CHAPTER 3

Complex Formation of
Whey Protein / Exocellular Polysaccharide EPS B40*

ABSTRACT
Whey proteins (WP) and the exopolysaccharide B40 (EPS B40) form electrostatic complexes under specific conditions. EPS B40 is a natural thickener in yogurt-like products. It is a phosphated polysaccharide and thus has a strong polyelectrolyte character. When the WP and the EPS B40 were mixed at pH values near or below the isoelectric point (pI) of the protein, soluble complexes were formed at pH\(_c\) and phase separation took place below pH\(_\phi\). The formation and the structure of those complexes were studied by various methods, including turbidity, dynamic and static light scattering (DLS and SLS), and viscosity measurements. The results showed that the strength of the interaction was strongly pH- and salt-dependent. The zeta-potential of the protein at pH\(_c\) and pH\(_\phi\) was linearly dependent on the square root of the ionic strength (√I), showing the electrostatic nature of the interaction. Light scattering and viscosity measurements provided new results on the behavior of the complexes at the molecular level. In the region where the complexes were still soluble and at low ionic strength, the DLS radius measured in the WP/EPS B40 mixture was smaller than the coil size in the EPS B40 solution but the apparent molar mass was increased. The increase of the molecular mass was attributed to the complexation of WP on the EPS B40 chain, which, at low salt, induced a reduction of the intramolecular repulsion and led to the compaction of the polysaccharide. Also, the ratio of protein to polysaccharide was varied in order to get more insights into the dynamics, the structure and the apparent stoichiometry of the EPS B40/WP complexes. The results illustrated that phase separation was a consequence of charge neutralization of the complexes and that the apparent stoichiometry of the complexes depends on the order of mixing of the compounds. In time, the complexes rearranged to form neutralized complexes and free EPS B40. The concept of cooperative binding was highlighted in the case studied.

*F. Weinbreck, H. Nieuwenhuijse, G. W. Robijn, C. G. de Kruif
Langmuir 2003, 19, 9404-9410.
Chapter 3

INTRODUCTION

Associative interactions between proteins and polysaccharides, \textit{i.e.} between amphiphiles and strong polyelectrolytes, are of relevance for many products in both food and pharmaceutical science [Dickinson, 1998; Doublier \textit{et al.}, 2000; Sanchez and Paquin, 1997; Tolstoguzov, 2002]. The interactions between proteins and polysaccharides can be either repulsive, or attractive. The last induces interbiopolymer complexing, which almost always arises from electrostatic interactions between opposite charges on the biopolymers. At pH values below their isoelectric point (pI), proteins carry positive charges and can interact with polysaccharides bearing carboxylic, phosphate or sulfate groups. The first empirical experimental studies were made by Bungenberg de Jong in the 1940’s (1949a, 1949b, 1949c), studying the complex coacervation of gelatin and gum arabic. The theoretical work on complexing oppositely charged polymers was initialized by Overbeek and Voorn (1957). They extended the Flory-Huggins theory with an extra term accounting for electrostatic interactions, for which they used the Debye-Hückel approximation. Later theories on complex coacervation were refinements of the Overbeek-Voorn theory. A number of recent theoretical papers deal with the complexation of homogeneously charged spheres and oppositely charged polyelectrolytes [Netz and Joanny, 1999; Nguyen and Shklovskii, 2001]. They could be applied to the complexation of oppositely charged proteins and polyelectrolytes. Recently, de Vries \textit{et al.} (2003) considered the binding of a polyelectrolyte to the surface of a protein by considering the protein as a sphere with random charge patches, explaining the complexation between similarly charged proteins and polyelectrolytes (above the pI of the protein).

Current work in the field reports the influence of various parameters (\textit{e.g.} charge density, pH, ionic strength, and biopolymer concentration) on the electrostatic interactions of polymers [Dubin and Oterie, 1983; Schmitt \textit{et al.}, 1998]. It is now well-known that pH and ionic strength are key parameters that can regulate the strength of the electrostatic interactions. Mattison \textit{et al.} (1995), for example, reported the phase boundaries of protein-polyelectrolyte systems. Using turbidimetric titrations, they determined the pH-induced structural transitions of synthetic polymer complexes. The formation of soluble protein-polyelectrolyte complexes was initiated at pH$_t$, which preceded the pH of visual phase separation at pH$_p$ [Mattison \textit{et al.}, 1995; Dubin \textit{et al.},...
Complex formation of whey protein / exocellular polysaccharide EPS B40

1987; Kaibara et al., 2000]. These pH-induced transitions were also determined for whey proteins and gum arabic [Weinbreck et al., 2003a; Weinbreck and de Kruif, 2003b]. Gum arabic is a polysaccharide that carries carboxylic groups and can be considered as a weak polyelectrolyte whose charge density is pH-dependent. The electrostatic interactions between whey proteins and gum arabic lead to the formation of coacervates in the pH window between 2.5 and 4.8 [Weinbreck et al., 2003a; Weinbreck and de Kruif, 2003b; Schmitt et al., 1999; Schmitt 2000b].

