

Kinetics of the Irreversible Thermal Denaturation and Disulfide Aggregation of α -Lactalbumin in Milk Samples of Various Concentrations

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ABSTRACT: α -lactalbumin denaturation was first order and was virtually unaffected by milk solids concentration, with a similar denaturation rate at all the concentrations for each temperature investigated. Similarly, disulfide aggregation of α -lactalbumin was first order and unaffected by milk solids concentration. Only about 70% of the denatured α -lactalbumin was involved in disulfide-aggregated complexes with other denatured whey proteins or with the casein micelles. Calculated thermodynamic parameters (activation energies, enthalpies of activation, entropies of activation and free energies of activation) for irreversible denaturation and for disulfide aggregation were similar for all milk concentrations.

KEY WORDS: milk, concentrated milk, α -lactalbumin, thermal denaturation

Introduction

SKIM MILK PRODUCTS CAN RANGE IN CONCENTRATION FROM that observed naturally (about 9.6% total solids, TS), through concentrated products (about 20 to 25% TS) to dehydrated powders (about 96% TS). During manufacture these milk products may be subjected to temperatures at which the whey proteins can denature. Although there have been numerous studies on the effect of heat treatment on the denaturation of whey proteins in milk (Gough and Jenness 1962; Lyster 1970; Hillier 1976; Hillier and Lyster 1979; Manji and Kakuda 1986; Dannenberg and Kessler 1988; Kessler and Beyer 1991; Anema and McKenna 1996), few have been conducted on the effect of milk concentration on the denaturation process; and the results appear to be inconclusive or contradictory.

An early report by Harland and others (1952) indicated that increasing milk solids concentration had little effect on the total whey protein denaturation. Guy and others (1967) reported a minimum whey protein denaturation at a milk concentration of 20% total solids (TS). McKenna and O'Sullivan (1971) reported that the denaturation of the whey proteins as a percentage of that initially present was markedly reduced at higher initial milk solids concentrations when milk samples were heated at 75 or 80 °C.

In a recent study it was shown that the rate of denaturation of β -lactoglobulin was markedly dependent on the milk solids concentration and heating temperature (Anema 2000). At 75 °C, the thermal denaturation of β -lactoglobulin 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. About 85% of the denatured β -lactoglobulin was involved in disulfide-aggregated complexes, either with other denatured whey proteins or with the casein micelles. There have been no similar studies on the effect of milk concentration on the thermal denaturation and disulfide aggregation of α -lactalbumin.

This paper continues the investigation of the effect of milk solids concentration on whey protein denaturation. The thermal denaturation and the disulfide aggregation of α -lactalbumin in reconstituted skim milk was examined over wide temperature, time and milk concentration ranges. These conditions allowed accurate estimations of key kinetic and thermodynamic parameters to be made for both the denaturation and disulfide aggregation reactions.

Materials and Methods

Milk supply

Reconstituted skim milk samples of 9.6 to 38.4% total solids (TS, wt/wt) were prepared by adding the appropriate quantity of low-heat skim milk powder (New Zealand Dairy Board, Wellington, New Zealand) to demineralized water. A small quantity (about 0.04%) of sodium azide was added to each of the milk samples as a preservative. The milk samples were stirred for at least 2 h before further use.

Heat treatment

Subsamples of each milk (200 mg) were transferred to small sealable plastic tubes and heated for 0 to 15 min in a thermostatically controlled water bath preset to temperatures in the range from 75 to 100 °C (± 0.1 °C). The heat-up time, estimated by inserting a thermocouple in selected sample tubes and monitoring the temperature change during heating, was found to be about 45 sec and was subtracted from the total heating times. After heat treatment, the milk samples were rapidly cooled in an ice bath for 5 min. Selected samples were heated and analysed in duplicate in order to determine the variation induced by the heat treatments and analyses involved in this study.

Dilution of milk samples

The concentrated milk samples were accurately diluted with water to a concentration comparable with that of the

9.6% TS milk samples. 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 temperatures; however, the redispersion method allowed a representative sample of the milk slurry to be collected.

Determination of denatured and disulfide aggregated α -lactalbumin concentrations

The level of native α -lactalbumin in the unheated and heat-treated milk samples was determined using native polyacrylamide gel electrophoresis (native-PAGE), as has been described previously (Anema and McKenna 1996). The casein and the 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 diluted in sample buffer before analysis by native-PAGE.

The level of α -lactalbumin not involved in disulfide-linked aggregations was determined by a non-reduced 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. The milk samples were diluted directly with sample buffer before analysis by NR-SDS-PAGE.

After electrophoresis, the gels were stained using 0.1% (wt/vol) amido black 10B in a solution containing 10% acetic acid and 25% isopropanol. After staining for 3 h, the gels were destained using a solution containing 10% acetic acid until a clear background was achieved. The gels were scanned using a Molecular Dynamics model P.D. computing densitometer (Molecular Dynamics Inc., Sunnyvale, Calif., U.S.A.) and the integrated intensities of the α -lactalbumin bands were determined using the Molecular Dynamics Imagequant integration software. The residual α -lactalbumin band intensities of the heated milk samples were compared with the α -lactalbumin band intensity of the average of two unheated samples, with corrections for differences induced from the various dilution steps in the sample preparations.

The concentrations of α -lactalbumin in the milk samples were determined by comparing the band intensities of α -lactalbumin in the milk samples with standard curves prepared from purified α -lactalbumin solutions of known concentrations.

Kinetic and thermodynamic evaluations

The reaction orders, rate constants and thermodynamic parameters for the irreversible denaturation and disulfide aggregation of α -lactalbumin were determined using the general kinetic and thermodynamic equations, which have been described previously (Dannenberg and Kessler 1988; Anema and McKenna 1996).

