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The effect of structural features of gelatin on its thermodynamic compatibility with locust bean gum in aqueous media

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Abstract

Thermodynamic compatibility and phase equilibria in aqueous systems containing locust bean gum (LBG) and acid or alkaline gelatin samples with similar molecular weights (∽ 100 kD), but different isoelectric points (7.9 and 4.9 respectively) were studied. The effects of the state of gelatin molecules (molecular-dispersed and colloidal-dispersed (∽ 0.4 μm)) and limited degradation of biopolymers on their incompatibility are investigated. Passing from partially degraded to molecular-dispersed and then to colloidal-dispersed gelatins results in a decrease of the total concentration in the threshold point (C_t*), and in increasing asymmetry of phase diagrams. Compatibility of LBG with gelatins is minimal at low ionic strength (μ) and pH close to their isoelectric points. In the two-phase region, the degree of compatibility, determined by the phase diagrams, was dependent on pH and ionic strength of mixtures, as well as on ionogenic properties of the gelatins studied, varying according to the change of gelatin–solvent interaction.

Keywords: Gelatin; Locust bean gum; Steric entropy

1. Introduction

The phase separation of mixtures of proteins and polysaccharides (PS) in a solvent is studied both for practical and fundamental reasons (Tolstoguzov, 1991). At definite conditions, these biopolymers are usually incompatible, which leads to phase separation, except in dilute solution (Antonov et al., 1975). Most studies in this area dealt with the effects of major physico–chemical parameters on the phase equilibria in mixed solutions of proteins and PS (Antonov et al., 1977, 1979a, 1979b, 1980, 1982). The role of structural features of these biopolymers (type of ionogenic groups and their concentrations, state of protein molecules, molecular-dispersed or associated colloidal dispersed), and the effect of limited degradation of the biopolymers on phase separation and phase equilibria were not investigated. In contrast, although the conditions of phase separation for many protein–PS systems are well known (incompatibility of proteins with PS shows itself only when the possibility of formation of intermacromolecular complexes is excluded, and at least one of the biopolymeric components is associated (Antonov et al., 1977, 1979a, 1979b), the situation is more complicated for mixtures comprising albumins which are important in industry, such as gelatin and bovine serum albumin, the self-association of which is more complex. Specific interactions of albumins with PS occur more often (Antonov et al., 1996a, 1996b; Braudo and Antonov, 1993; Grinberg and Tolstoguzov, 1972; Lashko et al., 1991; Semenova et al., 1991), and are strongly dependent on their aminoacid composition, and state of protein molecules (gelatin). The conditions of incompatibility in the albumin–PS systems depend strongly on the structural features of PS (Antonov et al., 1996).

The aim of this work is to study the effects of aminoacid composition and state of gelatin molecules (molecular-dispersed, associated colloidal dispersed, or partially degraded), as well as limited degradation of PS on phase separation and phase equilibria in aqueous gelatin–PS systems. The experiments involved acid and alkaline gelatin samples, similar in molecular size but different in isoelectric point (IEP), and commercial and partially degraded locust bean gum (LBG) samples. It is well known that LBG forms very viscous solutions at relatively low concentrations, which are almost unaffected by pH, salts, or heat processing. It was assumed that using uncharged LBG, the association behaviour of which does not depend on pH and ionic strength, would permit to obtain information on the effect of gelatin–solvent interaction on phase separation. To estimate the contribution of gelatin–solvent interaction on

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Table 1
The major physico-chemical characteristics of the gelatin samples studied

Sample	T (K)	IEP	iip	MW(kD)	$[\eta]$ (dl g^{-1})	Size (μm)
Gelatin type A ''SBI-SYSTEMS BIOINDUSTRIES''	313 21	7.90[a]	7.15[c] 7.00[d]	91[j]	—	0.018[i]
	291	7.90[b]	—	50000	—	0.48[k]
Gelatin type B[(1)] ''SBI-SYSTEMS BIOINDUSTRIES''	313	4.90[a] 4.90[b]	4.61[c] 4.60[d]	105[j]	—	0.021[i]
	291	—	—	40000	—	0.42[k]
Gelatin type B[(2)] ''Sigma''	313	5.4[e]	4.8[d]	243[h]	0.633[e,f]	—
Degraded gelatin type B[(2)] ''Sigma''	313	5.4[e]	4.8[d]	57[h]	0.258[e,g]	—

[a] Obtained by isoelectric focusing.
[c] After passing through a column PD-10.
[j] Determined by polyacrylamide gel electrophoresis.
[i] According to Williams et al. (1954).
[d] By determining the pH value of acid or alkaline solvent which does not change after the addition of dry gelatin sample.
[b] Determined as the pH at which a 0.5% solution of gelatin had a maximum turbidity at 490 nm.
[k] Determined by Malvern Mastersizer IP laser Granulometer.
[e] Determined by viscometry.
[h] Calculated from $[\eta] = 2.9 \cdot 10^{-2} \cdot M_n^{0.62}$.
[f] In 0.15 M NaCl.
[g] In 2M KSCN at 278 K.

biopolymers compatibility and to establish the mechanism of phase separation in these systems, the simple Rosenberg's method (Middaugh et al., 1979) was employed.

The data obtained have been analyzed using general theoretical concepts (Hsu and Prausnitz, 1974; Scott, 1949; Zeman and Patterson, 1972) dealing with the effects of polymer(1)–polymer(2) and polymer(1,2)–solvent interactions on phase separation in polymer(1)–polymer(2)–solvent systems.

2. Materials and methods

Gelatin. Three gelatin samples were used. Two of them, a pigskin gelatin type A, 200 Bloom 30 PS (Lot 19538), and an ossein gelatin type B, 200 Bloom 30 PS (Lot 33678), were produced by SBI-SYSTEMS BIO-INDUSTRIES, France. These samples, denoted as gelatin A and gelatin B[(1)] were used without additional purification. Their characterisation was done at LPCM, (INRA, Nantes, France) and are presented in Table 1.

