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Pressure-Induced Denaturation of β-Lactoglobulin in Skim Milk: Effect of Milk Concentration

Skelte G. Anema*

Fonterra Research Centre, Private Bag 11029, Dairy Farm Road, Palmerston North, New Zealand

ABSTRACT: The effect of milk concentration (10−40% TS) on the kinetics of the pressure-induced denaturation of β-lactoglobulin (β-LG) was studied. The denaturation was found to be a second-order process at all milk concentrations and pressures. There was a change in pressure dependence of the rate constants for denaturation at about 300 MPa, and this effect became more pronounced as the milk concentration increased. At pressures ≥300 MPa, a small effect of milk concentration was observed, with small decreases in the rate of denaturation as the milk concentration was increased above 20% TS. This was attributed to the lower pH as the milk concentration was increased. In contrast, at 200 MPa, β-LG denaturation was markedly retarded as the milk solids concentration was increased. This was attributed to the increased lactose concentration at higher milk concentrations. This would promote β-LG dimerization at this pressure and this would stabilize the β-LG to denaturation.

KEYWORDS: *high pressure, milk, concentration, β-lactoglobulin, denaturation, kinetics*

INTRODUCTION

When milk is pressure treated, many changes can occur, and these changes have been well documented in numerous review papers.[1−3] One pressure-induced change to milk that is of particular interest is the denaturation of the whey proteins and their subsequent aggregation reactions (irreversible denaturation). Irreversible denaturation of the whey proteins are key reactions leading to changes in the functionality of the milk.

Of the major whey proteins, α-lactalbumin (α-LA), bovine serum albumin (BSA), the immunoglobulins, and lactoferrin are reasonably resistant to irreversible denaturation, requiring pressures in excess of 500 MPa and extended holding times to induce significant levels of denaturation/aggregation.[1,2,4,5] In contrast, β-lactoglobulin (β-LG) is denatured at much lower pressures and the denaturation reaction is considered to be a multistage process.[1,6] Structural changes are observed at pressures as low as 100 MPa,[1,7−9] with significant denaturation/aggregation occurring at pressures of 200 MPa or higher.[4,5,10] The pressure lability of β-LG compared with the other whey proteins (and other typical globular proteins) is probably a consequence of the hydrophobic calyx in the tertiary structure. As pressure favors processes that minimize volume, the presence of a cavity in the structure would be unfavorable.[1,6]

When evaluating the kinetics for the pressure-induced denaturation of β-LG in milk, an unusual dependence on pressure was observed. At pressures below 300 MPa, the denaturation process had higher activation volumes (V_a) than at higher pressures and was less affected by the temperature at pressurization.[10] These results indicate that the pressure-induced irreversible denaturation of β-LG, like the heat-induced denaturation reactions,[11,12] has a complex multistep mechanism and may involve competing rate determining steps.[10]

For the thermal denaturation reactions, changing the concentration of the milk or individual components provided information that helped understand the mechanisms involved in the denaturation processes.[13−16] It is expected that similar systematic studies on changing milk concentration and composition will provide information useful in determining the mechanisms involved in the pressure-induced denaturation reactions. This study was therefore undertaken to examine the effect of milk concentration on the pressure-induced denaturation of β-LG. The study was conducted over a sufficiently wide pressure/time range to allow comparative kinetic studies to be completed.

EXPERIMENTAL SECTION

Milk Supply. A low heat skim milk powder (Fonterra Co-operative Group, New Zealand) was used in all experiments. This milk powder was prepared with minimal heating during manufacture and was experimentally found to have less than 2% denatured β-LG (results not shown). Reconstituted skim milk samples of 10−40% total solids (w/w) were prepared by adding skim milk powder to purified water. A small quantity (0.02%) of sodium azide was added to each of the milk samples as a preservative. The milk samples were stirred for at least 12 h at ambient temperatures (about 20 °C) before further use.

