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ORIGINAL CONTRIBUTION

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Effects of citrate on the composition and enzymic coagulation of colloidal bovine casein aggregates

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Abstract Colloidal casein aggregates (CCA) prepared from soluble whole bovine caseinates in the presence of Ca^{2+} and phosphate (Pi) ions by addition of different citrate (Cit) concentrations showed different mineral and proteic composition. Citrate concentration conditions the Ca and Pi concentrations incorporated into CCA, probably due to the complexing effect of this anion on calcium. A significant change in the incorporated Ca/Pi ratio at 8 mM citrate could very likely be associated to changes in CCA net charge. The incorporation of individual caseins to the colloidal particles obtained, as well as their average size and size distribution, depended also on the Cit concentration used $[Cit]_P$. α_S- and β-caseins assembled in the CCA structure sharply decreased at a $[Cit]_P$ higher than 15 mM, i.e., at a low Ca^{2+} concentration in the aggregates, showing that the presence of this cation is necessary for the incorporation of these caseins. An inverse relationship between the aggregation step rate in CCA enzymic coagulation and their average size was observed. The aggregation rate vs the average size curve obtained at $[Cit]_P$ 8 mM clearly differed from the curves obtained at 10 and 12 mM, respectively, a fact probably related to a change in the CCA net charge. This behavior showed the effect of citrate concentration on CCA functional properties.

Keywords Colloidal casein aggregates · Citrate · Colloidal stability · Enzymic coagulation

Introduction

Caseins, the major protein fraction in bovine milk, are highly hydrophobic proteins suspended in the aqueous phase of milk as colloidal aggregates known as casein micelles (CM). These aggregates present an almost spherical shape and a net negative charge at the milk pH values. Their principal components are caseins α_{S1}, α_{S2}, β and κ, in a molar proportion of 4:1:4:1.3, respectively. The CM "core" is rich in α_S-caseins, while κ-casein is preferentially located near the CM surface. This location enables the hydrophilic moiety of κ-casein (caseinomacropeptide) to project to the aqueous environment, forming an external "hairy" layer that contributes, together with the net negative charge, to CM electrostatic and steric stabilization in suspension [1–4]. The α_S-caseins appear to be assembled by participation of clusters of colloidal calcium phosphate (CCP), a mineral complex essentially formed by calcium (Ca) and phosphate (Pi) ions with a minor participation of magnesium and citrate (Cit). CCP interacts with the phosphoserine residues of the proteins and plays a fundamental role in maintaining the integrity of the CM structure [5–9].

CM functional properties essentially involve destabilization and coagulation by heat, acidification or rennet action for cheese manufacture. This last process, known as enzymic coagulation, is the most frequently used. In this case, CM destabilization is produced by specific enzymic cleavage of the caseinomacropeptide moiety of κ-casein, basically by loss of a great part of the steric component of colloidal stability [10, 11]. Structural changes in CM could very likely be associated with changes in their colloidal

stability. Hence, natural or induced CM structural modifications can result in variations of cheese quality and yield, with important consequences for cheese manufacture. Thus, variations in the micellar Ca content of milk that naturally occur by a number of factors such as the stage of lactation, the mother's nutritional condition, and environmental and genetic factors, could affect CM functional properties [12]. As some of these changes have been related to the action of Cit, presumably as a Ca-chelating agent [2], the study of the effect of Cit on the structural and functional properties of CM or similar colloidal casein aggregates appears to be an interesting objective to be considered.

Casein colloidal aggregates (CCA) can be obtained from soluble caseinates by addition of adequate salt solutions. Varying the proportion of Ca, Pi and Cit ions used, CCA can be prepared with different sizes and mineral and protein compositions [5, 13]. Keeping the component concentrations and proportions in a defined range, CCA structurally similar to CM can be obtained [14–16], thus, providing a useful model to study the relationship between CCA structural and functional properties [17].

Different Cit concentrations have been used in this work to obtain CCA, which differ in size, mineral and proteic composition. The effect of these differences on the aggregation step of enzymic coagulation has been studied, and the action of Cit on the CCA structure has been discussed.

