On the One-Phase State of Aqueous Protein-Uncharged Polymer Systems: Casein-Guar Gum System

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ABSTRACT: Although the majority of biopolymers are incompatible in water, systems containing casein molecules and a neutral polysaccharide (guar gum galactomannan) showed phase separation only at an ionic strength above 0.09–0.2. Static light scattering, circular dichroism spectroscopy, velocity sedimentation, viscosimetry, phase analysis in different solvents, and Rosenberg's method were used to estimate the effect of polymer–solvent and polymer–polymer interactions on the phase state of casein-guar aqueous systems. Different solvent conditions were used to try to clarify the nature (electrostatic or nonelectrostatic) of the interaction between the two macromolecular species. Data obtained show that the dominant mechanism controlling the single-phase state at low ionic strength (below 0.01) involves the formation of water-soluble weak interpolymer complexes, which may be destroyed by increasing ionic strength. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 71: 471–482, 1999

Key words: caseins; guar gum; interactions; compatibility

INTRODUCTION

The phenomenon of polymer incompatibility in blends and in organic solvents is a consequence of the generally unfavorable interactions between polymer species. Even small positive values of the Flory–Huggins parameter of interaction between different polymer species can result in a phase separation, due to the small entropy gain upon mixing these macromolecules.[1–3] In fact, most of the systems studied phase separated, with a comparatively small number of exceptions. Phase separation was observed even for such similar polymers as *cis* and *trans* isomers of polyisoprene.[4] Therefore, even minute differences in the structure of macromolecules result in their phase separation when they are mixed.

Similar behaviors are observed in aqueous mixtures of polymers or biological macromolecules and have been extensively described experimentally,[5–7] but are less well understood.[5] However, aqueous systems in which one of the macrocomponents, or both of them, are charged have been comparatively less investigated.[8] In such situations, it is known that the general tendency to demix is greatly decreased.[8,9]

The behavior of blends of neutral and polyelectrolyte chains in a solvent has been studied theoretically by Khokhlov and coworkers.[9,10] They studied the dependence of free energy on concentration fluctuations, systematically accounting for the translational entropy of the polymers and counterions, the interfacial tension, and the electrostatic interactions between the charged spe-

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cies. Their study showed that increasing the charge of the polyelectrolyte:

1. stabilizes the mixed phase, moving the spinodal point to lower temperatures, and
2. changes the character of the transition from macro- to microphase separation, the characteristic length of the microphase separation being dependent on the polyion charge and the amount of added salt.

The former effect is due to counterion entropy, whereas the latter results from the new length scale introduced by the electrostatic interactions, the Debye screening length. Similar effects have been noted by Joanny and Leibler[11] in a theoretical study of weak segregation of polyelectrolytes in poor solvent.

The thermodynamic behavior of aqueous ternary biopolymer systems, containing one or two polyelectrolytes (or polyions), is less clearly analyzable, because biopolymers have more complicated structures than synthetic polymers. Globular proteins constitute a special case among biopolymers, in that they show a compact corpuscular conformation (a few nanometers in diameter) instead of a polymer-like one. Liquid–liquid and solid–liquid phase separation processes in mixtures of colloidal particles with polymers, which have been extensively studied experimentally,[12–16] are explained on the basis of volume exclusion osmotic effects (polymer depletion between particles) and have been thoroughly analyzed theoretically in the case of colloidal particles larger than the polymer size.[16–18] The reverse situation (i.e., particles smaller than polymer), which is that of globular protein-polysaccharide (PS) mixtures, is less clear because the theoretical study predicts no phase separation,[19] although liquid–liquid phase separation is observed in most globular protein-water soluble polymer systems.[20] Here, again, incompatibility is the rule, but shows itself at relatively high concentrations of both components. Some proteins, of prime importance as regards industrial applications, are not of the globular type. Gelatin exists in solution as polymer-like polypeptidic chains at temperatures high enough ($> \sim 333$ K). When temperature decreases, aggregates appear through the formation of helix segments involving two or three chains (at high enough protein concentration and low enough temperature, this process leads to the formation of gelatin gel). In milk, the molecules of α-, β-, and κ-caseins are associated in spherical particles with a complex structure, $\sim$ 200 nm in size (the casein micelles). The structure of casein micelles can be more or less dissociated when solvent conditions are changed; it is completely destroyed when caseinate is prepared. However, α- and β-casein molecules form aggregates in aqueous solution, with the importance of the aggregation process depending on protein concentration, temperature, and solvent conditions. "Solutions" of gelatin or caseinate are therefore complex systems and phase behavior of their mixtures with PSs could result from different types of processes. Nevertheless, it was found that the conditions of incompatibility depend on the ionogenic properties of the PSs, but not on the state (molecular or colloidal dispersed states) of the protein molecules.[21]

Thermodynamic incompatibility between PSs and proteins shows itself only when the possibility of intermacromolecular complexes formation is excluded.[22–24] Although the majority of biopolymer mixtures show phase separation, those containing two linear anionic PSs remain single phase. The other systems, including mixtures of both charged and neutral PSs, as well as linear anionic PS-gelatin systems, were two-phase only at an ionic strength $>0.09-0.2$.[25,26] In this connection, attention should be paid to the role of weak intermacromolecular interactions on the phase state of aqueous protein–neutral PS mixtures.

