Colloidal Stability of Bovine Calcium Caseinate Suspensions. Effect of Protein Concentration and the Presence of Sucrose and Lactose

Manuel A. Mancilla Canales, María E. Hidalgo, Patricia H. Risso,* and Estela M. Alvarez

Departamento de Química-Física, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, (S2002LRK) Rosario, Argentina

Sodium caseinate suspensions (Cas) are stabilized as submicelles in a dynamic system consisting of caseins α, *β*, and *κ*. Their use in the food industry is quite widespread because of their functional properties and nutritional value. In the presence of calcium ions, Cas are able to form colloidal stable aggregates in suspension (CCA) at certain Ca^{2+} and Cas concentration ratios (Ca:Cas). The aim of this work was to study the effect of the protein concentration on the CCA solubility and viscosity at different Ca:Cas and to relate those two functional properties to protein composition and conformational changes. The stability of CCA was also evaluated in the presence of lactose and sucrose. The apparent solubility of the CCA was determined by applying a spectrophotometric method, and the stability test parameters were obtained using a model based on thermodynamic linked functions. The protein conformational changes were followed by using fluorescence techniques and were related to the partial specific volume and the intrinsic viscosity. The experimental data obtained allowed us to conclude that the stability of CCA depends on the initial protein concentration for the same Ca:Cas ratio. A model based on multiple-step equilibrium was proposed as an approach to explain the stability of Cas against Ca^{2+}. Furthermore, it was observed that the addition of Ca^{2+} to Cas generates changes in the protein conformation, leading to more compact and symmetrical structures. The colloidal stability of the CCA was favored by the presence of sugars, especially by lactose.

Introduction

Caseins (CN) represent the major protein component of bovine milk. These dairy phosphoproteins precipitate at pH 4.6, and they have a considerable tendency to self-associate either in the presence or in the absence of calcium ions.[1]

Among the different types of CN (αs_1-CN, αs_2-CN, *β*-CN, *κ*-CN), there are some important characteristics that make the difference between them, on the basis of their charge distribution and their sensitivity to be precipitated by Ca^{2+}. The *κ*-CN fraction, insensitive to Ca^{2+}, acts as protection that attempts to prevent the other CN from Ca^{2+}-induced precipitation.[2] The ability of binding Ca^{2+} is a reversible process and has an important nutritional impact on the bioavailability of Ca^{2+} associated with these proteins.[3]

The CN precipitate at pH 4.6 and may be resolubilized by increasing the pH. If the increase in the pH is carried out by the addition of NaOH, it is possible to end up obtaining sodium caseinate, while if this change in the pH is done with $Ca(OH)_2$ it is finally obtained as calcium caseinate.[4]

CN and caseinates (Cas) are widely used in the food industry because of their nutritional and functional properties, as well as solubility (even in presence of Ca^{2+}), viscosity, and stability to heat.[1,5] They stabilize emulsions and foams, form gels, interact with lipids, and block fat.[6]

CN and Cas undergo some modifications depending on the seasonal and feeding variations of cattle as well as on previous treatments and industrial processing. These modifications usually introduce significant changes, which in turn exert an important influence over their functional properties, which may represent either a problem or an improvement in the manufacturing of dairy products.[7] Changes on the ratio Ca^{2+}:Cas could modify functional properties, as well as viscosity, solubility, and stability. Consequently, the study of the conformational state of CN and Cas is needed as it is one of the most important determining factors as to how the functional property is affected.[8]

Nowadays consumers have increased the demand of healthy and highly nutritionally valued food products. As a result, the food industry has undergone an increment in the production of supplemented foods, for instance, with the addition of dietary supplements such as minerals. Although on the one hand it represents a benefit from a nutritional point of view, on the other hand the addition of minerals (e.g., Ca^{2+}) constitutes an inconvenience in the formulation of dairy products because of the reduction on the mineral and protein concentration due to the precipitation produced as a consequence of the technological treatments. This effect would be diminished by incorporating milk proteins which have the ability to bound Ca^{2+} and combine it with a certain contribution of soluble Ca^{2+} at levels that do not alter the stability of the whole. The stability of milk proteins and the availability of Ca^{2+} are conditioned by mineral equilibria between the CN and the ions in the soluble phase. Therefore, analyzing the different equilibria involved in the stability of the protein−cosolutes systems is of capital importance if it is considered that the successful enrichment of food formula intended for mass consumption will depend on the interactions between these two components.

Lactose only is naturally present in milk, and its proportion varies with genetics and seasonal and dietary factors. This sugar represents an energetic source of easy and rapid utilization and favors calcium and magnesium absorption. Sucrose is an

* Corresponding author. Tel.: 54-0341-4804592/97 (int. 253). Fax: 54-0341-4372704. E-mail: phrisso@yahoo.com.ar.

10.1021/je900932a
Published on Web 04/21/2010

essential ingredient in the formulation of many foods such as dairy products.

The addition of sugars may introduce a change to the thermodynamic properties of proteins in aqueous solutions, through direct biopolymer−cosolute interactions or through modification of the water structure surrounding the protein.[9] Such changes could modify the biopolymer interactions and, consequently, the system properties.

The aims of this work are to characterize calcium caseinate aggregates (CCA) in different ratios of Ca:Cas and to relate the solubility and the viscosity of CCA suspensions to their structural properties, with the purpose of optimizing the bioavailability of Ca^{2+} and CN for the consumption of products that contain them. Changes on CCA stability in the presence of lactose and sucrose were also evaluated.

Experimental Section

Materials. The bovine sodium caseinate powder, 8-anilino-1-naphthalenesulfonate (ANS), as an ammonium salt and imidazole were purchased from Sigma-Aldrich Co. (Steinheim, Germany). Sucrose, lactose, HCl, and NaOH were provided by Cicarelli SRL (San Lorenzo, Argentina). Calcium chloride and sodium azide were purchased from Mallinckrodt Chemical (St. Louis, USA). Sodium caseinate suspensions (NaCas) were prepared from dissolution of commercial drug in water, at about 20 $g \cdot L^{-1}$ NaCas final concentration, pH 6.8, and 25 °C. After concentration measurements, 0.2 $g \cdot L^{-1}$ sodium azide was added as a bacteriostatic agent, and the solutions were stored at 4 °C.

Determination of Colloidal Stability of CCA. Two milliliters of desired concentration [(0 to 40) $mmol \cdot L^{-1}$] of calcium chloride in 0.2 $mol \cdot L^{-1}$ imidazole buffer at pH 6.8 were added to 2 mL of (5, 10, and 15) $g \cdot L^{-1}$ protein solution in thick-walled centrifuge tubes. The tubes were inverted three times and allowed to stand in a 25 °C water bath for 1 h. Tubes were centrifuged at 1500 times gravity for 20 min in a Luguimac LC 10 centrifuge (Buenos Aires, Argentina).[10] Precipitates (insoluble casein aggregates) and supernatant (casein colloidal aggregates, CCA) were obtained. Each experiment was replicated at least in triplicate.