The third gelatin sample was a gelatin type B from bovine skin, purchased from Sigma Chemical CO. (Lot 63H0312), denoted here as gelatin B[(2)]. As the initial commercial product had an intensive yellow colour and contained excess peptides, it was used after additional purification by washing in deionised water during 3 h, at 278 K, according to Loeb (1924), and freeze-drying at 293 K. The purified sample had a slightly yellow colour, contained 86.6% of the protein and only traces of peptides ($< 0.1\%$), a small amount of mineral substances (ash = 0.46%), and hexose sugars (0.45%) regarded as impurities.

Gelatin aggregates (GelatinAG) were produced as described by Boedtker and Doty (1954): 0.6%–0.8% solutions of gelatin were prepared at 313 K and stored at 278 K; after one day, they were placed in a thermostated bath and maintained at the desired temperature (291 K) for 18 h. By following this procedure, it was possible to obtained reproducible particle size distribution.

A thermally degraded (348 K, pH = 10.5, 24 h) gelatin sample was obtained from the third gelatin sample according to Chikamai et al. (1977). The major characteristics of all the gelatin samples studied are given in Table 1.

Locust bean gum (LBG), ref. LA 22, (moisture 8.05%; ash 0.14%; protein 0.17%; mannose/galactose ratio 3.65; $[\eta] = 10.35$ dl g^{-1}; $Mv = 1.5 \cdot 10^6$ D) was obtained from SBI-SYSTEMS BIO-INDUSTRIES, France. A thermally degraded LBG sample ($[\eta] = 4.69$ dl g^{-1}; $Mv = 0.673 \cdot 10^6$ D) was obtained from commercial LBG according to Chikamai et al. (1996).

Identification and quantification of the sugar residues of LBG samples were carried out by GLC using a capillary column DB-225 according to Blakeney et al. (1983). The protein was determined by the Kjeldahl method using a nitrogen factor of 5.87 for LBG (Anderson, 1986) and of 5.55 for gelatin (Johnston-Banks, 1977).

Intrinsic viscosity. The intrinsic viscosity, $[\eta]$ was evaluated from classical Huggin's and Kraemer's plots, at 298 K, in water for LBG and in 2 M KSCN for gelatin. Viscosity measurements were performed using a Cannon–Fenske capillary viscometer; for each concentration, the average of three essays was taken.

Molecular weights. Molecular weights for LBG samples were estimated from Mark–Houwink parameters obtained

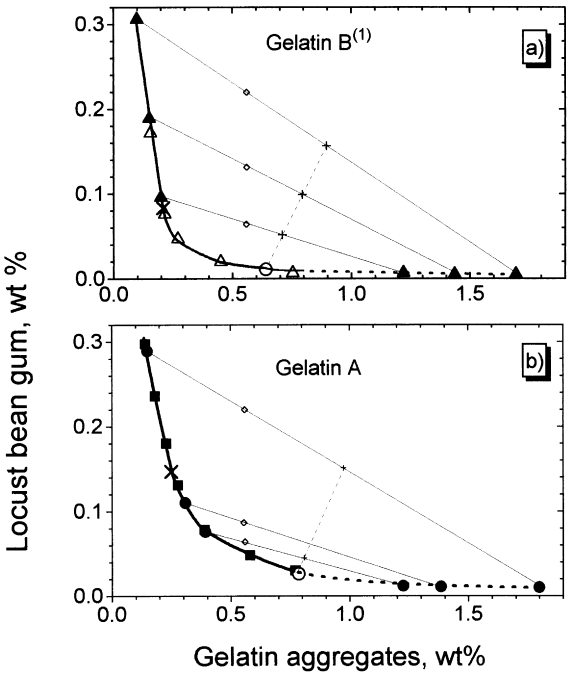

Fig. 1. Isothermal phase diagrams of gelatin (type $B^{(1)}$ AG–LBG (Fig. 2a) and gelatin (typeA) AG–LBG (Fig. 2b) systems. pH = 5.0; $\mu = 0.002$; $T = 291$ K. — binodal; —— the middle of tie lines; ---- tie lines; ●▲ composition of coexisting phases; ■△ other points on the phase diagram; ○ critical point; X threshold point.

for galactomannans by Doublier and Launay (1981) and, for gelatin $B^{(2)}$ sample, according to Williams et al. (1954).

The isoelectric (IEP) and isoionic (iip) points.The IEP and iip points of the gelatin samples, were estimated as described by Boedtker and Doty (1954).

The difference in IEP of the two types of gelatins (Table 1) results from the process used for their preparation, which involves either alkali or acidic pre-treatment of collagen (Chikamai et al., 1977).

Turbidimetric titration. The turbidimetric titrations of the solution of gelatinAG by LBG solution or by acid, alkaline and NaCl solutions, as well as those of the ternary water–gelatin–LBG system by acid, alkaline and NaCl solutions were performed according to Antonov et al. (1977). 40 ml of the gelatinAG solution or gelatin–LBG mixture at desired values of pH and ionic strength were placed in a thermostated mixing vessel and magnetically stirred and pumped to the spectrometer's thermostated flow cell. The temperature, in all experiments, was 291 K. A pH electrode was placed in the liquid in the mixing vessel. After the turbidity had reached a constant value, the necessary quantity of selected titrant was added step by step to achieve phase transition. Changes in turbidity were monitored at 490 nm and recorded as variations of the absorption coefficient, A_{490}, which is linearly proportional to the true turbidity for $A_{490} < 0.9$. The concentration of biopolymers in mixture, or pH and ionic strength for which A_{490} increased more than 2%–3% (clotting point) were regarded, according to Antonov et al. (1977), as the binodal point. When the binodal was crossed, the mixture was diluted with the appropriate solvent and a new portion of titrant was added to lead to phase transition at lower gelatin concentration. The process was repeated each time with more diluted gelatin solutions.