Pressure Treatment. Samples of skim milk were transferred to 3.5 mL tubes (Polyallomar Quick-Seal centrifuge tubes, Beckman Instruments Inc., Palo Alto, CA), heat sealed, and pressure treated at 200−700 MPa for times from 0 to 60 min and at 20 °C in a Stansted Fluid Power high-pressure food processor (Stansted Fluid Power Ltd., Stansted, Essex, UK), as has been described in detail previously.[10] During an experimental run, the temperature change during the pressurization, holding, and depressurization steps was monitored and the changes in temperature were similar to those described previously.[10] After pressure treatment, the samples were stored at room temperature for 24 h before analysis.

Polyacrylamide Gel Electrophoresis. The concentrated milk samples were accurately diluted with water to concentrations directly comparable with the 10% TS milk sample. The casein and the denatured whey proteins were precipitated by adding one part of milk to one part of sodium acetate buffer (0.2 M, pH 4.0), which adjusts the

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milk to pH 4.6. The precipitated casein and denatured whey protein were removed by centrifuging at 14000*g* for 5 min.

The supernatants samples were analyzed for native whey proteins using native-polyacrylamide gel electrophoresis. This method uses no dissociating or reducing agents and therefore separates only the monomeric "native" protein remaining in solution. The supernatant and milk samples were accurately diluted, by weight, with sample buffer. The electrophoresis, staining, and destaining were performed as previously described.[10,11,13] The gels were scanned and integrated using a Molecular Dynamics model PD computing densitometer and the associated Imagequant integration software (Molecular Dynamics Inc., Sunnyvale, CA). Integration was performed using a "volume integration method" where the intensity of each pixel in a desired band is summed to give the total band intensity. The changes in β-LG as a consequence of the pressure treatment were determined by comparing the residual β-LG band intensities of the pressure-treated milk samples with the β-LG band intensity of the average of at least two control samples.

Replicates and Statistical Evaluation. All samples were prepared, pressure treated, and analyzed on two occasions, and selected samples from each trial were also analyzed in duplicate. Error bars that represent the standard deviations of the repeated measurements are presented. A statistical analysis of key results was performed by an analysis of variance method using the EZAnalyze statistical analysis program,[17] and any results reported as significantly different had a $P \leq 0.05$.

RESULTS

In this study, it is the irreversible denaturation of β-LG that is monitored and this encompasses all possible reactions that result in a loss of the native protein but does not include β-LG that has refolded to be indistinguishable from the original native protein. This irreversible denaturation is monitored by measuring the level of β-LG remaining after a defined treatment, with comparisons to the level in untreated control samples.

Irreversible Denaturation of β-LG. Milk samples were pressure treated from 200 to 700 MPa at 20 °C for times up to about 60 min. After pressurization, the samples were analyzed for residual native proteins by native-PAGE. Selected gels are shown in Figure 1, and selected electrophoresis traces are shown in Figure 2. The electrophoresis traces are for illustrative purposes only; they were not used for integration. The gels and electrophoresis traces show the separation achieved for the various whey proteins in the milk as well as a visual impression of the loss of the proteins as a consequence of the pressure treatment. It is evident that the α-LA (peak 3) and BSA (peak 4) are hardly affected by the pressure treatment with no changes at 200 MPa (Figures 1 and 2A) and only small decreases at 600 MPa (Figures 1 and 2B). It appeared that at 600 MPa, both α-LA and BSA denatured somewhat faster at the lower milk concentrations than at the higher milk concentrations. The high barostability of α-LA[4,5,18] and BSA[18] is consistent with literature reports.

In contrast, both the A variant (peak 1) and B variant (peak 2) of β-LG were denatured by pressure treatment at both 200 MPa (Figures 1 and 2A) and 600 MPa (Figures 1 and 2B). The observation that β-LG is denatured at pressures of 200 MPa or above is consistent with literature reports.[4,5,10] From Figurse 1 and 2, it is evident that the β-LG denatured more extensively at low milk concentrations than at high milk concentrations, and these effects appeared more pronounced at 200 MPa than at 600 MPa. One further observation from the gels and electrophoretic traces is that at 200 MPa, the B variant of β-LG appeared to denature more rapidly than the A variant as the