For a thermodynamic study of the precipitation process of CN by Ca^{2+} and to gain a better understanding of colloidal stability of CCA, an approach based on the concepts of Wyman's thermodynamic linkage was used. The solubility study of CN was carried out at different protein concentrations and in the presence of lactose (Lac) or sucrose (Suc).

The approach assumed two kinds of binding sites for the Ca^{2+}, the first type of sites giving rise to nonsoluble complexes (salting-out), whereas the binding to the second type of sites would lead to the resolubilization (salting-in) of the mentioned complexes. Equilibria involved are formulated as:

$$p + n\mathrm{Ca} \overset{K_1^n}{\longleftrightarrow} p\mathrm{Ca}_n + m\mathrm{Ca} \overset{K_2^m}{\longleftrightarrow} p\mathrm{Ca}_n\mathrm{Ca}_m \qquad (1)$$

where p is the unbound protein concentration, n and m are the number of moles of Ca^{2+} bound to species $p\mathrm{Ca}_n$ and $p\mathrm{Ca}_n\mathrm{Ca}_m$, and K_1 and K_2 are the apparent salt-binding equilibrium constants. K_1 is the salting-out constant, and K_2 is the salting-in constant.

The amount of protein that was determined in the supernatant is named the apparent solubility (S_{ap}):

$$S_{ap} = f_p S_0 + f_{p\mathrm{Ca}_n} S_1 + f_{p\mathrm{Ca}_n\mathrm{Ca}_m} S_2 \qquad (2)$$

where S_0, S_1, and S_2 are the solubilities of p, $p\mathrm{Ca}_n$, and $p\mathrm{Ca}_n\mathrm{Ca}_m$, respectively. S_{ap} is a measure of colloidal stability at a given total calcium concentration, and f_i are the protein fractional components of species i. The incorporation of the salt-binding equilibrium constants to the last expression leads to the following:

$$S_{ap} = \frac{S_0}{1 + K_1^n[\mathrm{Ca}]^n} + \frac{S_1 K_1^n[\mathrm{Ca}]^n}{1 + K_1^n[\mathrm{Ca}]^n} + \frac{(S_2 - S_1)K_2^m[\mathrm{Ca}]^m}{1 + K_2^m[\mathrm{Ca}]^m} \qquad (3)$$

Equation 3 is valid when $K_1 > K_2$, when the n sites become saturated with calcium prior to significant binding of calcium to the m sites and when the sites do not interact between them. When the total concentration of protein is small compared to the free salt concentration ($[Ca^{2+}]$), under this circumstance the total salt concentration ($[Ca^{2+}]_T$) can replace the latter in the last expression and considering only the salting-out process gives rise to:

$$S_{ap} = \frac{S_0 + S_1 K_1^n[\mathrm{Ca}]_T^n}{1 + K_1^n[\mathrm{Ca}]_T^n} \qquad (4)$$

All solubility profiles were analyzed by nonlinear regression, fixing the value of n and calculating the best least-squares fit for the K_1 and S_1 values. The n value was then fixed to a new value, and the whole procedure was repeated. The n value which yielded the minimum root-mean-square and lowest error value for K_1 was then reported.[10] A kind of "shoulder" in the solubility profiles at $[Ca^{2+}]_T$ above 20 $mmol \cdot L^{-1}$ was observed, and then it was necessary to add a new term to the last equation:

$$S_{ap} = \frac{S_0 + S_1 K_1^n[\mathrm{Ca}]_T^n}{1 + K_1^n[\mathrm{Ca}]_T^n} + \frac{(S'_1 - S_1)K'^{n'}_1[\mathrm{Ca}]_T^{n'}}{1 + K'^{n'}_1[\mathrm{Ca}]_T^{n'}} \qquad (5)$$

The new value of n' was fixed, while repeating all of the procedure and using the n, K_1, and S_1 values estimated in the first calculation allowed us to calculate K'_1 and S'_1 values.[11]

Colloidal Stability of CCA in the Presence of Sugars. The system under study was prepared by adding to a NaCas (20 $g \cdot L^{-1}$) aqueous solution the following reagents: imidazole−HCl buffer at pH 6.8 and $CaCl_2$ solution to obtain a (1 to 40) $mmol \cdot L^{-1}$ $Ca^{2+}{}_T$ final concentration range, and (5, 10, and 15) $g \cdot L^{-1}$ Cas final concentration and tested sugars up to 20 $g \cdot L^{-1}$ when necessary. The tubes were let standing at room temperature for 1 h and then were centrifuged for 15 min at 1500 times gravity.[10] In the supernatant the [Cas] was determined by the Kuaye method which is based on the ability of strong alkaline solutions to shift the spectrum of the amino acid tyrosine to higher wavelength values in the UV region.[12]

CCA Average Size. NaCas and CCA possible size changes and/or degree of compactness were followed by the dependence of turbidity (τ) on the wavelength (λ). τ was measured as absorbance (A) in the (400 to 600) nm range, where there is no protein chromophore group absorbance.

For monodispersed particles of molecular weight M, concentration c, and with a refractive index close to that of the solvent, the turbidity is given by:

$$\tau = HcMQ \quad \text{where} \quad H = \frac{32\pi^2 n_0^2 \left(\frac{\partial n_1}{\partial c}\right)^2}{3N\lambda^4} \qquad (6)$$

where n_0 and n_1 are the refractive indexes of the pure solvent and the solution, respectively, N is the Avogadro's number, and $\delta n_1/\delta c$ is the specific refractive index increment. The dissipation factor Q results from the internal interference of light scattered

by the particle at all angles θ. The function Q depends on the particle size and can be defined as:

$$Q = \frac{3}{8}\int_0^{\pi} P(q,R)(1 + \cos^2\theta)\sin\theta\,d\theta \qquad (7)$$

where θ is the dispersion angle of light and $P(q,R)$ is a size factor function of the wavelength vector q and the ratio R of the particle.[13]

Simulation studies using different models for particle aggregation have highlighted the fractal nature of the colloidal aggregates obtained.[14] For an object with fractal structure, the expression for Q takes the form:

$$Q = \frac{3}{8}\int_0^{\pi} P(q,R)S(q)(1 + \cos^2\theta)\sin\theta\,d\theta \qquad (8)$$

where $S(q)$ is a structure factor that describes the special arrangement of the dispersion elements or monomers within the aggregate.[15]

From eq 6, we can obtain at constant c the derived equation:

$$\alpha = \frac{\partial \log \tau}{\partial \log \lambda} = \frac{\partial \log Q}{\partial \log \lambda} + 2\frac{\partial \log\left(n\frac{\partial n}{\partial c}\right)}{\partial \log \lambda} - 4 \qquad (9)$$

The parameter α is then related to the size, shape, and degree of compactness of the particles, and it can be used to detect changes in them under different conditions.