Determination of the phase diagrams of the gelatinAG–LBG systems. Initially, the method of turbidimetric titration was used to determine the binodal. Then, the position of the binodal curve being known, we used selected gelatinAG–LBG mixtures containing the same concentration of gelatinAG (0.45%) and different concentrations of LBG to determine their phase composition. After mixing for 1h, the mixtures were centrifuged at the same conditions (4000 rpm, 291 K, 1 h) of the gelatinAG solution with the same concentration as in the mixtures, and their phase state, and phase volume ratios were detemined. Phase composition of the separated systems was determined from both the material balance of the systems studied and the position of the binodal in the plot, using the correlation (Albertsson, 1971):

$$V_{LBG}/V_{GEL} = d_{GEL}\cdot X/d_{LBG}\cdot Y, \quad (1)$$

where V_{LBG} and V_{GEL} are the volumes of LBG enriched and gelatin enriched phases; d_{GEL} and d_{LBG} are the densities of the coexisting phases (less than 1.02), and X and Y are the distances on the plot between the points corresponding to (i) the composition of gelatin enriched phase and the composition of initial mixture (X), and (ii) the composition of LBG enriched phase and the composition of initial mixture (Y). Calculation of the phase compositions and plot of phase diagrams were performed using Origin 5.0 (Microcal, USA) software.

The compatibility of gelatin and LBG was characterised by the coordinates of the threshold (C^*_2; C^*_3), and critical ($C_{2,cr}$; $C_{3,cr}$) points, as well as by the values of maximum solubility of LBG in a concentrated solution of gelatin — $\overline{C}_3$ — and maximum solubility of gelatin in a concentrated solution of LBG — $\overline{C}_2$ [limit of solubility (Antonov et al., 1987)]. The threshold point was determined in the plot as the point where the line with the slope -1 is tangent to the binodal. The critical point of the system was defined as the point where the binodal intersects the rectilinear diameter (Koningsveld and Staverman, 1968).

2.1. State of gelatin in aqueous solutions.

The effect of ionic strength on solvent quality with respect to gelatin was estimated by the method of Middaugh et al. (1979), which consists in determining the dependence of protein solubility in the given aqueous solvent on the concentration of PEG in the Water (1)–Protein (2)–PEG (3) system. Extrapolation of this dependence to $C_{PEG} = 0$ gives the value for the effective activity of the protein in its saturated solution ($\ln C^0_{PEG}$). Evidence for the validity of this extrapolation includes (a) the experimentally observed

Table 2
Parameters of the phase diagrams obtained[a]

System	T (K)	pH	μ	$C_{2,cr}$(%)	$C_{3,cr}$(%)	C_{tcr}(%)	$K_s = C_{2,cr}/C_{3,cr}$	$\overline{C}_2$(%)	$\overline{C}_3$(%)	C_2*(%)	C_3*(%)	C_t*(%)
Water–Gelatin (type A) AG – LBG	291	5.0	2×10^{-3}	0.78	0.026	0.806	30	—	0.01	0.250	0.15	0.40
Water–Gelatin (type B$^{(1)}$) AG – LBG	291	5.0	2×10^{-3}	0.64	0.011	0.651	58	—	0.005	0.207	0.08	0.29
Water–Gelatin (type B$^{(2)}$) AG – LBG	291	5.0	2×10^{-3}	0.92	0.015	0.935	61	0.024	0.015	0.087	0.09	0.18
Water–Gelatin (type A) AG – LBG	291	9.0	2×10^{-3}	0.92	0.018	0.938	51	0.011	0.01	0.039	0.13	0.17
Water–Gelatin (type A) AG – LBG	291	4.3	2×10^{-3}	0.80	0.033	0.833	24	0.18	0.011	0.45	0.12	0.57
Water–Gelatin (type A) AG – LBG	291	4.0	2×10^{-3}	—	—	—	—	0.37	0.013	0.45	0.135	0.59
Water–Gelatin (type A) AG – LBG	291	5.0	0.1	0.76	0.062	0.822	12	—	0.01	0.218	0.215	0.433
Water–Gelatin (type B$^{(1)}$) AG – Degraded LBG	291	5.0	2×10^{-3}	0.91	0.047	0.957	19	0.065	0.035	0.109	0.129	0.238
Water–Gelatin (type B$^{(2)}$)– LBG	313	5.0	2×10^{-3}	1.66	0.35	2.01	4.5	0.29	0.09	0.55	0.92	1.47
Water–Gelatin (type B$^{(2)}$)– LBG	313	5.0	0.1	2.67	0.51	3.18	5.2	2.36	0.07	2.55	0.58	3.13
Water–Gelatin (type B$^{(2)}$) – Degraded LBG	313	5.0	2×10^{-3}	1.45	0.38	1.83	3.8	0.29	0.14	0.62	0.65	1.27
Water–Degraded Gelatin (type B$^{(2)}$) –LBG	313	5.0	0.1	3.01	0.45	3.46	6.6	2.37	0.16	2.97	0.43	3.40

[a] $C_{2,3,cr}$ — critical point concentration (2-Gelatin, 3-LBG, C_{tcr} -Gelatin + LBG), K_s — ratio of gelatin to LBG concentration at critical point, $\overline{C}_2$ — limit of solubility of gelatin in concentrated solution of LBG, $\overline{C}_3$ — limit of solubility of LBG in concentrated solution of gelatin, $C_{2,3}$* — concentration of biopolymer in the threshold point (2-Gelatin, 3-LBG), C_t* — total concentration of biopolymers in the threshold point.

