ABSTRACT

Much research dealing with the processing of milk by-products in heat exchangers has noted the key role of calcium in β-lactoglobulin (β-LG) fouling behavior. Nevertheless, the manner by which Ca affects β-LG denaturation has rarely been quantified using reliable kinetic and thermodynamic data. To this end, the influence of Ca on β-LG denaturation mechanisms in simulated lactoserum concentrates was studied on the laboratory-scale under 100°C by HPLC analysis. The heat-treated solutions were composed of 53.3 g/L β-LG and were enriched in Ca at various concentrations (0, 66, 132, and 264 mg/kg). The kinetic parameters (reaction order, activation energy, and frequency factor) associated with β-LG denaturation, along with the unfolding and aggregation thermodynamic parameters were deduced from these experiments and discussed with respect to Ca content. We found that the multistage process characterizing β-LG thermal denaturation is not greatly affected by Ca addition. In fact, the general model subdividing β-LG denaturation mechanisms in 2 steps, namely, unfolding and aggregation, remained valid for all tested Ca concentrations. The change in the predominant mechanism from unfolding to aggregation was observed at 80°C across the entire Ca concentration range. Moreover, the classical 1.5 reaction order value was unaffected by the presence of Ca. Interpretation of the acquired kinetic data showed that Ca addition led to a significant increase in kinetic rate, and more so in the aggregation temperature range. This indicates that Ca principally catalyzes β-LG aggregation, by lowering the Coulombian repulsion between the negatively charged β-LG reactive species, bridging β-LG proteins, or via an ion-specific conformational change. To a lesser extent, Ca favors β-LG unfolding, probably by disturbing the noncovalent binding network of native β-LG. Simultaneously, Ca has a slight protective role on the native and unfolded β-LG species, as shown by the increase in activation energy with Ca concentration. The calculation of thermodynamic parameters related to β-LG denaturation confirmed this observation. A threshold effect in Ca influence was noted in this study: no further significant kinetic rate change was observed above 132 mg/kg of Ca; at this concentration, the studied solution was an almost equimolar mixture of β-LG and Ca. Finally, we simulated the temporal evolution of β-LG species concentrations at diverse Ca contents at 3 holding temperatures. The simulations were based on the acquired kinetic parameters. This permitted us to highlight the greater effect of Ca on β-LG denaturation at high Ca content or for short-time heat treatments at temperatures near 100°C, as in heat exchangers. Key words: β-lactoglobulin, calcium, heat denaturation, thermodynamics

INTRODUCTION

Given that β-LG is the predominant whey protein in milk and dairy products, aggregation and fouling reactions under heat treatment are mainly governed by β-LG denaturation kinetics. Whey proteins such as β-LG are today regarded as milk by-products with high functional properties, such as gelation, foaming, water retention, emulsification, and thermal stability (Mulvihill and Donovan, 1987). These functionalities are governed by the mean aggregate size of the globular proteins, following from the denaturation process, which depends on the physicochemical properties of the heated dairy product and the manner in which heat transfer was conducted. Heat transfer is highly dependent on the heat process equipment and operating conditions used, such as residence time and temperature profile inside the heat exchanger.

In addition, the thermal treatment of milk by-products is complicated by the issue of fouling. Fouling is defined as the deposition of protein on the hot surfaces of heat exchangers and it causes decreases in heat exchange (René and Lalande, 1988; Benning et al., 2003) and increases in heat exchanger pressure drop (Grijspeerdt et al., 2004; Fryer et al., 2006) and in bacterial growth (Fryer et al., 2006; Jun and Puri, 2007). Fouling of industrial heat exchangers requires frequent cleanings, resulting in excessive use of water and harsh

KEYWORDS: β-lactoglobulin, calcium, heat denaturation, thermodynamics
Kinetic rates are highly dependent on temperature but also extrinsic factors, such as β-LG and total protein concentrations in the dairy solution, pH, ionic environment (e.g., Ca concentration), and so on. Unfortunately, data are lacking on β-LG denaturation kinetics in various physicochemical environments and thus, no predictive model is available to estimate a priori how a controlled chemical environment, such as the presence of Ca at a known concentration, will alter the β-LG denaturation kinetic parameters of raw dairy solutions.

The main findings concerning β-LG thermal behavior are summarized here, along with the effect of Ca on β-LG denaturation.

Bovine β-LG is one of the main whey proteins (along with α-LA and BSA) and accounts for about 50% of the whey proteins (Mulvihill and Donovan, 1987; Havea et al., 2001), which corresponds to about 3.5 g/L in milk (Lalande et al., 1989). Its molecular weight is about 18.3 kg/mol, its size approximately 3 nm (Mulvihill and Donovan, 1987), and it forms a native dimer at physiological pH and ambient temperature (Lalande et al., 1989; Sava et al., 2005). The isoelectric point of β-LG is around 5.3 (René and Lalande, 1988) and its denaturation temperature is estimated at about 77°C (Nielsen et al., 1995; Relkin, 1996; Havea et al., 2001; Linmark-Mansson et al., 2005), depending strongly on the chemical environment. The tertiary structure of β-LG is consolidated by 2 disulfide bonds. A thiol (–SH) group is hidden inside the hydrophobic core in the native state (Relkin, 1996; Labouré et al., 2004; Perez and Pilosof, 2004) and becomes exposed to solvent under heat treatment, allowing β-LG to form disulfide bonds with another whey protein via thiol group oxidation and disulfide interchange (Mulvihill and Donovan, 1987; Labouré et al., 2004). This latter phenomenon leads to the formation of β-LG aggregates.

The widely accepted model regarding β-LG heat-induced denaturation at temperatures between 60 and 100°C, Oldfield et al. (2005) proposed a 2-step reaction to model the denaturation mechanism: $N \rightarrow U \rightarrow A$. Under heat treatment, native β-LG ($N$) unfolds to form molten globule β-LG or reversibly unfolded β-LG ($U$). Then, these unfolded species can react with native or other unfolded β-LG via disulfide interchange or thiol oxidation to form denatured β-LG ($A$); that is, aggregates. Each step of the denaturation reaction (unfolding and aggregation) is considered kinetically driven.