Taking into account that some samples to be studied are not monodispersed, τ will be a function of the weight average of the molecular weight ($\bar{M}_w$) and of the z average of the dissipation factor (Q_z) that depends on the size distribution of the particles:

$$\tau = H\bar{M}_w Q_z \qquad (10)$$

From eq 10, it is possible to find the inverse relationship between α and the diameter average of the particle.[16]

τ was measured as the absorbance using a diode array diode-array spectrophotometer SPEKOL 1200 (Analytikjena, Belgium), pouring 3 mL of the different samples into a 1 cm spectrophotometer cuvette in a jacketed cuvette holder at a temperature maintained by water circulation. The α value is calculated from the slope of log τ versus log λ plots.

Protein Composition. Protein composition was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.[17]

The relative intensity of the stained bands was determined by gel scanning, and an analysis of the pixel densities of the digitized protein bands was determined using specially designed software (X-Gel) for this purpose, including deconvolution of the scanning pattern curves when necessary. Protein band identification was made using commercial α_s-, β-, and κ-CN (Sigma Chem. Co., Steinheim, Germany).

Further details on the method are given in the Supporting Information.

Viscosity (η) and Specific Volume ($\bar{v}_2$). For NaCas and CCA viscosity determinations a thermostatized Ostwald's capillary viscosimeter was used, which is suitable for Newtonian liquid viscosity determinations. In accordance with the Poiseuille law, the time for the meniscus solution to move between two marks was measured (t_{sol}) and the relative viscosity (η_r) was calculated as:

$$\eta_r = \frac{t_{sol} \cdot \rho_{sol}}{t_0 \cdot \rho_0} \qquad (11)$$

where ρ_{sol} is solution density, ρ_0 is buffer density without the protein, and t_0 is the time for meniscus buffer to move between two marks.

Intrinsic viscosities of samples ([η]), related to the protein conformation, are defined by the following equation:

$$[\eta] = \lim_{c_2 \to 0} \frac{(\eta/\eta_0) - 1}{c_2} \qquad (12)$$

where c_2 is the protein concentration in (0.006 to 0.014) g·mL^{-1} and η and η_0 are the solutions and solvent viscosities, respectively. The [η] was determined by η measurements of diluted solution series, plotting $((\eta/\eta_0) - 1)/c_2$ versus c_2 and extrapolating to zero concentration.[18]

The $\bar{v}_2$ of a component of a solution for macromolecules which usually interact with the solvent depends on the solution concentration and is defined by:

$$\bar{v}_2 = \frac{\partial V}{\partial m_2} \qquad (13)$$

Because solution concentration variations are related to density (ρ) variations, it would be possible to express $\bar{v}_2$ at any concentration in terms of ρ rate of change, $\partial\rho/\partial c_2$. Using the latter and c_2, we can write the following equation:

$$\bar{v}_2 = \left(\frac{1}{\rho_0}\right) \cdot \left(1 - \frac{\Delta\rho}{c_2}\right) \qquad (14)$$

where $\Delta\rho/c_2$ is the slope of the regression line of the experimental data and ρ_0 is density at zero concentration.

[η] and $\bar{v}_2$ are related by the following equation:

$$[\eta] = \nu \cdot \bar{v}_2 \qquad (15)$$

where ν is a shape-dependent constant, related directly with the macromolecule conformation.[19]

The NaCas solution and CCA suspensions at different Ca:Cas ratio densities were measured at pH 6.8 and 30 °C. Then, the values of ρ versus concentration were plotted. The resultant plots were linear in the protein concentration studied range, (6 to 14) g·L^{-1}. The Ca:Cas concentration ratio was kept always in a range where no precipitation was observed, (0 to 0.5) mmol·g^{-1}.

Spectrofluorimetric Determinations. Mixtures of 0.15 g·L^{-1} NaCas in water with $CaCl_2$, (0 to 4.2) mmol·L^{-1}, or with sugars, (0 to 20) g·L^{-1}, and isoionic pH 6.8 were put into quartz cells. Emission spectra were obtained every 0.2 nm, between (300 and 400) nm, with an excitation wavelength (λ_{ex}) of 281 nm and 25 °C, using a Aminco-Bowman series 2 spectrophotofluorometer (Thermo Fisher Scientific, USA).

Surface hydrophobicity (S_0) was calculated using the Kato and Nakai method,[20,21] with the ammonium salt of ANS as a fluorescent hydrophobic probe at the constant temperature of 25 °C.

The fluorescent relative intensity (FI) of samples containing 3 mL of 0.04 mmol·L^{-1} ANS with consecutive additions of NaCas or CCA at different Ca:Cas ratios (FI_b) was measured and also the protein alone in the correspondence buffer (FI_p) at the same protein concentration, with λ_{ex} and emission wavelength (λ_{em}) of (380 and 468) nm, respectively. The difference between FI_b and FI_p (ΔF) was calculated as the initial slope from ΔF versus the [NaCas] or [CCA] obtained curve.

Statistical Analysis. Several determinations were performed at least in triplicate. The data were reported as the average value ± their standard deviation. The statistical analysis was performed with Sigma Plot 8.0 software. The relationship between variables was statistically analyzed by correlation analysis using the Pearson correlation coefficient (r). The statistical significance was considered for $p < 0.05$ values.

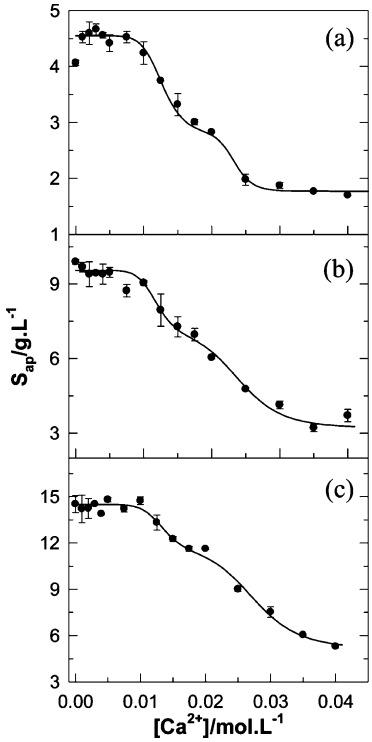

Figure 1. Mean experimental values ($n = 3$) of CCA suspensions solubility (S_{ap}) at different $[Ca^{2+}]_T$ (fitting from eq 5). NaCas concentrations: (a) 5 g·L^{-1}, (b) 10 g·L^{-1}, and (c) 15 g·L^{-1}. The error bars represent the standard deviation for each data point (normal distribution of errors).

