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ORIGINAL CONTRIBUTION

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Stability against aggregation of bovine casein micelles in the presence of acidic and alkaline gelatin

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Abstract The effect of the presence of colloidal dispersed and molecular dispersed acidic (type A) and alkaline (type B) gelatins with similar molecular weight and size but different isoelectric points (7.9 and 4.9) on the stability against aggregation of bovine casein micelles was investigated by turbidimetric titration and laser techniques, over a wide range of biopolymers concentrations, gelatin/casein ratio in the initial mixture (0.03–20), pH (4.9–6.7) and ionic strength (10^{-3}(milk salts)–1.0 NaCl), using glucono-δ-lactone (GL) as acidifier. Aggregates of acid gelatin A interact with the oppositely charged micellar casein at an ionic strength of around 10^{-3} (milk salts) and pH 6.7 resulting in the formation of an electroneutral complex by ionic bonds between the carboxyl groups of casein and the amino groups of the gelatin molecules. The complexes obtained are polynuclear, the aggregation of which is not as sensitive to pH as that of free casein micelles. Aggregation of such complexes is the result of bridging flocculation. The "molar" ratio gelatin aggregates/casein micelles in the mixed aggregates is 4/1. The complexes are formed and stabilised via electrostatic interaction rather than through hydrogen bonds or hydrophobic interaction. In the presence of an excess of gelatin molecules in the initial mixture a charged gelatin–casein complex forms and some dissociation of casein micelles occurs and, as a consequence, soluble complexes are obtained. During the addition of alkaline gelatin B aggregates to the micellar casein solution and subsequent acidification of the mixture by GL, no effect of the presence of gelatin B on the stability of micellar casein was observed.

Key words Bovine casein micelles · Gelatin aggregates · Interactions · Bridging flocculation

Introduction

Protein mixtures may exist in molecular dispersed and colloidal dispersed states depending on the properties of the individual proteins, the composition of the mixtures and the conditions in which the mixtures are obtained. Colloidal protein dispersions play a pivotal role in our everyday life and can be exploited for the efficient production of food [1]. The interaction between proteins and natural or synthetic polymers has been extensively investigated, in particular for the modulation of living processes in plant and animal cells, immobilisation or stabilisation of enzymes in complexes, modification of protein-substrate bridging affinity, changing properties of food products obtained by treatment of protein solution and gels [2–6]. These interactions may result in the formation of water-soluble complexes [7, 8], complex coacervation [9–11], formation of amorphous precipitates, stabilised by different type of bonds [12, 13] or in the thermodynamic incompatibility of the

macromolecules leading to liquid–liquid phase separation [14, 15].

However, until recently, comparatively little attention had been given to interactions in aqueous protein (1)–protein (2) systems, with both macromolecules in the colloidal dispersed state. It can be surmised that the compatibility and aggregation properties of a protein in the colloidal dispersed state can be different from these existing in the molecular dispersed state and that an increase in ''molecular'' weight and in the hydrophobicity of the accessible surface should cause a decrease in compatibility and an enhancement in stability against aggregation. However, data are lacking for a quantitative understanding of how structural features of the protein, such as net charge sign, charge density, conformation state, as well as the different physicochemical factors affect these properties.

We attempted to study the interaction of acidic and alkaline gelatins in the colloidal dispersed state with micellar casein at neutral pH and during acidification of these mixtures by glucono-δ-lactone (GL). Turbidimetric titration and particle size distribution determination were used to observe these effects.

Materials and methods

Materials

The samples of gelatin used were a pigskin gelatin, type B 200 Bloom 30 PS, (lot 33678), and an ossein gelatin type A 200 Bloom 30 PS (lot 19538), produced by SBI Systems Bio-Industries, France. The Bloom number of the gelatins reported by the manufacture was 200; this correspond roughly to a weight-average molecular mass of 1×10^5 g mol^{-1} [16]. The isoionic points of the gelatin samples, estimated as described by Boedtker and Doty [17], were 7.0 for the gelatin A and 4.6 for the gelatin B samples. The isoelectric points of the gelatin samples, determined by isoelectric focussing according to the procedure reported by Addeo et al. [18], were 7.9 for the gelatin A and 4.9 for the gelatin B samples.

Gelatin aggregates were produced as described Boedtker and Doty [17]. By following this procedure, it was possible to obtain reproducible particle size distributions. The diameter of aggregates of gelatin types B and A were 0.42 and 0.48 μm, respectively.

