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CHAPTER 2

Complex Coacervation of Whey Protein and Gum Arabic*

ABSTRACT

Mixtures of gum arabic (GA) and whey protein (whey protein isolate - WP) form an electrostatic complex in a specific pH-range. Three phase boundaries (pH_c, $pH\phi_1$, $pH\phi_2$) have been determined using an original titration method, newly applied to complex coacervation. It consists of monitoring the turbidity and light scattering intensity under slow acidification in situ with glucono-ä-lactone. Furthermore, the particle size could also be measured in parallel by dynamic light scattering. When the pH is lowered, WP and GA first form soluble complexes, this boundary is designated as pH_c. When the interaction is stronger (at lower pH), phase separation takes place (at $pH\phi_1$). Finally, at $pH\phi_2$ complexation was suppressed by the charge reduction of the GA. The major constituent of the WP preparation used was β-lactoglobulin (β-lg) and it was shown that β-lg was indeed the main complex forming protein. Moreover, an increase of the ionic strength shifted the pH boundaries to lower pH values, which was summarized in a state diagram. The experimental pH_c values were compared to a newly developed theory for polyelectrolyte adsorption on heterogeneous surfaces. Finally, the influence of the total biopolymer concentration (0-20% w/w) was represented in a phase diagram. For concentrations below 12%, the results are reminiscent to the theory on complex coacervation developed by Overbeek and Voorn. However, for concentrations above 12% phase diagrams surprisingly revealed a “metastable” region delimited by a percolation line. Overall, a strong similarity is seen between the behavior of this system and a colloidal gas-liquid phase separation.

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INTRODUCTION

The study of the attractive interactions between proteins and polysaccharides has relevance for many biological systems (*e.g.* organization of living cells), and also for pharmaceutical products and processed food (*e.g.* purification of macromolecules, microencapsulation of ingredients or cosmetics, fat substitutes, meat analogues, films, coatings, packaging, …) [Dickinson, 1998; Doublier *et al.*, 2000; de Kruif and Tuinier, 2001; Sanchez and Paquin, 1997; Tolstoguzov, 1996]. For instance, macromolecular interaction between biopolymers may affect the properties of food products (such as yogurts, acidified milk, yogurt drinks, juices, where *e.g.* added pectin and carrageenan form electrostatic complexes with casein micelles). Indeed, arising from electrostatic interaction, the two - weak or strong – oppositely charged polyelectrolytes form a complex, thereby releasing counterions and water molecules, contributing to an entropy gain in the system. The electrostatically bound complexes can be either soluble or insoluble. The insoluble complexes concentrate in liquid coacervate drops, leading to a phase separation of the mixture into two liquid layers. As a result, one phase is concentrated in the two polymers and the other phase contains mainly the solvent. This phenomenon has been studied since 1911, starting with the work of Tiebackx (1991), followed in 1929 by Bungenberg de Jong and Kruyt (1929) on mixtures of GA and gelatin; the term complex coacervation was then introduced to describe this phenomenon.

To gain more insights into the formation of these complexes, a number of studies have identified the influence of various parameters. It is now well known that the electrostatic interaction is determined by the physicochemical characteristics of each polymer (*e.g.* charge density and molar mass), their concentration and ratio, the solution conditions (*e.g.* pH, ionic strength, type of ions) [Schmitt *et al.*, 1998]. Furthermore, temperature, shear and pressure can affect the formation and the stability of complexes. Overbeek and Voorn (1957) proposed a theoretical treatment based on the results of Bungenberg de Jong (1949a, 1949b) and explain the spontaneous coacervation which occurred between gelatin and GA as a competition between the electrostatic attractive forces which tend to accumulate charged polyions and entropy effects which tend to disperse them [Overbeek and Voorn, 1957]. Later, Veis *et al*. (1960) suggested a modification of this theory, where they considered the formation of soluble complexes prior to

coacervation. The charge density of weak polyelectrolytes is governed mainly by the pH, which is, therefore, a significant parameter for complex coacervation. Furthermore, the addition of salt can reduce and even suppress complexation owing to a screening of the charged groups of the polymers [Bungenberg de Jong, 1949b]. However, as shown by Burgess (1990), coacervation can also be suppressed at low salt concentrations.

Some previous studies of the interactions between carboxymethylcellulose (CMC) and proteins (whey proteins, gelatin, …) showed that the formation of the complexes could be monitored by measuring the viscosity, the turbidity, or the coacervate volume [Ganz, 1974; Koh and Tucker, 1988a, 1988b]. Synthetic polymers in combination with bovine serum albumin (BSA) were studied in detail for protein purification using turbidity measurement and pH titration. The pH-induced structural transitions (pH transitions) were used to parameterize the phenomenological results and were determined as a function of several parameters (*e. g.* ionic strength). Two specific pH values, pH_c and $pH_{\phi 1}$, were identified. The formation of soluble protein-polyelectrolyte complexes was initiated at pH_c, which preceded the pH of visual phase separation called $pH_{\phi 1}$ [Dubin *et al.*, 1994; Kaibara *et al.*, 2000; Mattison *et al.*, 1995, 1999; Wang *et al.*, 1996; Wen and Dubin, 1997].

Former studies were made with synthetic polyelectrolytes, and systems with biopolymers currently used in food products have been poorly investigated so far. The novelty of this study lies in the use of a complex mixture of whey proteins (WP, whey protein isolate) together with a complex polysaccharide, GA. The acid-titration of the biopolymer mixtures is carried out in order to see whether pH-transitions found for synthetic polymers could also be detected for biopolymers, and for a weak polyelectrolyte like GA. The “standard” parameters influencing complex coacervation are studied (*e.g.* pH, ionic strength). However, an original titration method is used. With the classical titration method (*i.e.* addition of HCl solution), a dilution effect has to be considered, but in this study an in situ titration technique avoids the external perturbation of the system. The biopolymer mixtures are slowly acidified with glucono-ä-lactone (GDL) and their turbidity, scattering intensity, and particle size are monitored in parallel and reveal the formation of soluble and insoluble complexes. The determination of a state diagram – different from the one found with strong

polyelectrolytes – summarizes the effect of ionic strength and pH. Also, recently developed analytical estimates for pH_c that include the important effects of protein surface charge heterogeneity are applied to our experimental results [de Vries *et al.*, 2003]. This work aims to clarify whether pH-transitions and soluble complexes are formed for biopolymers just as for synthetic systems. Furthermore, the effect of using a weak polyelectrolyte is outlined. And finally, the study of the total biopolymer concentration is summarized in a phase diagram, which - to our opinion – was never done for such a large range of concentration (0.05% - 20% w/w).

The biomacromolecules used in this study are WP and GA. GA is a complex polysaccharide exuded from the African tree *Acacia Senegal*. It is an arabinogalactan-type polysaccharide composed of six carbohydrates moieties and a protein fraction. It was suggested that this polysaccharide has a “wattle blossom”-type structure with a number of polysaccharide units linked to a common polypeptide chain [Islam *et al.*, 1997]. This polysaccharide presents good emulsifying properties and a remarkable low viscosity. GA is a weak polyelectrolyte that carries carboxyl groups, and microelectrophoretic measurements showed that GA is negatively charged above pH 2.2, since at low pH (<2.2) the dissociation of the carboxyl groups is suppressed (with a minimum value at –2.2 $m^2s^{-1}V^{-1}$ for the pH range 5-10) [Burgess and Carless, 1984]. From the molecular structure of the GA, one can estimate the charge density of the polyelectrolyte as one carboxylic group per 5 nm [Islam *et al.*, 1997]. The WP isolate used is comprised of 75% of â-lactoglobulin (â-lg) and 15% of á-lactalbumin (á-la). Native â-lg has an isoelectric point (pI) at pH 5.2 and native á-la at pH 4.1 and they are thus positively charged below their pI. Since the pI of β-lg is higher than the pI of á-la and because β-lg is present in a much larger quantity than á-la, one can presume that the interaction between the WP and GA is dominated by the β-lg. As a result, complexation between WP and GA takes place in the pH range where they carry opposite charges. Schmitt *et al.* have already performed extensive work on this system and pointed out the large influence of WP aggregates on complex coacervation [Schmitt *et al.*, 1999, 2000a; Schmitt, 2000b]. Therefore, aggregates from our protein mixtures were removed.

