Effect of Milk Concentration on the Irreversible Thermal Denaturation and Disulfide Aggregation of β-Lactoglobulin

Skelte G. Anema†

Food Science Section, New Zealand Dairy Research Institute, Private Bag 11029, Palmerston North, New Zealand

The kinetics of β-lactoglobulin (β-LG) denaturation in reconstituted skim milk samples of various concentrations (9.6−38.4% total solids) over a wide temperature range (75−100 °C) was studied. The thermal denaturation of β-LG had a reaction order of 1.5 at all milk solids concentrations and at all temperatures. The rate of denaturation of β-LG was markedly dependent on the milk solids concentration and the heating temperature. At 75 °C, the thermal denaturation of β-LG was retarded at higher milk solids concentrations. However, this retardation was less pronounced at higher temperatures so that a similar rate of denaturation was observed at all milk solids concentrations at 100 °C. From an examination of the level of disulfide-aggregated β-LG, it was evident that most, but not all, of the denatured β-LG was involved in disulfide-aggregated complexes, either with other denatured whey proteins or with the casein micelles. As with β-LG denaturation, the rate of disulfide aggregation of β-LG was markedly dependent on the milk solids concentration.

Keywords: *Milk; concentrated milk; β-lactoglobulin; thermal denaturation*

INTRODUCTION

There are numerous studies on the effect of heat treatments on the denaturation of the whey proteins in heated milk, and several studies have completed full kinetic and thermodynamic evaluations (Gough and Jenness, 1962; Lyster, 1970; Hillier, 1976; Hillier and Lyster, 1979; Manji and Kakuda, 1986; Dannenberg and Kessler, 1988a−c; Kessler and Beyer, 1991; Anema and McKenna, 1996). Comparisons among these different studies show that there is a considerable variation in the kinetic and thermodynamic parameters obtained. This variation can be attributed to a number of factors such as variations in the methods and conditions of the heat treatment, variations in the methods for assaying the residual native whey protein levels, or a lack of sufficient data to determine key kinetic parameters.

Most of the studies have investigated the effect of heat on the denaturation of whey proteins in milk at its natural concentration. There have been very few studies on the effect of milk solids concentration on the thermal denaturation of the major whey proteins. Harland et al. (1952) reported that the concentration of skim milk from 9% total solids (TS) to 36% TS had only a small effect on total whey protein denaturation, as measured by using the Harland−Ashworth method (Harland and Ashworth, 1947). At 65.6 and 82.2 °C the same level of denaturation was observed at all milk solids concentrations, whereas at intermediate temperatures (71.1 and 76.7 °C) there was a small decrease in the degree of serum protein denaturation with increasing milk solids concentration. Guy et al. (1967) reported a minimum whey protein denaturation at a milk concentration of 20% TS, whereas McKenna and O'Sullivan (1971) showed that the denaturation of whey proteins, as a percentage of those initially present, was markedly reduced at higher initial milk solids concentrations when milks at various concentrations were heated at 75 or 80 °C. However, when the denaturation was measured as grams of whey protein denatured, the rate was found to be independent of the initial milk solids concentration.

No full study of the effect of milk solids concentration on individual whey protein denaturation and disulfide aggregation has been reported previously. This paper describes the results of a kinetic study into the thermal denaturation and disulfide aggregation of β-lactoglobulin (β-LG) in reconstituted skim milk over wide temperature, time, and milk solids concentration ranges.

MATERIALS AND METHODS

Milk Supply. Reconstituted skim milk samples of 9.6−38.4% TS (w/w) were prepared by adding the appropriate quantity of low-heat skim milk powder (New Zealand Dairy Board, Wellington, New Zealand) to water [purified through a Milli-Q apparatus (Millipore Corp., Bedford, MA)]. A small quantity (∼0.04%) of sodium azide was added to each of the milk samples as a preservative. The milk samples were allowed to stir for at least 2 h before further use.

Heat Treatment. Weighed aliquots (∼200 mg) of the milks at the various concentrations were transferred to small sealable plastic tubes. The milk samples were heated at temperatures in the range 75−100 °C (±0.1 °C) for times from 0 to 15 min in a thermostatically controlled water bath. The heat-up time was estimated by inserting a thermocouple in selected sample tubes and monitoring the temperature change during heating. The estimated heat-up time was subtracted from the total heating times. After heat treatment, the milk samples were rapidly cooled in an ice bath for 5 min.

Dilution of Milk Samples. The concentrated milk samples were accurately diluted, by weighing, with water to a concentration comparable with that of the 9.6% TS milk samples. Several small glass beads were added to each sample to aid redispersion. The samples were vigorously shaken to ensure homogeneous redispersion of the milk and allowed to stand for 24 h before analysis. Milk samples at high concentration aggregated during prolonged heat treatment at high temper-

† Fax +64 6 356 1476; e-mail skelte.anema@nzdri.org.nz.

10.1021/jf991173e CCC: $19.00
Published on Web 08/22/2000

atures; however, the redispersion method allowed a representative sample of the milk slurry to be collected.

Polyacrylamide Gel Electrophoresis. The level of native β-LG in the control and heat-treated milk samples was determined using native polyacrylamide gel electrophoresis (native PAGE), as has been described previously (Anema and McKenna, 1996). The level of β-LG not involved in disulfide-linked aggregations was determined by a nonreduced sodium dodecyl sulfate PAGE (NR-SDS−PAGE) technique that was similar to that described by Anema and Klostermeyer (1997) but with no reducing agent added to the samples. For native PAGE, the casein and denatured whey proteins were removed from the milk by adjusting the pH to 4.6 and centrifuging out the precipitate using a bench centrifuge. The resultant supernatant was used for analysis by native PAGE. For NR-SDS−PAGE, the milk samples were used directly.

The supernatant and milk samples were accurately diluted, by weight, with sample buffer. After electrophoresis, the gels were stained using 0.1% (w/v) amido black 10B in 10% acetic acid and 25% 2-propanol. After 3 h of staining, the gels were destained using a 10% acetic acid solution until a clear background was achieved. The gels were scanned using a Molecular Dynamics model P.D. computing densitometer (Molecular Dynamics Inc., Sunnyvale, CA), and the integrated intensities of the β-LG bands were determined using the Molecular Dynamics Imagequant integration software. No attempt was made to separate the two variants of β-LG, as these behaved similarly under the reaction conditions. The changes in β-LG as a consequence of heat treatment were determined by comparing the residual β-LG band intensities of the heated milk samples with the β-LG band intensity of the average of two unheated samples, with corrections for differences induced from the various dilution steps in the sample preparations.