Experimental sections

Materials and methods

Preparation of CCA

CCA were prepared according to the method of Knoop et al. [13]. Whole bovine caseins were prepared from suspensions of commercial, nonfat dried milk (MOLICO, Societé des Produits Nestlé S. A., Vevey, Switzerland) reconstituted to 10% (w/v) in distilled water, by isoelectric 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. The caseins were dissolved in distilled water at room temperature (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. The CCA were prepared at room temperature by adding to the casein solution the amount of 1 M sodium citrate necessary to reach the desired final Cit concentration ($[Cit]_P$). The mixture was stirred for 15 min. The prescribed Ca and Pi final concentration were obtained by five additions of 0.2 M $CaCl_2$ and 0.2 M $K_2H(PO_4)_3$, stirring continuously, with 15 min intervals between them. The volume and the pH were adjusted to reach a final casein concentration of 14 g L^{-1} and the final pH was adjusted to 6.8 by addition of 1 M HCl or 1 M NaOH, as necessary, followed by further 2 h stirring. The Ca and Pi concentrations used in CCA preparation were 25 and 20 mM, respectively. The $[Cit]_P$ varied in the range 5 to 25 mM. Sodium azide was added at the rate of 0.01% (w/v) to CCA as a preservative. After a 24 h storage at 4°C, the CCA samples were equilibrated at working temperature, and their pH was measured and corrected when necessary. All the studies performed were carried out after a CCA equilibration at working temperature.

Exclusion chromatography on controlled pore glass

Chromatography on controlled pore glass (CPG) was used both to separate the CCA obtained from the free caseins remnant in the solution and to obtain fractions of CCA of different sizes. CPG-10 (300 nm mean pore diameter, 120/200 mesh size, Sigma Chemical), was cleaned by treatment with 5% (w/v) HNO_3, 1 h at 90°C [18], washed thoroughly with distilled water, and allowed to equilibrate for 24 h in 1% (w/v) PEG 20,000 (Sigma Chemical) solution [19, 20]. Before each chromatographic run, CPG was equilibrated for 24 h with three column volumes of the correspondent elution solutions. Column dimensions were 1.6×144 cm. The chromatographic conditions were: 25°C temperature and a flow rate of 2.5 mL/min. The eluent solutions were prepared by adding 0.04% (w/v) PEG to solutions having the same mineral composition as each of the media in which the CCA were obtained. The compositions of such media were determined in ultrafiltrates obtained by centrifugation at 1,200 g for 30 min of the CCA suspensions in dialysis tubing loops suspended in 50-mL conical centrifuge tubes at working temperature [21]. The purpose of using this kind of eluents equilibrated with CCA suspensions was to minimize mineral and protein dissociation from the CCA during chromatography. Furthermore, the use of the CPG rigid matrix, which allows high eluent flow rates, produces a good resolution in short running times, contributing in this way to the preservation of the CCA composition [22, 23]. The protein concentration of the fractions was determined by Kuaye's method [24].

CCA size variations followed by turbidity measurements

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

$$\tau = \frac{32\pi^2 n^2 \left(\frac{dn}{dc}\right)^2 c\, M\, Q}{3N\lambda^4} \quad (1)$$

where τ is the turbidity of the suspension, λ is the wavelength, N is Avogadro's number and dn/dc is the specific refractive index increment. The function Q results from the internal interference of light scattered by the particle at all angles. From Eq. 1 we can obtain the derivate:

$$\frac{d\ \log \tau}{d\ \log \lambda} = \frac{d\ \log Q}{d\ \log \lambda} + 2\frac{d \log \left(n\frac{dn}{dc}\right)}{d \log \lambda} - 4 = \beta + \gamma - 4 \quad (2)$$

CMs do not meet all the requirements for the application of Eq. 1. Nevertheless, it was found that with a value of 0.2 for (and for τ measurements in the range of 400 to 800 nm, β can be used to detect and easily follow rapid size changes in CM submitted to different conditions, and also to calculate the fractal dimension of such particles [25].

In the case of the CCA prepared in this work, β can be applied for the same purposes, taking into account that such particles had a similar chemical composition and size to native CM. β values were obtained from the slope of log τ vs log λ plots, in the 400 to 700 nm range. τ was measured as absorbance using a Jasco V-550 and a Spekol 1200 spectrophotometers.

To verify if β was actually related to the average size of the aggregates, CCA average $D_{6,5}$ diameters were determined by dynamic light scattering for CPG chromatography fractions of CCA obtained with 10 mM Cit, applying the method of cumulants [26], and the relationship of the values obtained with the correspondent β values was studied. Dynamic light scattering measurements were performed on a Brookhaven BI-2005 M equipment, with a 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.