Table 1. Average ($n = 3$) Parameter Values of CCA Colloidal Stability at Different Cas Concentrations at 25 °C and pH 6.8[a]

NaCas	S_1	K_1		S'_1	K'_1	
g·L^{-1}	g·L^{-1}	L·mol^{-1}	n	g·L^{-1}	L·mol^{-1}	n'
5	2.78 ± 0.01	78.2 ± 0.4	8	1.77 ± 0.05	43 ± 1	18
10	6.9 ± 0.2	82 ± 3	8	3.2 ± 0.2	41 ± 1	8
15	11.4 ± 0.3	76 ± 4	8	5.1 ± 0.3	36 ± 1	8

[a] Note: errors are standard errors derived from the fitting of eq 5.

Results and Discussion

Colloidal Stability of CCA. Collected results for the S_{ap} determinations at different Ca^{2+} concentrations and the corresponding nonlineal regression curve are presented in Figure 1 (parts a, b, and c).

In Table 1 the calculated values for parameters S_1 and S'_1, K_1 and K'_1, and n and n' at constant temperature and pH are shown.

It is possible to observe that in the salting-out first stage n_1 and K_1 values remained constant, but in the salting-out second stage there is a reduction in the value of K'_1 and a constant value of n', except for a [NaCas] of 5 g·L^{-1}. Therefore, this would indicate that the calcium-induced salting-out first stage is not affected by changes in [NaCas] (K_1 almost constant). In this first stage pre-existing aggregates would present the same accessibility to the calcium ion,and this ion would interact with the same population of binding sites. On the other hand, in the first step, when the Ca^{2+} concentration is equal to the inverse of K_1, the apparent solubility (S_{ap}) is determined by eq 16:

$$S_{ap} = \frac{S_0 + S_1}{2} \tag{16}$$

The S_{ap} value obtained under this condition ranged from (11.8 to 13.9) mmol·L^{-1}.

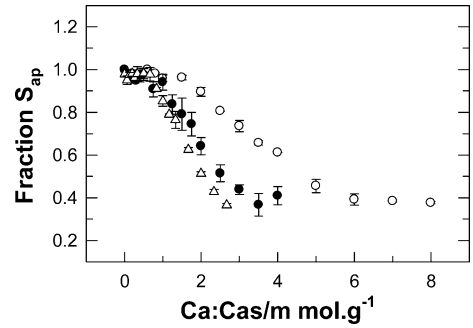

Figure 2. Mean experimental values ($n = 3$) of fractional apparent solubility (S_{ap}) of CCA versus Ca:Cas ratio/mmol·g^{-1}. NaCas concentration: ○, 5 g·L^{-1}; ●, 10 g·L^{-1}; and △, 15 g·L^{-1}. The error bars represent the standard deviation for each data point (normal distribution of errors).

The salting-out second stage equilibrium constant K'_1 showed a slight decrease indicating that the salting-out second stage is being altered by the NaCas concentration ([NaCas]) utilized. For higher concentrations, the corresponding lesser value of K'_1 indicates an increase in CCA stability. This could be caused by a variation in the population of binding sites of the Cas available and susceptible to associate with Ca due to structural changes. The protein composition may be different if a higher amount of protein is present. The n and n' binding sites calculated are mean values of population of sites, and they would be in some manner related to the number of phosphoserine and carboxilate residue groups of the CN which act as the aforementioned binding sites.

Since Cas conformation and composition in the presence of Ca^{2+} could change as the protein concentration is modified, it is interesting to evaluate the S_{ap} at different [NaCas] for a given Ca:Cas ratio. In Figure 2, it is shown that for a certain Ca:Cas ratio the fractional S_{ap} decreases at the same time that values of [NaCas] increase, indicating that a higher amount of present protein would favor the calcium precipitation equilibrium.

The fitting parameters values for 10 g·L^{-1} [NaCas] were lower than those obtained in a previous work.[11] These differences could be attributed to the different source of the Cas samples. In this work we used commercial Cas, and in the previous study it was prepared from the dissolution in NaOH of acid casein obtained by isoelectric precipitation of bovine nonfat milk.

Composition. The α-, β-, and κ-CN composition of commercial NaCas and of CCA, (5, 10, and 15) g·L^{-1}, was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the corresponding samples, and the fractional percent composition was calculated using a software especially developed for the analysis of pixel density of the digitalized protein bands (X-GEL).

Using this method we collected the following mass fraction of CN composition for the solutions of NaCas: 0.44 α-CN, 0.33 β-CN, and 0.24 κ-CN. These values are similar to that published in the literature by Mora-Gutierrez.[10]

For 15 g·L^{-1} NaCas, an equal precipitation for all of CN (Figure 3a) was evidenced. For 10 g·L^{-1} NaCas a significant α- and κ-CN protein precipitation and consequently rising β-CN fraction in the CCA (Figure 3b) were observed. For 5 g·L^{-1} NaCas, a remarkable α-CN protein precipitation and a constant composition for κ-CN resulting in the rise of the β-CN mass fraction (Figure 3c) were found. The higher precipitation of α-CN could be related to the appearance of a kind of "shoulder" in the solubility curve.

In the salting-out second stage, a diminution in α-CN mass fraction and an increase in the β- and κ-CN ones under all of

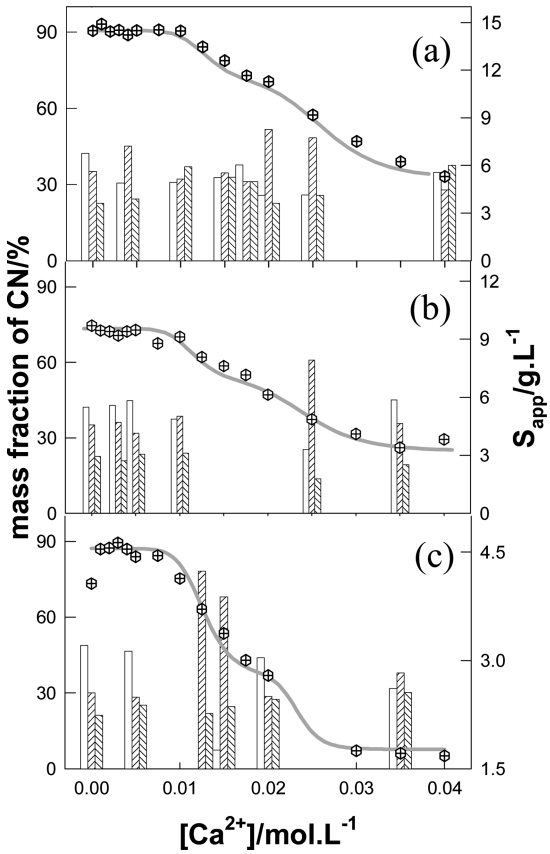

Figure 3. Protein composition (bars): (empty) α-CN, (increasing diagonal) β-CN, and (decreasing diagonal) κ-CN; and protein solubility (○ with +) of CCA versus [Ca^{2+}] at different Cas concentrations: (a) 15 g·L^{-1}, (b) 10 g·L^{-1}, and (c) 5 g·L^{-1}. The gray lines represent the fitting from eq 5.

