J. Dairy Sci. 105:3871–3882
https://doi.org/10.3168/jds.2021-21444

Kinetics of heat-induced interactions among whey proteins and casein micelles in sheep skim milk and aggregation of the casein micelles

Zheng Pan,[1] Aiqian Ye,[1]* Anant Dave,[1] Karl Fraser,[1,2] and Harjinder Singh[1]
[1]Riddet Institute, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand
[2]AgResearch, Private Bag 11 008, Palmerston North 4442, New Zealand

ABSTRACT

The interactions among the proteins in sheep skim milk (SSM) during heat treatments (67.5–90°C for 0.5–30 min) were characterized by the kinetics of the denaturation of the whey proteins and of the association of the denatured whey proteins with casein micelles, and changes in the size and structure of casein micelles. The relationship between the size of the casein micelles and the association of whey proteins with the casein micelles is discussed. The level of denaturation and association with the casein micelles for β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) increased with increasing heating temperature and time; the rates of denaturation and association with the casein micelles were markedly higher for β-LG than for α-LA in the temperature range 80 to 90°C; the Arrhenius critical temperature was 80°C for the denaturation of both β-LG and α-LA. The casein micelle size increased by 7 to 120 nm, depending on the heating temperature and the holding time. For instance, the micelle size (about 293 nm) of SSM heated at 90°C for 30 min increased by about 70% compared with that (about 174.6 nm) of unheated SSM. The casein micelle size increased slowly by a maximum of about 65 nm until the level of association of the denatured whey proteins with casein micelles reached 95%, and then increased markedly by a maximum of about 120 nm when the association level was greater than about 95%. The marked increases in casein micelle size in heated SSM were due to aggregation of the casein micelles. Aggregation of the casein micelles and association of whey protein with the micelles occurred simultaneously in SSM during heating.
Key words: whey protein, casein micelle, protein composition, structure, kinetics

INTRODUCTION

Milk is commonly exposed to commercial thermal processing, which induces some physicochemical changes, such as whey protein denaturation and an increase in casein micelle size (Raynal et al., 2007; Dalgleish and Corredig, 2012). Most of the denatured whey proteins (especially β-LG) bind covalently to the casein micelles via thiol–disulfide bond exchange with κ-CN (Van Hooydonk et al., 1987; Donato and Dalgleish, 2006). In addition, the presence of β-LG during heating helps α-LA to connect with κ-CN; α-LA cannot associate directly with κ-CN because of the lack of the thiol group (Baer et al., 1976; Elfagm and Wheelock, 1978; Wijayanti et al., 2019). These interactions between whey proteins and caseins/casein micelles lead to structural changes in the casein micelles and an increase in the size of the casein micelles (Anema and Li, 2003). However, these changes may vary to some degree in heated milks from different species because of different compositions and structures of the proteins. Studies have shown that sheep milk has lower heat stability than cow milk (Park et al., 2007; Raynal et al., 2007). In comparison with cow milk, sheep milk showed a greater extent of whey protein denaturation after heating at 80 to 90°C for 0.5 to 10 min (Law, 1995; Raynal and Remeuf, 1998).

Previous studies suggested that the casein micelle size increased from its initial value by 25 to 75% in sheep milk upon heating at 75 to 90°C for 0.5 to 10 min but remained unchanged in cow milk (Raynal and Remeuf, 1998). The different effects of heating on the casein micelle size between sheep milk and cow milk might be caused by the differences in protein content and the extents of dissociation or aggregation of the casein micelles (Raynal and Remeuf, 1998). Previous studies in cow milk have shown that heating can induce dissociation of caseins from casein micelles, leading to an increase in the proportion of small-sized protein particles (Singh and Creamer, 1991; Anema and Li, 2000); the extent of casein dissociation may be different for milks from different species (Raynal and Remeuf, 1998). The casein micelles in sheep milk are more mineralized micelles than cow milk; this is responsible for

Received October 17, 2021.
Accepted January 17, 2022.
*Corresponding author: A.M.Ye@massey.ac.nz

the greater extent of micelle aggregation at high temperature (Van Hooydonk et al., 1987; Muir et al., 1993). In addition, sheep milk has higher protein content than cow milk, which probably increases the micelle–micelle interactions during heating and thus leads to aggregation of the casein micelles (Raynal and Remeuf, 1998). However, no studies to date demonstrate whether the changes in casein micelle size in heated sheep milk are caused exclusively by the denatured whey proteins associating with the casein micelles or are also caused by the partial aggregation of the casein micelles that accompanies this association behavior.

Many studies on the kinetics of the whey protein denaturation of cow milk over wide ranges of heating temperature and time have been conducted (Dannenberg and Kessler, 1988; Anema and McKenna, 1996; Oldfield et al., 1998b, 2005), and several models to describe the mechanisms of whey protein denaturation and aggregation in cow milk have been proposed. For instance, Oldfield et al. (1998b) investigated the kinetics of denaturation and aggregation of the whey proteins in cow skim milk over a wide range of temperature–time (70–130°C for 3–1,800 s); they found that the aggregation of β-LG involved the dissociation of the dimer, unfolding, and the formation of intermolecular disulfide linkages, whereas the aggregation of α-LA appeared to involve hydrophobic interactions. These kinetic models provide useful information for understanding the mechanisms of the denaturation of the whey proteins and the association of the denatured whey proteins with the casein micelles in heated milk. To date, little information on the kinetics of the denaturation of the whey proteins in sheep milk is available and no studies on the association of denatured whey proteins with the casein micelles in sheep milk have been reported.

The objective of this study was to investigate the kinetics of the irreversible denaturation of β-LG and α-LA and the association of denatured β-LG and α-LA with the casein micelles in sheep skim milk (**SSM**) at natural pH in the temperature range 67.5 to 90°C for 0.5 to 30 min, and to understand how interactions between whey proteins and casein micelles affect the casein micelle size. The structural changes in the casein micelles after heat treatment were also determined using transmission electron microscopy.

MATERIALS AND METHODS

Milk Supply and Heat Treatment

Mid-lactation sheep milk (pH 6.56 ± 0.01) was obtained from Neer Enterprises Limited (Carterton, New Zealand). The main composition of 3 batches of sheep milk was analyzed using a MilkoScan FT1 (FOSS) and by SDS-PAGE as described by Ye et al. (2016), and is shown in Table 1. A small amount of sodium azide (0.01%) was added to the unheated milk as a preservative. The whole sheep milk was skimmed at 3,000 × *g* for 15 min at 25°C using a bench centrifuge (Heraeus Multifuge X3R; Thermo Fisher Scientific Inc.). The skimmed sheep milk (6 mL) was transferred into 10 mL sealable glass tubes and the well-sealed tubes were then heated at a range of temperatures (67.5–90°C) and times (0.5–30 min) with continuous rocking in a thermostatically controlled water bath. After heat treatment, the milk samples were immediately immersed in cold running water for cooling to room temperature. The heated milk samples were kept at room temperature for 6 h before further analyses.

Table 1. Composition of raw sheep milk

Parameter	Sheep milk[1]
Total solids (g·100 g^{-1})	17.7 ± 0.3
Fat (g·100 g^{-1})	6.0 ± 0.2
Protein (g·100 g^{-1})	6.1 ± 0.2
Casein (g·100 g^{-1})	4.8 ± 0.2
α_{S2}-CN (%)[2]	19.2
α_{S1}-CN (%)[2]	7.2
β-CN (%)[2]	64.6
κ-CN (%)[2]	9.0
Whey protein (g·100 g^{-1})	1.3 ± 0.0
α-LA (%)[3]	26.1
β-LG (%)[3]	62.3

[1]Values are reported as mean ± SD from 3 batches of sheep milk.
[2]Percentage of total caseins.
[3]Percentage of total whey proteins.