THEORY

Primary soluble complexes between protein and polyelectrolyte are formed at a specific pH, called pH_c, which varies with the ionic strength. In a number of cases it was found that at low ionic strength, soluble complexes also form at pH values for which the proteins and polyelectrolytes carry the same net charge. This is also observed for the system WP/GA. The phenomenon has been ascribed to the attraction between polyelectrolyte charges and oppositely charged "patches" on the protein surface [Dubin *et al.*, 1994; Wen *et al.*, 1997]. A number of recent theoretical papers deal with the complexation of homogeneously charged spheres and oppositely charged polyelectrolytes [see for example: Netz and Joanny, 1999; Nguyen and Shklovskii, 2001]. These could be applied to complexation between oppositely charged proteins and polyelectrolytes. However, complexation between similarly charged proteins and polyelectrolytes cannot be explained by these theories. Ellis *et al.* (2000) performed Monte Carlo simulations of polyelectrolyte adsorption on randomly charged surfaces and found complexation for polyelectrolytes and surfaces of the same net charge. Carlsson *et al*. (2001) performed Monte Carlo simulations of polyelectrolytes complexing with heterogeneously charged spheres (with a surface charge distribution mimicking that of lysozyme). These authors found that it was necessary to include a small nonelectrostatic protein-polyelectrolyte attraction in order to account for complexation of similarly charged proteins and polyelectrolytes. Finally, Grymonpré *et al.* (2001) used computer modeling of protein electrostatics to identify a "charge patch" on serum albumin that could act as an electrostatic binding site.

Recently, de Vries *et al.* (2003) have developed analytical estimates for pH_c, that include the effects of charge patches, or more generally, the protein surface charge heterogeneity. The theory gives simple expressions for the dependence of the measurable quantity pH_c on the ionic strength and the linear charge density of the flexible polyelectrolyte. However, these are obtained at the expense of neglecting many protein and polyelectrolyte structural details. Therefore, at best, this theory can be expected to give correct order-of-magnitude estimates. In the Results section, we compare our own experimental data for pH_c to these analytical estimates. Below, we outline the essential ingredients of the theory.

A first crucial observation is that that there is virtually no correlation in the sign of neighboring charges on the surface of globular proteins. This was demonstrated

through a statistical analysis of the surface charge distribution for β-lg. A continuous (but heterogeneous) protein surface charge density is introduced by coarse-graining the discrete protein surface charge distribution at the level of the Kuhn segment length l_K of the flexible polyelectrolyte. Roughly speaking, the protein surface is divided into "sites" of area l_K^2 having a statistically independent random surface charge density that may vary between $\sigma-\Delta\sigma$ and $\sigma+\Delta\sigma$, where σ is the average protein surface charge density and $\Delta\sigma$ are the root-mean-square variations of the local protein surface charge density. Each of these sites is large enough to accommodate one segment of the flexible polyelectrolyte. Note that in such a model, there is a finite probability of finding charged patches of various sizes that have a charge opposite to the net protein charge. Hence, this model is not inconsistent with the idea of charged patches, but rather tries to approximate the statistics of how often these charge patches typically occur.

Existing approaches for polymer adsorption on randomly interacting surfaces were used to estimate critical adsorption conditions [Odijk, 1990; Andelman and Joanny, 1991]. The electrostatic interaction potential between polymer segments and the protein surface is approximated by a "random square well" potential. The width of the well, *i.e.* the range of the interaction, is the Debye length κ^{-1} (which is a function of the ionic strength). This approximation leads to expressions for the polyelectrolyte adsorption that are only accurate up to a numerical factor of order unity, but do retain the correct dependence on the salt concentration. The average depth V of the well (in units of the thermal energy k_BT) is proportional to the product of the polymer linear charge density ν (number of elementary charges e per Kuhn segment length l_K) and the average protein surface charge density σ (number of elementary charges e per unit area):

$$V = 4\pi l_B \kappa^{-1} \sigma \nu, \qquad \text{(Eq. 1)}$$

where $l_B = e^2/\varepsilon k_BT$ is the Bjerrum length and ε is the solvent dielectric constant. In aqueous solutions, and at room temperature, $l_B \approx 0.7$ nm. In terms of the concentration c_s of added monovalent electrolyte, the Debye length is given by $\kappa^{-1} = (8\pi\, l_B\, c_s)^{-1/2}$ The root-mean-square fluctuation of the well depth due to the variations of the local protein surface charge density is ΔV. It is proportional to the polymer linear charge density and to the root-mean-square variation $\Delta\sigma$ of the (local) protein surface charge density. In units of the thermal energy k_BT:

$$\Delta V = 4\pi l_B \kappa^{-1} \Delta\sigma \nu \qquad \text{(Eq. 2)}$$

In the interesting limit of strong fluctuations ($\Delta V^2 >> V$), the random square well model predicts polymer adsorption for

$$V - 0.5\Delta V^2 < 0.95(\kappa l_K)^2 \qquad \text{(Eq. 3)}$$

In the general case, the critical conditions ($V, \Delta V^2, \kappa l_K$) for the onset of polymer adsorption follow from the full analytical solution of the random square well model [de Vries *et al.*, 2003].

To estimate values of V and ΔV for globular proteins around the isoelectric point pI, we set:

$$\sigma \approx \left.\frac{\partial \sigma}{\partial pH}\right|_{pH=pI} (pH - pI) \qquad \text{(Eq. 4)}$$

The derivative can be deduced from titration data, assuming some reasonable estimate for the protein surface area. The magnitude of the root-mean-square variations of the local surface charge density is roughly pH independent around the isoelectric point and is left as an adjustable parameter [de Vries *et al.*, 2003]:

$$\Delta\sigma^2 \approx \frac{m}{\pi l_K^2}\left.\frac{\partial \sigma}{\partial pH}\right|_{pH=pI} \qquad \text{(Eq. 5)}$$

for some numerical constant μ of order 1. Estimates for the critical pH for the formation of soluble complexes are deduced by finding the pH for which the random square well model predicts the onset of polymer adsorption.

EXPERIMENTAL SECTION

Materials

Bipro is a whey protein isolate (WP) comprised mainly of â-lactoglobulin (â-lg), and á-lactalbumin (á–la) - from Davisco Foods International (Le Sueur, USA). The WP aggregates were removed by acidification (at pH = 4.75) and centrifugation (1h at 33000 rpm with a Beckman L8-70M ultracentrifuge, Beckman instruments, The Netherlands). The supernatant was then freeze-dried (in a Modulo 4K freeze-dryer from Edwards High Vacuum International, UK). Finally, the resulting powder was stored at 5°C. The final powder contained (w/w) 88.1% protein (N x 6.38), 9.89% moisture, 0.3% fat and 1.84% ash (0.66% Na^+, 0.075% K^+, 0.0086% Mg^{2+}, and 0.094% Ca^{2+}). The protein content of the treated Bipro is: 14.92% á-la, 1.46% BSA, 74.86% β-lg, and 3.21% immunoglobuline (IMG).

Batches of > 90% pure β-lg and > 90% pure α-la were provided by NIZO food research (Ede, The Netherlands). The powder of β-lg contained (w/w) 92% β-lg, 2% á-la, 2% of nonprotein nitrogen compounds, and 2.1% ash (including 0.75% Na^+, 0.02% K^+, 0.008% Mg^{2+}, and 0.12% Ca^{2+}) on a dry basis. The amount of moisture was 4.0%. The composition of á-la powder is not exactly known but contains 93.8% of á-la (determined by reverse HPLC).