However, many of the polysaccharides used in food systems are of the strong polyelectrolyte-type, for example, the carrageenans and the exopolysaccharide B40 (EPS B40) from lactic acid bacteria, carrying sulfate and phosphate groups, respectively. The objective of this work was to study the interaction between whey proteins and a phosphated polysaccharide naturally present in yogurt-like products, the exopolysaccharide EPS B40. The EPS B40 is excreted by the lactic acid bacterium Lactococcus lactis subsp. cremoris NIZO B40 during fermentation and thus acts as a natural thickener in acidified milk products. EPS B40 has already been extensively studied at NIZO food research from a genetic point of view [van Kranenburg, 1999]; the physical properties of the EPS and its role in fermented milks have been studied by van Marle et al. (1999) and Ruas-Madiedo et al. (2002), and the segregative phase separation between EPS B40 and dairy proteins at neutral pH was studied by Tuinier et al. (1999, 2000). Thus, the anionic polysaccharides play an important role in the texture and stability of dairy/food products. Since almost all food products have an acidic pH, it is clear that the complex formation between proteins and polysaccharides is of prime interest. This model study aims to clarify, first, whether pH transitions and soluble/aggregated complexes are formed for a mixture of whey proteins and EPS B40 in acidic conditions at various ionic strengths, using turbidity measurements. The formation of soluble complexes was explored at the molecular level using three different methods (i.e. dynamic and static light scattering and viscosity measurement). Finally, the influence of the protein to polysaccharide (Pr:Ps) ratio and the order of polymer mixing allowed more insights into the structure, the properties, and the apparent stoichiometry of the complexes which were previously unknown.
EXPERIMENTAL SECTION

Materials
Bipro is a whey protein (WP) isolate consisting mainly of 75% \( \alpha \)-lactoglobulin (\( \alpha \)-lg), and 15% \( \alpha \)-lactalbumin (\( \alpha \)-la) - from Davisco Foods International (Le Sueur, MN). Residual whey protein aggregates were removed by acidification as previously described [Weinbreck et al., 2003a]. The exopolysaccharide EPS B40 was produced and isolated from \textit{Lactococcus lactis} subsp. \textit{cremoris} NIZO B40 at NIZO food research (The Netherlands) as described by Tuinier et al. (1999). The freeze-dried powder was stored at 5°C. It contains 63% EPS B40, 18% protein, 8% ash, 6% mannan-rich material and 5% water. The weight-averaged molar mass (\( M_w \)) and the average radius of gyration (\( R_g \)) were determined with SEC MALLS (\( M_w = 1620 \) kg/mol and \( R_g = 86 \) nm). The ionic strength was adjusted by addition of reagent-grade NaCl. Deionized water was used in all experiments. Control measurements with only WP and only EPS B40 were systematically carried out under the same conditions as the mixtures of biopolymers. The zeta-potential of the WP and EPS B40 mixture was measured as a function of pH with a Zetasizer 2000 (Malvern, USA).

Turbidimetric titration under acidification
Mixtures of WP and EPS B40 (initial pH = 7) were acidified by dropwise addition of HCl. The influence of the ionic strength ([NaCl] = 0 – 1M), the protein to polysaccharide ratio (Pr:Ps = 1:1 – 25:1 w/w) and the total biopolymer concentration (\( C_p = 0.05\% - 0.5\% \)) were studied by varying one parameter at a time. The turbidity of each sample was measured as a function of the pH with a Cary 1E spectrophotometer (Varian, The Netherlands) at a wavelength of 514.5 nm (similar to previous measurements [Weinbreck et al., 2003a]). The samples were put in a 1 cm path-length cuvette and the turbidity was then measured as a function of time at 25°C. The turbidity (\( \tau \)) was defined as:

\[
\tau = - \ln \left( \frac{l}{l_0} \right),
\]

where \( l \) is the light intensity that passes through a volume of solution of 1 cm length and \( l_0 \) the incident light intensity. (The turbidity corresponds thus to the optical density multiplied by 2.3).
Turbidimetric titration under salt addition
The turbidity of WP / EPS B40 mixtures was followed upon addition of NaCl at pH 3 and pH 4 for Cp = 0.1% and at a Pr:Ps ratio of 2:1. The initial mixture was adjusted to pH 4 or pH 3. Then, a known amount of NaCl was added in powder form to the mixture and at each salt addition, the turbidity of the mixture was measured. Blanks of mixtures at pH 7, WP at pH 3 and EPS B40 at pH 3 were also titrated in the same way.

