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Denaturation of β-Lactoglobulin in Pressure-Treated Skim Milk

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The kinetics of β-lactoglobulin (β-LG) denaturation in pressure-treated reconstituted skim milk samples over a wide pressurization range (100−600 MPa) and at various temperatures (10−40 °C) was studied. Denaturation was extremely dependent on the pressure and duration of treatment. At 100 MPa, no denaturation was observed regardless of the temperature or the holding time. At higher pressures, the level of denaturation increased with an increasing holding time at a constant pressure or with increasing pressure at a constant holding time. At 200 MPa, there was only a small effect of changing the temperature at pressurization. However, at higher pressures, increasing the temperature from 10 to 40 °C markedly increased the rate of denaturation. The two major genetic variants of β-LG (A and B) behaved similarly to pressure treatment, although the B variant appeared to denature slightly faster than the A variant at low pressures (≤400 MPa). The denaturation could be described as a second-order process for both β-LG variants. There was a marked change in pressure dependence at about 300 MPa, which resulted in markedly different activation volumes in the two pressure ranges. Evaluation of the kinetic and thermodynamic parameters suggested that there may have been a transition from an aggregation-limited reaction to an unfolding-limited reaction as the pressure was increased.

KEYWORDS: Milk; high pressure; β-lactoglobulin; denaturation; kinetics

INTRODUCTION

Heat treatment of milk at temperatures above about 70 °C results in the (reversible) denaturation and subsequent irreversible aggregation of whey proteins. Unless otherwise stated, all references to denaturation reactions in this paper are for the irreversible denaturation reactions, which comprise the denaturation process and subsequent aggregation reactions.

These denaturation reactions, especially that of β-lactoglobulin (β-LG), the major whey protein, are of extreme importance to the dairy industry as they can be used to manipulate the physicochemical and functional properties of the resultant dairy products. As a consequence, there have been numerous studies examining the denaturation of whey proteins during the heat treatment of milk, the interactions of the denatured whey proteins with other milk components, and the effect of these reactions on the physical and functional properties of the milk products (*1*−*5*). Full kinetic and thermodynamic studies on whey protein denaturation have been conducted and allow the prediction of the degree of denaturation under most processing conditions (*6*−*8*). Coupling these denaturation studies with those examining the interactions of the denatured whey proteins with other milk components has provided more detailed information that aids in the prediction of the functional properties of milk products based on the compositional properties of the milk and the processing conditions applied (*3*, *4*, *9*−*12*).

Pressure is a fundamental physical state that influences kinetic and thermodynamic parameters and as such is at least as important as temperature. However, experiments examining the properties of milk products at various pressures have been relatively limited, especially when compared with those on the heat treatment of milk. This has been due to the expense of high-pressure equipment and the limited potential for commercialization of the technology. In recent years, many of the technological hurdles in using high-pressure treatment on a large scale have been overcome, and commercial pressure treatment plants are now available. This has been accompanied by a surge in research interest in the use of high pressures in food-based systems, including dairy products. This interest is reflected by the large number of recent review papers on the effect of high pressure in dairy-based systems (*13*−*16*).

High-pressure treatment of milk can denature the whey proteins in a similar, but not identical, manner to heat treatment. However, as compared with heating, fewer studies on the pressure-induced denaturation of whey proteins in milk are available. In an early study, Johnston et al. (*17*) showed that the amount of nonsedimentable nitrogen decreased with increasing pressure treatment, and this was attributed to the denaturation of the whey proteins. Lopez-Fandino et al. (*18*) showed that the level of native β-LG in milk decreased as a consequence of

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pressure treatment, with a more rapid denaturation at higher pressures. Hinrichs et al. (*19*) completed a full kinetic study on the pressure-induced denaturation of β-LG and α-lactalbumin (α-lac) in whey protein isolate solutions. Denaturation of β-LG was observed at pressures above about 150 MPa, whereas denaturation of α-lac was not observed until the pressure was increased to about 500 MPa. No difference in the denaturation rates of the A and B variants was observed. In a recent study, Huppertz et al. (*20*) showed that the denaturation of β-LG and α-lac was more rapid in milk than in sweet whey, indicating that the presence of casein micelles and colloidal calcium phosphate may play an important role in the irreversible denaturation reactions of the whey proteins.

High pressure in addition to temperature in the study of reactions in solutions offers an additional dimension to obtain information for the elucidation of underlying reaction mechanisms. A few studies have examined the effect of both temperature and pressure on whey protein denaturation, although there are some contradictory results. Lopez-Fandino and Olano (*21*) studied the effect of pressure (100−400 MPa for 15 min) and temperature (25, 40, 50, and 60 °C) on the denaturation of whey proteins in milk. No denaturation was observed for β-LG and α-lac at pressures of 100 and 300 MPa, respectively. At higher pressures, the denaturation reaction of both β-LG and α-lac proceeded more rapidly as the temperature was increased. In a similar study, Garcia-Risco et al. (*22*) found almost complete denaturation when milk was pressure treated for 15 min at 400 MPa at temperatures above 40 °C, whereas about 75% of the β-LG was denatured when the temperature was reduced to 25 °C. However, Huppertz et al. (*23*) observed an increase in the denaturation rate when the temperature was increased from 5 to 20 °C, but little further change in the level of denaturation was observed when the temperature was raised to 40 °C.

Hinrichs and Rademacher (*24*) examined the kinetics of whey protein denaturation in whey protein isolate solutions over a wide pressure and temperature range using a one-step nonlinear regression method. This method allowed for the global fit of all data, and the data could be analyzed as a function of temperature or pressure. Kinetic studies of reactions under pressure allow the activation volumes (V_a) to be calculated (*25*, *26*). The V_a for the pressure-induced denaturation of β-LG decreased as the temperature was increased, indicating that denaturation proceeded more rapidly at higher temperatures. The activation energy (E_a) for the denaturation was similar at all pressures, at about 70−100 kJ/mol. This was interpreted as indicating that aggregation was rate limiting at all pressures.