Micellar casein was prepared by I.N.R.A.-LRTL (Rennes, France). Its composition is as follows: protein content 87.04%, nonprotein nitrogen 0.37%, lactose 1.81%, total mineral substances 8.46% (including calcium 3.2%, phosphorus 1.7%, chloride 0.2%, sodium 0.08%). The initial pH of the micellar casein dispersions was 6.8 and the water activity at 25 °C was −0.450. Casein micelles in water presented a narrow size distribution, nearly Gaussian, with a 0.30-μm diameter, very close to that of literature data [19–21]; this size corresponds to colloidal particles with a molecular weight of about 160,000 kDa [22].

Skimmed milk (protein: 35.5%, lipids: 0.8%, calcium: 1.29%) was a commercial food product.

The GL was obtained from Aldrich Chemie, Germany, catalogue no. G-201. Dry micellar casein or skimmed milk powder was rehydrated to obtain dispersions at the same concentration of micellar casein as in fresh skimmed milk (2.5%). Micellar casein was first dispersed in deionised water or buffer by magnetic stirring for 5 min at 5 °C The dispersion was then sonicated for 5 min at the same temperature. The colloidal dispersions obtained were centrifuged at 1460g for 60 min at room temperature to remove aggregated micelles. To prevent bacterial growth, 0.05% sodium azide was added when necessary.

The protein concentration in the dispersions was determined in triplicate by drying at 104 °C for 12 h, taking into account the nitrogen content (18.30% for the gelatin type A and 18.15% for the gelatin type B) which does not depend on the isolation method [16] and the protein content of micellar casein.

Aggregation of casein micelles and their association with gelatins

Two methods were used to follow the aggregation of casein micelles during acidification, during heating or after addition of gelatin: the measurement of the size distribution of colloidal particles and turbidimetric titration.

Size distribution

The size distribution of the casein micelles, the gelatin aggregates and the particles in mixed casein–gelatin systems was determined at 5 °C with a Malvern Mastersizer IP laser Granulometer using a 45-mm lens. The minimum size of particles measured was 0.08 μm. The results were expressed in terms of volume/surface average diameters. For each experimental trial, the average of three essays was taken.

Turbidimetric titration

Forty millilitres of the solvent was placed in a thermostatic mixing vessel and was magnetically stirred and pumped to the thermostated spectrometer flow cell. The vessel and the cell were thermostated separately at the desired temperature (from 2 to 70 °C). A pH electrode was placed in the liquid in the mixing vessel. Changes in turbidity were monitored at 590 nm and recorded as variations of the absorption coefficient (A_{590}), which is linearly proportional to true turbidity for $A_{590} < 0.9$. Solutions of micellar casein at 4.8% (w/w) protein concentration were placed in the mixing vessel and diluted with the appropriate solvent (buffer or gelatin solutions in buffer) so as to get a final A_{590} value of 0.3–0.4 (approximately 0.08 gdl^{-1} of casein concentration); in the case of the turbidimetric titration of micellar casein–gelatin mixtures, the dilution of micellar casein dispersions was made with colloidal dispersed gelatin solutions so as to get gelatin/casein ratios varying from 0.03 to about 13. After the turbidity had reached a constant value, the necessary quantity of GL was added to acidify the dispersion to the desired final pH value (pH 4.9). However, GL was unable to acidify casein–gelatin mixtures at high ionic strength; the titration was done in such cases by addition of 0.01–0.5 M KOH. The pH value below which A_{590} begins to increase was taken as the clotting point of casein micelles. The higher the GL concentration, the higher the clotting point and the stronger the aggregation. This kinetic effect disappeared when acidification took 1 h and more. The clotting points were therefore determined in all subsequent experiments using different amounts of GL added step by step so as to achieve acidification in about 2 h.

Interprotein interactions

Circular dichroism spectra of solutions of gelatins, micellar casein and their mixtures were recorded with a Jobin Yvon Mark VI dichrograph in 1-mm quartz cells at 10 °C over the wavelength range 190–250 nm. The method was used to spot the possible formation of gelatin–micellar casein molecular associations within the one-phase region. It was assumed that in the absence of such associations the spectrum of the mixture must be the sum of the spectra of the solutions of the individual components at the same concentrations as in the mixture; a departure from additivity should then be due to ''complex'' formation.