GA (IRX 40693) was a gift from the Colloides Naturels International Company (Rouen, France). The powder contained (w/w) 90.17% dry solid, 3.44% moisture, 0.338% nitrogen, and 3.39% ash (0.044% Na^+, 0.76% K^+, 0.200% Mg^{2+}, and 0.666% Ca^{2+}). Its weight average molar mass (M_w = 520 000 g/mol) and its average radius of gyration (R_g = 24.4 nm) were determined by size exclusion chromatography followed by multiangle laser light scattering (SEC MALLS). SEC MALLS was performed using TSK-Gel® 6000 PW + 5000 PW column (Tosoh Corporation, Tokyo, Japan) in combination with a precolumn Guard PW 11. The separation was carried out at 30°C with 0.1 M $NaNO_3$ as eluent at a flow rate of 1.0 mL min^{-1}.

Stock solutions were prepared by dissolving the powder in deionized water (concentrations were varied from 0.5% - 25% w/w). For concentrations of 1% and below, the mixtures were stirred for 2 h at room temperature and the resting time was one night at 5°C. For concentrations of 5% and more, the mixtures were stirred 24h (5h at room temperature and overnight at 5°C).

Preparation of the mixtures

Various concentrations of WP and GA mixtures were obtained by diluting the stock solutions in deionized water at the desired pH and ionic strength. The concentration of the total biopolymer (Cp) varied from 0.05% to 25% and the ratio of WP to GA (Pr:Ps) was set at 2:1 (w/w). The mixtures were slowly acidified using glucono-ä-lactone (GDL) powder, which is an internal ester. Once added to the mixture, the GDL dissolves and hydrolyses slowly to form gluconic acid (GH), a weak acid, and further dissociation leads to the formation of G^- and H^+ [de Kruif, 1997]. To cover the desired pH range, 0.07% to 0.16% (w/w) of GDL was added to the mixture. Occasionally the mixtures were also acidified using 1 M HCl. Comparisons of acidification with GDL and HCl showed no difference in the experimental pH_c and $pH_{\phi 1}$ values. After mixing proteins

and polysaccharides, the ionic strength was adjusted using NaCl, and finally the pH was brought to the desired initial value (pH = 7.0 ± 0.5) with 0.5 M NaOH. Then, the sample was put in a water bath at 25ºC. Next, GDL powder was added and the mixture was stirred for 1 min to allow a homogeneous dissolution of the GDL. A sample was subsequently taken out for pH measurements, another one for turbidity measurements and another for static light scattering measurements. Of the same sample, pH, turbidity, and scattering intensity were followed in time. The measurements were repeated at least three times at each condition. The values of the pH_c and $pH_{\phi 1}$ had a variation within the accuracy of the pH-meter (±0.05 pH-units). Controls with only WP and only GA were systematically performed in the same conditions as those for the mixtures of biopolymers.

Turbidity measurement

Turbidity measurements were carried out with a Cary 1E spectrophotometer (Varian, USA) at a wavelength of 514.5 nm (*i.e.* similar to the laser light wavelength used for static light scattering measurements). The samples were put in a 1 cm path length cuvette and the turbidity was then measured as a function of time at 25ºC. The turbidity (τ) was defined as:

$\tau = - \ln (I/I_0)$, with I the light intensity that passes through a volume of solution in 1 cm cube and I_0 the incident light intensity.

Static and dynamic light scattering measurement

The static and dynamic light scattering experiments were performed on mixtures of 0.05% - 1% w/w biopolymer concentration, using a Spectra Physics 275 mW Argon laser with a wavelength of 514.5 nm. The light beam was focused on the axis of the goniometer using a lens. The sample was initially filtered with a 0.45 ìm filter and centrifuged for 30 seconds to remove all impurities and air bubbles. The sample was placed in the cuvette housing, which was kept at a temperature 25°C in a toluene bath. The goniometer was set at 45° (90° for Figure 2.1b). The detected intensity was processed by a digital ALV-5000 correlator. Finally, the scattered light intensity was measured and its average was recorded every minute. The second order cumulant fit was used for the determination of the particle size and the averaged intensity was used as the scattering intensity value each minute.

The filtration was performed at pH values higher than pH_c to avoid any removal of material. Experiments performed with and without filtration showed that the pH-transitions were found at the same value, proving that the filtration step did not spoil the results.

Building the phase diagrams

Addition and removal of salt

For each total concentration of biopolymer (Cp) in the range (0.05% - 25%), the concentration of NaCl was varied. By slowly adding NaCl to a mixture of WP and GA (Pr:Ps = 2:1) at pH 3.5, one could see at which concentration of NaCl, or/and at which conductivity, the turbidity of the mixture started to decrease. Then, by adding ion exchange resins (mixed bed) into the solution, one could remove the salt without varying the pH and measure the increase of turbidity. This measurement was done for high Cp values (from 12% to 20%). However, one cannot exclude the possibility of protein adsorption on the ion-exchange resins, therefore another method of building phase diagram was used, *i.e.* the dilution method.

Dilution method

The concentration of NaCl was maintained constant and the concentration of biopolymer was varied. A mixture of WP and GA (Pr:Ps = 2:1) was prepared at pH 3.5, at a defined [NaCl], and at a high biopolymer concentration (Cp around 25%). This mixture was then diluted slowly with water of the same pH and ionic strength and the turbidity was measured as the mixture was diluted. Finally, the concentration of biopolymer could be calculated for each measurement. As a result, the Cp of phase separation was determined. This measurement was repeated for all added [NaCl] in the range 0 mM-150 mM.

Conductivity measurement

The conductivity of the mixtures was measured at T=25°C with a conductivity hand-held meter LF 340 and a standard conductivity cell TetraCon® 325 (Wissenschaftlich – Technische Werkstätten GmbH, Germany).

RESULTS AND DISCUSSION

The strength of complexation depends on the charge density of both biopolymers. Therefore pH and ionic strength play a fundamental role in the formation of complexes between WP and GA. We determined under which conditions complexation occurs using turbidity and static light scattering measurements as a function of pH and salt concentration. The complexity of the protein mixture has been investigated as well, by titrating mixtures of > 90% pure β-lg/GA and > 90% pure á-la/GA. Furthermore, the total biopolymer concentration and the ratio of the two biopolymers also influence complexation. The effect of each parameter will be systematically discussed.

Effect of the pH

By adding GDL to a mixture of WP and GA, complexation of biopolymers could be studied in the pH range 7.0 – 4.0. Controls with only WP or GA were measured as a background measurement. During acidification, the turbidity of the mixtures was monitored simultaneously by spectrophotometry and static light scattering. The latter technique is more sensitive to the detection of small particles than the turbidity measurement, especially in highly transparent systems. Dynamic light scattering experiments performed in parallel allowed the size evolution of the particles to be followed as a function of pH. Initially, measurements were carried out on mixtures containing a total biopolymer concentration (Cp) of 0.1% and 0.3% with a ratio of protein to polysaccharide (Pr:Ps) of 2:1 (w/w) at [NaCl] = 12.5 mM and [NaCl] = 0 mM. The initial pH of the mixture was set at 7.0 and, by adding 0.07(w/w)% GDL, it decreased slowly to pH 4.0 in approximately 1.5 h.