Determination of Denaturation of Whey Proteins

The native whey proteins were obtained by removing the caseins and denatured whey proteins from the skim milk samples using acetic acid precipitation as described by Vasbinder et al. (2003). A 0.4 mL subsample of SSM was mixed well with 0.8 mL of MilliQ water and 0.1 mL of acetic acid (10%) in an Eppendorf tube (2 mL) and left to stand for 10 min. Then, 0.6 mL of MilliQ water and 0.1 mL of sodium acetate (1 *M*) were added into the solution, which was mixed again. The mixed solution reached an equilibrium pH of 4.6 and was kept in an ambient environment for 1 h. The caseins and denatured whey proteins were precipitated after centrifugation at 3,000 × *g* for 5 min. The amount of native whey proteins in the supernatant was determined by HPLC. The percentage of denatured whey proteins was calculated by subtracting the percentage of native whey proteins in heated samples from that in unheated samples.

Determination of Denatured Whey Proteins Associated with Casein Micelles

To determine the association of the denatured whey proteins with the casein micelles, the unheated and heated SSM were ultracentrifuged at 63,000 × g for 1 h at 20°C using a Sorval WX 80+ Ultracentrifuge (Thermo Fisher Scientific Inc.) to remove all denatured whey proteins that had associated with casein micelles and all casein micelles. The serum-phase whey proteins were defined as those whey proteins that did not sediment after the ultracentrifugation. The resultant supernatant (serum phase) containing nonsedimentable proteins was carefully collected and analyzed by HPLC. The quantity of each protein in the supernatant of the heated SSM was presented as a percentage of that in the supernatant of the unheated SSM. The level of denatured whey proteins that had associated with casein micelles was calculated by subtracting the level of serum-phase whey proteins in heated samples from that in unheated samples. This centrifugal method had been proved to be the minimum centrifugation speed required to effectively sediment the whey proteins associated with casein micelles and to reduce the possibility of centrifuging down soluble whey protein aggregates (Anema and Li, 2003).

Analysis of Protein Composition

Milks and the supernatants obtained from acid-precipitated and ultracentrifuged milk samples were analyzed by reversed-phase HPLC using a reversed-phase C18 column (Aeris Widepore 3.6 μm XB-C18 RP; Phenomenex) to determine the protein composition, as described by Bobe et al. (1998). The quantity of native whey proteins (β-LG and α-LA) in heated SSM was calculated by comparing the relative peak areas of the heated SSM with the original unheated SSM. The quantity of whey proteins in the ultracentrifugal supernatants was determined by comparing the relative peak areas of the supernatant fractions of the heated SSM with the original unheated SSM. All peak areas of these chromatograms were determined using peak integration algorithm LabSolutions software (Shimadzu Corporation).

Kinetic Analysis for Whey Protein Denaturation

The order of thermal denaturation of the whey proteins was determined using a general rate equation:

$$-\frac{\mathrm{d}C_t}{\mathrm{d}t} = k_n {C_t}^n. \quad [1]$$

For $n \neq 1$, this equation yields

$$\left(\frac{C_t}{C_0}\right)^{1-n} = 1 + (n-1) k_n {C_0}^{n-1} t. \quad [2]$$

When $n = 1$, this equation yields

$$\ln\left(\frac{C_t}{C_0}\right) = -k_n {C_0}^{n-1} t, \quad [3]$$

where n = reaction order, C_0 (g·L^{-1}) = concentration of native protein before heat treatment, C_t (g·L^{-1}) = concentration of native protein at time t (s), and k_n (g^{1-n}·L^{n-1}·s^{-1}) = rate constant. Equations 2 and 3 were used to calculate the reaction order n at different temperatures for different whey proteins. The rate constant k_n was calculated when $\left(\frac{C_t}{C_0}\right)^{1-n}$ was plotted against t.

The temperature dependence of the rate constant k_n can be defined using the Arrhenius equation:

$$\ln(k_n) = \ln(k_{n,0}) - \frac{E_a}{RT}, \quad [4]$$

where $k_{n,0}$ = frequency factor (g^{1-n}·L^{n-1}·s^{-1}), E_a = activation energy (J·mol^{-1}), R = universal gas constant (8.314 J·mol^{-1}·K^{-1}), and T = absolute temperature (K). The activation energy E_a was obtained as the logarithm of the rate constant (ln k_n) resulting from Equations 2 or 3 versus the reciprocal of the absolute temperature.

Kinetic Analysis for Association of Whey Proteins with Casein Micelles. The reaction kinetics of β-LG and α-LA associating with the casein micelles were fitted using a site-filling model (Equations 5 and 6; Sharma and Dalgleish, 1994). Presumably, the reactive sites available for the association of β-LG and α-LA are κ-CN and α_{S2}-CN.

When $n \neq 1$,

$$\left(\frac{C_{max} - C_t}{C_{max}}\right)^{1-n} = 1 + (n-1) k_n {C_{max}}^{n-1} t, \quad [5]$$

where C_{max} is the maximum amount (g·L^{-1}) of β-LG or α-LA, which is the total concentration of β-LG or α-LA that could associate with the casein micelles, C_t is the amount (g·L^{-1}) of β-LG or α-LA associated with the casein micelles at time t (s), and k_n is the apparent reaction rate constant (g^{1-n}·L^{n-1}·s^{-1}) for β-LG or α-LA associating with the casein micelles.

When n = 1,

$$\ln\left(\frac{C_{max}-C_t}{C_{max}}\right)=-k_1t, \quad [6]$$

where k_1 is the first-order reaction rate constant (s^{-1}).

Determination of Casein Micelle Diameter

The method for casein micelle diameter measurement was based on the method described by Anema (2018). Briefly, the unheated and heated SSM were diluted 1:50 in Ca–imidazole buffer (20 m*M* imidazole, 5 m*M* $CaCl_2$, and 30 m*M* NaCl, pH 7.0) and measured by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd.).

Microstructural Changes in Casein Micelles

The microstructural changes in SSM after heat treatment were observed using transmission electron microscopy, as has been described previously by Mittal et al. (2015). Briefly, milk samples were mixed in tubes with warm melted (30°C to 40°C) 3% low-temperature-gelling agarose at a ratio of 1:1. The mixture was fixed primarily with 3% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2) at 5°C for 24 h. Secondary fixation (overnight at room temperature) was done in 1% osmium tetroxide cacodylate buffer. The samples were rinsed with cacodylate buffer after each fixation. Dehydration of the samples was performed in a series of acetone before embedding in resin (Procure 812). Ultrathin sections (100 nm) were obtained using an ultramicrotome (Leica), followed by staining with uranyl acetate (2%, wt/vol) and lead citrate (2.5%, wt/vol). Samples were examined in a transmission electron microscope (FEI Tecnai G2 Biotwin), operated at 60 kV.

Statistical Analysis

All experiments reported were fully triplicated on 3 batches of sheep milk, and the results are presented as the mean ± standard deviation. Although there were some variations between different batches, the same trends and relationships as reported here have been observed for all samples examined to date. The data were plotted using GraphPad Prism 8.4.0 (GraphPad Software). Statistical analysis was performed using 1-way and 2-way ANOVA and Tukey's multiple comparison test at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Denaturation of Whey Protein in Heated SSM

Figure 1 shows the percentages of native β-LG and native α-LA in the supernatants obtained from differently heat-treated SSM after isoelectric precipitation of the denatured whey proteins and the caseins at pH 4.6. The percentages of native β-LG and native α-LA decreased as both the heating temperature and the holding time increased. At heating temperatures from 67.5 to 75°C, the percentage of native β-LG decreased relatively slowly to a maximum of about 24% as the heating time increased up to 30 min (Figure 1A). In the high temperature range (80–90°C), the percentage of native β-LG decreased rapidly during the first 5 min of heating and tended to plateau with further heating. After heat treatment at 90°C for 10 min, the percentage of native β-LG had decreased to 0.4%, indicating that almost all of the β-LG in the SSM had been denatured. A similar pattern was also found for native α-LA (Figure 1B). The percentage of native α-LA decreased slowly in the temperature range 67.5 to 75°C, and decreased more rapidly at 80 to 90°C. The percentages of native α-LA in the SSM heated for 10 min at 80, 85, and 90°C were 16.3, 6.4, and 3.8%, respectively. These results indicate that nearly all the whey proteins (β-LG and α-LA) were denatured when heated at 85 to 90°C for 10 min.