In spite of this simplification of the mechanism, Tolkach and Kulozik (2007) have shown that this novel
reaction kinetic approach, associated with HPLC quantification of soluble proteins, allows the evaluation of native, unfolded, and denatured β-LG concentrations as a function of heating time. Consequently, it can also be used for the kinetic characterization of β-LG unfolding and aggregation reactions. The denaturation reaction mentioned in numerous papers (Dannenberg and Kessler, 1986; De Wit, 1990; Gotham et al., 1992; Roefs and De Kruijff, 1994; Anema and McKenna, 1996; De Wit, 1990; Oldfield et al., 2005; Tolkach and Kulozik, 2007) corresponds to the pathway from the native to the aggregated state ($N \rightarrow A$) with the reaction mechanism adopted in this paper (see equation [1]).

The β-LG denaturation reaction is influenced by many parameters, such as solution chemistry, thermal history during heat treatment (Foster et al., 1989; Schraml and Kessler, 1996; Santos et al., 2006b), hydrodynamics (Delplace et al., 1997; Zumaeta et al., 2007), and heat exchanger surface type (Kim and Lund, 1998; Rosamininho et al., 2007).

The role of ionic Ca is important in β-LG denaturation and aggregation. According to the literature, Ca is involved in 2 aspects of β-LG denaturation and aggregation mechanisms. On one hand, Ca is involved in growth and deposition of β-LG aggregates (Mulvihill and Donovan, 1987); it is expected to form covalent bridges between β-LG monomers, aggregates, and surface minerals (Anema and McKenna, 1996; Changani et al., 1997), which strengthens the aggregate or deposit structure (Britten et al., 1988). Consequently, aggregates formed in a Ca-enriched solution will be denser and their size will generally increase (Mulvihill and Donovan, 1987; Xiong, 1992); however, under particular environmental conditions, it has been reported that Ca decreases the aggregate size (Simmons et al., 2007). Calcium also participates in the denaturation mechanism by lowering the temperature at which β-LG begins to denature (De Wit, 1990; Simmons et al., 2007). On the other hand, an increase in Ca concentration helps to raise the ionic strength. The presence of numerous ions in a β-LG solution favors aggregates growth (Verheul et al., 1998; Allen and Smith, 2001; Schmitt et al., 2007) and surface fouling (De Wit, 1990; Relkin, 1996; Simmons et al., 2007) by neutralizing the protein surface charge and thus reducing drastically Coulombian repulsion.

The interaction between Ca and β-LG has been extensively investigated recently and it was established that Ca is able to influence β-LG denaturation and aggregation mechanisms in 3 different ways (Jeyarajah and Allen, 1994; Simons et al., 2002; O’Kennedy and Mounsey, 2009), depending on the environmental conditions. First, Ca can cause the intermolecular cross-linking of adjacent negatively charged groups (such as carboxylic groups) of β-LG, leading to β-LG aggregation by bridging. Second, ionic Ca takes part in the intramolecular electrostatic shielding of β-LG negative charges, which tends to lower Coulombian repulsion between β-LG proteins and favors β-LG aggregation by hydrophobic bonds. Third, Ca can induce specific conformational changes in the β-LG tertiary structure, leading to local unfolding of β-LG and exposition of its free thiol group.

By studying Ca-binding features of β-LG at ambient temperature or after heat treatment at various temperatures, some authors have found that the role of Ca concerns principally the shielding of β-LG charge (O’Kennedy and Mounsey, 2009) and the ion-specific interaction (Jeyarajah and Allen, 1994; Simons et al., 2002). Indeed, irreversibly denatured β-LG have a greater affinity for Ca than native or molten globule β-LG (Jeyarajah and Allen, 1994), which is explained by the higher exposition of carboxylic groups in the denatured β-LG species (Simons et al., 2002). In consequence, Ca would be expected to affect essentially β-LG aggregation. Furthermore, the concentration of Ca required to trigger β-LG aggregation increases when more Ca ions are bound to β-LG, which makes the intermolecular β-LG–Ca$^{2+}$–β-LG cross-linking hypothesis unlikely (Simons et al., 2002).

Because divalent anions such as Ca$^{2+}$ have a specific action on β-LG denaturation and aggregation mechanisms that cannot be reproduced with monovalent cations, it was deduced that Ca acts on β-LG not only by shielding its electric charges but also via an ion-specific interaction (Jeyarajah and Allen, 1994). In fact, Ca induces a local unfolding of β-LG, which exposes the previously hidden free thiol group and allows the formation of intermolecular disulfide bonds. This effect is concomitant with an increase in β-LG hydrophobicity, which further increases the propensity of β-LG to aggregate (Jeyarajah and Allen, 1994).

Many dairy fouling studies were performed with milk by-products containing different Ca concentrations, protein types and concentrations, and ionic environments. Consequently, some trends can be deduced about the role played by Ca in β-LG unfolding, aggregation, and deposition reactions. However, specific studies conducted on the laboratory scale and dedicated to quantifying the effect of Ca on the chemical behavior of β-LG have not been carried out. To quantify the role of Ca in β-LG denaturation and fouling processes, the evolution of the kinetic parameters with Ca concentration has to be determined.

In this study, kinetic and thermodynamic parameters related to heat-induced β-LG denaturation under 100°C were determined by HPLC for a 53.3 g/L β-LG solution.
containing various concentrations of calcium chloride at neutral pH. The evolution of these kinetic parameters with temperature and Ca concentration was discussed and the associated thermodynamic data were calculated. These experiments enabled us to determine the influence of Ca on β-LG unfolding and aggregation at temperatures below 100°C. Denaturation β-LG at various Ca concentrations and temperatures was simulated to illustrate the effect of Ca on β-LG denaturation as a function of temperature.

**MATERIALS AND METHODS**

**Chemical and Reagents**

For the whole experiment, the 53.3 g/L β-LG solutions with various Ca concentrations were prepared by mixing 6 g of β-LG powder (industrial powder: β-LG 88.85%, α-lactalbumin <0.01%, Ca 0.03%, phosphate 0.07%, sodium 0.76%, lactose 0.4%) in 94 g of deionized water (Millipore, Bedford, MA) at 40°C. Then, different quantities (ranging from 0 to 660 μL) of a molar Ca chloride (CaCl₂ 96%, Prolabo, VWR International, West Chester, PA) solution were added to the β-LG solution to obtain stock solutions containing 5.33% (wt/wt) β-LG and 0, 66, 132, or 264 mg/kg Ca (i.e., 0, 1.65, 3.3, and 6.6 mmol/L). These solutions were maintained at 40°C for 2 h for complete dissolution of the β-LG powder. The pH of these solutions was close to 6.8.