As illustrated in Figure 2.1a, the increase of scattering intensity of the biopolymer mixture reveals three phases (A, B, C) as compared to the blanks with only WP or GA, which intensity remains almost constant over the whole pH-range. The turbidity curve corresponding to the mixture of biopolymers presents only one strong increase. The scattering intensity of the WP/GA mixture is almost constant and low at a pH above a critical pH value (pH_c) (region A). The second phase is comprised between pH_c and a pH of phase separation (called $pH_{ö1}$) where the scattering intensity increases slightly with decreasing pH (region B). This small increase is clearly perceptible with the light scattering but the turbidity value remains low. Then at $pH_{ö1}$ (and below), the scattering intensity increases abruptly as shown by both the static light scattering measurement

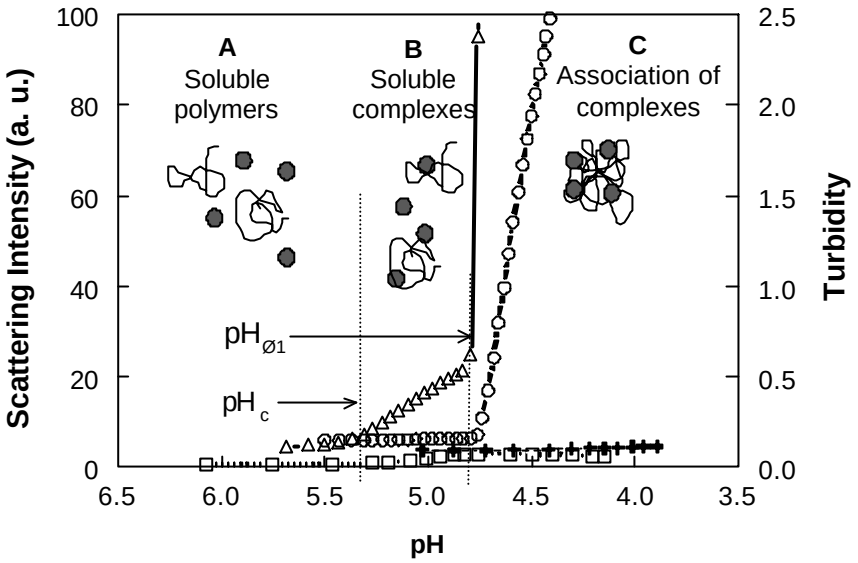

Figure 2.1a: Stability and instability regions (A, B, C) as a function of pH. ( ): Scattering intensity and ( ): Turbidity of a mixture of WP and GA (Cp = 0.1% and Pr:Ps = 2:1). ( ): Scattering intensity of WP (Cp = 0.07%). (**+**): Scattering intensity of GA (Cp = 0.03%). [NaCl] = 12.5 mM, [GDL] = 0.07%.

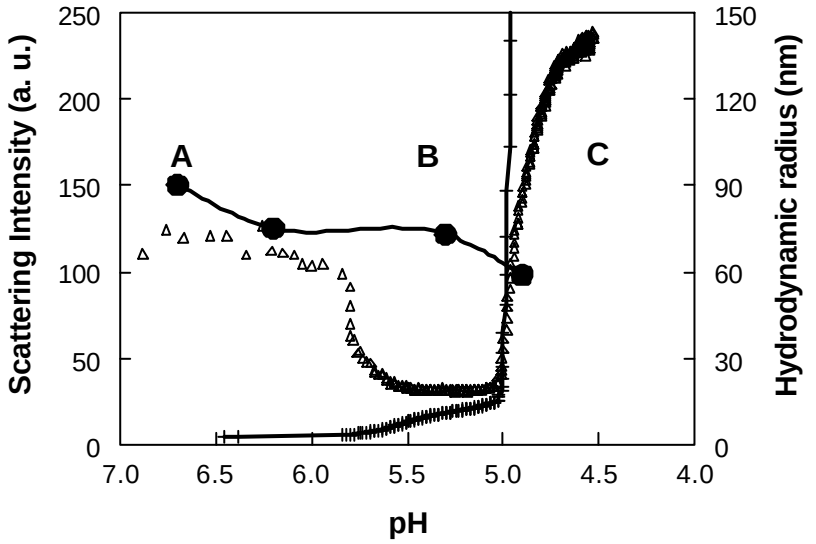

Figure 2.1b: Stability and instability regions (A, B, C) as a function of pH. (**+**): Scattering intensity at 90° and ( ) Hydrodynamic radius of a mixture of WP and GA (Cp = 0.3%, [NaCl] = 0 mM, and Pr:Ps = 2:1, [GDL] = 0.07%). ( ): Hydrodynamic radius of GA (Cp = 0.1%, [NaCl] = 0 mM).

and the turbidity measurement (region C). The values of pH_c and $pH_{\phi 1}$ were measured graphically as the intersection point of two tangents to the curve. In Figure 2.1a, the values are pH_c = 5.3 and $pH_{\phi 1}$ = 4.8. Figure 2.1a illustrates that in the presence of only one biopolymer (WP or GA), the scattering intensity remains flat compared to the one of the WP/GA mixtures. This result proves that a pH-dependent two-step increase of scattering intensity in the biopolymer mixture is due to an electrostatic interaction between the GA and the WP. No aggregation of GA takes place, and a very limited protein-protein association is visible at pH 4.75. This slight aggregation of protein could be induced by the freeze-drying step or by some residual aggregates that were not removed.

In Figure 2.1b, the evolution of the scattering intensity and the hydrodynamic radius of the particles are plotted as a function of the pH for a mixture of WP and GA with a Cp = 0.3%, Pr:Ps = 2:1, and a [NaCl] = 0 mM. A blank of 0.1% GA shows the size evolution of the polysaccharide alone as a function of pH ([NaCl] = 0 mM). This result illustrates that at a pH > pH_c (region A), the initial radius of the complex is similar to the radius of the GA alone. At pH_c, when the scattering intensity increases, the radius of the complex strongly decreases to a value close to 20 nm, whereas the radius of the GA alone remains between 90 and 60 nm on the whole pH range. The particle size of the complexes is constant from pH_c until $pH_{\phi 1}$ (region B), and increases abruptly at $pH_{\phi 1}$ together with the scattering intensity (region C).

The presence of the pH transitions (pH_c and $pH_{\phi 1}$) is comparable to the results of Mattison *et al*. (1995) for a system of BSA-poly(dimethyldiallylammonium chloride) (PDADMAC). However, the difference is that PDADMAC is a highly charged cationic polyelectrolyte. At pH > pH_c (region A in Figure 2.1a and 2.1b) proteins and polysaccharides are both negatively charged and then repulsive Coulombic forces prevent the complexation. This means that biopolymers are soluble in the aqueous solvent, and consequently the scattering intensity measured is low and constant. In this region, the particle size in the WP/GA mixture - determined by the DLS - (region A Figure 2.1b) corresponds to the size of the GA measured in the blank. (The proteins are very small (~3 nm) and, therefore, they do not contribute to the measurement of the hydrodynamic radius.) This result gives us an indication of the size range of the polymer, but cannot be taken as the absolute value of the polymer radius, since the measurements were only performed at one angle (90° in this case) in view of the polydispersity of the sample. The apparent hydrodynamic radius of the GA blank and

the initial radius of the mixture (region A) is high compared to the radius of gyration determined with the SEC MALLS (R_g = 24.4 nm). This result is attributed to the fact that the mixtures were prepared without adding any salt. The GA being negatively charged, the polymer will be in an expanded form due to intramolecular electrostatic repulsions.
In a pH window between pH_c and $pH_{\phi 1}$, the scattering intensity increases but the radius of the particles in the solution decreases strongly compared to the blank of GA (region B Figure 2.1b). If the particle size decreases in the presence of WP, the increase of the scattering intensity is induced by an increase in molecular mass of the particles. The result can be explained by the formation of soluble complexes of WP and GA. The decrease of the GA radius could be due to the shrinkage of the molecule, which becomes less expanded when WP interacts with the carboxylic groups of the polysaccharide, leading to a reduced intramolecular repulsion. The blank of GA does not show such a strong decrease of its radius, the slight decrease of the radius of GA alone can be explained by the addition of microions induced by the acidification. Between pH_c and $pH_{\phi 1}$ (region B), the soluble complexes still carry a net negative charge, and are thus soluble in the solvent [Kaibara *et al.*, 2000]. Furthermore, at low ionic strengths, pH_c appears at a pH above the isoelectric point of the protein (5.2 in the case of native â-lg). Here, it is proposed that soluble complexes are formed by the attraction between some positively charged protein “patches” and the negatively charged polysaccharide [Dubin *et al.*, 1994; Wen and Dubin, 1997]. Finally, macroscopic phase separation is reached in the third region (C), where $pH < pH_{\phi 1}$. The primary complexes tend towards electroneutrality, which allows their association, and phase separation takes place, as illustrated by the strong increase in scattering intensity, turbidity and particle size.

