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Phase behavior of gelatin in the presence of pectin in water-acid medium

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Summary

Phase separation of alkaline gelatin in water-acid solutions in the presence of low etherified pectin (ED 38%) were investigated. The effects of the pectin weight fraction in pectin/gelatin mixture (q_o) as well as two conditions of complex formation, namely, mixing of the binary biopolymer-solvent systems at pH 3.5 ('mixing conditions'), or preparation of the ternary gelatin-pectin-water systems at pH 7.5 and their subsequent acidification up to pH 3.5 ('titration conditions'), on phase equilibrium and macrostructure of the concentrated complex phase were established using phase analysis, and optical microscopy. At $q_o < 0.5$ the aggregative phase separation was observed in both conditions of complex formation leading to the almost complete concentration of both biopolymers in the bottom phase at $q_o = 0.3$ ('mixing conditions') and at $q_o = 0.5$ ('titration conditions'). At $q_o > 0.5$ unusual three phase separation took place in the 'mixing conditions', leading to formation of supernatant (phase 1), complex coacervate (phase 2) and concentrated pectin solution (phase 3). Possible mechanism of such phenomenon was discussed in term of segregative and aggregative phase separations.

Keywords

Pectin, gelatin, complex formation, phase separation

Introduction

The complex coacervation of polymers and their thermodynamic incompatibility are fundamental physico-chemical phenomena, which determine the structure and physical properties of biopolymers mixtures [1-3] and play an important role in protein processing [4-6]. Therefore, to predict the properties of mixed biopolymer systems, their phase behavior and phase diagrams should be known over a wide range of physico-chemical parameters.

Although the majority of biopolymer mixtures show phase separation, this is not totally clear for those containing similarly charged gelatin and linear anionic polysaccharide particularly pectin or alginate [7, 8]. These systems turned out to be

two-phasic at pH values above isoelectric point (iep) of gelatin only at high ionic strength [7]. In this connection, attention should be paid to possible intermacromolecular interactions in such systems and their effect on phase separation.
Inter-biopolymer complexes are frequently formed in aqueous media between biopolymers. They can be both, soluble or insoluble. The occurrence of water soluble electrostatic complexes depends largely on the balance of hydrophobic and coulomb interactions between these species. However, the concentration of the biopolymers, as well as pH, ionic strength, temperature and other minor variables can play an important role [1, 2, 9, 10]. Complex coacervation of gelatin with pectin turned out to be of special interest. These biopolymers and the complex coacervates formed by gelatin and pectin are widely used in microencapsulation for biomedical and food purposes [11]. In such biological systems specific interactions between macromolecules are quite important. Recently Morris and co-workers [11] using DSC showed evidence of the presence of two thermal transitions for mixtures prepared at pH 3.0 , one coincident with formation and melting of gelatin helices (below 35°C) and the other centred at higher temperature (~50° C) coincident with additional cross linking of gelatin through pectin.
In the present work we are particularly dealing with the questions how the conditions of complex formation, namely, mixing of the binary biopolymer-solvent systems at pH 3.5 (below iep of gelatin ('mixing conditions'), or preparation of the ternary gelatin-pectin-water systems at pH values above iep and their subsequent acidification up to pH 3.5 ('titration conditions'), affect on the phase separation mechanism, phase equilibrium and macrostructure of the concentrated complex phase. Although the systems containing two interacting polyelectrolyte's in general can be sensitive to the conditions of their preparation, no cases of this influence on phase separation mechanism have so far reported for ternary water -gelatin-pectin systems according to our knowledge.

Experimental

Materials

The gelatin B 250 bloom sample from porcine skin (isoionic point 5.2), average molecular weight 160 kDa was provided by Fluka (Lot 375858/1). The apple pectin AU-701, etherification degree 38%, was purchased from the Herbstreith & Fox, Germany.