Static and dynamic light scattering measurement
The static light scattering (SLS) and dynamic light scattering (DLS) experiments were carried out on mixtures of 0.1% w/w biopolymer concentration at various pH values using a 22 mW HeNe laser with a wavelength of 632.8 nm. The light beam was focused on the axis of the goniometer using mirrors and a lens. The stock solutions of WP and EPS B40 were initially filtered with a 0.45 μm filter separately to avoid the filtration of some initial complexes, and the mixture of WP / EPS B40 was then centrifuged for 30 s to remove all impurities and air bubbles. The sample was placed in the cuvette housing, which was kept at a temperature of 25°C in a toluene bath. The scattering angle was varied from 30° to 150° in 10° steps. The measurement was repeated three times at each angle. The detected intensity was processed by a digital ALV-5000 correlator. From the intensity as a function of angle, the molar mass and the radius of gyration of the particles could be determined as a function of pH.

Viscosity measurement
The viscosity of the WP/EPS B40 mixtures was obtained using a capillary viscosimeter Ubbelhode (Schott Geräte, Germany), using a capillary No. Ic that can contain 20 mL of dispersion. The viscosity measurements were carried out at 25°C on samples containing 0.1% WP/EPS B40 at a Pr:Ps ratio of 2:1, and at [NaCl] = 0 and 25 mM. The samples were acidified with glucono-δ-lactone (GDL), and their viscosity was related to the pH of the sample. Calibration was done with water.

Titration of one polymer by the other
The titration was performed by i) slowly adding under stirring 0.1% WP into 0.1% EPS B40 (titration A) or ii) adding 0.1% EPS B40 into 0.1% WP (titration B). These titrations were done at pH 3 and at pH 4 on mixtures of low ionic strength. The Pr:Ps ratio was then recalculated and at each ratio, the turbidity of the sample was measured.
High-Performance Liquid Chromatography (HPLC) measurements

The amount of residual WP and EPS B40 in the upper phase was followed in time for a mixture of WP / EPS B40 (Cp = 0.1%, Pr:Ps = 4:1, pH = 4). The samples were prepared with titrations A and B and they were left under continuous stirring for 10 days. Every day, a sample was taken out. After 2h of natural phase separation, the amount of β-lg, α-la, and EPS B40 was analyzed by HPLC, using a Biosep Sec 2000 column at a pump flow of 0.7 mL/min. The proteins were detected with the UV at 280 nm (Applied Biosystems) and the EPS B40 was detected by refraction index (RI, Erma-7510, Betron Scientific) and the concentration was extrapolated from a calibration plot.

RESULTS AND DISCUSSION

Behavior of the system as a function of pH and salt

Because the interaction between WP and EPS B40 was expected to be mainly electrostatic in nature, pH and ionic strength would play a key role in the complex formation [Schmitt et al., 1998]. Indeed, pH affects the ionization degree of the amino groups of the protein, and electrostatic complexing will take place below the pI of the protein. If microions are present in the solution, they can interact with the charged groups of the polymers and affect complex formation. Therefore, the influence of pH and ionic strength on the interaction between WP and EPS B40 was first investigated by means of turbidity measurements and macroscopic observations. Figure 3.1 illustrates the turbidity of mixtures titrated with HCl for ionic strengths ranging from 0 to 200 mM.

Under acidification, the mixtures of WP/EPS B40 showed an increase of turbidity, while the turbidity of control samples with only WP or EPS B40 remained constant at a low turbidity over the whole pH range (results not shown here). The turbidity increase of WP / EPS B40 mixtures was attributed to the formation of electrostatically bound protein – polysaccharide complexes, which initially occurred at a pH close to the pI of the protein (pI = 5.2). Specifically, for [NaCl] < 75 mM, the turbidity curves increased in two steps. These two pH transitions were designated as pH\(_c\) and pH\(_φ\). The first increase appeared at pH\(_c\) (in the pH window 5.4 – 4.5), and the second at pH\(_φ\) (between pH 4.5 and pH 3), below which macroscopic phase separation took place. For [NaCl] > 75 mM, only one increase in turbidity was recorded (at pH\(_c\)) and no macroscopic phase separation occurred.
Figure 3.1: HCl titration at various ionic strengths of a mixture of WP / EPS B40, total biopolymer concentration = 0.25%, Pr:Ps ratio = 2:1, temperature = 25°C; (+): [NaCl] = 0 mM; ( ) : [NaCl] = 10 mM; ( ) : [NaCl] = 25 mM; ( ) : [NaCl] = 40 mM; ( ) : [NaCl] = 50 mM; ( ) : [NaCl] = 75 mM, ( ) : [NaCl] = 100 mM, ( ) : [NaCl] = 200 mM.

