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ORIGINAL CONTRIBUTION

Calcium-induced aggregation of bovine caseins: effect of phosphate and citrate

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Abstract The formation of colloidal particles by Ca^{2+} precipitation of whole caseinates in the presence of phosphate (Pi), citrate (Cit), or both of the anions in concentrations found to be effective in previous works was followed comparing the colloidal particle size and the ionic and proteic composition of the precipitates obtained. Ca^{2+} was incorporated to the precipitate and colloidal particles in a different way than Pi, differences which were related to the presence of Pi and/or Cit in the media. A sequential salting-out process due to progressive Ca^{2+} binding to at least two kinds of sites was observed. The precipitation curves were fitted, and the affinity constants and binding site numbers were calculated with a modification of the Farrell's equation based on the concept of Wyman's linked functions. Precipitates obtained at low total Ca^{2+} concentrations in different conditions varied their casein composition. Colloidal particles appeared at the beginning of the second salting-out step, in different amount, and in average size according to the presence or absence of Pi and/or Cit in the media. Consideration of these differences showed that Cit favored the formation of bigger colloidal particles, acting especially in the first steps of the casein aggregation and conditioning the mechanism of this process.

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Introduction

Caseins and caseinates are extensively used in food industries because of their physicochemical, nutritional, and functional properties, which make caseinates useful ingredients in complex food preparations. Thus, they are added to different products because of their high emulsifying, water-binding, and gelation capacities, their resistance to heat treatments, and their contribution to the food texture and juiciness. Some of these properties make caseinates useful ingredients both in the preparation of bakery and confectionery products, where they can be used as milk substitutes [1].

From a nutritional point of view, caseins have all the essential amino acids and an important function in the transport of calcium and phosphate, representing an easily digestible source of nutrition, contributing to a carefully balanced diet especially for specific consumer groups (infant formulas, sport foods, etc.) [2].

Caseins occur in milk as stable colloidal aggregates known as casein micelles (CM), mainly composed by α_{S1}-, α_{S2}-, β-, and κ-casein (α_{S1}-, α_{S2}-, β-, and κ-CN) linked together with the contribution of colloidal calcium phosphate (CCP), a mineral complex involving mainly calcium and phosphate ions, with magnesium and citrate in much lower proportion [3]. Numerous studies, performed mainly on CM, i.e., on aggregates formed in vivo, in physiological conditions [4], have found that CCP is present in these micellar structures as clusters of different size. Although it is difficult to assign a defined stoichiometry to such kind of aggregates, Holt has calculated that they could be formed by inorganic clusters

containing in average 66 Ca^{2+}, 44 inorganic phosphate moieties, and 132 water molecules, linked to 22 phosphate groups from the phosphorylated side-chain residues of five casein molecules [3]. Different average stoichiometry relationships have been proposed by other authors [5–8].

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 according to the associated cations. Particularly, calcium ions produce a number of important effects on casein solubility and colloidal stability. Therefore, calcium binding to caseins is important not only in the formation of CM in vivo but also in food processing when calcium caseinate is used as an ingredient [9]. 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. On the contrary, this latter component is able to stabilize the other casein components against the flocculating action of calcium ions by the formation of stable colloidal aggregates [10]. When obtained in the presence of adequate phosphate and citrate concentrations, these casein colloidal aggregates (CCA) show physicochemical and functional properties similar to those of CM [11]. Although solubility and colloidal stability of caseins in the presence of calcium ions have been studied by different authors from thermodynamic and kinetic approaches [12, 13], the role of phosphate and citrate in these phenomena has merited lower attention. In this work, the precipitation by Ca^{2+} alone of whole bovine caseinates was compared with the precipitation in the presence of either phosphate (Pi), citrate (Cit), or both. These processes were studied from a thermodynamic approach using the concept of Wyman's linked functions [14] to characterize the interactions involved in them, following also the proteic composition of the precipitates obtained and the formation of stable colloidal particles, in order to gain insight on the role of such anions on the Ca^{2+}–caseins interaction.

