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SHORT COMMUNICATION

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The effect of the melting of the collagen-like gelatin aggregates on the stability against aggregation of the bovine casein micelles

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Abstract The effect of the melting of the collagen-like acid and alkaline gelatin aggregates on the stability against aggregation of bovine casein micelles was investigated by turbidimetry, DSC and circular dichroism in the wide range of biopolymers concentrations, gelatin/casein ratio (R) in initial mixture ($R = 0.03$–20), pH (4.9–6.7), ionic strength ($I = 10^{-3}$ to 1.0/NaCl/), and temperature (10°–70 °C), using glucono-δ-lactone (GL) as acidifier. At low ionic strength (10^{-3}/milk salts/) and neutral pH interaction between gelatin molecules and casein micelles is suppressed significantly above 36 °C. The melting of the collagen-like acidic gelatin above this temperature shifts the pH at which the complex formation is maximal to the acidic range. The cause may be that some of the functional ionized groups of gelatin molecules are inaccessible due to the conformational changes. The presence of gelatin B molecules had no effect on the aggregation stability of micellar casein in the range $0.03 < R < 20$. At very high total protein concentration (above 10%) depletion flocculation of casein micelles was observed. The reason for the very high stability of casein micelles in this case cannot be explained by volume exclusion.

Key words Bovine casein micelles · Gelatin · Interactions · Depletion flocculation

Introduction

Phase separation and flocculation in colloidal dispersions, induced by the addition of nonadsorbing or adsorbing polymers, are phenomena of fundamental interest and considerable technological importance [1]. This is also true for aqueous protein colloidal dispersions [2, 3]. However, whereas the effect of the presence of polymer on the stability of nonprotein and protein colloids was the subject of attention of many researchers (see, for example, [1]), little work has considered aggregative stability of protein colloids in the presence of other biopolymers. Even less work has been focused on aqueous protein (1)-protein (2) systems, with one of the macromolecule in the colloidal-dispersed state. We refer to the study by Polyakov et al. [4] which showed thermodynamic compatibility of native proteins (ovalbumin and bovine serum albumin) with soluble thermotropic ovalbumin aggregates produced by heat treatment of the protein solution. Using ovalbumin as an example, it was shown that compatibility of bovine serum albumin with ovalbumin sharply decreases when ovalbumin molecules were replaced by ovalbumin thermoaggregates.

In the previous study [5], we investigated the interaction of acidic and alkaline gelatin aggregates with micellar casein at neutral pH and after acidification and established formation (at low ionic strength) of mixed (acidic gelatin aggregates-casein micelles) particles and aggregation by bridging flocculation.

The aim of the present work is to provide a better insight into the relationship between the molecular structure of gelatins and its influence on the stability against aggregation of casein micelles. We studied the

effect of melting of the collagen-like acidic and alkaline gelatin aggregates on the stability against aggregation of the bovine casein micelles at neutral pH and after acidification by glucono-δ-lactone (GL).

Material and methods

Materials

The major characteristics of the studied objects and methods of preparation of their solutions were described in a preceding paper [5].

Turbidimetric titration of micellar casein dispersions and micellar casein-gelatin systems were performed as described previously [5].

Interprotein interactions

Two methods were used to follow interprotein interactions: measurement of the circular dichroism spectra and calorimetric measurements.

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 at wavelengths 190–250 nm as described in a preceding paper [5].

Calorimetric measurements were carried out with a highly sensitive differential scanning microcalorimeter (Microcal, USA) within 5–60 °C at a heating rate of 1 °C/min and under 2 atm excess pressure. Data processing was carried out using Origin software (Microcal Software, Inc.). The thermal properties of the micellar casein-gelatin complexes were compared to that of gelatin alone in the following way. Water insoluble micellar casein-gelatin complexes were produced as described above. Complex gel-like precipitates were solubilized at 50 °C over 1 h and were diluted so that no separation was observed in the stirred medium after cooling to 4 °C. One part of the solution of casein-gelatin complexes was stored overnight at 4 °C before the DSC run.

To the other part, solid NaCl was added to 0.5 mol/l concentration, and the pH was brought to the IEP of casein (pH 4.6) by addition of 5 mol/l HCl; it was then centrifuged under 1200 g for 20 min at 5 °C. The casein precipitate was washed twice with deionized water, centrifuged for 20 min at 5 °C under 3900 g and dissolved at room temperature in water at pH 6.8 over 12 h. The thermogram of this solution was subtracted from that of diluted gelatin-casein complex.