Figure 3.1 also highlights the strong effect of the addition of salt microions to the mixture. Indeed, the addition of NaCl caused a shift of pHₙ and pHₚ to more acidic values and the maximum turbidity decreased. Figure 3.2 summarizes the results shown in Figure 3.1 by plotting the values of pHₙ and pHₚ as a function of the ionic strength. The values of pHₙ and pHₚ at various Cp values are also reported in the figure (Cp = 0.05%, 0.1%, 0.25%, and 0.5%). The resulting state diagram illustrates that the pH boundaries (pHₙ and pHₚ) seemed to be independent of the total biopolymer concentration, but they were strongly dependent upon ionic strength.

At pH > pHₙ, the dispersions were fully transparent; both polymers were negatively charged, which prevented their complexation. At pHₙ, the turbidity of the mixture increased, showing that some attraction between the polymers took place. However, no macroscopic phase separation occurred. This result would suggest that the formation of primary complexes between WP and EPS B40 was first induced at pHₙ. Then, at pHₚ, the turbidity abruptly increased, illustrating further aggregation of the complexes, which will require that they approach a neutrality condition; the mixture became unstable and phase-separated in time. The pH transitions are salt dependent, which is
a well-known phenomenon. The microions screen the charges of the polymers and thus reduce the range of their associative interaction [Bungenberg de Jong, 1949b; Overbeek and Voorn, 1957; Schmitt et al., 1998]. As a result, the polymers interact at a lower pH, where the protein carries more positive charges. If the [NaCl] was high enough (> 50 mM), the charges of the biopolymers were screened and the electrostatic interaction was insufficient to induce electroneutrality of the complexes and thus aggregation. Varying the total biopolymer concentration (Cp) did not seem to affect the interaction between the WP and the EPS B40 (i.e. pH$_c$ and pH$_\phi$). As demonstrated by Mattison et al. (1995), the Cp only influences the amount of complexes formed. And since the charge balance between WP and EPS B40 is not modified by increasing Cp, the phase boundaries will occur at the same pH. These results are in agreement with results previously found for synthetic polymers and other biopolymers, where two pH transitions (pH$_c$ and pH$_\phi$) were also reported [Mattison et al., 1995; Weinbreck et al., 2003a; Weinbreck and de Kruif, 2003b].

![Figure 3.2](image.png)

**Figure 3.2:** State diagram as a function of [NaCl] of a mixture of WP / EPS B40, Pr:Ps ratio = 2:1, temperature = 25°C, for various total biopolymer concentrations (Cp) ( / ) Cp = 0.05%, ( / ) Cp = 0.1%, ( / ) Cp = 0.25%, (V/ ) Cp = 0.5%, open symbols: pH$_c$ and filled symbols: pH$_\phi$.

Next, to check whether the aggregated complexes were reversible, the titration upon salt addition was carried out on mixtures of WP / EPS B40 at a pH where aggregation of the complexes already occurred (Figure 3.3). The turbidity of mixtures containing
initially insoluble complexes (at pH 3 and pH 4) was monitored as a function of NaCl concentration. As the ionic strength increased, the turbidity of the mixture dropped to lower turbidity values, which occurred faster at pH 4 than at pH 3 (Figure 3.3). At [NaCl] = 200 mM, the turbidity values were similar to the values of the soluble complexes in Figure 3.1. Since the turbidity of the blanks (WP and EPS B40) was low (<0.5), it indicated that the turbidity at high salt concentrations was not due to a salting out of the protein (blanks not shown here). This result would suggest that at high salt concentration, soluble complexes were still present in the mixture, which is consistent with the result of the state diagram (Figure 3.2). The addition of salt weakened the interactions between the biopolymers and the precipitates dispersed spontaneously into soluble complexes. The first breaking point of the turbidity occurred at [NaCl] = 20 mM at pH 4 and [NaCl] = 50 mM at pH 3 for Cp = 0.25%. These values corresponded to the salt concentration at which the phase boundary was crossed in Figure 3.2 (denoted as the point of salt resistance) [Bungenberg de Jong, 1949b]. The formation / dissociation of the aggregated complexes was thus reversible. The soluble complexes remained in solution in the salt concentration range studied.

Figure 3.3: Salt titration of WP / EPS B40 mixtures, Pr:Ps = 2:1, temperature = 25°C, ( / ) Cp = 0.1%, ( / ) Cp = 0.25%, open symbols: pH 3, filled symbols: pH 4.
The effect of pH on the complex formation arises from the dependence of protein charge density on pH [de Vries et al., 2003; Mattison et al., 1999]. The influence of ionic strength can easily be understood if the complexation is mainly electrostatic in nature. Indeed, by addition of microions, electrostatic screening causes pH\(_c\) and pH\(_φ\) to decrease with increasing ionic strength. In a previous study, the value of the zeta-potential of the protein has been determined as a function of pH (results not shown here). Therefore, another representation of the state diagram would be to convert pH (pH\(_c\) and pH\(_φ\)) to protein zeta-potential and plot it as a function of the square root of the ionic strength (\(\sqrt{I}\)) (Figure 3.4).

