Generality and Specificity of the Binding Behaviour of Lysozyme with Pectin Varying in Local Charge Density and Overall Charge

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Abstract We examined the similarities and differences of the binding behavior of lysozyme (lys) with pectin varying in local charge density (blockwise distribution and statistical distribution of methoxyl groups, BP and SP) and similar degree of methoxylation (DM). The interaction at ionic strength $I=0.01$ and pH 5.1 was found to be mainly electrostatic, associated with an exothermic enthalpy change. BP which is more inclined to self-association binds lys forming strongly associated complex particles with average sizes much larger (10-20 μm) than that of SP. The latter formed with lys small (0.6-3 μm) complex particles or liquid droplets depending on DM. The critical I_{set} and pH_{set} values for BP/lys systems, above which complexes do not form, are not sensitive to DM (approximately 0.1 and 11.5 respectively), whereas for SP/lys systems the critical I_{set} and pH_{set} values decrease significantly with increasing DM. The effect of I on complexation (CP) of lys with BP and SP has a nonmonotonic character. The distribution of BP and SP within the complex particles is irregular and is different for pectin with low and high DM values. The binding constant and stoichiometry ratio (lys/pectin) of the systems with SP are slightly higher

than those of BP at the same DM, and these values decrease sharply (50 times) with increasing DM. The large positive changes in entropy in SP/lys systems suggest that the interactions in these systems tend to be more hydrophobic in character at high DM. On the other hand, with BP, a negative enthalpy change, especially for pectin with low charge, suggests the formation of a large quantity of hydrogen bonds or van der Waals interactions during CP.

Keywords: Pectin; lysozyme; complexation; confocal microscopy; ITC; structure.

1. INTRODUCTION

Proteins, polysaccharides and some synthetic and modified polymers are widely used in the food, pharmaceutical, and cosmetic industries in the form of multicomponent systems (Stephen, Phillips, Williams, 2006). At present, it is generally accepted that intermolecular interactions and the phase state of these systems determine their functional properties (Tolstoguzov, 1986). Phase separation in aqueous protein–polyelectrolyte (PE) systems can be the result of the manifestation of two different physicochemical phenomena: thermodynamic incompatibility of the (bio)polymers and formation of water insoluble protein–polyelectrolyte complexes. These phenomena are frequently described as segregative and associative phase separation (Piculell, Lindman, 1992; De Kruif, C. G., & Tuinier, R. 2001). There is a large number of original studies and reviews concerning interaction and complex formation in protein-synthetic PE systems (Tsuboi, et al, 1996; Xia, Dubin, Dautzenberg, 1993; Mattison, Brittain, Dubin, 1995; Cooper, Dubin, Kayitmazer, & Turksen, 2005; Da Silva, Lund, Jönsson, & Akesson, 2006; Kaibara, Okazaki, Bohidar, & Dubin, 2000; Mattison, Dubin, & Brittain, 1998; Seyrek, Dubin, Tribet, & Gamble, 2003; Kayitmazer et al., 2013; Cooper, Dubin, Kayitmazer, & Turksen, 2005) and protein-polysaccharide systems (Tolstoguzov, 1997, 1986, 2003; Antonov, Zhuravleva, Cardinaels, Moldenaers, 2015; Antonov, Cardinaels, Moldenaers, 2017; Antonov, Zhuravleva, Cardinaels, Moldenaers, 2018; Galazka, Smith, Ledward, & Dickinson, 1999; Tolstoguzov, 1986; Serov, Antonov, Tolstoguzov, 1985; Gurov, Gurova, Leontiev, Tolstoguzov, 1988; Laneuville, Paquin, Turgeon, 2000; Dickinson, & Pawlowsky, 1998; Weinbreck, De Vries, Schrooyen, & De Kruif, 2003; Doublier, Garnier, Renard, & Sanchez, 2000; Kabanov, 1994). The nature of protein/polysaccharide complexes is influenced by entropic factors, that depend on parameters such as the structure and the molecular weight of the biopolymers. The complexes are also influenced by enthalpic forces, which are regulated by the protein/polysaccharide ratio and the nature and density of charges as well as the type and distribution of functional groups on the biopolymers (Girard, Turgeon and Gauthier 2003).