Kinetics of Denaturation of Whey Proteins

Order of Reactions. The reaction order n and the rate constant k_n for the thermal denaturation of β-LG and α-LA were calculated from the best fittings of the experimental data using Equations 2 and 3 (Table 2). For β-LG, a reaction order of 1.7 was found when the SSM was heated at 67.5–90°C (Table 2), and a linear relationship was obtained when $(C_t/C_0)^{-0.7}$ was plotted against the heating time (data not shown). A linear relationship for α-LA in the heated SSM was also observed when $\ln(C_t/C_0)$ was plotted against the heating time (data not shown), suggesting that the denaturation of α-LA followed first-order reaction kinetics. The reaction orders for the denaturation of β-LG and α-LA that were determined for the heated SSM are essentially in agreement with those from previous research on cow milk, which showed reaction orders of 1.5 and 1 for the denaturation of β-LG and α-LA, respectively determined by linear regression (Dannenberg and Kessler, 1988; Kessler and Beyer, 1991; Anema and McKenna, 1996; Anema et al., 2006) and reaction orders of 1.3 ± 0.3 and 1.0 ± 0.4 for the denaturation of β-LG

Table 2. Kinetic parameters for denaturation of β-LG and α-LA in heated sheep skim milk

Whey protein	Temperature (°C)	Rate constant k_n (10^{-3} $g^{1-n}\cdot L^{n-1}\cdot s^{-1}$)	Activation energy E_a ($kJ\cdot mol^{-1}$)	$\ln(k_{n,0})$	R^2
β-LG;	67.5	0.012	429.3	144.3	0.999
n = 1.7	70	0.039			
	72.5	0.104			
	75	0.306			
	80	10.238	207.7	70.3	0.990
	85	33.256			
	90	71.664			
α-LA;	67.5	0.096	306.3	102.9	0.993
n = 1.0	70	0.179			
	72.5	0.472			
	75	0.880			
	80	2.728	152.0	50.0	0.992
	85	6.303			
	90	11.335			

and α-LA, respectively determined by nonlinear regression (Oldfield et al., 1998b). For instance, Anema et al. (2006) investigated the influence of the concentrations of protein, nonprotein-soluble components, and lactose on the irreversible denaturation of β-LG and α-LA in reconstituted skim milk heated at temperatures ranging from 75 to 100°C, and showed that the reaction orders for the irreversible thermal denaturation of β-LG and α-LA were 1.5 and 1, respectively, in all systems and under all conditions.

Rate Constants for Whey Protein Denaturation. The rate constants for the denaturation of β-LG and α-LA in SSM heated at different temperatures are shown in Table 2. The values of the rate constant k_n for β-LG denaturation at 67.5 to 90°C were between 0.01 and 4.208 10^{-3} $g^{1-n}\cdot L^{n-1}\cdot s^{-1}$, and those for α-LA denaturation at 67.5 to 90°C were between 0.006 and 0.680 10^{-3} $g^{1-n}\cdot L^{n-1}\cdot s^{-1}$. The calculated k_n values for β-LG were markedly higher than those for α-LA in SSM heated at 80 to 90°C but were comparable with those for α-LA at 67.5 to 75°C, indicating a faster denaturation rate for β-LG than for α-LA at 80 to 90°C and comparable denaturation rates for both β-LG and α-LA at 67.5 to 75°C in SSM. This finding is in agreement with previous reports that β-LG was less heat stable than α-LA in cow milk when the heating temperature was higher than 80°C (Oldfield et al., 1998a; Anema, 2020).

The Arrhenius plots for the denaturation of β-LG and α-LA were drawn using logarithms of the rate constants ($\ln k_n$) resulting from Equations 2 and 3 at different temperatures (the reciprocal of the absolute temperature, $1/T$; Figure 2). The activation energies E_a and the frequency factor logarithms $\ln (k_{n,0})$ for the denaturation of β-LG and α-LA were calculated using the equation resulting from the Arrhenius plot obtained by least-squares linear regression. Linear relationships in 2 temperature regions were found for both β-LG and α-LA; the activation energies (E_a) and the

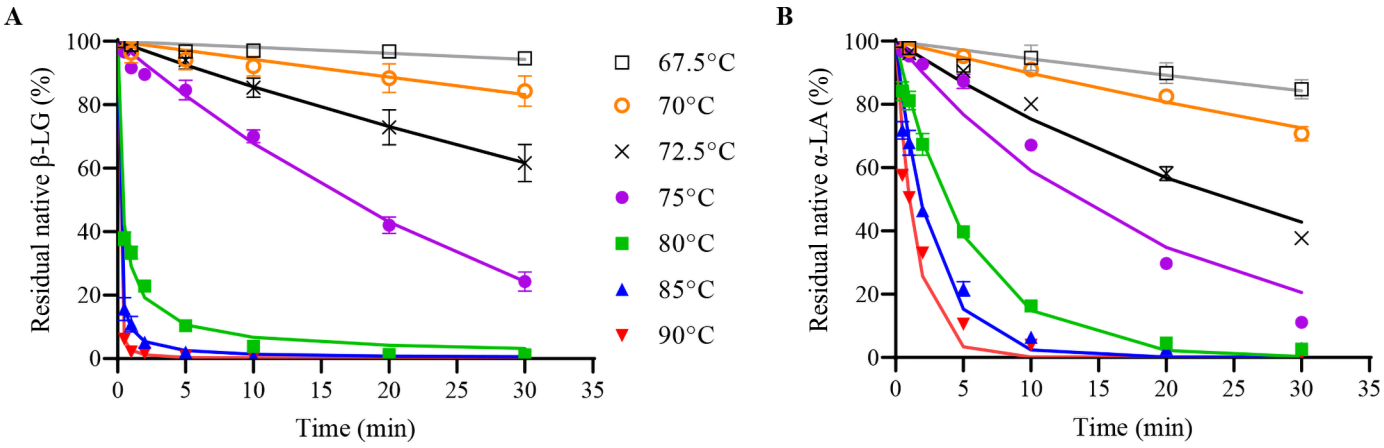

Figure 1. Percentage of residual native β-LG (A) and α-LA (B) in sheep skim milk heated at different temperatures for different holding times. The lines represent the predicted denaturation from the model. Each data point represents the mean ± SD of the results from 3 different batches of sheep milk.