Results

Irreversible thermal denaturation of α -lactalbumin

Heated and unheated milk samples were analyzed by native-PAGE to determine the residual α -lactalbumin concentration. For all four milk concentrations, the levels of native α -lactalbumin remaining after the various heat treatments are shown in Figure 1. As expected, for each milk concentration, the level of native α -lactalbumin decreased with increasing heating time at each temperature and the rate of loss increased as the heating temperature was increased. Using linear regression, the denaturation data were analysed

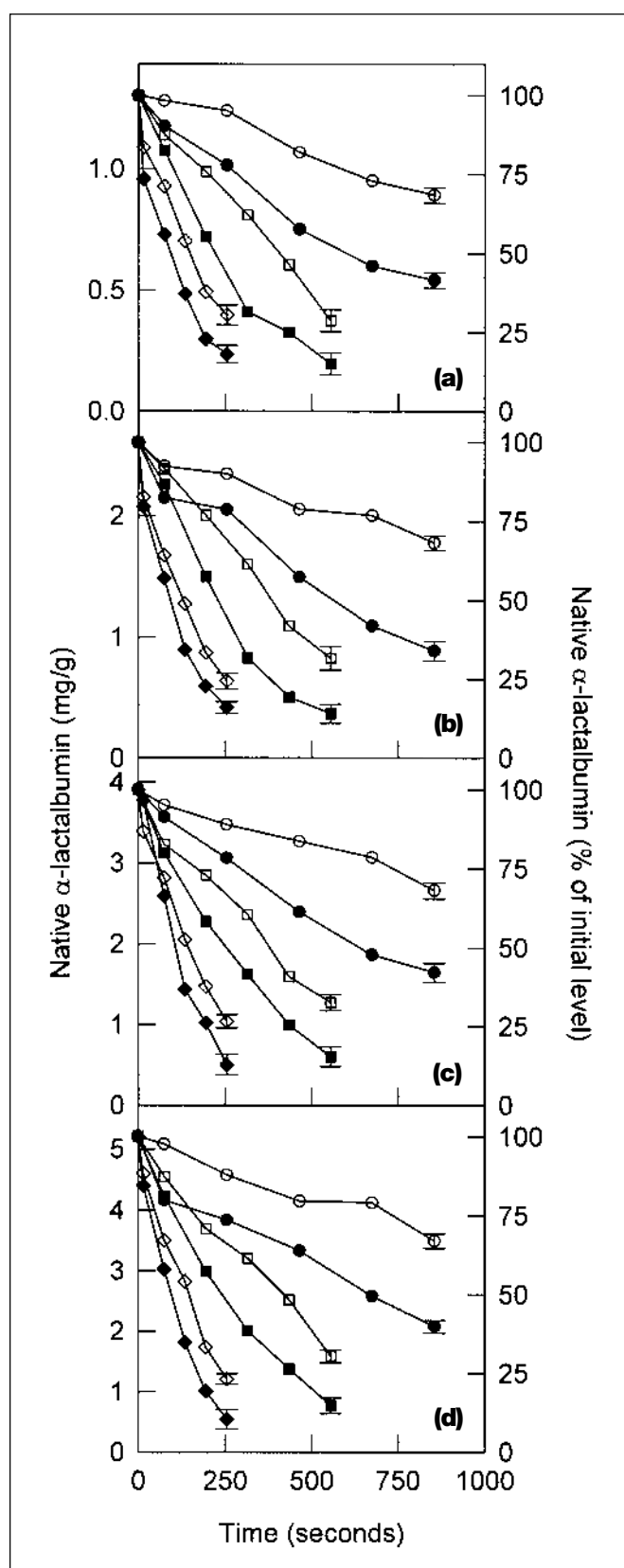


Figure 1—Temperature dependence of the thermal denaturation of α -lactalbumin (native-PAGE results). a: 9.6% TS milk; b: 19.2% TS milk; c: 28.8% TS milk; d: 38.4% TS milk. $\circ$: 75 °C; $\bullet$: 80 °C; $\square$: 85 °C; $\blacksquare$: 90 °C; $\diamond$: 95 °C; $\blacklozenge$: 100 °C. Error bars, included on the last points, represent the maximum standard deviation observed between duplicate measurements within the series.

using the standard integrated rate equations to determine the overall order and rate constants for the denaturation reaction, as has been described previously (Dannenberg and Kessler 1998; Anema and McKenna 1996). The denaturation of α -lactalbumin was best described as a first-order reaction at all temperatures and milk concentrations investigated (Figure 2).

According to Arrhenius, the logarithms of the rate constants obtained from the straight lines in Figure 2 were plotted against the reciprocal of absolute temperature (Figure 3). The relationship between the rate constants and $1/T$ was linear within certain temperature ranges with an apparent change in temperature dependence at about 80 °C. This is in agreement with previous reports (Dannenberg and Kessler 1998; Anema and McKenna 1996). The linearity within the two temperature ranges allowed the activation energies (E_a), the enthalpies of activation ($\Delta H^\ddagger$), the entropies of activation ($\Delta S^\ddagger$) and the free energies of activation ($\Delta G^\ddagger$) to be calculated. These are given in Table 1.

The results in Figures 1 to 3 clearly demonstrate that the

concentration of the milk prior to heat treatment had no effect on the irreversible denaturation of α -lactalbumin. Similarly, the E_a , $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values were relatively unaffected by the milk solids concentration (Table 1).