Experimental sections

Materials and methods

Preparation of caseinate

Whole bovine sodium caseinate was prepared from suspensions of commercial, nonfat dried milk (MOLICO, Société des Produits Nestlé S.A., Vevey, Switzerland) reconstituted to 10% (*w*/*v*) in distilled water by acid precipitation at pH 4.6 with 1 M HCl. The precipitated caseins were separated by centrifugation at 1,000×*g* for 10 min and washed several times with 0.1 M sodium acetate–acetic acid buffer, pH 4.6, and finally with distilled water, and dissolved in distilled water at room temperatures (23–25 °C) at a concentration of 28 g L^{-1} by gradual addition of 1 M NaOH, ensuring that the pH did not exceed 7.0. The pH was finally adjusted to 6.8 [15].

Colloidal stability test

Sodium caseinate (about 20 g L^{-1}) was dissolved in water, adjusted to pH 6.8 with 0.1 N NaOH, and equilibrated in a water bath at 25 °C for 15 to 20 min. Two milliliters of calcium chloride of desired concentration in solution imidazole–HCl buffer at pH 6.8, in the 0 to 25 mM range, was added to 2 mL of protein solution in thick-walled centrifuge tubes. The tubes were inverted twice and allowed to stand in a 25 °C water bath for 30 min. The tubes were centrifuged at 1,500×*g* for 15 min [9, 10]. Precipitates (insoluble casein aggregates) and supernatant (CCA) were obtained. Mixtures that were allowed to stand 24 h before centrifugation did not show appreciable differences in the amount of precipitated protein or in the turbidity values of the supernatants to those obtained after the 30 min equilibration, thus showing that this time was enough to reach equilibrium conditions. Each experiment was replicated at least three times.

Precipitation of casein by Ca^{2+}

Caseinate precipitation by Ca^{2+} was interpreted by Farrell et al. [9, 10, 13] using the concept of Wyman's linked functions [14], assuming that the cation binding to the protein is followed by the precipitation or salting-out of the less soluble calcium caseinate formed:

$$p + nCa \xleftarrow{K_1^n} pCa_n \tag{1}$$

where p is the unbound protein, n is the number of Ca^{2+} moles bound to the specie pCa_n, and K_1^n is the equilibrium constant of the process of precipitation or salting-out. The mathematical relationship representing the above stoichiometry will be:

$$S_{app} = S_0 f(p) + S_1 f(pCa_n) \tag{2}$$

where S_{app} is the apparent protein solubility at a given Ca^{2+} concentration, $f(i)$ is the protein fractional component of species i, S_0 is the initial concentration of soluble caseinate, and S_1 is the apparent solubility of pCa_n. Incorporation of the equilibrium constant K_1^n to Eq. 2 leads to the following:

$$S_{app} = \frac{S_0 p}{p + K_1^n p\left[Ca^{2+}\right]^n} + \frac{S_1 K_1^n p\left[Ca^{2+}\right]^n}{p + K_1^n p\left[Ca^{2+}\right]^n} \tag{3}$$

where p is the concentration in percentage of the unbound protein, and $[Ca^{2+}]$ is the concentration of unbound cation. Cancellation of common terms yields:

$$S_{app} = \frac{S_0}{1 + K_1^n \left[Ca^{2+}\right]^n} + \frac{S_1 K_1^n \left[Ca^{2+}\right]^n}{1 + K_1^n \left[Ca^{2+}\right]^n} \quad (4)$$

Moreover, if the total protein concentration is small with regard to $[Ca^{2+}]$, the total Ca^{2+} concentration can be used in Eq. 4 instead of the free cation concentration. According to previous experimental results, Eq. 4 was developed to obtain Eq. 5, which results in the addition of a term, correspondent to the second group of salting-out sites [16]:

$$S_{app} = \frac{S_0}{1+K_1^n \left[Ca^{2+}\right]^n} + \frac{S_1 K_1^n \left[Ca^{2+}\right]^n}{1+K_1^n \left[Ca^{2+}\right]^n} + \frac{\left(S_1' - S_1\right)\left(K_1'\right)^m \left[Ca^{2+}\right]^m}{1+K_1' \left[Ca^{2+}\right]^m} \quad (5)$$

