Effect of Calcium on Ovine Caseinate Functional Properties

Cássia R. Nespolo,[†,‡] Anselmo D. Reggiardo,[†] Manuel A. Mancilla Canales,[†] Jorge R. Wagner,[§] Estela M. Alvarez,[†] Adriano Brandelli,[‡] and Patricia H. Risso*[,†]

Departamento de Química-Física, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, (2000) Rosario, Argentina, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal de Rio Grande do Sul, Av. Bento Gonçalves 9500, (91501-970) Porto Alegre, Brasil, and Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, (1876) Bernal, Argentina

The solubility of colloidal particles of ovine caseinate in the presence of calcium was followed by analyzing the colloidal particle size and the protein composition of colloidal particles remaining in suspension. A comparison between the behavior of bovine and ovine caseinate was carried out. A sequential two-step salting-out process, due to progressive Ca^{2+} binding to at least two kinds of sites, was observed for both caseinates. The precipitation curves were fitted, and the affinity constants and binding site numbers were calculated with an equation based on the concept of Wyman's linked functions. Ovine caseinate colloidal aggregates obtained in the presence of calcium turned out to be less stable and bigger than the bovine ones. Calcium binding to protein residues modifies the composition and the conformation of caseinates. An aggregation process at low ovine caseinate concentrations and a gelation process at high protein concentrations induced by glucono-δ-lactone hydrolysis were studied in the presence of calcium concentrations where no precipitation is observed. The presence of calcium affects the kinetics of both processes and the final state of aggregates and gels network formed. The degree of compactness, average size of the aggregates, and rheological properties of gels formed at the end of the acidification process depend on the calcium concentration added.

Introduction

The production of typical dairy products from sheep can provide a profitable alternative to cow milk products due to their specific taste, texture, and their natural and healthy properties. Some major reviews exist concerning the biochemical composition of sheep milk. In fact, it is well-known that sheep milk contains approximately a 2-fold concentration of proteins, fats, and a higher energetic value than cow milk.[1] It also contains vitamins and is an important source of macrominerals and oligoelements. In sheep cheese, a major product derived from sheep milk, the amount of these nutrients is even higher than those observed in untreated sheep milk due to their concentration during the cheese-making process.[2] Sheep milk is a more suitable source for cheese production compared to cow milk as it is characterized by a lower colloidal stability, a key factor that favors its fabrication.[3]

The main protein fraction in ovine milk is represented by caseins ((76 to 83) % of total proteins). These proteins and their anionic form, caseinates, are extensively used in food industry because of their physicochemical, nutritional, and functional properties that make them valuable ingredients in complex food preparations.[1] Caseinates are commonly employed as additives in a great variety of food products because of their high emulsifying, water-binding, and gelation capabilities, their heat stability, and their contribution to the food texture and juiciness. Some of these properties make caseinates useful and desirable ingredients in the preparation of bakery and confectionery products, where they can be used as milk substitutes.[4] Cheese making, that is, renneting properties, of sheep milk are affected by its physicochemical properties, including pH, casein micelle size, calcium amount per casein weight, and other mineral concentrations in milk, which cause differences in coagulation time, coagulation rate, curd firmness, and amount of rennet needed.[1,5] However, in-depth research on these physicochemical properties of ovine caseins is still needed.

From a nutritional point of view, caseins have all of the essential amino acids and play an important role in the transport of calcium and phosphate, representing an easily digestible source of nutrients, contributing to a carefully balanced diet.[6] Caseins occur in milk as stable colloidal aggregates known as casein micelles, mainly composed by α_{S1}-, α_{S2}-, β-, and κ-casein (α_{S1}-, α_{S2}-, β-, and κ-CN) linked together in association with clusters of colloidal calcium phosphate, a mineral complex involving mainly calcium and phosphate ions.[7] Regarding the aminoacidic composition of ovine and bovine caseins, no difference is observed between those of α_{S1}-CN residues, while ovine α_{S2} and κ-CN possess a higher amount of residues. On the other hand, ovine β-CN contains a lesser number of residues than the bovine counterpart.[1] It is interesting to point out that the amount and position in the polypeptide chain of phosphoserine and phosphothreonine residues in caseins of both species are similar, except for ovine α_{S1}-, α_{S2}-, and κ-CN that contain one more phosphoserine residue.[1,8−10]

Industrial caseinates are obtained from milk caseins in different ways and are generally associated with sodium, potassium, calcium, or magnesium ions. Their functional properties are different depending on the associated cations. Particularly, calcium ions exert important effects on casein solubility and colloidal stability. Therefore, calcium binding to

* Corresponding author. Tel.: 54-0341-4804592/97 (int. 253). Fax: 54-0341-4372704. E-mail: phrisso@yahoo.com.ar.
[†] Universidad Nacional de Rosario.
[‡] Universidade Federal de Rio Grande do Sul.
[§] Universidad Nacional de Quilmes.

10.1021/je1001335
Published on Web 10/11/2010

caseins has to be considered in food processing when calcium caseinate is used as an ingredient.[11] Studies with purified caseins showed that individual α_{S1}-, α_{S2}-, and β-CN are readily precipitated by calcium ions, whereas κ-CN is not. Consequently, this last component is responsible for the stabilization of the other casein components against the flocculating action of calcium ions by favoring the formation of stable colloidal aggregates.[12] Although solubility and colloidal stability of bovine caseins in the presence of calcium ions have been studied by different authors from thermodynamic and kinetic approaches,[13,14] research regarding ovine caseins has merited lower attention.