Although there have been some studies on the pressure-induced denaturation of whey proteins in skim milk, and a few have examined the effect of temperature, most have examined the denaturation at a single holding time with variable pressures and temperatures or at a single pressure with variable holding times and temperatures. To allow the development of equations for the prediction of the level of denaturation, full kinetic studies at a range of pressures, holding times, and temperatures are required. As β-LG and α-lac denature over markedly different pressure ranges (*19*, *24*, *27*), this study concentrated on the denaturation of β-LG and examined the effect of temperature and pressure on the denaturation reaction in reconstituted skim milk over the 100−600 MPa pressure range. The denaturation studies were conducted in sufficient detail to obtain kinetic parameters, and as two pressure units of different sizes were available, the effect of scale-up on the denaturation reactions was also investigated.

MATERIALS AND METHODS

Milk Supply. Reconstituted skim milk samples of 12% total solids (w/w) were prepared by adding low heat skim milk powder (Fonterra Cooperative Group, New Zealand) to water (purified through a Milli-Q apparatus (Millipore Corp., Bedford, MA)). 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 15 mL transparent tubes (Ultra-Clear centrifuge tubes, Beckman Instruments Inc., Palo Alto, CA) and heat sealed. For kinetic experiments, the sealed samples were pressure treated in a Stansted Fluid Power Micro-lab FPG5740-11 high-pressure food processor (cylindrical chamber of 17 mm (diameter) by 132 mm (length) and a volume of 30 mL). For the experiments on the effect of scale-up, the sealed samples were pressure treated in a Stansted Fluid Power FPG5500HL high-pressure food processor (cylindrical chamber of 65 mm (diameter) by 220 mm (length) and a volume of 730 mL). Both units were supplied by Stansted Fluid Power Ltd., Stansted, Essex, UK. The temperatures of the sample, the chamber, and the pressurization fluid were pre-equilibrated to the desired temperature (10, 20, 30, or 40 °C) for at least 1 h before treatment. Samples were treated from 0 to 600 MPa for times from 0 to 60 min. The temperature and pressure were monitored throughout the run using data logging equipment. After pressure treatment, the samples were stored at room temperature for 24 h before analysis.

Polyacrylamide Gel Electrophoresis. The level of native β-LG in the control and pressure-treated milk samples was determined using native polyacrylamide gel electrophoresis (native-PAGE). The casein and denatured whey proteins were precipitated by adding one part milk to one part sodium acetate buffer (0.2 M, pH 4.0), which adjusts the milk to pH 4.6. The precipitated casein and denatured whey protein were removed by centrifuging at 14 000*g* for 5 min. The supernatants were analyzed for residual native whey proteins using native-PAGE.

The supernatant and milk samples were accurately diluted, by weight, with sample buffer, and electrophoresis was performed as previously described (*7*, *28*). After electrophoresis, the gels were stained using 0.1% (w/v) amido black 10B in 10% acetic acid. After staining for 3 h, the gels were destained using a 10% acetic acid solution until a clear background was achieved. The gels were scanned using a Molecular Dynamics model P.D. computing densitometer (Molecular Dynamics Inc., Sunnyvale, CA), and the integrated intensities of the β-LG bands were determined using the Molecular Dynamics Image-quant integration software. The two variants of β-LG were separated by the electrophoresis technique and were analyzed separately. 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 two control samples, with corrections for differences induced from the various dilution steps in the sample preparations. Selected samples were prepared, pressure treated, and analyzed on at least three occasions, and error bars that represent the standard deviations of the repeated measurements are presented.

RESULTS

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

Temperature and Pressure Changes during Pressure Treatment. The change in temperature and pressure during 10 min pressurization cycles at various pressures is shown in **Figure 1**. The pressure increased at a rate of about 300 MPa/min and was consistent between runs. Pressure correction, if required, was automatically performed every 45 s throughout the duration