Here, S_1' is the apparent solubility of pCa_nCa_m, and K_1' corresponds to the second salting-out process. The parameters involved were calculated as follows: in a first step, the Ca^{2+} induced 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. 5 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. 5, and the same approach was applied for m, S_1', and K_1' calculation.

When the caseinate aggregates were obtained in the presence of Pi, Cit, or both, the information about the structural features of similar aggregates obtained in vivo or in vitro previously described in the "Introduction" section suggests that the participation of these ions in the structure of such aggregates could not be discarded. Applying a similar approach to that proposed by Farrell et al. [13], the participation of Pi, Cit, or both in the process should be reflected in the modifications of the values of K_1^n, n, or both in Eq. 5.

Urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Urea-SDS-PAGE)

The CCA supernatants obtained from the colloidal stability test were analyzed by Urea-SDS-PAGE using a vertical gel system, according to the method of Laemmli [17]. Thirty-microgram protein samples were dissolved in 1 mL of buffer containing 0.1 M Tris–HCl, pH 6.8, 2% (*w*/*v*) SDS, 10% (*v*/*v*) β-mercaptoethanol, 10% (*v*/*v*) glycerol, and 0.01% (*w*/*v*) bromophenol blue. The separating gel was composed of 20% (*w*/*v*) acrylamide and 0.53% (*w*/*v*) *bis*-acrylamide dissolved in 0.38 M Tris–HCl buffer, pH 8.8, containing 7–9 M urea and 0.5% (*v*/*v*) SDS. The stacking gel was composed of 7.5% (*w*/*v*) acrylamide and 0.16% (*w*/*v*) *bis*-acrylamide dissolved in 0.12 M Tris–HCl buffer, pH 6.8, containing 0.1% (*v*/*v*) SDS. Migration was run for 2 h at 25 °C and under 100 V constant voltage conditions.

Proteins were stained with Coomassie brilliant blue R250 staining solution and destained with 10% (*v*/*v*) methanol, 10% (*v*/*v*) acetic acid destaining solution. The relative intensity of the stained 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 (X-GEL). Deconvolution of the scanning pattern curves was performed, when necessary, by means of the GRAMS program. The protein bands were identified using commercial α_S-, β-, and κ-CN (Sigma). These same standards were used not only to determine the range of protein concentrations at which the relation pixel density–concentration remains linear but also the reproductiveness of the method. The *w*/*v* concentration of each of the caseins in the caseinate solution or in the supernatants from the colloidal stability test was calculated from the total casein concentration in each of the samples and the fraction of the total stained surface occupied for each of the caseins in the correspondent chromatographic line. The difference between the concentration of each of the caseins in the caseinate solution and in the supernatants allowed us to calculate the *w*/*w* concentration of the precipitates. Each Urea-SDS-PAGE electrophoresis was replicated at least two times.

Composition of the precipitates

The precipitates of insoluble casein aggregates obtained from the colloid stability test were separated at 25 °C by centrifugation at 1,500×*g*, 30 min, using 20 mL conical centrifuge tubes at room temperature. 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 the Eq. 5, in order to interpret the experimental curves obtained and the relative concentration of each of the components determined by PAGE.

Size variations of the CCA

The changes in the CCA size were followed by the wavelength (λ) dependence of turbidity (τ) of the suspensions, measured as $\alpha = -d(\log\tau) / d(\log\lambda)$. α is a parameter which has an inverse relationship to the average size of the

particles and can be used to detect and easily follow rapid size changes, and was obtained from the slope of log τ vs log λ plots in the 400 to 700 nm range [18]. τ was measured as absorbance using a Spekol 1200 spectrophotometer with a diode arrangement. α determinations were the average of at least three replicates.