Disulfide aggregation of α -lactalbumin

Each milk sample was also analyzed by NR-SDS-PAGE, which uses a dissociating agent (SDS) but no reducing agent. NR-SDS-PAGE should allow measurement of the loss of α -lactalbumin due to disulfide interactions (or other covalent interactions). Figure 4 shows the level of unreacted α -lactalbumin as a function of heating time, as estimated using the NR-SDS-PAGE system. The level of unreacted α -lactalbumin decreased with an increase in heating time at each temperature and the rate of loss increased as the heating temperature was increased. As with the denaturation of α -lactalbumin, the aggregation of α -lactalbumin by disulfide interactions could be described as a first order reaction at all milk concentrations and temperatures (Figure 5). At higher temperatures, the fit of the aggregation data to the first order

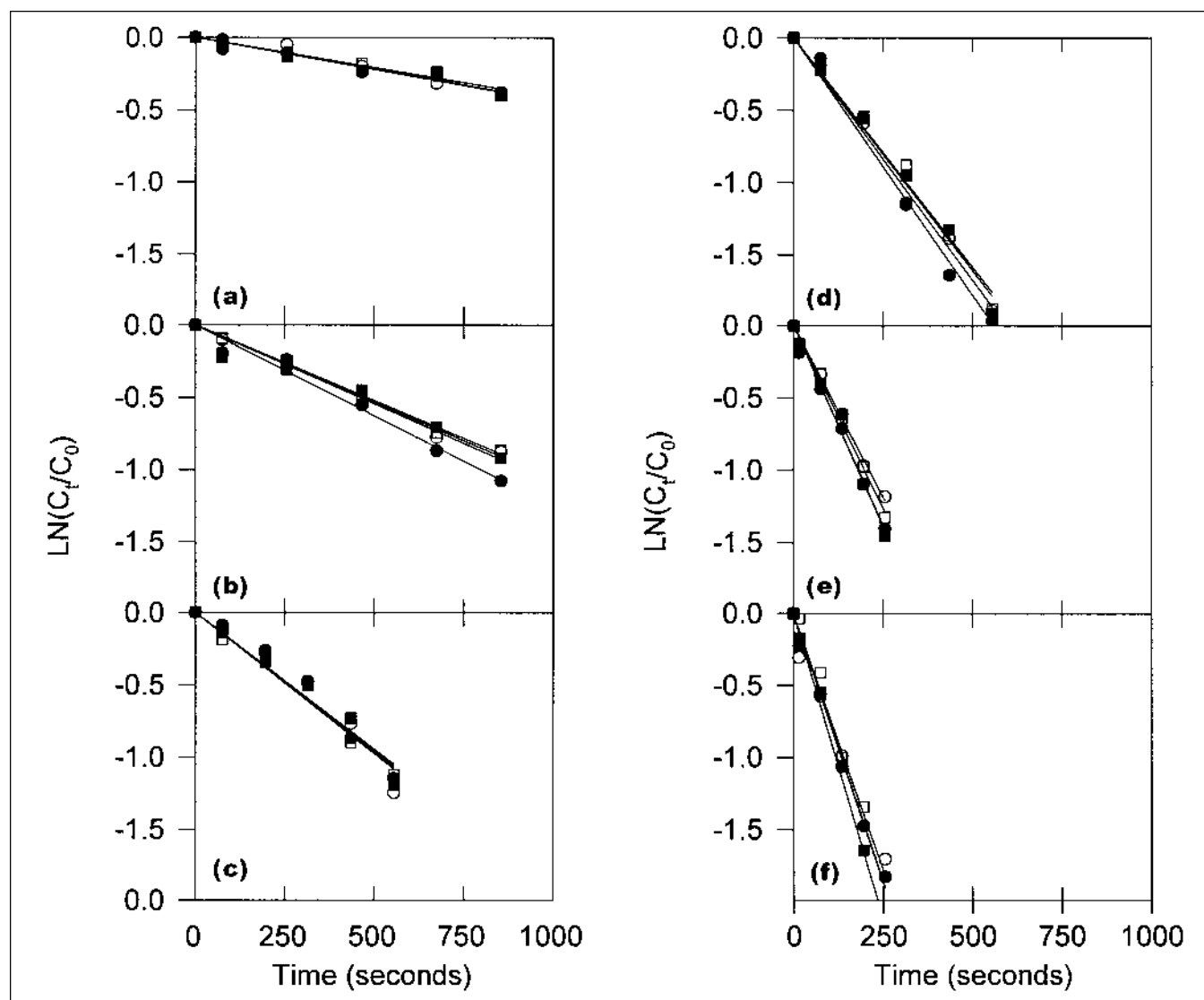


Figure 2—Denaturation of α -lactalbumin as a first-order reaction. a: 75 °C; b: 80 °C; c: 85 °C; d: 90 °C; e: 95 °C; f: 100 °C. ○: 9.6% TS; ●: 19.2% TS; □: 28.8% TS; ■: 38.4% TS

Table 1—Thermodynamic parameters for the thermal denaturation of α-lactalbumin (the standard error for E_a and $\ln k_0$ for the 80 to 100 °C temperature range are given in parentheses)

Milk conc.	Temp. range (°C)	E_a (kJ/mol)	$\ln k_0$	Average $\Delta H^\ddagger$ (kJ/mol)	Average $\Delta S^\ddagger$ (kJ/mol.K)	Average $\Delta G^\ddagger$ (kJ/mol)
9.6% TS	75–80	186.5	56.7	183.6	0.22	107.6
	80–100	102.1 (5.4)	28.0 (1.8)	99.1	−0.02	107.1
19.2% TS	75–80	214.1	66.2	211.2	0.30	107.4
	80–100	101.5 (6.1)	27.9 (2.0)	98.4	−0.02	106.9
28.8% TS	75–80	189.2	57.6	186.3	0.22	107.7
	80–100	107.4 (4.3)	29.8 (1.4)	104.4	−0.01	107.0
38.4% TS	75–80	182.3	55.2	179.4	0.21	107.6
	80–100	109.8 (2.2)	32.3 (0.7)	106.8	−0.0002	106.9

rate equation was not as good as that observed for the denaturation reaction (Fig 2). This may be attributed to the greater difficulty in analysing low levels of residual α-lactalbumin by the NR-SDS-PAGE technique rather than to a change in reaction mechanism at these higher temperatures.