Results and discussion

The results of turbidimetric titration of micellar casein alone by GL at different temperatures are summarized in Fig. 1. Changing the temperature from 10 °C to 70 °C increases the stability noticeably, and the clotting point is shifted from pH ~5.5 to pH ~5.2.

As shown in our previous study [5] the addition of even a small amount of gelatin A aggregates to micellar casein solutions at neutral pH causes large changes in turbidity due to the complex formation and bridging flocculation. The effect of melting of collagen-like gelatin aggregates on the behavior of casein micelles is

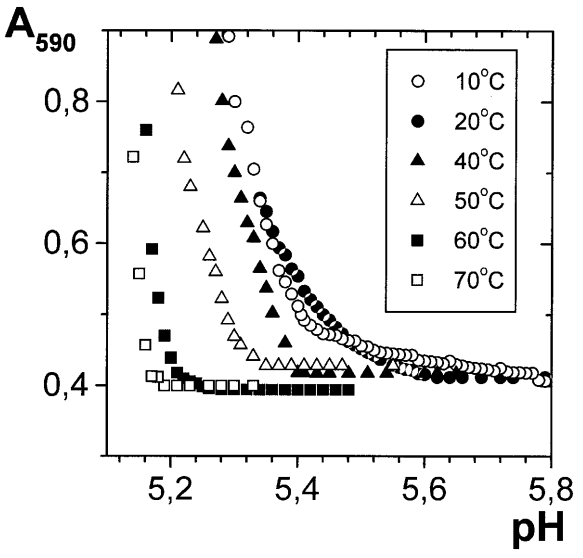

Fig. 1 Dependence of the turbidity of a 0.06% micellar casein solution at different temperatures on pH. I = 0.09 (phosphate buffer). Acid titration by δ-gluconolactone

presented in Fig. 2. When the temperature increases from 10 °C to 40 °C, the turbidity of the mixture decreases sharply to a value close to the initial turbidity of micellar casein solution; the change occurs within a narrow temperature range, essentially between 27 °C and 47 °C (inflection of the curve at ~36 °C, Fig. 2). The gelatin/casein ratio of mixture at 40 °C has no significant effect on the turbidity curve (Fig. 3). It is important to note that a the decrease of the temperature

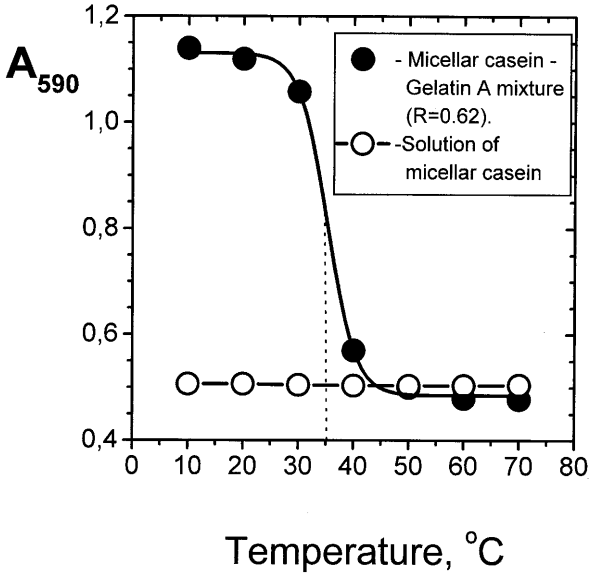

Fig. 2 Variation of the turbidity of the micellar casein-gelatin A mixture with temperature during heating. R = 0.62

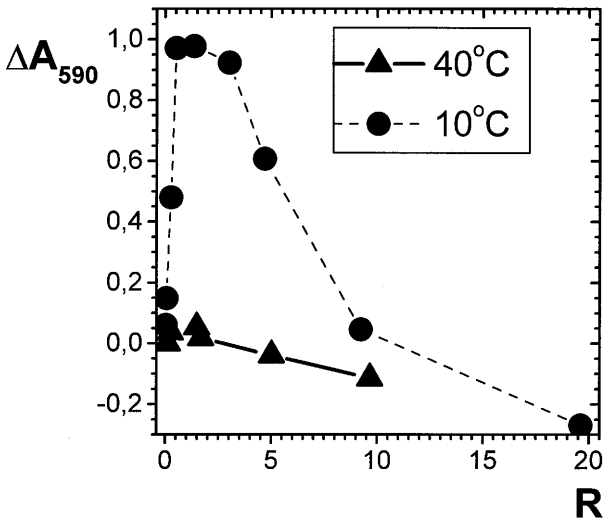

Fig. 3 Effect of gelatin/casein ratio R on turbidity of a micellar casein solution (0.03 wt%) after addition of a gelatin solution (0.5 wt%) at 40 °C. I = 0.001 (milk salts); pH = 6.7. The dependence at 10 °C is given for comparison

from 60 °C to 10 °C reaches the initial value of the turbidity.