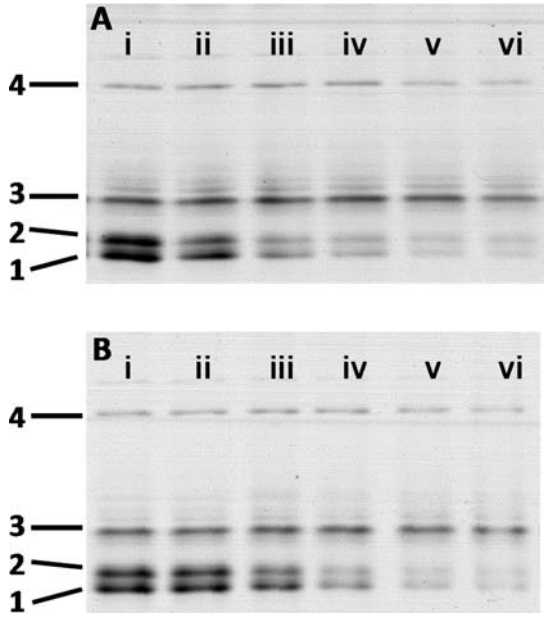

Figure 1. (A) Native PAGE patterns for 10% TS milk samples pressure treated at 200−600 MPa for 60 min. (B) Native PAGE patterns for 40% TS milk samples pressure treated at 200−600 MPa for 60 min. The samples in each lane were: (i) untreated milk or milks treated at (ii) 200 MPa, (iii) 300 MPa, (iv) 400 MPa, (v) 500 MPa, (vi) 600 MPa. The bands identified are (1) β-LG A, (2) β-LG B, (3) α-LA, and (4) BSA.

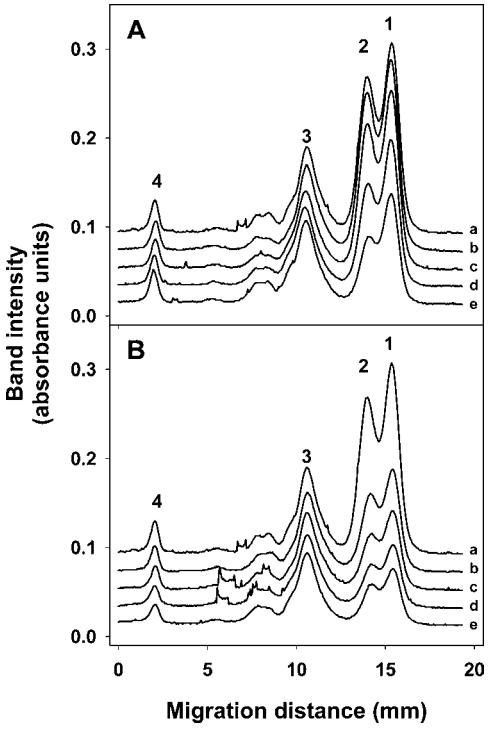

Figure 2. Electrophoretic traces for the native whey proteins from pressure treated skim milk with comparison to untreated skim milk: (A) 200 MPa for 60 min or (B) 600 MPa for 15 min. The skim milk samples were: (a) untreated 10% TS skim milk (control) or pressure treated skim milks of (b) 40% TS, (c) 30% TS, (d) 20% TS, or (e) 10% TS. All milks were diluted after pressure treatment to be comparable to the 10% TS control skim milk. The identified peaks are (1) β-LG A, (2) β-LG B, (3) α-LA, and (4) BSA.

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relative intensities and peak heights the two variants changed as the β-LG denatured especially at the lower milk concentrations (Figures 1 and 2A). At 600 MPa, the two variants appeared to denature at similar rates as the band intensities and relative peak heights were similar in all samples at this pressure (Figures 1 and 2B).

The two variants of β-LG differ through two amino acid substitutions where the Asp^{64} and Val^{118} in the A variant are replaced by Gly^{64} and Ala^{118} in the B variant. Botelho et al.[7] calculated that the replacement of the Val by Ala at position 118 would introduce a small cavity in the B variant structure and suggested that the presence of this cavity explained the higher pressure sensitivity of the B variant when compared with the A variant. As the cavity is small, it may only have a significant effect on denaturation at low pressures where denaturation is slower (Figures 1 and 2).