Using the Arrhenius relationship (Dannenberg and Kessler 1998; Anema and McKenna 1996), the logarithms of the rate constants obtained from the straight lines in Figure 5 were plotted against the reciprocal of absolute temperature (Figure 6) and the E_a , $\ln k_0$, $\Delta H^\ddagger$, $\Delta S^\ddagger$ and $\Delta G^\ddagger$ were calculated (Table 2).

As with the thermal denaturation reaction, the Arrhenius plot displayed a break in temperature-dependence at about 80 °C. The disulfide aggregation reaction of α-lactalbumin was relatively unaffected by the milk solids concentration at every temperature investigated, and the E_a , $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ were relatively constant for all milk concentrations.

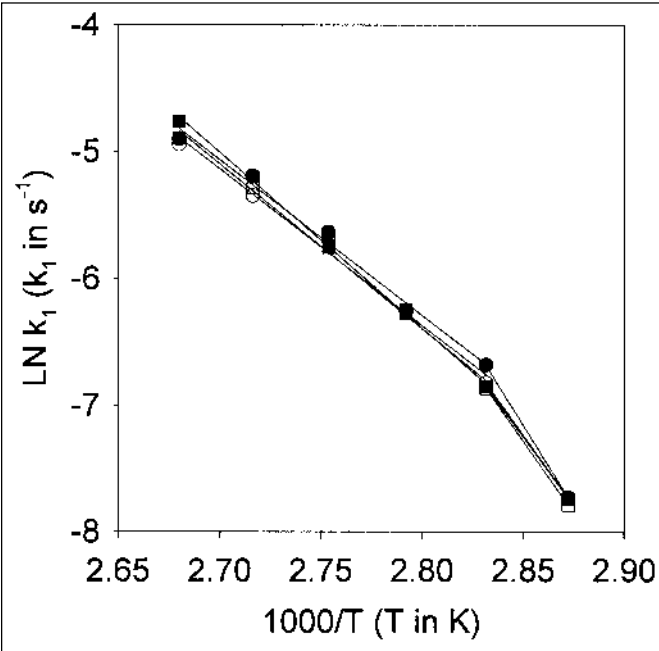


Figure 3—Arrhenius plot for α-lactalbumin denaturation. ○: 9.6% TS; ●: 19.2% TS; □: 28.8% TS; ■: 38.4% TS

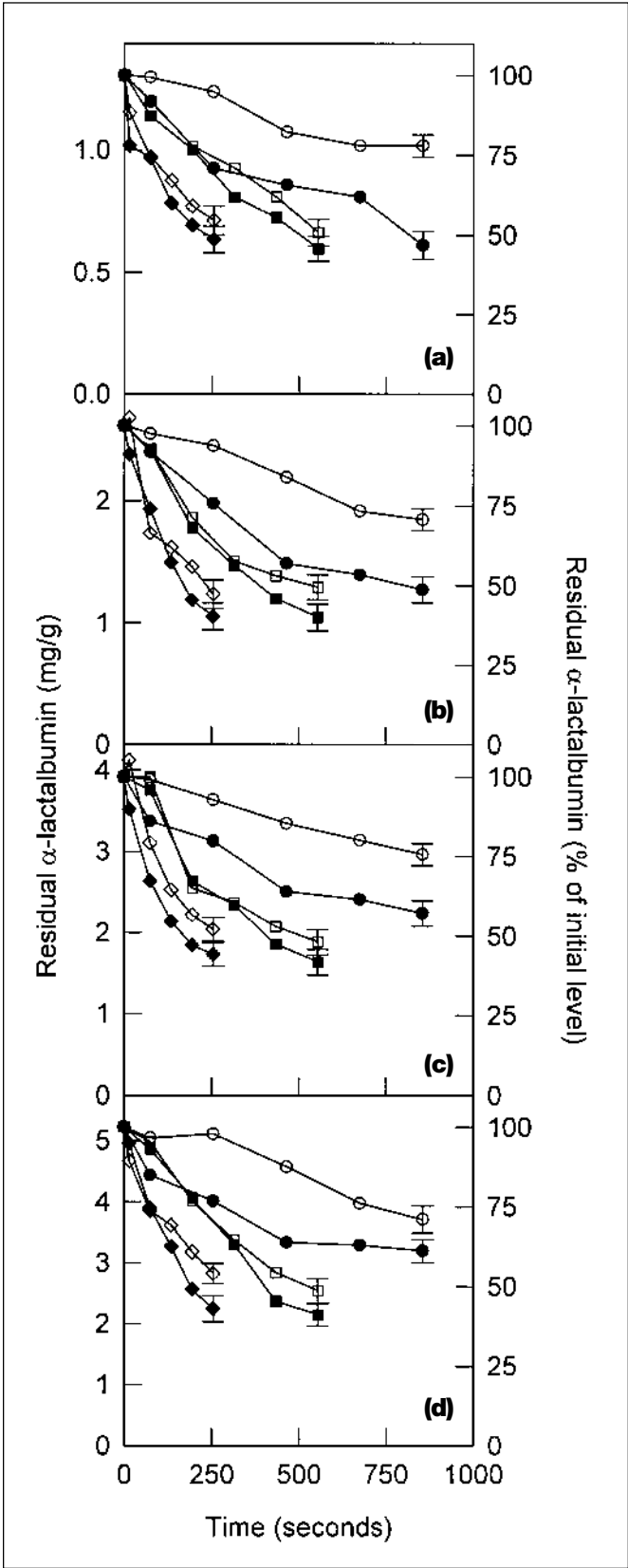


Figure 4—Temperature dependence of the disulfide aggregation of α-lactalbumin (NR-SDS-PAGE results). (a) 9.6% TS milk; (b) 19.2% TS milk; (c) 28.8% TS milk; (d) 38.4% TS milk. ○: 75 °C; ●: 80 °C; □: 85 °C; ■: 90 °C; ◇: 95 °C; ◆: 100 °C. Error bars, included on the last points, represent the maximum standard deviation observed between duplicate measurements within the series.