The heat-treated samples were prepared for HPLC analysis according to the following protocol: after dilution with deionized water when necessary, small quantities of molar acetic acid (Normapur, Prolabo) were added until the pH reached 4.6 to precipitate aggregates. After 1 h at rest, the samples were centrifuged (6K15, Sigma, St. Louis, MO) at 9,056 × g and 4°C for 30 min. The native β-LG containing supernatant was finally collected for HPLC analysis.

The mobile phases used in HPLC were 0.1% (vol/vol) trifluoroacetic acid (99%, Acros Organics, Thermo Fisher Scientific, Waltham, MA) in deionized water, and 0.1% trifluoroacetic acid in a mixture of 80% acetonitrile (HPLC grade, Thermo Fisher Scientific) and 20% deionized water. Calibration standards in the range from 1 to 5 g/L were prepared by dissolving the β-LG powder in deionized water.

**Heat Treatment**

All thermal denaturation experiments were conducted on twelve 1.4-mL samples for each Ca concentration. Before submitting samples to the desired holding temperature, the samples were preheated to 60°C (below the β-LG denaturation temperature of 77°C; Nielsen et al., 1995; Linmark-Mansson et al., 2005). This was achieved by putting samples of stock solution in polypropylene tubes (Eppendorf, Hamburg, Germany) in the first water bath at 62°C for 30 min. The temperature increase from the preheating temperature (60°C) to the desired holding temperature was performed by placing the samples in a second water bath whose temperature was maintained 10°C higher than the holding temperature to shorten the heat increase time. The first sample was acquired when the sample temperature was equal to the holding value. The sample temperature was monitored by following the temperature evolution of a reference vial filled with the stock solution, in which a temperature probe was inserted.

The holding temperature, ranging from 68 to 96°C, was maintained for a period sufficient for significant denaturation by means of a third water bath whose temperature was fixed at 2°C higher than the desired temperature (e.g., 98°C for a holding temperature of 96°C). Immersion of a sample in this last water bath corresponded to time zero. At different times, the samples were removed from the last water bath and cooled immediately in a beaker half filled with melting ice.

**HPLC**

The native β-LG concentration in the samples after pH adjustment and centrifugation was evaluated with HPLC. The chromatographic system (Waters, Milford, MA) included a 717 Plus autosampler, a 616 quadratic pump system, a Jones Model 7971 column oven, a CLHP ACE 300 Å C4 separation column (25 cm × 4.6 mm), and the associated guard column (Advanced Chromatography Technologies, Aberdeen, UK), a 486 UV-visible spectrophotometer, and acquisition software (Millenium 3.2, Waters).

The HPLC analyses were carried out in the following conditions: flow rate 1 mL/min, injection volume of 10 μL, temperature of 30°C, detection wavelength of 214 nm, and gradient elution. Analyses were repeated 3 times for each standard or sample. The calibration was carried out in the range 1 to 5 g/L, which involved diluting the samples before pH adjustments. A daily column conditioning was achieved by eluting acetonitrile at 1 mL/min for 1 h to restore the column properties.

The sample concentrations were calculated by averaging the 3 measured chromatographic areas and converting this area value into a β-LG concentration by means of the HPLC calibration curve.

**Determination of Kinetic Parameters**

The reaction model used in this study is derived from the work of Oldfield et al. (2005) and Anema and McK-
Determination of the Denaturation Rate Constant. First, an analytical technique such as HPLC is solely able to evaluate the β-LG total soluble concentration \( C_s \); however, native and unfolded β-LG concentrations \( C_N \) and \( C_U \) remain inaccessible with this analytical technique. Nevertheless, the denaturation reaction concerns the transformation of soluble species (native and unfolded β-LG) into insoluble species (aggregates), which is described by the chemical equation:

\[
\text{β-LG (native and unfolded)} \rightarrow \text{aggregates}, \]

and defined in equation 2:

\[
\frac{\text{d}C_s}{\text{d}t} = k_n C_s^n, \tag{2}
\]

where \( C_s \) is soluble β-LG concentration (mol/L) equal to \( C_N + C_U \), \( k_n \) is denaturation rate constant for a reaction order equal to \( n \) \((g^{1-n} L^{n-1} s^{-1})\), and \( n \) is denaturation reaction order.

The denaturation equation resolution provides different expressions depending on the reaction order value: For \( n \neq 1 \),

\[
\frac{\left( \frac{C_s(t)}{C_s^n} \right)^{1-n} - 1}{(n-1)(C_s^n)^{n-1}} = k_n t. \tag{5}
\]

And for \( n = 1 \),

\[
-\ln \left( \frac{C_s(t)}{C_s} \right) = k_t t. \tag{6}
\]

Rewriting the previous equations allows the calculation of the denaturation rate constant at a given temperature.

For \( n \neq 1 \),

\[
\ln \left( \frac{C_s(t)}{C_s^n} \right) = \frac{E_{A_n}}{R T_{n}} t,
\]

where \( E_{A_n} \) is unfolding activation energy \((J/mol)\), and \( T \) is temperature \((K)\).

An example of Arrhenius plot obtained with 264 mg/kg Ca is represented in Figure 2. The Arrhenius plot of Figure 2 draws the evolution of the denaturation rate constant logarithm with inverse temperature. As expected from equation 7, the denaturation rate constant logarithm varied linearly with \( 1/T \), with a slope equal to \(-E_{A_n}/R\), but in the case of β-LG denaturation, 2 mechanisms appear on this graph, separated by an Arrhenius critical temperature \( T_c \) of about 80°C. This slope break delimits 2 temperature ranges: below the critical temperature, the denaturation reaction is limited by the unfolding reaction (the unfolding reaction is slower than aggregation) and unfolded β-LG represents a fraction of total soluble β-LG, which increases faster with temperature. For temperatures above the critical temperature, β-LG denaturation is limited by the aggregation reaction and in that case, aggregation is the slower reaction.

Consequently, linear regressions of the experimental values in the 2 temperature ranges permit us to determine the kinetic parameters (frequency factors and activation energies) related to the unfolding (Eq. 8) and aggregation (Eq. 9) phenomena:

\[
\ln(k_{\text{unf}}) = \ln(k_{\text{unf}}^o) - \frac{E_{A_{\text{unf}}}}{RT}, \tag{8}
\]

and

\[
\ln(k_{\text{agg}}) = \ln(k_{\text{agg}}^o) - \frac{E_{A_{\text{agg}}}}{RT}, \tag{9}
\]

where \( k_{\text{unf}} \) is unfolding constant rate, depending on \( n \) \((g^{1-n} L^{n-1} s^{-1})\), \( k_{\text{unf}}^o \) is unfolding frequency factor \((g^{1-n} L^{n-1} s^{-1})\), and \( E_{A_{\text{unf}}} \) is unfolding activation energy \((J/mol)\); and

\[
\ln(k_{\text{agg}}) = \ln(k_{\text{agg}}^o) - \frac{E_{A_{\text{agg}}}}{RT}, \tag{9}
\]

otherwise were plotted as a function of time, and linear regressions of experimental data were performed. The adequate reaction order corresponded to the linear regression that best fits the experimental data plotted in this way. For each temperature, the corresponding denaturation rate constant was deduced from the linear regression slope.