Representative denaturation curves for the A variant of β-LG after the various pressure treatments are shown in Figure 3. Similar results were obtained for the B variant (results not shown). The level of denaturation of both variants of β-LG was dependent on the magnitude of the pressure, the duration of the pressure treatment, and the concentration of the milk (Figures 1−3). The level of denaturation increased when the pressure was increased (at a given holding time and milk concentration), when the holding time was increased (at a given pressure and milk concentration), and at lower milk concentrations (at a given pressure and holding time). At a pressure of 200 MPa, the level of β-LG denaturation was lower as the milk concentration was increased from 10 to 40% TS; however, at pressures ≥300 MPa, the denaturation of both variants of β-LG were similar at milk concentrations up to 20% TS and was only reduced when the milk concentration was above 20% TS (Figures 1−3).

Reaction Order for the Denaturation of β-LG. The reaction order for the pressure-induced denaturation of β-LG was determined using a similar process to that reported previously.[4,10] The denaturation of β-LG, as shown for selected samples in Figure 3, were analyzed using eqs 1 and 2 to determine the most appropriate reaction order (best linear fit), and it was found that the pressure-induced irreversible denaturation reactions for both variants were best described with a reaction order of 2.0 when all pressures and milk concentrations were considered (selected results for β-LG A are shown in Figure 4). This reaction order is consistent with literature reports for the pressure induced denaturation of β-LG in skim milk at its natural concentration.[4,10]

$$\ln(C_t/C_0) = -kt \quad (\text{when } n = 1) \tag{1}$$

$$(C_t/C_0)^{1-n} = 1 + (n-1)k(C_0)^{n-1}t \quad (\text{when } n \neq 1) \tag{2}$$

(where n = reaction order, k = rate constant, C_0 = initial native protein concentration, and C_t = concentration of native protein at time t).

The rate constants (k) were obtained from the slopes of the straight lines, examples of which are shown in Figure 4. When the $\ln(k)$'s were plotted against the pressure used, linear relationships were obtained within certain pressure ranges; however, a marked change in dependence was observed at about 300 MPa for both variants of β-LG (Figure 5). A statistical analysis of the results where several measurements at selected pressures were performed showed that, for both variants of β-LG, the rate constants obtained at 200 MPa

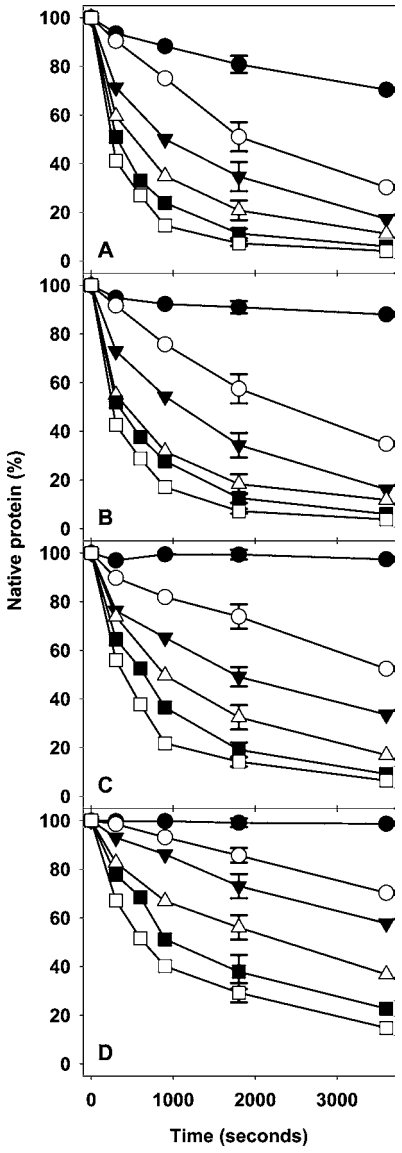

Figure 3. Denaturation of β-LG A in pressure treated skim milk samples. The milk concentrations were: (A) 10% TS, (B) 20% TS, (C) 30% TS, and (D) 40% TS. Milk samples were treated at pressures of: (●) 200 MPa, (○) 300 MPa, (▼) 400 MPa, (△) 500 MPa, (■) 600 MPa, and (□) 700 MPa. Error bars on selected points represent the standard deviations of duplicate measurements.

significantly decreased as the milk concentration was increased from 10 to 40% TS. In contrast, in the 300−700 MPa range, at any given pressure the rate constants for the 10−20% TS milk samples were not significantly different from each other, whereas at higher milk concentrations, the rate constants significantly decreased as the milk concentration increased from 20 to 40% TS.