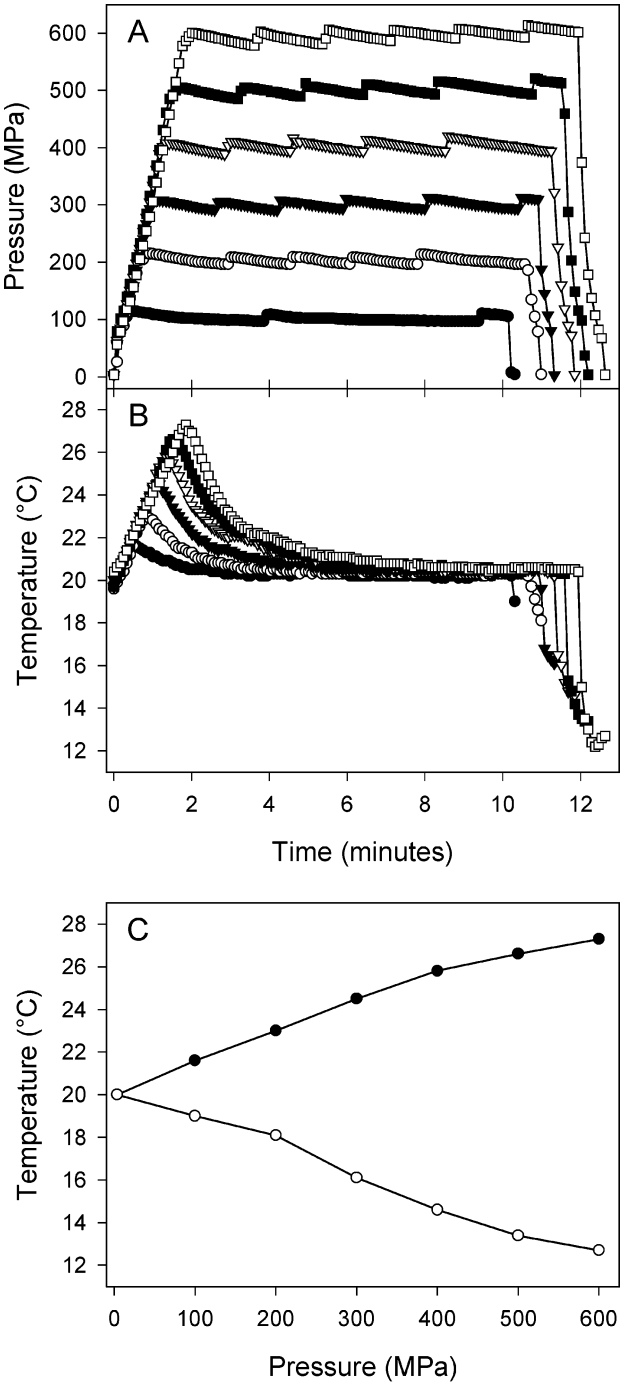

Figure 1. (**A**) Pressure changes during typical pressurization cycles. (**B**) Temperature changes during typical pressurization cycles. ●: 100 MPa; ○: 200 MPa; ▼: 300 MPa; ▽: 400 MPa; ■: 500 MPa; and □: 600 MPa. (**C**) Maximum temperature increases on pressurization (●) and maximum temperature decreases on depressurization (○).

of a run (**Figure 1A**). The temperature increased by about 1.3−1.8 °C per 100 MPa during pressurization as a result of adiabatic heating (**Figure 1B**,**C**). This increase was lower than the theoretical ∼3 °C for aqueous solutions in a closed system (*29*), and this difference was presumably due to heat loss to the surrounding environment. The temperature reequilibrated back to the set temperature within about 5 min of achieving the desired pressure (**Figure 1B**). The pressure decrease on depressurization was rapid as compared with the rate of pressure increase, and the temperature decreased by about 1.3−1.8 °C per 100 MPa as a result of adiabatic cooling, which was of a similar magnitude to the increase observed on pressurization.

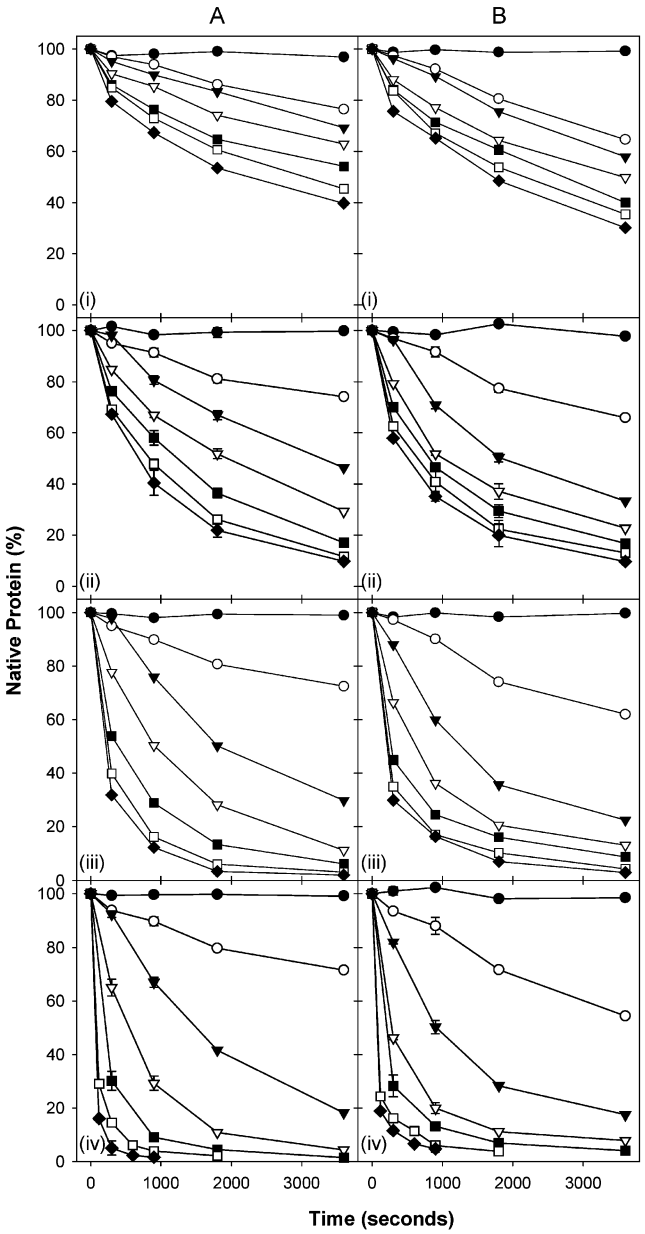

Figure 2. (**A**) Denaturation of β-lactoglobulin A. (**B**) Denaturation of β-lactoglobulin B. (**i**): 10 °C; (**ii**): 20 °C; (**iii**) 30 °C; and (**iv**): 40 °C. ●: 100 MPa; ○: 200 MPa; ▼: 250 MPa; ▽: 300 MPa; ■: 400 MPa; □: 500 MPa; and ◆: 600 MPa. Error bars on selected points are the standard deviation of triplicate measurements (in many cases, the error bars are smaller than the symbols).

The temperature increase on pressurization may cause some increase in the rate of denaturation in the early stages of each pressure cycle, especially when the initial temperature/pressure combination was just below the denaturation transition. Similarly, it was recognized that the pressure increase was not instantaneous and that some denaturation could occur during the pressurization process. No attempt was made to compensate for these temperature and pressurization effects as they were considered to be part of the experimental procedure and were reproducible between cycles.