![Figure 3.4: Data of Figure 3.2 presented as the zeta-potential of WP as a function of the ionic strength (I\(^{1/2}\)) for a WP / EPS B40 mixtures, Pr:Ps = 2:1, Temperature = 25°C, ( ): Zeta-potential at pH\(_c\) ( ): Zeta-potential at pH\(_φ\).](image)

Both zeta-potentials at pH\(_c\) and pH\(_φ\) were linear with \(\sqrt{I}\), showing that the critical surface charge density was proportional to the Debye-Hückel parameter (\(\hat{e}\), \(\hat{e}\)- \(\sqrt{I}\)) and that the system is thus controlled by electrostatic forces. This result is consistent with several theoretical treatments [Muthukumar, 1987, 1995; Evers et al., 1986], and this relation has already experimentally been described for a complexation between proteins and a synthetic strong polyelectrolyte [Mattison et al., 1995]. If the linear relation is extrapolated, the presence of soluble complexes would then be suppressed at [NaCl] =
0.75 M at pH 4 and 1.5 M at pH 3. From general properties of the Debye-Hückel term, it is evident that adding salt promotes decomplexation.

**Events at the molecular level**

To get more insights into the complexation at the molecular level, light scattering experiments (SLS and DLS) were carried out on a mixture of 0.1% WP/EPS B40 at Pr:Ps = 2:1 - and on EPS B40 blanks - as a function of pH. The values of the $R_g$ and $M_w$ of the mixture were normalized to the values of the $R_{g0}$ and $M_{w0}$ of the EPS B40 blank (Figure 3.5).

![Figure 3.5](image)

**Figure 3.5:** Normalized molecular weight ($M_w / M_{w0}$) and radius of gyration ($R_g / R_{g0}$) of a mixture of WP / EPS B40 compared to an EPS B40 blank, Pr:Ps = 2:1, total biopolymer concentration = 0.1%, temperature = 25°C; [NaCl] = 0 mM; (): $M_w$, ( ): $R_g$.

At pH > pH$_c$, $R_g$ was close to the value of $R_{g0}$ ($R_g / R_{g0} = 1$) and the $M_w$ in the mixture was equal to the $M_{w0}$ of the EPS B40 blank ($M_w / M_{w0} = 1$). Between pH$_c$ and pH$_φ$, the $R_g$ of the EPS B40 in the mixture decreased by 12% of its initial size and the $M_w$ of the polymer increased by a factor of 4. In view of the fact that Pr:Ps = 2:1, an $M_w$ increase of a factor of 3 would be expected. Indeed, if we estimate that all the WP interacts with the EPS B40 (maximum interaction), the $M_w$ of the complex will be $2 \times M_w + 1 \times M_w$, since the Pr:Ps ratio is expressed in w/w. The experiments showed an increase of a factor of 4, which is, concerning the uncertainties, in good agreement with the
estimation. At pH sub{phi}, the M_w strongly increased and the sample was very turbid. Since the experiments were carried out at low salt concentrations, long-range interactions took place in the system and the values of R_g and M_w will therefore be uncertain. Besides, because of the polydispersity of the sample and the difficulty of interpreting light scattering experiments on turbid systems (for pH > pH sub{phi}), viscosity measurements were carried out in order to check if the same trend is found as with the light scattering.

Figure 3.6 represents the evolution of the viscosity of a mixture of WP/EPS B40 (Cp = 0.1%, Pr:Ps = 2:1) as a function of pH, compared to a blank of EPS B40, at [NaCl] = 0 and 25 mM. For [NaCl] = 0 mM, the viscosity of the EPS B40 blank was larger than the viscosity of the WP / EPS B40 mixture, which could be explained by the fact that the ionic strength was increased when acid was added to the mixture. The viscosity of the WP / EPS B40 mixture decreased as a function of pH and especially at pH sub{c} and pH sub{phi} (determined by turbidity measurements), where two breaking points could be noticed. On the other hand, for [NaCl] = 25 mM, both the viscosity of the blank of EPS B40 and the viscosity of the mixture of WP / EPS B40 remained constant until pH sub{phi}, where the viscosity of the mixture of WP / EPS B40 decreased. The viscosity of a dilute polymer mixture is directly related to the size of the particle. Therefore, the viscosity measurement is another means of measurement of the particle size, which is less influenced by the polydispersity (and turbidity) of the sample. Bungenberg de Jong (1949b) used this type of measurement, and he attributed the decrease in the viscosity before and during the actual complexation to a reduction of the amount of liquid occluded inside the complexes. The decrease in viscosity of polyelectrolyte systems has been used to determine the optimum conditions of complexation [Ganz, 1974; Koh and Tucker, 1988a] and it was reported that the low viscosity close to the point of complexation is consistent with intrapolymer condensation [Dubin and Oterie, 1983].