To verify if α was actually related to the average size of the aggregates, CCA average $D_{6,5}$ diameters were determined by dynamic light scattering, applying the method of cumulants [19], and the relationship of the values obtained with the correspondent α values was studied [20]. Dynamic light scattering measurements were performed on a Brookhaven BI-2005 M equipment, with an He–Ne laser (λ_0= 632.8 nm) with a maximal power of 15 mV, and using 90° as measuring angle during 420 s for each determination. The results were the average of at least three determinations.

CCA samples were diluted in the correspondent buffer which had previously been filtered through Millipore of 1 μm pore diameter to eliminate dust particles. Then they were transferred to glass cuvettes in a jacketed cuvette holder immersed in decaline and maintained at the desired temperature by a MGW LAUDA RC3 circulation bath.

Casein and ion determinations

Casein, Ca^{2+}, and Pi concentrations were determined in the initial caseinate solution and in the supernatants of the colloidal stability test. Casein was measured according to the Kuaye's method [21]. Ca^{2+} was measured by atomic absorption spectrophotometry (Metrolab RC 250 AA). Pi was measured by a standard colorimetric method based on the formation of phosphomolybdate in acidic medium.

Cit was determined in the initial caseinate solution by the colorimetric method of Marier and Boulet using TCA filtrates as described by White and Davies [22].

All the values used were the average of at least three determinations.

Results and discussion

Colloidal stability test

Figure 1a shows the protein remaining in solution or in colloidal suspension in the supernatant when sodium caseinates were precipitated by increasing total calcium concentrations (TCC) in the absence or presence of 15 mM Pi and/or 10 mM Cit (the ion/casein concentration ratios used were similar to those found in bovine milk) and centrifuged at 1,500×*g*. The remaining protein was plotted against TCC in the medium.

In absence of added Pi and/or Cit, the precipitation was observed from about 5 mM Ca^{2+}, progressing with the increase of TCC until about 50% of the initial protein concentration remained in the supernatant at 25 mM Ca^{2+}. The shape of the curve obtained presented certain interesting features which will be analyzed below. The values of τ and α for the protein suspension in the supernatants were plotted in Fig. 1b and c, respectively. The average size of the particles remaining in suspension, as estimated by α values, decreased during the precipitation at increasing TCC, but it increased again when both the precipitation and the amount