The composition of water–insoluble micellar casein-gelatin complexes was determined for the mixtures in deionised water at 10 °C. A solution of gelatin aggregates (0.2% w/w, obtained by dilution of 0.5% of gelatin aggregates) and a 2.5% w/w solution of micellar casein were prepared separately and mixed at pH 6.8 and 20 °C to get gelatin/casein w/w ratio ratios (R) from 0.2 to 2.0. The mixed solutions were stored for 2 h and at 10 °C to reach equilibrium, decanted and submitted to low-speed centrifugation for 20 min under 1200g at 10 °C in order to separate free proteins (which did not take part in the complex formation) from the complex. The precipitated complex was washed twice with deionised water to remove traces of low-molecular-weight compounds and were solubilised at 40 °C. The concentration of gelatin in the complex was determined by subtraction of the dry weight of casein from the dry weight of the casein–gelatin complex; a second method consisted in measuring the absorption at 240 nm after dissociation of the complex and removal of micellar casein by precipitation at pH 4.6 in 0.5 M NaCl.

Results and discussion

Solutions of gelatin aggregates show an extremely low turbidity at 590 nm. This makes it possible to study the behaviour of the casein micelles in mixed micellar casein–gelatin solutions. We consider the behaviour of casein micelles in water and NaCl solutions in the presence of colloidal dispersed gelatin. The problem of the bridging flocculation and depletion flocculation in weakly charged micellar casein–gelatin mixtures will be approached in terms of complex formation and incompatibility of biopolymers.

Since the behaviour of casein micelles in the presence of gelatins is extremely dependent on the sign of the net charge of the gelatin molecules, we will examine the effect of acidic gelatin and of alkaline gelatin on the stability of casein micelles against aggregation separately.

The effect of acidic gelatin at pH 6.7

Figure 1 presents the results of the turbidimetric titration of micellar casein solution in water (0.03% w/w) by aqueous solutions, containing aggregates of gelatin A (0.5% w/w), at pH 6.7 and at different temperatures from 2 to 20 °C. The addition of even a small amount of gelatin aggregates to micellar casein solution causes large changes in turbidity. They strongly depend on the gelatin/casein ratio, R: as R increases, the turbidity first increases steeply, reaches a sharp maximum for $R \cong 1.26$ and then decreases to values lower than its initial level when $R > 5$–10. In theory, such a bell-shaped turbidity curve could be the result of a depletion flocculation mechanism of casein micelles induced by gelatin molecules, the flocculation of the colloidal particles being reversed at high concentrations of the destabilising polymer [1, 23]. Such a dependence on R is also typical for interpolymer complex formation [24, 25]. The latter hypothesis seems more probable since the system studied contains two kinds of protein colloids with opposite net charge sign; an additional argument in favour of a bridging flocculation process is that in the case of depletion stabilisation one would expect that turbidity of such a system should be close to that of micellar casein. The electrostatic interaction between the two species causes formation of mixed casein–gelatin aggregates with compensated charges and, as a consequence, bridging flocculation would occur; precipitation would be a maximum for $R = 1.26$ (maximum of turbidity), and at $R \geq 10$ charged complexes (soluble complexes) could be formed with partial dissociation of casein micelles.

As a matter of fact, we do observe coprecipitation of casein and gelatin as the formation of a protein-enriched gel-like phase. The effect of the gelatin/casein ratio in the initial mixture on the composition and yield of the proteins in the protein-enriched gel is shown in Fig. 2. An increase in R increases the yield of the precipitate, and at $R = 1.26$ almost all the proteins are precipitated. The gelatin/casein ratio in the precipitate and the dry weight of the precipitate did not depend on the gelatin/casein ratio in the initial mixture; they were equal to 1.26 ± 0.05 and 6.65 ± 0.15%, respectively. The mixed aggregates coexist together with free casein micelles at low R values, but the number of free micelles decreases strongly as R increases. Above $R = 0.94$ free casein micelles are absent. These results demonstrate that coprecipitation is the result of bridging flocculation. Taking into account that the molecular weight of gelatin A aggregates in the conditions used is about 50,000 kDa and that of micellar casein is about 160,000 kDa, we evaluate the molar ratio gelatin aggregates/micellar casein micelles in the mixed aggregates of gelatin and micellar casein. A simple calculation shows that this ratio is 4/1.