Temperature Dependence of the Denaturation Rate Constant: Use of the Arrhenius Plot. The relation between the denaturation kinetic rate and the heat treatment temperature is given by the Arrhenius equation:

\[
\ln(k_n) = \ln(k_n^o) - \frac{E_{A_n}}{RT}, \tag{7}
\]

where \( k_n \) is denaturation frequency factor \((g^{1-n} L^{n-1} s^{-1})\), \( E_{A_n} \) is denaturation activation energy \((J/mol)\), \( R \) is universal gas constant \((\approx 8.314 \text{ J·K}^{-1}·\text{mol}^{-1})\), and \( T \) is temperature \((K)\).

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where $k_{\text{agg}}$ = aggregation constant rate, depending on $n \left(g^{1-n} \cdot L^{n-1} \cdot s^{-1}\right)$, $k_{\text{agg}}^0$ = aggregation frequency factor \( \left(g^{1-n} \cdot L^{n-1} \cdot s^{-1}\right) \), and $E_{\text{Agg}}$ = aggregation activation energy (J/mol).

These kinetic parameters fully define β-LG denaturation in each temperature range and could be used to simulate the evolution of concentrations of the different β-LG species in a given temperature profile, as illustrated later in this paper.

**Calculation of Thermodynamic Denaturation Parameters**

Thermodynamic denaturation parameters can be calculated for the unfolding and aggregation reactions, which provides information about the structural changes occurring in the 2 temperature ranges in the Arrhenius plot. The equations used to evaluate the free energy $\Delta G$, enthalpy $\Delta H$, and entropy $\Delta S$ of activation of the β-LG denaturation reaction are listed by Anema and McKenna (1996) and reported here:

$$k = \frac{k_B T}{h} \exp \left( -\frac{\Delta G}{RT} \right),$$  \hspace{1cm} [10]

$$\Delta H = E_A - RT,$$  \hspace{1cm} [11]

$$\Delta G = \Delta H - T \Delta S,$$  \hspace{1cm} [12]

$$k = k_n \cdot C_s^{on-1},$$  \hspace{1cm} [13]

where $k$ = denaturation kinetic rate that is independent of the reaction order (s$^{-1}$), $k_B$ = Boltzmann constant \( \approx 1.38 \times 10^{-23} \text{ J/K} \), and $h$ = Planck constant \( \approx 6.62 \times 10^{-34} \text{ J s} \).
In this context, the thermodynamic constants are specific to each Arrhenius temperature range and should be determined separately for unfolding and aggregation reactions by using the same formalism as in equations [10] to [13].

**Computation of the Evolution of Denaturation Level with Time and Ca Concentration at Different Temperatures**

The temporal evolution of the β-LG denaturation level (i.e., the aggregate fraction) for the 4 studied Ca concentrations and 3 holding temperatures (70, 80, and 90°C) were simulated by using the Scilab software (Digiteo, Saclay, France). These simulations were based on the classical denaturation mechanism and its associated kinetic parameters obtained with the HPLC method, along with the temporal discretization of the simulated temperature profiles. We fixed the time step at 1 s, which provided sufficient accuracy. The total heating time was equal to 60 min in our simulation.

The simulated heat treatment profiles were similar to those recorded when performing the denaturation experiments. These temperature profiles were approximately linear in 2 successive time ranges: after a rapid and linear temperature increase from 60°C to the holding temperature (the second water bath), the temperature remained almost constant at the holding value (the third water bath). The approximate times needed to heat the β-LG samples from 60°C to the holding temperatures in the second water bath were 15 s at 70°C, 45 s at 80°C, and 60 s at 90°C. With heating times less than 1 min, β-LG began to denature significantly from 70 to 75°C. This justified our choice to set the initial temperature at 60°C in all experiments, and to simulate the denaturation level evolution only from 60°C. The mean heating rate ranged from 0.33 to 0.67°C/s in these experiments.

**RESULTS AND DISCUSSION**

**Influence of Ca on the β-LG Denaturation Reaction Order**

The β-LG denaturation reaction order found in the literature was generally 1.5, although in some conditions it should be taken equal to 2 (Mulvihill and Donovan, 1987; Relkin, 1996). To settle this issue with respect to the various Ca concentrations, we performed the same heat treatment to the 53.3 g/L β-LG solutions containing different Ca amounts. Figure 3 shows the experimental data acquired with the previously described method applied to study the effect of Ca up to 264 mg/kg on β-LG denaturation at about 75°C; that is, in the unfolding limited range. Furthermore, this figure shows the models obtained with the 1.5 and 2 reaction orders at Ca concentrations of 0, 66, 132, or 264 mg/kg.

As seen in Figure 3, the 1.5 reaction order model was the best able to describe β-LG denaturation at 75°C. Indeed, the 1.5 reaction order model precisely fitted the experimental data at all Ca concentrations. Regarding the 2 reaction order model, the theoretical curves diverged noticeably from the experimental points, which confirms the 1.5 denaturation reaction order hypothesis. The Ca concentration did not seem to affect the denaturation reaction order in the unfolding limited range. Thus, no significant change in the unfolding mechanism reaction order can be attributed to the presence of Ca.