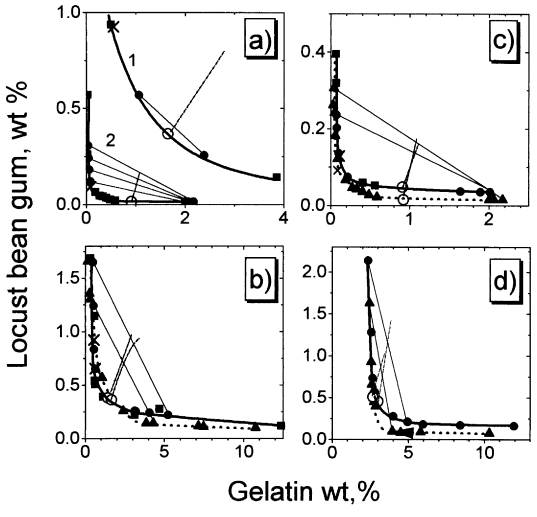

Fig. 2. Isothermal phase diagrams of some gelatin –LBG systems. pH = 5.0. — binodal; – – – the middle of tie lines; — tie lines: (a) $\mu = 0.002$. Curve 1 — gelatin (type B$^{(2)}$)– LBG system, $T = 313$ K. Curve 2 — gelatin (type B$^{(2)}$) AG–LBG system, $T = 291$ K, (b) $\mu = 0.002$. Gelatin (type B$^{(2)}$)–degraded LBG system at $T = 313$ K. Phase diagram of the gelatin (type B$^{(2)}$)–LBG system at 313 K is shown for comparison (dotted line), (c) $\mu = 0.002$. Gelatin (type B$^{(2)}$) AG–degraded LBG system at 291 K. Phase diagram of the gelatin (type B$^{(2)}$) AG–LBG system at 291 K is shown for comparison (dotted line), (d) $\mu = 0.1$. Degraded gelatin (type B$^{(2)}$) –LBG system at 313 K. Phase diagram of the gelatin (type B$^{(2)}$)–LBG system at $\mu = 0.1$ and 313 K is shown for comparison (dotted line).

linearity of log solubility versus PEG concentration plots, (b) the extrapolation of such plots to correct activities in the situation where protein activities can be experimentally determined, and (c) the independence of the extrapolated activities on protein concentration over a wide range.

A more detailed analysis (Polyakov et al., 1985) makes it possible to relate the activity to the value of the second virial protein coefficient characterising the protein–solvent interaction. The original concentrations of gelatin and PEG were 6% and 40%, respectively. After mixing for 60 min, the mixtures were centrifuged at 380000g for 40 min. until a complete separation of phases occured. The weight concentration of the protein in the supernatant (C_{PEG}) was determined spectrophotometrically at 240 nm, taking PEG solution as the reference. According to the data obtained, ln C_{PEG} was plotted against PEG concentration (% w/w) and, extrapolating this dependence to the zero PEG concentration, the value of ln C^0_{PEG}, was determined.

3. Results

3.1. Effect of ionogenic properties of gelatin on its incompatibility with LBG.

The isothermal phase diagrams of the systems, comprising aggregates of gelatin molecules similar in size but

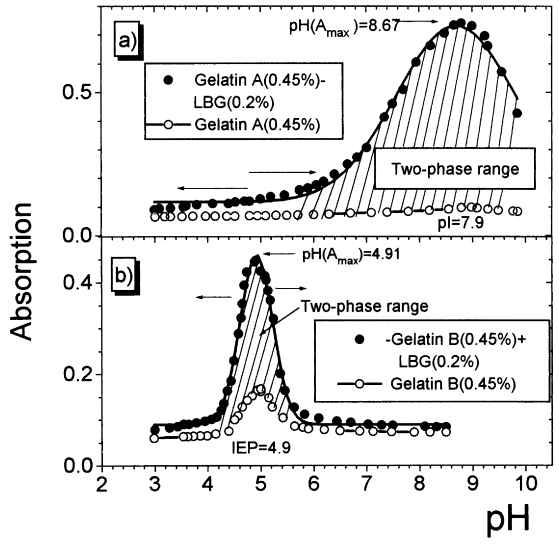

Fig. 3. Effect of pH on the change in absorption at 490 nm of the binary and ternary systems. $\mu = 0.002$; 291 K. ← direction of titration: (a) water–gelatin (type A) AG (0.45%) and water–gelatin (type A) AG (0.45%)–LBG(0.2%) systems, (b) water–gelatin (type B$^{(1)}$) AG (0.45%) and water–gelatin (type B$^{(1)}$) AG(0.45%)–LBG(0.2%) systems.

differing in IEP (see Table 1) are given in Fig. 1. Both diagrams were determined in the same conditions: pH = 5.0; $\mu = 0.002$ (acetic acid–sodium hidroxide buffer), $T = 291$ K.

As can be see from Fig. 1, the thermodynamic behaviour of acid and alkaline gelatines with LBG in the selected conditions is similar. The total concentration of biopolymers in the critical point is less than 0.9%, whereas the total concentration in the threshold point (C_t*) is even less than 0.5%. These are extremely low values of the critical parameters if compared with other aqueous protein–PS systems studied before (Antonov et al., 1975, 1977, 1979a, 1979b, 1980, 1982; Grinberg and Tolstoguzov, 1972). Phase diagrams obtained are very asymmetric ($K_s = 58$ and 30 for the systems comprising gelatin B and A respectively); the critical point corresponds to a much lower concentration of LBG compared to that of gelatin. The concentrations of biopolymeric components in the equilibrium phases are differently affected by the increase of biopolymer content in these systems. The concentration of LBG in a LBG enriched phase increases faster than that of gelatinAG in a gelatin enriched phase. The values of (C_t*) and (C_{tcr}) for the system comprising gelatin A are slightly higher and assymetry of phase diagram is less than those for the gelatin B–LBG system (see Table 2).

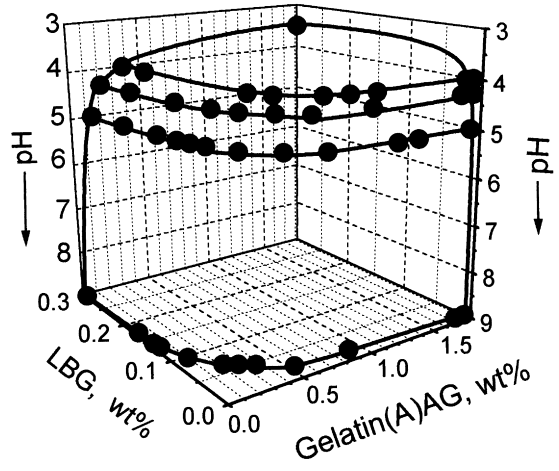

Fig. 4. The cross-section of the separation body of the gelatin (typeA) AG–LBG system at $\mu = 0.002$ and 291 K in coordinates:concentration of gelatin (type A) AG–concentration of LBG–pH.