CCA samples were diluted in the correspondent buffer which had previously been filtered through Millipore of 1 mm 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.

Incorporation of minerals and proteins into the CCA

The amount of the mineral components in the colloidal phase was calculated using the difference between their total concentration and the concentration of ultrafiltrates obtained as explained in the "Exclusion chromatography on controlled pore glass" section. The concentrations of the ultrafiltrates were found to be coincident with the concentrations in supernatants obtained by ultracentrifugation, which shows that the incorporation of minerals into protein fractions remaining in the supernatants was negligible.

The incorporation of proteins into the CCA was studied by precipitating the colloidal phase by ultracentrifugation, and by determining the protein concentration (Kuaye's method) in the supernatant [24]. CCA samples were centrifuged at 250,000 g for 90 min using a Beckman L8-80 M ultracentrifuge, and the amount of protein in the CCA was obtained as the difference between the total protein in the sample and the value for the supernatant. The different caseins incorporated were identified by urea-polyacrylamide gel electrophoresis of the supernatants, previously concentrated by ultrafiltration (Spectrum Molecular/Pore Membrane C Type).

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

The CCA supernatants were analyzed by Urea-SDS-PAGE using a vertical gel system, according to the method of Laemmli [27]. 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, 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 brillant 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 of the stained gels and analysis of the pixel densities of the digitized protein bands, using software specially designed for this purpose. 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-casein (Sigma Chemical). 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.

Chemical determinations

Ca was measured by atomic absorption spectrophotometry (Metrolab RC 250 AA) and Pi by a standard colorimetric method based on the formation of phosphomolybdate in acidic medium. Cit was determined by the colorimetric method of Marier and Boulet using TCA filtrates, as described by White and Davies [28].

Enzymic coagulation studies

The enzymic coagulation process of CM includes three well-defined steps [29, 30]:

1. Proteolysis of the hydrophilic moiety of surface κ-casein by chymosin
2. Aggregation of the partially hydrolyzed CM (pCM) to give coagula
3. Coagula compaction and syneresis

CCA obtained in the conditions used in this work presented a similar functional behavior when submitted to rennet action. Working in conditions of enzyme excess, the proteolytic step can be practically completed before the start of aggregation [29–31]. Under these conditions, the aggregation kinetics can be studied after β changes. β values were obtained from absorption spectra recorded at different times using a Spekol 1200 spectrophotometer with a diode arrangement. An aliquot of the CCA suspension, previously diluted to 0.3 g L^{-1} with 10 mM Tris–HCl, 10 mM $CaCl_2$ buffer, pH 6.4, and equilibrated at working temperature (35°C), was poured into a 3-mL spectrophotometer cuvette in a jacketed cuvette holder, maintained at working temperature by water circulation. Coagulation was started by addition of 0.1 mL of a 1/8 (v/v) rennet dilution to the cuvette. The mixture was gently stirred with a Teflon stirrer for 5 s and its absorbance spectra, between 400 and 700 nm, were recorded as a function of time. The concentration of rennet used was enough to produce the κ-casein proteolysis from 10 to 30 s. Commercial liquid rennet with a strength of 100 RU was a gift from C.O.T.A.R. S.A. (Argentine).

Statistical analysis

Data were reported as mean value±standard deviations for all data points. All the experiments were carried out three times at least. Standard ANOVA analysis was used to determine significant differences between variables.

Correlation analysis The strength of the linear relationship between the values of β_0 and average $D_{6,5}$ for CCA_{10}, was calculated using the Pearson coefficient of correlation r. Statistical significance was considered at p values below 0.05.

Results

Mineral composition of CCA

Figure 1 shows the amount of Ca and Pi incorporated to the CCA by protein gram at different $[Cit]_P$. At increasing $[Cit]_P$, incorporated Ca and Pi increased up to 8 mM Cit. At higher $[Cit]_P$, whereas Pi diminished with regularity from 8 mM to 12 mM Cit, Ca remained practically constant between 8 and 10 mM Cit, decreasing later. The incorporated Ca/Pi ratio showed a sharp increase between 8 and 12 mM $[Cit]_P$.