The results of the circular dichroism study of the micellar casein-gelatin A in water at 50 °C, pH = 6.7, and R = 20 showed little difference in optical rotation compared to gelatin alone (Fig. 4). The temperature and the specific enthalpy of denaturation of gelatin were not significantly modified by micellar casein at I = 0.001 (Fig. 5A), and remained about the same for the

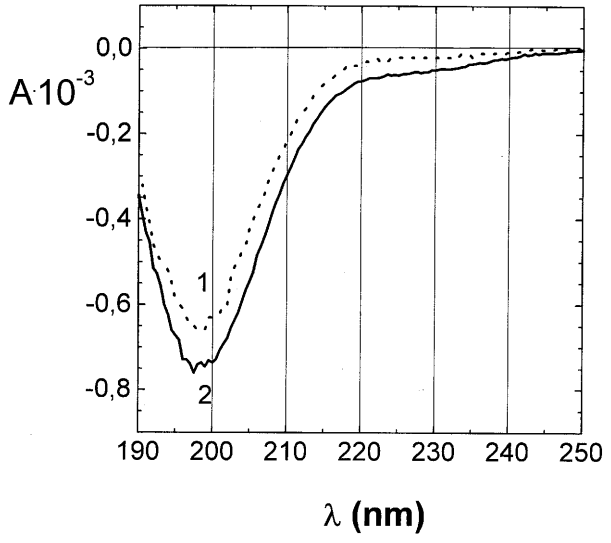

Fig. 4 Circular dichroism spectra of gelatin A solution (*curve 2*) and those of gelatin A solution in presence of micellar casein for R = 20 (*curve 1*), obtained at 50 °C. Concentration of gelatin = 0.02 wt%; pH = 6.7; cell length = 0.1 cm

precipitated complex obtained from the micellar casein (0.03%)-gelatin A (0.6%) at 10 °C (Fig. 5B). The only significant difference is a sharp decrease of the heat capacity of the mixture and complex at 32 °C as compared to gelatin (see also Fig. 2).

These results show that the interaction between gelatin molecules and casein micelles is suppressed above 36 °C, as a consequence of the dissociation of ionic bonds.

The stability against aggregation of micellar casein-gelatin A during acidification at 50 °C and 60 °C is shown in Fig. 6A, B. At low R values (0.17–0.4) a difference to the micellar casein alone (see Fig. 2) was not observed. At higher R values (0.7–6) the stability against aggregation of the mixture differed from that at low temperature (10 °C). At R = 0.7 the maximum in turbidity occurred at pH ≅ 5.6. This maximum decreased in intensity and moved in the direction to pH ~7.0 when R increased. At R values much higher than the equilibrium ratio (R > 10), the turbidity of the mixtures did not change during acidification. Thus, the gelatin molecules in solution interact with micellar