Dissociation and a further aggregation step of casein fractions due to caseinate acidification results in the formation of a gel structure. A possible explanation to this observation is that as the pH is adjusted toward the isoelectric point it causes a decrease of the repulsive interactions, resulting in a destabilization of the colloidal aggregates as the pH drops slightly below 5 at a given temperature.[15,16] Nowadays, a process that has gained the attention of food industry is direct acidification by the addition of a lactone, such as glucono-δ-lactone (GDL) which allows us to overcome some of the difficulties associated with the traditional process of using bacteria. In fact, the final pH of the system is a function of the amount of GDL added, whereas starter bacteria produce acid until they inhibit their own growth as the pH becomes lower.[15,17]

Casein gels are responsible for most of the rheological and textural properties (i.e., stretch, fracture) of sheep cheese and other dairy products. Rheological properties are evaluated as a quality control method in the food industry to perform research on the structure and texture of food products. Although rheological characteristics and functional properties of cow milk and its dairy products have been extensively studied, the information available regarding those of sheep milk products is still scarce.[18]

In this work, an overall study of the physicochemical parameters of whole ovine caseinates in the presence of calcium was performed. The solubility of ovine casein in the presence of calcium was studied from a thermodynamic approach using the concept of Wyman's linked functions.[19] Using a fluorescence method, the possible conformational changes exhibited by casein due to the effect of calcium was investigated. Acid aggregation of calcium caseinate (CaCas) was analyzed on the basis of spectrophotometric measurements and a particle size analysis, whereas gelation was investigated by following the rheological properties of the system.

Experimental Section

Materials. The bovine sodium caseinate powder, the acidulant glucono-δ-lactone (GDL), imidazole, and 8-anilino-1-naphthalene sulfonate (ANS) as ammonium salt were commercially acquired from Sigma-Aldrich Co. (Steinheim, Germany). HCl, NaOH, acetone, and chloroform were provided by Cicarelli SRL (San Lorenzo, Argentina). Calcium chloride and sodium azide were purchased from Mallinckrodt Chemical (St. Louis, USA).

An experimental procedure was carried out for the preparation of ovine casein from milk of Lacaune breed sheep from southern Brazil. After centrifugation at 10 000 times gravity for 10 min at 4 °C to remove fats as much as possible, the skim milk was acidified at pH 4.5 with 1 mol·L^{-1} acetic acid under continuous stirring at 25 °C to cause isoelectric precipitation of caseins. After 30 min at 40 °C, the mixture was filtered through Whatman No. 40 paper (Kent, UK) using a vacuum pump. The precipitated casein was washed with distilled water, dissolved with the addition of 10 g·L^{-1} NaOH until it reached pH 7.0, and precipitated again. Four successive cycles of precipitation and washing were carried out. The final precipitate was washed with acetone and chloroform to remove the residual fat globules.[20,21]

Ovine sodium caseinate (10 g·L^{-1}) was prepared from the previously obtained ovine casein, by dissolving 1 g of casein in 50 mL of 0.1 mol·L^{-1} NaOH. The dissolution of ovine casein was adjusted to a final pH 6.8 by adding small volumes of 0.1 mol·L^{-1} HCl and taken to a final volume of 100 mL with distilled water.[22]

Bovine sodium caseinate solution was prepared from the dissolution of commercial product in distilled water (pH 6.8) at 25 °C.

Casein concentration was determined in the initial caseinate solutions and was measured according to the Kuaye's method which is based on the ability of strong alkaline solutions (0.25 mol·L^{-1} NaOH) to shift the spectrum of the amino acid tyrosine to higher wavelength values in the UV region.[23] All of the values obtained were the average of two determinations. After concentration measurements, the solutions were stored in the dark at 4 °C after the addition of sodium azide at 0.2 g·L^{-1} as a bacteriostatic agent.

Colloidal Stability Test. Two milliliters of desired concentration (0 to 30) mmol·L^{-1} of calcium chloride in 0.2 mol·L^{-1} imidazole buffer pH 6.8 were added to 2 mL of protein solution in thick-walled centrifuge tubes. The tubes were inverted three times and allowed to stand at 25 °C in a water bath for 1 h. Then the tubes were centrifuged at 1500 times gravity for 20 min in a Luguimac LC 10 centrifuge (Buenos Aires, Argentina).[11,12] Precipitates (insoluble casein aggregates) and supernatant (casein colloidal aggregates, CCA) were obtained. Each experiment was replicated in triplicate.

Precipitation of Casein by Ca^{2+}. Caseinate precipitation by Ca^{2+} was interpreted by Farrell et al.,[11−13] using the concept of Wyman's linked functions,[19] assuming that cation binding to the protein is followed by the precipitation or salting-out process of the less soluble calcium caseinate formed:

$$p + n\mathrm{Ca} \overset{K_1^n}{\longleftrightarrow} p\mathrm{Ca}_n + n'\mathrm{Ca} \overset{K_1^{n'}}{\longleftrightarrow} p\mathrm{Ca}_n\mathrm{Ca}_{n'} \qquad (1)$$

where p is the unbound protein and n and n' are the number of Ca^{2+} moles bound to the species $p\mathrm{Ca}_n$ and $p\mathrm{Ca}_n\mathrm{Ca}_{n'}$, respectively. K_1^n and $K'^{n'}_1$ are the equilibrium constants for the process of precipitation or salting out. The mathematical relationship representing the above stoichiometry will be:

$$S_{app} = S_0 f(p) + S_1 f(p\mathrm{Ca}_n) + S'_1 f(p\mathrm{Ca}_n\mathrm{Ca}_{n'}) \qquad (2)$$

where S_{app} is the apparent protein solubility at a given Ca^{2+} concentration, $f(i)$ the protein fractional components of species i, S_0 the initial concentration of soluble caseinate, S_1 the apparent solubility of $p\mathrm{Ca}_n$, and S'_1 the apparent solubility of $p\mathrm{Ca}_n\mathrm{Ca}_{n'}$.