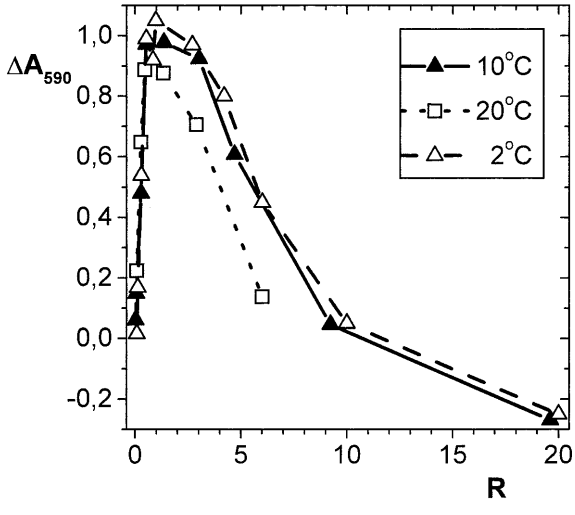

Fig. 1 Effect of gelatin/casein ratio (R) on the turbidity (ΔA_{590}) of a micellar casein solution (0.03% w/w) after addition of a solution of gelatin aggregates (0.5% w/w). $I = 0.001$ milk salts; pH 6.7

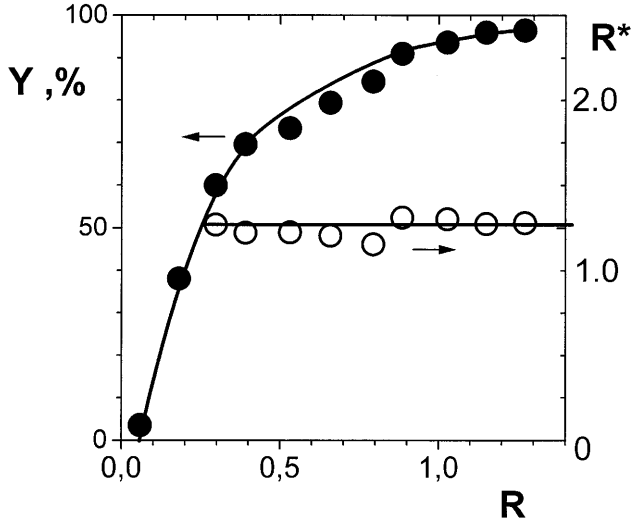

Fig. 2 Yield (Y) and composition (R^*) of the water-insoluble gelatin A–micellar casein complex obtained for different gelatin/micellar casein ratios (R) in the initial mixture. pH 7.0; concentration of micellar casein: 0.28%; 10 °C

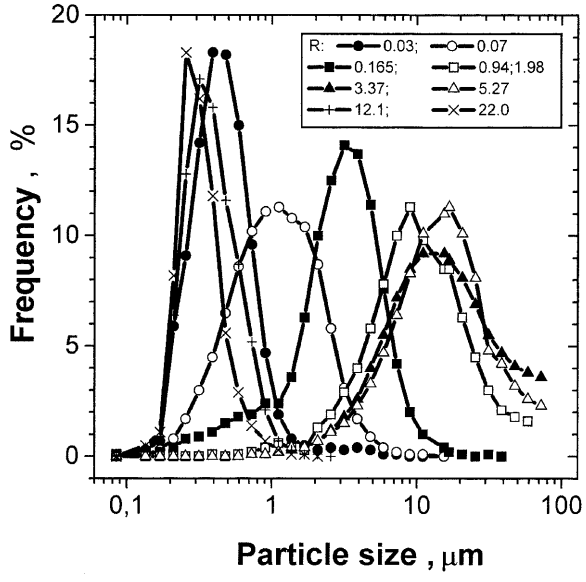

Fig. 3 Effect of gelatin/ casein ratio (R) in gelatin A–micellar casein mixtures on the size distribution of the colloidal particles. pH 6.7; $I = 0.001$ mol/l milk salts; 18 °C

In the case of systems containing flexible macroions such as gelatin and caseins in the molecular state, the composition of the concentrated phase is conditioned by the stoichiometry of the water-insoluble electroneutral complex and depends on the charge ratio of macromolecular components [24, 26–29]. For systems containing rigid particles such as globular proteins this rule does not apply [24, 29].