The possibility of complex formation in such systems has been discussed by Woodside and colleagues[26,27] and by Grinberg and Tolstoguzov.[28] These authors analyzed the thermodynamic behavior of gelatin-D-glucan mixtures. The existence of gelatin-PS complexes was inferred from the considerable solubility of D-glucans in acidic ethanol in the presence of gelatin[26,27] and from nephelometric and viscosimetric data.[28]

Assuming that the question of weak interactions between different macromolecular species is important for understanding the phenomena of the incompatibility of biopolymers, and taking into account the lack of experimental data in this area, the present study deals with the relationship between the phase state of protein–neutral PS mixtures and the interactions between the two types of macromolecules. The protein was sodium caseinate or β-casein, and the PS was guar gum—a typical neutral PS of linear structure, having 1–4 linked β-D-mannopyranosyl backbone with single β-D-galactopyranosyl side groups randomly distributed along the chain.[29] The ratio of

galactose to mannose is $\sim 1:2$.[30] The aim was to establish or to exclude formation of protein–PS complexes in the single-phase region of the phase diagram. Different solvent conditions were used to try to clarify the nature (electrostatic or non-electrostatic) of the interaction between the two macromolecular species.

EXPERIMENTAL

Materials

Sodium caseinate (95.62% protein; 0.76% fat; 3.78% ash; 580 mg calcium per kg) was provided by Kerry Ingredients (Ireland). The β-casein-enriched fraction (97% protein; 3.0% ash) was prepared by the National Dairy Products Research Centre (Moorepark, Ireland).

Guar gum sample was MEYPRO purified guar from Meyhall Chemical AG (Switzerland). Its intrinsic viscosity in water at 293 K was 1,130 ml g^{-1}. Polyethyleneglycol (PEG-20000), from Fluka Chemie AG (Switzerland), $M_r > 17{,}000$, was used without additional purification.

Urea, purity > 99.9%, was from BDH Chemicals Ltd. (UK).

Sample Preparation: Phase Diagram Determination

Stock solutions of caseinate or β-casein, guar gum, and PEG were prepared at room temperature at fixed values of NaCl concentration and pH. The solutions of casein and guar were then centrifuged for 60 min under 100,000g at 303 K to remove undissolved particles and air bubbles and dialyzed against solvent for 24 h at 277 K.

Phase diagrams of the ternary systems were constructed at fixed values of pH, concentration of NaCl and temperature as described elsewhere,[31] and illustrated schematically in Figure 1. In these Cartesian coordinate diagrams, the horizontal axis corresponds to casein concentrations, and the vertical one corresponds to guar concentrations. The procedure is adapted from Koningsveld and Staverman[32,33] and Polyakov and colleagues.[34,35] The separately prepared protein and PS solutions (concentrations C_2° and C_3°, respectively) were mixed at 20°C in various weight ratios, yielding mixtures with protein and PS concentrations C_2 and C_3 (wt %), respectively, which are represented on the phase diagram by points lining on the secant $\{C_2^\circ, C_3^\circ\}$. The phase state of the systems was determined by eye after centrifugation (50,000g for 1 h at 20°C) following a rest period of 1 h at 20°C. When phase separation occurred, the volumes of the two coexisting liquid phases were determined by weighing, assuming the density of the phases to be equal to 1.0. The concentrations of the protein in the two phases were determined from the optical density at 280 nm after suitable dilutions in glycin buffer at pH 10 (condition chosen to avoid turbidity), and those of the PS were calculated from dry weight determinations (104°C overnight) and material balance. The points where the binodal intersects the secant $\{C_2^\circ, C_3^\circ\}$ are given by extrapolation of the relation $r = V'/(V' + V'') = f(C_2/C_2^\circ)$ to $r = 0$ and to $r = 1$; V' and V'' represent volumes of the protein-enriched (lower phase) and the PS-enriched (upper phase) phases, respectively. The value $r = 0.5$ gives the position of the middle of the tie lines. By repeating the procedure for a series of values of C_2° and C_3°, the phase diagram can be reconstructed from the set of points corresponding to the binodal and centers of the tie lines.

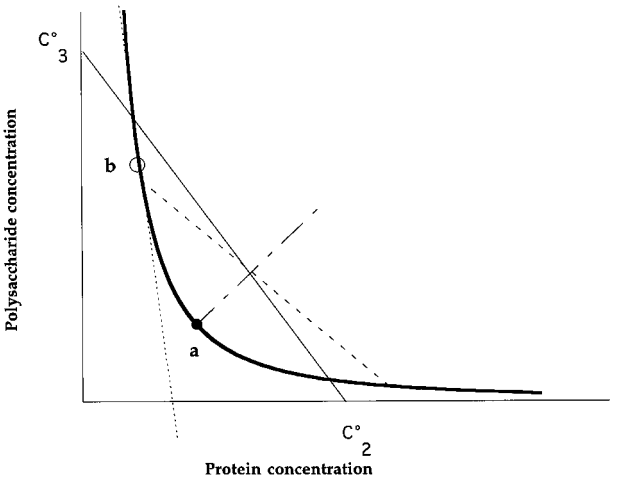

Figure 1 Schematic figure illustrating the determination of the phase diagrams (see text). (——) Binodal. (——) Secant. (-----) Tie line. (– – –) Rectilinear diameter. (· · · · ·) Tangent to the binodal with slope −1. (a) Critical point. (b) Phase separation threshold.

The degree of compatibility of caseinate with guar gum was characterized by the coordinates of the phase separation threshold and critical point. The separation threshold was determined on the plot as the point where the line with the slope −1 is tangent to the binodal (dotted line in Fig. 1). The critical point of the system was defined as the point where the binodal intersects the rectilinear diameter, which is the line joining the center of the tie lines (Fig. 1).