Alvarez et al.[24] developed the following equation, which is used to fit the experimental data of solubility as a function of concentration of Ca^{2+} as it was found that a second salting-out step takes place as calcium concentration increases (the second group of salting-out sites n'):

$$S_{app} = \frac{S_0}{1 + K_1^n[Ca^{2+}]^n} + \frac{S_1 K_1^n[Ca^{2+}]^n}{1 + K_1^n[Ca^{2+}]^n} + \frac{(S_1' - S_1)(K_1')^{n'}[Ca^{2+}]^{n'}}{1 + K_1'[Ca^{2+}]^{n'}} \quad (3)$$

where K_1 and K_1' correspond to the first and second salting-out process, respectively. Caseinate solubility data were subject to nonlinear regression using the Levenberg−Marquard algorithm. The parameters involved were calculated as follows: in a first step, the Ca^{2+} solubility profiles were analyzed by fixing the value of n and calculating the values of K_1 and S_1, which gave the best least-squares to fit eq 3 in the first moiety of the curve; n was then fixed to a new value, and the whole process was repeated. The values of n, which gave the minimum root-mean-square and the lowest K_1 error, and the correspondent S_1 and K_1 values were introduced in eq 3, and the same approach was applied for n', S_1', and K_1' calculation. The parameters S_0, S_1, and S_1' when possible were calculated averaging the first, the middle, or the last set of data points, respectively. Then the K_1 and K_1' were obtained from the nonlinear regression analysis.

Urea-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (Urea-SDS-PAGE). The CCA that remained in the supernatant fraction after the colloidal stability test were analyzed on their qualitative composition by Urea-SDS-PAGE using a vertical gel system ($n = 2$), according to the method of Laemmli.[25] The protein bands were identified using commercial α_S-, β-, and κ-CN (Sigma Chemical Co., Steinheim, Germany).

The relative intensity of the bands was determined by scanning the stained gels and analyzing the pixel densities of the digitized protein bands, using specially designed software for this purpose (WinXgel). WinXgel is software specially developed to analyze digital images of electrophoresis gels. Software functions include the determination of a profile distribution for each one of the samples loaded into the gel and the background correction. Besides, it allows us to calculate the area under the curves obtained and their peak point position. WinXgel makes it possible to estimate the total area of the experimental curves through the addition of Gaussian distributions which in turn allows to analyze the profiles obtained by the deconvolution of the overlapping zones. Moreover, results might be after imported into another data analysis and graphing software such as MS-Excel and SigmaPlot among others.

Composition of the Precipitates. The precipitates of insoluble casein aggregates obtained from the colloidal stability test were separated at 25 °C by centrifugation at 1500 times gravity for 30 min. The amount of the different components in the solid phase was obtained from the difference between the total concentrations and the average concentrations in the supernatants. These last concentrations were calculated from the solubility values of the correspondent precipitation curves, obtained by fitting these ones using eq 3, to render an interpretation of the experimental curves obtained, and the relative concentration of each of the components determined by PAGE.

Size Variations of the CCA. Changes in the CCA average size were followed by the dependence of turbidity (τ) on wavelength (λ) of the suspensions, determined according to:

$$\beta = 4.2 + \frac{d(\log \tau)}{d(\log \lambda)} \quad (4)$$

β is a parameter that has a direct relationship with the average size of the particles, can be used to easily detect and follow rapid size changes, and was obtained from the slope of log τ versus log λ plots, in the (450 to 650) nm range, where the absorption due to the protein chromophores is negligible allowing the estimation of τ as absorbance in the (400 to 800) nm range.[26] On the other hand, it has been shown that β, for a system of aggregating particles of the characteristics of caseinates, tends, upon aggregation, toward an asymptotic value that can be considered as a fractal dimension (D_f) of the aggregates.[27,28]

τ was measured as absorbance using a Spekol 1200 spectrophotometer (Analytikjena, Belgium), with a diode arrangement. Determinations of β were the average of measurements in duplicate.

Casein Determination. Casein concentration was determined in the initial caseinate solution and in the supernatants of the colloidal stability test. Casein was measured according to Kuaye's method.[23] All of the values obtained were the average of two determinations.

Effect of Calcium in Acid Aggregation of Casein. Calcium chloride was added to the ovine sodium caseinate solution to obtain a final Ca^{2+} concentration ranging from (0 to 2.5) mmol·L^{-1}. After the addition of calcium chloride, the system was maintained under continuous stirring for about (1 to 2) h.

Acid Aggregation of Ovine Caseinate. A previously prepared solution of caseinate (5 g·L^{-1}) was used. Kinetics of calcium caseinate aggregation induced by the acidification with GDL was analyzed by measuring turbidity (τ) in the range of (450 to 650) nm, in a Spekol 1200 spectrophotometer with a thermostatized cell.

The amount of GDL added was calculated using the following relation:

$$R = \frac{\text{GDL mass fraction}}{\text{protein mass fraction}} \quad (5)$$

R used for this experiment was 0.5, at a temperature of 35 °C.

Acidification was initiated by the addition of solid GDL to 10 g of calcium caseinate suspension. Absorption spectra (450 to 650 nm) and absorbance at 650 nm (A_{650}) were registered as a function of time until a maximum and constant value of A_{650} was reached; simultaneously the pH decrease was measured. The determinations were performed in triplicate. Values of the parameter β were calculated using eq 4.