Methods

To prepare molecularly dispersed gelatin solutions, distilled water was gradually added to the gelatin, and stirred first at 320K for 1 hour. The required pH values of the solutions were adjusted by addition of 0.1-0.5 M NaOH or HCl. These were added in small amounts avoiding changes in ionic strength. The solutions were centrifuged at 50.000 g for 1 hour at 313 K to remove insoluble particles. Subsequently, the concentrations of the biopolymer were determined by measuring the dry weight residue. Pectin solutions were prepared by dispersing the gum in water under stirring for 1 hour at 318 K. Subsequent manipulations were the same as those described for the preparation of the gelatin solutions. Gelatin solutions show almost Newtonian behaviour (at temperatures of 313 K and above) up to concentrations of 30% where the hydrodynamic volumes occupied by the chains overlap [12].

The ternary water-gelatin-pectin systems were prepared using two methods ; a) by mixing solutions of each biopolymer at pH 3.5 and 320 K for 1 hour (mixing conditions), and b) by mixing solutions of each biopolymer at pH 7.0 and 320 K for 1 hour and subsequent titration of the ternary system to pH 3.5. The systems were centrifuged at 9500 g for 35 min at 313 K using a temperature-controlled rotor in order to induce phase separation, cooled up to room temperature and kept in a refrigerator at 278 K for 24 hours to reach equilibrium, decanted and then submitted to phase analysis.
The concentrations of biopolymers in coexisting phases were determined by spectrophotometric measurements at two wavelengths of λ_1=260 nm and λ_2=300 nm according to Vierordt's procedure described before by Heilmeyer [13]. The solvent used for solution preparation was acetate buffer with a high ionic strength, (μ = 1.0 /NaCl/) to dissociate water insoluble complex. All measurements were performed after equilibrating the coexisting phases at 293 K for 15 h using Specord UV VIS, Carl Zeiss, Jena, Germany.
Microscopy experiments have been performed on a NU 2 Carl Zeiss, Jena optical microscope, using different magnifications. The digital camera Canon EQS 300 was used to capture the images.

Results and discussion

It has been shown [7] that the effects of a weak inter macromolecular interactions at pH values above iep are insignificant to form a stable water insoluble complexes between these biopolymers but they are quite enough to be responsible for the single phase state of the system. The question is arise: whether mixing of the binary biopolymer-solvent systems at pH 3.5 (‘mixing conditions’) or preparation of the ternary gelatin-pectin-water systems at pH values above iep and their subsequent acidification to pH 3.5 (‘titration conditions’) lead to formation of water-insoluble complexes with the same composition and yield, or mechanism of formation of such complexes, their composition and morphology will be quite different?

Phase equilibrium

Gelatin is soluble in water in a wide range of pH and ionic strength. It forms water-insoluble complexes with pectin in diluted and semi diluted solutions at pH values below iep of gelatin. Our previous experiments shown (data are not presented) that the maximal yield of gelatin and pectin in the concentrated complex phase was observed at pH value close to 3.5. At pH values above iep, gelatin form homogeneous mixtures with pectin in water.
Figure 1 presents the dependences of the protein and polysaccharide yields in the concentrated complex phase of the system, on the pectin weight fraction in this system (q_o). With one hand, the yields of biopolymers in concentrated phase depend very much on the pectin weight fraction in initial mixture (q_o); as q_o increases, yields of biopolymers increases considerably, shows a sharp maximum for q_o = 0.3 ‘mixing conditions’ and $q_o \sim 0.5$ ‘titration conditions’, and then decreases to values close its initial level when sharply. At these q_o value almost all the protein is concentrated in the bottom phase. In theory, such a dependence on q_o is typical of interpolymer complexes formation [14-16]. The electrostatic interaction between the two species causes formation of water insoluble complexes, their association and liquid-liquid

phase separation. As a matter of fact, we do observe co-concentration of pectin and gelatin and the formation of a biopolymers-enriched liquid phase. Stoichiometry of such complexes is (mainly) ruled by the charge density of the polyanions and polycations (i.e. as a function of pH). At a mixing ratio where coacervation is maximal the complexes formed are neutral and stoichiometric (1:1).
On the other hand, in the 'mixing conditions', at q_o values close to 1, (significant an excess of pectin in ternary mixture) abnormally high content of gelatin in concentrated complex phase is observed. This phenomenon was not observed in 'conditions of titration' (Fig. 1b) and is indicative on considerable change in composition of complex coacervate at given conditions.