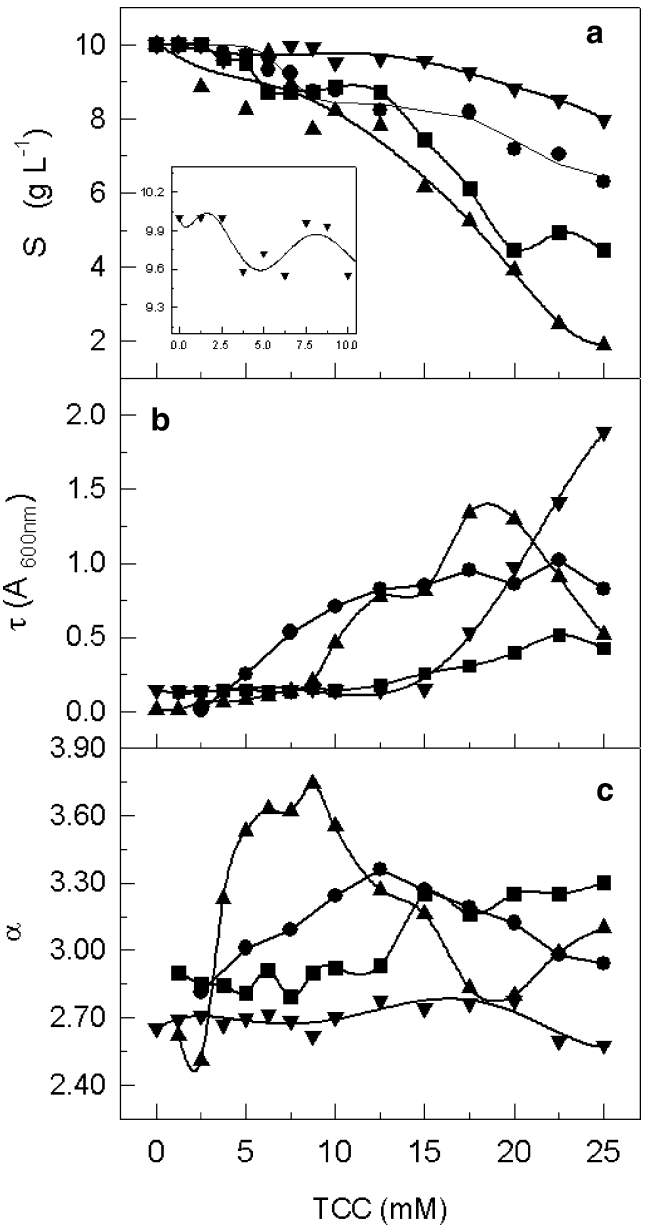

Fig. 1 **a** Remnant caseinate concentration (*S*), **b** turbidity (τ), and **c** parameter $\alpha = -d(\log \tau) / d(\log \lambda)$ in the supernatants of mixtures of 10 g L^{-1} sodium caseinate with Ca^{2+} in a range of 0 to 25 mM (*filled circles*) and in the presence of 15 mM Pi (*filled triangles*), 10 mM Cit (*inverted filled triangles*), or 15 mM Pi and 10 mM Cit (*filled squares*) as a function of the concentration of total Ca^{2+} (*TCC*), pH 6.8, temperature 25 °C

of colloidal particle in suspension were maximal. The initial increase of α values simultaneously with an initial increase of τ, at TCC where no precipitation was yet observed, could be indicating that Ca^{2+} addition to the medium started the aggregation of caseins to form initially small colloidal particles. Further increase of TCC to values higher than 15 mM produced particles of progressively bigger average size, as shown by the decrease of α values.

Figure 1 also shows the results obtained by Ca^{2+} addition when 15 mM Pi was added to the mixtures for the study of the precipitation of caseinates. Precipitation (Fig. 1a) started at low TCC (<5 mM) and progressed until an important protein fraction was precipitated (80% of total protein at 25 mM of TCC). τ (Fig. 1b) increased to reach a maximum at 20 mM TCC, and then decreased faster. α values (Fig. 1c), considered together with τ values, showed an initial formation of a low amount of relatively small particles, followed by a second step with further formation of progressively bigger particles, which finally precipitated at high TCC (25 mM).

Figure 2 shows the Ca^{2+} and Pi incorporated to the precipitate at different TCC, in a medium containing 15 mM Pi. According to these data, Ca^{2+} and Pi were progressively incorporated until 10 mM total Ca^{2+}. For higher TCC, the incorporated cation remained almost constant until 20 mM total Ca^{2+}, but it increased again (Fig. 2a). The anion was incorporated in a lower amount, reaching a maximal value of 1 mM, remaining almost constant for TCC higher than 10 mM (Fig. 2b).

Precipitation of calcium phosphates is to be expected under the working conditions used. The behavior observed, however, suggests that only a minor and constant fraction of the precipitated Ca^{2+} was associated with Pi, the major part of it being increasingly bound to the proteins.

The curves obtained when 10 mM Cit was added to the caseinates are also shown in Fig. 1. The concentration at which the precipitation started (Fig. 1a) was higher (10 mM) than in the two previous cases. Protein was precipitated in lower amount (20% at 25 mM total Ca^{2+}) than in all the other conditions. These observations suggested that the complexing action of Cit on the cation reduced the activity of Ca^{2+} free to interact with caseins and Pi. A marked increase of τ values (Fig. 1b) started at 15 mM total Ca^{2+}, as a consequence of the presence of increasing amounts of colloidal particles in suspension. According to the values of α (Fig 1c), the average size of these particles did not evidence important variations, remaining relatively big.