Analysis of Conformational Changes of CaCas. Excitation and emission spectra of the CaCas (5 g·L^{-1}) were obtained with the aim of detecting any spectral shift and/or changes in the intensity of fluorescence (FI) as the calcium concentration becomes higher. Previously, the excitation wavelength (λ_{exc}) and the range of concentration with a negligible internal filter effect were determined. The samples (3 mL) used for the spectral analysis and FI measurements were poured into a fluorescence cuvette with a light path length of 1 cm and placed into a cuvette holder maintaining temperature at desired constant values. Values of FI ($n = 3$) were registered within the range of (300 to 400) nm at 35 °C using a λ_{ex} of 286 nm.

Surface Hydrophobicity (S_H). S_H was estimated according to Kato and Nakai method,[29,30] using the ammonium salt of amphiphilic ANS as a fluorescent probe, in an Aminco Bowman Series 2 spectrofluorometer (Thermo Fisher Scientific, USA). The measurements were carried out using λ_{exc} and emission wavelength (λ_{em}) set at (396 and 474) nm, respectively, at a constant temperature of 35 °C. As mentioned above, both

wavelengths were previously obtained from emission and excitation spectra.

The FI of samples containing ANS and different concentrations of caseinate (FI_b) as well as the intrinsic FI of the calcium caseinate (FI_p) were determined ($n = 3$). The difference between FI_b and FI_p (ΔF) was calculated, and S_H was determined as the initial slope in the ΔF versus protein concentration ($mL \cdot g^{-1}$) plot.

Size Distribution of CaCas Particles. The average size of CaCas particles and size distribution measurements were performed in a Mastersizer 2000 HYDRO 2000 MU model (Malvern Instruments Ltd., Worcestershire, U.K.). This instrument is based on low angle laser light scattering (LALLS), which determines that the diffraction angle is inversely proportional to size particle.

Experimental data were obtained as a function of diameter size versus volume distribution, and the De Brouckere mean diameter ($D_{4,3}$) was also calculated by software processing.

To carry out this determination, an amount of solid GDL was added to 550 mL of 2 $g \cdot L^{-1}$ CaCas solution to obtain an R of 0.5 according to eq 5. This mixture was maintained at a constant and slow stirring of 600 rpm. Measurements at different times were monitored until the maximum of obscuration (turbidity) allowed by the instrument was reached, in triplicate. Simultaneously, the registration of pH values was followed.

Rheological Properties of Acid Gels of CaCas. Rheological properties of CaCas samples (30 $g \cdot L^{-1}$) were determined in a stress and strain controlled rheometer TA Instruments, AR G2 model (Brookfield Engineering Laboratories, Middleboro, MA, USA) using a cone geometry (diameter: 40 mm, cone angle: 2°, cone truncation: 55 mm) and a system of temperature control with a recirculating bath (Julabo model ACW 100) connected to a Peltier plate.

An amount of solid GDL according to an R of 0.5 was added to initiate the acid gelification. Measurements were performed each 20 s during 100 min with a constant oscillation stress of 0.1 Pa and a frequency of 0.1 Hz. The Lissajous figures at various times were plotted to make sure that the determinations of storage or elastic modulus (G') and loss or viscous modulus (G'') were always obtained within the linear viscoelastic region. The determinations were performed in duplicate.

The $G'-G''$ crossover times (t_g) of acidified caseinate systems were considered here as the gel times, since most studies of milk or caseinate gelation have adopted this criterion.[15,31] pH at t_g was also determined considering the pH value at the $G'-G''$ crossover (pH_g).

Statistical Analysis. The data are reported as the average values ± their standard deviations. The statistical analysis was performed with Sigma Plot 10.0, OriginPro 8, and SYSTAT 12 software. The relationship between variables was statistically analyzed by correlation analysis using the Pearson correlation coefficient (r). The differences were considered statistically significant at $p < 0.05$ values.

Results and Discussion

Colloidal Stability Test. The protein concentration remaining in solution, that is, in colloidal suspension in the supernatant, when precipitation of ovine sodium caseinate was induced by increasing total calcium concentration (TCC) and centrifuged at 1500 times gravity is shown in Figure 1A.

Precipitation started at low TCC (~3 $mmol \cdot L^{-1}$) and continued until an important protein fraction was precipitated (50 % of total protein at 20 $mmol \cdot L^{-1}$ of TCC) (Figures 1A and 2A). Comparing these results with those obtained for bovine caseinate (Figure 1B), it can observe for the latter that precipitation starts approximately at 2-fold TCC values. In addition, only 35 % of bovine caseinate was precipitated at 20 $mmol \cdot L^{-1}$ of TCC (Figure 2A). Nonetheless, the shape of the curves obtained for both caseinates showed certain similarities. In fact, they exhibit the presence of a two well-defined steps of salting-out.

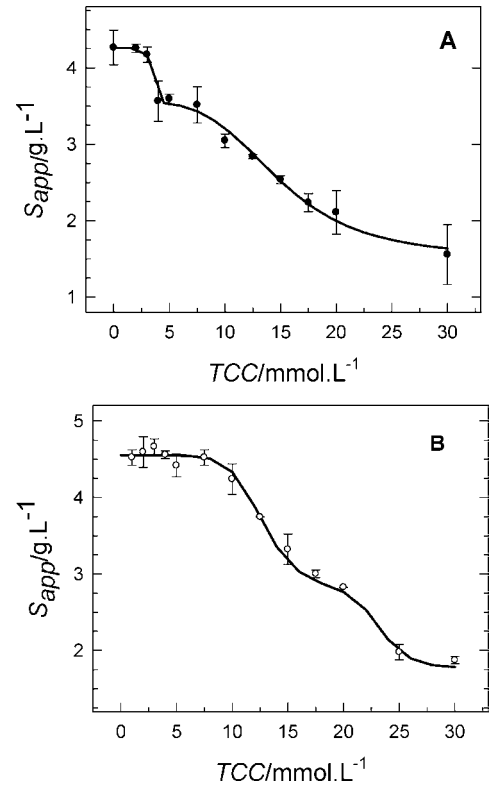

Figure 1. Mean experimental values ($n = 3$) of remnant caseinate concentration (S_{app}) in the supernatants of mixtures of 5 $g \cdot L^{-1}$ ovine (A) or bovine (B) sodium caseinate as a function of the total calcium concentration (TCC) at pH 6.8 and a temperature of 25 °C. The error bars represent the standard deviation for each data point (normal distribution of errors). The gray lines represent the fitting from eq 3.