The concentration of β-LG in the milk samples was determined by comparing the band intensity of β-LG in the milk samples with standard curves prepared from purified β-LG solutions of known concentrations.

RESULTS AND DISCUSSION

Irreversible Denaturation of β-LG. Milk samples of 9.6−38.4% TS were heated at temperatures from 75 to 100 °C for times up to ∼15 min before analysis by native PAGE. Typical native PAGE patterns are shown in Figure 1A,B. For all four milk concentrations, the levels of native β-LG remaining after the various heat treatments are shown in Figure 2. Equations 1 and 2

$$(C_t/C_0)^{1-n} = 1 + (n-1)k_f C_0^{n-1} t \quad (\text{when } n \neq 1) \qquad (1)$$

$$\ln(C_t/C_0) = -k_f t \qquad (\text{when } n = 1) \qquad (2)$$

(where n = reaction order; k_f = rate constant; C_0 = initial native protein concentration; and C_t = concentration of native protein at time t) were used to analyze the results at each temperature and thereby obtain the overall order, n, for the thermal denaturation reaction for β-LG at each milk solids concentration.

The denaturation of β-LG was best described as a reaction order of 1.5 at all milk solids concentrations and at all temperatures (Figure 3). The determined order of 1.5 for the thermal denaturation of β-LG in milk is in agreement with that reported by Dannenberg and Kessler (1988a−c), Kessler and Beyer (1991), and Anema and McKenna (1996). Oldfield et al. (1998a), using nonlinear regression analysis, reported reaction orders of 1.0−1.6 for β-LG denaturation in heated skim milk.

The relationship between the observed rate constants and the temperature of the reaction was analyzed using the Arrhenius equation (Dannenberg and Kessler, 1988a; Anema and McKenna, 1996). The logarithms of the rate constants, obtained from the straight lines in Figure 3, were plotted against the reciprocal of absolute temperature [Figure 4; note that the observed rate constant ($k_f C_0^{0.5}$) is obtained directly from the experimental results, whereas the true rate constant (k_f) is corrected for the initial β-LG concentration]. The relationship between the rate constants and $1/T$ was linear within certain temperature ranges with a marked change in temperature dependence at ∼90 °C. This observation is in agreement with earlier reports (Manji and Kakuda, 1986; Dannenberg and Kessler, 1988a−c; Kessler and Beyer, 1991; Anema and McKenna, 1996).

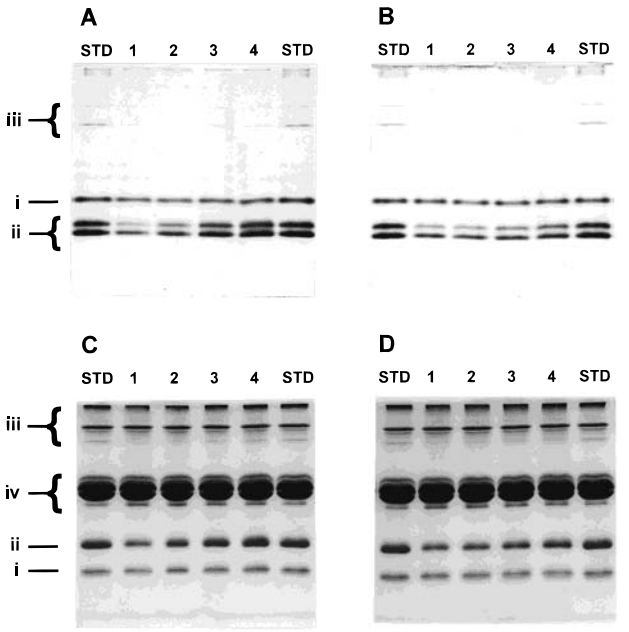

Figure 1. (A) Native PAGE patterns for milk samples heated at 80 °C for 435 s; (B) native PAGE patterns for milk samples heated at 95 °C for 45 s; (C) NR-SDS−PAGE patterns for milk samples heated at 80 °C for 435 s; (D) NR-SDS−PAGE patterns for milk samples heated at 95 °C for 45 s: STD, unheated milk; lane 1, 9.6% TS milk; lane 2, 19.2% TS milk; lane 3, 28.8% TS milk; lane 4, 38.4% TS milk; i, α-lactalbumin; ii, β-lactoglobulin; iii, high molecular weight whey proteins; iv, casein proteins. Milk samples were diluted to account for concentration differences.

The linearity within the two temperature ranges allowed the activation energies (E_a), the enthalpies of activation ($\Delta H^{\#}$), the entropies of activation ($\Delta S^{\#}$), and the free energies of activation ($\Delta G^{\#}$) to be calculated using the appropriate equations (Dannenberg and Kessler, 1988a; Anema and McKenna, 1996). These are presented in Table 1. For the 9.6% TS milk, the E_a, $\Delta H^{\#}$, $\Delta S^{\#}$, and $\Delta G^{\#}$ for β-LG denaturation were comparable with those reported previously for fresh milk samples (Hillier and Lyster, 1979; Manji and Kakuda, 1986; Dannenberg and Kessler, 1988c; Oldfield et al., 1998a). Anema and McKenna (1996) have shown that the kinetic and thermodynamic parameters for the thermal denaturation of β-LG in reconstituted milk were similar to those in fresh milk. This indicates that modifications to β-LG induced through the drying and/or reconstitution processes either are negligible or do not have a major effect on the denaturation reaction on subsequent heating of the reconstituted milk.