When 15 mM Pi was added to the medium in the presence of 10 mM Cit, the TCC at which precipitation started (Fig. 1a) was higher than in the case of 15 mM Pi alone (10 mM). Although the behavior of τ and α (Fig.1b and c, respectively) was similar to the one observed

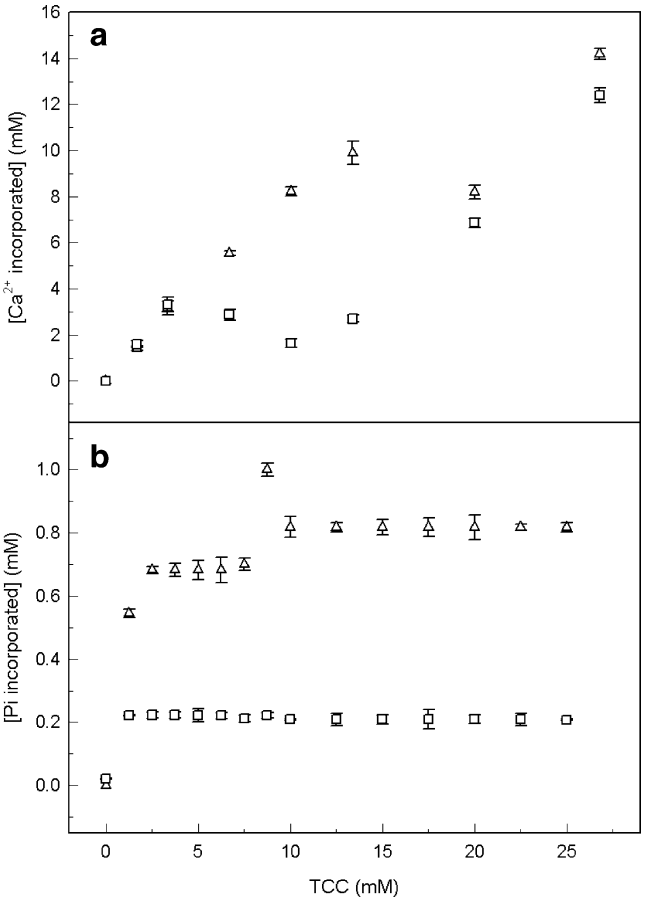

Fig. 2 **a** Incorporated Ca^{2+} concentration to the precipitate and **b** incorporated Pi concentration to the precipitate in a medium containing 15 mM Pi (*empty triangles*) or 15 mM Pi and 10 mM Cit (*empty squares*) as a function of the concentration of total Ca^{2+} (*TCC*), pH 6.8, temperature 25 °C

in the absence of Cit, the maxima in the τ vs TCC curves occurred at higher concentrations, and both the reached maximal τ and the amount of protein precipitated at a given TCC were lower. The incorporation of Ca^{2+} and Pi to the precipitate presented similar features to those obtained in the presence of 15 mM Pi alone (Fig. 2a and b), although the amount of the incorporated ions were both lower especially in the case of Pi. Besides, the changes observed in the Ca^{2+} incorporation appeared at lower TCC.

The curves of protein remaining in solution vs TCC (Fig. 1a) showed, in all the cases, the presence of a salting-out process, similarly to the results reported by Mora-Gutierrez et al. [9, 10] for different caseins. These authors described also a following salting-in process, which was not observed in our case probably because the Ca^{2+} concentrations used were not high enough. In our TCC range, the salting-out curves obtained for caseinates presented an inflection point especially in the presence of Pi. This inflection in the precipitation curves was coincident with changes observed in the turbidity plots (Fig. 1b). Following the rationale exposed by Farrell et al. [13], this

behavior was probably due to the presence of two kinds of sites which were able to produce precipitation by sequential Ca^{2+} binding. Therefore, the curves of Fig. 1a were fitted using Eq. 5, and the parameters involved were calculated as explained in the "Materials and methods" section. The results obtained were shown in Table 1.