Irreversible Denaturation of β-LG. Milk samples were pressure treated from 100 to 600 MPa at temperatures from 10 to 40 °C for times up to about 60 min. After pressurization, the samples were analyzed for native β-LG by native-PAGE. The levels of native β-LG A and B remaining after the various pressure treatments are shown in **Figure 2**. No irreversible

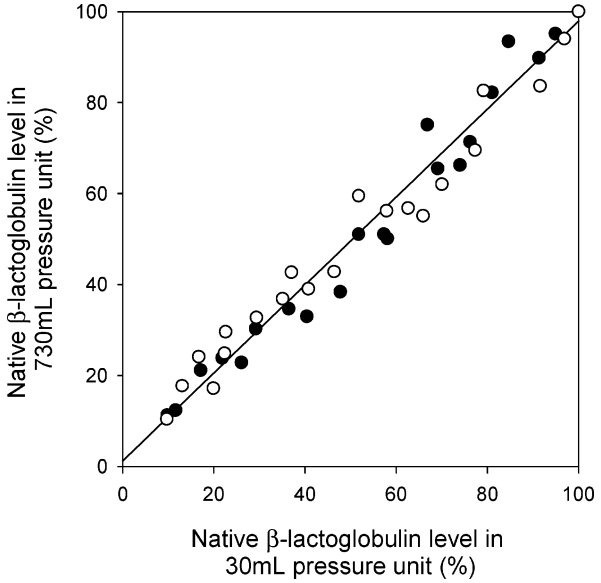

Figure 3. Comparison of the denaturation levels of β-lactoglobulin A (●) and β-lactoglobulin B (○) between laboratory-scale and pilot-scale high-pressure units.

denaturation of β-LG was observed at 100 MPa under all pressure treatment conditions. At higher pressures, β-LG irreversibly denatured, and the level of denaturation was dependent on the temperature, pressure, and duration of the pressure treatment. In general, the level of denaturation increased when the pressure was increased (at constant holding time and temperature), when the holding time was increased (at constant pressure and temperature), and when the temperature was increased (at constant holding time and pressure). As can be seen in **Figure 2**, the effect of temperature was relatively small for the samples treated at 200 MPa when compared with those at higher pressures.

To examine the effect of scale, milk samples prepared from the same milk powder were treated at selected pressures in two high-pressure units of different capacities, and the levels of β-LG denaturation were monitored and compared (**Figure 3**). The results indicate that, at any particular pressure/time combination, similar levels of denaturation were observed in both high-pressure units. This indicates that, in the two pilot scale units available, the denaturation was unaffected by the scale of the pressure treatment equipment (although this will need to be confirmed by comparing denaturation results between samples processed in pilot scale equipment (up to 1 L) and those processed in commercial-scale equipment of about 35 L). As there were some differences in pressurization and depressurization rates and in the temperature change during pressure treatment, these results suggest that the changes in temperature and pressure during the pressurization phase had only a small effect on the level of denaturation, when compared with the holding phase. This may not be surprising as the holding phase (up to 60 min) was considerably longer than the pressurization and depressurization phases (up to ~2−3 min at 600 MPa) and the time for temperature reequilibration (about 5 min at 600 MPa).

Determination of Reaction Order. The kinetics of whey protein denaturation under high-pressure conditions can be evaluated using similar processes to those used in studying the thermal denaturation (*6*, *7*). In these cases, the calculated kinetic and thermodynamic parameters are for the forward reaction from native protein, through the activated complex to the irreversibly aggregated complexes. The reaction order for the pressure-induced denaturation of β-LG was determined to allow a comparison of the rate constants for the denaturation of β-LG at different temperatures and pressures and to calculate kinetic and thermodynamic parameters over the entire temperature and pressure range. It is preferable to use a single reaction order for the entire pressure and temperature range, as this allows for the direct comparison of kinetic parameters. The denaturation results, as shown in **Figure 2**, were analyzed using eqs 1 and 2 to determine the most appropriate reaction order, as has been described previously (*6*, *7*, *19*, *28*). The pressure-induced irreversible denaturation for both variants of β-LG was best described with a reaction order of 2.0 when all pressures and temperatures were considered, as this order consistently produced reasonably straight line fits with good correlation coefficients (**Figure 4**). Higher or lower reaction orders tended to produce distinctly curved plots, especially at temperature/

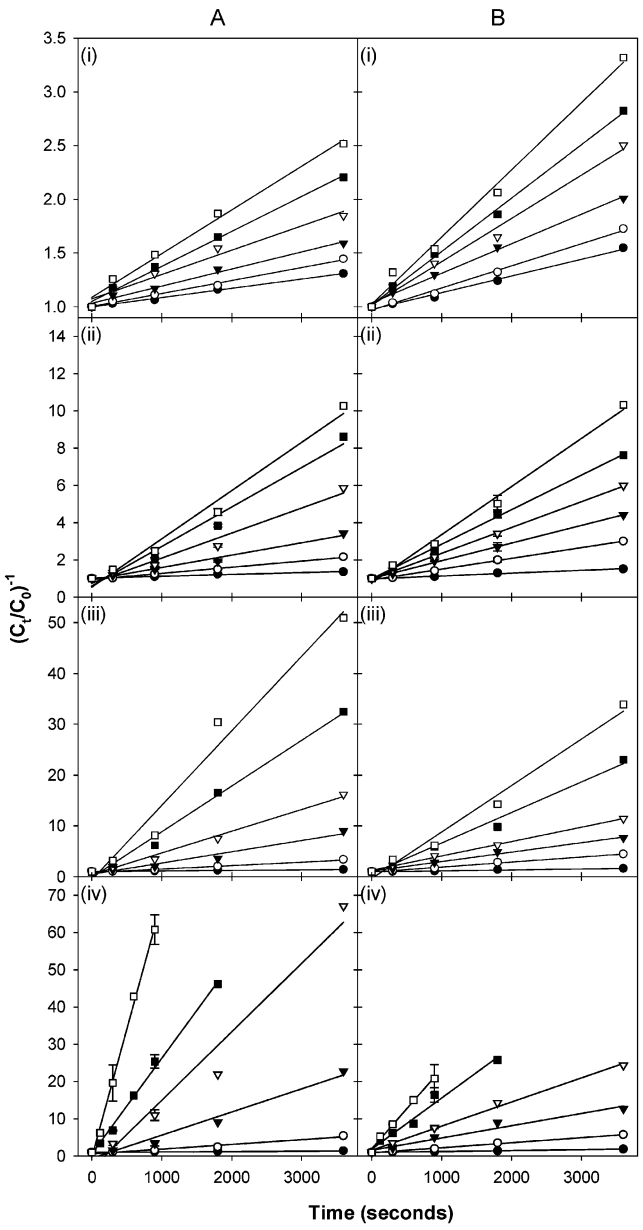

Figure 4. (**A**) Denaturation of β-lactoglobulin A as a reaction order of 2.0. (**B**) Denaturation of β-lactoglobulin B as a reaction order of 2.0. (**i**): 10 °C; (**ii**): 20 °C; (**iii**) 30 °C; and (**iv**): 40 °C. ●: 200 MPa; ○: 250 MPa; ▼: 300 MPa; ▽: 400 MPa; ■: 500 MPa; and □: 600 MPa. Error bars on selected points are the standard deviation of triplicate measurements (in many cases, the error bars are smaller than the symbols).