$\ln(k_{n,0})$ values were different in each region (Table 2). There was a break in the Arrhenius plot at 80°C for both β-LG and α-LA. These findings are in agreement with previous findings in cow milk reported by Dannenberg and Kessler (1988) and Anema et al. (2006), who showed that abrupt changes in the temperature dependence of the rate constants were observed for the denaturation of both β-LG and α-LA in the temperature range 75 to 100°C. The abrupt changes observed in the Arrhenius plots were probably due to changes in the rate-determining step from the denaturation process in the low-temperature range to association reactions in the high temperature range (Anema et al., 2006). The temperature for the abrupt change for the denaturation of α-LA (80°C) reported here is in agreement with that reported previously in cow skim milk; that is, the break was observed at 80°C for the denaturation of α-LA (Anema and McKenna, 1996; Oldfield et al., 1998b). However, the findings for the denaturation of β-LG in SSM do not support the previous research on cow skim milk, which showed that the break was found at 90°C for the denaturation of β-LG (Dannenberg and Kessler, 1988; Oldfield et al., 1998a). These differences indicated that the rate-determining step for the denaturation of β-LG changed at a lower temperature in sheep milk than in cow milk; however, the mechanism for these differences between sheep milk and cow milk is unclear. Previous studies on cow skim milk showed that the critical Arrhenius temperature of 90°C for the denaturation of β-LG was independent of the total protein concentration (Law and Leaver, 1997), the pH (Kessler and Beyer, 1991; Anema and McKenna, 1996; Law and Leaver, 2000), and the milk composition (i.e., nonprotein-colloidal/soluble components) (Anema et al., 2006), despite the rate constants k_n for the denaturation of β-LG being affected by these factors. Several reports indicated that the physicochemical properties of β-LG could be affected by its gene sequences and structures (Erhardt et al., 1989; Loch et al., 2014). Loch et al., (2014) showed different β-LG gene sequences and 3-dimensional structure between sheep milk and cow milk, and demonstrated that those differences could influence the physicochemical properties of β-LG. Therefore, it is hypothesized that the lower Arrhenius critical temperature for β-LG observed in sheep milk compared with cow milk may be related to the different gene sequences and structures of β-LG.

The activation energies E_a were calculated in 2 temperature regions using the Arrhenius plots, with values of 429.3 and 306.3 kJ·mol^{-1} at 67.5 to 75°C for β-LG and α-LA, respectively, and values of 207.7 and 152.0 kJ·mol^{-1} at 80 to 90°C for β-LG and α-LA, respectively (Table 2). The results presented here were higher than those reported previously by Dumitraşcu et al. (2013), who showed single values of 137 and 156 kJ·mol^{-1} in the whole heating temperature range of 72.5 to 90°C for the activation energies E_a for the denaturation of β-LG (based on a reaction order of 1.5) and α-LA (based on a first-reaction order), respectively. The differences in the activation energies E_a may have been due to the different milk sources used. Dumitraşcu et al. (2013) used milk from Merino sheep, whereas this study used milk from East Friesian sheep; the gene sequences and structures for β-LG of the 2 breeds could be different (Staiger et al., 2010; Mastrangelo et al., 2012), which may result in different kinetic parameters for the denaturation of β-LG. Additionally, no break was observed in the Arrhenius plots for the denaturation of both β-LG and α-LA in the temperature range 72.5 to 90°C in their results. A possible explanation for the discrepancy between the present results and their results might be that they worked with relatively fewer temperature and time points, resulting in the kinetic parameters being somewhat uncertain.

Thermodynamics of Whey Protein Denaturation

The average thermodynamic parameters for the denaturation of β-LG and α-LA were calculated based on the Eyring equation described by Anema and McKenna (1996) and are shown in Table 3. The changes in enthalpy ($\Delta H^{\ddagger}$, where $\ddagger$ designates parameters related to the transition state) values were about 428 and 305 kJ·mol^{-1} for β-LG and α-LA, respectively, in the low-temperature range (67.5–75°C), which were higher than those (about 206 and 150 kJ·mol^{-1} for β-LG and α-LA, respectively) in the high temperature range (80–90°C).

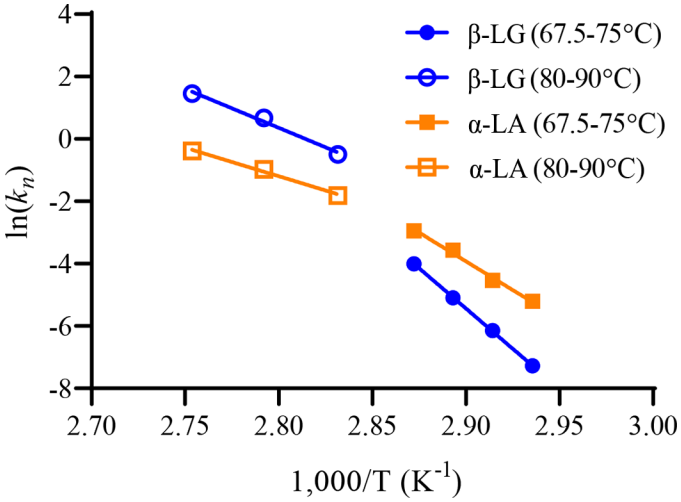

Figure 2. Arrhenius plots for the denaturation of β-LG and α-LA in heated sheep skim milk. Rate constant k_n has units of $g^{1-n}\cdot L^{n-1}\cdot s^{-1}$.

Table 3. Enthalpy ($\Delta H^{\ddagger}$), free energy ($\Delta G^{\ddagger}$), and entropy ($\Delta S^{\ddagger}$) obtained for denaturation of β-LG and α-LA in heated sheep skim milk[1]

Whey protein	Temperature (°C)	$\Delta H^{\ddagger}$ (kJ·mol^{-1})	$\Delta G^{\ddagger}$ (kJ·mol^{-1})	$\Delta S^{\ddagger}$ (kJ·mol^{-1}·K^{-1})
β-LG	67.5–75	428.1	100.9	0.950
	80–90	205.7	86.6	0.333
α-LA	67.5–75	305.1	96.4	0.606
	80–90	150.0	91.7	0.164

[1]The ‡ symbol designates parameters related to the transition state.

In addition, the $\Delta H^{\ddagger}$ values for β-LG and α-LA in both temperature ranges were higher than those reported previously for cow skim milk; that is, $\Delta H^{\ddagger}$ values of 260–300 kJ·mol^{-1} (70–90°C) for β-LG, and 190 to 270 kJ·mol^{-1} (70–80°C) and 50 to 70 kJ·mol^{-1} (85–115°C) for α-LA (Dannenberg and Kessler, 1988; Anema and McKenna, 1996; Oldfield et al., 1998b). The discrepancy between the present study and the data reported previously for cow skim milk can be attributed to the different Arrhenius critical temperatures (Figure 2) and the different milk compositions (Akkerman et al., 2016), as discussed above.

All the entropy $\Delta S^{\ddagger}$ values for both β-LG and α-LA were positive and were higher in the 67.5 to 75°C range than in the 80 to 90°C range. This is consistent with the findings on cow skim milk reported by Anema et al. (2006), who showed a decrease in the value of the entropy $\Delta S^{\ddagger}$ from the low-temperature range (75–90°C) to the high temperature range (90–100°C). The reduced $\Delta S^{\ddagger}$ values at higher temperatures suggested a decrease in disorder, indicating that association reactions were becoming the rate-determining step. In comparison, irreversible denaturation reactions were the rate-limiting step in the low-temperature range (Oldfield et al., 1998b).

At all heating temperatures, the values of the free energy $\Delta G^{\ddagger}$ were relatively constant, at 85 to 100 kJ·mol^{-1} for β-LG and 90 to 100 kJ·mol^{-1} for α-LA. These results match the data reported in earlier studies on both sheep milk and cow milk, and what is expected for protein unfolding (Dannenberg and Kessler, 1988; Anema and McKenna, 1996; Dumitraşcu et al., 2013).

Association of Denatured Whey Proteins with Casein Micelles

The percentages of serum-phase β-LG and α-LA decreased with increases in both the temperature and the duration of heat treatment (Figure 3). At 75°C, the percentage of serum-phase β-LG decreased relatively slowly throughout the heating time, reaching about 34% after 30 min of heating (Figure 3A). At higher heating temperatures (80–90°C), the decrease in serum-phase β-LG was more rapid and reached a plateau at a longer holding time. After 10 min of heating at 85 to 90°C, only about 3% of the β-LG remained in the serum phase. Serum-phase α-LA (Figure 3B) showed a similar pattern to serum-phase β-LG. When SSM was heated for 30 min, the percentages of serum-phase α-LA decreased to around 23, 11, 9, and 7% at 75, 80, 85, and 90°C, respectively. These results suggested that nearly all the whey proteins had associated with the casein micelles after heating at 80 to 90°C for 30 min.