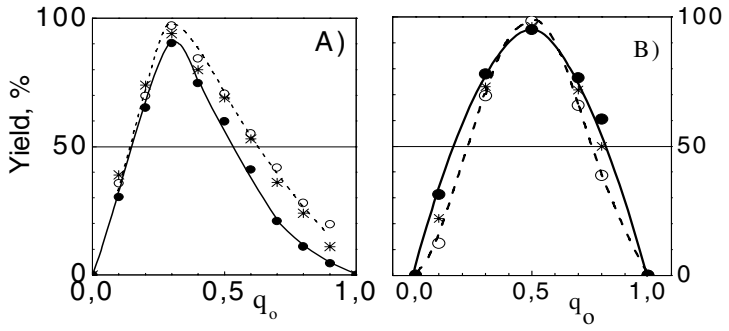

Figure 1. The yields of gelatin (●), pectin (o) and both biopolymers (*) in the concentrated complex phase as a function of q^o measured at pH 3.5 and 293 K. The total concentration of biopolymers is 2 wt%. a)' mixing conditions', b) 'titration conditions'.

The nature of complex aggregation

An important property of the complexes described above is their high thermal stability. Thus, for the mixtures with different composition we observed constancy of absorption values at 500 nm in processes of their heating from +5°C to 70°C. We can formulate important question: what is the nature of complex aggregation? Many scientists suppose [17-20] that nonelectrostatic forces, hydrophobic and (or) hydrogen bonds, play a determinant role in this process.
The interaction energy in the presence of salt has been considered by de Kruif et all [21]. It was expected that the protonated amino groups of the protein associate with de-protonated carboxyl group of the polysaccharide. Ball et al [22] argued that this requires energy to be put into the system, because the average distance between the macro ions is larger due to steric hidrances.
The low ionic strength will suppress dissociation of the carboxylic groups (the effective pK_a increases) and may be suppresses protonation of the protein, an energy which is released on mixing. So it seems that at low ionic strength the lowering of the free energy is due to enthalpic effects while at higher ionic strength entropic effects largely contribute [21]. Such a conclusion is in line with the observed independence of temperature of the coacervation. This entropy gain is more probably due to the 'liberation' of the salt ions as in the Voorn-Overbeek theory [15] than due to the delocalization of the polyelectrolyte in the concentrated coacervate phase as in the

theory developed by Veis [16]. It seems that entropy gain is the main driver in complex coacervation as follows from temperature independence of the phase transition.