The results in Figures 2−4 and Table 1 demonstrate the effect of milk solids concentration on the kinetic and thermodynamic properties of β-LG denaturation. It was

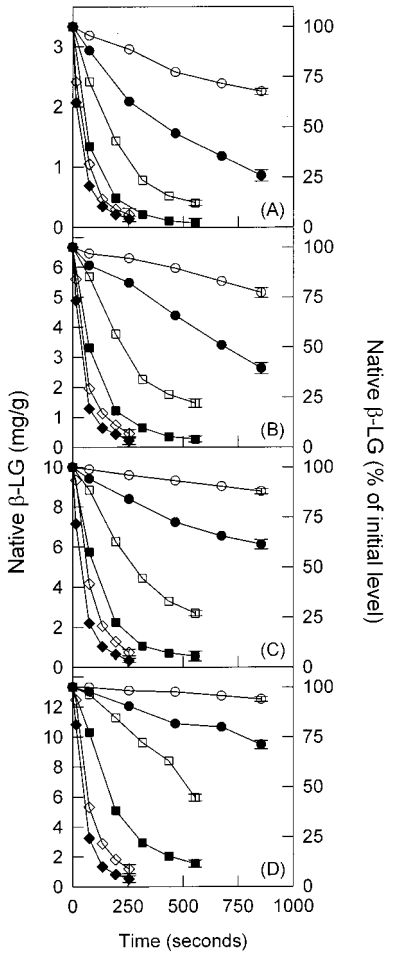

Figure 2. Temperature dependence of the thermal denaturation of β-LG (native PAGE results): (A) 9.6% TS milk; (B) 19.2% TS milk; (C) 28.8% TS milk; (D) 38.4% TS milk; ○, 75 °C; ●, 80 °C; □, 85 °C; ■, 90 °C; ◇, 95 °C; ◆, 100 °C. Error bars, included on the last points, represent the maximum standard deviation observed between duplicate measurements within the series.

evident that the rate of denaturation of β-LG was retarded at higher milk solids concentrations; however, this retardation became less pronounced as the temperature of the reaction was increased so that the level of denaturation was similar for all four milk solids concentrations at 100 °C. The results in Table 1 show that the E_a, $\Delta H^{\#}$, $\Delta G^{\#}$, and $\Delta S^{\#}$ values increased with increasing milk solids concentration in both temperature ranges.

Disulfide Aggregation of β-LG. Each milk sample was also analyzed by NR-SDS−PAGE. This system contains a dissociating agent (SDS) but no reducing agent and allows for the measurement of the loss of β-LG due to disulfide interactions (or other covalent interactions) as native β-LG and β-LG associated by noncovalent interactions will be measured by NR-SDS−PAGE.

Typical NR-SDS−PAGE patterns are shown in Figure 1C,D. The level of unreacted β-LG remaining after each temperature and time combination is shown in Figure 5. The overall order, n, for the disulfide aggregation of β-LG was found to be 1.5 at all milk solids concentrations and at all temperatures (Figure 6). Oldfield et al. (1998a), using nonlinear regression analysis, reported a reaction order of 1.6−1.7 for the disulfide aggregation of β-LG; however, direct comparison is complicated as

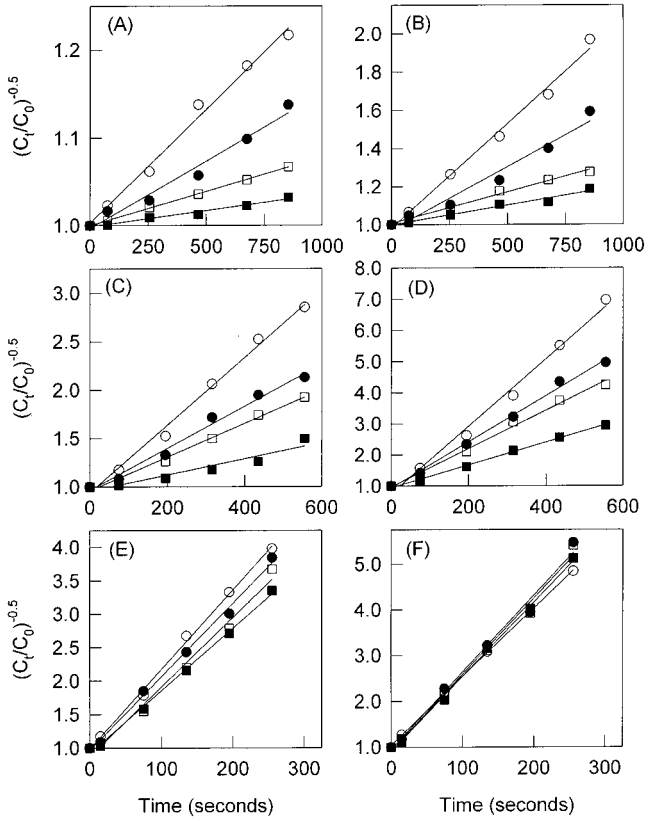

Figure 3. Denaturation of β-LG as a reaction order of 1.5: (A) 75 °C; (B) 80 °C; (C) 85 °C; (D) 90 °C; (E) 95 °C; (F) 100 °C; ○, 9.6% TS; ●, 19.2% TS; □, 28.8% TS; ■, 38.4% TS.

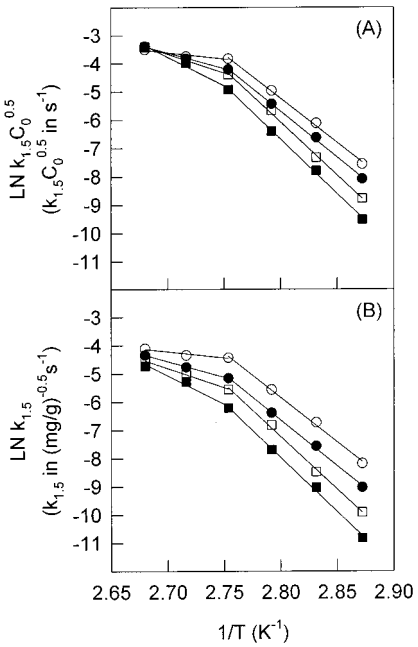

Figure 4. Arrhenius plot for β-LG denaturation: (A) raw data; (B) data corrected for β-LG concentration; ○, 9.6% TS; ●, 19.2% TS; □, 28.8% TS; ■, 38.4% TS.

the latter study analyzed only the β-LG remaining in the ultracentrifugal supernatants and this may not be representative of the total disulfide-aggregated β-LG in the heated milk.