Casein incorporation to CCA

The proteic composition of CCA obtained at $[Cit]_P$ ranging from 5 to 25 mM is shown in Fig. 2. Incorporation of the α_S-casein fractions (α_{S1} and α_{S2} caseins) to the CCA showed a slight increase when 15 mM $[Cit]_P$ was reached but dropped between 20 and 25 mM. β-casein incorporation dropped between 15 and 20 mM $[Cit]_P$, while an opposite behavior was observed for κ-casein. A maximal value for the $(\alpha_S+\beta)/\kappa$ incorporated casein ratio was found at 15 mM Cit.

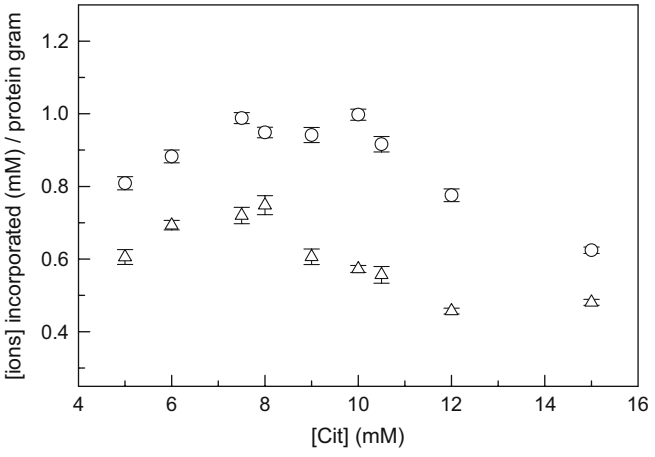

Fig. 1 Concentration of Ca (○) and Pi (△) incorporated into the CCA by protein gram, at different Cit concentrations used in CCA preparation. Temperature 25°C, pH 6.8. Each point is the average of three independent determinations

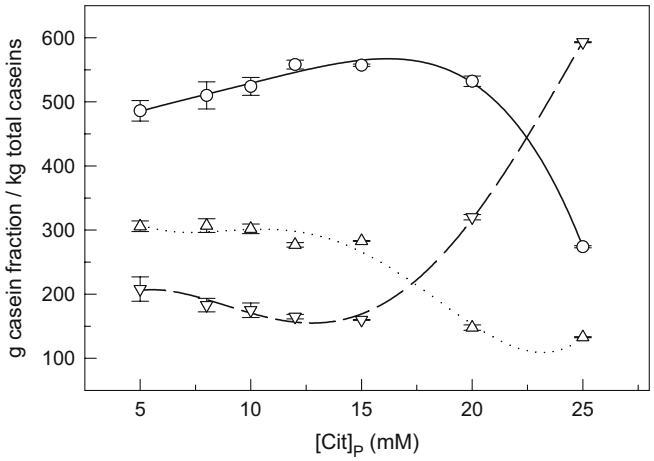

Fig. 2 Proteic composition of CCA correspondent to the fraction (○) α_S-casein, (△) β–casein and (▽) κ–casein, obtained from 14 g L^{-1} caseinate, 20 mM Pi, 25 mM Ca and different $[Cit]_P$ (range 5 to 25 mM). Each point is the average of three independent determinations

Size fractionation of CCA

Figure 3 shows the kinetic curves obtained for the aggregation rate in the enzymic coagulation of CCA prepared using $[Cit]_P$ of 5, 8, 10, 12, 15, 20 and 25 mM. A sigmoid form was observed for some of these curves, especially at high Cit concentrations. In these conditions, the aggregation rate and the associated turbidity increase were slow enough to allow the observation of an initial turbidity decrease due to the first step of enzymic coagulation, i.e., the CCA partial proteolysis. The aggregation of CM obtained from reconstituted skim milk (RCM) in the presence of 10 mM Cit, working in similar conditions of initial particle concentration and enzyme excess, is also shown. From these results, it is evident that the CCA obtained from $[Cit]_P$ 8, 10 and 12 mM (CCA_8, CCA_{10} and CCA_{12}, respectively) produced aggregation curves similar to that of RCM, with initial aggregation rate constant values close to that of the reconstituted micelles. Regarding this fact, it is interesting to remark that these CCA have a similar proteic composition to RCM (not shown).