Comparison between denatured and disulfide-aggregated α-lactalbumin

The level of denatured α-lactalbumin correlated with the level of α-lactalbumin aggregated by disulfide bonding (Figure 7a; $R = 0.96$; $p \leq 0.001$). However, the levels of denatured α-lactalbumin were markedly higher than the levels of disulfide-aggregated α-lactalbumin with about 20 to 30% more denatured α-lactalbumin than disulfide-aggregated α-lactalbumin. This can also be seen when comparing the rate constants, as the rate constants for the thermal denaturation of α-lactalbumin were markedly higher than those for the disulfide aggregation reaction (Figure 7b; $R = 0.98$, $p = 0.001$).

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. All four milk powders were low-heat and differed only in the

Table 2—Thermodynamic parameters for the disulfide aggregation of α-lactalbumin (the standard error for E_a and $\ln k_0$ for the 80 to 100 °C temperature range are given in parentheses)

Milk conc.	Temp. range (°C)	E_a (kJ/mol)	$\ln k_0$	Average $\Delta H^\ddagger$ (kJ/mol)	Average $\Delta S^\ddagger$ (kJ/mol.K)	Average $\Delta G^\ddagger$ (kJ/mol)
9.6% TS	75–80	196.0	59.1	193.1	0.24	108.3
	80–100	74.9 (1.0)	18.4 (2.7)	71.9	−0.10	108.9
19.2% TS	75–80	169.2	50.7	166.3	0.17	107.9
	80–100	81.1 (0.9)	19.8 (2.4)	78.1	−0.08	108.4
28.8% TS	75–80	166.8	49.6	163.9	0.16	108.6
	80–100	84.8 (1.1)	21.8 (3.0)	81.8	−0.07	108.6
38.4% TS	75–80	128.8	36.6	125.9	0.05	108.6
	80–100	84.9 (1.0)	21.7 (2.6)	81.9	−0.07	108.6