The dependence of the particle size of mixed aggregates upon the gelatin/casein ratio in the initial system is shown in Fig. 3. At small R values (0.03–0.165) relatively small mixed aggregates are formed and the average particle size in the system increases with R; the peak of the size distribution curve shifts from 0.3 to 3 μm. With R values between 0.94 and 5, the central particle size did not depend on R and was 12–14 μm. When R exceeds 10, the size of the particles drops suddenly to small values, of the order of 0.2–0.3 μm, smaller than the size of casein micelles, indicating that not only the mixed aggregates dissociate but to some extent the casein micellar structure also (Fig. 3). These results are coherent with those of the turbidity measurements reported in Fig. 1.

Thus, complexes of casein micelles and aggregates of acid gelatin seem to be polynuclear aggregated complexes, similar to most interpolymer complexes formed by mixing two polymers with opposite charges at a given pH [30]. According to the theory of Veis [31], the first stage is the formation of electroneutral complexes by electrostatic interactions between the oppositely charged polyions. At neutral pH, acid gelatin bears a positive net charge owing to the cationic amino acids: lysine, arginine and histidine. The pK_a value of histidine is low (6.5–7.0) and the amount of this amino acid is very small (0.011 mmol/g) [32]; therefore, the arginine (0.53 mmol/g, pK_a > 12) and lysine (0.30 mmol/g, pK_a = 10–10.4) groups of gelatin are mainly responsible

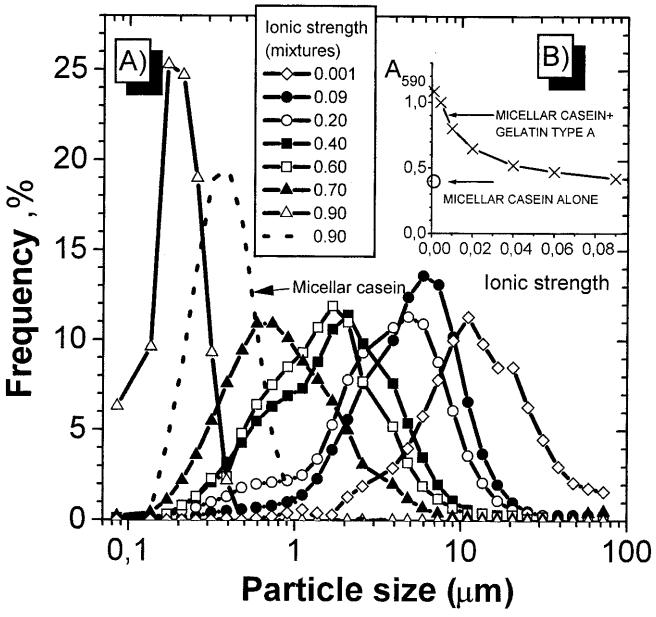

Fig. 4 Effect of ionic strength on the formation of gelatin A–micellar casein complexes and B effect of ionic strength (NaCl concentration) on the turbidity of the gelatin A–micellar casein system. pH 6.7; concentration of micellar casein: 0.28%; $R = 0.6$. **A**: $T = 18$ °C. *Dotted line*: micellar casein alone, $I = 0.9$ NaCl. **B**: *point 1*: turbidity of the initial solution of micellar casein alone

for the formation of ionic bonds with the negatively charged side groups of the casein micelles. These side groups are mainly glutamic acid residues (12–25%, $pK_a = 4.5$), since caseins are relatively poor in aspartic acid residues (2–4%) [33]. The second stage of complex formation consists of the aggregation of electroneutral complexes and the formation of a concentrated phase. The typical composition of the complex precipitate, obtained from the water–micellar casein (2.4%) aggregates of gelatin A (0.25%) at 10 °C and pH 6.7 contains 2.36% of caseins, 2.98% of gelatin and 1.276% of mineral salts.