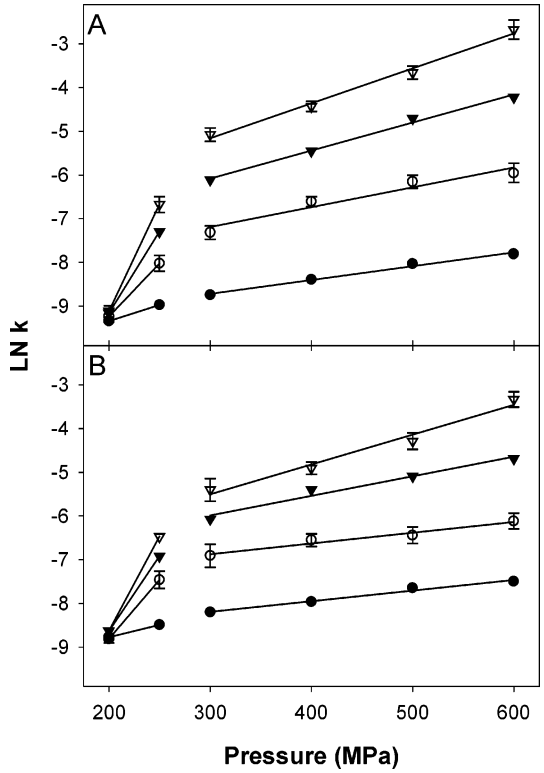

Figure 5. (**A**) Relationship between ln *k* and pressure for the denaturation of β-lactoglobulin A. (**B**) Relationship between ln *k* and pressure for the denaturation of β-lactoglobulin B. ●: 10 °C; ○: 20 °C; ▼: 30 °C; and ▽: 40 °C. Error bars on selected points are the standard deviation of triplicate measurements.

pressure combinations that produced high levels of denaturation (results not shown).

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

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

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

Calculation of V_a. The denaturation reactions were carried out at a range of pressures; therefore, the V_a values at each temperature could be determined using eq 3. The natural logarithms of the rate constants (ln *k*), obtained from the slopes of the straight lines in **Figure 4**, were plotted against the pressure (**Figure 5**). Interestingly, the relationship between ln *k* and the pressure was linear within certain pressure ranges; however, a marked change in pressure dependence was observed at about 300 MPa for both variants of β-LG. This change in dependence has not previously been reported for the pressure-induced denaturation of β-LG and suggests that there may be a change in the reaction mechanism within the two pressure ranges. A temperature-dependent change in reaction mechanism has been reported for the thermal denaturation of β-LG (*6*, *7*, *30*, *31*).

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

where k_f = rate constant, k_0 = frequency factor, R = universal gas constant, T = absolute temperature, p = pressure, and V_a = activation volume.

There were small temperature- and pressure-dependent differences in the rates of denaturation between A and B variants of β-LG. The B variant denatured more rapidly than the A variant at pressures ≤400MPa. At higher pressures, the rate of denaturation for the two variants was similar, although the A variant appeared to denature slightly faster than the B variant, particularly at the higher temperatures (**Figures 2**, **4**, and **5**).

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

pressure range (MPa)	temperature (°C)	β-lactoglobulin A		β-lactoglobulin B	
		V_a (mL/mol)	ln k_0	V_a (mL/mol)	ln k_0
200–300	10	–17.26	–10.81	–13.07	–9.88
200–300	20	–59.10	–14.09	–65.89	–14.22
200–300	30	–93.96	–16.62	–86.54	–15.50
200–300	40	–127.29	–18.90	–112.21	–17.24
300–600	10	–7.48	–9.67	–5.73	–8.92
300–600	20	–11.05	–8.55	–6.05	–7.62
300–600	30	–16.13	–8.01	–11.24	–7.32
300–600	40	–20.79	–7.56	–17.78	–7.56

The linearity within the two pressure ranges (**Figure 5**) allowed the V_a values to be calculated using eq 3. However, as only two data points were available for the low-pressure range (200−300 MPa), the calculated V_a should be treated with some caution and should be considered as an apparent V_a based on this limited data. The V_a values and frequency factors (ln k_0) are presented in **Table 1**. In all cases, negative V_a values were observed, which indicate that the rate of denaturation of β-LG will increase under pressure, as was observed. In the low-pressure range (200−300 MPa), V_a decreased markedly as the temperature at pressurization was increased from 10 to 40 °C. In the high-pressure range (300−600 MPa), V_a still decreased with increasing temperature; however, the decrease was less marked. The decrease in V_a indicates that the pressure-induced denaturation of β-LG is more rapid at higher temperatures and that the low-pressure range is more temperature sensitive than the high-pressure range.

Calculation of E_a Values and Thermodynamic Parameters. As the denaturation reactions were carried out at a range of temperatures, the E_a values at each pressure could be determined from the Arrhenius equation (eq 4). The natural logarithms of the rate constants (ln *k*), obtained from the slopes of the straight lines in **Figure 4**, were plotted against the reciprocal of absolute temperature (1/*T*) (**Figure 6**). The relationship between ln *k* and 1/*T* was essentially linear at each pressure, which allowed the E_a value to be calculated using eq 4. The E_a values and the frequency factors are given in **Table 2**. For both variants of β-LG, the E_a was low at 200 MPa (about 5−10 kJ/mol), increased markedly as the pressure was increased to 300 MPa (about 90 kJ/mol at 300 MPa), and then increased more slowly at higher pressures to about 110−125 kJ/mol at 600 MPa (**Table 2**).

$$\ln k_f = \ln k_0 - E_a/RT \quad (4)$$

where k_f = rate constant, k_0 = frequency factor, R = universal gas constant, T = absolute temperature, and E_a = activation energy.