Because of the results described above, CCA_8, CCA_{10} and CCA_{12} were chosen to further study the relationship between CCA size and aggregation kinetics. For this purpose, these CCA were submitted to CPG chromatography to isolate fractions of colloidal particles with more homogeneous size and composition. Figure 4 shows the CPG chromatography elution patterns obtained. A main component was present as a large peak in the first fractions eluted at all the $[Cit]_P$, containing the bigger colloidal particles obtained. The protein content of this peak was higher in the case of CCA_{10}, with a dispersion slightly lower than that observed in the case of either CCA_8 or CCA_{12}. The peak elution volumes suggested that the average size of particles can be ranked in the order of $CCA_{10}>CCA_8\cong CCA_{12}$. CCA_{12} particles appear to be the most heterogeneous colloidal particles. Fractions containing even smaller particles and free proteins can be observed at the three $[Cit]_P$ used.

The average $D_{6,5}$ diameter of the fractions of CCA_{10} particles determined by dynamic light scattering showed a good linear correlation (r=0.96; p<0.05) with the values of β_0 presented by these fractions (Fig. 5), allowing us to assume that this parameter can be used as one related to the average CCA size of fractions of similar composition.

Enzymic coagulation

Fractions of the first peaks collected from CPG chromatograms were diluted to concentrations adequate for the coagulation tests with the correspondent buffer, as explained in the Materials and methods section. Slight modifications were observed in the average size of CCA fractions, detected by initial β (β_0) variations, because of

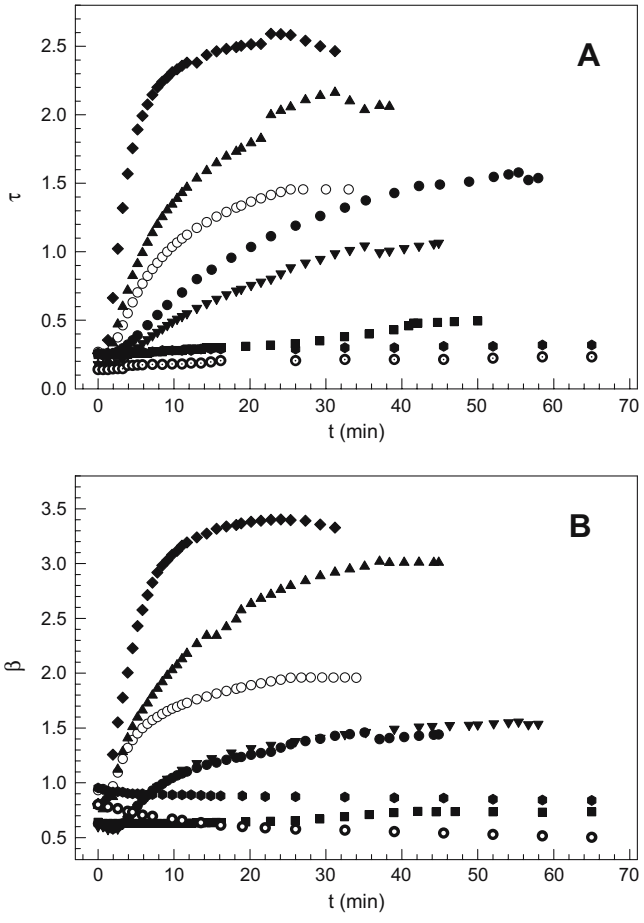

Fig. 3 (**a**) τ as A to 600 nm and (**b**) β during aggregation step of enzymic coagulation of RCM (○) and CCA obtained from 14 g L^{-1} caseinate, 20 mM Pi, 25 mM Ca and different $[Cit]_P$: (♦) 5 mM, (▲) 8 mM, (●) 10 mM, (▼) 12 mM, (■) 15 mM, (⬟) 20 mM and (◘) mM. Each point is the average of three independent determinations

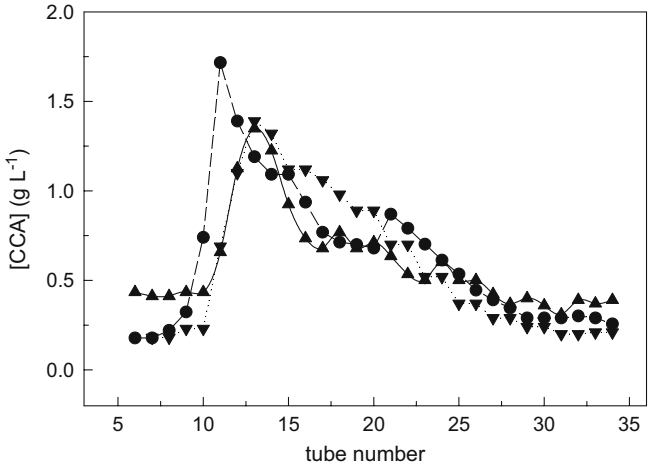

Fig. 4 CPG chromatography elution patterns of CCA prepared at 8 mM (▲), 10 mM (●) and 12 mM (▼) $[Cit]_P$. Each point is the average of three independent determinations