Using the Arrhenius relationship (Dannenberg and Kessler, 1988a; Anema and McKenna, 1996), the logarithms of the rate constants (both k_f and $k_f C_0^{0.5}$) obtained from the straight lines in Figure 6 were plotted against

Table 1. Kinetic and Thermodynamic Parameters for the Thermal Denaturation of β-LG (Standard Error for E_a and ln k_0 Given in Parentheses)

milk concn (% TS)	temp range (°C)	E_a (kJ/mol)	ln k_0	av $\Delta H^{\#}$ (kJ/mol)	av $\Delta S^{\#}$ [kJ/(mol·K)]	av $\Delta G^{\#}$ (kJ/mol)
9.6	75−90	259.8 (8.8)	82.3 (2.9)	256.86	0.43	106.70
	90−100	36.3 (9.7)	8.2 (3.2)	33.27	−0.19	103.67
19.2	75−90	269.5 (7.2)	85.1 (2.4)	266.55	0.58	109.18
	90−100	92.4 (3.4)	26.4 (1.1)	89.31	−0.17	105.07
28.8	75−90	310.5 (9.8)	98.5 (3.3)	307.57	0.63	111.34
	90−100	112.4 (1.9)	32.8 (0.6)	109.34	0.03	105.95
38.4	75−90	319.1 (8.1)	100.8 (2.7)	316.15	0.64	113.67
	90−100	128.3 (3.1)	38.0 (3.9)	125.27	0.14	107.05

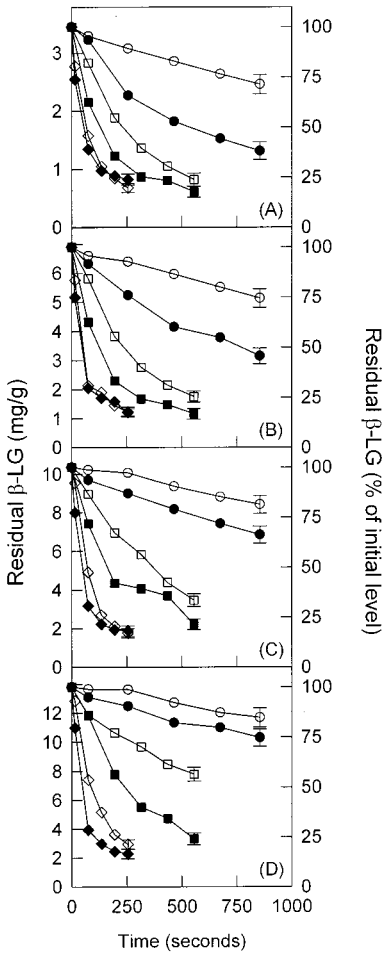

Figure 5. Temperature dependence of the disulfide aggregation of β-LG (NR-SDS−PAGE results): (A) 9.6% TS milk; (B) 19.2% TS milk; (C) 28.8% TS milk; (D) 38.4% TS milk; ○, 75 °C; ●, 80 °C; □, 85 °C; ■, 90 °C; ◇, 95 °C; ◆, 100 °C. Error bars, included on the last points, represent the maximum standard deviation observed between duplicate measurements within the series.

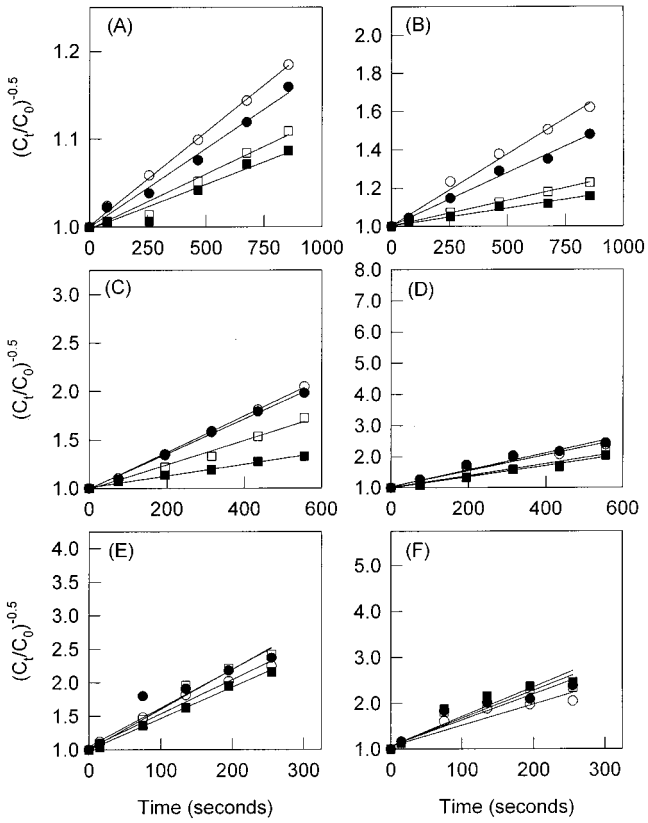

Figure 6. Disulfide aggregation of β-LG as a reaction order of 1.5: (A) 75 °C; (B) 80 °C; (C) 85 °C; (D) 90 °C; (E) 95 °C; (F) 100 °C; ○, 9.6% TS; ●, 19.2% TS; □, 28.8% TS; ■, 38.4% TS.

the reciprocal of absolute temperature (Figure 7). As with the native PAGE system, the relationship between the rate constants and 1/T was linear within certain temperature ranges with a marked change in temperature dependence at ∼90 °C. The E_a, $\Delta H^{\#}$, $\Delta G^{\#}$, and $\Delta S^{\#}$, calculated using the appropriate equations (Dannenberg and Kessler, 1988a; Anema and McKenna, 1996), are given in Table 2.

As with β-LG denaturation, the disulfide aggregation of β-LG was retarded at higher milk solids concentrations (Figures 5−7 and 9B), and this retardation became less pronounced as the temperature of the reaction was increased. Although the rate of reaction was influenced by the milk solids concentration, the observation that the reaction order was 1.5 suggests that the overall mechanisms for both the thermal denaturation and the disulfide aggregation of β-LG were likely to be the same at all milk solids concentrations. Unlike the denaturation reaction, the E_a, $\Delta H^{\#}$, $\Delta G^{\#}$, and $\Delta S^{\#}$ for the disulfide aggregation of β-LG showed no particular trends with increasing milk solids concentration.