In the case of the systems where only 10 mM Cit was added, the second step did not appear at the TCC used, and a dip could be observed at low concentrations (insert in Fig. 1a). This dip, in fact, could be also observed in the other systems, although it was not taken into account for the fitting of the curves because it was less pronounced in these cases. This dip resulted from the fact that the earlier precipitation of the caseinates was followed by a salting-in process [23]. In the presence of 10 mM Cit, these sequential salting-out and salting-in processes appeared as the most important features of the curves, although they were very irregular. Only the first salting-out process was considered in this case, with results reported in Table 1.

Two kinds of Ca^{2+} binding sites were observed in the precipitation plots, presenting apparent average binding constants differing in one order of magnitude, the stronger ones (K_1) corresponding to the initial step of the precipitation process. K_1 values were similar to the values reported by Farrell et al. [13] for average Ca^{2+} binding constants linked to the precipitation of α_{S1}- and β-CN genetic variants. Because these values were comparable to Ca^{2+} binding constants for model phosphate compounds, the authors mentioned assumed that this binding involves mainly casein phosphoserine residues. In our case, the average binding site number obtained (Table 1) exceeded the number of such residues in the caseins, suggesting the participation of other anionic residues, such as carboxylates. Similar results have been reported by other authors [24, 25] for Ca^{2+} binding to α_{S1}-CN. The parameters of this initial binding appeared to be affected neither by the presence of 15 mM Pi nor by the addition of 10 mM Cit to such mixtures. Lower values were obtained for K_1', the average association constant for the second step of salting-out (Table 1), suggesting the participation of weaker affinity sites in this process.

Although the estimation of the binding parameters in the presence of Cit was difficult, the results obtained indicate an increase of the binding constants, with a reduction of the number of sites involved in the process. Because Ca^{2+} activity is strongly reduced by the complexing action of Cit, Ca^{2+} binding in an appreciable amount will be possible only on higher affinity sites. Cit produces, in this way, a certain site selection likely related to a particular composition and structure of the casein aggregates.

Table 2 shows the composition of the precipitates obtained in the presence of 15 mM Pi, with or without 10 mM Cit, at TCC (10 and 25 mM) in which the first and second salting-out steps have been respectively completed. These results were calculated as explained in the "Materials and methods" section. The precipitates obtained at 25 mM total Ca^{2+} have, in all the cases, a composition similar to that of the original caseinates. At 10 mM total Ca^{2+}, however, the precipitated fractions were less rich in α_S-CN especially in the case of mixtures containing only Pi.

Conclusions

These differences can be interpreted as logical consequences of the aggregation mechanism currently accepted for caseins [9, 10, 20, 26]. It is assumed that for aggregation to occur, a fraction of the casein charge has to be neutralized by Ca^{2+} binding. This reduction in repulsive potential between particles enables a colliding pair of them to approach enough to be linked by hydrophobic or other short-range interactions, provided that they have been moving with the adequate orientation.

Because α_S-caseins, at the pH used, have a negative charge higher (at least twice) than that of β-CN [27], their repulsive potential at low Ca^{2+} activity will remain high enough to maintain the fraction of their collisions resulting in aggregation to be relatively lower. The aggregates obtained in these conditions will then have proportionally more β- and κ-CN.