Turbidimetric titrations of pectin by gelatin, performed at pH = 3.5 in the presence of 0.5 M NaCl or 6 M urea are an attempt to answer to this question (data are not presented). Sodium chloride suppress only electrostatic interactions between biopolymers, while 6 M urea suppress both hydrophobic and hydrogen interactions in biopolymer systems. This allow us to analyse a contributions of different intermacromolecular bonds in the process of complex formation. Introduction of NaCl in water results in full insensitivity of the pectin solutions to the presence of gelatin in all the q_o range studied. On the other hand, an addition of 0.5 M NaCl in the ternary pectin-gelatin system (pH = 3.5, q_o = 0.3) after a 24 h storage results in a sharp decrease of the system absorption to that of gelatin solution alone. This shows that the complexes are formed and stabilized via electrostatic interaction, rather than through hydrogen bonds formation or hydrophobic interaction. The role of salt is to "soften" the interactions, which is equivalent to making the electrostatic binding constant smaller. Note that the dissociation effect of salts is frequently used as a formal proof of the fact that the complexes are formed and stabilized via electrostatic interaction. However, some authors [23, 24] argue that this fact only proofs that electrostatic interaction is an essential but not always a sufficient condition for the formation of such complexes. The results of turbidimetric titration of pectin by gelatin performed in 6 M urea make this situation more clear. First it was established that 6 M urea doesn't prevent complex coacervation and a liquid-liquid phase separation is observed in a wide range of q_o values. However the degree of aggregation of the concentrated complex phase in the presence of 6 M urea is less than that of the concentrated complex phase without urea. For example, the light transmittance of the gelatin-pectin system in 6 M urea for 1cm cell at 500 nm, A_{500} (1cm) = 0.32, while A_{500} (1 cm) values determined in the absence of urea is higher than 1.0. In other words, the presence of 6 M urea in the binary solvent-biopolymer system do not prevent the complex formation in the ternary system but it partially prevent aggregation of the concentrated complex phase. Aside from this, the light transmittance for the system in 6 M urea, after the removal of urea by three days dialysis opposite to distilled water is close to that obtained in the absence of 6 M urea. This indicates that the presence of 6 M urea is not a factor which significantly weakens the gelatin-pectin interaction, and consequently the interaction is mainly electrostatic. Since the generally accepted idea is that the hydrophobic and hydrogen interactions between biopolymers and their complexes are suppressed in urea, formation of the water insoluble gelatin-pectin complexes should be explained by interaction of charged functional groups of these biopolymers. The absence of any effect of the change of temperature from 278 K to 323 K on the light transmittance of the systems (Fig. 2) is an additional evidence of insignificant influence of hydrophobic and hydrogen interactions in the formation of the complex. On the other hand, appreciable aggregation of the complex after removal of urea from solution can be an indication of some role of nonelectrostatic forces in the process of complex aggregation.

Phase diagrams

Conditions of complexing have a determinant effect on the mechanism of phase separation and composition of coexisting phases (Figures 2 and 3). Note, that the

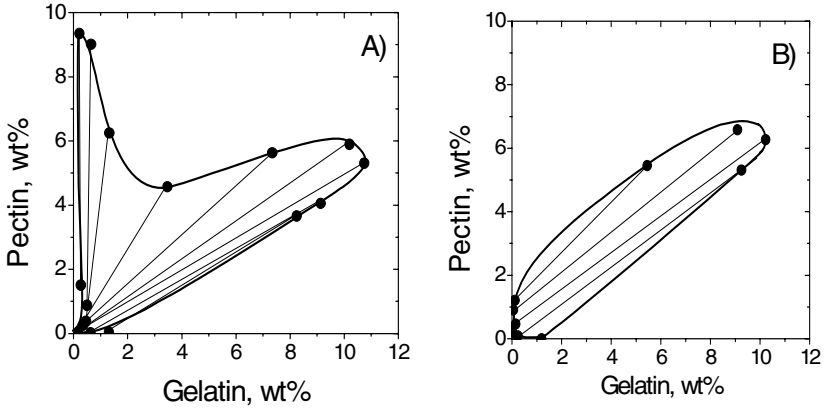

Figure 2. Isothermal phase diagrams of water-pectin-gelatin system determined at pH 3.5 and 293 K. A) 'titration conditions', B) 'mixing conditions'. The total concentration of biopolymer in the initial mixtures is 2 wt %.

water content in the majority of food systems is greater than the content of biopolymers, therefore, rectangular co-ordinates are most often used to describe the phase diagrams in ternary biopolymer systems.

In the case of 'titration conditions', the phase diagram obtained is a typical for a systems containing flexible macro ions such as gelatin and Arabic gum described by Bungenberg de Jong [14]. The polysaccharide/gelatin ratio in the concentrated phase (R_o*) depends on the charge ratio of macromolecular components and doesn't depend on q_o, and it is conditioned by stoichiometry of the water-insoluble electroneutral complex. On the contrary, phase diagram of the system formed in 'mixing conditions' became "bimodal" when the q_o value exceed 0.3 (Fig. 2 b). For q_o > 0.3 the pectin/gelatin ratio in the concentrated phase depend strongly on weight fraction of pectin in the ternary system (Figure 3). The higher q_o values the greater content of