The precision in the determination of the binodal is in the range of 5–9%, depending on the concentration levels.

Light Scattering Experiments

The refractive index increments for β-casein and guar gum were determined using a Shimadzu differential refractometer. We obtained the following results (pH 6.8, $I = 0.002$/NaCl, $\lambda = 436$ nm): $\nu_2 = 0.191\ \mathrm{cm^{-3}\,g^{-1}}$ and $\nu_3 = 0.163\ \mathrm{cm^{-3}\,g^{-1}}$ (indices 2 and 3 refer to casein and guar, respectively).

The light scattering experiments were conducted at 293 K on the individual solutions of β-casein and guar gum, and on the single-phase ternary systems using a nephelometer FPS-3M (Unique Design Bureau of Scientific Instruments, Academy of Science, Russia). The wavelength of nonpolarized light was $\lambda = 436$ nm. Dust-free benzene was used as the calibration standard; its Rayleigh ratio was taken as: $R_{90} = 47.4 \cdot 10^{-6}$ $\mathrm{cm^{-1}}$ (ref. 36). The intensity of scattered light was corrected using the refractive index ratio $n_{\mathrm{water}}/n_{\mathrm{benzene}} = 0.785$ and the variation in the light scattering volume. The exit wall of the sample cells had traps to minimize the effect of reflections. The intensity of scattered light was calculated for every scattering angle as the difference in intensities of light scattered by the solution and the pure solvent.

All solvents and solutions were filtered through PVH membranes (Selectron, Germany; pore size: 0.45 μm) into the cell.

In the case of β-casein solutions, there is no angular dependence of the scattered intensity. The excess light scattering ΔR_{90} of the protein solutions at 90° scattering angle was measured and $KC_2/\Delta R_{90}$ ($K = 2\pi^2 n_1^2/N_A\lambda^4$; n_1 is the refractive index of solvent, N_A is Avogadro's number, and λ is the wavelength of incident light in vacuum) was plotted against the concentration C_2 of the protein. The molecular weight M_2 and the second virial coefficient A_{12} of the protein in the solvent were obtained in the usual way from the extrapolation to zero concentration and from the slope, respectively, of the linear plot. For the pure guar gum solutions, the scattering angle varied from 40° to 140°, and the Zimm plot was established. The classical double extrapolation procedure at 0 angle and 0 concentration gives the weight-average molecular weight M_3 and the second virial coefficient A_{13} of guar gum in the solvent.

In the case of casein–guar mixtures, we followed the same procedure as for guar solutions. The initial mixture was diluted by successive additions of the pure solvent, keeping constant and equal to 1 the ratio of the concentrations of the two macromolecular constituents and allowing operation in dilute conditions. The cross-second virial coefficient A_{23} was calculated from the slope of the linear concentration dependence of the ratio $K(C_2 + C_3)/[\Delta R_{\theta=0}^{(c)}]$ according to the following expression[37]:

$$\frac{K(C_2+C_3)}{[\Delta R_{\theta=0}^{(c)}]} = \frac{1}{\nu_2^2 M_2 X_2 + \nu_3^2 M_3 X_3} + 2\,\frac{\nu_2^2 M_2^2 X_2^2 A_{12} + 2\nu_2\nu_3 M_2 M_3 X_2 X_3 A_{23} + \nu_3^2 M_3^2 X_3 A_{13}}{(\nu_2^2 M_2 X_2 + \nu_3^2 M_3 X_3)^2}(C_2+C_3) \tag{1}$$

where subscripts 1, 2, and 3 relate to the solvent, the protein, and the PS, respectively; $K = 2\pi^2 n_1^2/N_A\lambda^4$; n_1 is the refractive index of solvent; N_A is Avogadro's number; λ is the wavelength of incident light in the vacuum; M_2 and M_3 are the weight-average molecular weights of protein and guar, respectively; ν_2 and ν_3 are their refractive index increments; C_2 and C_3 are the concentrations (g $\mathrm{ml^{-1}}$); X_2 and X_3 are the mass fractions of protein and guar in the mixture; $[\Delta R_{\theta=0}^{(c)}]$ is the excess light scattering at 0 angle; A_{12} and A_{13} are the second viral coefficients characterizing the interaction of the protein and the PS, respectively, with the solvent, the values of which were obtained as explained previously; and A_{23} is the second virial coefficient characterizing the mutual interaction of macromolecules 2 and 3 (cross-second virial coefficient).

Experimental errors are 12% on A_{12} and A_{13}, and 30% on A_{23}.

State of Casein in Aqueous "Solutions"

Method of Rosenberg

The effect of ionic strength on solvent quality with respect to caseinate was estimated by the method of Middaugh and colleagues,[38] which consists of determining the dependence of protein solubility in the given aqueous solvent on the concentration C_{PEG} of PEG in the water-protein-PEG system. Extrapolation of this dependence to $C_{\mathrm{PEG}} = 0$ gives the value for the effective activity of the protein in its saturated solution (C_2°). The as-

sumption is that the chemical potential of the protein in the solid precipitate phase is constant. A more detailed analysis makes it possible to relate the activity to the value of the second virial coefficient A_{12} characterizing the protein–solvent interaction.[39] The chemical potential μ_2 of the protein in the water-protein-PEG system can be written as follows[40]:

$$(RT)^{-1}\mu_2 = (RT)^{-1}\mu_2^\circ + \ln(m_2) - (1 - m_2)a_{12} + a_{23}m_3 \quad (2a)$$