This unusual change in pressure dependence was subjected to a sensitivity analysis to indicate what level of β-LG denaturation would be required in order to shift the rate constants at 200 MPa to be on the same line as that observed at 300−700 MPa (Figure 5). For the 10% TS milk and for β-LG A, $\ln(k)$ would need to increase from about −9.0 to about −8.0. This would require the level of native protein remaining after pressure treatment for 60 min to decrease from about 70% to about 50%. Similarly, for the 30%TS milk, $\ln(k)$ would need to increase from about −11.9 to about −9.0, and this would

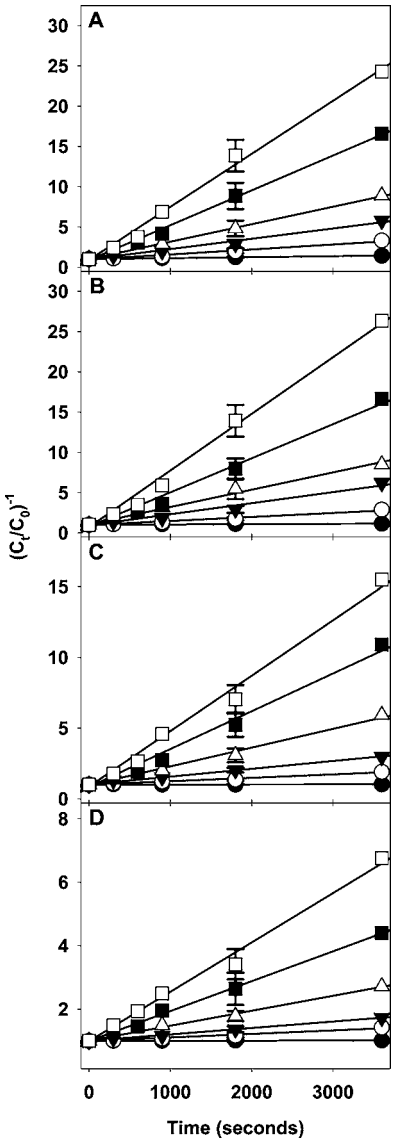

Figure 4. Denaturation of β-LG A as a reaction order of 2.0. The milk concentrations were: (A) 10% TS, (B) 20% TS, (C) 30% TS, and (D) 40% TS. Milk samples were treated at pressures of: (●) 200 MPa, (○) 300 MPa, (▼) 400 MPa, (△) 500 MPa, (■) 600 MPa, and (□) 700 MPa. Error bars on selected points represent the standard deviations of duplicate measurements.

require the level of native β-LG remaining after pressure treatment for 60 min to decrease from over 95% to less than 70%. These are very marked changes in denaturation levels and, as the method for analyzing native whey protein had an error typically less than 5%, it appears very unlikely that change in behavior of the denaturation β-LG at about 300 MPa can be attributed to errors in the experimental procedures.

Calculation of Activation Volumes, V_a. As the plots of $\ln(k)$ against pressure were linear within the two pressure ranges (Figure 5), the V_a values could be calculated using eq 3. As only two data points were available for the low pressure range (200−300 MPa), the calculated V_a should be considered as an "apparent V_a" based on this limited data and only gives an indication of the changes with increasing milk concentrations. The V_a and the frequency factors ($\ln(k_0)$) are presented in Table 1. The V_a was negative at all milk concentrations and in both pressure ranges. This indicates that the denaturation of β-LG will be favored under pressure, as is experimentally observed. In the low pressure range (200−300 MPa), the V_a for both variants of β-LG decreased markedly as the milk concentration increased from 10 to 40% TS. However, in the high pressure range (300−700 MPa), the V_a for both variants of β-LG were essentially constant (Table 1). The V_a and $\ln(k_0)$ obtained for the 10%TS milks in both pressure ranges were comparable with those in literature reports for milks of similar concentrations.[4,10]