three [NaCas] analyzed can be observed. We could infer that even if ultimately all CN precipitate, the α-CN does it in a greater extent. These results are consistent with those obtained by Pitkowski et al.[22]

Evaluation of CCA State. Change in the Average Size of the CCA. The degree of aggregation of the CCA was studied by τ determination of suspensions of NaCas at (5, 10, and 15) g·L^{-1}.

In Figure 4 we can appreciate, in all cases, an initial increase of τ values with a following decrease of this τ value without reaching back its initial value. This would indicate an increase in the number of particles as a result of the increase in the [Ca^{2+}]$_T$. It could be assumed that Ca^{2+} would have the ability to dissociate pre-existing aggregates of NaCas. Because τ depends on the concentration of particles, this last factor would explain the increase in the τ value for a given Ca:CAS ratio.

A study of the variation of average size of the CCA was also performed. In Figure 5 it can be seen that for all studied [NaCas] in the presence of a raising [Ca^{2+}]$_T$, the average size of the CCA decreases initially (increase in α) until it reaches a minimum followed by an slight increase in the average size but, in all cases, being below its initial value. When [Ca^{2+}]$_T$ increases, the pre-existing aggregates undergo a sharp drop in its average size until they reach a minimum at (7.5 to 10) mmol·L^{-1} [Ca^{2+}]$_T$ range, prior to reaching half of the destabilization of the salting-out first stage. As a consequence, the size of aggregates increases up to a constant value.

Intrinsic Viscosity and Partial Specific Volume. Intrinsic viscosity and partial specific volume were determined from η and ρ measurements in a [NaCas] range from (6 to 16) g·L^{-1}

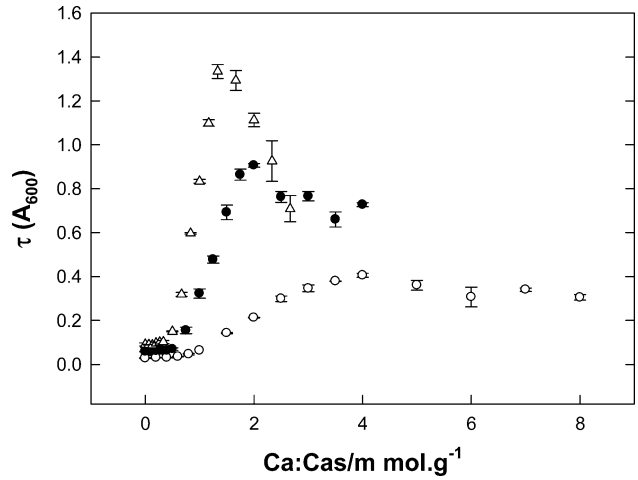

Figure 4. Mean experimental values ($n = 3$) of the CCA turbidity (τ) as A at 600 nm versus Ca:Cas ratio/mmol·g^{-1}. NaCas concentration: ○, 5 g·L^{-1}; ●, 10 g·L^{-1}; and △, 15 g·L^{-1}. The error bars represent the standard deviation for each data point.

and under different constant ratios of Ca:Cas, (0, 0.2, 0.25, and 0.5) mmol·g^{-1} (Figures 6 and 7).

In Table 2 the corresponding values for [η], $\bar{v}_2$, and ν are shown.

Results reveal a reduction in [η] and in $\bar{v}_2$ as the Ca:Cas ratio increases, probably due to a diminution in the degree of hydration of the CCA. The values of ν decrease significantly, indicating that the increase in the Ca:Cas ratio also would cause an increment in the symmetry of the CCA.

[η], $\bar{v}_2$, and ν determinations lead us to infer that the caseinate aggregates in the presence of Ca^{2+} reach a higher symmetry and a lower hydration degree (more compact structure) than in the absence of the cation.

Spectrofluorimetric Determinations. S_0 of the CCA was determined for 5 g·L^{-1} NaCas and for different Ca:Cas ratios. In the presence of Ca^{2+}, the values for S_0 turned out to be higher, but varying with regard to Ca:Cas ratio (Table 3). The results would point to the formation of CCA with a more compact structure caused by action of Ca^{2+} but rendering hydrophobic patches exposed on its surface that probably belong to κ-CN which would be responsible for the stability of the CCA.

Emission spectra of intrinsic fluorescence of aqueous suspensions of NaCas in the absence and in the presence of $CaCl_2$ were also obtained and are presented in Figure SI1 (Supporting Information).

Effect of Sugars on the CCA Colloidal Stability. To evaluate the colloidal stability of the CCA in the presence of sugars, the solubility of mixtures of 10 g·L^{-1} NaCas and different Ca^{2+}, Suc, and Lac concentrations were analyzed. NaCas solutions were prepared by dissolution of acid casein in NaOH and subsequently adjusted at a pH of 6.8.

A previous study about the effect of both sugars on the initial average size and conformation of NaCas suspensions as well as measurements of the viscosity of the media were carried out. The initial average size of NaCas suspensions obtained in the aforementioned study was constant in the presence of sugars at all assayed concentrations.

The effect of both sugars on the intrinsic FI of Cas can be observed in Figure SI2 (see Supporting Information).