The effect of the state of gelatin molecules (colloidal-dispersed, or molecular-dispersed associated states) on their incompatibility with LBG is demonstrated in Fig. 2a for the gelatin B$^{(2)}$– LBG system (pH = 5.0; $\mu = 0.002$). The change from colloidal-dispersed to molecular-dispersed state results in a strong increase in biopolymers compatibility (C_t* increases eight times) and decrease in the phase diagram asymmetry (K_s value decreases 13.5 times).

The effect of the limited degradation of LBG or gelatin (in other words, the effect of molecular weight of gelatin and LBG) on their incompatibility is shown in Fig. 2b,c,d, for the systems comprising gelatin B$^{(2)}$ aggregates (Fig. 2c) and molecular-dispersed gelatin B$^{(2)}$ (Fig. 2b,d).

As can be seen, decrease in the molecular weight of the gelatin sample (from 243000 to 57000) or decrease in molecular weight of LBG to half the original results in a noticeable increase of the limit of LBG solubility in concentrated gelatin solution, and of the total concentration in the threshold point, while an increase of the limit of gelatin solubility in concentrated LBG solution was not noticeable.

3.2. Effect of pH and ionic strength on the compatibility of LBG with gelatins

3.2.1. Gelatin (type A) AG–LBG system

A study has been done on the effect of pH and ionic strength on the compatibility in gelatinAG–LBG systems. In the coordinates of the protein and polysaccharide concentrations (C_2, C_3), pH and ionic strength μ, the equation of the surface of the separation body can be expressed as follows Antonov, 1977:

$$\Sigma = F(C_2, C_3, \mu, \mathrm{pH}, T) = 0. \quad (2)$$

The most characteristic section of the separation body of the gelatin–LBG systems was determined using phase diagrams.

$$\Sigma_1 = F(C_2, C_3, \mathrm{pH}) = 0, \qquad \mu, T - \mathrm{const.} \quad (3)$$

To find the characteristic values of pH and sodium chloride concentration at which the systems undergo a considerable change in phase equilibrium, acid–alkaline turbidimetric titrations of some selected gelatinAG–LBG systems were performed.

The results of the turbidimetric titrations of solutions of gelatinAG (0.45%) alone and of gelatinAG (0.45%)–LBG

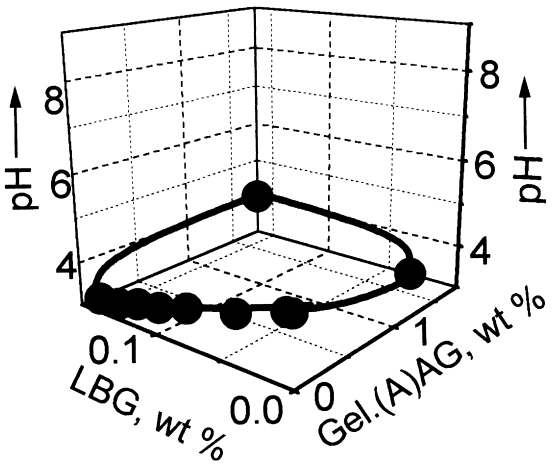

Fig. 5. The cross-section of the separation body of the gelatin (typeA) AG–LBG system at $\mu = 0.1$ and 291 K in coordinates: concentration of gelatin (type A) AG–concentration of LBG–pH.

(0.2%) mixture, at $\mu = 0.002$ and 291 K, are presented in Fig. 3a. The absorption of gelatin (type A) AG solutions in the visible range was practically independent of pH. A very small maximum was observed near the IEP (pH = 7.9) in Fig. 3a. On the contrary, absorption of the Gelatin (type A) AG–LBG system strongly depended on pH with a maximum at pH = 8.67. At pH values below 4.0, the difference in the absorption between the solution of gelatin aggregates and its mixture with LBG is minimal while, at pH 8.67, this difference is maximal. Centrifugation (4000 rpm, 291 K, 1 h) of the mixed gelatin (type A) AG–LBG solutions performed at pH 3.0, 3.5, 4.0, 5.0, 8.7 and 10.0, showed that almost all of them, excluding the mixtures at pH 3.0 and 3.5, are separated in two coexisting phases.

Fig. 4 displays a cross-section of the separation body of the gelatin (type A) AG–LBG system at $\mu = 0.002$ and $T = 291$ K, in the following coordinates: concentration of gelatinAG–concentration of LBG–pH. The decrease of pH from 9.0, leads to an increase in the compatibility of LBG with gelatin A aggregates. These features are reflected in the coordinates of the threshold points for pH = 9.0; 5.0; 4.3 and 4.0 (Table 2). The decrease in the pH value of the mixture from 9.0 to 5.0 results in a minor increase in compatibility. Further decrease in pH from 5.0 to 4.0 leads to a marked increase in compatibility with a significant narrowing of the section of the separation body. However, up to pH 4.0, the system remains two-phase. The separation body is asymmetric. $\overline{C}_3$ keeps a low value while $\overline{C}_2$ increases when the pH of the system decreases from 9.0 to 4.0. In all cases $\overline{C}_2 > \overline{C}_3$. At pH below 3.5, the gelatin (type A) AG–LBG system is single phase in the whole concentration range studied. Hence the system has the lower critical point. As can be seen, the results of the acid–alkaline titration of the system are in good agreement with the separation bodies obtained.

Increase in ionic strength of the system from 0.002 to 1.0 (NaCl) performed at pH 5.0 results in a sharp decrease in its absorption starting at $\mu = 0.1$, which is an indication on an increase of biopolymer's compatibility (data are not presented).