The effect of NaCl on the aggregative behaviour in mixed micellar casein–gelatin A aggregates at $R = 0.6$ is shown in Fig. 4A and B. Introduction of a very minute salt concentration causes a considerable increase in turbidity, but then the turbidity decreases steadily as the ionic strength increases and at $I = 0.09$ it becomes similar to that of initial system, even if the ionic strength is increased only after a 24-h storage of the turbid system. This shows that the complexes are formed and stabilised via electrostatic interaction, rather than

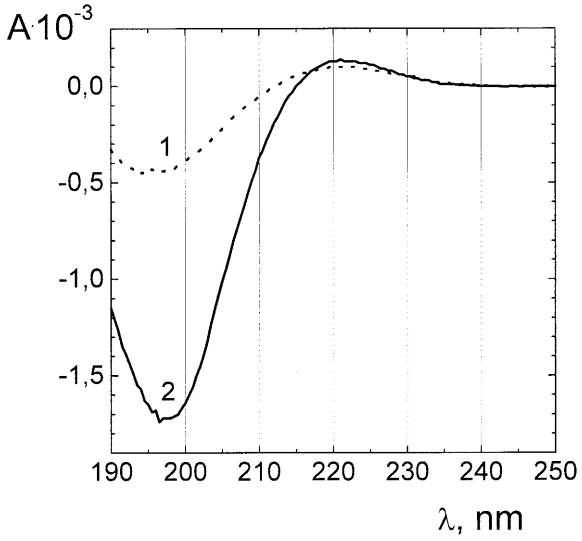

Fig. 5 Circular dichroism spectra of gelatin A solution (*curve 2*) and those of gelatin A solution in presence of micellar casein for $R = 20$ (*curve 1*), obtained at 10 °C. Concentration of gelatin: 0.02% (w/w); pH 6.7; cell length: 0.1 cm

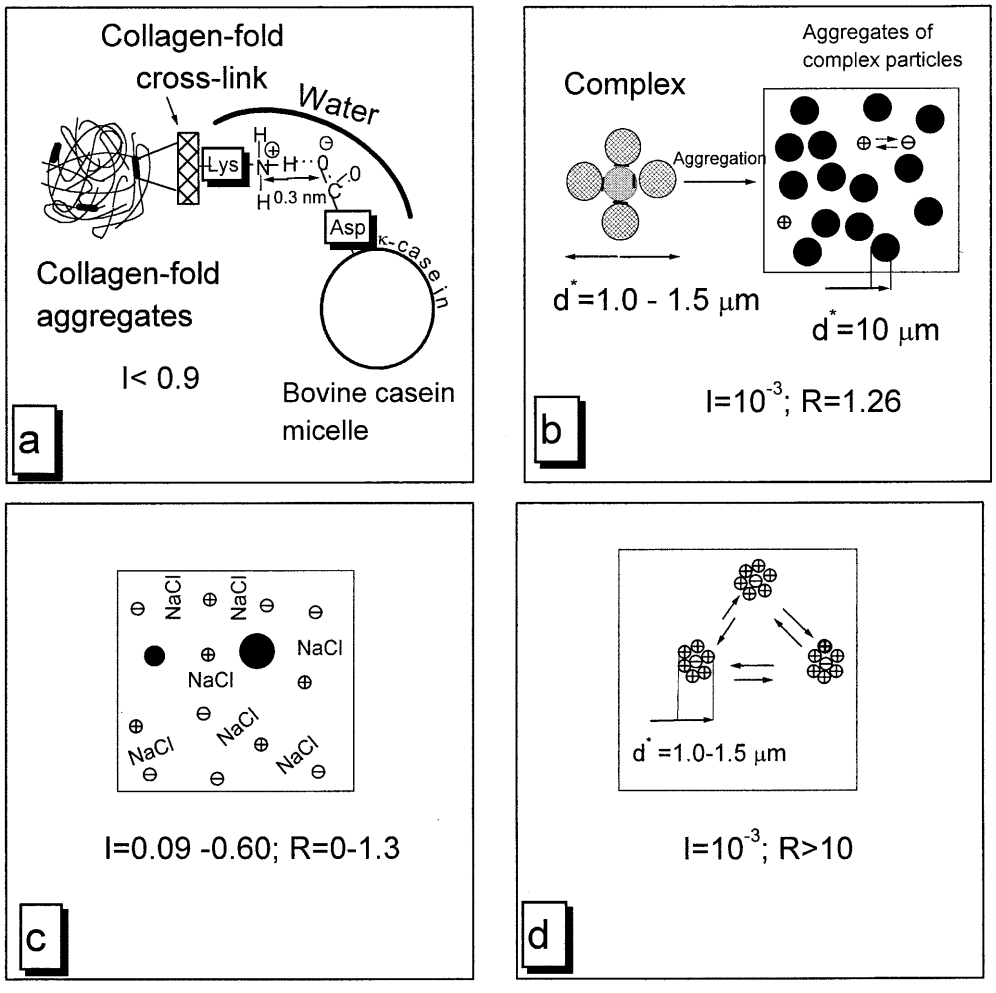

Fig. 6A–D Schematic illustration the effect of aggregates of acid gelatin on the aggregative behaviour of bovine casein micelles