Comparison with pure b-lactoglobulin and á-lactalbumin

The proteins used in this system are WP isolate and their main compound is β-lg, but some molecules of á-la are also present in the powder. It is more likely that the β-lg will mostly determine the complexation with GA. This hypothesis was checked by comparing the behavior of WP with >90% pure β-lg and >90% pure á-la. Figure 2.2 represents the turbidity as a function of pH for WP/GA, β-lg/GA, and á-la/GA. The turbidity of the mixture of WP/GA and the mixture of pure β-lg/GA strongly increases at the same $pH_{\phi 1}$ ($pH_{\phi 1}$ = 4.7). However, the $pH_{\phi 1}$ of á-la/GA shifts to $pH_{\phi 1}$ = 4.1. This result confirms that the interaction between WP/GA is mainly driven by the interaction

of β-lg with GA, and especially concerning the pH_c and $pH_{\phi 1}$ values. The difference between the $pH_{\phi 1}$ of β-lg/GA and á-la/GA is easily understandable since the pI of á-la is 4.1 and the pI of β-lg is 5.2.

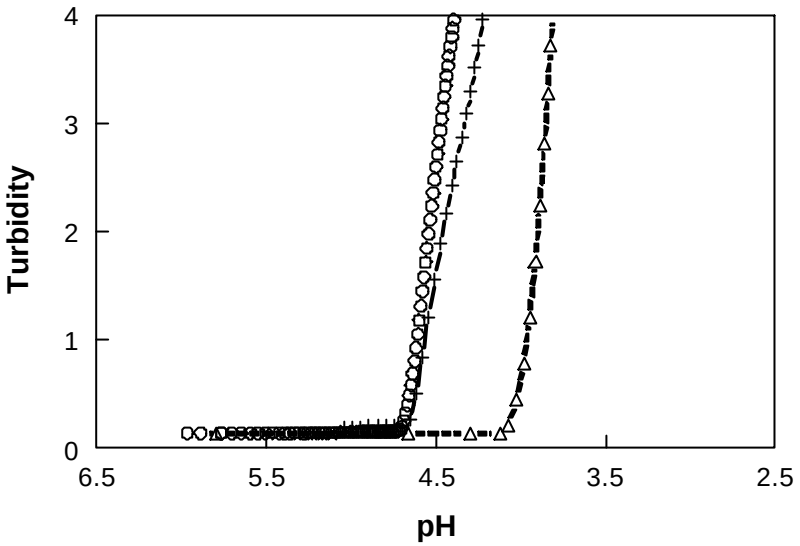

Figure 2.2: Mixture of proteins and gum arabic, Cp = 0.1%, Pr:Ps ratio = 2:1, [NaCl] = 7 mM, [GDL] = 0.13%, Temperature = 25°C, ( ): WP/GA, (+) β-lg/GA; (Δ): α-la/GA.

Therefore, one can conclude that the pH boundaries pH_c (not shown here) and $pH_{\phi 1}$ are due to the formation of a complex between the β-lg and the GA mainly. Of course á-la might form coacervates with the GA as well at lower pH values (pH<4.1) where the β-lg/GA coacervates are already formed. Then the structure of the coacervates, in the two phase region, might be changed or at least influenced by the presence of other proteins in the WP isolate. Furthermore, the β-lg molecule is mainly present as a dimer in solution. However, if some monomers are present, their pI being lower, they might also interact with the GA at lower pH values. However, all this will not influence the main results of this paper.

The values of pH_c and $pH_{\phi 1}$ depend on several parameters such as the pI of the protein, the biopolymer ratio (for the $pH_{\phi 1}$ only), the ionic strength, the molar masses of the biopolymers, but does not depend – or very little - on total biopolymer concentration [Mattison *et al.*, 1995, 1999]. Some previous experiments (not reported here) showed that $pH_{\phi 1}$ is influenced by the Pr:Ps ratio (by increasing the Pr:Ps, the $pH_{\phi 1}$ shifts to

higher pH values, since more proteins are available per polysaccharide chain). In this work the Pr:Ps ratio was kept constant at 2:1, since the pH window where complexation occurs was easily reachable with GDL titration. In the present study, special attention was given to the influence of the ionic strength and the total biopolymer concentration on the phase boundaries. The temperature was kept constant at 25°C.

Effect of the ionic strength

The influence of the ionic strength was studied by adding various amounts of microions (NaCl) to a mixture of 0.1% WP and GA (Cp = 0.1%, Pr:Ps = 2:1). Similarly to the previous experiment, the turbidity and the scattering intensity of the mixtures were monitored during acidification. Figure 2.3a highlights the strong effect of salt concentration on complex formation, since the addition of NaCl causes a significant decrease of the $pH_{\phi 1}$ to lower values. Besides, with increasing ionic strength, the intensity corresponding to the soluble complexes phase decreases.

The effect of ionic strength on complex coacervation is a well-known phenomenon. The microions present in the solution screen the charges of the polymers and thus reduce the range of their associative interactions [Bungenberg de Jong, 1949b; Schmitt *et al.*, 1998; Overbeek and Voorn, 1957]. As a result, the polymers interact at a lower pH, where the protein carries more positive charges. The screening of the charges reduces also the number and the stoichiometry of soluble complexes formed, and the scattering intensity between pH_c and $pH_{\phi 1}$ is therefore lower.

However, it should be noticed here that complexation is influenced by salt concentration above a certain value only. Indeed, as displayed in Figure 2.3a, $pH_{\phi 1}$ has the same values between I=7.6 mM and I=20.3 mM. The addition of small amounts of microions even slightly enhances the coacervation by promoting the solubility of the polymers (coiling of the molecule); the access to the charge is then favored, and so is the electrostatic attraction [Burgess, 1990]. On the other hand, a high salt concentration inhibits complexation by total screening of the charges.

The interaction between GA and WP is a function of their charge density. This charge density is dependent on the pH for the WP but also for the GA since it is a weak (pH-dependent) polyelectrolyte. The electrophoretic mobility measurements made by Schmitt *et al.* (1999) showed that GA tended to electroneutrality at pH 3.5. This method was previously used by Burgess and Carless (1984) for the same polysaccharide and they measured a zero mobility at pH = 2.2. Schmitt *et al.* (1999) explained the

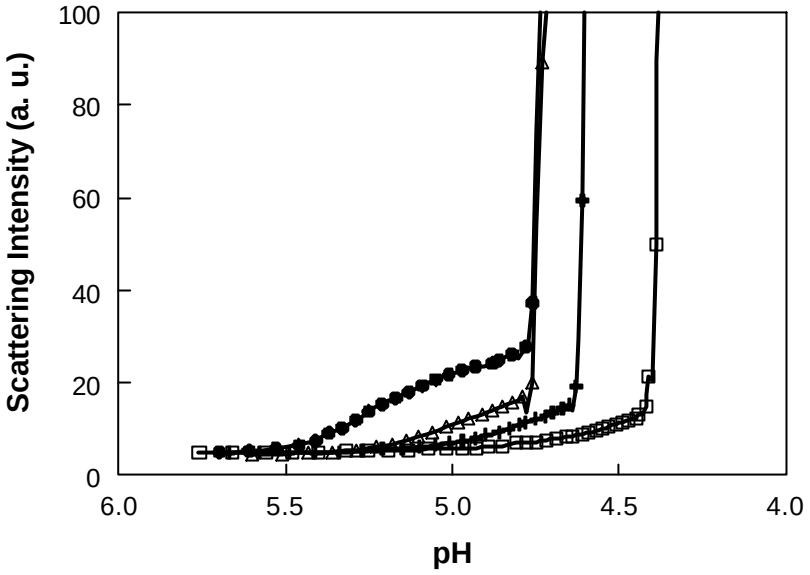

Figure 2.3a: Influence of salt on a mixture of WP/GA, total biopolymer concentration = 0.1%, Pr:Ps ratio = 2:1, [GDL] = 0.07%, Temperature = 25°C, ( ): I_{ini} = 4 mM, I_{fin}= 8 mM; ( ) : I_{ini} = 17 mM, I_{fin}= 20 mM; (**+**) : I_{ini} = 26 mM, I_{fin}= 30 mM; ( ) : I_{ini} = 35 mM, I_{fin}= 39 mM.