Previous studies have shown that not all the denatured whey proteins associate with the casein micelles in cow milk; a maximum of about 70 to 80% of the denatured whey proteins associated with the micelles in the heating temperature range 75 to 90°C (Anema and Li, 2003). However, in sheep milk, it appeared that nearly all the denatured whey proteins associated with the casein micelles after heat treatments of 80 to 90°C for 30 min. This was probably due to the higher content of α_{S2}-CN in sheep milk. It has been reported that α_{S2}-CN can provide 2 cysteines for β-LG to interact with via thiol–disulfide exchange reactions (Farrell et al., 2009). Thus, the higher content of α_{S2}-CN may result in more denatured whey proteins associating with the casein micelles (Rasmussen et al., 1992; Patel et al., 2006).

Kinetics of Association of Denatured Whey Proteins with Casein Micelles

The kinetic parameters for the association of denatured β-LG and α-LA with the casein micelles were calculated using Equations 5 and 6 and are shown in Table 4. The associations of β-LG and α-LA with the casein micelles at 80 to 90°C had reaction orders of 2.6 and 1.8, respectively, which were higher than the reaction orders for denaturation of 1.7 for β-LG and first-order for α-LA. The results suggested that a more complex reaction occurred for the association process during heating, which may have been due to the involvement of the association between β-LG and α_{S2}-CN. Additionally, sheep milk has a higher content of α_{S2}-CN (19.2% of total caseins, Table 1) than cow milk (10.3% of total caseins; Balthazar et al., 2017), which may affect the association process of β-LG during heat-

Table 4. Kinetic parameters for associations of β-LG and α-LA with the casein micelles in heated sheep skim milk

Whey protein	Temperature (°C)	Rate constant k_n (10^{-3} $g^{1-n}{\cdot}L^{n-1}{\cdot}s^{-1}$)	Activation energy E_a ($kJ{\cdot}mol^{-1}$)	$\ln(k_{n,0})$	R^2
β-LG; n = 2.6	80	1.275	241.73	80.0	0.980
	85	6.516			
	90	14.412			
α-LA; n = 1.8	80	1.299	61.77	21.3	0.999
	85	1.743			
	90	2.319			

ing and thus lead to changes in the kinetic parameters for SSM compared with cow milk.

The rate constants k_n at 80 to 90°C for β-LG and α-LA association were lower than those for β-LG and α-LA denaturation (Table 2). Additionally, the value of the rate constant k_n for association with the casein micelles was lower for α-LA than for β-LG, indicating a slower association with the casein micelles for α-LA than for β-LG during heating. The findings of the slower association rates for α-LA than for β-LG are in agreement with previous findings in cow milk, which showed that a great amount of β-LG was denatured and associated with the casein micelles in skim milk in the temperature range 80 to 130°C before α-LA began to denature and complex with β-LG to any significant extent (Oldfield et al., 1998a). It has been reported that α-LA associates with casein micelles by forming a complex with β-LG, as α-LA cannot associate with casein micelles on its own (Elfagm and Wheelock, 1978). Therefore, the slower association rates of α-LA with the casein micelles were probably caused by the less accessible thiol group of denatured β-LG that had already associated with the casein micelles (Oldfield et al., 1998a).

Casein Micelle Size

The Z-average diameters of the casein micelles in unheated and heated SSM are shown in Figure 4A. The Z-average diameters of the casein micelles in SSM heated at 75 and 80°C had a sharp increase in the first 2 min and then increased slowly to about 208 and 221 nm, respectively, with further heating to 30 min. For SSM heated at 85 and 90°C, the diameters of the casein micelles showed similar patterns to those for SSM heated at 75 and 80°C in the first 20 min, but the size increased markedly at 30 min to about 290 nm, which was about 1.7 times higher than that in the unheated milk (174.6 nm). The increases in the casein micelle size in heated SSM, especially at 85 and 90°C, were generally in agreement with the result reported by Raynal and Remeuf (1998), who showed that the casein micelle size increased from its initial value by 75% in SSM heated for 1 min at 85 and 90°C.

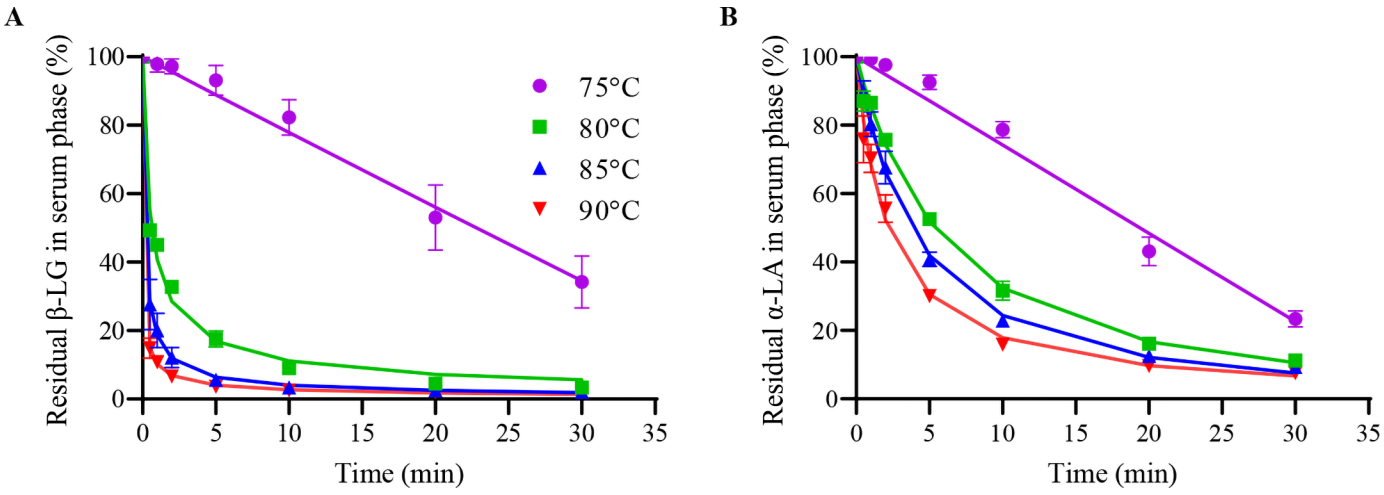

Figure 3. Percentages of residual β-LG (A) and α-LA (B) in the serum phase of sheep skim milk heated at different temperatures for different holding times. The lines represent the predicted associations of denatured β-LG and α-LA with the casein micelles from the model. Each data point represents the mean ± SD of the results from 3 different batches of sheep milk.