through hydrogen-bond formation or hydrophobic interaction. The determination of the particle size distribution (Fig. 4A) confirms these results: at $I = 0.001$, mixed aggregates are formed with a size distribution centred around 10–20 μm; the size distribution is shifted progressively to smaller sizes as the ionic strength increases above this value; at $I = 0.9$, the average size drops to a value slightly below those of casein micelles and gelatin A aggregates, meaning that the initial mixed aggregates are dissociated.

When R was very high (above 10), we observed at $I = 0.001$ that the turbidity of the mixed system was lower than that of the micellar casein dispersion (Fig. 1) and that the corresponding size distribution curves are shifted to sizes below that of pure micellar casein (Fig. 4A).

By analogy with the formation of electrostatic complexes of casein and polysaccharides [30] we assumed that in the presence of an excess of gelatin molecules in the initial mixture (i.e. an excess of positively charged groups) a charged gelatin–casein complex forms but that electrostatic repulsion between similarly charged groups causes some dissociation of casein micelles and, as a consequence, soluble complexes are obtained. This surmise finds some support in the results of circular dichroism measurements on gelatin A solution and its mixture with micellar casein: at 10 °C, $R = 20$ and pH 6.8, after the subtraction of the spectrum of micellar casein obtained of the same conditions, the absolute amplitude of the negative dichroic peak at around 198 nm of gelatin A aggregates is considerably reduced in the presence of micellar casein (Fig. 5), indicating the existence of interactions between the two macromolecular components. The presence of a very small amounts of micellar casein in the solution of gelatin A aggregates decreases the optical rotation of gelatin solution. It is an indication of some interaction between the two macromolecular species in this system. All the results obtained allow a schematic representation of the process of interaction of casein micelles with the aggregates of gelatin A (Fig. 6A, B, C, D), including electrostatic interaction between positively charged lisine side groups of gelatine aggregates and negatively charged aspartic acid residues of casein micelles (Fig. 6A), aggregation of electroneutral complexes (Fig. 6B), dissociation of the complexes at high ionic strength (Fig. 6C) and the formation of soluble complexes in the presence of an excess of gelatin molecules in the initial mixture (Fig. 6D).

The effect of aggregates of gelatin A on the behaviour of casein micelles during acidification at very low ionic strength

Figure 7A, B and C presents the results of the turbidimetric titration by GL of the aqueous micellar casein-acid gelatin aggregates at $I = 0.001$ in the temperature range from 3 to 20 ° for different R values. At R values less than the equilibrium ratio ($R = 1.26$), acidification increases the turbidity of the mixed systems at pH 5.5 ± 0.1 (Fig. 7). This increase becomes less significant as R increases, and at R values close to the equilibrium ratio the turbidity does not change significantly down to pH 5.2–4.8, but drops markedly when the pH becomes more acidic (Fig. 7). This behaviour can be ascribed to the formation of the weakly aggregated electroneutral complexes, the aggregation of which is not as sensitive to pH as that of free casein micelles. The size distribution of the complexes does not change significantly in the pH range from 6.7 to 4.8 (Fig. 8). In other words, the complex particles obtained at an equilibrium ratio of R do not aggregate during acidification. At pH < 4.8, the turbidity of the mixture decreased (Fig. 7), probably as a consequence of the dissociation of the complexes owing to the charge reversal of casein. At R values (10 or greater) much larger than the equilibrium ratio, the decrease in pH had very little effect on the turbidity of the mixture. Similar