As illustrated in Figures 3.5 and 3.6, the size reduction of the polymer with a simultaneous increase of the molecular weight highlights the complexation of WP molecules to the EPS B40 chain, indicating the formation of soluble complexes. The shrinkage of the EPS B40 molecule, occurring at low ionic strength and not at [NaCl] = 25 mM, could be understood as a reduction of the intramolecular repulsion induced by the interaction of the WP with the phosphate group of the EPS B40. At low ionic strength, this interaction could even occur at a pH above the pl of the protein (pl = 5.2)
because of the presence of positive “patches” on the WP [de Vries et al., 2003; Dubin et al., 1994; Wen and Dubin, 1997]. In this pH window, the mixture did not phase separate; therefore, the complexes are called “soluble complexes”. Furthermore, the increase of turbidity in the soluble complexes region in Figure 3.1 can now be attributed to an increase of the molecular weight of the compounds and not an increase of their size. At pH$_0$ the complexes aggregate together, as shown by the dramatic increase of the molecular weight and the strong decrease of the viscosity due to two-phase flow / phase separation.

![Graph](image)

**Figure 3.6:** Viscosity measurement of (): a blank of EPS B40, Cp = 0.03%, [NaCl] = 0 mM; (): a mixture of WP / EPS B40, Cp = 0.1%, Pr:Ps ratio =2:1, [NaCl] = 0 mM; (+): a blank of EPS B40, Cp = 0.03%, [NaCl] = 25 mM; (): a mixture of WP / EPS B40, Cp = 0.1%, Pr:Ps ratio =2:1, [NaCl] = 25 mM; temperature = 25°C.

The results presented here for a strong polyelectrolyte were reminiscent of the results found previously for a weak polyelectrolyte (gum arabic) [Weinbreck et al., 2003a]. The influences of parameters such as pH and salt were similar. However, some soluble complexes were still present at high salt concentrations for WP/EPS B40, which was not the case for WP/gum arabic. The interaction between WP and EPS B40 is stronger than that of WP and gum arabic, because of the higher charge density of the EPS B40.
Protein to polysaccharide ratio and dynamics of complex formation

The influence of the protein to polysaccharide ratio (Pr:Ps) and the order of mixing the biopolymers provided information on the structure, apparent stoichiometry, and dynamics of the complexes.

The state diagram of Figure 3.7 was determined using turbidimetric titration under acidification as a function of the Pr:Ps ratio. It represents the region of stability and instability of a mixture of WP/EPS B40 as a function of the Pr:Ps ratio (Cp = 0.1% and [NaCl] = 0 mM). The pH\(_c\) remained constant around pH 5.3 for all Pr:Ps ratios. On the other hand, the pH\(_φ\) increased as the Pr:Ps ratio increased up to Pr:Ps = 9:1, where the pH\(_φ\) then stabilized.

**Figure 3.7**: State diagram as a function of Pr:Ps ratios of a mixture of WP / EPS B40, total biopolymer concentration = 0.1%, [NaCl] = 0 mM, temperature = 25°C, ( ): pH\(_c\), ( ): pH\(_φ\).