where μ_2° is the chemical potential of the protein in the standard state; m_2 and m_3 are the concentrations in the system of the protein and PEG, respectively, expressed as moles of macromolecule per mole of solvent; and a_{12} and a_{23} are the second virial coefficients characterizing protein–solvent and protein–PEG interactions, respectively. At the equilibrium of phases in the water-protein-PEG system, μ_2 is equal to the chemical potential μ_2' of the protein in the precipitated solid phase; because $m_2 \ll 1$, eq. (2a) can be written:

$$\ln(m_2^*) = (RT)^{-1}(\mu_2' - \mu_2^\circ) + a_{12} - a_{23}m_3 \quad (2b)$$

where m_2^* is the concentration of the protein in the supernatant (moles of protein per mole of solvent); and $(\mu_2^\circ - \mu_2')$ is the free energy of transfer of the protein from the solid to the liquid phase. Therefore, if the logarithm of the concentration C_2 of the protein in the supernatant varies linearly with the concentration C_3 of PEG over a range of C_3 large enough, the slope of the straight line reflects protein–PEG interactions and its extrapolation to $C_3 = 0$ measures the sum of the two first terms on the right side of eq. (2b) and thus reflects the quality of the solvent for the protein.

Two series of water-caseinate-PEG mixtures were prepared by mixing binary solutions of each macromolecule at pH 6.86 and 20°C. For the first series, the solvent was water and for the second, 0.25*M* NaCl. The initial concentration of caseinate was 2%, and the PEG concentration range was 10–40%. After mixing for 1 h, the mixtures were centrifuged at 50,000*g* for 30 min until a complete separation of phases. The weight concentration of the protein in the supernatant (C_2) was determined spectrophotometrically at 280 nm, with the relevant PEG solution taken as the reference.

Sedimentation

The sedimentation coefficients were measured at 293 K at 50,000 rpm using a MOM 3170 B ultracentrifuge (Hungary) equipped with a refractometric measuring system. These coefficients were determined at different protein concentrations and extrapolated to 0 concentration; in fact, below 0.5% casein concentration, the apparent sedimentation coefficients did not differ practically from the extrapolated values, as usual for dilute protein solutions.[41] The effect of diffusion was negligible.

The apparent molecular weight of β-casein was approximately estimated from the data of sedimentation velocity. According to ref. 39, the dependence of the sedimentation constant on molecular weight for globular proteins is described by the following equation:

$$\ln(s^\circ) = -5.67 + 0.65 \cdot \ln(M_w) \quad (3)$$

where s° is the sedimentation constant of protein and M_w is the effective protein molecular weight.

At pH 6.86 and $I = 0.15$/NaCl, β-casein solutions showed two sedimentation peaks, with sedimentation coefficient s_1° and s_2°, respectively. Application of eq. (3) gave the corresponding molecular weights M_{w1} and M_{w2}. The apparent average molecular weight was then taken as:

$$M_w = M_{w1}x + M_{w2}(1 - x) \quad (4)$$

where x and $(1 - x)$ are the weight fractions of the fast and slow components, respectively, which were derived from the areas under the peaks on the sedimentograms. The Johnston–Ogston effect was not taken into account explicitly; it was considered as negligible, because the results did not depend significantly on concentration.

Circular Dichroism Measurements

Circular dichroism spectra of solutions of β-casein, guar gum, and their mixtures were recorded with a Jobin Yvon Mark VI dichrograph in 1-mm quartz cells at 293 K over the wavelength range of 190–250 nm. The method was used to spot the possible formation of β-casein-galactomannan 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 concentra-

Table I Phase State of β-Casein-Guar Gum Mixtures in Different Solvents

No.	Solvent (% wt)	Phase State
1	pH 7 Distilled water or NaCl solutions $< 0.01M$ (80–99%)	Single phase
2[a]	pH 7 Distilled water (5–10%)	Single phase
3	pH 7 NaCl solutions ($>0.01M$)	Two-phase
4	Dilute HCl (pH < pI)	Two-phase
5	Dilute NaOH (pH > pI)	Single phase
6	pH 7 $8M$ urea + $0.25M$ NaCl	Two-phase
7	Aqueous ethanol solution (5–7% ethanol)	Single phase

[a] Systems obtained by drying system 1.

tions as in the mixture; departure from additivity should then be due to "complex" formation.

Flow Curve Determination

Flow curves of caseinate solutions, guar gum solutions, and their mixtures in the single-phase region were established at 293 K using a Rheometrics RFS2 rheometer with a cone-plate geometry (cone diameter: 5 cm; cone angle: 0.04 rad). Shear rates ranged between 0.01 and 100 s^{-1}. For each shear rate, the shear stress was recorded after equilibrium was reached.

RESULTS

Phase State of Casein-Guar Mixtures

We studied the phase state of aqueous β-casein-guar gum dispersions over a wide range of pH, ionic strength, and temperature (Table I). At pH > pI, at low ionic strength (in distilled water), all systems were single phase. The phase state of these mixtures was not changed after drying at room temperature (the systems remained optically transparent) or in the presence of $8M$ urea or 5–7% ethanol. On the contrary, in the presence of neutral salt ([NaCl] $\geq 0.01M$), all of the mixtures studied were two phase when the concentration of the macrocomponents was high enough. This was the case even in the presence of $8M$ urea or at pH > 9, solvent conditions for which casein is dissociated as individual protein molecules.[42]

The effect of NaCl on the phase equilibria in pH 7 sodium caseinate-guar gum systems is shown in Figure 2. In the range of NaCl concentrations from 0.25 to $0.15M$, compatibility changes relatively little. A further decrease in NaCl concentration results in a considerable rise in biopolymer compatibility, with a significant narrowing of the section of the miscibility gap. Below $0.01M$ NaCl, sodium caseinate is fully compatible with guar gum at pH 7.