dilution (Fig. 6). A certain degree of size increase was observed for both CCA_8 and CCA_{10}, but size changes appeared to be more important in CCA_{12} fractions, which clearly showed the presence of bigger particles. In all the cases, β_0 variations were accompanied by τ_0 increases, suggesting the presence of association processes. Since in a simple association–dissociation equilibrium dilution is expected to produce a shift to the formation of smaller particles, this behavior could better be attributed to an association process driven by an increase in the Ca^{2+} binding because of dilution in a buffer with a Ca^{2+} concentration higher than the equilibrium value correspondent to the casein concentration (6.8 mM, 8.3 mM and 9.5 mM for CCA_8, CCA_{10} and CCA_{12}, respectively).

The results obtained for CCA aggregation kinetics are presented in Fig. 6 as β vs time plots. Aggregation rates

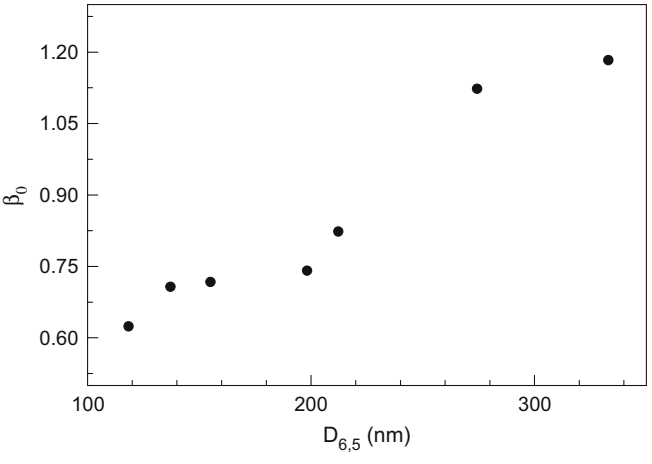

Fig. 5 Correlation between the values of β_0 and the average $D_{6,5}$ diameter determined by dynamic light scattering for the fractions of CCA_{10} (r=0.96; p<0.05)