This finding may differ in the upper temperature range, where aggregation is the limiting reaction, as Ca is expected to be highly involved in the aggregation mechanism (Mulvihill and Donovan, 1987) and structure (Britten et al., 1988) by increasing the size of aggregates (Verheul et al., 1998; Allen and Smith, 2001; Schmitt et al., 2007) and lowering the β-LG denaturation temperature, which favors aggregate formation (De Wit, 1990; Simmons et al., 2007). Figure 4 displays the acquired results at an approximate temperature of 90°C for the different experimental solutions. Once again, the 1.5 reaction order led to the more accurate modeling of the experimental data. Indeed, the results at Ca concentrations of 0 and 66 mg/kg were properly fitted by the 1.5 reaction order model, as were the first points at 132 mg/kg of Ca. However, the few points that seemed aberrant at this latter concentration could not be fitted by the same trend, indicating the lack of precision concerning the determination of β-LG concentration. It is interesting to note that the 2 reaction order model permitted a better fit of the 264 mg/kg concentration results, even though the 1.5 reaction order was also suitable. However, the 1.5 reaction order correctly modeled the whole experimental set. The influence of Ca in the aggregation-limited temperature range appeared to be more important at higher Ca concentrations, because the aggregation mechanism was better described with a 2 reaction order. This latter finding confirms results found by some researchers (Dannenberg and Kessler, 1986; Verheul et al., 1998; Santos et al., 2006a; Tolkach and Kulozik, 2007; De Wit, 2009), particularly that of De Jong et al. (1992). Nevertheless, the observation made in this study may be the consequence of the lack of precision at the lower β-LG concentrations, as the samples over 1.5 min at a Ca concentration of 264 mg/kg had a concentration below the HPLC standard lowest concentration of 1 g/L.
INFLUENCE OF CALCIUM ON DENATURATION OF β-LACTOglobulin

The imprecision of β-LG sample concentrations over 3 min at a Ca concentration of 132 mg/kg illustrated this fact.

Moreover, by comparing Figures 3 and 4 it can be seen that denaturation rates follow an increasing trend with temperature: for instance, a $C_\alpha/C_s$ of 3 was achieved in approximately 10 min at 75°C and Ca concentration of 0 or 66 mg/kg, whereas it took only 1 to 2 min at 90°C.

**Temperature Dependence of the Unfolding and Aggregation Kinetic Rates**

The Arrhenius plot for the denaturation reaction at various Ca concentrations has been drawn in Figure 5. This figure depicts the temperature influence on the β-LG denaturation kinetic rate in the temperature range from 68 to 96°C. Each symbol refers to one experiment at fixed temperature and Ca concentration. On Figure 5, the associated uncertainties (i.e., the kinetic rate logarithm prediction intervals) were also represented. They were calculated by using the integral of Student’s probability density function at 95% confidence level and spreading the errors.

As expected, a sharp bend can be observed in the Arrhenius plot around 80°C (more precisely, between 79 and 82°C depending on the Ca concentration). This critical temperature splits the Arrhenius plot in 2 temperature ranges: below 80°C, the β-LG denaturation reaction is unfolding limited, which means that aggregation is faster than unfolding, and over 80°C, all β-LG molecules are quickly converted to unfolded species and aggregation is thus the limiting reaction. The literature indicates that the Arrhenius critical temperature is found between 85 and 95°C (Dannenberg and Kessler, 1986; Lalande et al., 1989; Anema and McKenna, 1996;
Galani and Apenten, 1999; Sava et al., 2005; Tolkach and Kulozik, 2007). Sava et al. (2005), working on β-LG surface thiol groups, found a result (80°C) similar to that in the present study.

The discrepancy in critical temperature encountered here may be explained by the multiple factors influencing β-LG denaturation, such as the nature of the heat-treated solution, chemical environment, β-LG concentration, heat temperature profile, hydrodynamics, analytics, and so on. An accurate determination of the Arrhenius critical temperature would require additional trials, but this was not the main goal of the current study.

With regard to the influence of Ca on β-LG denaturation kinetics, Figure 5 shows that the kinetic rates increased strongly with Ca concentration. In terms of ln \(k_n\), this increase was equal to 0.92 at 70°C, 1.27 at 80°C, and 1.54 at 95°C, when the Ca concentration was increased from 0 to 264 mg/kg. The kinetic rate increase with Ca concentration is more important in the aggregation-limited range. This indicates that Ca acts principally on β-LG aggregation. Indeed, in these conditions, the free Ca ion (Ca\(^{2+}\)) is expected to reduce the repulsion between the β-LG species, which are negatively charged at this pH above the β-LG isoelectric point. It is well known in the literature that Ca forms bridges that strengthen the aggregates structure (Verheul et al., 1998; Allen and Smith, 2001; Schmitt et al., 2007), but this work clearly shows that β-LG unfolding kinetics are also influenced by the Ca concentration. That is, both unfolding and aggregation kinetics are enhanced by Ca addition, this effect being more intense in the upper temperature range.

The kinetic rate increase with Ca concentration is not linear: it is more important in the low Ca concentra-
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Figure 5. Arrhenius plot for the β-LG denaturation reaction with 1.5 reaction order at various Ca concentrations. The error bars represent the kinetic rate values and their respective prediction interval. The solid lines correspond to the linear regressions of experimental data. T = temperature in Kelvin (K); $k_n$ = denaturation reaction rate ($g^{1-n} L^n s^{-1}$).

In Figure 5, the curves corresponding to Ca concentrations of 132 and 264 mg/kg were very close, with a maximum kinetic rate discrepancy equal to 0.25 logarithmic units. This, and the fact that the kinetic rates increased markedly at low Ca concentrations, may indicate a threshold in the Ca effect on β-LG denaturation: a 2-fold increase in Ca concentration over 264 mg/kg would lead to a very small increase in kinetic rate. This can be explained by the β-LG and Ca concentrations in solution: 53.3 g/L β-LG equals 2.9 mmol/L, whereas 132 mg/kg Ca corresponds to 3.3 mmol/L; in other words, the studied solution at a Ca concentration of 132 mg/kg is an almost equimolar mixture of β-LG and Ca. The Ca concentration required to trigger β-LG aggregation is known to increase with the number of Ca ions bound to β-LG (Simons et al., 2002). Thus, above a certain concentration, Ca is expected to have antagonistic effects on β-LG aggregation: on one hand, Ca accelerates β-LG denaturation and aggregation by charge shielding, bridging, or by inducing conformational changes in β-LG structure (Simons et al., 2002; O’Kennedy and Mounsey, 2009); on the other hand, β-LG structure is strengthened by binding Ca, because more Ca is required to initiate β-LG aggregation (Simons et al., 2002). Consequently, the increase in β-LG aggregation rate with Ca concentration should be drastically decreased at a certain Ca concentration, which was observed in our experimental conditions at 132 mg/kg Ca.

For each Ca concentration, linear regressions of experimental data in the 2 temperature ranges (unfolding and aggregation limited) were performed and indicated that denaturation kinetic rates varied with temperature. In the upper temperature range (between
80 and 96°C), β-LG denaturation was limited by the aggregation reaction, so that the aggregation kinetic rate and its evolution with temperature were directly obtained by linear regression of experimental data acquired above 80°C. The same method was used for data acquired below 80°C to determine the kinetic parameters of the unfolding reaction. The obtained frequency factor logarithms (ln $k^\circ$) and activation energies ($E_A$) are shown in Table 1 and some values found in the literature have been added to allow comparison of our experimental results.