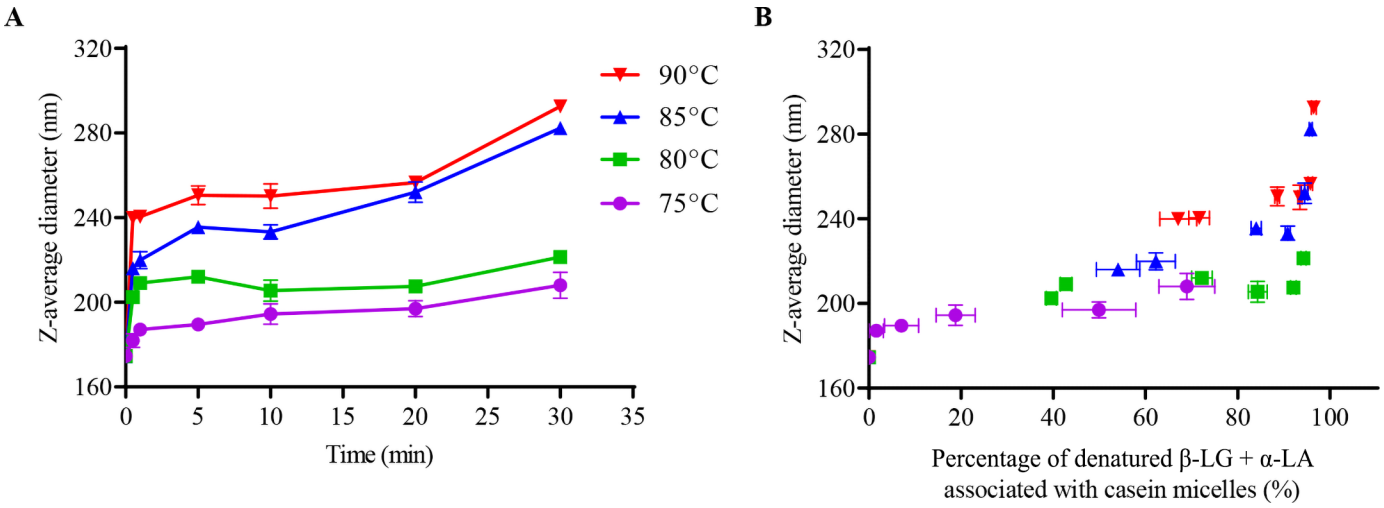

Figure 4. (A) Effect of heating temperature and time on the average size (Z-average diameter) of the particles in sheep skim milk; (B) relationship between the percentage of denatured whey proteins (β-LG + α-LA) associated with the casein micelles and the casein micelle size in heated sheep skim milk at different temperatures. Each data point represents the mean ± SD of the results from 3 different batches of sheep milk.

Relationship Between Casein Micelle Size and Association of Denatured Whey Proteins with Casein Micelles

The relationship between the casein micelle diameter (the particle size of sheep milk) and the percentage of denatured whey proteins (β-LG and α-LA) associated with the casein micelles in heated SSM is shown in Figure 4B. For all heating temperatures, the size of particles in the SSM increased slowly and steadily as the percentage of denatured whey proteins associated with the casein micelles increased until the association level reached almost 95%, with a maximum increase of about 25 to 30% for the average casein micelle diameter compared with that in unheated SSM. However, different particle sizes were observed when the heated SSM had similar percentages of denatured whey proteins associated with the casein micelles (Figure 4B). For instance, when the association level was about 70%, the particle size was significantly higher ($P < 0.01$) for the SSM heated at 90°C (about 240 nm) than for that heated at 75°C (about 208 nm). One possible explanation is that the aggregation of the casein micelles and the association of the whey proteins with the casein micelles occurred simultaneously during heating. Previous studies have stated that it is unclear whether the changes in the casein micelle size of cow milk during heating are due exclusively to the association of the denatured whey proteins with the casein micelles or are also due to the partial aggregation of the casein micelles that occurs at the same time as the whey proteins associating with the casein micelles (Anema and Li, 2003; Anema, 2007). Additionally, aggregation of the casein micelles might occur to different extents at different temperatures. Therefore, the different sizes of the casein micelles with the similar association level found in the present study may be a consequence of aggregation of the casein micelles to different extents under different heating conditions. Another possible explanation is that the denatured whey proteins formed larger aggregates at higher heating temperatures and subsequently associated with the casein micelles (Oldfield et al., 1998b), resulting in different particle sizes of the casein micelles/whey protein complex with the similar association level.

The particle size increased markedly by 70% compared with unheated SSM when the association level exceeded about 95% (Figure 4B), indicating that the casein micelle size increased significantly with little further association of the denatured whey proteins with the casein micelles. Previous studies on cow milk have noted that moderate heat-induced increases in casein micelle size are caused mainly by the association of denatured whey proteins with the casein micelles (Anema, 2007), but that the casein micelle size of milk samples heated at 75 to 90°C for 0.5 to 30 min would not increase by more than 30% compared with that in unheated cow milk at natural pH (Raynal and Remeuf, 1998; Anema, 2018; Li et al., 2019). For example, Anema and Li (2003) reported that heat treatment (75–100°C for up to 60 min) of cow skim milk resulted in a maximum of about 80% of the whey proteins associating with the casein micelles, leading to a maximum of only about a 15% increase in the casein micelle size. These reports

on heat-treated cow skim milk suggest that, even if all the whey proteins associated with the casein micelles, the increase in the casein micelle size of heated milk would not exceed 30% compared with unheated cow milk (Raynal and Remeuf, 1998; Anema, 2008). Moreover, the ratio of caseins to whey proteins (3.7:1) in SSM, presented here (Table 1), is close to that in cow milk (4:1) (Anema, 2021). It can thus be concluded that the denatured whey proteins associating with the casein micelles cannot induce such marked increases in the size of the casein micelles in heated SSM. Therefore, the significant increases in the casein micelle size in heated SSM when the extent of association exceeded about 95% could have been caused by aggregation of the casein micelles. The mechanism of casein micelle aggregation in this temperature range (75–90°C) is not clear. It is possible that heating SSM at 85 and 90°C might induce casein (particularly κ-CN) dissociation from the micelles, creating unstable hydrophobic areas on the surface of the casein micelles so that other casein micelles might be able to get closer and aggregate (Van Hooydonk et al., 1987). Additionally, the protein content was higher in the SSM (Table 1) than in cow skim milk, which may result in more micelle–micelle interactions during heating and thus more aggregation of the casein micelles (Singh and Creamer, 1991). An alternative explanation is that the large whey protein aggregates at the surface of the casein micelles at high temperatures tend to form bridges between the casein micelles at higher protein content. However, further studies to investigate how aggregation of the casein micelles occurs during the heating of SSM are needed.

Microstructural Changes in Casein Micelles

Figure 5 presents the microstructures of the casein micelles in unheated SSM and SSM heated at 75 to 90°C for 30 min. In unheated samples, the casein micelles had mainly a spherical structure with a smooth outline and were separated from neighboring micelles (Figure 5A). When the SSM was heated at 75°C for 30 min, the casein micelle structure showed a similar appearance to that in the unheated SSM, but some irregular projections associated with the surface of the casein micelles were observed (Figure 5B). This suggested that denatured whey proteins had associated with the casein micelles. At 80°C, a greatly increased number of irregular projections surrounded the surface of the micelles (Figure 5C). Similar changes were also found in the SSM heated at 85 and 90°C for 30 min (Figures 5D and 5E). In addition, large numbers of casein micelles with different sizes appeared to aggregate together when the SSM was heated at 85 to 90°C for 30 min (Figures 5D and 5E). This observation is in line with the findings presented above; that is, aggregation of the casein micelles occurred synchronously with association of the whey proteins with the casein micelles. Therefore, this observation further supports that the