The curves were fitted using eq 3, and the affinity constants (K_1, K_1') and binding site numbers (n, n') were calculated with a model based on the concept of Wyman's linked functions by using the Gauss−Newton algorithm (Table 1). In both regression analyses the nonlinear model was statistically significant. An adjusted R_2 value of 0.996 and 0.96734 for bovine and ovine caseinates was obtained, respectively. Also the normal error distribution assumption was checked by the Shapiro−Wilks test in both cases.

It was possible to observe that in the first stage these parameters (affinity constants, number of binding sites) exhibited higher values for ovine caseinate than in the bovine counterpart. Two kinds of Ca^{2+} binding sites are proposed in the precipitation plots (Figure 1). Apparent average binding constants differed in 1 order of magnitude with the stronger ones (K_1) corresponding to the initial step of the precipitation process. Since it is well-known that the affinity of phosphoserine residues for Ca^{2+} is higher than any other binding sites,[13,24] we assume that this first step involves most of the casein phosphoserine residues as evidenced by higher K_1 values. However, we cannot disregard the involvement of other anionic residues, such as carboxylate groups; they cannot be ruled out in this stage. Lower values were obtained for the average association constant for the second salting-out step (Table 1), suggesting the participation of weaker

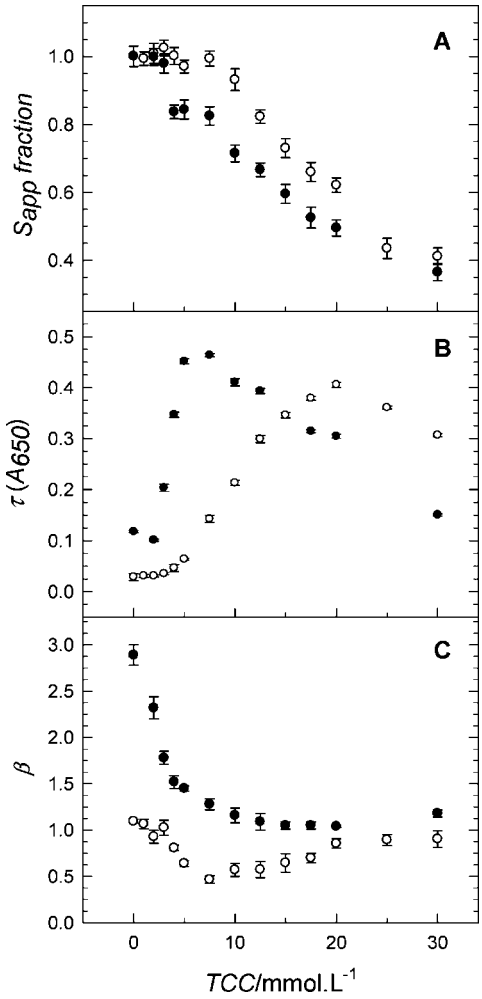

Figure 2. Mean experimental values ($n = 3$) of fractional apparent solubility (S_{app}) (A), turbidity (τ) as absorbance at 650 nm (B), and parameter β (C) in the supernatants of mixtures of ●, ovine and ○, bovine sodium caseinate (5 g·L^{-1}) as a function of the TCC at pH 6.8 and a temperature of 25 °C. The error bars represent the standard deviation for each data point (normal distribution of errors).

Table 1. Average ($n = 3$) Parameter Values of Calcium Binding to Sodium Caseinates Calculated by the Fitting of Equation 3[a]

	K_1		S_1	K'_1		S'_1
parameters	L·mol^{-1}	n	g·L^{-1}	L·mol^{-1}	n'	g·L^{-1}
ovine caseinate	300 ± 22	18	3.6 ± 0.1	68 ± 2	4	1.6 ± 0.1
bovine caseinate	72 ± 2	4	2.4 ± 0.1	43 ± 1	17	1.7 ± 0.1

[a] Note: errors are standard errors derived from the fitting of eq 3. Protein concentration 5 g·L^{-1}, 25 °C, and pH 6.8.

affinity sites. K_1 and K'_1 values were higher for ovine caseinate compared to the bovine ones.

On the other hand, in the first step when the TCC is equal to the inverse of K_1, the apparent solubility (S_{app}) is determined by eq 3:

$$S_{app} = \frac{S_0 + S_1}{2} \quad (6)$$

The value obtained for ovine caseinate (3.2 mmol·L^{-1}) was much lower than the one calculated for bovine caseinate (12.8 mmol·L^{-1}).

The values of S_{app} fractional change, τ, and β for CCA suspensions in the supernatants were plotted (Figure 2A, B, and C, respectively) as a function of TCC. For a given TCC, the S_{app} of ovine caseinate is always lower than the bovine caseinate one (Figure 2A). The average size of particles remaining in suspension, as estimated by β values, decreased during the precipitation as TCC increased (Figure 2C). On the contrary, bovine CCA β values diminished at low TCC but rose again when both the precipitation and the amount of colloidal particles in suspension reached a limit value. Nevertheless, despite the shape of the β profiles, it can be always observed that average sizes of the ovine CCA were higher, especially at low TCC values.