Because of the fact that aggregation is limited by particles diffusion, we determined the effect on the viscosity caused by the addition of sucrose or lactose. Figure 8 shows a regular

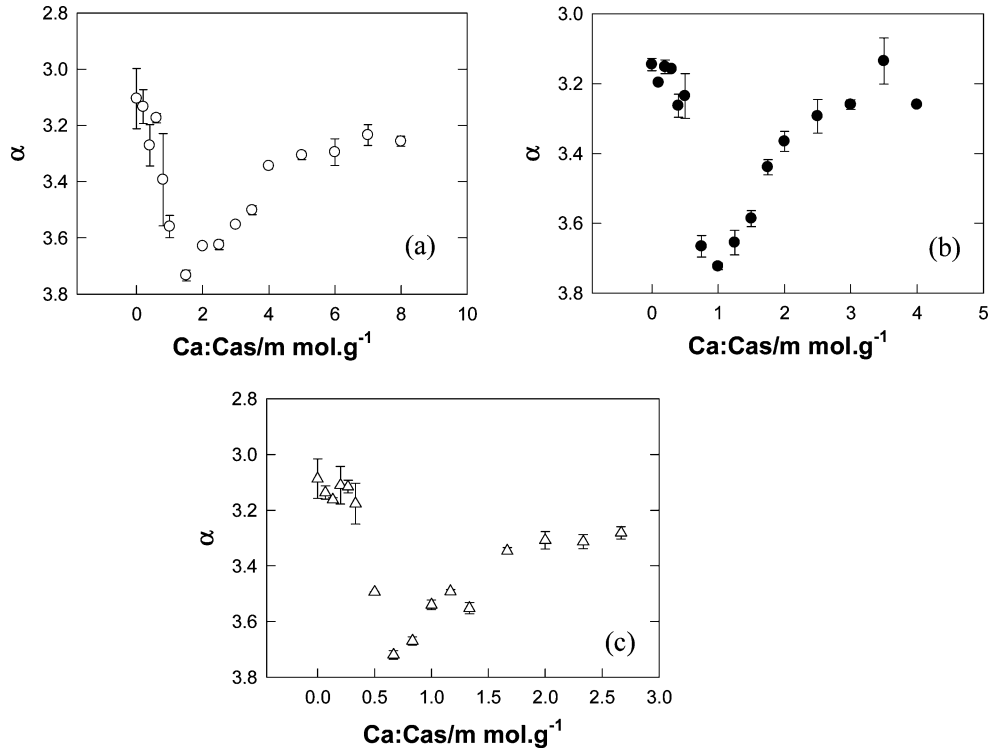

Figure 5. Mean experimental values ($n = 3$) of the CCA parameter α versus Ca:Cas ratio/mmol·g^{-1}. NaCas concentration: ○, 5 g·L^{-1}; ●, 10 g·L^{-1}; and △, 15 g·L^{-1}. The error bars represent the standard deviation for each data point.

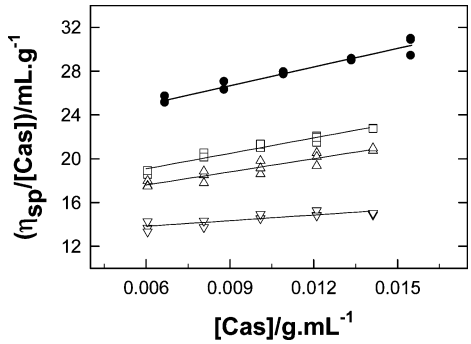

Figure 6. Mean experimental values ($n = 3$) of η_{sp}/[Cas] as function of [Cas] for Ca:Cas ratio/mmol·g^{-1}: ●, 0; □, 0.2; △, 0.25; ▽, 0.5. The lines represent the fitting according Richards.[19]

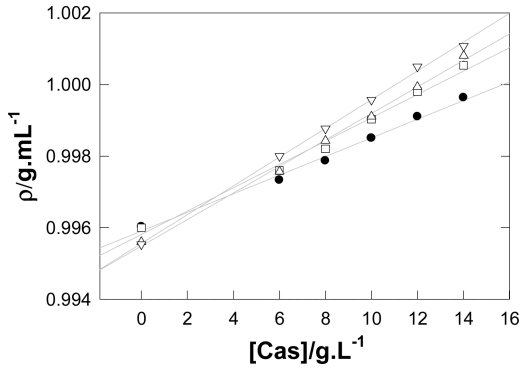

Figure 7. Mean experimental values ($n = 3$) of density (ρ) vs Cas concentration for Ca:Cas ratio/mmol·g^{-1}: ●, 0; □, 0.2; △, 0.25; ▽, 0,5.

increment of η_r with the concentration of tested sugars, in a greater extent for lactose.

Average ($n = 3$) parameter values after fitting the experimental data for the colloidal stability of the CCA under the different concentrations of Suc and Lac analyzed are shown in Table 4.

Table 2. Calculated Values of [η], $\bar{v}_2$, and ν Obtained from Equations 12, 14, and 15, Respectively, at Different Ca:Cas Ratios/mmol·g^{-1}

Ca:Cas ratio	$[\eta]^a$	$\bar{v}_2{}^a$	
mmol·g^{-1}	mL·g^{-1}	mL·g^{-1}	ν^a
0.0	21.6 ± 0.3	0.73 ± 0.02	29.6 ± 0.1
0.2	16.3 ± 0.5	0.66 ± 0.02	24.7 ± 0.1
0.25	15.2 ± 0.4	0.64 ± 0.02	23.6 ± 0.1
0.50	12.8 ± 0.2	0.60 ± 0.02	21.2 ± 0.1

[a] Note: errors are standard errors derived from the fitting of eqs 12, 14, and 15, respectively.

Table 3. Average ($n = 3$) Parameter Values of Surface Hydrophobicity (S_0) in Mixtures of 0.15 g·L^{-1} NaCas at Different Ca:Cas Ratios, 25 °C, and pH 6.8[a]

Ca:Cas ratio	S_0
mmol·g^{-1}	mL·g^{-1}
0.0	1.35 ± 0.07
1.5	1.37 ± 0.04
2.5	1.37 ± 0.01
4.0	1.49 ± 0.01
5.0	1.40 ± 0.02

[a] Note: errors are standard deviations of S_0.

In the first stage at a certain [Ca^{2+}], and in the presence of disaccharides, a decrease in K_1 values was observed. This effect was more significant in the presence of Lac. Once the sites with a stronger affinity were occupied, a subsequent decrease in the K'_1 value was also revealed, this effect also being more pronounced for Lac. These results would indicate that the presence of disaccharides affects both binding stages of Ca^{2+} to Cas and this effect would be dependent on Suc and Lac concentrations in the range of concentrations studied. The decrease of K_1 and K'_1 constant values is related to a stabilizer effect of both sugars even though for a given concentration Lac gives rise to more stable CCA than Suc. This effect can be observed in the Figure 9, where we plotted the Ca^{2+} concentra-

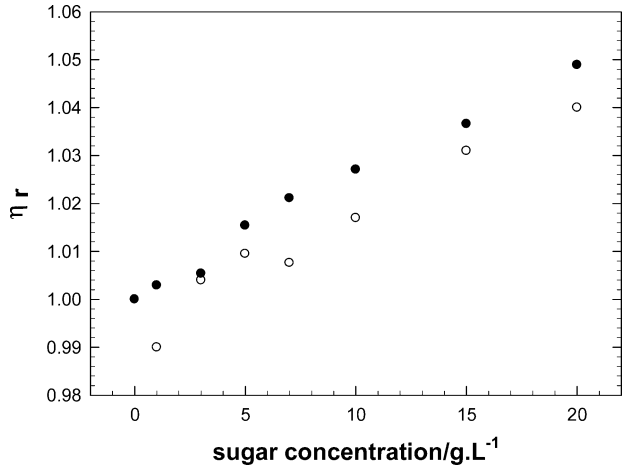

Figure 8. Mean experimental values ($n = 3$) of relative viscosity (η_r) variations in the presence of ●, Lac or ○, Suc. Cas concentration was 5 g·L^{-1} and temperature 25 °C.