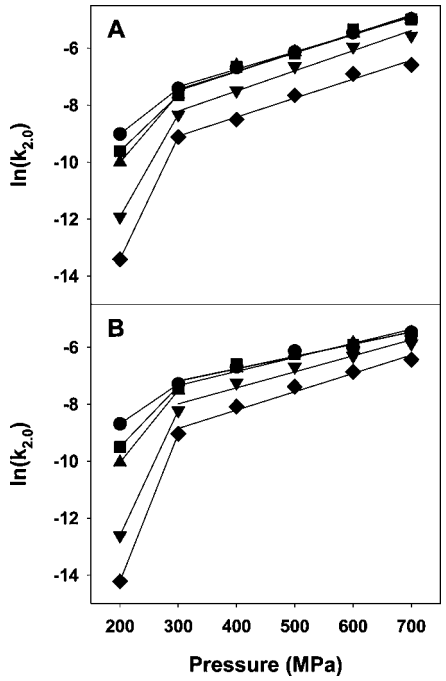

Figure 5. Relationship between $\ln(k)$ and pressure for the denaturation of: (A) β-LG A and (B) β-LG B. The concentration of the milk samples were: (●) 10% TS, (■) 15% TS, (▲) 20% TS, (▼): 30% TS, and (◆) 40% TS.

Table 1. Activation Volumes (V_a) and Frequency Factors ($\ln k_0$) for the Pressure-Induced Denaturation of β-Lactoglobulin

		β-lactoglobulin A		β-lactoglobulin B	
pressure range (MPa)	concentration (% TS)	V_a (mL/mol)	$\ln k_0$	V_a (mL/mol)	$\ln k_0$
200−300	10	−39	−12	−34	−12
200−300	15	−48	−14	−52	−14
200−300	20	−58	−15	−62	−15
200−300	30	−88	−19	−107	−21
200−300	40	−105	−22	−126	−25
300−600	10	−14	−9	−9	−8
300−600	15	−14	−9	−8	−8
300−600	20	−13	−9	−10	−8
300−600	30	−16	−10	−11	−9
300−600	40	−16	−11	−13	−10

$$\ln k = \ln k_0 - pV_a/RT \quad (3)$$

(where k = rate constant, k_0 = frequency factor, R = universal gas constant, T = absolute temperature = 273.15K, p = pressure, and V_a = activation volume.)

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DISCUSSION

The change in dependence of the rate constants with pressure for β-LG denaturation (Figures 1−5) has been reported previously for unconcentrated skim milk.[10] It was suggested that there was a transition from "aggregation" to "unfolding" as the rate determining step as the pressure was increased.[10] Increasing the milk concentration had a markedly greater effect on the denaturation of β-LG in the 200−300 MPa range than at higher pressures (Figures 3−5). When considering the effects of milk concentration on the denaturation of β-LG, the effects of milk components on the interactions involved in maintaining the native structure need to be taken into account. Hydrogen bonding is promoted under high pressure, whereas hydrophobic interactions and ion pairings are diminished under pressure.[19−21] Globular proteins are denatured under sufficiently high pressures as their native conformations involve hydrophobic interactions and ion pairs; however, as the elements of secondary structures involve hydrogen bonds, some secondary structural characteristics may be maintained after pressure-induced denaturation.[20,21]

Increasing the milk concentration will increase the protein, lactose, and the milk mineral concentrations. In addition, the milk pH will decrease from about 6.7 at 10% TS to about 6.2 at 40% TS. All these changes can affect the denaturation of β-LG to some extent, as has been shown for the thermal denaturation of this protein in milk.[14−16]

The pressure-induced irreversible denaturation reaction of β-LG is complex and will involve many consecutive and/or concurrent steps. As described previously,[10] a reaction scheme as shown in eq 4 can be used to discuss the denaturation reactions. The first steps are reversible and involve the dissociation of dimeric β-LG to monomers followed by the unfolding of the monomeric native structure. This is followed by irreversible aggregation reactions that can involve other denatured whey proteins or the casein proteins.