Table 1 shows that our experimental results concerning the unfolding reaction were consistent with the literature values. In fact, in the unfolding-limited temperature range, the calculated frequency factor logarithms and unfolding activation energies at a Ca concentration of 264 mg/kg were in agreement with the values published by Tolkach and Kulozik (2007), whereas the values from De Jong et al. (2002) and Dannenberg and Kessler (1986) were closer to the results acquired at 66 mg/kg of Ca. This discrepancy between literature values may result from the different dairy solutions used to study β-LG denaturation and particularly from the Ca concentration. Indeed, it has been noted previously that the kinetic rates increased with Ca concentration. Dannenberg and Kessler (1986) and De Jong et al. (2002) must have used β-LG solutions with Ca concentrations <264 mg/kg, which may explain the observed differences with the work of Tolkach and Kulozik (2007). This hypothesis is confirmed by the very low frequency factor logarithms obtained in the present work for β-LG solutions containing lower Ca concentrations (0 and 66 mg/kg), which were even inferior to the literature values.

In the aggregation-limited range, we obtained results consistent with those of Tolkach and Kulozik (2007), De Jong et al. (2002), and Dannenberg and Kessler (1986), although our values were a little greater. This means that β-LG aggregation was faster under our conditions, most likely because of the relatively high β-LG concentration (5.33% wt/wt) of the investigated β-LG concentrate. This was confirmed by the greater discrepancy between our values and the literature results for the aggregation reaction compared with the unfolding reaction: the higher the β-LG concentration, the more frequent are the collisions between reactive β-LG. Thus, increases in β-LG concentration are expected to affect principally the aggregation behavior.

With regard to the influence of Ca on β-LG denaturation kinetics, we can deduce from the values in Table 1 that Ca contributes to increase the kinetic parameters, both in the unfolding and aggregation limited temperature ranges. However, it should be noted that the few measures at each Ca concentration except 264 mg/kg (about 6 or 7 experiments between 68 and 96°C) can generate imprecision in the evaluation of kinetic parameters. Nevertheless, these results may give a correct insight into the role of Ca concentration in the β-LG denaturation mechanism.

On one hand, the great increase in unfolding activation energy between 0 and 264 mg/kg of Ca indicates that the unfolding reaction needs more energy to initiate at higher Ca concentrations, which means that the native β-LG is more difficult to unfold in the presence of Ca. Therefore, the Ca ions should have a protective effect against β-LG unfolding, from a purely thermodynamic point of view. This can be explained by the fact that Ca alters the native β-LG tertiary structure and forms intramolecular bonds with some (previously hidden) negatively charged groups, leading to reinforcement of the β-LG native structure. This hypothesis is consistent with the increase in Ca-binding ability of
unfolded proteins (Jeyarajah and Allen, 1994). The measured unfolding activation energies have high values (~300 kJ/mol); that is, β-LG unfolding is a strongly endothermic reaction. This indicates that the unfolding reaction corresponds to the break of numerous hydrogen or hydrophobic bonds (each releasing a few kilojoules per mole). The increase in β-LG unfolding activation energy with Ca concentration is a sign of a greater degree of binding in β-LG proteins containing Ca, which confirms our previous hypothesis of β-LG structure reinforcement being due to Ca binding.

On the other hand, our data indicate that the unfolding kinetic rate at a given temperature increases with Ca concentration, because of the significant increase of the frequency factor (its logarithm moved from 73 to 100 in the Ca concentration range of this work). From a kinetic point of view, β-LG unfolding is facilitated by Ca addition. This can be related to the partial β-LG unfolding induced by Ca, which leads to thiol exposure and enhances β-LG denaturation, via the formation of intermolecular disulfide bonds (Jeyarajah and Allen, 1994).

In the aggregation-limited temperature range, activation energy increased slightly from 0 to 264 mg/kg of Ca, which is surprising given that aggregation is known to be favored in the presence of Ca (Verheul et al., 1998; Allen and Smith, 2001; Schmitt et al., 2007) by means of charge shielding, bridging, or by inducing conformational changes in β-LG structure (Simons et al., 2002; O’Kennedy and Mounsey, 2009). The observed increase in β-LG aggregation activation energy may derive from the trigger effect due to Ca-binding: the greater the number of Ca ions bound to β-LG, the higher the Ca concentration required to induce significant β-LG aggregation (Jeyarajah and Allen, 1994). The higher activation energy in the presence of Ca may be related to the additional noncovalent interactions devoted to Ca binding.

As for the unfolding reaction, the aggregation frequency factor increased strongly with Ca concentration (from 25 logarithm units without Ca to 32 logarithm units at 264 mg/kg of Ca), which compensates the higher activation energy required to initiate the aggregation reaction; thus, this allows the aggregation kinetic rate to increase slightly with Ca content.

Therefore, Ca has a similar effect on the unfolding and aggregation mechanisms: from the single thermodynamic point of view, Ca seems to protect the native and unfolded β-LG species by increasing the activation energies required to initiate the unfolding and aggregation reactions, whereas our kinetic results indicate that Ca enhances the denaturation kinetic rates. These conclusions may appear contradictory at first glance, as we showed that Ca both favors and limits β-LG denaturation, according to the obtained thermodynamic and kinetic results. However, it is not aberrant that both denaturation activation energies and kinetic rates increase with Ca concentration. In fact, the activation energy represents the required energy to initiate β-LG denaturation. In our work, β-LG denaturation was studied between 68 and 96°C (i.e., a temperature range in which β-LG denaturation is observable). Therefore, by applying heating temperatures over β-LG denaturation temperature, we provided enough energy to the β-LG solution to initiate β-LG heat denaturation. This explains why the protective effect of Ca on β-LG denaturation was only observable by calculation of thermodynamic parameters (and not by following β-LG denaturation). One way to illustrate the role of Ca in β-LG denaturation thermodynamics could be to measure the temperature at which β-LG begins to denature as a function of the Ca content. In our case, Ca would be expected to increase the temperature at which β-LG begins to denature, as the activation energy increased with Ca content.