However, even at high ionic strength ($\mu = 1.0$), the thermodynamic behaviour of the system is strongly dependent on pH. The cross-section of the separation body of the gelatin (type A) AG–LBG system at $\mu = 1.0$, in coordinates concentration of gelatin–concentration of LBG–pH, is presented in Fig. 5. In the pH range from 9.0 to 4.5, phase separation of the system did not occur. Further decrease in pH of the system results in its phase separation. In other words, the system has the upper critical point.

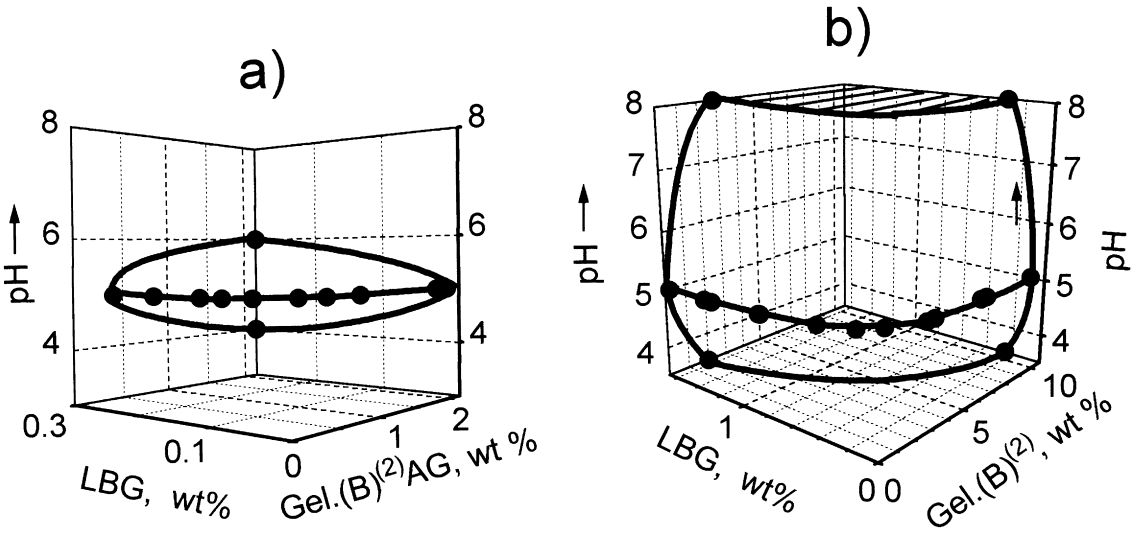

Fig. 6. The cross-section of the separation bodies of the gelatin (type $B^{(2)}$) AG–LBG and gelatin (type $B^{(2)}$)–LBG systems at $\mu = 0.002$ in coordinates: concentration of gelatin–concentration of LBG–pH: (a) 291 K, (b) 313 K.

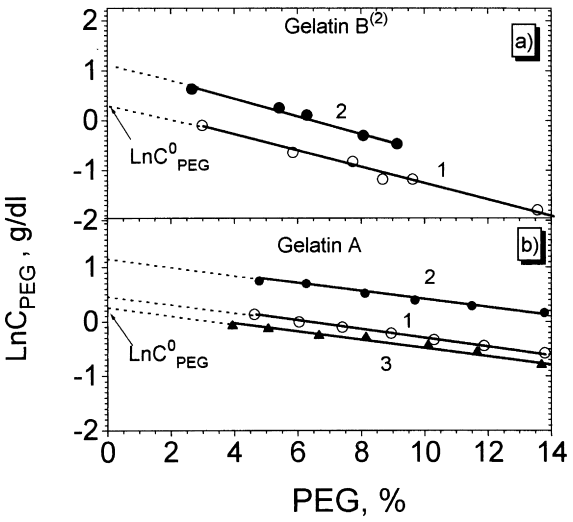

Fig. 7. Effect of PEG on the solubility of alkaline (a) and acid (b) gelatins. Measurements were made at 313 K. $\mu = 0.002$ and pH = 5.0 (curves 1), $\mu = 1.0$ and pH = 5.0 (curves 2), $\mu = 0.002$ and pH = 9.0 (curve 3). The *solid line* is a least squares fit of the data to a straigth line. The *dotted line* gives a linear extrapolation of the solubility in the absence of PEG.

3.2.2. Gelatin (type B) AG–LBG systems

Thermodynamic behaviour of the system comprising gelatin (type $B^{(1)}$) AG is quite different from that including gelatin (typeA) AG. The results of acid–alkaline titrations of the solutions of gelatin (type $B^{(1)}$) AG (0.45%) alone and gelatin (type$B^{(1)}$) AG (0.45%)–LBG (0.2%) mixture at $\mu =$ 0.002 and 291 K are shown in Fig. 3b. Both curves exhibit a maximum in absorption exactly at pH = IEP of gelatin $B^{(1)}$. Phase separation is observed only in a narrow range of pH (from pH ≅ 4.2 to pH ≅ 5.8). Outside this range of pH, the systems were single phase and optically transparent in all the concentration range studied (0%–0.6% for gelatin B and 0%–0.3% for LBG). Hence the system has both the lower, and the upper critical points.

Increase in ionic strength of the system from 0.002 to 1.0 (NaCl) performed at pH 5.0 results in a sharp decrease in its absorption and, at $\mu > 0.1$, the absorption values of the ternary solvent–gelatin (type B) AG(0.45%)–LBG(0.2%) system and the binary solvent–gelatin type B(0.45%) system were the same (data are not presented). Phase separation of the solvent–gelatin (typeB) AG–LBG system, at $\mu = 1.0$ and 291 K, was not observed in all the pH range studied (from 3.0 to 9.0), contrarily to what was observed with gelatin (type A) system.

The effects of pH and ionic strength on the phase equilibria of the systems, comprising molecular-dispersed gelatin are similar to those observed for the mixtures with gelatin in colloidal-dispersed state, when total biopolymer concentration does not exceed 4–6 wt.%. In other words, at moderately high concentration phase separation of the systems comprising molecular-dispersed gelatin only occurs at low ionic strength, in the range of IEP of gelatins. At higher total concentrations the effects of pH and ionic strength on phase separation and phase diagram become less expressed. The typical comparative data for both types of systems, characterising the effect of pH, are presented in Fig. 6a,b. As can be seen, change of pH from pH = 5.0 (close to IEP of gelatin $B^{(2)}$), in any region, results in a considerable increase of biopolymer's compatibility, but for the mixtures with molecular-dispersed gelatin, change in the phase state does not occur.