Casein Dispersions: Protein/Protein and Protein/Solvent Interactions

Analytical ultracentrifugation and the method of Rosenberg and colleagues were used to examine the effect of ionic strength and pH on the interaction of casein with the solvent. Figure 3(a,b) shows the results of sedimentation analysis in the case of a 0.5% β-casein solution. At pH 6.86 and $I = 0.002$/NaCl (conditions where β-casein-guar mixtures remain single phase at all concentrations), β-casein exhibits a single symmetrical

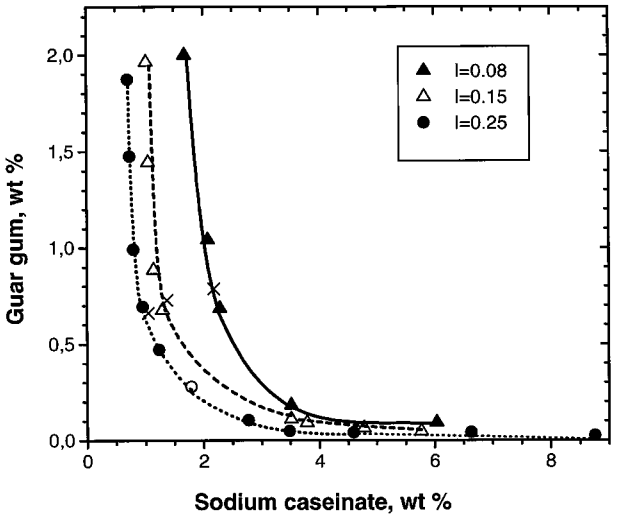

Figure 2 Effect of ionic strength on the binodal of water-sodium caseinate-guar gum system (pH 6.86, T = 293 K). (○) Critical point. (×) Phase separation threshold.

peak [Fig. 3(a)]. At the same pH, when salt concentration is increased to 0.15M (conditions where liquid–liquid phase separation occurs in β-casein-guar mixtures at concentrations high enough), a second peak, rapidly sedimenting, appears [Fig. 3(b)]. An increase in the pH value from 6.86 to 9.5 at I = 0.15 leads to the disappearance of the rapidly sedimenting peak. The nearly symmetrical character of the single peak observed in 0.002M NaCl at pH 6.86 is consistent with its negligible concentration dependence; the corresponding sedimentation coefficient is 1.55 S, in good agreement with the literature value (1.57 S) for the β-casein monomer.[42] This corresponds to an apparent molecular weight of ~ 20 kDa for the β-casein molecule, in agreement with literature data obtained by various methods[43]; we obtained a similar M_w value (25 kDa) by light scattering (Table II). The sedimentation coefficient of the fast peak observed in 0.15M NaCl at pH 6.86 is 13 S, corresponding to an apparent molecular weight of 318 kDa; the weight-average molecular weight of β-casein in these conditions is 128 kDa. The fast peak can be therefore ascribed to β-casein aggregates. Casein aggregates dissociate at high pH values, consistently with the disappearance of the fast-moving peak at pH 9.5.

Figure 4 shows the effect of PEG on the solubility of caseinate at pH 6.86 and I = 0 (curve 1) and at pH 6.86 and I = 0.25 (curve 2). Caseinate solubility decreases as PEG concentration increases in both solvent conditions. The linearity of the curves of caseinate concentration in the supernatant *vs.* PEG concentration indicates that eq. (2b) can be used. Their parallelism shows that ionic strength does not affect sensibly protein–PEG interactions, but that an increase in ionic strength leads to a dramatic drop in activity of saturated casein solutions (ln C_2°), apparently in consequence of protein aggregation. This is consistent with the fact we have seen before that an increase in ionic strength in aqueous caseinate-guar gum systems decreases considerably the compatibility of the two macromolecules.

β-Casein-Guar Interaction

The possibility of interaction of β-casein and guar gum molecules in water at low ionic strength was studied by sedimentation, viscometry, light scattering, and circular dichroism (CD) spectroscopy in the single-phase state of the mixtures.

Figure 3(d,e) represents the results of sedimentation analysis of 0.25% β-casein-0.25% guar gum mixtures at pH 6.86 and I = 0.002 (conditions in which β-casein-guar mixtures do not undergo