When Ca^{2+} activity increases, further neutralization of the net negative charge by Ca^{2+} binding will produce a further decrease of repulsive potential, increasing in this way the fraction of effective collisions in which α_S-CN takes part and thereby the percentage of these caseins in the precipitates. Finally, if the caseins reach similar probabilities

Table 1 Calcium binding parameters for sodium caseinates in media with different ionic composition

Parameters	S_1 (g L^{-1})	K_1 (M^{-1})	n	S_1' (g L^{-1})	K_1' (M^{-1})	n'
Mixtures						
Caseinate	8.10±0.01	114±3	11±1	5.12±0.02	44±2	11±1
Caseinate and 15 mM Pi	7.88±0.02	136±1	13±2	1.35±0.01	54±2	11±1
Caseinate and 10 mM Cit	9.50±0.01	297±2	8±1	–	–	–
Caseinate, 15 mM Pi, and 10 mM Cit	8.75±0.02	196±3	19±2	4.65±0.02	61±1	19±2

Sodium caseinate concentration 10 g L^{-1}, pH 6.8, temperature 25 °C

Table 2 Comparison of α_S-, β-, and κ-casein content in precipitates of sodium caseinate by Ca^{2+} in different conditions

	Ca^{2+} (mM)	α_S-Casein (% *w*/*w*)	β-Casein (% *w*/*w*)	κ-Casein (% *w*/*w*)	*S* (g L^{-1})
Caseinate	–	45.80±0.02	31.10±0.01	23.10±0.02	10.00±0.03
Caseinate and 15 mM Pi	10	7.40±0.02	45.50±0.03	47.10±0.01	7.90±0.02
	25	46.30±0.02	31.50±0.01	22.20±0.03	1.30±0.01
Caseinate, 15 mM Pi and 10 mM Cit	10	25.45±0.01	33.10±0.02	41.50±0.01	8.75±0.01
	25	44.60±0.01	29.10±0.02	26.30±0.02	4.60±0.01

Sodium caseinate concentration 10 g L^{-1}, pH 6.8, temperature 25 °C

to be aggregated in a collision, then their participation in the precipitates will depend only on their initial concentration in the suspension, as shown in the results described above.

It is interesting to note that when 10 mM Cit was added to the mixtures, the precipitates obtained in the first salting-out step contained more α_S-CN. This increase, produced in spite of the decrease of Ca^{2+} activity due to the Cit presence, suggests a Ca^{2+} binding process different from that produced in the presence of only Pi probably with Cit participation.

The comparison of the three kinds of plots (remaining protein, τ, and α) for each of the cases allows us to realize certain general features of the process of casein aggregation and precipitation in the presence of Ca^{2+}. Thus, it is interesting to remark that in the first precipitation step, only a very low amount of the aggregates formed remained in colloidal suspension. The precipitates obtained at this low TCC, on the other hand, are relatively rich in β- and κ-CN. Although we do not have any information about the degree of heterogeneity of the precipitated particles, their global composition suggests that the presence of homogeneous β- and κ-CN aggregates should not be discarded. The average size of the suspended particles generally showed a decrease, at least at the end of this stage, reflecting perhaps the precipitation of a low amount of the bigger particles and formation of smaller ones, especially in the presence of only Pi. τ increased sharply at TCC at which the second step of salting-out has not started yet. When Cit was not present, the increase of τ was simultaneous with an average size increase, suggesting in this case the presence of either an aggregation or growth process of the particles formed. Conversely, in the presence of Cit, τ increase was accompanied by an average size reduction, suggesting the formation of smaller particles. A similar behavior was observed in media containing only Cit, where a higher amount of relatively big colloidal particles were obtained, showing only slight variations in their average size. The presence of Cit appeared then as conditioning the initial colloidal particle formation mechanism, perhaps by a simple effect of reduction of Ca^{2+} activity. Shoulders in the τ plots in media with only Pi could be showing the sequential formation of different colloidal particles by progressive Ca^{2+} binding. According to the τ plots obtained, these particles grow and then precipitate also in a sequential way, although this behavior could not be appreciated in the precipitation curves. When Cit was present in the media, this sequential formation of colloidal particles was not observed possibly because the Ca^{2+} activity in these cases was not high enough to produce the whole process. In summary, while Pi appeared to be favoring the sequential formation and precipitation of smaller particles, Cit favored the formation of bigger colloidal particles, acting especially in the first steps of the casein aggregation and conditioning the mechanism of this process.

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