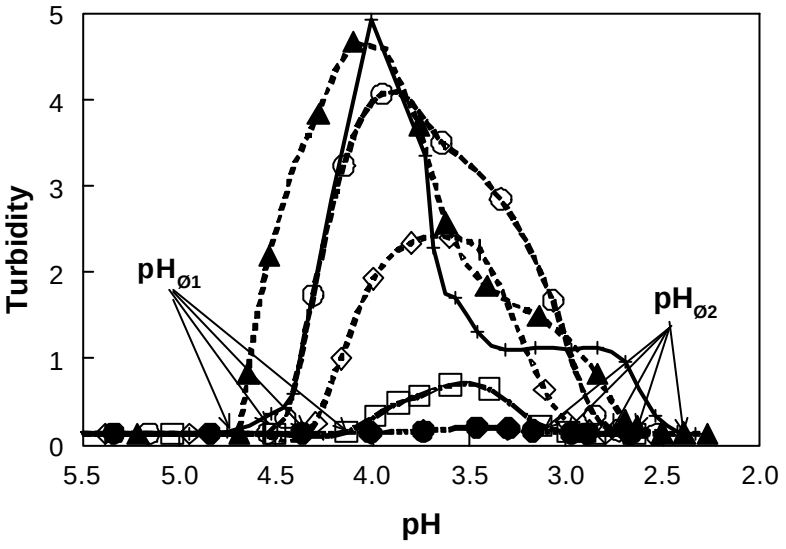

Figure 2.3b: Titration with HCl at different ionic strengths of a mixture of WP/GA, total biopolymer concentration = 0.1%, Pr:Ps ratio = 2:1, Temperature = 25°C; (+): I_{ini} = 0 mM; ( ): I_{ini} = 17 mM; ( ): I_{ini} = 30 mM; ( ): I_{ini} = 40 mM; ( ): I_{ini} = 50 mM; ( ): I_{ini} = 60 mM.

difference by the probable polydispersity and the origin of the GA sample. From our following results, a zero mobility measurement at pH 2.2 seems more probable, since complexation was observed until pH 2.5.

The behavior of the mixture (Cp = 0.1%, Pr:Ps = 2:1) was followed across the pH range 6.0 – 2.0 by HCl titration (this experiment could not be performed with GDL down to this low pH). Examining the $pH_{\phi 1}$ determined with the GDL acidification method gave results identical with those determined by HCl acidification. The influence of ionic strength on the turbidity of the system was studied in a broader pH range. As illustrated in Figure 2.3b, below a certain pH (<3.0), the biopolymer mixture exhibits a loss of turbidity, indicating that phase separation is suppressed at $pH_{\phi 2}$. A maximum in turbidity and two limiting pH values of complexation ($pH_{\phi 1}$ and $pH_{\phi 2}$) are observed, and the region between $pH_{\phi 1}$ and $pH_{\phi 2}$ narrows when the ionic strength is increased. The erratic shape of the curves is due to a phase separation of the sample during the measurement, since at these pH values the mixtures are highly unstable.

By plotting the pH_c, $pH_{\phi 1}$ and $pH_{\phi 2}$ as a function of the ionic strength, the phase boundaries of the 0.1% mixture of WP and GA are identified (Figure 2.4). This resulting state diagram is consistent with the three phases already explained in Figure 2.1, and depends on the main parameters influencing charge density (*i.e.* pH and ionic strength). Above pH_c the large overall negative charges of the biopolymers provide stable mixtures, whereas between pH_c and $pH_{\phi 1}$, the electrostatic associations are becoming favorable to the formation of soluble complexes but the mixture does not demix (region B in Figures 2.1 and 2.3). A further decrease in pH, between $pH_{\phi 1}$ and $pH_{\phi 2}$, leads to phase separation between insoluble complexes of oppositely charged biopolymers and the solvent. As outlined previously, the ionic strength is the other relevant parameter reported on the state diagram. The shrinking of the pH window (between $pH_{\phi 1}$ and $pH_{\phi 2}$) - from low to high NaCl concentration - is due to the screening by microions of the biopolymer charges. Plotting the turbidity value (within the two-phase region) as a function of ionic strength allows us to determine the point of salt resistance. This term was introduced by Bungenberg de Jong (1949b), and is the amount of added salt necessary to prevent coacervation. Here, a high concentration of salt (>54 mM) causes sufficient charge compensation and screening to prevent complexation of the biopolymers.

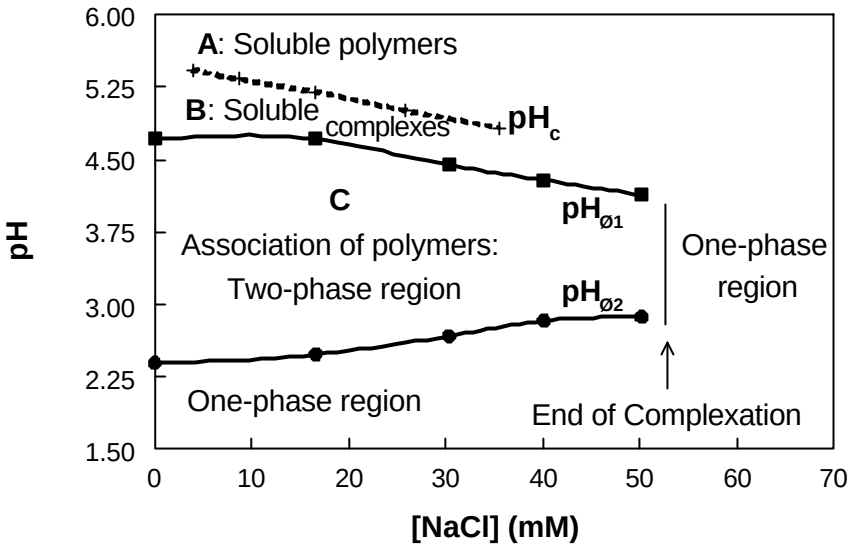

Figure 2.4: State diagram of a mixture of WP/GA, total biopolymer concentration = 0.1%, Pr:Ps ratio = 2:1, Temperature = 25°C, (+) pH_c, ( ) $pH_{\phi 1}$, ( ) $pH_{\phi 2}$.

The existence of a symmetric phase of soluble complexes was studied under highly acidic conditions (below $pH_{\phi 2}$) using static light scattering, but soluble complexes were not observed at this low pH. The transition between the unstable and the stable region seems to be very sharp owing to the neutrality of GA, which suppresses complexation completely. Below $pH_{\phi 1}$ the attraction takes place between a fully negatively charged GA and slightly positively charged proteins. Maybe no soluble complexes can be found between highly positively charged proteins and almost neutral GA. The shape of the GA might be changed so that the interaction is not favorable anymore. However, these ideas should be checked.

Turbidity measurement, while slowly acidifying a mixture of oppositely charged biopolymers such as GA and WP, permits identification of its phase boundaries of demixing. Analytical estimates for pH_c, as discussed in the Theory section, were calculated for our system from the full solution of the random square well model and compared to the experimental data [de Vries *et al.,* 2003]. The Kuhn segment length (l_K) of the polymer was set to l_k = 3nm. Assuming a protein surface area of $2\pi R_p^2$ with a radius $R_p \gg$ 2.5 nm gives $\partial\sigma/\partial pH \approx$ -0.25 nm^2 at the isoelectric point of pI =5.2 for the β-lg dimer, as deduced from titration data for β-lg [Fogolari *et al.*, 2000]. Note that around

the isoelectric point there is considerable self-association of β-lg [Verheul *et al.*, 1999]. This might result in a lower protein surface area available for complexation, but this effect is not taken into account here. Comparing the experimental data with the analytical estimates gives an estimated polyelectrolyte charge density of one elementary charge per 3 nm, which is of the right order of magnitude. Indeed, one can estimate the charge density from the structure of the GA of one carboxylic group per 5 nm [Islam *et al.*, 1997]. The estimate for the proportionality constant μ for the magnitude of the surface charge density variations deduced from the experimental data is m=0.35, which is of order 1, as it should be.