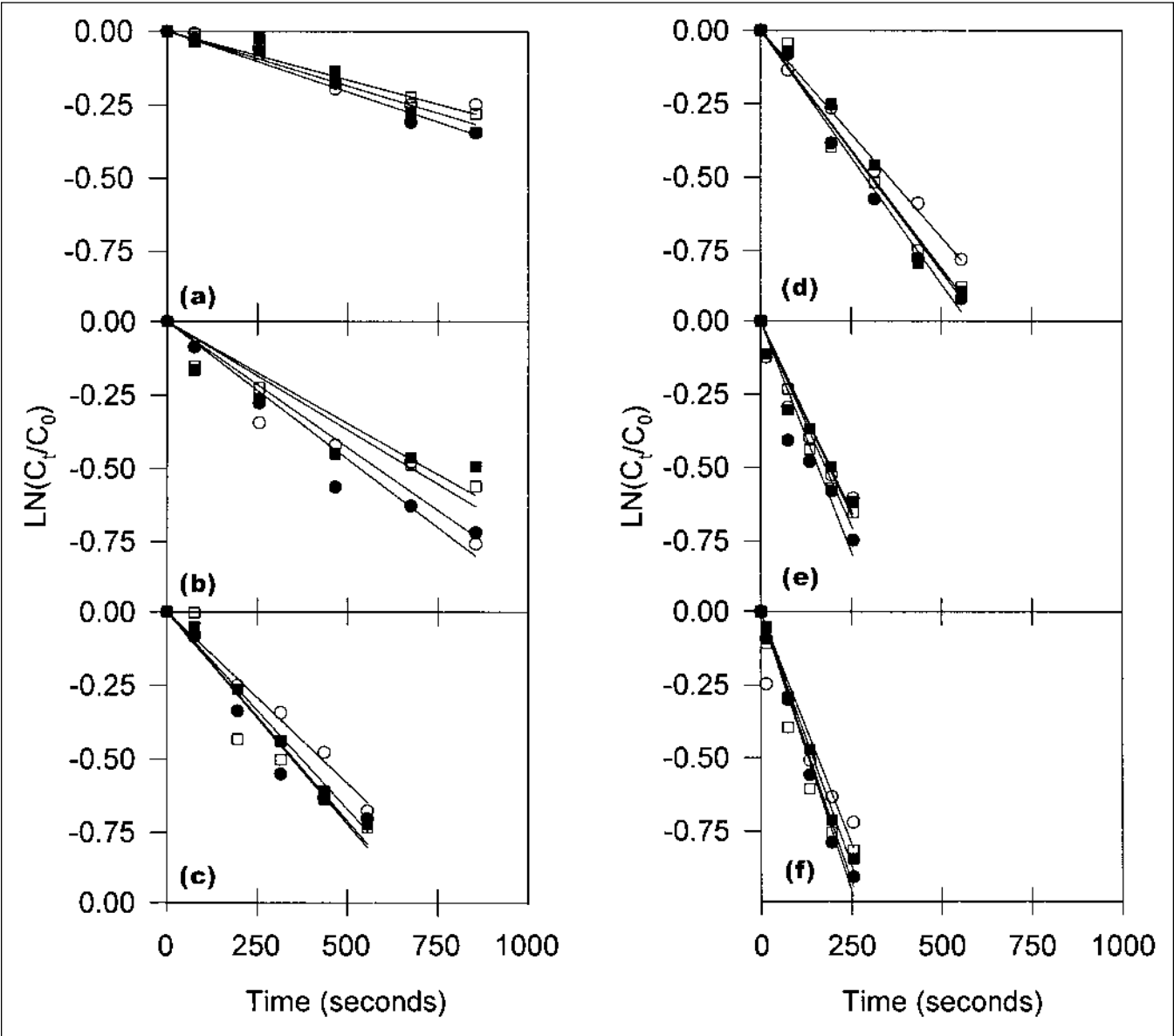


Figure 5—Disulfide aggregation of α-lactalbumin as a first-order reaction. (a) 75 °C; (b) 80 °C; (c) 85 °C; (d) 90 °C; (e) 95 °C; (f) 100 °C. ○: 9.6% TS; ●: 19.2% TS; □: 28.8% TS; ■: 38.4% TS

date of production. Selected results (80 °C for 435 s and 95 °C for 45 s) are shown in Figure 8a (native-PAGE) and Figure 8b (NR-SDS-PAGE). All four milk samples behaved similarly, although there was some variation in the denaturation and disulfide aggregation of α -lactalbumin between samples, which is probably related to compositional/pH differences in the milk sources. At both 80 °C and 95 °C, increasing milk solids concentration had little effect on the denaturation and disulfide aggregation of α -lactalbumin.

Discussion

THE OBSERVATION OF A FIRST-ORDER REACTION FOR THE thermal denaturation of α -lactalbumin in milk is in agreement with all previous studies on α -lactalbumin denaturation regardless of whether the protein is in buffer solution (Baer and others 1976), sweet whey (Kessler and Beyer 1991) or milk (Dannenberg and Kessler 1988; Anema and McKenna 1996). There are no reported studies examining the kinetics for the disulfide aggregation of α -lactalbumin in heated milk.

The E_a , $\Delta H^\ddagger$, $\Delta S^\ddagger$ and $\Delta G^\ddagger$ for the thermal denaturation of α -lactalbumin in the 9.6% TS milk samples in the low temperature range (75 to 80 °C) were comparable with those in the previous reports of Anema and McKenna (1996) and Oldfield and others (1998), although markedly lower than those reported by Dannenberg and Kessler (1988). The E_a , $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values in the high-temperature range (80 to 100 °C) were comparable, although slightly higher, than previously reported ones (Manji and Kakuda 1986; Dannenberg and Kessler 1988; Kessler and Beyer 1991; Anema and McKenna 1996). The E_a , $\Delta H^\ddagger$, $\Delta S^\ddagger$ and $\Delta G^\ddagger$ for the disulfide aggregation of α -lactalbumin were comparable with those for the thermal denaturation reactions.

The marked change in the temperature dependence of

the rate constants for both the denaturation (Figure 3) and the disulfide aggregation (Figure 6) of α -lactalbumin has been ascribed to a multi-step reaction mechanism with different rate-determining steps in the two temperature ranges. The thermodynamic parameters (Tables 1 and 2) are consistent with a mechanism in which the denaturation reaction (unfolding) is rate limiting in the low temperature range (75 to 80 °C) and aggregation reactions involving denatured whey proteins are rate limiting at higher temperatures (Kessler and Beyer 1991; Anema and McKenna 1996; Oldfield

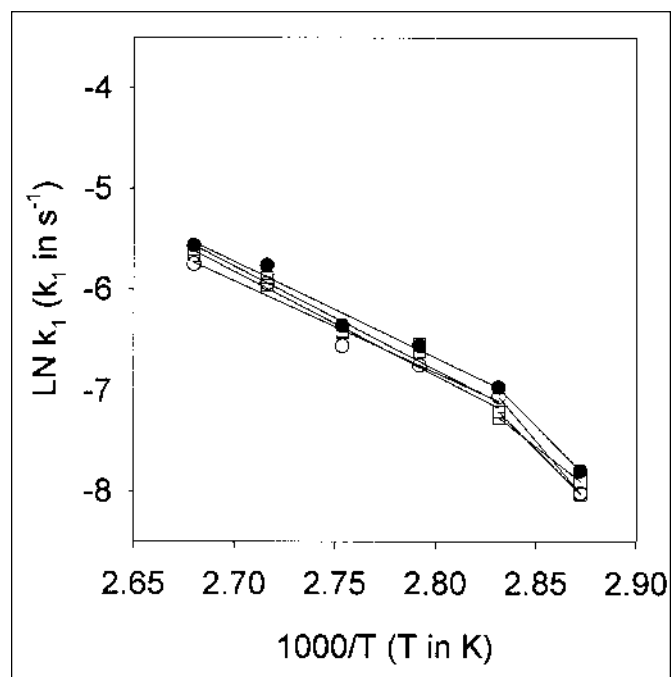


Figure 6—Arrhenius plot for the disulfide aggregation of α -lactalbumin. ○: 9.6% TS; ●: 19.2% TS; □: 28.8% TS; ■: 38.4% TS

