for β-lg, 95–130°C (○) and 75–90°C (●); and for α-la, 85–130°C (○) and 70–80°C (●).

treatment of milk,

$$\beta\text{-N} \rightleftharpoons \beta\text{-U} \quad \text{(I)}$$

$$\beta\text{-U} \rightarrow \beta\text{-U}^{SH} \quad \text{(II)}$$

$$\beta\text{-U} + \beta\text{-U}^{SH} \rightarrow (\beta\text{-A})_{s-s} \rightleftharpoons (\beta\text{-A})^{*}_{s-s} \quad \text{(III)}$$

where, β-N represents native β-lg, β-U represents partially unfolded β-lg, β-U$^{SH}$ represents β-lg with an exposed thiol group, and $(\beta\text{-A})_{s-s}$ and $(\beta\text{-A})^{*}_{s-s}$ represent various disulphide-linked aggregates of β-lg.

When milk is heated above 70°C, the native structure of β-lg (β-N) is transformed into an activated state (β-U)

which then unfolds further to expose a thiol group ($\beta$-$U^{SH}$). Consequently, $\beta$-$U^{SH}$ can react with a disulphide bond of another $\beta$-lg molecule, the transferred thiol becoming available for further thiol-disulphide interchange reactions with a new $\beta$-lg molecule.

Since the unfolding of $\beta$-lg is also likely to expose some hydrophobic residues, it is possible that the following reactions could occur on heating under some conditions, e.g. at temperatures below 75°C.

$$\beta\text{-}N \rightleftharpoons \beta\text{-U}, \quad \text{(IV)}$$

$$\beta\text{-U} \rightarrow (\beta\text{-A})_x, \quad \text{(V)}$$

$$(\beta\text{-A})_x \rightarrow (\beta\text{-A})_{s-s} \rightleftharpoons (\beta\text{-A})^*_{s-s}, \quad \text{(VI)}$$

where, $(\beta\text{-A})_x$ represents hydrophobically associated aggregates of $\beta$-lg. However, electrophoretic analysis did not provide clear evidence for the formation of hydrophobic aggregates of $\beta$-lg $(\beta\text{-A})_x$. A possible explanation is that if the hydrophobic aggregates were formed, they were rapidly converted into disulphide-bonded aggregates. If two or more $\beta$-lg molecules can first form hydrophobic aggregates, then it is possible that thiol-disulphide interchange reactions could occur more readily within the low-polarity environment [reaction (VI)]. The mechanism proposed by McSwiney *et al.* (1994) for $\beta$-lg aggregation appears to be covered by eqns (IV) to (VI).

The reactions involving $\alpha$-la may be depicted as follows:

$$\alpha\text{-N} \rightleftharpoons \alpha\text{-U} \quad \text{(VII)}$$

$$\alpha\text{-U} + \beta\text{-}U^{SH} \rightarrow (\alpha/\beta\text{-A})_{s-s} \rightleftharpoons (\alpha/\beta\text{-A})^*_{s-s} \quad \text{(VIII)}$$

$$\alpha\text{-U} + \beta\text{-U or } \beta\text{-}A_x \rightarrow (\alpha/\beta\text{-A})_x \quad \text{(IX)}$$

$$(\alpha/\beta\text{-A})_x \rightarrow (\alpha/\beta\text{-A})_{s-s} \rightleftharpoons (\alpha/\beta\text{-A})^*_{s-s} \quad \text{(X)}$$

where $\alpha$-N represents native $\alpha$-la, $\alpha$-U represents partially unfolded $\alpha$-la, $(\alpha/\beta\text{-A})_x$ represents hydrophobic aggregates of $\alpha$-la and $\beta$-lg, and $(\alpha/\beta\text{-A})_{s-s}$ and $(\alpha/\beta\text{-A})^*_{s-s}$ represent various disulphide-linked forms of $\alpha$-la and $\beta$-lg aggregates.

$\alpha$-La has been shown to undergo conformational changes at about 64°C, but $\alpha$-la does not readily form aggregates because of the lack of a thiol group (Ruegg *et al.*, 1977; Calvo *et al.*, 1993; Gezimati *et al.*, 1997). However, the presence of $\beta$-lg in the heated milk system enables hydrophobic aggregates containing both $\alpha$-la and $\beta$-lg to be formed $(\alpha/\beta\text{-A})_x$ and at appropriate temperatures, the thiol group of $\beta$-lg can initiate sulphydryl-disulphide interchange between the two proteins $(\alpha/\beta\text{-A})_{s-s}$ (Gezimati *et al.*, 1997). The present study clearly showed evidence for the formation of hydrophobic aggregates containing $\alpha$-la in milks heated below 80°C. In contrast, at temperatures above 80°C, the aggregates are predominantly disulphide-linked; presumably, at the higher temperatures the greater availability of thiol group of $\beta$-lg (Kirchmeier *et al.*, 1984), together with its faster rate of denaturation (unfolding), allow more sulphydryl-disulphide interchange reactions to occur between $\alpha$-la and $\beta$-lg.

To further understand the mechanisms of whey protein denaturation and aggregation, it would be necessary to isolate and identify the types of $\beta$-lg and $\beta$-lg/$\alpha$-la aggregates formed during heating. This may require working with model systems where the whey protein concentration and other parameters (e.g. pH, milk salts concentration, casein micelles) can be more easily manipulated.

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