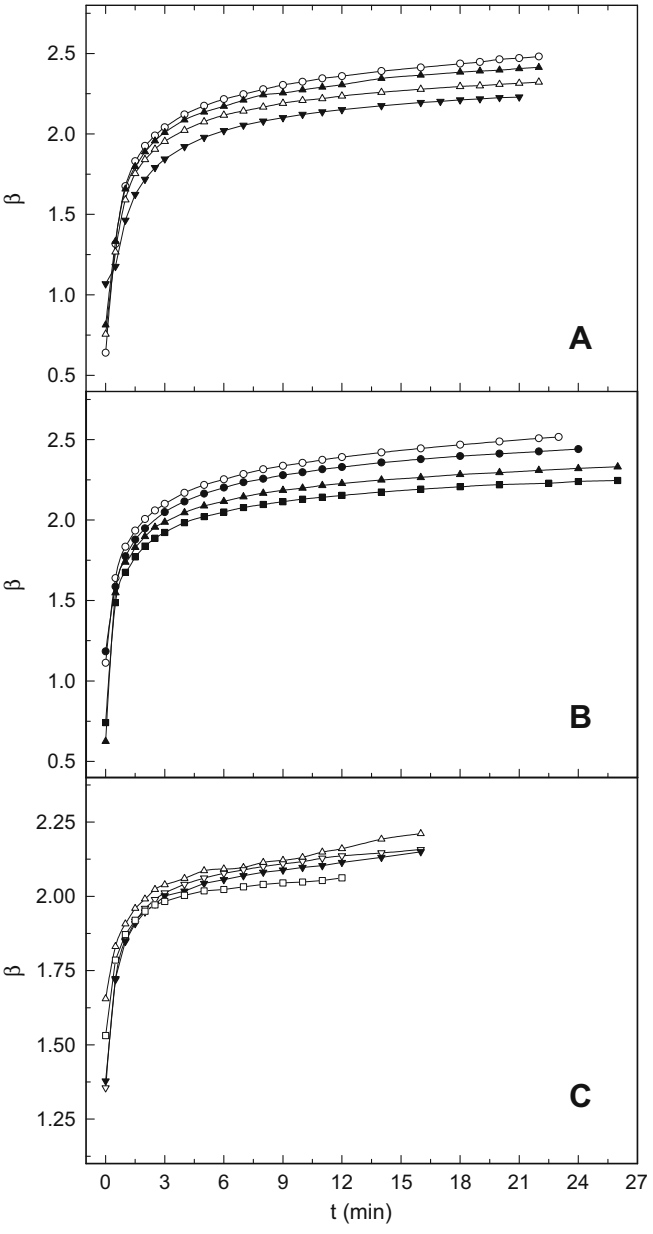

Fig. 6 β as a function of aggregation time for different fractions (Fig. 4) of CCA suspension prepared at 8 mM (**a**), 10 mM (**b**) and 12 mM (**c**) $[Cit]_P$. Fraction numbers: (●) 10, (○) 11, (▲) 12, (△) 13, (▼) 14, (▽) 15, (■) 16, (□) 18. Protein concentration 0.3 g L^{-1}, in 10-mM Tris–HCl, 10-mM $CaCl_2$ buffer, pH6.4, temperature 35°C. Each point is the average of two independent determinations

were estimated as the maximal value of $\partial\beta/\partial t$, and plotted as function of the β_0 values, related to the initial average size of the aggregates (Fig. 7). The curves obtained showed, as a general tendency, an inverse relationship between aggregation rate and average initial size. When these curves were fitted with linear regressions, CCA_{10} and CCA_{12} showed good correlation coefficients (0.999 and 0.998, respectively). Furthermore, the origin intercept and

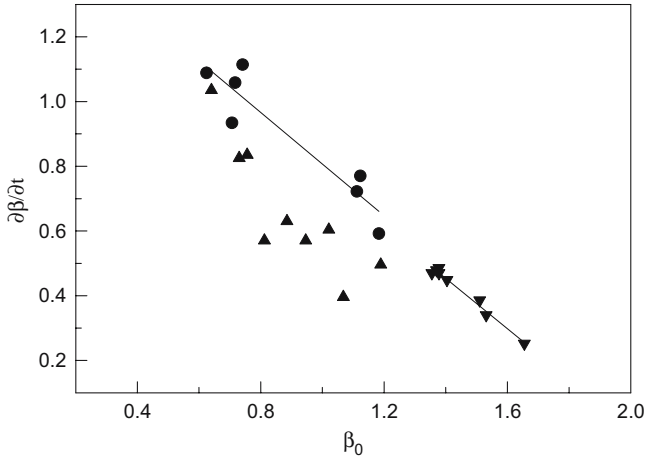

Fig. 7 Aggregation rates estimated as the maximal value of $\partial\beta/\partial t$, plotted as function of β_0, 8 mM (▲), 10 mM (●) and 12 mM (▼) $[Cit]_P$. Protein concentration 0.3 g L^{-1}, in 10-mM Tris–HCl, 10 mM $CaCl_2$ buffer, pH 6.4, temperature 35°C. Each point is the average of two independent determinations

slope of these two lines do not show statistically significant differences ($p>0.01$). The curve correspondent to CCA_8, on the contrary, was clearly different, showing a non-linear behavior.

Conclusion

The results obtained in this work and in a previous one showed that, at constant Ca^{2+} and Pi concentrations, both the mineral and proteic CCA composition depended on the Cit concentration used in CCA preparation [21]. Finally, the presence of Cit conditions the amount of CCP formed, likely through the reduction of Ca^{2+} activity related with the Cit complexing action on this cation. These results can be interpreted on the basis of the existence of different kinds of sites for Ca^{2+} binding, either on the CCP complex or directly on the caseins, as suggested by different authors [5, 32, 33]. Increasing $[Cit]_P$ produces Ca^{2+} activity decrease, thus conditioning the sites occupation and, thereby, the casein aggregation process. At $[Cit]_P$ higher than 8 mM, the Ca incorporated into the aggregates did not diminish at the same rate that Pi did; instead, it remained almost constant until a $[Cit]_P$ of 10 mM was reached. This difference could reflect the Ca^{2+} directly bound to casein phosphoserine residues [5]. It is interesting to remark that the change in the Ca/Pi ratio at 8 mM $[Cit]_P$ must be very likely related to changes in the net charge of the colloidal particles obtained and, therefore, to changes in the aggregation rate of these particles when they were partially proteolyzed by chymosin.