With regard to the denaturation kinetic rate, it represents the reaction rate related to β-LG denaturation once this reaction is initiated. Indeed, a reaction must be able to proceed before its kinetic rate can be defined. Whereas the ability to react is described by thermodynamics, how the reaction proceeds once initiated concerns kinetics. Thus, our results, indicating a strong increase in the denaturation kinetic rates with Ca concentration, show that Ca favors β-LG denaturation once this reaction is initiated, which was the case in our operating conditions, as denaturation was detected for all temperatures and Ca concentrations of our experimental set. This explains how Ca can have both protective and destabilizing influences regarding β-LG denaturation.

The threshold effect of the Ca concentration, which was observed in the Arrhenius plot of Figure 5, can be deduced from the results in Table 1, as the kinetic parameters for the unfolding and aggregation kinetics reached a plateau from 132 mg/kg of Ca. Increasing the concentration beyond 132 mg/kg led to a very small increase in the denaturation kinetic rate.

Denaturation Thermodynamic Parameters

Thermodynamic parameters for the β-LG denaturation reaction are shown in Table 2. They were calculated by applying equations [10] to [13] to the experimental data acquired in this study. Thermodynamic data on β-LG denaturation are rare and the main contribution to these aspects is from Anema and McKenna (1996), which built upon the pioneer work of Dannenberg and Kessler (1986).
By examining Table 2, good agreement is seen between the results obtained in this work at 66 mg/kg and that of Anema and McKenna (1996), for which we estimated the Ca concentration to be about 80 mg/kg, especially with the A genetic variant of β-LG. Indeed, the free energy of activation ΔG, activation enthalpy ΔH, and activation entropy ΔS were almost identical in the unfolding temperature range and very close in the aggregation limited range, except for ΔH over the Arrhenius critical temperature. The differences observed in the aggregation area between our study and data from the literature can be explained by the difference in Arrhenius critical temperature (80°C in this study versus 85°C in Anema and McKenna, 1996) and by the use of dissimilar β-LG solutions (β-LG powder solution in our study vs. reconstituted whole milk in the latter study) and different Ca concentrations.

The free energy of activation values were, for all temperatures very close to 100 kJ/mol, corresponding to what is expected for protein unfolding. Anema and McKenna (1996) discussed that such a ΔG range is common for this kind of reaction, with data based on older research in which 18 different proteins were studied.

The evolution of activation entropy with temperature reinforced the idea that the overall denaturation reaction is a 2 step-mechanism (unfolding and aggregation) governed mainly by temperature. Indeed, we can see that the entropy was drastically lower in the aggregation temperature range. In fact, high activation energy and entropy values are indicators of a high degree of β-LG intramolecular binding. This point is also reinforced by the enthalpy decrease between the unfolding and aggregation temperature ranges. Such an enthalpy decrease indicates that the break of intramolecular chemical bonds under heating is shackled over the Arrhenius critical temperature.

In the present work, the determined value for the enthalpy of activation was reduced by a factor of 3 when the temperature was increased above 80°C and the entropy of activation was reduced to one-tenth. This implies that β-LG intramolecular bonds are broken mainly in the first denaturation step, which corresponds to β-LG unfolding. This is related to the conformational change occurring in this lower temperature range: β-LG unfolds, which involves the breaking of several noncovalent bonds that were essential to stabilization of the native structure. Additionally, the high positive value of activation entropy observed under the Arrhenius critical temperature indicates a significant reduction of β-LG state of order, supporting the thesis of the intramolecular bonds breakage.

Regarding the upper temperature range, the activation enthalpy ΔH, entropy ΔS, and energy $E_A$ were

<table>
<thead>
<tr>
<th>Denaturation mechanism</th>
<th>Enthalpy of activation ΔH (kJ/mol)</th>
<th>Free energy of activation ΔG (kJ/mol)</th>
<th>Entropy of activation ΔS (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfolding: $T &lt; T_c$</td>
<td>252.5 331.8 308.2 291.8 280.6 (A)</td>
<td>92.0 100.5 110.2 108.0 104.6 (A)</td>
<td>38.7 47.9 47.9 47.9 47.9 (A)</td>
</tr>
<tr>
<td>Aggregation: $T ≥ T_c$</td>
<td>103.5 102.3 100.6 99.6 98.3 (A)</td>
<td>101.9 100.2 97.8 97.6 97.6 (A)</td>
<td>−0.03 −0.03 −0.03 −0.03 −0.03 (A)</td>
</tr>
</tbody>
</table>

where $T_c$ = Arrhenius critical temperature.

Table 2. Mean enthalpies, free energies, and entropies of activation and comparison with those reported by Anema and McKenna (1996).
lowered, which denote a strong change in the denaturation mechanism. This temperature range mechanism involves almost no breakage or formation of intramolecular bonds, because of the almost zero activation entropy. Consequently, the β-LG state of order remained similar, which is consistent with the aggregation mechanism expected to occur above the Arrhenius critical temperature.

With regard to the influence of Ca on β-LG denaturation thermodynamic parameters, ΔG was almost unaffected by a Ca concentration increase, whereas the ΔH and ΔS values were significantly increased. Therefore, on one hand, the observed reaction type remained the same at various Ca concentrations: it consisted of β-LG denaturation, as indicated by the almost constant ΔG value. On the other hand, the ΔH and ΔS increases are attributable to the reinforcement of the native β-LG intramolecular bond network because of the increased presence of Ca. The β-LG native structure is also expected to be strengthened by the fact that Ca binds 2 parts of the β-LG polypeptide chain, by bridging 2 negatively charged groups such as carboxylates (Simons et al., 2002). Thus, the presence of Ca significantly stabilizes the tertiary structure of β-LG, as attested in the unfolding limited temperature range by the clear ΔH increase from 233 to 302 kJ/mol between 0 and 264 mg/kg Ca. The same trend was shown in the ΔS values (from 0.37 to 0.58 kJ/mol), which confirms that the native β-LG state of order increases with Ca concentration, as expected in case of increased noncovalent bonds in the β-LG structure.

Similar observations were made in the aggregation limited temperature range, but the Ca effect was less intense: ΔH increased from 92 to 108 kJ/mol in the studied Ca concentration range, while ΔS remained quasi constant, which indicates that Ca had little protective effect on the β-LG molten globule state. Calcium also strengthens the network of noncovalent binding interactions of unfolded β-LG, even though its role is more important in stabilization of the β-LG native structure. This result may derive both from the trigger effect of Ca on β-LG aggregation (Simons et al., 2002) and from the partial β-LG unfolding induced by Ca ions, which favors β-LG denaturation and aggregation (Jeyarajah and Allen, 1994).