The dependence of pH\(_φ\) on the Pr:Ps ratio can be explained if phase separation is induced by the neutralization of the soluble complex. If more proteins are available per polysaccharide chain (large Pr:Ps), then phase separation takes place at a higher pH. At a Pr:Ps ratio close to 9:1, the pH\(_φ\) leveled off (from pH\(_φ\) = 4.8). From zeta-potential measurements performed on WP and EPS B40 (not presented here), the zeta-potential value of the protein at pH = 4.8 was -9 to -10 times smaller than the zeta-potential value of the polysaccharide. This result was in good agreement with the Pr:Ps ratio at
which pH₆ leveled off. The use of zeta-potential measurements for the prediction of complex coacervation was already reported by Burgess et al. (1984) in order to determine the optimum pH and ionic strength. They showed that at the electrical equivalence pH (EEP), where the electrophoretic mobility of the complex is zero, the electrophoretic mobility of the separated polyions was inversely related to the composition of the complex. In other words, if the Pr:Ps composition is 9:1, then one will find that the electrophoretic mobility at the EEP is 1:9. A saturated ratio of Pr:Ps = 9:1 (w/w) would correspond to a ratio of 0.5:0.000617 (mol/mol) (with the $M_w$ of EPS B40 = 1620 kg/mol and the $M_w$ of WP = 18 kg/mol). This means that 810 molecules of WP interact with one EPS B40 chain. The EPS B40 consists of 1680 repeating units, which corresponds to a complexation of one protein per two repeating units of EPS B40 [Tuinier et al., 1999]. This situation corresponds to a close packing of the WP on the chain of EPS B40, which is consistent with the hypothesis that the EPS B40 is saturated. The pH₆ was not dependent on the Pr:Ps ratio, which could suggest that the formation of soluble complexes was the result of the interaction between a single polysaccharide chain and a defined amount of protein. By comparing these results with some typical pH₆ values of WP/gum arabic, it appeared that the pH₆ was higher for the WP/EPS B40 system than for WP/gum arabic. The pI of the WP is 5.2 and the soluble complexes were already formed at pH 5.3 because the protein already carried some positive patches [Dubin et al., 1994; Wen and Dubin, 1997]. Furthermore, in the two-phase region, the WP/EPS B40 complexes looked more like a precipitate than a coacervate, unlike WP/gum arabic. The explanation lies in the fact that the charge density of EPS B40 is much higher than that of gum arabic.

Next, the order of biopolymer mixing was studied (Figure 3.8). When the WP was slowly added to a mixture of EPS B40 at pH 3 (titration A), the formation of complexes was instantaneous as indicated by the strong initial increase of turbidity, and phase separation occurred slowly in time. At a Pr:Ps = 1.35:1, the complexes precipitated, which was indicated by a sharp decrease of turbidity due to the high instability of the sample in the cuvette. At pH 4, the initial addition of WP induced a slight increase of the turbidity, which remained constant between a Pr:Ps = 0.3:1 and Pr:Ps = 0.9:1. In this region, the mixture did not phase separate after one week. Then the turbidity increased slowly until a Pr:Ps = 5.3:1, above which precipitation occurred. At Pr:Ps < 5.3:1, the mixture slowly phase separated in the following days. On the other hand, for Pr:Ps >
5.3:1, the mixture precipitated instantaneously. When the EPS B40 was added to the mixture of WP (titration B), the mixtures behaved differently. Indeed, they precipitated immediately, and the complexes stayed as a precipitate even at low Pr:Ps ratios. Experimentally, the turbidity was difficult to measure because of a fast phase separation in the cuvette, the data could, thus, not be plotted in Figure 3.8. From titration A, one can conclude that the interaction is much stronger at pH 3 than at pH 4, which is in agreement with the previous results of Figure 3.3. In Figure 3.3, the salt titration was performed at Pr:Ps = 2:1, which means that the complexes were in a precipitate form at pH 3 but not at pH 4.

![Figure 3.8: Titration of EPS B40 by WP (titration A) ( ): pH 3, (+): pH 4. Total biopolymer concentration = 0.1%, [NaCl] = 0 mM, temperature = 25°C.](image)

The results of titration B showed that the complexes formed at high ratios (precipitates) were not reversible upon decreasing the Pr:Ps ratio. The way the complexes were formed seemed to influence their nature. To understand which of the two states would be the most favorable for the complexes, two mixtures were prepared, one with titration A and the other with titration B, at a Pr:Ps ratio of 4:1 and at pH = 4. Under those conditions, the amount of EPS B40 would be in excess compared to the WP. The mixtures were left under continuous stirring for 2 weeks and the behavior of the samples was followed in time. As illustrated in Figure 3.9, the sample prepared with titration A (addition of WP into the EPS B40) was initially turbid, but after 4 days, the
Complex formation of whey protein / exocellular polysaccharide EPS B40

Sample contained some precipitates, like the sample made with titration B. It seemed that the system rearranged to form precipitates. To check this hypothesis, the experiment was repeated and the upper phase of the mixtures was taken out every day. After 2 h of phase separation, the upper phase was analyzed by HPLC and the amount of unbound $\beta$-lg, $\alpha$-la, and EPS B40 was determined in time (Figure 3.10). The results were similar for both mixtures (titrations A and B); therefore, only the results of titration A are shown. The concentration of unbound $\alpha$-la seemed to be unchanged in time. In contrast, the amount of $\beta$-lg in the upper phase decreased in the first 4 days and then remained stable. The amount of soluble EPS B40 was reduced in the first 2 days, and then increased. These results support the evidence that the complexes rearranged in time. During the first 4 days, some soluble complexes were still present in the mixture, as indicated by the initial decrease of $\beta$-lg and EPS B40. Then, the complexes rearranged to form fully neutralized EPS B40 and some free EPS B40 chains were expelled into the aqueous phase. Besides, the initial amount of $\alpha$-la before complexation was 0.012%, and the residual amount of unbound $\alpha$-la after complexation was close to that value. The pH of the mixture was close to the pI of $\alpha$-la ($pI = 4.1$), meaning that the $\alpha$-la was less charged than the $\beta$-lg. The complexes were thus mainly formed between EPS B40 and $\beta$-lg. These results could be understood if WP binds cooperatively to the EPS B40. The final state of the mixture consisted of neutral complexes and of free EPS B40.