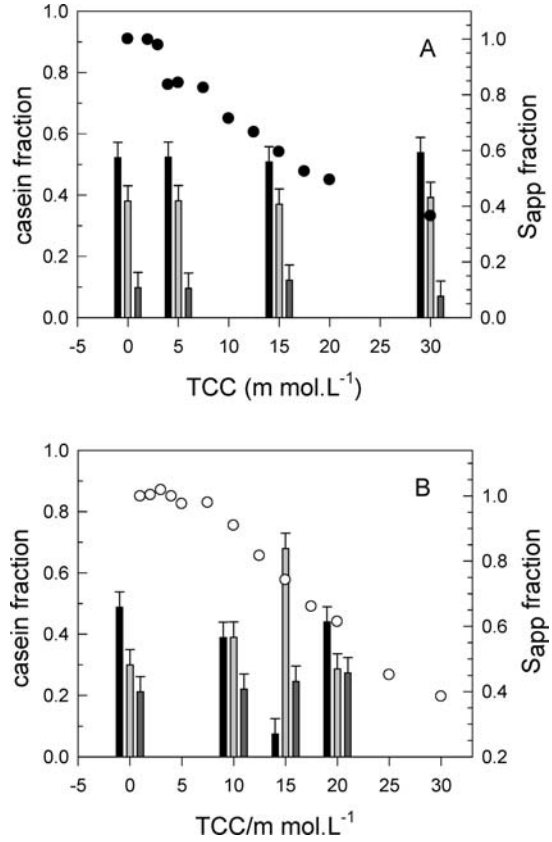

Figure 3. Protein composition (bars) and ●, protein solubility of CCA versus TCC for ovine (A) and bovine (B) caseinate at 5 g·L^{-1}. black ■, α-CN; light gray ■, β-CN; and dark gray ■, κ-CN. $\alpha = 0.01$.

As regards τ (Figure 2B), it increased up to a maximum at 20 mmol·L^{-1} TCC in the case of bovine caseinate and at 7.5 mmol·L^{-1} TCC for the ovine caseinate with a following evident decrease in both cases. For ovine caseinate, β values underwent an abrupt decrease until ~5 mmol·L^{-1} of TCC and then continued to decrease slowly to lower values (Figure 2C). These results would indicate the disappearance of the biggest CCA at high TCC. For bovine caseinate, β values showed an initial formation of a low amount of quite small particles, followed by a second step with the further formation of colloidal particles that progressively grow in size.

A qualitative and quantitative analysis of SDS-PAGE showed for all TCC a higher content of α- and β-CN fractions in the CCA compared to the amount of κ-CN in the case of ovine caseinate (Figure 3A). On the other hand, the ovine κ-CN fraction is always lower than the bovine, thus suggesting an explanation to the higher average size of ovine CCA observed under all TCC.

For bovine CaCas, it was observed a significant α-CN protein precipitation, that is, a small electrophoretic band, and a practically constant composition for κ-CN, leading consequently

Table 2. Average ($n = 3$) Parameter Values of Surface Hydrophobicity (S_H) in Mixtures of 5 g·L^{-1} Ovine NaCas at Different TCC Values, at 35 °C[a]

TCC / mmol·L^{-1}	S_H / mL·g^{-1}
0.0	1.5 ± 0.1
0.5	0.86 ± 0.03
1.0	0.74 ± 0.02
1.5	1.06 ± 0.02
2.0	0.85 ± 0.01
2.5	0.60 ± 0.03

[a] Note: errors are standard deviations of S_H. α = 0.01.

to an increase of the fractional percentage of β-CN (Figure 3B). The higher decrease of α-CN content could be related to the moment when a kind of "shoulder" appears in the solubility curve. In the second salting-out stage, it can be observed a decrease of α-CN percentage and an increase in the β- and κ-CN percentage with regard to the NaCas. We could assume that, even though all the CN precipitate, the α-CN seems to do it in a higher proportion. These results are consistent with those obtained by Pitkowski et al.[32] This change of composition was not detected in the ovine system probably due to the difference in α_S-CN phosphorylated residues between both species.

According to the results reported above, at a given temperature and pH, ovine calcium CCA seems to be less stable than the bovine counterpart. The differences observed might be related to the composition and different proportion of caseins in cow[24] and sheep milk. Sheep milk presents a higher amount of α- and β-casein that in turn have a higher amount of phosphoserine residues capable of Ca^{2+} binding,[1] which makes ovine caseins more sensitive to precipitation. Moreover, ovine casein is characterized by a smaller content of κ-CN fraction which is known to act in the stabilization of casein colloidal aggregates. Therefore, a lower amount of κ-CN could be also responsible for the decrease on the ovine particle stability.

Analysis of Conformational Changes and Surface Hydrophobicity. Emission spectra of ovine sodium caseinate at different Ca:Cas ratios are presented in Figure SI1 (Supporting Information).

S_H of the ovine CCA was determined for different Ca^{2+} concentrations, and it is listed in Table 2. S_H was higher in the absence of Ca^{2+}, varying as calcium was added, but with general tendency to diminish when Ca^{2+} increases.

These results would indicate the formation of CCA with a more compact structure when calcium is present, according to that reported for bovine CCA.[33]

In the studied range of calcium concentrations ((0 to 2.5) mmol·L^{-1}), in which the ovine caseinate solubility is not affected (Figure 1A), two contrary effects upon S_H may be inferred from the data obtained. On the one hand, as TCC increases, the average size of the aggregates in suspension decreases (Figure 2C) as a direct consequence of a dissociation process undergone by CCA, which favors the exposure of hydrophobic patches. On the other hand, an increase of TCC introduces a conformational change in CCA which makes the fluorophore residues of the protein (Trp and Tyr) to project onto a less polar surrounding environment (Figure SI1, Supporting Information). As a result, the values obtained for S_H depend on a balance between those opposite effects.