Table 4. Average ($n = 3$) Parameter Values of Colloidal Stability of CCA in the Presence or Absence of Sucrose (Suc) or Lactose (Lac) at 25 °C and pH 6.8[a]

sugar	S_1	K_1		S'_1	K'_1	
g·L^{-1}	g·L^{-1}	L·mol^{-1}	n	g·L^{-1}	L·mol^{-1}	n'
without sugar	5.3 ± 0.6	114 ± 5	5	5.1 ± 0.2	55 ± 1	7
Suc 3	5.7 ± 0.4	97 ± 2	7	1.8 ± 0.1	52 ± 1	7
Suc 6	5 ± 0.4	98 ± 2	7	1.7 ± 0.1	50 ± 1	7
Suc 9	6.1 ± 0.3	101 ± 3	7	1.9 ± 0.1	50 ± 1	7
Suc 12	4.6 ± 0.4	85 ± 1	7	1.7 ± 0.1	46 ± 1	7
Suc 15	5.4 ± 0.6	99 ± 3	7	1.8 ± 0.1	50 ± 1	7
Lac 3	5.6 ± 0.4	92 ± 2	8	1.9 ± 0.1	54 ± 1	10
Lac 6	5.6 ± 0.4	85 ± 2	9	1.9 ± 0.2	48 ± 1	10
Lac 9	4.4 ± 0.5	82 ± 1	9	1.8 ± 0.1	47 ± 1	11
Lac 12	5.1 ± 0.3	75 ± 2	11	2.0 ± 0.2	45 ± 2	10
Lac 15	4.1 ± 0.4	91 ± 1	5	1.7 ± 0.1	44 ± 1	9

[a] Note: errors are standard errors derived from the fitting using eq 5.

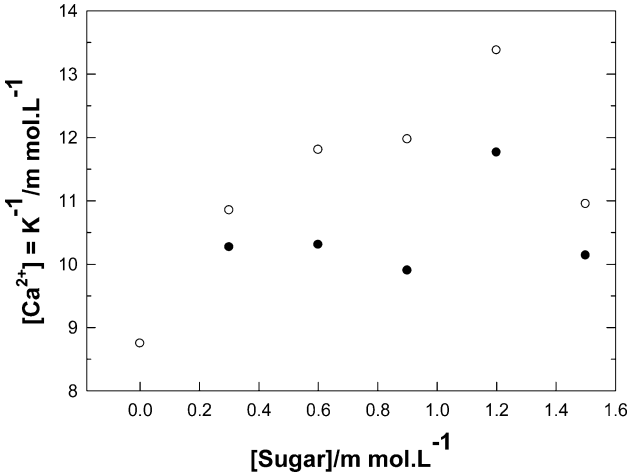

Figure 9. Mean experimental values ($n = 3$) of calcium concentration which is equal to $1/K_1$ as function of ●, Lac or ○, Suc concentrations. Cas concentration was 10 g·L^{-1}, temperature 25 °C, and pH 6.8.

tion values which are the inverse of K_1 (eq 16) against sugar concentrations.

The results obtained for protein−calcium−sugars systems, in the assayed range of concentrations at constant pH and temperature indicate that caseinate particles are involved in multiple equilibria. Therefore, we propose a model in an attempt to explain these equilibria. Further details about this model are given in the Supporting Information.

The first equilibrium occurs between associated protein (p_n) and dissociated protein (p):

$$p_n \rightleftarrows np$$

This is based on the study of Lee and Timasheff[23] about the stabilization of proteins by sucrose, low-molecular-weight sugars excluded, at least partially, from the protein domain. This effect might be due to a specific repulsion between the protein and the sugar molecules or possibly as a result of an unspecific mechanism where the cosolute is accumulated in the bulk of solution instead of in the interfacial surface. The introduction of these sugars into the protein solution tends to drive the system toward a state where the contact surface between the protein and the solvent is reduced.

On the other hand, Belyacova et al.[24] suggest that the sugars promote protein dissociation. The porous nature of caseinate submicelles favors the accessibility of sucrose molecules and the formation of direct hydrogen bond between sugars and proteins. The multiple hydrogen bonds with low-molecular-weight sugars could weaken the original intramolecular interactions in the interior of the protein associates.

The last part of the model proposed attempts to explain the changes on the parameters of Ca^{2+} binding in the presence of sugars. The second equilibrium would correspond to the incorporation of calcium to caseinates (first stage of salting-out). This cosolute would initially bind to the dissociated protein because of its easy access to the binding sites available:

$$p + Ca^{2+} \rightleftarrows p \sim Ca$$

where p represents casein molecules (α, β, and κ), and p∼Ca casein particles bound to calcium. The binding of Ca to the protein would move the position of the first equilibrium, producing a decrease in the amount of aggregated particles and the average size of particles in suspension. In the presence of sugars, an increase of n and a decrease of K_1 were observed. A shift of first equilibrium toward the dissociation allows us to explain the rise of n. A drop of K_1 could be due to a lower accessibility of Ca^{2+} to protein affinity sites because of interactions between the protein and the sugars. Lactose, which has a free anomeric carbon, may open its ring structure and generate a more significant steric impediment for Ca^{2+} binding. On the other hand, the increment of the medium viscosity induced by lactose is higher than the effect caused by sucrose, making the diffusion of Ca^{2+} toward binding sites more difficult in its presence.

Moreover, the diminution of protein intrinsic viscosity would indicate that calcium generates more compact and smaller CCA as Ca:Cas increases. During this stage, the turbidity of samples increases, indicating an increment in the amount of smaller particles until p ∼ Ca becomes higher (α decrease) as it is shown in the third equilibrium involved:

$$n(p \sim Ca) \rightleftarrows (p \sim Ca)_n$$

where $(p \sim Ca)_n$ stands for the p∼Ca biggest aggregates. This aggregation process would be favored by the higher exposure of hydrophobic patches.