Using the relationships shown in eqs 5−7, the enthalpies of activation ($\Delta H^{\ddagger}$), the free energies of activation ($\Delta G^{\ddagger}$), and the entropies of activation ($\Delta S^{\ddagger}$) were calculated. The average $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$, and $\Delta S^{\ddagger}$ values are presented in **Table 2**. For both genetic variants of β-LG, $\Delta H^{\ddagger}$ was very low at 200 MPa (about 3−8 kJ/mol) and increased markedly when the pressure was increased from 200 to 300 MPa (to about 85−90 kJ/mol). The $\Delta H^{\ddagger}$ value increased more gradually as the pressure was increased from 300 to 600 MPa (to about 123 kJ/mol for β-LG A and about

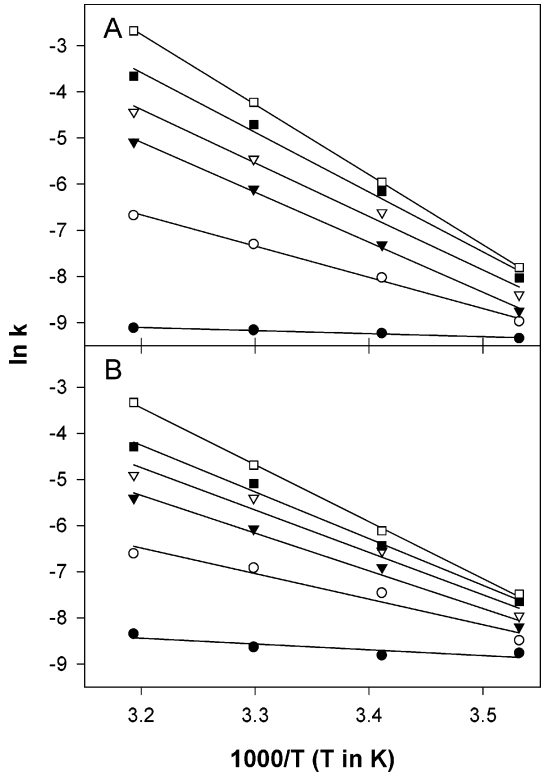

Figure 6. (**A**) Arrhenius plots for the denaturation of β-lactoglobulin A. (**B**) Arrhenius plots for the denaturation of β-lactoglobulin B. ●: 200 MPa; ○: 250 MPa; ▼: 300 MPa; ▽: 400 MPa; ■: 500 MPa; and □: 600 MPa.

Table 2. Activation Energies (E_a), Activation Enthalpies ($\Delta H^{\ddagger}$), Activation Entropies ($\Delta S^{\ddagger}$), and Activation Free Energies ($\Delta G^{\ddagger}$) for the Denaturation of β-Lactoglobulin

β-lactoglobulin A					
pressure (MPa)	E_a (kJ/mol)	ln k_0	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol/K)	$\Delta G^{\ddagger}$ (kJ/mol)
200	5.4	−7.0	2.9	−312	95.9
250	56.3	15.0	53.8	−129	92.1
300	90.1	29.6	87.6	−7	89.8
400	96.4	32.7	93.9	19	88.3
500	107.7	37.9	105.2	61	86.9
600	126.3	45.9	123.8	128	85.7
β-lactoglobulin B					
pressure (MPa)	E_a (kJ/mol)	ln k_0	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol/K)	$\Delta G^{\ddagger}$ (kJ/mol)
200	10.4	−4.5	7.88	−290	94.43
250	56.3	15.5	53.85	−125	91.23
300	89.1	29.6	86.58	−9	89.40
400	91.4	30.9	88.93	2	88.30
500	99.9	34.5	97.37	33	87.45
600	102.4	36.0	99.96	46	86.28

100 kJ/mol for β-LG B). For both genetic variants of β-LG, $\Delta S^{\ddagger}$ was negative at 200 MPa and progressively increased with increasing pressure. The $\Delta S^{\ddagger}$ value became positive at pressures above about 300 MPa. For both genetic variants of β-LG, $\Delta G^{\ddagger}$ decreased slightly as the pressure was increased.

$$k_f = (k_b T/h) \exp(-\Delta G^{\ddagger}/RT) \quad (5)$$

$$\Delta H^{\ddagger} = E_a - RT \quad (6)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (7)$$

where k_b = Boltzmann's constant and h = Planck's constant.

A statistical analysis of the results where several measurements were performed showed that, for both variants of β-LG, the rate constants obtained at 200 MPa were not significantly different when the experiments were performed at 20 or 40 °C. In contrast, the rate constants at higher pressures (≥250 MPa) were significantly different between 20 and 40 °C ($P \leq 0.01$). In addition, a sensitivity analysis was performed to indicate what level of β-LG denaturation would be required at 200 MPa to shift the rate constants at this pressure to be on the same line as observed at 300−500 MPa (**Figure 5**). At 40 °C and for β-LG A, ln k would need to increase from about −9.25 to about −6.0. This would require the level of native protein remaining after pressure treatment for 60 min to decrease from about 75% to about 10%. Similarly, at 20 °C, ln k would need to increase from about −9.25 to about −7.75, and this would require the level of native β-LG remaining after pressure treatment for 60 min to decrease from about 75% to about 30%. 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 a change in behavior of the denaturation β-LG at about 300 MPa can be attributed to errors in the experimental procedures.