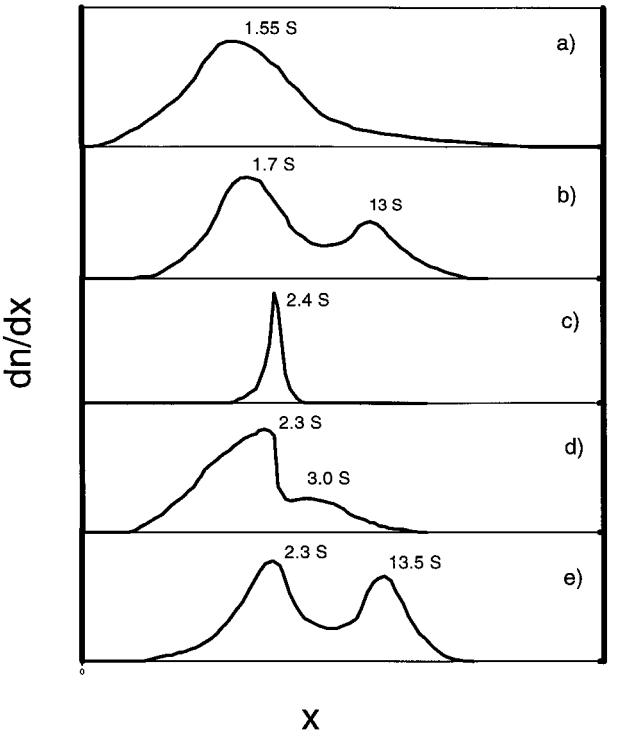

Figure 3 Sedimentation diagrams of β-casein, guar gum, and their mixture at pH 6.86 and 293 K. β-casein 0.5%: I = 0.002 (a); I = 0.15 (b); guar gum 0.5%, I = 0.002 (c); β-casein 0.25%-guar gum 0.25%: I = 0.002 (d); I = 0.15 (e).

Table II Light Scattering Results on β-Casein, Guar Gum, and Their Mixture in Aqueous Solution (I = 0.002/NaCl, pH 6.8, T = 293 K)

	M_w (kDa)	Second Virial Coefficient, Weight Units 10^4 (cm^3 mol g^{-2})	Second Virial Coefficient, Molal units[a] 10^{-5} (cm^3 mol^{-1})
β-casein	25 ± 3	A_{12} = 1.9 ± 0.10	a_{12} = 0.0024
Guar gum	350 ± 35	A_{13} = 5.9 ± 0.15	a_{13} = 1.4 ±0.15
β-casein + guar gum	—	A_{23} = − 18.5 ± 2	a_{23} = −0.32 ±0.05

[a] $a_{1i} = 2A_{1i}M_{wi}/1000$; $a_{23} = 2\,A_{23}\,M_{w2}M_{w3}/1000$.

phase separation) and at pH 6.86 and I = 0.15 (conditions in which incompatibility is observed above the critical concentration), as compared with the sedimentation data for β-casein [Fig. 3(a,b)] and guar gum [Fig. 3(c)]. Similar sedimentation diagrams were obtained for equimass mixtures at different concentrations in the dilute range. At high ionic strength [Fig. 3(e)], two sedimentation peaks are observed in the mixture; the second one (s = 13.5 S) is visibly the same as the peak of β-casein aggregates observed in the case of the protein alone in the same solvent conditions; the first one (s = 2.3 S), very close in position to guar peak but broader and extending to lower sedimentation coefficients, results probably from the superimposition of guar with protein molecules or small aggregates. At low ionic strength [Fig. 3(d)], the mixed solution exhibits two slowly sedimenting peaks. The first peak, very unsymmetrical, seems to be a composite one formed by the partial overlap of two components: the fast component (apparent sedimentation coefficient of 2.3 S) corresponds probably to guar (2.4 S), whereas the slow one appears as a shoulder and can be apparently ascribed to β-casein molecules. As for the second peak, its apparent sedimentation coefficient (3.0 S) is higher than those of guar and β-casein molecules, but much lower than that of casein aggregates.

Results of the light scattering experiments for guar and β-casein solutions at low ionic strength (pH 6.8) are illustrated in Figures 5 and 6. Both the angular and concentration dependences are linear over the whole measuring range for guar solutions. This was also the case for the equimass

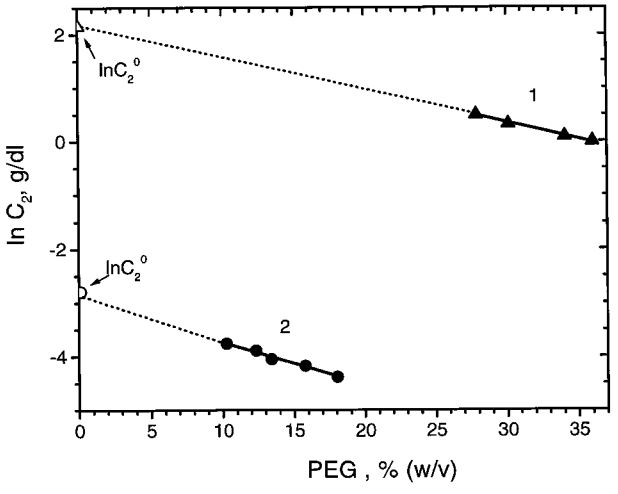

Figure 4 Effect of PEG on solubility of sodium caseinate at 293 K and pH 6.86 in distilled water (curve 1), and at 293 K and pH 6.86 in 0.25M NaCl solution (curve 2). The solid line is a least-squares fit of the data to a straight line. The dashed line gives a linear extrapolation of solubility in the absence of PEG.

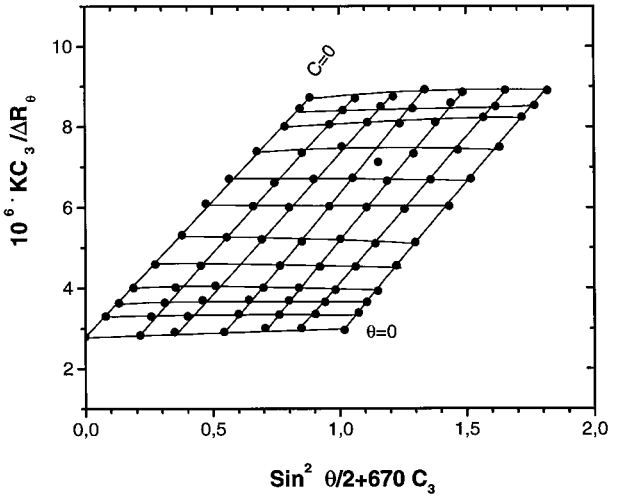

Figure 5 Zimm plot of guar gum solution (pH 6.86, I = 0.002/NaCl, T = 293 K).