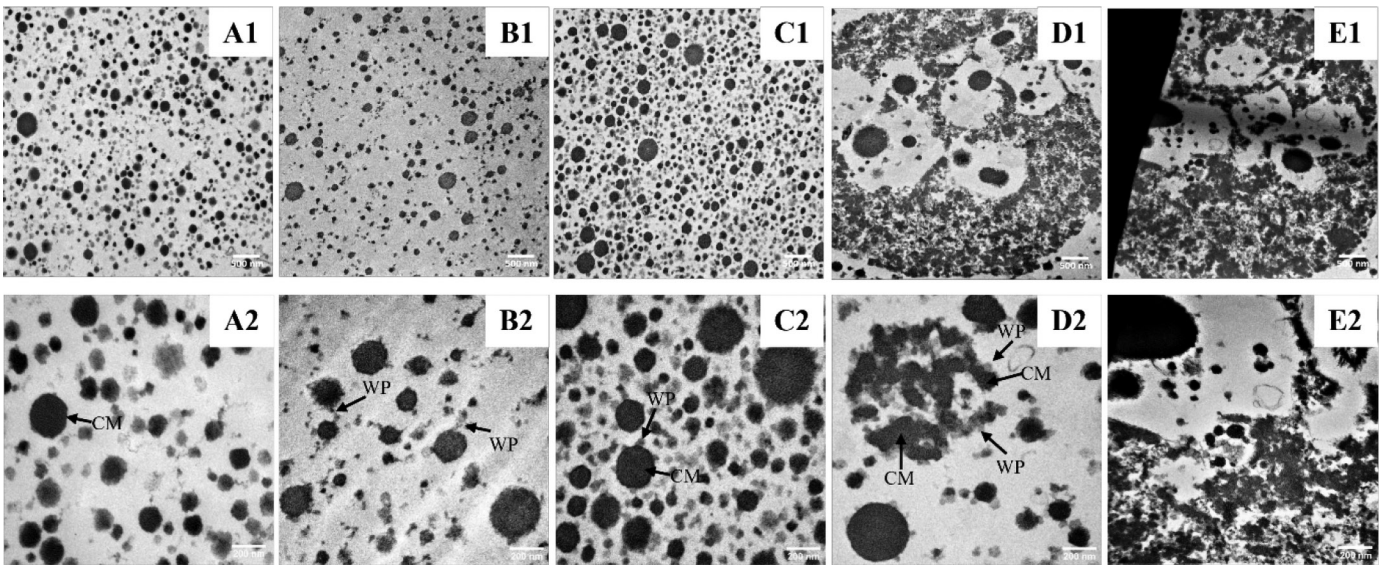

Figure 5. Transmission electron micrographs of the proteins in unheated sheep skim milk (A1 and A2) and sheep skim milk heated for 30 min at 75°C (B1 and B2), 80°C (C1 and C2), 85°C (D1 and D2), and 90°C (E1 and E2). The scale bars represent 500 nm (upper row) and 200 nm (lower row). CM = casein micelle; WP = whey protein.

marked increase in the casein micelle size of SSM at 85 to 90°C is due to both aggregation among the casein micelles and association of denatured whey proteins with the casein micelles.

CONCLUSIONS

In general, this study confirmed that the casein micelle size in SSM increased markedly under heat treatment (75–90°C for 0.5–30 min); the kinetics of the denaturation of β-LG and α-LA and of the associations of β-LG and α-LA with the casein micelles were explored. Kinetic analysis showed that the denaturation processes for both β-LG and α-LA in SSM were similar to those reported for cow milk. However, a break in the Arrhenius plot was observed at the lower temperature of 80°C for the denaturation of β-LG in SSM, compared with those reported for cow milk, which may have been due to the different gene sequences and structures of β-LG. The kinetic parameters showed that the β-LG in SSM denatured and associated with the casein micelles at a faster rate than α-LA in the temperature range 80–90°C. The microstructural changes in the casein micelles and the relationship between the casein micelle size and the level of denatured whey proteins associated with the casein micelles confirmed that the increases in casein micelle size could be attributed to the denatured whey proteins associating with the casein micelles and to aggregation of the casein micelles. Further study to validate how the casein micelles form large aggregates and their relationship with the heat stability of sheep milk should be carried out.

ACKNOWLEDGMENTS

This study was supported by Ministry of Business, Innovation and Employment–New Zealand Milks Mean More (NZ3M), Massey University–Doctoral Scholarship, New Zealand, and the Riddet Institute Centre of Research Excellence (CoRE) funded by the Tertiary Education Commission. The authors acknowledge the support of Neer Enterprises Limited (Carterton, New Zealand) for supplying the sheep milk, and Yanyu He and Raoul Solomon of Manawatu Microscopy and Imaging Centre, New Zealand, for training and providing access to transmission electron microscopy. The authors also thank Siqi Li (Riddet Institute, Massey University, Palmerston North, New Zealand) for his insightful discussion when analyzing the data and Claire Woodhall (Havelock North, New Zealand) for proofreading the manuscript. The authors have not stated any conflicts of interest.

REFERENCES

Akkerman, M., V. M. Rauh, M. Christensen, L. B. Johansen, M. Hammershøj, and L. B. Larsen. 2016. Effect of heating strategies on whey protein denaturation—Revisited by liquid chromatography quadrupole time-of-flight mass spectrometry. J. Dairy Sci. 99:152–166. https://doi.org/10.3168/jds.2015-9924.

Anema, S. G. 2007. Role of κ-casein in the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. J. Agric. Food Chem. 55:3635–3642. https://doi.org/10.1021/jf062734m.

Anema, S. G. 2008. On heating milk, the dissociation of κ-casein from the casein micelles can precede interactions with the denatured whey proteins. J. Dairy Res. 75:415–421. https://doi.org/10.1017/S0022029908003555.

Anema, S. G. 2018. Effect of whey protein addition and pH on the acid gelation of heated skim milk. Int. Dairy J. 79:5–14. https://doi.org/10.1016/j.idairyj.2017.11.008.

Anema, S. G. 2020. The whey proteins in milk: thermal denaturation, physical interactions, and effects on the functional properties of milk. Pages 325–384 in Milk Proteins. 3rd ed. M. Boland and H. Singh, ed. Academic Press.

Anema, S. G. 2021. Heat-induced changes in caseins and casein micelles, including interactions with denatured whey proteins. Int. Dairy J. 122:105136. https://doi.org/10.1016/j.idairyj.2021.105136.

Anema, S. G., S. K. Lee, and H. Klostermeyer. 2006. Effect of protein, nonprotein-soluble components, and lactose concentrations on the irreversible thermal denaturation of β-lactoglobulin and α-lactalbumin in skim milk. J. Agric. Food Chem. 54:7339–7348. https://doi.org/10.1021/jf061508+.

Anema, S. G., and Y. Li. 2000. Further studies on the heat-induced, pH-dependent dissociation of casein from the micelles in reconstituted skim milk. Lebensm. Wiss. Technol. 33:335–343. https://doi.org/10.1006/fstl.2000.0665.

Anema, S. G., and Y. Li. 2003. Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. J. Dairy Res. 70:73–83. https://doi.org/10.1017/S0022029902005903.

Anema, S. G., and A. B. McKenna. 1996. Reaction kinetics of thermal denaturation of whey proteins in heated reconstituted whole milk. J. Agric. Food Chem. 44:422–428. https://doi.org/10.1021/jf950217q.

Baer, A., M. Oroz, and B. Blanc. 1976. Serological studies on heat-induced interactions of α-lactalbumin and milk proteins. J. Dairy Res. 43:419–432. https://doi.org/10.1017/S0022029900016009.

Balthazar, C. F., T. C. Pimentel, L. L. Ferrão, C. N. Almada, A. Santillo, M. Albenzio, N. Mollakhalili, A. M. Mortazavian, J. S. Nascimento, M. C. Silva, M. Q. Freitas, A. S. Sant'Ana, D. Granato, and A. G. Cruz. 2017. Sheep milk: Physicochemical characteristics and relevance for functional food development. Compr. Rev. Food Sci. Food Saf. 16:247–262. https://doi.org/10.1111/1541-4337.12250.

Bobe, G., D. C. Beitz, A. E. Freeman, and G. L. Lindberg. 1998. Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. J. Agric. Food Chem. 46:458–463. https://doi.org/10.1021/jf970499p.

Dalgleish, D. G., and M. Corredig. 2012. The structure of the casein micelle of milk and its changes during processing. Annu. Rev. Food Sci. Technol. 3:449–467. https://doi.org/10.1146/annurev-food-022811-101214.

Dannenberg, F., and H.-G. Kessler. 1988. Reaction kinetics of the denaturation of whey proteins in milk. J. Food Sci. 53:258–263. https://doi.org/10.1111/j.1365-2621.1988.tb10223.x.

Donato, L., and D. G. Dalgleish. 2006. Effect of the pH of heating on the qualitative and quantitative compositions of the sera of reconstituted skim milks and on the mechanisms of formation of soluble aggregates. J. Agric. Food Chem. 54:7804–7811. https://doi.org/10.1021/jf060961i.