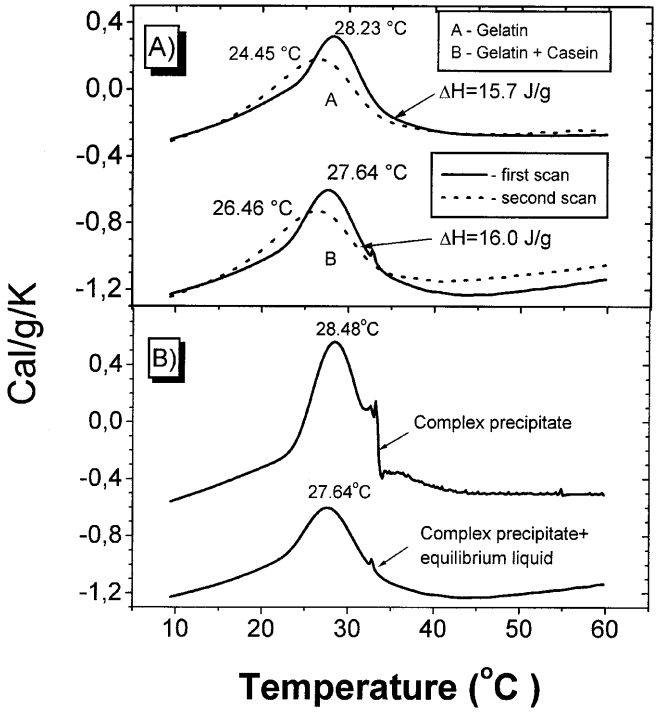

Fig. 5 **A** Thermograms of the gelatin A solution and the micellar casein (0.03%) – gelatin A (0.6%) system after subtraction of the thermogram of micellar casein solution obtained under the same conditions of concentration, pH and ionic strength. **B** Thermograms of the micellar casein (0.03%) – gelatin A (0.6%) system and of the complex precipitate after the subtraction of the thermogram of the micellar casein solution obtained in the same conditions of concentration, pH and ionic strength. pH = 6.7; I = 0.001 trace of milk salts

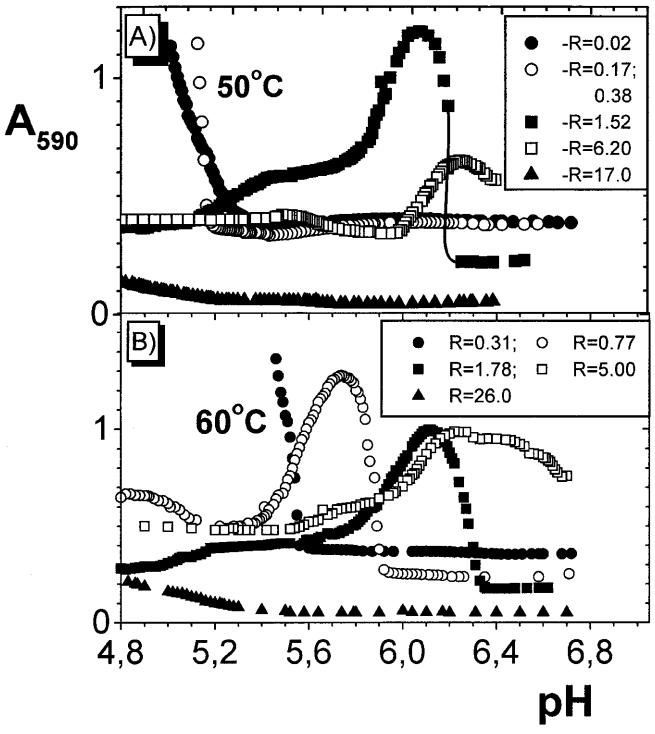

Fig. 6A, B Variation of the turbidity of skimmed milk-gelatin A with pH at different values of the gelatin/casein ratio (R) in the initial mixture. I = 0.001 mol/l milk salts: **A** 50 °C; **B** 60 °C

casein like gelatin aggregates at low temperature, but in the first case the interaction occurs only in the acidic region.

At ≥40 °C some of the functional ionizable groups of gelatin molecules cannot interact with negative charged micelles of casein due to the coil conformation, i.e. charge density of gelatin molecules should be less than that of the collagen-like structure of gelatin aggregates. The complex obtained is electroneutral. Thus, we can suppose that the shift of the absorption peak at moderately high R (0.7–6) in the acid region shows the tendency of system to neutralize the excess charge of casein micelles. An increase of R stimulates the increase of the total amount of positive charged macroions in the mixture and it should change the pH at which the complex formation is maximal in the direction of decreasing of total charge of gelatin, i.e., to the neutral region of pH.

The effect of alkaline gelatin on the behavior of casein micelles

Turbidimetric titration of a micellar casein solution (0.09 wt%) in water by 10 wt% molecular-dispersed solution of alkaline gelatin did not show an appreciable change of turbidity with R, when R = 0.03–20 (at 40–60 °C). During system acidification of the micellar casein-alkaline gelatin by GL, no effect of the presence of alkaline gelatin on the stability against aggregation of micellar casein was observed for $0.03 < R < 2.67$ (data are not presented). Finally, the turbidimetric titration of micellar casein solution by concentrated albumin solutions (bovine serum albumin and ovalbumin) as well as the behavior of micellar casein-globular protein systems during acidification at R = 2.8 were similar to those of micellar casein alone.