Figure 2.5 shows that with these parameter values, the analytical estimates can indeed account for the experimental data. Note that for low ionic strengths complexation is predicted at pH values above the isoelectric point of the protein, as observed experimentally. The parameters values are perfectly reasonable, which further supports the interpretation of complexation above the isoelectric point as being due to strong variations of the local protein surface charge density, or, in other words, due to positively charged patches on the protein surface.

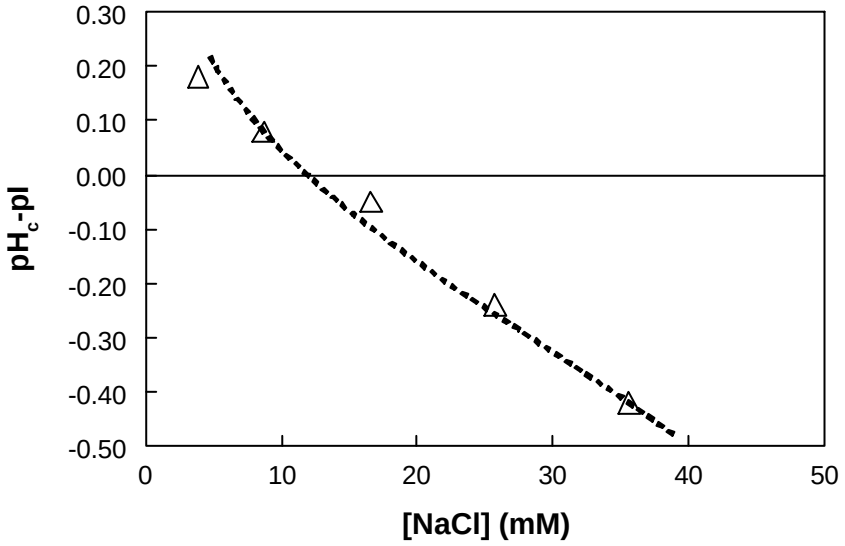

Figure 2.5: Comparison of theoretical model and experimental data of pH_c (for a mixture with Cp = 0.1%, Pr:Ps ratio = 2:1, Temperature = 25°C). ( ): experimental points and dashed line: theoretical model by de Vries *et al.* (2003) which estimates pH_c at which weakly charged polyelectrolytes and globular proteins start forming soluble complexes.

Effect of the total biopolymer concentration

The determination of the transition pH, previously described for Cp = 0.1%, was performed for other biopolymer concentrations (in the range 0.05% - 1%), and the results are presented in Figure 2.6. Although the general trend is that by increasing Cp (from 0.05% to 1%) the $pH_{\phi 1}$ shifts to higher values, the investigation shows slightly less profound effects at low ionic strengths. Indeed, for Cp =0.05% and 1% and [NaCl] <20 mM, the $pH_{\phi 1}$ is initially constant and then decreases with increasing [NaCl], whereas for a Cp of 0.5% and 1%, no significant difference in $pH_{\phi 1}$ is noticeable. Owing to experimental limitations, the pH_c was difficult to determine for all ionic strengths, especially for Cp<0.1% and Cp>1%. However, when no NaCl was added to the mixtures, the pH_c had the same value (pH_c ~ 5.5) for all the total biopolymer concentration.

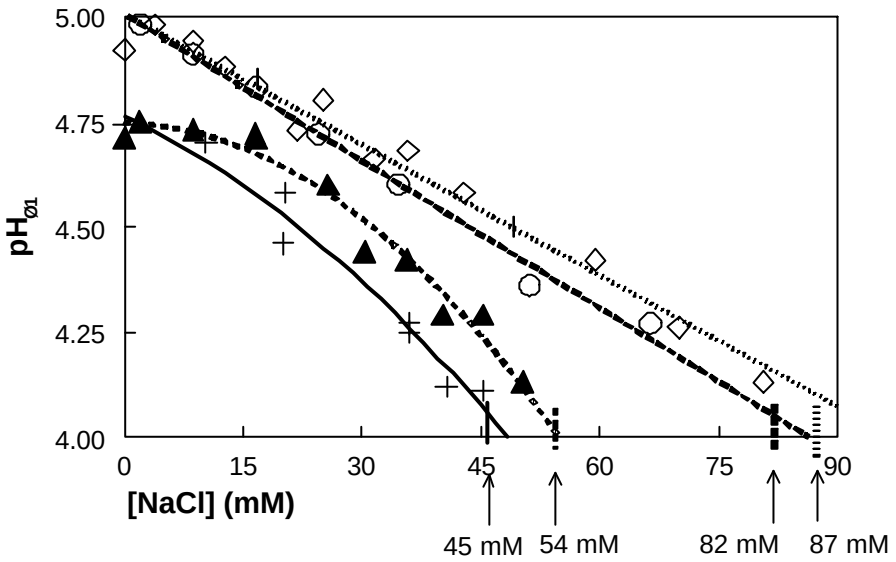

Figure 2.6: State diagram ($pH_{\phi 1}$) of a mixture of WP/GA at various total biopolymer concentrations (Cp), Pr:Ps ratio = 2:1, Temperature = 25°C, (+): Cp = 0.05%; ( ): Cp = 0.10%; ( ): Cp = 0.50%; ( ): Cp = 1.00%

Examining the stable values of pH_c for various Cp supports the hypothesis that soluble complexes are formed between a single polysaccharide and a given amount of proteins, therefore independently of the total amount of biopolymers. This result is in good agreement with the particle size results (Figure 2.1b) that shows that the size of

the soluble complex shrinks upon addition of WP and remains constant between pH_c and $pH_{\phi 1}$. The independence of the pH_c value of concentration was already reported by Mattison *et al.* (1995, 1999) for a system of BSA-PDADMAC, where the total solute concentration, at constant Pr:Ps ratio, had no effect on either pH_c or $pH_{\phi 1}$. These results are consistent with ours on pH_c but not on $pH_{\phi 1}$, probably due to our use of biopolymers at higher concentrations. Indeed Mattison *et al.* reported no mass-action law on his system in a Cp window 0.01% - 0.3%, whereas the Cp window in this study is broader and higher (0.05% - 1%). Besides, biopolymers were used instead of synthetic polymers. For a similar system (â-lg and GA), Schmitt (2000b) found that increasing biopolymer concentration reduces the influence of pH on complex coacervation, which is in accordance with our study. At Cp = 0.5% and 1%, the shift in the $pH_{\phi 1}$ values is not as significant as for lower concentrations.

The binary phase diagram, obtained at pH 3.5, provides information on the influence of the concentration of microions as a function of total biopolymer concentration. Here, higher values of Cp were measured (up to 20%). In Figure 2.7, the phase diagram built with various methods clearly illustrates the boundaries above which the biopolymer mixture remains stable. Conductivity measurements indicate the amount of salt present in the mixture and are another measure of the ionic strength. In the main graph, the conductivity due to the presence of the biopolymers itself was subtracted and only the influence of NaCl was plotted. However, the conductivity due to the presence of all ions is reported in the inset of Figure 2.7. The shift of the phase line to higher conductivity values is due to the presence of the counterions.

Using the “adding salt” method, the salt concentration ([NaCl]) at which the complexes dissociated was determined. A high ionic strength inhibits complex coacervation and this critical [NaCl], also called salt resistance, varies with Cp. Indeed, as Cp increases up to 1%, a larger increment of NaCl is required to reach the point of salt resistance, but for Cp > 1% the critical [NaCl] decreases finally to zero for Cp = 15%. An optimum Cp could then be determined at Cp = 1%. And at Cp = 15%, the loss of turbidity is indicative of a stable mixture, phase separation does not take place (with the “adding salt” method).