Conclusions

The stability of CCA depends on initial protein concentration for the same Ca:Cas ratio. A higher amount of protein would favor the salting-out process due to the increment of effective collision probability. Moreover, for the same Ca:Cas ratio, an increase of Ca^{2+} concentration diminishes the energetic barrier produced by the electrostatic repulsion of negative protein

surface, favoring a consequent drop of solubility. On the other hand, the addition of Ca^{2+} to NaCas leads to conformational changes in the protein, giving rise to more compact and symmetrical structures.

The presence of sugars produces significant modifications on the protein stability. Sucrose and lactose increase the protein solubility in the presence of calcium for a particular caseinate concentration. Lactose is responsible for a more effective stabilization than sucrose.

The results of this work would allow us to know the calcium availability with regards to caseinate concentration and in the presence or absence of lactose and sucrose. These considerations could be taken into account when these cosolutes are used to supplement the food formula containing calcium.

Acknowledgment

We would like to express our gratitude to Prof. Mirta Armendariz of Universidad Nacional de Rosario for offering valuable advice in the mathematical fittings. The authors would like to thank Anselmo Reggiardo for the English revision.

Supporting Information Available:

SDS-PAGE and emission spectra and multiple equilibria model of protein−calcium systems details are given in the Supporting Information section. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Walstra, P.; Jenness, R. Composición y estructura de la leche. In *Dairy chemistry and physics*; Acribia, S. A., Zaragoza, Eds.; John Wiley & Sons: New York, 1984; pp 1−225.
(2) Qi, P. X.; Brown, E. M.; Farrell, H. M., Jr. New views on structure-function relationships in milk proteins. *Trends Food Sci. Technol.* **2001**, *12*, 339–346.
(3) Farrell, H. M., Jr.; Kumosinski, T. F.; Malin, E. L.; Brown, E. M. The caseins of milk as calcium binding proteins. In *Calcium binding proteins*; Vogel, Ed.; Humana Press: Totowa, NJ, 2002; pp 91−140.
(4) Mulvihill, D. M.; Fox, P. F. Caseins and manufactured. In *Development in Dairy Chemistry*; Fox, P. F., Ed.; Elsevier Applied Science: London, 1989; Vol. 4, pp 97−130.
(5) Torresani, M. Enfoque nutricional en la prevención de la osteoporosis. *Actual. Osteol.* **2007**, *3*, 76–80.
(6) Walstra, P.; de Roos, A. Proteins at air-water and oil-water interfaces: static and dynamic aspects. *Food Rev. Int.* **1993**, *9*, 503–525.
(7) Dickinson, E. Stability and rheological implications of electrostatic milk protein-polysaccharide interactions. *Trends Food Sci. Technol.* **1998**, *9*, 347–354.
(8) Euston, S.; Hirst, R. Comparison of the concentration-dependent emulsifying properties of protein products containing aggregated and non-aggregated milk protein. *Int. Dairy J.* **1999**, *9*, 693–701.
(9) Antipova, A. S.; Semenova, M. G.; Belyakova, L. E. Effect of sucrose on the thermodynamic properties of ovalbumin and sodium caseinate in bulk solution and at air-water interface. *Colloids Surf., B* **1999**, *12*, 261–270.
(10) Mora-Gutierrez, A.; Farrell, H. M., Jr.; Kumosinski, T. F. Comparison of calcium-induced associations of bovine and caprine caseins and the relationship of α_{S1}-casein content to colloidal stabilization: a thermodynamic linkage analysis. *J. Dairy Sci.* **1993**, *76*, 3690–3697.
(11) Alvarez, E. M.; Risso, P. H.; Gatti, C. A.; Burgos, M.; Suarez Sala, V. Calcium-induced aggregation of bovine caseins: effect of phosphate and citrate. *Colloid Polym. Sci.* **2007**, *285*, 507–514.
(12) Kuaye, A. Y. An ultraviolet spectrophotometric method to determine milk protein content in alkaline medium. *Food Chem.* **1994**, *49*, 207–211.
(13) Horne, D. S. Determination of the fractal dimension using turbidimetric thechniques. Application to aggregatting protein systems. *Faraday Discuss. Chem. Soc.* **1987**, *83*, 259–270.
(14) Worning, P.; Bauer, R.; Øgendal, L.; Lomholt, S. A novel approach to turbidimetry of dense systems: An investigation of the enzymatic gelation of casein micelles. *J. Colloid Interface Sci.* **1998**, *203*, 265–277.
(15) Teixeira, J. Fractal and non-fractal Patterns in Physics. In *On growth and form*; Stanley, Ostrowsky, Eds.; Martinus Nijhoff Publisher: Dordrecht, The Netherlands, 1986; pp 145−162.
(16) Holt, C.; Parker, T. G.; Dalgleish, D. G. Measurement of particle sizes by elastic and quasi-elastic light scattering. *Biochim. Biophys. Acta* **1975**, *400*, 283–292.
(17) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(18) Alvarez, E.; Risso, P.; Mancilla Canales, M.; Pires, M.; Gatti, C. Hydrodynamic properties-structure relationship for sodium caseinates in presence of calcium. *Colloids Surf., A* **2008**, *327*, 51–56.
(19) Richards, E. The equilibrium thermodynamics of solutions of large molecules. In *An introduction to Physical Properties of Large Molecules in Solution*; Cambridge University Press: Cambridge, U.K., 1980; pp 37−39.
(20) Kato, A.; Nakai, S. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13–20.
(21) Haskard, C. A.; Li-Chan, E. C. Y. Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRODAN) and anionic (ANS) fluorescent probes. *J. Agric. Food Chem.* **1998**, *46*, 2671–2677.
(22) Pitkowski, A.; Nicolai, T.; Durand, D. Stability of caseinate solutions in the presence of calcium. *Food Hydrocolloids* **2009**, *23*, 1164–1168.
(23) Lee, J. C.; Timasheff, S. N. The stabilization of proteins by sucrose. *J. Biol. Chem.* **1981**, *256*, 7193–7201.
(24) Belyakova, L. E.; Antipova, A. S.; Semenova, M. G.; Dickinson, E.; Merino, L. M.; Tsapkina, E. N. Effect of sucrose on molecular and interaction parameters of sodium caseinate in aqueous solution: relationship to protein gelation. *Colloids Surf., B* **2003**, *31*, 31–46.

Received for review October 30, 2009. Accepted March 16, 2010. This work was supported by grants from the Universidad Nacional de Rosario, Argentina (PID BIO 167).

JE900932A