4. Discussion

As the phase separation of colloidal dispersed gelatin–LBG systems is reversible, it may result of depletion flocculation (Napper, 1983) of gelatin aggregates by LBG.

De Hek and Vrij (1979) established that results of numerous experiments concerning depletion flocculation can be well described on the basis of general theoretical concepts (Hsu and Prausnitz, 1974; Scott, 1949; Zeman and Patterson, 1972) of phase separation in ternary–solvent–polymer 1–polymer 2 systems. The most important factors determining the features of phase equilibria in mixed polymer solutions, and in solutions of neutral nonadsorbing polymer and protein colloids are affinity of polymers to each other and to the solvent, molecular weight and molecular weight ratio of polymers.

A first look on the conditions of phase separation of the systems studied here, at relatively low total biopolymer concentration, allows to regard the possibility of existence of specific interaction (at low ionic strength in the acid pH range) between LBG and both types of gelatin (acid and alkaline). Really, in these conditions, phase separation was not observed. This supposition finds some support in the results of many studies of interactions and compatibility of gelatin with PS. First, Doyle et al. (1967), Woodside et al. (1968) have discussed the possibility of formation of gelatin–D-glucan complexes. Then, Grinberg and Tolstoguzov (1972), Lashko et al. (1991), Braudo and Antonov (1993), using different methods, indicated the presence of some kind of intermacromolecular interaction between gelatin B and poly-D-glucans at pH below IEP of gelatin B (below pH 4.5).

However, the fact that the phase separation in aqueous systems comprising LBG and acid or alkaline gelatins occurs at high total biopolymer concentration independently of pH and ionic strength, gives indication on the absence of any specific interaction between these biopolymers. Probably, the structural features of PS are the key factor which determine the possibile formation of week protein–PS complexes.

The sharp increase in the compatibility of gelatins with LBG, when ionic strength increases or pH of the systems decreases, can be explained by the disappearance of the difference in the thermodynamic interaction parameters between each of the biopolymers and water (Hsu and Prausnitz, 1974; Scott, 1949; Zeman and Patterson, 1972). Therefore, the method of Middaugh et al. (1979) was

Table 3
Dependence of the total concentration of biopolymers in the threshold point (C_t*) of the system on the molecular weight of gelatin B$^{(2)}$

Molecular weight (kD)	C_t* (wt%)	C_t*$M^{0.4}$	C_t*$M^{0.5}$
∽ 40, 000	0.18	12.5	36.0
243	1.47	13.2	22.9
57	3.40	17.1	25.7

employed here to examine the effect of ionic strength and pH on the interaction of gelatin with the solvent.

Fig. 7 shows the effect of PEG on the solubility of gelatin B$^{(2)}$ at pH 5.0 and $\mu = 0.002$ or $\mu = 1.0$ (curves 1 and 2 on Fig. 7a) and the effect of PEG on the solubility of gelatin A at $\mu = 0.002$ and pH = 5.0 and 9.0, (curves 1 and 3 on Fig. 7b) and at $\mu = 1.0$ and pH = 5.0 (curve 2 on Fig. 7b). The dependences obtained proved to be rectilinear. We have seen before that an increase in ionic strength of all the systems studied, as well as an increase of pH of the systems, with gelatin A from 5.0 to 9.0, increases considerably the compatibility of the two macromolecules. The results obtained show that a decrease in ionic strength leads to a dramatic decrease in activity of saturated gelatin solutions ($\ln C_2^0$) apparently in consequence of gelatin self-association, whereas decrease of pH of the gelatin A solution from 9.0 to 5.0 results in a relatively small increase in activity of saturated gelatin solution.

Thus, taking into account that the LBG–water interaction does not depend on pH and ionic strength, the considerable increase in compatibility of LBG with both gelatins (types A and B), when ionic strength increases, is the result of a sharp decrease in the difference in the thermodynamic interaction parameter of each of the biopolymers with water. Similar effect, but less expressed, was observed when the pH of the gelatin A–LBG system was increased from 5.0 to 9.0 (Fig. 7).

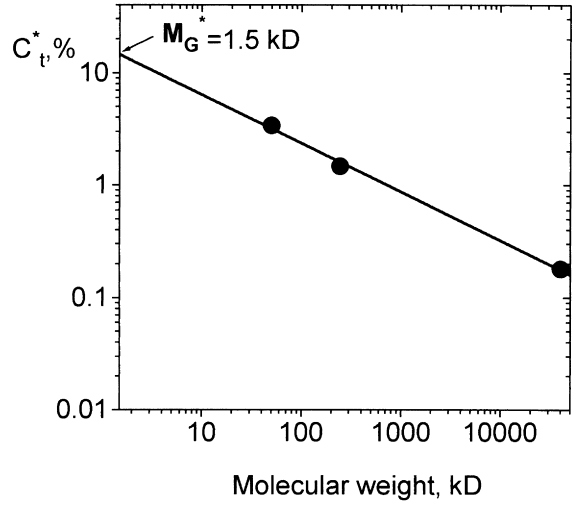

Fig. 8. Dependence of the total concentration of biopolymers in the threshold point of the gelatin B(2)–LBG system on the effective molecular weigth of gelatin.