Dumitraşcu, L., E. Moschopoulou, I. Aprodu, S. Stanciu, G. Râpeanu, and N. Stănciuc. 2013. Assessing the heat induced changes in major cow and non-cow whey proteins conformation on kinetic and thermodynamic basis. Small Rumin. Res. 111:129–138. https://doi.org/10.1016/j.smallrumres.2012.12.019.

Elfagm, A. A., and J. V. Wheelock. 1978. Heat interaction between α-lactalbumin, β-lactoglobulin and casein in bovine milk. J. Dairy Sci. 61:159–163. https://doi.org/10.3168/jds.S0022-0302(78)83572-3.

Erhardt, G., J. Godovac-Zimmermann, and A. Conti. 1989. Isolation and complete primary sequence of a new ovine wild-type β-lactoglobulin C. Biol. Chem. Hoppe Seyler 370:757–762. https://doi.org/10.1515/bchm3.1989.370.2.757.

Farrell, H. M. Jr., E. L. Malin, E. M. Brown, and A. Mora-Gutierrez. 2009. Review of the chemistry of α_{S2}-casein and the generation of a homologous molecular model to explain its properties. J. Dairy Sci. 92:1338–1353. https://doi.org/10.3168/jds.2008-1711.

Kessler, H.-G., and H.-J. Beyer. 1991. Thermal denaturation of whey proteins and its effect in dairy technology. Int. J. Biol. Macromol. 13:165–173. https://doi.org/10.1016/0141-8130(91)90043-T.

Law, A. 1995. Heat denaturation of bovine, caprine and ovine whey proteins. Milchwissenschaft 50:384–388.

Law, A. J. R., and J. Leaver. 1997. Effect of protein concentration on rates of thermal denaturation of whey proteins in milk. J. Agric. Food Chem. 45:4255–4261. https://doi.org/10.1021/jf970242r.

Law, A. J. R., and J. Leaver. 2000. Effect of pH on the thermal denaturation of whey proteins in milk. J. Agric. Food Chem. 48:672–679. https://doi.org/10.1021/jf981302b.

Li, S., A. Ye, and H. Singh. 2019. Seasonal variations in composition, properties, and heat-induced changes in bovine milk in a seasonal calving system. J. Dairy Sci. 102:7747–7759. https://doi.org/10.3168/jds.2019-16685.

Loch, J. I., M. Molenda, M. Kopeć, S. Świątek, and K. Lewiński. 2014. Structure of two crystal forms of sheep β-lactoglobulin with EF-loop in closed conformation. Biopolymers 101:886–894. https://doi.org/10.1002/bip.22471.

Mastrangelo, S., M. T. Sardina, V. Riggio, and B. Portolano. 2012. Study of polymorphisms in the promoter region of ovine β-lactoglobulin gene and phylogenetic analysis among the Valle del Belice breed and other sheep breeds considered as ancestors. Mol. Biol. Rep. 39:745–751. https://doi.org/10.1007/s11033-011-0794-2.

Mittal, V. A., A. Ellis, A. Ye, S. Das, and H. Singh. 2015. Influence of calcium depletion on iron-binding properties of milk. J. Dairy Sci. 98:2103–2113. https://doi.org/10.3168/jds.2014-8474.

Muir, D., D. Horne, A. R. Law, and A. M. Sweetsur. 1993. Ovine milk. II: Seasonal changes in indices of stability. Milchwissenschaft 48:442–445.

Oldfield, D. J., H. Singh, and M. W. Taylor. 1998a. Association of β-lactoglobulin and α-lactalbumin with the casein micelles in skim milk heated in an ultra-high temperature plant. Int. Dairy J. 8:765–770. https://doi.org/10.1016/S0958-6946(98)00127-7.

Oldfield, D. J., H. Singh, and M. W. Taylor. 2005. Kinetics of heat-induced whey protein denaturation and aggregation in skim milks with adjusted whey protein concentration. J. Dairy Res. 72:369–378. https://doi.org/10.1017/S002202990500107X.

Oldfield, D. J., H. Singh, M. W. Taylor, and K. N. Pearce. 1998b. Kinetics of denaturation and aggregation of whey proteins in skim milk heated in an ultra-high temperature (UHT) pilot plant. Int. Dairy J. 8:311–318. https://doi.org/10.1016/S0958-6946(98)00089-2.

Park, Y. W., M. Juárez, M. Ramos, and G. F. W. Haenlein. 2007. Physico-chemical characteristics of goat and sheep milk. Small Rumin. Res. 68:88–113. https://doi.org/10.1016/j.smallrumres.2006.09.013.

Patel, H. A., H. Singh, S. G. Anema, and L. K. Creamer. 2006. Effects of heat and high hydrostatic pressure treatments on disulfide bonding interchanges among the proteins in skim milk. J. Agric. Food Chem. 54:3409–3420. https://doi.org/10.1021/jf052834c.

Rasmussen, L. K., P. Hojrup, and T. E. Petersen. 1992. Localization of two interchain disulfide bridges in dimers of bovine α_{S2}-casein. Parallel and antiparallel alignments of the polypeptide chains. Eur. J. Biochem. 203:381–386. https://doi.org/10.1111/j.1432-1033.1992.tb16561.x.

Raynal-Ljutovac, K., Y. W. Park, F. Gaucheron, and S. Bouhallab. 2007. Heat stability and enzymatic modifications of goat and sheep milk. Small Rumin. Res. 68:207–220. https://doi.org/10.1016/j.smallrumres.2006.09.006.

Raynal, K., and F. Remeuf. 1998. The effect of heating on physico-chemical and renneting properties of milk: A comparison between caprine, ovine and bovine milk. Int. Dairy J. 8:695–706. https://doi.org/10.1016/S0958-6946(98)00112-5.

Sharma, S. K., and D. G. Dalgleish. 1994. Effect of heat treatments on the incorporation of milk serum proteins into the fat globule membrane of homogenized milk. J. Dairy Res. 61:375–384. https://doi.org/10.1017/S002202990003079X.

Singh, H., and L. K. Creamer. 1991. Influence of concentration of milk solids on the dissociation of micellar κ-casein on heating reconstituted milk at 120°C. J. Dairy Res. 58:99–105. https://doi.org/10.1017/S0022029900033549.

Staiger, E. A., M. L. Thonney, J. W. Buchanan, E. R. Rogers, P. A. Oltenacu, and R. G. Mateescu. 2010. Effect of prolactin, β-lactoglobulin, and κ-casein genotype on milk yield in East Friesian sheep. J. Dairy Sci. 93:1736–1742. https://doi.org/10.3168/jds.2009-2630.

Van Hooydonk, A., P. De Koster, and I. Boerrigter. 1987. The renneting properties of heated milk. Neth. Milk Dairy J. 41:3–18.

Vasbinder, A. J., A. C. Alting, and K. G. de Kruif. 2003. Quantification of heat-induced casein–whey protein interactions in milk and its relation to gelation kinetics. Colloids Surf. B Biointerfaces 31:115–123. https://doi.org/10.1016/S0927-7765(03)00048-1.

Wijayanti, H. B., A. Brodkorb, S. A. Hogan, and E. G. Murphy. 2019. Thermal denaturation, aggregation, and methods of prevention. Pages 185–247 in Whey Proteins. H. Deeth and N. Bansal, ed. Academic Press.

Ye, A., J. Cui, D. Dalgleish, and H. Singh. 2016. The formation and breakdown of structured clots from whole milk during gastric digestion. Food Funct. 7:4259–4266. https://doi.org/10.1039/C6FO00228E.

ORCIDS

Zheng Pan https://orcid.org/0000-0002-9208-8358
Aiqian Ye https://orcid.org/0000-0003-1048-8858
Karl Fraser https://orcid.org/0000-0002-1136-4024
Harjinder Singh https://orcid.org/0000-0002-8807-3241