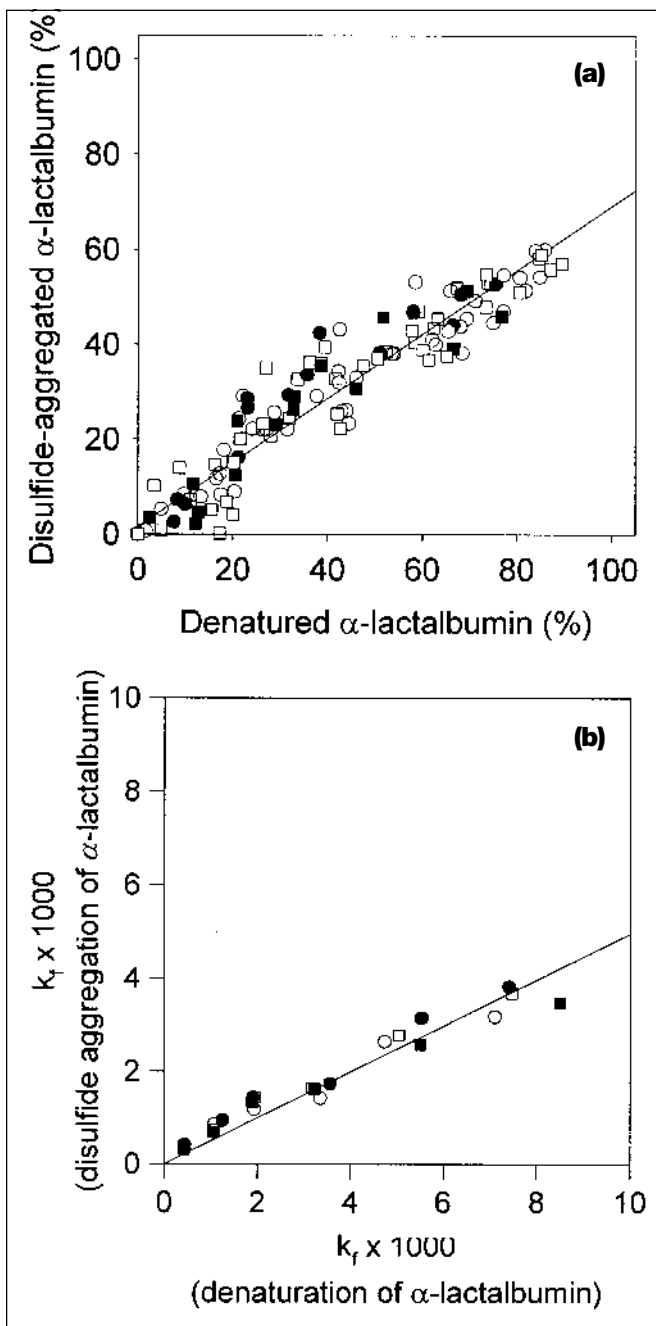


Figure 7—(a) Comparison between the levels of denatured and disulfide-aggregated α -lactalbumin in heated milk. (b) Comparison between the rate constants for the denaturation and disulfide aggregation of α -lactalbumin. ○: 9.6% TS; ●: 19.2% TS; □: 28.8% TS; ■: 38.4% TS

and others 1998).

Both the thermal denaturation reaction and the disulfide aggregation reaction of α -lactalbumin were unaffected by the milk solids concentration at all temperatures investigated. Similarly the E_a , $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ for both thermal denaturation and disulfide aggregation were relatively constant for all milk concentrations. Law and Leaver (1997) showed that the rate of thermal denaturation of α -lactalbumin and β -lactoglobulin in skim milk was increased when the total protein content was increased by ultrafiltration or the total whey protein was increased by addition of freeze dried whey proteins. In this system, the concentration of total protein or whey protein is increased whereas the concentration of diffusible components remains constant. Hillier and others (1979) showed that α -lactalbumin denaturation in cheese

whey, at a constant pH of 6.0, was enhanced at higher whey solids concentrations. In addition, Hillier and others (1979) noted that the rate constant for α -lactalbumin denaturation was increased by increasing the protein concentration or pH of the sweet whey but retarded by increasing the lactose, calcium or both lactose and calcium concentrations of the whey before heating. Plock and others (1998a) investigated the effect of totals solids content on the denaturation of α -lactalbumin in heated sweet whey. The denaturation of α -lactalbumin was found to have a reaction order of 1.0 at all whey concentrations and the rate of α -lactalbumin denaturation was unaffected by the concentration of the sweet whey. This is a finding that is comparable with our results. In a subsequent study, Plock and others (1998b) showed that the denaturation rate of α -lactalbumin was unaffected by changes in lactose concentration at all temperatures examined.

This contrasts with the irreversible denaturation and disulfide aggregation reactions of β -lactoglobulin which were markedly retarded at higher milk solids concentrations, although the retardation became less pronounced at higher temperatures (Anema 2000). Plock and others (1998a) also observed a retardation of β -lactoglobulin denaturation when the concentration of sweet whey was increased. McKenzie (1971) and Iametti and Bonomi (1996) reported that the first step in the denaturation reaction is the dissociation of dimeric β -lactoglobulin to a monomeric form. Anema (2000) proposed that the stabilization of β -lactoglobulin to denaturation at increasing milk solids concentration was a consequence of the increased self-association of β -lactoglobulin to form dimers. As α -lactalbumin has no tendency to self-associate, no retardation in denaturation as a consequence of self-association can occur. This may explain why the irreversible denaturation of α -lactalbumin is unaffected by milk solids concentration.

A significant proportion (about 30%) of the denatured α -lactalbumin was not involved in disulfide aggregation and was, therefore, aggregated via hydrophobic interactions. Oldfield and others (1998) reported that almost all of the denatured α -lactalbumin in ultracentrifuged supernatants of heated milk was in the form of disulfide-linked aggregates. However, only about 60% of the denatured α -lactalbumin associated with the casein micelles (in ultracentrifuged pellets) was in the form of disulfide-linked aggregates. Our present study measured the total (both soluble and colloidal) disulfide-aggregated α -lactalbumin. The results of these independent studies taken together indicate that a significant proportion of the denatured α -lactalbumin is involved in aggregates involving non-covalent interactions.