Comparison between Denatured and Disulfide-Aggregated β-LG. Figure 8A shows that the level of denatured β-LG (native PAGE results) was strongly correlated ($r = 0.99$; $p \leq 0.001$) with the level of β-LG aggregated by disulfide bonding (NR-SDS−PAGE results). However, the levels of denatured β-LG were, on average, ∼15% higher than the levels of disulfide-aggregated β-LG. This indicates that not all of the denatured β-LG was aggregating via disulfide interactions and that a small amount must be aggregated via noncovalent (e.g., hydrophobic) interactions. The rate constants for β-LG denaturation were strongly correlated with the rate constants for the disulfide aggregation of β-LG ($r = 0.97$; $p \leq 0.001$); however, the rate

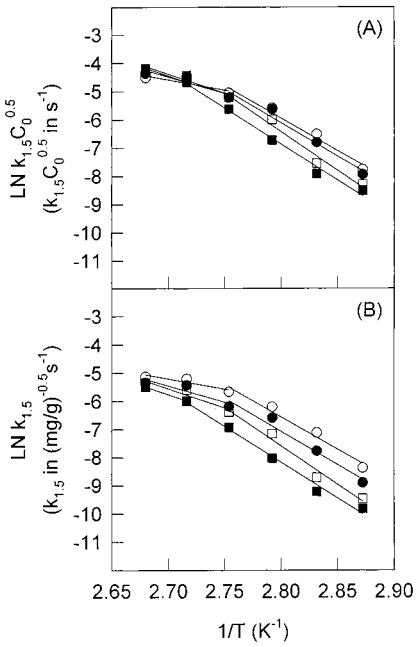

Figure 7. Arrhenius plot for the disulfide aggregation of β-LG: (A) raw data; (B) data corrected for β-LG concentration; ○, 9.6% TS; ●, 19.2% TS; □, 28.8% TS; ■, 38.4% TS.

constants for the denaturation reaction were higher than those for the disulfide aggregation reactions (Figure 8B).

Oldfield et al. (1998a) reported a close correlation between denatured and disulfide-aggregated β-LG in the supernatant whey protein of heated milk, although a close examination of their results indicated that the level of denatured β-LG was ~15% higher than the level of disulfide-aggregated β-LG, which is similar to that reported in this study. For purified β-LG in buffer solutions, the rate for the disulfide aggregation of β-LG was considerably slower than the rate of denaturation at all pH values and temperatures investigated (McSwiney et al., 1994a,b; Manderson et al., 1998). Similarly, Havea et al. (1998) reported that the rate of disulfide aggregation was markedly slower that the rate of denaturation in heated whey protein concentrate solutions at all protein concentrations investigated. A comparison of results indicates that, in the absence of casein, a greater proportion of the denatured β-LG is aggregated via noncovalent (e.g., hydrophobic) interactions than in the milk system where casein proteins are present. Presumably a substantial proportion of the denatured β-LG in milk is aggregated via thiol−disulfide exchange reactions with κ-casein and α_{S2}-casein. Several studies have shown that the denatured β-LG in heated milk will undergo thiol−disulfide exchange with other denatured whey proteins and with the casein micelles (Weichen and Knoop, 1974; Oldfield et al., 1998a,b).

Comparison of Denaturation and Disulfide Aggregation in Four Different Milk Samples. The experiments were repeated on four separate milk samples prepared from four different milk powder sources. Selected results (80 °C for 435 s and 95 °C for 45 s) are shown in Figure 9A (native PAGE) and Figure 9B (NR-SDS−PAGE). All four milk samples behaved similarly, although there was some variation in the denaturation and disulfide aggregation of β-LG between samples, which is probably related to compositional/pH differences in the milk sources. At 80 °C, a strong retardation of β-LG denaturation and disulfide aggregation was observed with increasing milk solids concentration. At 95 °C, increasing milk solids concentration was less effective in stabilizing β-LG to denaturation and disulfide aggregation.

No similar study of the effect of milk concentration on β-LG denaturation has been previously reported. However, Hillier et al. (1979) showed that the rate of β-LG denaturation in cheese whey, at a constant pH of 6.0, was retarded at higher whey concentrations and that this retardation was less pronounced at higher temperatures. More recently, Plock and Kessler (1993) and Plock et al. (1998a,b) reported that β-LG denaturation in sweet whey was retarded by increasing the total solids concentration, whereas α-lactalbumin denaturation was unaffected. This retardation was not due to increases in the viscosity of the concentrated systems and therefore could not be explained by changes in the rate of diffusion-controlled reactions (Plock et al., 1998a). Plock et al. (1998b) demonstrated that the reduced rate of β-LG denaturation was due to the increase in the lactose level as the whey was concentrated and proposed that the stabilization may be due to the direct interaction between lactose and β-LG. It was suggested that protein-bound lactose groups increase the active radius of the β-LG molecules and thereby reduce the rate of thiol−disulfide exchange reactions, which are integral in the irreversible denaturation mechanism of β-LG.

Recent studies have demonstrated that β-LG is susceptible to glycosylation by lactose (Burr et al., 1996; Nacka et al., 1998). However, α-lactalbumin is reported to be more susceptible than β-LG to glycosylation by lactose (Nacka et al., 1998). If glycosylation was responsible for the retardation in β-LG denaturation, then a similar retardation in α-lactalbumin denaturation would also be expected, but this is not observed (Plock et al., 1998a,b; Anema, unpublished results). As the glycosylation of β-LG would be irreversible, the denaturation rate of β-LG in dilute milk prepared from heated milk concentrate should be retarded when compared with that in dilute milk prepared from unheated concentrate; this aspect has not been studied.