To find the effect of alkaline gelatin on the aggregative stability of casein micelles, we prepared (at 40 °C and I = 1.0/NaCl/) a series of highly concentrated mixed solutions of skimmed milk and alkaline gelatin at concentrations of casein of 6.6%, 4.8%, 3.2%, and 2.5%, and at different gelatin concentrations. These mixtures were centrifuged at low acceleration (470 g, 20 min at 40 °C) to exclude a possible precipitation of micellar casein from skimmed milk. Stability against aggregation of micellar casein in the presence of alkaline gelatin was determined by the phase volume ratio method [6]. The results are presented in Fig. 7; they show a very high stability of casein against aggregation. The critical concentration of flocculation for skimmed milk containing 6.6% of micellar casein is about 3%. This value is much higher than polysaccharide concentrations (0.01–0.1%) required in the case of skimmed milk-polysaccharide [7–9] but not so far from the critical concentration for phase separation of other aqueous protein 1-protein 2 systems containing protein aggregates [4]. Flocculation is weak and reversible: dilution of the micellar casein enriched phase below the initial concentration in milk led to rapid redispersion although mild agitation was required to accomplish this. Since the

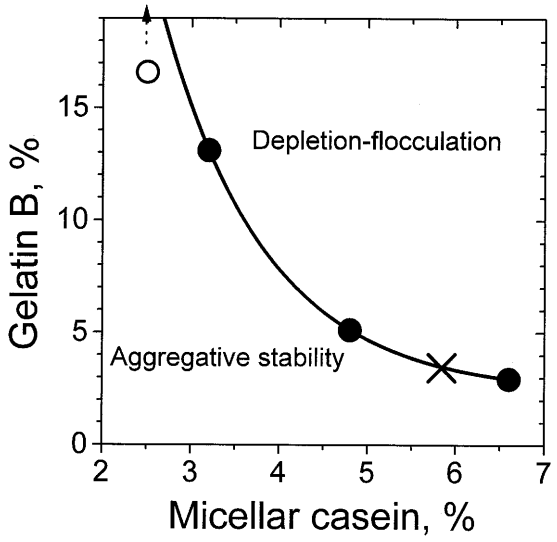

Fig. 7 Phase diagram of the skimmed milk-gelatin B mixtures at pH = 6.7; I = 1.0 NaCl, and 20 °C. × = threshold point

aggregation of casein micelles is reversible it corresponds most probably to a depletion flocculation mechanism [1].

The reason for the very high stability against aggregation of casein micelles in a presence of alkaline gelatin is not quite clear. The most general theory of depletion flocculation was developed for systems containing colloidal particles and free polymer. The polymer molecules compete with the colloidal particles for the available solvent, i.e., the flocculation process is primarily due to excluded volume effects. According to this view, protein colloids are sterically excluded from regions of the solvent occupied by the inert polymer and are concentrated until their solubility is exceeded and flocculation occurs [10].

An attempt to assess quantitatively the excluded volume concept [11] revealed (except the case of low concentration of free polymer) that the exclusion of protein from the solution cannot be explained simply by volume exclusion. At higher concentrations, the amount of volume excluded per molecule is expected to diminish because of the overlap between the covolume radii [12]. Neglecting this effect leads to unrealistically large interaction coefficients. However, it is not clear how such effects can be incorporated into analysis without higher virial terms.

Polyakov et al. [13] reported on the phase equilibrium in water-protein 1-protein 2 systems, including partially colloidal-dispersed and molecular-dispersed proteins, and the interaction of the protein with the solvent and with each other. They concluded that the greater the difference in the intensities of interaction between the proteins and the solvent, and the greater the positive contribution to the interaction between proteins of different types to dilution enthalpies, the higher is the probability of the system to be two-phasic. In our opinion this approach is more relevant for the characterization of specific affinity of polymers with each other than for the determination their phase state. For example, *cis*-polybutadiene-*cis*-polyisoprene mixtures are two-phase systems although these polymers have practically the same polymer-solvent affinity [14].

In this connection it is of interest to pay attention to the ideas of Dobry and Boyer-Kawenoki [15] concerning the mechanism of phase separation of polymers. They supposed that macromolecules in solution are surrounded by solvent molecules which form a solvation layer with a definite structure which is the template for the structure of polymer molecules. When similar macromolecules come close to each other, the regular solvation layers do not disturb each other but reconstruct in such a way as to form molecular associates with similar structure, i.e., a topotactic relation must exist between the two species. This would be an analogy between the formation of mixed crystalline structures in mixtures of crystalline polymers and the aggregation of protein macromolecules.

Conclusion

Stability against aggregation of the bovine casein micelles in the presence of melting aggregates of acidic and alkaline gelatins was investigated over a wide range of compositions, pH, and ionic strengths. Melting of collagen-like acidic gelatin results in the shift of pH optimal for complex formation to the acidic range due to the inaccessibility of some positively charged amino groups. The experiments show a very high stability against aggregation of casein micelles in the presence of alkaline gelatin which cannot be explained by volume exclusion effects.

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