DISCUSSION

The covalent bonds that stabilize protein structures are relatively unaffected by pressure treatments, at least up to 1000 MPa (*25*). The dissociation of ion pairs and the disruption of hydrophobic interactions are associated with negative volume changes, and therefore, these interactions are unfavorable under high-pressure conditions (*32*−*33*). In contrast, most studies suggest that the hydrogen bonds involved in maintaining the protein structure are shortened under high pressure, leading to a smaller volume, and thus are promoted under pressure (*33*, *34*). However, other reports suggest that very small changes in the reaction volume are associated with the formation of hydrogen bonds and that these changes may be negative (favored) or positive (unfavored) depending on the model system investigated (*32*). Considering that the tertiary structures of globular proteins are stabilized by hydrophobic interactions and ion pairing, it is evident that proteins will be denatured under high-pressure conditions. However, elements of secondary structure, particularly those stabilized by hydrogen bonding, may be maintained (*35*, *36*).

The results in **Figure 2** demonstrate that the treatment of skim milk at pressures of 200 MPa or greater results in the irreversible denaturation of β-LG, and this is in agreement with literature reports on the pressure treatment of milk (*18*, *21*, *22*), sweet whey (*20*), and whey protein isolate solutions (*19*, *24*). Some studies suggest structural changes to purified β-LG at pressures below 200 MPa, although these effects are dependent on the composition of the buffers (including pH and ionic strength) and are reversible to various extents (*37*−*40*).

The comparative rates of irreversible denaturation of β-LG A and B indicated that the A variant denatured at a slower rate at pressures ≤400MPa, whereas at higher pressures, the denaturation rates were similar, although the A variant appeared to denature slightly faster especially at the higher temperatures (**Figures 2**, **4**, and **5**). Hinrichs et al. (*19*) and Hinrichs and Rademacher (*24*) indicated that both variants of β-LG in whey protein isolate solutions denatured at a similar rate at all pressures between 200 and 800 MPa and at temperatures up to

70 °C. Botelho et al. (*39*) showed that β-LG A was more stable than β-LG B when pure protein solutions were treated at 100−350 MPa at 23 °C, which is in agreement with this study for a similar temperature and pressure range. For the thermal denaturation of β-LG in milk, some reports suggest that the B variant denatures more rapidly than the A variant at all temperatures (*6*, *31*, *41*), whereas other reports suggest that there is a crossover, with the B variant denaturing faster than the A variant at temperatures between 70 and 100 °C and the A variant denaturing faster than the B variant at temperatures above about 100 °C (*7*, *42*).

Botelho et al. (*39*) suggested that the substitution of the valine in the A variant with an alanine in the B variant creates a cavity in the protein matrix of the B variant, which allows for a greater volume change on unfolding and therefore a greater pressure sensitivity. Although this may explain the differences between A and B variants at lower pressures, changes in temperature and pressure can affect many aspects of protein stability, including the monomer−dimer equilibrium, hydrophobic interactions, ion pairing, hydrogen bonding, and subsequent aggregation reactions (*32*−*34*). As these reactions will have different temperature and pressure dependences, changes in their relative rates between the different genetic variants of β-LG may explain the changes in the relative rates of denaturation of the different genetic variants at different temperatures and pressures. In addition, irreversible denaturation (aggregation end-product) may have some different dependence at different pressures when compared with reversible denaturation (unfolding).

The pressure-induced denaturation of β-LG was best described with a reaction order of 2.0. A reaction order of 2.5 (*19*) or 3 (*24*) has been reported for the pressure-induced denaturation of β-LG in whey protein isolate solutions. In skim milk, the thermal denaturation of β-LG is generally observed to follow a reaction order of 1.5 (*6*, *7*, *28*, *31*, *41*). The unusual value for the reaction order for both thermal and pressure-induced denaturation of β-LG has been ascribed to a complex reaction mechanism involving many consecutive and/or concurrent steps (*6*, *7*, *19*, *24*).

The pressure-induced denaturation of β-LG showed a marked change in pressure dependence at about 300 MPa when ln k was plotted against the pressure, and this change became more pronounced as the temperature at pressurization was increased (**Figure 5**). This resulted in markedly different V_a values in the two pressure ranges at each temperature, with a substantially lower V_a value in the low-pressure range (200−300 MPa) than in the high-pressure range (300−600 MPa) at each temperature. Nonlinear plots of ln k against pressure can be attributed to a change in the compressibility of the reactants or activated states or to a change in the rate-determining step of the reaction (*33*, *43*). It is unknown what changes in the compressibility of β-LG occur at different pressures, as it was not possible to measure this parameter. It is possible that there are changes in the rate-determining step with changes in pressure, and examination of the kinetic and thermodynamic parameters may give insights into the reaction mechanism, especially when both pressure and temperature effects are considered.

The irreversible denaturation reactions of the whey proteins have complex mechanisms involving many consecutive and/or concurrent steps. The kinetic and thermodynamic parameters presented in **Table 1** are for the overall denaturation-aggregation process induced by the pressure treatment. A simplified reaction mechanism, exemplifying the major steps including aggregation reactions involving hydrophobic interactions (eq 8c) and thiol−disulfide interchange reactions (eq 8d), is shown in eq 8. In both cases, the first steps in the reaction involve the formation of monomeric β-LG (eq 8a) from the dimer and the unfolding of the tertiary structure exposing hydrophobic regions (β-LG_u) and/or the free thiol group (β-LG_{-SH}) of β-LG (eq 8b). These first steps in the reactions are reversible. Subsequent irreversible aggregation reactions involving other denatured whey proteins or κ-casein from the casein micelles can occur simultaneously and/or consecutively so that a mixture of hydrophobic (eq 8c) and disulfide-bonded (eq 8d) aggregate species can be formed. Although the overall mechanism is similar to that proposed for the thermal denaturation reaction (*7*), the observation of different reaction orders between thermal and pressure-induced processes suggests differences in the dominance of individual steps in the overall mechanism. For example, as it is generally reported that hydrophobic interactions are not favored under high-pressure conditions (*33*, *34*), the hydrophobic step in eq 8c is not expected to proceed while under pressure. However, there is the possibility that, on pressure release, hydrophobic interactions of the unfolded protein may take place.