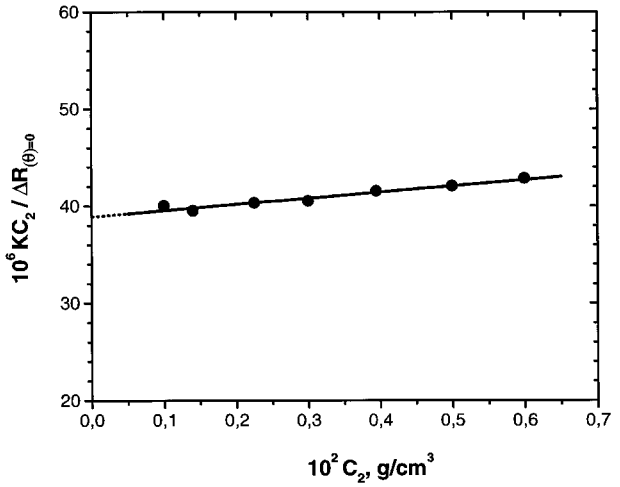

Figure 6 Zero angle light scattering diagram of β-casein solutions as a function of protein concentration (pH 6.86, I = 0002/NaCl, T = 293 K).

mixtures of the two components in the same solvent conditions (results not shown). As previously stated, the scattered intensity showed no angular dependence in the case of β-casein solutions. Results are summarized in Table II. As expected, at low ionic strength and neutral pH, β-casein occurs as monomers (M_w = 25 kDa). Molecular weight of guar gum estimated by light scattering (350 kDa) is lower than the values reported by Robinson and colleagues[44] for guar gum and lower than what could be expected from the intrinsic viscosity of the guar sample we used. The reason for the discrepancy could be a systematic overestimation of the concentration; in this case, the value of the apparent virial coefficient would not be affected. Whereas the second virial coefficients of individual components are positive (although quite small in the case of β-casein), the value of the cross-second virial coefficient is negative, pointing to an attraction between the two macrocomponents.

Figure 7 shows the flow curve of a caseinate-guar gum mixture in the single phase region, compared with the flow curve of the solution of guar at the same concentration as in the mixture; the solvent was 0.25*M* NaCl (pH 6.86) in both cases. The shear thinning behavior of the two systems is identical, but the mixture shows an initial Newtonian plateau (0.99 Pa s) appreciably higher than that of the guar solution (0.73 Pa s). This increase in viscosity cannot result from the additive contribution of caseinate, which is completely negligible (~ 0.0011 Pa s), compared with the contribution of guar at the concentrations considered. It reflects that some interaction between the two components exists, even at high ionic strength.

CD spectra of β-casein in the far UV region at pH 5.6 and I = 0.008 (K_2HPO_4 buffer) are characterized by a negative Cotton effect (Fig. 8, curve 2), with the minimum of the peak at 200 nm, which corresponds to the disordered structure, and by a small "shoulder" at 220 nm indicative of the presence of a small amount of α-helix.[45–47] Literature data on CD[48] and Raman spectroscopy[49] suggest that β-casein contains 10–13% α-helix and 13–22% β-structure. Guar gum spectra in the far UV region show a characteristic simple dichroic band, with a positive peak ~ 203 nm (Fig. 8, curve 1). Mixing β-casein solution with guar gum solution, in conditions such that the system remains into the single phase region, resulted in the disparition of the protein CD spectrum (Fig. 8, curve 3) over the whole range of PS concentrations examined (from 0.004 to 0.06%).

DISCUSSION AND CONCLUDING REMARKS

The effects of the main physicochemical factors on the phase state of aqueous casein-guar gum mixtures show that the conditions of thermodynamic incompatibility of these biopolymers at pH > pI

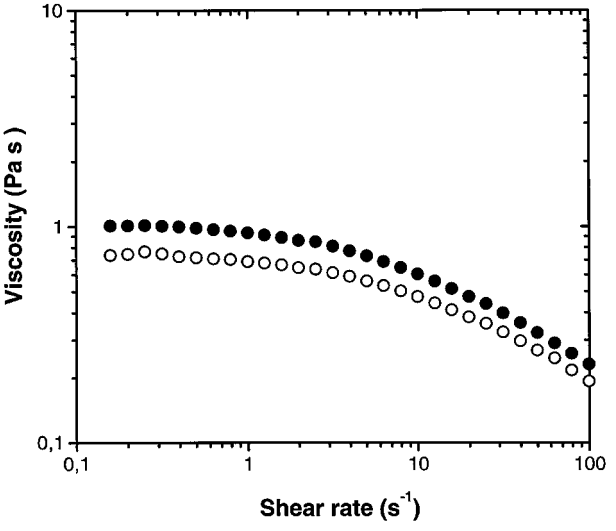

Figure 7 Comparison of the flow curves of the caseinate 0.5%-guar gum 0.8% system (filled circles) and the 0.8% guar solution (empty circles) (pH 6.86, I = 0.25/NaCl, T = 293 K).