The results of this work showed that CCA prepared at 15 mM $[Cit]_P$ showed a higher proportion of α_S- and β-caseins and a lower proportion of κ-casein. Conversely, at higher $[Cit]_P$, the incorporation of α_S- and β-caseins decreased, while κ-casein incorporated into the particles increased sharply, suggesting a change in the nature of the particles formed. This behavior confirms that calcium binding was necessary for the association of the first two kinds of caseins to initiate the formation of CCA. The incorporation into the aggregates of κ-casein, which cannot give further hydrophobic associations or calcium bridges because it has only one phosphoserine residue and one hydrophobic region by molecule, could limit the particle growth, regulating, in this way, the final size of the CCA. κ-casein is not precipitated by Ca^{2+} but could be aggregated in conditions of low Ca^{2+} activity by a self-association process through hydrophobic interactions to give κ-casein micelles a different kind of particles [7, 8]. The possible Cit incorporation to the CCA structure, possibly as a Ca–Cit complex forming part of CCP, as in native CM, should not be discarded, specially at high $[Cit]_P$ concentrations [21].

The results of CPG chromatography have also shown that the use of different Cit concentrations for CCA preparation resulted in the formation of different amounts of colloidal particles with different size distributions; 10 mM $[Cit]_P$ produced the highest yield with the lowest size dispersion.

A general inverse relationship between the aggregation rate measured as $(\partial\beta/\partial t)_0$ and β_0 was found, showing that the hydrolyzed CCA aggregation rate measured in this way, was partially determined by the average size of the initial monomeric particles (Fig. 7).

CCA aggregation, as well as CM aggregation, can be considered as a diffusion limited aggregation process (DLA). Monomeric particles without any kind of colloidal stabilization would aggregate, in DLA, at the maximal rate (k_2^0), independently of the initial monomeric particle size, as proposed by Smoluchowski:

$$k_2^0 = \frac{8kT}{3\eta} \tag{3}$$

where k is the Botzmann's constant, T the temperature and η the medium viscosity [34].

CCA, however, can present both electrostatic and steric stabilization. The presence of these effects will be taken into account in W, the stability factor, which relates k_2, the actual aggregation rate, to k_2^0:

$$k_2 = \frac{k_2^0}{W} \tag{4}$$

W can be interpreted as the ratio of the particle collision frequency to the frequency with which collisions result in aggregation.

The experimental results obtained showed that at higher β_0 values, the aggregation rates were lower, and therefore the W values should be higher. This could be due either to changes in the CCA size or in their net charge, or both, which affect the electrostatic stabilization. Another possibility, which must be simultaneously considered, could be the presence of differences in the proteolyzed particles (pCCA) surface structure able to modify the steric component of the colloidal stabilization. For example, as surface p-κ-casein groups could act as hydrophobic sites for the formation of intermicellar hydrophobic bonds during the aggregation [35], differences in surface density of p-κ-casein related to differences in pCCA size could modify the fraction of effective collisions. In this case, higher pCCA sizes related to lower surface para-κ-casein densities [36, 37] could show lower aggregation rates, in accordance with our experimental results. Furthermore, the smallest CCA contained a higher proportion of κ-casein, which has a negative net charge smaller than those of α_S- or β-casein at the working pH, a fact that could give a smaller net negative charge to those particles, with the consequent diminution in electrostatic stabilization of pCCA.

Apart from the general tendency observed in the relationship between $(\partial\beta/\partial t)_0$ and β_0, the curve obtained for CCA_8 differed from those obtained for CCA_{10} or CCA_{12}, which did not show any statistical difference between them. This fact appears to be consistent with the change in Ca/Pi ratio experimented by the CCA_8, as a variation in the net charge of the particles must produce a variation in the kinetic constant of the aggregation process.

In summary, the Cit concentration used in the CCA preparation conditions the interactions of Ca and Pi with the caseins, which gives rise to different amounts of colloidal particles that differ in size distribution and in mineral and proteic composition. At certain $[Cit]_P$ (8 mM in this work), a sharp variation in the ratio between incorporated Ca and Pi probably resulted in an important modification of the CCA net charge, producing a perceptible change in their functional properties, enzymic coagulation, in this case.

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