$$(\beta\text{-LG}_n)_2 \rightarrow 2(\beta\text{-LG}_n) \quad (8a)$$

$$\beta\text{-LG}_n \rightarrow \beta\text{-LG}_u \rightarrow \beta\text{-LG-SH} \quad (8b)$$

$$x(\beta\text{-LG}_u) \rightarrow (\beta\text{-LG})_x \quad (8c)$$

$$\beta\text{-LG-SH} + \text{PS-S} \rightarrow \beta\text{-LG-S-S-P-SH} \quad (8d)$$

The observation of negative V_a is in agreement with the V_a value expected for protein unfolding (eq 8b), and the observed values for V_a (from −5 to −130 mL/mol, **Table 1**) were in the range observed for the pressure-induced unfolding of a range of different proteins (*44*). Chemical reactions can have positive and negative V_a values that range in magnitude to several tens of mL/mol (*45*). Schmid et al. (*46*) showed that the V_a for the dissociation of lactic dehydrogenase was about −60 mL/mol and that the V_a for the irreversible aggregation reaction of this protein was about −100 mL/mol. Both of these V_a values are in the range observed for the low-pressure range of this study, indicating that V_a alone is not sufficient to give mechanistic information on the rate-determining step of the irreversible denaturation reactions. Interestingly, Royer (*44*) indicated that all studies on the pressure-induced unfolding of proteins showed that V_a increased with increasing temperature, whereas in this study, V_a decreased with increasing temperature in both pressure ranges (although the magnitude of the decrease was lower in the higher-pressure range; **Table 1**). This may indicate that the unfolding step (eq 8b) is not rate limiting in the irreversible denaturation reactions or that the observed V_a is obtained from the cumulative effects of unfolding and aggregation.

Analyzing the denaturation as a function of temperature allows E_a, $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$, and $\Delta S^{\ddagger}$ to be calculated at each pressure, and these parameters may give insights as to whether there is a change in the rate-determining step at different pressures. The reversible protein dimer to monomer dissociation and unfolding reactions (eqs 8a,b) and the subsequent irreversible aggregation reactions (eqs 8c,d) would be expected to have markedly different kinetic and thermodynamic parameters for denaturation. The denaturation process (eq 8b) involves the rupture of a large number of intramolecular bonds, producing an unfolded molecule with increased rotational and translational entropy. This process is expressed with high values for E_a and $\Delta H^{\ddagger}$ and a positive $\Delta S^{\ddagger}$, reflecting the lower state of order of the unfolded protein molecules. In contrast, aggregation reactions (eqs 8c,d)

involve the formation of a few intermolecular bonds, and the order of the system is increased as the aggregated molecules have less rotational and translational freedom than the non-aggregated species, resulting in lower E_a and $\Delta H^{\ddagger}$ and a negative $\Delta S^{\ddagger}$.

Examination of the results in **Table 2** suggests that, at 200 MPa, the aggregation reactions were likely to be rate determining, as E_a and $\Delta H^{\ddagger}$ were low and $\Delta S^{\ddagger}$ was negative for both variants of β-LG. At 600 MPa, these parameters were more consistent with the denaturation step as the rate-determining step, as E_a and $\Delta H^{\ddagger}$ were markedly higher than at 200 MPa and $\Delta S^{\ddagger}$ was positive. However, there appeared to be a transition from an aggregation reaction as rate determining to the denaturation reaction as rate determining as E_a, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ progressively increased as the pressure was increased. For example, at 300−400 MPa, E_a and $\Delta H^{\ddagger}$ were intermediate between those observed at 200 and 600 MPa, and $\Delta S^{\ddagger}$ was virtually zero.

In a general reaction scheme with multiple steps, if one reaction step is considerably slower than all the other steps, this is the rate-determining step and controls the rate of the overall reaction. However, if several steps have similar rates, then the reaction rate will be a composite of the kinetic constants of these elementary steps in the reaction. The thermal denaturation of β-LG shows a temperature-dependent change in reaction mechanism, with the kinetic and thermodynamic parameters being consistent with denaturation (unfolding) as the rate-determining step at temperatures between 70 and 90 °C (high E_a, high $\Delta H^{\ddagger}$, and positive $\Delta S^{\ddagger}$), whereas these parameters are consistent with aggregation as the rate-determining step above 90 °C (low E_a, low $\Delta H^{\ddagger}$, and negative $\Delta S^{\ddagger}$; refs *6* and *7*). However, unlike the step change in thermal denaturation, in pressure-induced denaturation, it appears that the rates for denaturation and aggregation are similar, and as a consequence, there is a transition from the aggregation reaction being rate determining at low pressures (~200 MPa) and unfolding being rate determining at high pressures (600 MPa).

Pressure-induced changes in rate-determining steps have been noted previously for chemical reactions and for enzyme-catalyzed reactions. For example, the rate-determining step in the reaction of carbon monoxide with protohemes changed from bond formation-limited to diffusion-limited as the pressure was increased, and this produced ln k versus pressure plots with markedly different slopes (*47*). Similarly, a change in the rate-determining step was observed for the hydrolysis of substrates (benzoylcholine and benzoylthiocholine) by butyrylcholinesterase, with deacylation as the rate-determining step at lower pressures and acylation as the rate-determining step at higher pressures. As deacylation has a negative volume change and acylation has a positive volume change, the rate of reaction initially increases with increasing pressure when deacylation is rate limiting and then decreases with further increases in pressure when acylation becomes rate limiting (*43*).

In conclusion, this study has demonstrated that β-LG denatures when milk is pressure treated and that the denaturation rate is dependent on the pressure applied and the temperature at pressurization. The kinetics for the denaturation reaction indicate that the reaction is complex, with a marked change in pressure dependence at about 300 MPa. Analysis of the kinetic and thermodynamic parameters suggests that there may be two competing rate-determining steps. These kinetic and thermodynamic parameters are consistent with aggregation as the rate-determining step at low pressures; however, there appears to be a transition to unfolding as the rate-determining step as the pressure increases as these parameters progressively change from those consistent with aggregation to those consistent with unfolding.

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