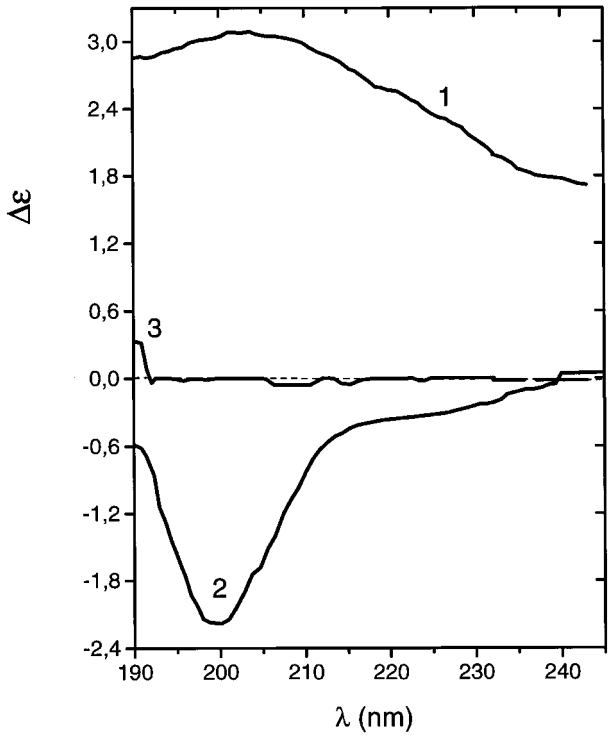

Figure 8 Comparison of the circular dichroism spectra of guar gum (curve 1), β-casein (curve 2), and a β-casein-guar gum mixture after subtraction of the PS spectrum (curve 3). Potassium phosphate buffer: pH = 5.6; $I = 0.008$; 2923 K. Protein concentration: 0.02%. Guar gum concentration: 0.004%.

depend on the ionic strength (see Table I). Let us consider the effect of ionic strength on the phase state from the point of view of the general theoretical concepts relative to the relations between the intensity of polymer–solvent and polymer–polymer interactions, and the phase state of polymer/polymer/solvent systems.[2,50,51]

Compatibility between caseinate and guar in water at low ionic strength when the pH is above the isoelectric point of casein can be ascribed to two causes: the formation of weak soluble interpolymer complexes, which dissociate when ionic strength rises; and/or the vanishing of the difference in the thermodynamic interaction parameter between each of the biopolymers and water. The second one seems unlikely, because caseinate is thermodynamically incompatible with PEG in the absence, as well as in the presence, of salt (Fig. 4). The first explanation finds support in the data of light scattering, sedimentation, viscosimetry, and CD spectroscopy of β-casein, guar gum, and their mixtures in water.

The negative sign of A_{23} points to the existence of an attractive interaction between molecules of β-casein and guar gum at pH 7. The presence of a component sedimenting more rapidly than β-casein molecules and guar in the mixed solution at $I = 0.002$, ionic strength at which compatibility is observed at all concentrations at pH 7, and the absence of this component at $I = 0.15$—ionic strength at which the liquid/liquid phase separation is observed above a concentration threshold—is a strong argument in favor of the formation of a water-soluble interpolymer complex. The fact that the sedimentation coefficient of this peak is low (3.0 S), compared with the aggregates of casein (13 S), and that the intensity of light scattering in the guar gum-β-casein system in these conditions is of the same order as the light scattered by each component, indicate that the complex is formed with casein molecules and not with casein aggregates. The fact that some kind of association takes place between the protein and the PS within the single-phase region agrees with the observed excess viscosity of casein-guar mixtures in the single-phase region, compared with the PS solutions. That the formation of weak complexes between protein and PS results in a single-phase state of these systems was shown previously.[22–24,28,52,53]

The nature of the forces stabilizing these complexes is not known. Hydrogen bonding between chain segments belonging to polymer 1 and polymer 2 has been suggested to interpret the miscibility observed for a few polymer pairs in aqueous solvents,[5] but this hypothesis does not seem to be able to explain the effects of pH and ionic strength on the compatibility of casein-guar systems we have described. Our results indicate indeed that the ionization state of the protein is involved in the formation of casein-guar complexes. In the case of water-gelatin-dextran systems, studied by light scattering, it has been proposed that complex formation would be due to a weak ionic interaction between the charged protein and a very small number of charges carried by the "neutral" PS.[21,53] Such a mechanism would require, in our case, the hypothesis of the existence of a small number of positively charged functional groups on guar gum molecules. Complex formation would be due to the weak ionic interaction between the negatively charged casein (pH > pI) and the positively charged guar interaction that weakens off in the presence of salt. Complexes could not form when the net charge of casein is positive (pH < pI); at pH = pI, they would dissociate as soon as the ionic strength is not negligible. At pH > pI, they would dissociate progressively as the ionic

strength is increased, because of the screening effect of the salt ions; this is in agreement with the observed compatibility/incompatibility behavior of casein-guar gum systems.

The CD results seem to show that the formation of the β-casein-guar gum complex causes the disruption of the small amount of secondary structure displayed by the protein. A loss of the secondary structure of globular proteins on their interaction with PSs has been reported long ago.[54–56] In the absence of any other possible explanation, this suggestion that the dominant mechanism controlling compatibility of casein and guar in water involves the creation of weak water-soluble electrostatic complexes could be considered as a hypothesis for further work. It finds some support in the theoretical work of Khokhlov and colleagues[9,10] on blends of neutral and polyelectrolyte chains. They showed that the presence of even a small fraction of charges on a polymer leads in many cases to a substantial increase of compatibility.

However, the hypothesis does not explain the absence of the effect of ionic strength on the phase state of some protein–neutral PS mixtures (e.g., two-phase gelatin-locust bean gum systems).[57]

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