Acid Aggregation and Gelation of Ovine Caseinate: Effect of Calcium. The acid aggregation was evaluated by following how the parameter β is modified as a function of pH after adding GDL, either in the absence or in the presence of

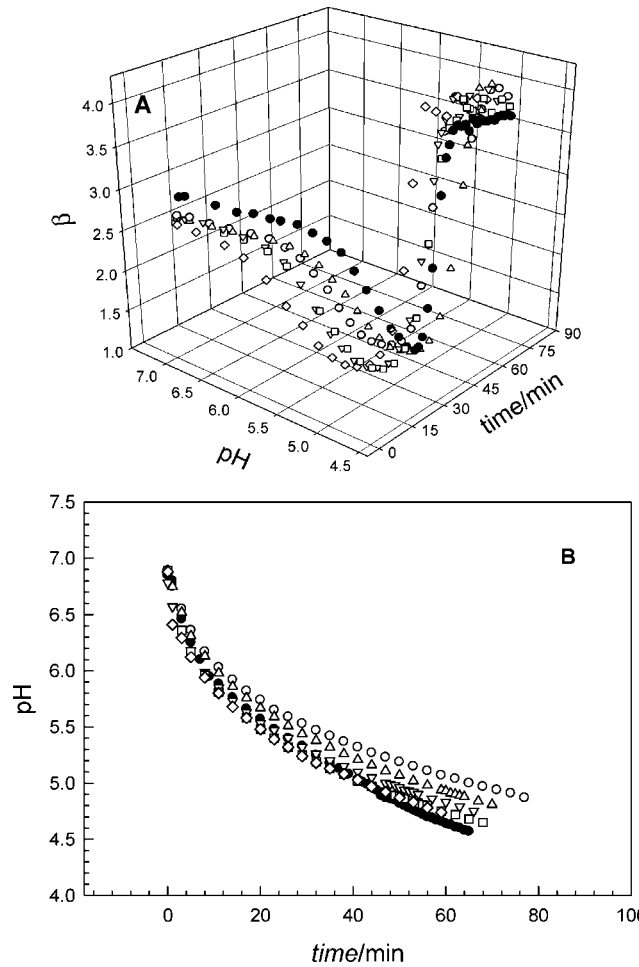

Figure 4. Parameter β variations ($n = 3$) as a function of the time (t) and the pH, after the addition of GDL (A) and pH values as a function of time (B), during the acid aggregation ($R = 0.5$, 35 °C) of ovine sodium caseinate solutions (5 g·L^{-1}) in ●, the absence and in presence of different calcium concentrations (mmol·L^{-1}): ○, 0.5; △, 1.0; ▽, 1.5; □, 2.0; ◇, 2.5.

Ca^{2+} concentrations but always under conditions where no caseinate precipitation is observed (< 3 mmol·L^{-1}). The variation of the parameter β as a function of pH and time is presented in Figure 4A.

Results showed, under all circumstances, the existence of two well-defined steps. At the beginning, a slow phase with a decrease of β values is observed. The second step presents a sharp increase in the average size of particles due to formation of colloidal aggregates (t_{ag}) that grow until β reaches a limit value, that is, D_f.

In the presence of calcium, TCC-dependent changes on t_{ag} can be observed. The t_{ag} increased in the presence of up to 1 mmol·L^{-1} calcium concentration and decreased at higher TCC. This may be partly linked to a reduction on the rate at which pH becomes lower in the presence of calcium (Figure 4B) but could also be related to a decrease on the aggregation rate of caseinate particles in the presence of the cation. Calcium does not alter the hydrolysis rate of GDL (data not shown); therefore, we assume that the presence of this cation affects the initial state of the colloidal particles leading to changes in the aggregation process.

Furthermore, the presence of calcium increases the pH_{ag}. Because the colloidal particles of caseinate in suspension have a negative net charge, an addition of calcium would decrease its electrostatic stability favoring their aggregation by a consequent reduction of the net charge of the soluble particles and

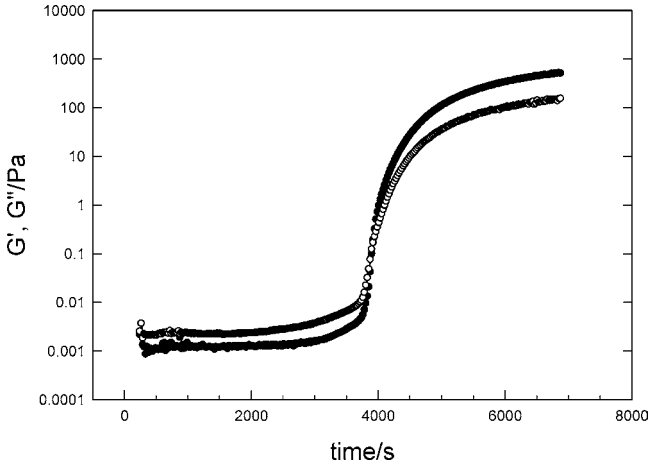

Figure 5. Variations of ●, elastic modulus G' and ○, viscous modulus G'', as an example, during acidification process after addition of GDL (R = 0.5) to obtain $G'-G''$ crossover. Caseinate concentration 30 g·L^{-1}, TCC 0.5 mmol·L^{-1}, temperature 35 °C.