![Figure 3.9: Pictures of mixtures of WP / EPS B40, Cp = 0.1%, Pr:Ps = 4:1, [NaCl] = 0 mM, pH = 4; tube A: WP added to EPS B40 (titration A); tube B: EPS B40 added to WP (titration B).]
Considering all the results, a mechanism is proposed to understand the structure and the formation of the complexes formed. When the mixture is slowly acidified from pH 7 to pH 2, the complexes are initially formed by interaction between a certain amount of WP per polysaccharide chain. Indeed, the independence of the pH upon the Pr:Ps ratio suggests that soluble complexes are formed between a single polysaccharide chain and a fixed amount of WP. Those complexes could be called homogeneous complexes. Upon further acidification, the WP becomes more charged and progressively saturates the EPS B40 chain that will precipitate. When the compounds are directly mixed at acidic pH, the results can be different. First, if the EPS B40 is added to an excess of WP, one could easily understand that there are enough WP molecules to saturate the polysaccharide chain; therefore, a precipitate is formed immediately. And upon further addition of EPS B40, the WP molecules saturate the EPS B40 one by one and do not redistribute over all EPS B40 chains. However, if the WP is added to the EPS B40 under stirring, the WP will be distributed on all the EPS B40 chains in excess, but with time and under continuous stirring, the WP will more likely be redistributed in order to neutralize one EPS B40 chain at a time. Such an effect can be called cooperative binding [Mattison et al., 1999; Dubin et al., 1990;
Complex formation of whey protein / exocellular polysaccharide EPS B40

Currie et al., 2000; Tolstoguzov et al., 1985; Antonov and Gonçalves, 1999]. Indeed, it seems that if one WP binds to an EPS B40 molecule, the next WP will bind more effectively due to an increasing binding constant. These results are consistent with previous studies of Gurov et al. (1977) on the sorption of bovine serum albumin (BSA) by dextran sulfate. When the BSA and the dextran sulfate are mixed under the condition of intense complexing, the complexes obtained hardly dissolve in water. On the contrary, when the mixture is obtained by slow titration, the interaction is gradually increased and the resulting complexes are soluble. The latter complexes correspond to a uniform distribution of protein molecules among the dextran sulfate chain, and when the pH is increased, the distribution becomes nonuniform. This phenomenon is attributed to the cooperative nature of the BSA sorption on the polysaccharide chain [Tolstoguzov et al., 1985; Olins et al., 1967; McGhee and von Hippel, 1974]. Similar results were found for the system WP/EPS B40.

CONCLUSIONS

Electrostatic interactions between EPS B40 and WP led, under appropriate conditions of pH and ionic strength, to the formation of soluble or aggregated complexes. When the mixtures were slowly acidified, instability arose from a two-step process. First, soluble complexes were formed at pH\textsubscript{c} and then macroscopic phase separation took place at pH\textsubscript{φ}. The values of these pH transitions were strongly salt dependent. The addition of microions weakened the interactions between the particles by screening the charges, and this phenomenon depended linearly on √I, that is, the ionic strength. In the pH region where soluble complexes were formed, viscosity and SLS measurement showed that the complexation of the WP with the phosphate groups of the EPS B40 reduced the intramolecular repulsion and the “stiffness” of the molecule, allowing a decrease in particle size. Finally, the order of biopolymer mixing is important to determine the structure and the apparent stoichiometry of the complexes. The rearrangement of the complexes was followed in time, showing that the energetically most favorable state for both biopolymers is to form few neutralized EPS B40 chains rather than distributing the WP over all the EPS B40 chains. In other words, the WP bound cooperatively to EPS B40 in the condition studied (low salt). This work gave a better understanding of the formation and the structure of soluble complexes. The results are consistent with previous studies involving carboxylated polysaccharide. In
the future, the interaction of WP with a sulfated polysaccharide will be studied to test the hypothesis.

ACKNOWLEDGEMENTS

Friesland Coberco Dairy Foods (FCDF) is acknowledged for sponsoring this work. The authors thank Pascale Louis-Louisy, Roelie Holleman and Jan van Riel for their experimental assistance, and Dr. Patricia Ruas Madiedo, Dr. Hans Tromp, and Dr. Renko de Vries for encouraging discussions.