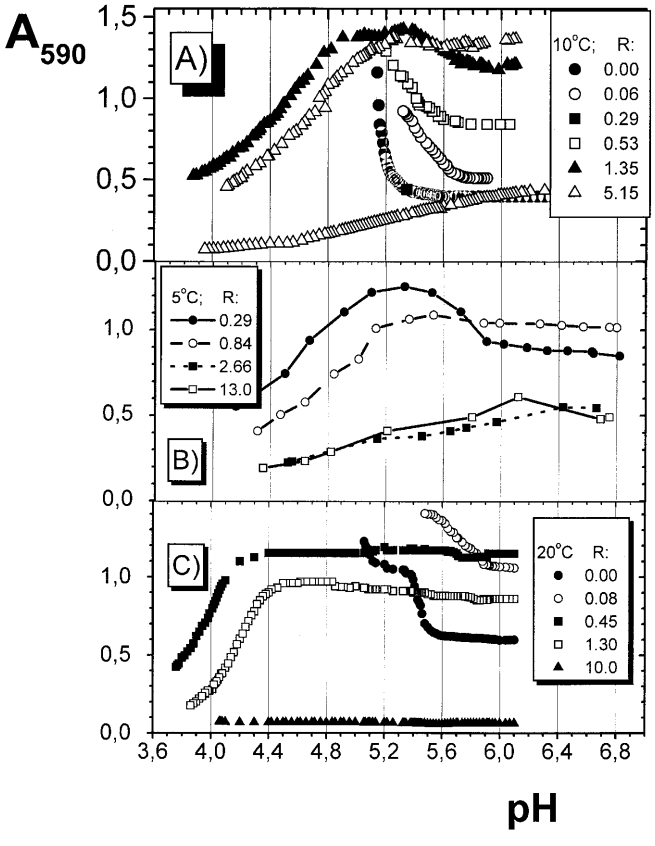

Fig. 7A–C Variation of the turbidity of gelatin A–micellar casein systems with pH at different values of the gelatin/casein ratio (R) in the initial mixture. $I = 0.001$ salts. $T = 10$ °C (**A**); 5 °C (**B**); 20 °C (**C**)

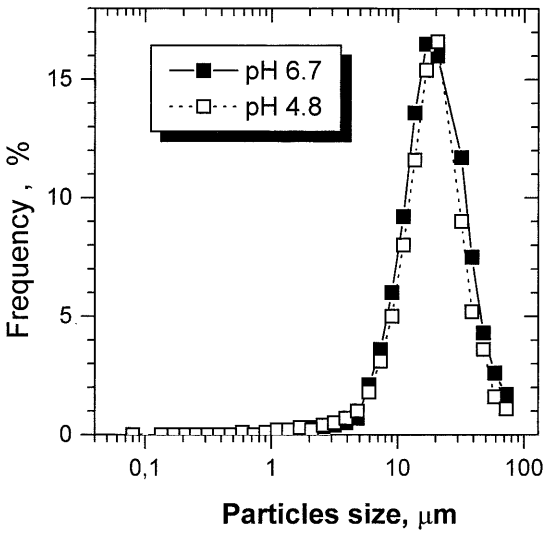

Fig. 8 Particle size distribution of colloidal particles in the micellar casein–aggregates of gelatin A mixture before and after acidification. Gelatin/casein ratio $R = 1.0$; $I = 0.001$ mol/l NaCl; 18 °C

behaviour was observed long ago for solutions of polysaccharide and globulin or polysaccharide [30] and was explained by the formation of charged complexes which do not aggregate readily.

The effect of alkaline gelatin B on the behaviour of casein micelles

Turbidic titration of the micellar casein solution (0.09% w/w) in water by a 0.5% solution of gelatin B aggregates did not show an appreciable change of turbidity with R, for R up to 2.7. The particle size distribution in micellar casein–alkaline gelatin aggregates at 1% micellar casein concentration (10 times the concentration used in the turbidimetric titration) did not show any significant change for R values from 0.02 to 0.63 (data not presented). During acidification of the micellar casein–alkaline gelatin B aggregates by GL, no effect of the presence of alkaline gelatin on the stability against aggregation of micellar casein was observed in the range $0.03 < R < 2.67$ (data not presented). Such behaviour of alkaline gelatin aggregates–micellar casein indicates the absence of specific polymer interactions.

Conclusion

At neutral pH and low ionic strength casein micelles interact with acidic gelatin aggregates by creation of ionic bonds between carboxyl groups of casein and oppositely charged amino groups of gelatin. The complexes formed are polynuclear aggregated electroneutral complexes. Aggregation of such complexes is the result of bridging flocculation. The dispersity of the complex aggregates of acidic gelatin and casein micelles can be predicted on the basis of the knowledge of the sizes of the interacting aggregates. In the presence of an excess of positively charged gelatin molecules in the initial mixture a charged gelatin–casein complex forms but electrostatic repulsion between similarly charged groups causes some dissociation of casein micelles and, as a consequence, soluble complexes are obtained.

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