Table 3. $G'-G''$ Crossover Times (min) and pH$_g$ Values (n = 2) for Formulations Containing Ovine Sodium Caseinate (30 g·L^{-1}) with Different TCC Values, after Adding GDL (R = 0.5) at 35 °C[a]

TCC	t_g		G'_{max}
mmol·L^{-1}	min	pH$_g$	Pa
0.0	31.5 ± 0.1	4.99 ± 0.01	167 ± 2
0.5	65.4 ± 0.2	4.56 ± 0.01	518 ± 4
1.0	32.0 ± 0.2	4.99 ± 0.03	277 ± 1
2.0	26.8 ± 0.1	5.14 ± 0.02	228 ± 1

[a] Note: errors are standard deviations of the measured parameters. α = 0.01.

by increasing ionic strength of the medium. In addition, as described in Colloidal Stability Test Section, the average size of initial colloidal particles decreases as TCC becomes higher. Since the rate of aggregation is limited by the diffusion of particles, larger particles have a slow motion giving rise to an increase of t_{ag}.

It was observed that in presence of calcium the aggregates formed showed a higher degree of compactness (higher D_f). To confirm the direct relationship between the parameter β and the average size of particles, measurements of the $D_{4,3}$ and the distribution of sizes of the same ones were performed using the Mastersizer 2000 HYDRO 2000 equipment. Results are reported in the Supporting Information (Figures SI2 and SI3). Variations of $D_{4,3}$, which indicates the mean diameter in volume of particles in a system, are shown in Figure SI4, where the concentration of calcium, pH, and time were modified.

Figure 5 shows, as an example, the curves obtained for variations of G' and G'' during the process of acidification. There is a slow stage where both moduli have very low values followed by a sharp increment of G' before $G'-G''$ crossing. Results of rheological properties during gelation of caseinate samples (30 g·L^{-1}) revealed that the t_g, pH$_g$, and G'_{max} varied depending on the system studied (Table 3).

In the presence of calcium, an increase in its concentration was accompanied by an increase of pH$_g$ and a decrease of t_g (Table 3), probably due to a loss of the net charge loss of the particles, which reduces their electrostatic stability and made them more susceptible to flocculate. These gels reached higher values of G'_{max} than gels formed in the absence of Ca^{2+} at the same protein concentration and amount of GDL. Therefore, calcium would favor the intermolecular forces during the formation of the gel mesh, especially at low cation concentrations. At a calcium concentration of 0.5 mM, the gelation process is slow enough to allow rearrangement of the gel mesh, resulting in more elastic gels.

The decrease in gelation time among different samples containing calcium was around (51.6 to 59) %, indicating that the gel time was also heavily dependent on the higher intensity of association or dissociation of casein particles which, in turn, depend on changes in the ionic and solubility characteristics of the casein molecules.[15]

Conclusions

A two-step well-defined salting-out process with apparent average binding constants differing in 1 order of magnitude was observed when calcium was added to ovine sodium caseinate suspensions. Precipitation started at low calcium concentrations (~3 mmol·L^{-1}) and continued to increase until an important protein fraction precipitated, as calcium concentrations became higher, while the average size of particles remaining in suspension decreased.

At constant temperature and pH, ovine casein colloidal aggregates generated in the presence of calcium are less stable and bigger than bovine ones. These differences would be related to casein composition of colloidal aggregates; ovine particles have higher proportion of α and β-CN, a lower fraction of κ-CN, and a higher amount of phosphoserin groups which act as Ca^{2+} binding sites. Furthermore, the surrounding environment of protein fluorophores in caseins becomes more hydrophobic, and protein structures are more compact, decreasing the exposure of hydrophobic sites as the cation concentration in the system becomes higher.

In both cases, for either ovine or bovine caseinates, during the aggregation or gelation processes, before GDL addition, the profiles obtained from turbidity and LALLS experiments at low caseinate concentrations and from rheological measurements at high caseinate concentrations present certain similarities. They reveal two stages: the first one was characterized by a diminution of particles average size, limited turbidity of suspensions, and predominant viscous modulus. The second one was characterized by a sudden increase of particle average size and turbidity and a sharp increment of elastic modulus.

Calcium decreases the electrostatic stability of colloidal particles as a consequence of the reduction of its net charge and, in that way, would favor the intermolecular forces during the formation of the gel mesh but is accompanied by a decrease of surface hydrophobicity which to same extent diminishes the participation of hydrophobic interactions in the rearrangements during gel formation. The degree of compactness and the hardness of gels depend on the balance of these two effects which, in turn, show a dependence on Ca^{2+} concentration.

The compactness and average size of the aggregates formed at the end of the acidification process of ovine caseinate depend also on the distribution of the initial sizes and on the kinetics of the aggregation phenomena. As the aggregation process becomes slower, the more easily a polypeptide chain could acquire different orientations, leading to the formation of a more compact aggregates and gels with more elasticity and hardness.

As a conclusion, too many variables may affect the functional properties of dairy proteins. Enrichment of milk-derived products with mineral ions, such as calcium, strongly affects solubility and gelation properties of ovine casein, leading to important effects on the bioavailability of minerals and rheological properties of the final product.

Acknowledgment

Thanks to Dr. Gonzalo Palazolo and MSc. Luis Martínez of Universidad Nacional de Quilmes for their advice in the LALLS and rheological measurements. Thanks to Cesár Araujo for his advice on the use of the WinXgel software.

Supporting Information Available:

SDS-PAGE, emission spectra, and multiple equilibria model of protein−calcium system details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review February 6, 2010. Accepted September 7, 2010. This work was supported by grants from the Universidad Nacional de Rosario (Argentine) and Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES), of Ministry of Education (Brazil). A.B. and J.R.W. are research awardees of CNPq (Brazil) and CONICET (Argentine), respectively.

JE1001335