Comparison of the phase diagrams comprising molecular-dispersed and colloidal-dispersed gelatin, obtained at the same values of pH and ionic strength, shows a significant role of the state of gelatin on the phase equilibria in these systems. Passing from molecular-dispersed to colloidal-dispersed gelatin results in a significant decrease in biopolymers compatibility and in an increase in asymmetry, as stressed by the comparison between the values of C_t* and the values of K_s, respectively, relative to gelatin–LBG and gelatinAG–LBG systems (Fig. 2a). At the same time, compatibility of biopolymers, especially in the region of high protein concentrations, increases considerably when passing from the molecular-dispersed gelatin B to the partially degraded gelatin. Table 3 shows the effect of molecular mass of gelatin B$^{(2)}$ on the total concentration in the threshold point of the systems (C_t*). As gelatin aggregates are soluble in the presence of salt (Boedtker and Doty, 1954) comparative data were evaluated at low ionic strength. The results for degraded gelatin can be regarded roughly as, in the process of alkaline degradation of gelatin and subsequent neutralization of its solution, ionic strength is about 0.1. One can see that, the higher the molecular weight of gelatin, the lower the value of C_t* The dependence obtained is as follows

$$C_t^* \propto M_W^{-(0.4-0.5)}. \quad (4)$$

The correlation obtained is in accordance with the theoretical dependence of flocculation of colloidal particles by nonsorbing polymers (Napper, 1983)

$$\gamma_2 \propto M^{-(0.4-0.5)} \quad (5)$$

where γ_2 is the critical concentration of flocculation; and M is the molecular weight and also with the results of many experimental studies (see for example Clarke and Vincent (1981)).

The dependence of C_t* from molecular weigth of gelatin, plotted in double logarithm coordinates in Fig. 8 is rectilinear. This gives us the possibility to evaluate roughly the critical value of molecular weigth of gelatin (M_G*) at which phase separation of the degraded gelatin–LBG systems doesn't occur. In fact, taking into account that the limit of solubility of LBG in water doesn't exceed 2.0% wt (Whistler, 1973), and assuming that the relative content of LBG in the threshold point for the systems comprising high degraded gelatin C_3*/C_t* = 0.13 does not depend from the degree of degradation of gelatin, we can suppose that the value C_t* = 2.0/0.13 = 15% is the characteristic one at which phase separation is impossible to occur. Simple extrapolation shows that, if the average molecular weight of degraded gelatin is less than 1.5 kD, its mixture with LBG will be compatible in all the concentration ranges (Fig. 8); obviously, molecular weight of gelatin samples is not the critical parameter which determines the phase separation in the studied systems.

High asymmetry of the phase diagrams determined at pH

5 and low ionic strength (Fig. 1, Table 2) gives indication on significant differences between the second virial coefficients, characterising gelatin–solvent and LBG–solvent interactions, in these conditions (Scott, 1949; Zeman and Patterson, 1972).

As the average size of gelatin (type A) AG (0.48 μm) is slightly larger than that of gelatin (type B) AG (0.42 μm), it can be expected, for similar conditions, to observe phase separation of mixtures comprising gelatin (type A) AG at a slightly lower concentration. However, this is not observed here, for the gelatin (type A) AG–LBG system (see Table 2). This discrepancy can be because of different self-association behaviour of gelatin A and gelatin B, at pH = IEP. Probably, aggregates of gelatin A at pH 5.0 have a slightly less pronounced self-association.

The fact that compatibility of gelatin A and gelatin B with LBG, at low ionic strength, is minimal at pH close to IEP of these proteins, indicates that self-association of these proteins is maximal in this pH range. This result agrees with the data on the compatibility of gelatin type B with D-glucans in isoionic conditions (Grinberg and Tolstoguzov, 1972; Lashko et al., 1991). In these works, the low gelatin–D-glucan compatibility is attributed to the fact that the above conditions are favourable to the association of the electrically neutral macromolecules because of their electrostatic interaction caused by charge fluctuations. The interaction is suppressed when the pH is shifted from the isoionic point of the polyampholyte, as well as when the ionic strength is sufficiently increased (generally up to 0.1 mol/l) (Enrilich and Doty, 1954). The results obtained in this study demonstrate the accuracy of this supposition for mixtures comprising acid gelatin, the IEP of which is placed far from that of alkaline gelatin.

By analogy with the results of the effect of ionic strength and pH on the activity of saturated gelatin solutions we can suppose that the difference in the thermodynamic behaviour of acid and alkaline gelatins with LBG, at high ionic strength in the acid range (incompatibility of acid gelatin and compatibility of alkaline gelatin in this conditions), reflects the difference in their self-association behaviour. Probably, in these conditions, the macromolecules of gelatin A develop a higher capacity of self-association as compared to those of gelatin B. It is interesting to note that the phase transition for the gelatin (type A) AG–LBG systems takes place at a pH close to pH 4–4.5, that is near the pK_a of the main anionic (aspartic and glutamic acids) side groups of gelatin. The amount of these amino acids in gelatin A is much less than in gelatin B (Johston-Banks, 1977), and this can be the reason for their stronger self-association at given conditions.

5. Conclusion

The experimental data obtained show that, at high total biopolymer's concentration, phase separation of gelatin–LBG systems occurs independently from pH, ionic strength and the state of protein molecules (degraded, dissociated, or associated). At moderately high total biopolymer's concentration, phase separation of the systems comprising degraded, dissociated, associated or colloidal dispersed gelatin molecules occurs only at definite values of pH and ionic strength. In other words, the steric entropy factor is the main factor which determines the possibility of phase separation of the studied systems.

In the two-phase region, the degree of compatibility determined by the phase diagrams depends on pH and ionic strength of the mixtures, as well as on the ionogenic properties of the studied gelatins, changing with the change of gelatin–solvent interaction.

The dependence of threshold point, C_t*, from molecular weight of gelatin is the same as that obtained from theories of phase separation of solvent–polymer 1–polymer 2 systems and depletion flocculation.

The state of the gelatin molecules is an important factor which determines the values of the critical parameters of phase separation and especially the process of water transfer between coexisting phases.

Finally, all the experimental data obtained on the compatibility of gelatins with LBG were found to be in good agreement with the theoretical concepts (Hsu and Prausnitz, 1974; Scott, 1949; Zeman and Patterson, 1972) which establish the dependence of the compatibility of polymers on the intensity of the interactions between them, as well as on the difference in the intensities of the interaction between each polymer and the solvent, i.e. on the difference of biopolymer hydrophilicities.

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