Gezimati and others (1997) showed that a proportion of the denatured β -lactoglobulin and α -lactalbumin in heated admixtures was hydrophobically aggregated. Similarly Havea and others (1998) noted that significant levels of denatured α -lactalbumin, β -lactoglobulin and bovine serum albumin were hydrophobically aggregated when whey protein concentrate solutions were heated. These authors concluded that, when α -lactalbumin and β -lactoglobulin admixtures are heated, stable hydrophobic aggregates containing both β -lactoglobulin and α -lactalbumin are formed. After prolonged heating and under appropriate conditions, the free thiol group of β -lactoglobulin can initiate thiol-disulfide exchange reactions that incorporate both the denatured α -lactalbumin and the denatured β -lactoglobulin.

It is likely that a similar series of reactions occurs in heated milk samples; however, there is the added complexity of interactions that can involve the casein micelles. A possible

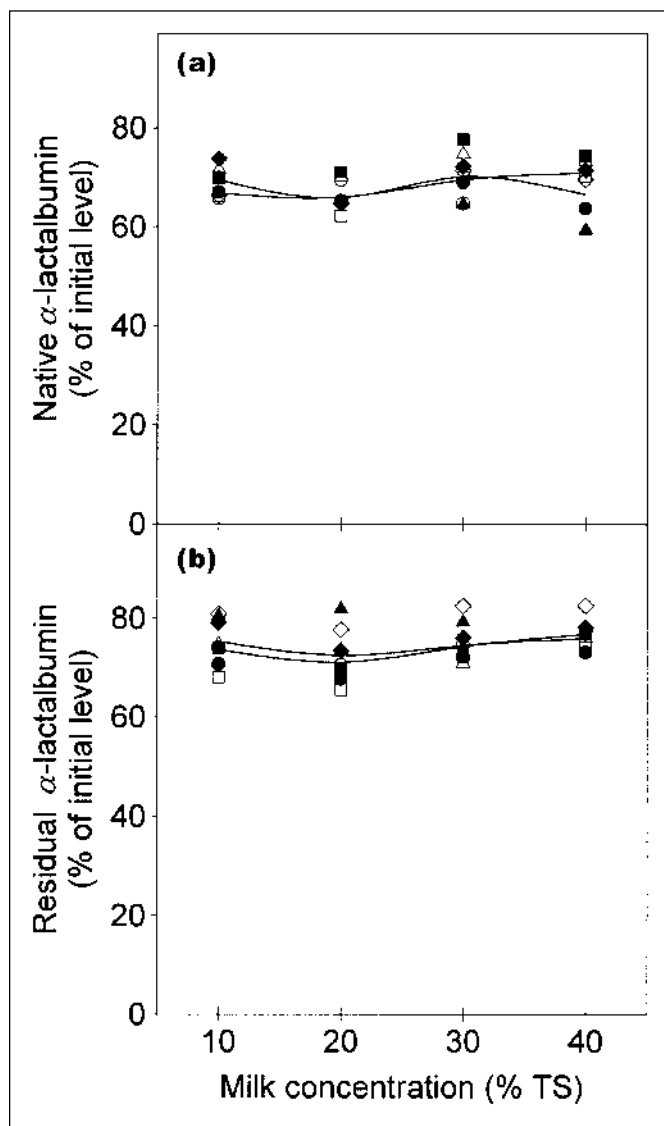


Figure 8—(a) Comparison of the denaturation of α -lactalbumin for four reconstituted skim milk samples; (b) Comparison of the disulfide aggregation of α -lactalbumin for four reconstituted skim milk samples. $\circ$ ●: milk 1; $\square$ ■: milk 2; $\diamond$ ◆: milk 3; $\triangle$ ▲: milk 4. Open symbols: samples heated at 80 °C for 435 s; filled symbols: samples heated at 95 °C for 45 s

sequence of events is the initial unfolding of α -lactalbumin and monomeric β -lactoglobulin which aggregate together via hydrophobic interactions. These aggregates may also interact, via hydrophobic interactions, with the surface of the casein micelles. The exposure of the free thiol group of β -lactoglobulin initiates thiol-disulfide exchange reactions involving the denatured β -lactoglobulin, denatured α -lactalbumin, κ -casein and, possibly, α_{s2} -casein. The occurrence of thiol-disulfide exchange reactions requires the free thiol of one protein to come into close proximity with the disulfide bond of another protein and, therefore, this reaction may be slower than the hydrophobically driven aggregation reactions. Some of the hydrophobically aggregated proteins may be spatially located so that thiol-disulfide interchange is not achieved on the reaction time scale and these remain as hydrophobically linked aggregates.

Conclusions

THE IRREVERSIBLE THERMAL DENATURATION AND DISULFIDE aggregation reactions of α -lactalbumin were unaffected by milk concentration. At any particular temperature, similar denaturation and disulfide aggregation rates were observed at all milk concentrations. About 30% of the denatured α -lactalbumin was not involved in disulfide-aggregated complexes and, therefore, associated via hydrophobic interactions. In contrast, it was previously shown that the denaturation and disulfide aggregation of β -lactoglobulin was retarded by increasing the milk solids concentrations and that about 85% of the denatured β -lactoglobulin was involved in disulfide-aggregated complexes (Anema 2000). These results suggest that significant denaturation of whey protein can occur when concentrated milk samples are heated and that α -lactalbumin will be more susceptible to heat-induced damage than β -lactoglobulin at high milk solids concentrations. Further studies are required to determine why β -lactoglobulin denaturation is markedly retarded by increasing milk concentrations whereas α -lactalbumin denaturation is unaffected.

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