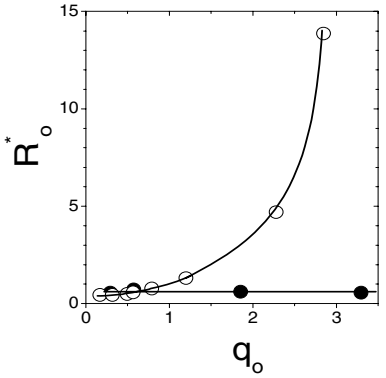

Figure 3. The dependences of R_o* values on q_o. Calculated from the phase diagrams. (o) - 'mixing conditions'; (•) 'titration conditions'.

pectin in the concentrated phase. Such character of the phase diagram directly indicate on existence of two competitive factors (mechanisms) determining phase equilibrium in such system; at $q_o < 0.3$-0.5 and at $q_o > 0.5$. Since character of the bimodal curve of the phase diagrams determined in the 'conditions of mixing and titration ' at $q_o < 0.3$ is practically the same, we can suppose that phase separation in these conditions is determined by aggregative association leading to formation of the water-insoluble electroneutral complex. The phase behavior of the system prepared in conditions of mixing at $q_o > 0.3$ is more complicated and could not be easy explained. In this connection the important question arises: what is the second factor determining the phase separation of such system at $q_o > 0.3$? Two possible reasons can be responsible for deviation of the aggregative phase separation behavior at $q_o > 0.5$ from that described earlier [14]. First, formation of complexes with uncompensated charges in conditions of an excess of pectin (in excess of the negatively charged macroions). This explanation looks unlikely because complexes with uncompensated charges are usually soluble ones while the pectin-gelatin system preparing in the 'conditions of mixing' at $q_o > 0.3$ is a two phase one. Moreover, ratio of biopolymers in of the charged complexes changes normally from 3/1 to 1/3 and rarely from 6/1 to 1/6 (only for the systems containing one weak and one strong polyelectrolyte) [1, 2, 6] whereas for the concentrated phase of the pectin –gelatin system at $q_o = 2.7$ the content of pectin is 14 times higher than that of gelatin (Fig.1).

The other possible reason for "bimodality" of the binodal curve could be that at high content of the pectin fraction in the initial mixture (at $q_o > 0.3$), self association of the partially deionized pectin macroions (segregative association) lead to the segregative phase separation of the pectin molecules with the other macromolecular components of the system (gelatin, and or pectin-gelatin electroneutral complex). In other words, at $q_o > 0.3$ phenomenon of complex coacervation is going to be suppressed while phenomenon of segregative phase separation of pectin with other macromolecular components of system become show itself. In this case, a high content of pectin in the concentrated phase could be quite explainable. This conclusion finds a support in the results of morphological study of the systems prepared in the 'conditions of mixing', and their concentrated phases (Figure 4 and 5).

Macrostructure of the complex coacervates

One can see (Fig. 4) that at $q_o = 0.3$ the concentrated complex phase has a white color which is more or less similar in all volume of this phase. Such ternary two phase system contains dispersed particles 5-10 um in size (Fig. 5 A). These coacervate droplets are partially coalesced into a larger droplets with the sizes of 15-25 um.They coalesce more or less fatly because they are fully charge balanced. The concentrated phase of such system (Fig. 5 B) has a high optic density indicating on a high degree of aggregation of water-insoluble complex. Morphology of the concentrated phase of the ternary system at $q_o = 0.7$ is quite different (Fig. 5 C, D). It is characterized by presence of a very small particles (1-2 um) and some amount of larger droplets, 5-8 um in size. The optic density of such phase is smaller than that described above. The concentrated complex phase was less compact and the coacervate less structured. Such ternary system after centrifugation was separated into a three phases (Fig. 4). The upper part of the concentrated complex phase has a color and composition similar to that obtained for the concentrated phase of the ternary system at $q_o = 0.3$, and the lower part of the complex phase was almost optical transparent and represented itself