There have been numerous studies on the thermal denaturation of pure β-LG in buffer systems including studies on the effect of pH, protein concentration, ionic strength, and the relative importance of covalent/noncovalent interactions (Cairoli et al., 1994; Roefs and DeKruif, 1994; Iametti et al., 1996; Hoffmann and van Mil, 1997, 1999; Verheul et al., 1998). Initiation of the irreversible denaturation reaction of β-LG consists of a number of steps in which the monomer to dimer dissociation is the first essential process. This dissociation occurs at temperatures much lower than those at which modifications to the tertiary structure are observed (Cairoli et al., 1994; Iametti et al., 1996). This is followed by the rearrangement of the native β-LG conformation to a state in which the free thiol is exposed and becomes reactive. Roefs and De Kruif (1996) have provided an elegant model, based on free radical polymerization reactions, to quantitatively describe the denaturation and aggregation of pure β-LG in buffer solutions. In this model, the reactive thiol group of unfolded β-LG acts as the free radical and the thiol−disulfide exchange reaction propagates the aggregation reactions of unfolded β-LG. At low ionic strength, at near neutral pH, and over a moderate temperature range (60−70 °C), this model correctly predicts the

Table 2. Kinetic and Thermodynamic Parameters for the Disulfide Aggregation of β-LG (Standard Error for E_a and ln k_0 Given in Parentheses)

milk concn (% TS)	temp range (°C)	E_a (kJ/mol)	ln k_0	av $\Delta H^{\#}$ (kJ/mol)	av $\Delta S^{\#}$ [kJ/(mol·K)]	av $\Delta G^{\#}$ (kJ/mol)
9.6	75−90	191.1 (22.2)	58.4 (7.5)	188.20	0.23	106.37
	90−100	60.6 (24.8)	15.1 (8.1)	57.54	−0.13	105.23
19.2	75−90	196.7 (25.5)	60.1 (8.6)	236.82	0.36	109.73
	90−100	100.9 (22.0)	26.8 (13.8)	97.83	−0.03	108.10
28.8	75−90	227.5 (23.4)	70.2 (7.9)	224.51	0.33	108.31
	90−100	112.7 (35.5)	32.2 (11.6)	109.63	0.01	104.92
38.4	75−90	243.1 (10.9)	74.6 (3.7)	209.04	0.27	113.45
	90−100	162.7 (28.2)	48.4 (9.2)	109.90	0.002	109.56

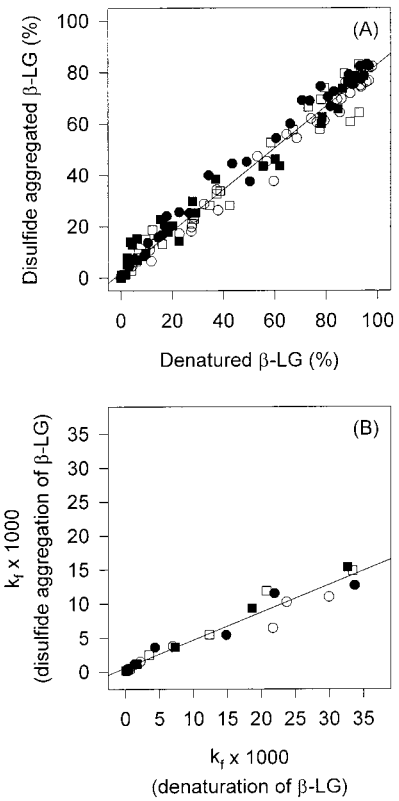

Figure 8. (A) Comparison between the levels of denatured and disulfide-aggregated β-LG in heated milk; (B) comparison between the rate constants for the denaturation and disulfide aggregation of β-LG: ○, 9.6% TS; ●, 19.2% TS; □, 28.8% TS; ■, 38.4% TS.

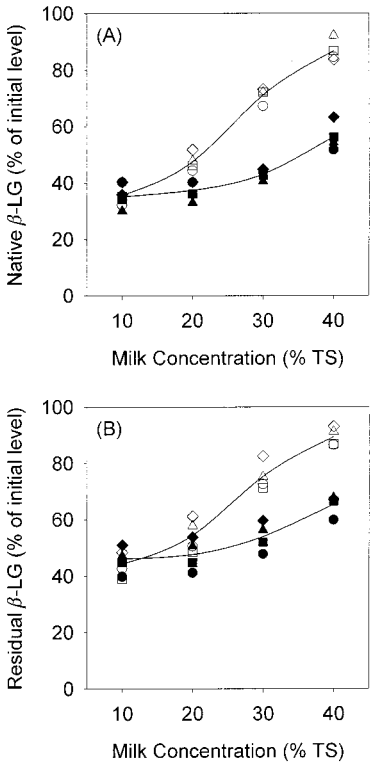

Figure 9. (A) Comparison of the denaturation of β-LG for four reconstituted skim milk samples; (B) comparison of the disulfide aggregation of β-LG for four reconstituted skim milk samples: ○, ●, milk 1; □, ■, milk 2; ◇, ◆, milk 3; △, ▲, milk 4; open symbols, samples heated at 80 °C for 435 s; solid symbols, samples heated at 95 °C for 45 s.

fractional reaction order for β-LG denaturation and the size of the aggregated protein polymer particles.

However, at higher ionic strength (such as those found in milk), lower pH, or higher temperature, the free radical model is no longer applicable. A simplified reaction scheme consisting of a first-order denaturation step and a second-order aggregation step can be used to model the denaturation of β-LG under these conditions (Verhuel et al., 1998). This model explains the fractional order of the irreversible denaturation (or aggregation) of β-LG as the apparent order would be between 1 and 2 depending on the ratio of the reaction rates of the two steps. In these pure protein solutions, the reaction order was found to be between 1 and 1.7 under all conditions, with the order increasing with pH and decreasing with ionic strength. This suggests that the aggregation step becomes rate limiting at higher pH and the unfolding step becomes rate limiting at higher ionic strength (Verhuel et al., 1998).