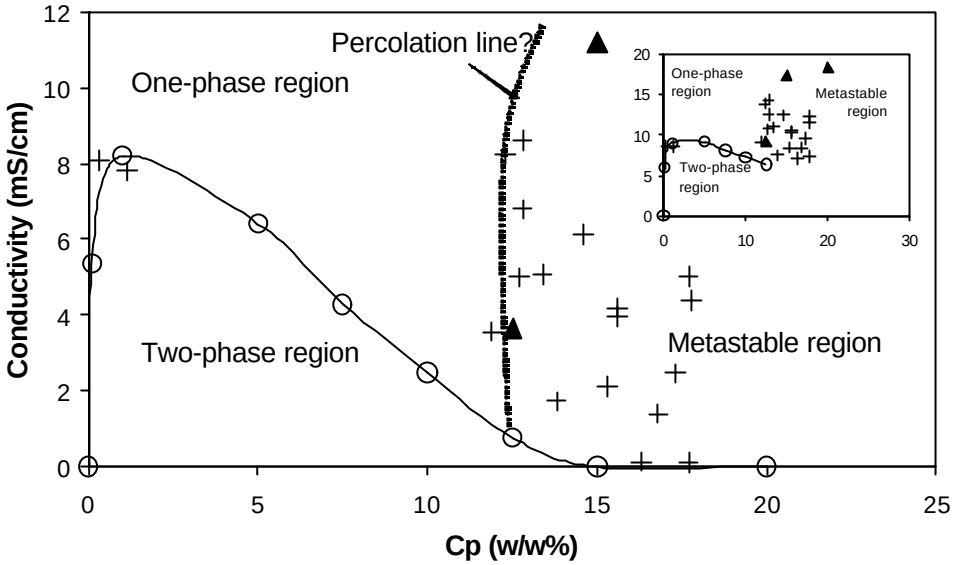

Figure 2.7: Phase diagram of mixtures of WP/GA, Pr:Ps ratio = 2:1, pH 3.5, Temperature = 25°C, with the "adding salt method" ( ), "removing salt method" ( ), and the "dilution method" (+). In the inset, the conductivity due to the presence of all ions is plotted.

These results are in agreement with the previous results shown in Figure 2.6, where a $pH_{\delta 1}$ shifts to higher values as the Cp increases to 1%. Furthermore, at low Cp and for a conductivity of 6 mS/cm, the system is in the one-phase region, since the microions are screening the charges of the biopolymers. If one increases the Cp to 0.5%, there are not enough microions to screen the additional biopolymer added; the system is moved to the two-phase region.

The effect of the total biopolymer concentration is not extensively reported in the literature, and it varies depending on the polyelectrolytes used. As discussed by Burgess (1990) for a system of gelatin-BSA, the salt resistance of a mixture varies with the total polymer concentration (the amount of microions required for suppression of complexation increases with increasing the concentration, until the maximum intensity is reached, and then decreases). Our results are in agreement with hers. Moreover, an optimum Cp of 2%, was already observed for a pectin-gelatin system by McMullen *et al.* (1982), who explained it in terms of a mass action effect. Then, above this optimum Cp, the efficiency of coacervation is decreased, as illustrated in the phase diagram (Figure 2.7). Indeed, increasing the total biopolymer concentration favors the release of more counterions in solution, which screen the charges of the biopolymers, suppressing coacervation, and increasing the solubility of the coacervates. The phase

diagram built by the “adding salt” method (Figure 2.7) points out the characteristic features of complex coacervation as described in the theory of Overbeek and Voorn (1957). Below a critical salt concentration and a critical polymer concentration, the mixture demixes in a polymer rich phase and an aqueous phase poor in polymers. The composition of the two phases becomes closer when the concentration of microions is increased, and finally reaches a critical point, beyond which phase separation no longer occurs.

The phase diagram was also built using different methods at high Cp, by using a second method (“removing salt”). For Cp > 12.5% the mixtures become much more unstable and exhibit a high turbidity even at high concentration of microions. Since one cannot exclude the possibility of protein adsorption of the resins, a third method was used. This “dilution method” provides further evidence for this unstable region. It seems that above 12.5% of biopolymers the system is in a “metastable” state. Indeed, the measurements were difficult to duplicate (they were unstable at different Cp values) but they all show instability in these regions for the “dilution method” and the “removing salt method”. However, this region was not found using the “adding salt” method, which means that it is path-dependent. Furthermore, this instability occurs only when both biopolymers are in solution. Controls with only WP and only GA stayed clear, refuting the hypothesis that this high turbidity would be induced by the aggregation of one of the biopolymer.

Our tentative and imaginative explanation of the results is as follows. As shown in Figure 2.7 WP/GA form complexes, which can be viewed as new and separate entities. These new particles are dynamic in nature; *i.e.* the WP and the microions can be exchanged. On a time average scale these “WP/GA particles” have an effective interaction potential (not to be confused by the potential between the WP and the GA). The interaction potential is probably repulsive just below pH_c but is strongly attractive at $pH\phi_1$. In the two-phase region (*e.g.* at pH 3.5) the interaction potential between the WP/GA complexes is also a function of salt concentration.

Now, the shape of the phase diagram in Figure 2.7 is reminiscent of a gas-liquid demixing of a polymer or colloid in solution, where salt concentration here would play the role of temperature in a gas-liquid demixing [Poon *et al.*, 1996]. Therefore, Figure

2.7 can be understood as the coexistence of two phases: a dilute phase and a concentrated phase of WP / GA complexes. The analogy goes even further. Just as in gas-liquid demixing, a solid (crystal) phase appears at high concentrations. For colloid mixtures, this phase is usually hidden under a glass phase delimited by a percolation line. The position of the percolation line depends very much on the range of the colloidal attractions [Poon *et al.*, 1996]. Short-ranged interactions tend to shift the glass transition to lower concentrations. Possibly therefore complexes of strong polyelectrolytes form “precipitates”. For the WP/GA system interactions seem to be relatively weak (low charge density and screening by uncharged polymer) and then the glass transition would be expected at a higher concentration. The unpredictable behavior in the glass state region shows similarities to systems that show delayed creaming or delayed phase separation. The dynamics of the system are time and preparation path dependent. It is therefore suggested that much of the phase behavior can easily be understood by considering the WP/GA complexes as separate entities and the strength of their attractive interactions depends on salt concentration. Sure enough the description is speculative, but the parallels with colloidal demixing are striking.

CONCLUSIONS

The in situ titration method – using GDL – combined to light scattering, turbidity and particle size measurement allowed us to follow the formation of soluble and insoluble complexes. The complexation of WP and GA seems to be mainly driven by the interaction of β-lg (main component of the WP) and GA. The effect of various parameters on the complex coacervation between WP and GA has been investigated. A state diagram was built, where the two main parameters influencing complex coacervation are taken into account (*i.e.* pH and ionic strength). It revealed the region of stability and instability of the system. Below a certain pH_c, WP and GA form electrostatic (stoichiometric) complexes as predicted by a theoretical model. A lower boundary ($pH\phi_2$) was found due to the neutrality of the GA. The pH window where complex coacervation occurs shrinks with increasing ionic strength, due to the screening of the charges by the microions. Although the effect of total biopolymer concentration is certainly less evident than the influence of pH and ionic strength, it is, however, relevant, since an optimum concentration exists, where complex coacervation

is maximum. The complexes can be viewed as separate entities, where interaction depends on salt concentration. At low salt concentration, the complexes demix into a complex rich and a complex poor phase, just as in a gas-liquid coexistence. At high concentrations, a glassy state with poorly defined kinetics is formed. Future work includes a further experimental confirmation and a better understanding of the formation of the soluble complexes and the structure of the coacervate phase. Other polysaccharides will be tested as well.

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