$$(\beta\text{-LG}_n)_2 \rightleftharpoons 2(\beta\text{-LG}_n) \quad (4a)$$

$$\beta\text{-LG}_n \rightleftharpoons \beta\text{-LG}_u \quad (4b)$$

$$x(\beta\text{-LG}_u) \rightarrow (\beta\text{-LG})_x \quad (4c)$$

Increasing sugar concentrations increase the thermal stability of proteins.[14,22,23] This has been attributed to the ordering of water molecules around the protein at high sugar concentrations. This excludes the sugars from the protein environment and therefore increases the free energy of the system[22,23] and reduces the propensity of the native protein to unfold (eq 4b). This may partially account for the lower rate of pressure-induced denaturation of β-LG at higher milk concentrations. This effect has been reported to account for the stabilization of some proteins to pressure when sugars or polyol concentrations are increased[8,24,25] and may account for the increased stability of β-LG in the presence of sucrose.[8]

However, this cannot account for the different effects in the low and high pressure ranges (Figures 3−5, Table 1). The first step in the denaturation of β-LG involves the dissociation of dimers to monomers (eq 4a). Pressures of only a few hundred megapascals can dissociate oligomeric proteins to monomers.[26−28] It has been shown that β-LG dimers are dissociated to monomers at moderate pressures.[9] The dimeric state of β-LG is maintained by hydrophobic interactions, and hydrophobic interactions are less favorable under pressure. Dimeric β-LG has a lower surface area than the monomers, therefore, in the presence of sugars, the dimer state would be more favorable as this would reduce the free energy of the system.[22]

As a result, there are opposing effects as the high sugar concentration would favor dimerization of β-LG, whereas high pressures would make dimerization less favorable. It is possible that at low pressures (<300 MPa) the effect of high sugar concentration dominates and β-LG dimerization is favored and denaturation is substantially retarded as milk concentration increases. However, at higher pressures (≥300 MPa), the hydrophobic interactions are substantially diminished so that dimerization becomes unfavorable and the denaturation is less affected by milk concentration. This hypothesis would also account for the different effects of milk concentration on the V_a in the low and high pressure ranges (Table 1).

In the high pressure range, the denaturation is still retarded at higher milk concentrations, although less so than at low pressures. This could be due to the effect of sugars making the unfolded state less favorable than the native structure, as described above. However, other factors could also be important. For example, increasing milk concentration from 10 to 40% TS will decrease the pH of the milk from ∼pH 6.7 to about pH 6.2. A pH reduction of this magnitude in normal concentration milk retards the pressure-induced denaturation of β-LG.[5,29] For example, Huppertz et al.[5] showed that denaturation level of β-LG after treatment of milk at 400 MPa/30 min decreased from ∼93% to only 60% when the pH was decreased from pH 6.7 to pH 6.2. These changes in pH and denaturation level were comparable to those observed when the milk concentration was increased from 10 to 40% (Figure 3). However, other changes such as increasing the ionic strength, increasing the protein concentrations, and reducing the calcium activity may also affect the denaturation of β-LG. Detailed studies would be required to elucidate the relative importance of different components on pressure-induced denaturation of β-LG, as has been completed for the heat-induced denaturation.[14−16]

In conclusion, this study has shown that β-LG denaturation is retarded as milk concentration is increased, with a greater effect at low pressures (200−300 MPa) than at high pressures. The greater effect at low pressures was attributed to the baroprotective effect of the increased lactose concentration, which would promote β-LG dimerization and thus retard β-LG denaturation. Although the increased lactose concentration could also partially account for the higher stability of β-LG in the higher pressure range, the decreased pH of the concentrated milks is also expected to contribute to this increased stability.

AUTHOR INFORMATION

Corresponding Author

*Phone: +64 (6) 350 4649. Fax: +64 (6) 356 1476. E-mail: skelte.anema@fonterra.com. Address: Fonterra Research Centre Private Bag 11029 Palmerston North New Zealand.

Notes

The authors declare no competing financial interest.

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