In pure β-LG solutions and over moderate concentration ranges, the rate for the irreversible denaturation of β-LG is not strongly influenced by the initial protein concentration. At very high initial β-LG concentrations, the rate is observed to increase due to the interaction between protein intermediate species and aggregated species (Roefs and De Kruif, 1994; Hoffmann et al., 1997). Iametti et al. (1995) reported that the irreversible changes to the structure of β-LG are not dependent on the protein concentration and the stabilization of aggregates by intermolecular disulfide bonds was only concentration dependent at temperatures <75 °C. The reaction rate for the irreversible denaturation of β-LG was found to increase with increasing pH. This is probably related to the increase in the concentration of thiolate ions at higher pH, which would increase the rate of thiol−disulfide exchange reactions and therefore the overall irreversible denaturation of β-LG (Verhuel et al., 1998; Hoffmann and van Mil, 1999). Increasing the ionic strength with NaCl above 0.1 M stabilizes the native conformation of β-LG, reduces the rate of protein unfolding, and therefore decreases the overall rate for irreversible denaturation (Verhuel et al., 1998). It is

possible that the stabilizing effect of increased milk concentration may be related to the combined stabilizing effects of increased ionic strength and reduced pH as the milk is concentrated. However, increasing ionic strength and decreasing pH would be expected to exert a similar stabilizing effect on α-lactalbumin denaturation, but this is not observed (Plock et al., 1998a,b; Anema, unpublished results).

There are alternative reasons why increased milk solids concentration/lactose concentration could stabilize β-LG to thermal denaturation. Several studies have shown that the thermal stability of proteins is increased by the addition of sugars and polyhydric alcohols (Back et al., 1979; Arakawa and Timasheff, 1982; Kristjansson and Kinsella, 1991). Arakawa and Timasheff (1982) suggested that the stabilizing effect of sugars was due to the preferential hydration of the protein in the presence of the sugars. It is proposed that there is an ordering of water structure around the protein molecules which effectively excludes the sugar from the protein environment. This results in unfavorable increases in the free energy of the system. As these effects increase with increases in the surface area of the proteins, unfolded proteins will have more unfavorable protein−water interactions than native proteins. Therefore, the native structure of the protein is stabilized. However, as with the glycosylation theory of Plock et al. (1998b), if the stabilizing effect of increased milk solids concentration on β-LG denaturation was due to the effect of lactose on water structure and hydrophobic interactions, then some stabilizing effect in the thermal denaturation of α-lactalbumin may also be expected, but this is not observed (Plock et al., 1998a,b; Anema, unpublished results).

An important consequence of the preferential hydration theory of Arakawa and Timasheff (1982) is that, for proteins that can undergo self-association, the presence of sugars favors the associated state as the formation of contacts between the protein molecules decreases the total surface area and therefore the free energy of the system. β-LG is known to undergo self-association under certain conditions of pH, concentration, and temperature (McKenzie, 1971). It has been suggested that the first step in the denaturation of β-LG involves the separation of dimeric β-LG to the monomeric form as the temperature is raised (McKenzie, 1971; Iametti and Bonomi, 1996). If the denaturation/unfolding reaction is rate determining in the low-temperature range (<90 °C), then the stabilizing effect of increased milk solids/lactose concentration could be due to a shift in the equilibrium between the monomer and dimer to a state that favors the dimer at the elevated temperatures. As this self-association effectively reduces the concentration of the reactive species (β-LG monomer), the rate of the reaction is reduced and no change in reaction mechanism would be necessary. At higher temperatures (>90 °C), aggregation reactions involving the unfolded β-LG are proposed to be rate determining (Dannenberg and Kessler, 1988a−c; Anema and McKenna, 1996). Therefore, the dimerization of β-LG, as induced by an increase in milk solids concentration, may be less effective in stabilizing the denaturation at these higher temperatures.

This explanation is consistent with the observation that α-lactalbumin denaturation is unaffected by milk solids or lactose concentration (Plock et al., 1998a,b; Anema, unpublished results). As α-lactalbumin exists as a monomeric species, no retardation in denaturation through molecular associations can occur.

The results of this study on the effect of milk solids concentration have shown that β-LG denaturation is markedly retarded at higher milk solids concentrations; however, the effect becomes less pronounced at higher temperatures. The stabilization is probably a consequence of the increased lactose concentration in the milk; however, the exact mechanism by which lactose stabilizes β-LG to denaturation remains unclear. Further studies on β-LG denaturation in milk concentrates or model systems, particularly on thiol reactivity, glycosylation level, and monomer/dimer interactions, are required to elucidate the mechanism for the stabilization of β-LG denaturation at higher milk solids concentrations.

ACKNOWLEDGMENT

I thank Friederike Lugt and Michael Li for technical assistance, Lawrie Creamer, Jeremy Hill, and Wayne Thresher for useful comments and discussions, and Claire Woodhall for proofreading the manuscript.

LITERATURE CITED

Anema, S. G.; Klostermeyer, H. Heat-induced, pH-dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100 °C. *J. Agric. Food Chem.* **1997**, *45*, 1108−1115.

Anema, S. G.; McKenna, A. B. Reaction kinetics of thermal denaturation of whey proteins in heated reconstituted whole milk. *J. Agric. Food Chem.* **1996**, *44*, 422−428.

Arakawa, T.; Timasheff, S. N. Stabilization of protein structure by sugars. *Biochemistry* **1982**, *21*, 6536−6544.

Back, J. F.; Oakenfull, D.; Smith, M. B. Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry* **1979**, *18*, 5191−5196.

Burr, R.; Moore, C. H.; Hill, J. P. Evidence of multiple glycosylation of bovine β-lactoglobulin by electrospray ionisation mass spectrometry. *Milchwissenschaft* **1996**, *51*, 488−492.

Cairoli, S.; Iametti, S.; Bonomi, F. Reversible and irreversible modifications of β-lactoglobulin upon exposure to heat. *J. Protein Chem.* **1994**, *13*, 347−354.

Dannenberg, F.; Kessler, H.-G. Reaction kinetics of the denaturation of whey proteins in milk. *J. Food Sci.* **1988a**, *53*, 258−263.

Dannenberg, F.; Kessler, H.-G. Application of reaction kinetics to the denaturation of whey proteins in heated milk. *Milchwissenschaft* **1988b**, *43*, 3−7.

Dannenberg, F.; Kessler, H.-G. Thermodynamic approach to kinetics of β-lactoglobulin denaturation in heated skim milk and sweet whey. *Milchwissenschaft* **1988c**, *43*, 139−142.

Gough, P.; Jenness, R. Heat denaturation of β-lactoglobulins A and B. *J. Dairy Sci.* **1962**, *45*, 1033−1039.

Guy, E. J.; Vettel, H. E.; Pallansch, M. J. Denaturation of cottage cheese whey proteins by heat. *J. Dairy Sci.* **1967**, *50*, 828−832.