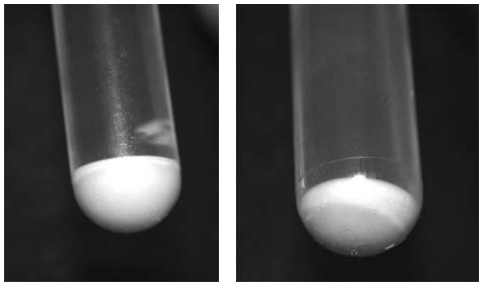

Figure 4. Photos of the concentrated complex phase of pectin-gelatin system formed in the 'conditions of mixing' at q_o = 0.3 (Left) and q_o = 0.7 (right). The total concentration of biopolymers in gelatin-pectin system is 2 wt%. The full length of each image corresponds to 35 mm.

a concentrated pectin solution. The greater was a q_o values the higher was a content of this liquid phase.

Thus, our assumption that at $q_o > 0.3$, phenomenon of complex coacervation is going to be suppressed while phenomenon of segregative phase separation of pectin with other macromolecular components of system become show itself find a support in the results of morphological study of water pectin-gelatin system. In the 'conditions of mixing' at pH 3.5 and in an excess of pectin in the ternary system, phenomenon of segregative phase separation induced by self association of pectin molecules and, or, aggregation of the electroneutral complex can to complete with the phenomenon of aggregative phase separation. It is well known [25] that at pH 3.5 pectin molecules in semidilute solutions are partially neutralized, and the polyelectrolyte character of the polymer is reduced promoting association of the system.

The absence of "bimodality" of the binodal curve for the system formed in 'conditions of titration' could be explained by a smaller degree of association of pectin molecules in such conditions. Really it is known [7] that at pH 5 (above iep) pectin forms water soluble complexes with gelatin. It is probable cause that association degree of pectin in such ternary system after it titration to pH 3.5 could be less that of the binary pectin solution prepared initially at pH 3.5.

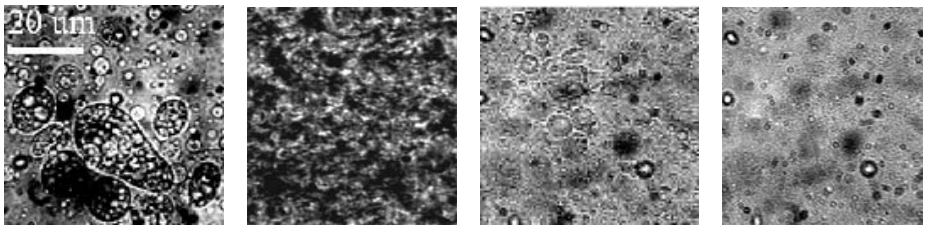

Figure 5. Microphotography's of pectin-gelatin system formed in the 'conditions of mixing' before separation (A and C) and their concentrated complex phases containing 1 vol. % of supernatant (B and D). q_o = 0.3 (A, B); q_o = 0.7 (C, D).

Conclusion

According to the results obtained in this study, the main conclusions are:
The interaction between low etherified pectin and alkaline gelatin is mainly electrostatic. The complexes are formed and stabilized via electrostatic interaction, rather than through hydrogen bonds formation or hydrophobic interaction. Nonelectrostatic forces play an appropriate role in the process of complex aggregation.
Conditions of complexing have a determinant effect on the mechanism of phase separation and composition of coexisting phases. In the case of 'titration conditions', the phase diagram obtained is a typical for a systems containing flexible macroions. Phase diagram of the system prepared in 'mixing conditions' became "bimodal" when the q_o value exceed 0.5. At $q_o > 0.5$ unusual three phase separation take place in the 'mixing conditions', as a result of two competitive factors (mechanisms) determining phase equilibrium in such system.
In the 'conditions of mixing' at pH 3.5 and in an excess of pectin in the ternary system, phenomenon of segregative phase separation induced by self association of pectin molecules and, or, aggregation of the electroneutral complex can to complete with the phenomenon of aggregative phase separation.

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