The β-LG concentrate used in this study has been kinetically qualified and the calculation of thermodynamic parameters has shed light on the denaturation mechanisms (unfolding and aggregation), their respective predominance ranges, and their modification in the presence of various Ca concentrations. In the last part of this paper, we will illustrate the Ca influence at different temperatures by examining the temporal evolution of β-LG denaturation at different fixed Ca concentrations and temperatures.

**Influence of Ca on the Temporal Evolution of Denaturation at Different Temperatures**

The last part of the experimental section will discuss the effect of Ca on β-LG denaturation at various temperatures. We have chosen to illustrate the role of Ca in the unfolding and aggregation limited temperature ranges (at 70 and 90°C, respectively) and at the Arrhenius critical temperature of our system (80°C). The computed results are given in Figure 6, which shows the evolution of denaturation at 0, 66, 132, and 264 mg/kg Ca and at 70, 80, and 90°C. The denaturation level represents the proportion of β-LG proteins that have been converted into aggregates; that is, $1 - \frac{C_s}{C_s^0}$.

As expected from the form of the Arrhenius equation (equation [7]) and in the conclusions built on the results in Figure 5, the denaturation level increased faster (i.e., the denaturation rate was higher) when the temperature increased. Near full denaturation (a denaturation level >90%), in the absence of Ca, was reached in about 6 min at 90°C and around 30 min at 80°C, whereas at 70°C, a heat treatment of 60 min was insufficient to denature almost all of the native β-LG (data not shown).

The denaturation level curves had a characteristic shape with a high denaturation rate from the beginning of the heat treatment (see equation [2] with high $C_i$ values) until the β-LG native concentration was reduced by half. Then, the denaturation rate decreased even more and converged on very low values when the denaturation level was approaching 80 to 90%.

The computed evolutions of denaturation level related to the whole Ca concentration range were distributed in well-separated curves, except at 90°C and 0 mg/kg Ca, whose results were very similar to those at 80°C and the 2 highest Ca concentrations. This confirms the strong increases in denaturation rate and level with temperature. In each set of curves, the denaturation level increased with the Ca concentration, which indicates that the denaturation mechanisms are catalyzed by the presence of Ca. At 70°C, the discrepancy between the curves was significant and regular, whereas at higher temperatures (80 and 90°C), the modification of the Ca concentration seems at first glance to have a lesser effect on the denaturation level. At the latter temperatures, the threshold effect in the Ca influence on β-LG denaturation, already discussed in the denaturation kinetic parameters determination, is very apparent in Figure 6, as the denaturation levels at 132 and 264 mg/kg of Ca...
were very close or identical. In view of these elements, it could be suggested that Ca has a greater influence on β-LG denaturation at low temperatures. Therefore, Ca would be expected to interfere with the unfolding mechanism, which would be accelerated from small Ca additions. In actuality, although Ca had a more apparent effect on β-LG denaturation at low temperatures (Figure 6; the denaturation level increased progressively with the Ca concentration), Ca influenced the aggregation mechanism more, as can be deduced from the kinetic rate measurements. Indeed, the maximal denaturation level difference between the 2 extreme Ca concentrations (0 and 264 mg/kg) was greater at higher temperatures. At 90°C, the denaturation level at about 0.8 to 1 min moved from 10 to 80% between 0 and 264 mg/kg; at 80°C and 5 min, from 40 to 90%; and at 70°C, only from 20 to 60%. Thus, we concluded that Ca alters aggregation more than denaturation, especially at short heat treatment times, as in industrial pasteurization facilities, for instance. Therefore, these findings explain why Ca initially seemed to be more significant at high time ranges and low temperatures (i.e., in the unfolding limited range), whereas its role in the aggregation temperature range is actually predominant.

**CONCLUSIONS**

The calculation of the kinetic parameters allowed us to evaluate the reaction order value that was the best fit to the β-LG denaturation behavior: 1.5.

The critical temperature that splits the Arrhenius plot in 2 linear parts was estimated at 80°C, each temperature range being related to the predominance of the unfolding or aggregation mechanisms, which constitute the 2-step reaction path of the β-LG denaturation. The Ca effect was more important in the aggregation limited temperature range, indicating that Ca acts principally on β-LG aggregation by lowering the Coulombian barrier between 2 negatively charged molten globule or aggregated β-LG, by bridge formation, or via an

Figure 6. Evolution of β-LG denaturation level at various Ca concentrations and temperatures (dotted line = 70°C, dashed line = 80°C, and solid line = 90°C).
ion-specific conformational interaction. Its role in β-LG unfolding was limited to the reinforcement of the native β-LG tertiary structure. This was consistent with Ca trigger effect previously described in the literature.

The calculation of the unfolding and aggregation thermodynamic parameters showed good agreement with literature data. The activation enthalpy and entropy obtained in our work were lower in the aggregation range than in the unfolding one. This signifies that the unfolding mechanism involves the break of numerous intramolecular bonds (high ΔH), reducing the β-LG state of order (high ΔS), contrary to the high temperature mechanism, whose low ΔH and ΔS are characteristic of an aggregation reaction preserving the β-LG state of order, in which few (here, only one) covalent bonds are formed.

From a thermodynamic point of view, Ca has a protective influence principally on native β-LG but also on its molten globule species; however, from a kinetic point of view, Ca catalyzes both unfolding and aggregation reactions, because the associated kinetic rates were strongly increased in the presence of Ca. This discrepancy between kinetics and thermodynamics shows that β-LG denaturation is a complex process, catalyzed by high Ca concentrations, with a 2-step mechanism highly dependent on the Ca content. The thermodynamic characterization of β-LG denaturation is not sufficient to predict the behavior of the different β-LG species in complex environments, which may explain why the kinetic evaluation is so common in β-LG studies, whereas thermodynamics are rarely discussed, despite the additional information that it provides.

We confirmed by computing the kinetic data that an increase in the Ca concentration promoted β-LG denaturation mechanisms; namely, unfolding and aggregation. We showed that the results from simulations of β-LG concentration can be wrongly interpreted, as Ca seemed graphically to enhance β-LG denaturation particularly at low temperatures. Nevertheless, this interpretation was due to a deceptive impression: Ca influence is maximal at short treatment times and high temperatures. This last point emphasizes the importance of addition of Ca when the temperature is high, especially in heat exchangers where β-LG solutions are subjected to short-time heat treatments with a final temperature near 100°C. Monitoring the Ca concentration is a prerequisite for prediction of β-LG species concentration within heat exchangers and other chemical facilities.

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