Harland, H. A.; Ashworth, U. S. A rapid method for estimation of whey proteins as an indication of baking quality of nonfat dry-milk solids. *Food Res.* **1947**, *12*, 247−251.

Harland, H. A.; Coulter, S. T.; Jenness, R. The effect of the various steps in the manufacture on the extent of serum protein denaturation in nonfat dry milk solids. *J. Dairy Sci.* **1952**, *35*, 363−368.

Havea, P.; Singh, H.; Creamer, L. K.; Campanella, O H. Electrophoretic characterization of the protein products formed during heat treatment of whey protein concentrate solutions. *J. Dairy Res.* **1998**, *65*, 79−91.

Hillier, R. M. The quantitative measurement of whey proteins using polyacrylamide-gel electrophoresis. *J. Dairy Res.* **1976**, *43*, 259−265.

Hillier, R. M.; Lyster, R. L. J. Whey protein denaturation in heated milk and cheese whey. *J. Dairy Res.* **1979**, *46*, 95−102.

Hillier, R. M.; Lyster, R. L. J.; Cheeseman, G. C. Thermal denaturation of α-lactalbumin and β-lactoglobulin in cheese whey: effect of total solids concentration and pH. *J. Dairy Res.* **1979**, *46*, 103−111.

Hoffmann, M. A. M.; van Mil, P. J. J. M. Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulfide bonds. *J. Agric. Food Chem.* **1997**, *45*, 2942−2948.

Hoffmann, M. A. M.; van Mil, P. J. J. M. Heat-induced aggregation of β-lactoglobulin as a function of pH. *J. Agric. Food Chem.* **1999**, *47*, 1898−1905.

Iametti, S.; Bonomi, F. Transient modification of the association equilibria in heated β-lactoglobulin. In *Heat Treatments and Alternative Methods*; IDF Special Issue 9602; International Dairy Federation: Brussels, Belgium, 1996a; pp 341−349.

Iametti, S.; De Gregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin. *Eur. J. Biochem.* **1996**, *237*, 106−112.

Kessler, H. G.; Beyer, H. J. Thermal denaturation of whey proteins and its effect in dairy technology. *Int. J. Biol. Macromol.* **1991**, *13*, 165−173.

Kristjansson, M. M.; Kinsella, J. E. Protein and enzyme stability: structural, thermodynamic, and experimental aspects. *Adv. Food Nutr. Res.* **1991**, *35*, 237−316.

Lyster, R. L. J. The denaturation of α-lactalbumin and β-lactoglobulin in heated milk. *J. Dairy Res.* **1970**, *37*, 233−243.

Manderson, G. A.; Hardman, M. J.; Creamer, L. K. Effect of heat treatment on the conformation and aggregation of β-lactoglobulin A, B, and C. *J. Agric. Food Chem.* **1998**, *46*, 5052−5061.

Manji, B.; Kakuda, Y. Thermal denaturation of whey proteins in skim milk. *Can. Inst. Food Sci. Technol. J.* **1986**, *19*, 163−166.

McKenna, B. M.; O'Sullivan, A. C. Whey protein denaturation in concentrated skimmilks. *J. Dairy Sci.* **1971**, *54*, 1075−1077.

McKenzie, H. A. β-Lactoglobulins. In *Milk Proteins. Chemistry and Molecular Biology*; McKenzie, H. A., Ed.; Academic Press: New York, 1971; Vol. II, pp 257−330.

McSwiney, M.; Singh, H.; Campanella, O. H. Thermal aggregation and gelation of bovine β-lactoglobulin. *Food Hydrocolloids* **1994a**, *8*, 441−453.

McSwiney, M.; Singh, H.; Campanella, O. H.; Creamer, L. K. Thermal gelation and denaturation of bovine β-lactoglobulins. *J. Dairy Res.* **1994b**, *61*, 221−232.

Nacka, F.; Chobert, J.-M.; Burova, T.; Leonil, J.; Haertle, T. Induction of new physicochemical and functional properties by the glycosylation of whey proteins. *J. Protein Chem.* **1998**, *17*, 495−503.

Oldfield, D. J.; Singh, H.; Taylor, M. W.; Pearce, K. N. Kinetics of denaturation and aggregation of whey proteins in skim milk heated in an ultrahigh temperature (UHT) pilot plant. *Int. Dairy J.* **1998a**, *8*, 311−318.

Oldfield, D. J.; Singh, H.; Taylor, M. W. Association of β-lactoglobulin and α-lactalbumin with the casein micelles in skim milk heated in an ultrahigh temperature plant. *Int. Dairy J.* **1998b**, *8*, 765−770.

Plock, J.; Kessler, H. G. Valuation of commercial whey proteins by HPLC and gelation in relation to the denaturation inhibiting effect of lactose. In *Protein and Fat Globule Modifications by Heat Treatment, Homogenisation and Other Technological Means for High Quality Dairy Products*; IDF Special Issue 9303; International Dairy Federation: Brussels, Belgium, 1993; pp 138−146.

Plock, J.; Spiegel, T.; Kessler, H. G. Influence of the dry matter on the denaturation kinetics of whey proteins in concentrated sweet whey. *Milchwissenschaft* **1998a**, *53*, 327−331.

Plock, J.; Spiegel, T.; Kessler, H. G. Influence of the lactose concentration on the denaturation kinetics of whey proteins in concentrated sweet whey. *Milchwissenschaft* **1998b**, *53*, 389−393.

Roefs, S. P. F. M.; De Kruif, K. G. A model for the denaturation and aggregation of β-lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883−889.

Verheul, M.; Roefs, S. P. F. M.; De Kruif, K. G. Kinetics of heat-induced aggregation of β-lactoglobulin. *J. Agric. Food Chem.* **1998**, *46*, 896−903.

Wiechen, A.; Knoop A. M. Studies on the reaction of β-lactoglobulin and α-lactalbumin during heat treatment of milk by means of J 131-labeled whey proteins. *Milchwissenschaft* **1974**, *29*, 65−70.

Received for review October 28, 1999. Revised manuscript received March 22, 2000. Accepted April 26, 2000. This research was partially funded by the Foundation for Research, Science and Technology, Contract DRI801.

JF991173E