Effects of experimental parameters such as pH and ionic strength, as well as that of many structural parameters (polyion and polyampholyte types, their flexibility) on complexation in protein/PE systems have been extensively studied for many types of proteins. On the contrary, the effects of the local structural changes of the PE (the number of charged and uncharged groups, and their distribution along the chain) have only been investigated for a limited amount of proteins. In this connection we can refer to studies on the complexation between pectin and β -lactoglobulin (Sperber, Schols, Stuart, Norde, Voragen, 2009; Girard, Turgeon, & Gauthier 2003a, b; Xu, Melton, Jameson, Williams, Mc Gillivray 2015; Xu, Melton, Ryan, 2018). In principle local structural changes can significantly alter the charge density of the PE and its chain stiffness and can thus have a great impact on protein/PE complexation (CP). Lys (E.C.3.2.17, N-acetylmuramic-hydrolase) is a bacteriolytic enzyme commonly found in nature in many plant and animal sources, which is especially abundant in hen egg whites. It is a compact basic (pI=11.32) globular protein having an ellipsoidal shape with dimensions of 4.5 x 3.0 x 3.0 nm and a molecular weight M_w =14.3 kDa. Lys is found mainly as a monomer, but like many other natural compounds, it might even be more active in the dimeric or polymeric form. Bacteriostatic and bactericidal properties of lys are used to preserve various types of food (Cegielska-Radziejewska, Leśnierowski, Kijowski, 2008; Proctor, and Cunningham, 1988). Pectin at first approximation is a linear polysaccharide comprising galacturonic acid residues. It is mainly found in the middle lamella of the plant cell membrane. Some of its galacturonate units are esterified as methyl galacturonate, whose extent is reported as the degree of methoxylation (DM). These methylesters can be present with a statistical or blockwise distribution (Kohn, and Luknar, 1977; Kohn, Markovič, Machová, 1983; Kohn, 1987; Williams, Buffet, Foster, Norton, 2001; Daas Meyer – Hansen, Schols, De Ruiter, Voragen, 1999; Ngouémazong, Tengweh, Duvetter, Fraeye, Van Loey, Moldenaers, Hendrickx 2011). It is well-known that pectin with a blockwise distribution of methylesters is more inclined to self-association than pectin with a statistical distribution of methylesters (Antonov et al., 1996). Its excellent functional properties make pectin a widely used additive in the food industry e.g. as jam, jelly, emulsion stabilizer and thickener in sauces.

In our recent study we characterized the CP of pectin with lysozyme (lys) at different physico-chemical conditions and compositions of the system (Antonov et al, 2019). The effect of the overall charge of pectin (DM) on the complexation behavior was investigated. The work presented in the present paper focusses on the binding behavior of lys with pectin varying in local charge density (block wise distribution and statistical distribution of methoxyl groups denoted as BP and SP) but being similar in overall charge. Analysis of the lys protein surface reveals a close to homogeneous distribution of positive charges over the surface (Van de Weert, Andersen and Frokjaer, 2004), suggesting that the interaction with pectin does not occur at a very specific site on the lysozyme

molecule. This fact combined with the potential applications of pectin/lysozyme systems provides a strong motivation for this study.

Turbidimetric titration, dynamic light scattering (DLS), confocal and optical microscopy, and isothermal titration calorimetry (ITC) were applied. Turbidimetric titration and DLS allowed to determine the sizes of the complex particles, and the main phase transitions of the system in terms of composition, pH and ionic strength. Confocal and optical microscopy allowed to monitor changes of the system morphology and to determine the distribution of the biopolymers within the complex particles. ITC allowed accurate measurements of the thermodynamic parameters, such as the binding constant, enthalpy, entropy, and stoichiometry of the interactions.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Biopolymers and reagents

The lys from chicken egg white (dialyzed, lyophilized powder) was purchased from Sigma-Aldrich and used without further purification. High methylesterified commercial citrus pectin with molar mass of 56.1 kDa and DM of 95.1 was obtained from Sigma-Aldrich (Belgium). Pectins with a blockwise distribution of the methyl ester groups (BP) and various degrees of methoxylation (DM) namely 16.9% (BP16.9), 33.1% (BP33.1) and 68.2% (BP68.2) were produced enzymatically using plant pectin methyl esterase (PME). The enzymatic demethylesterification included an incubation of highly methyl esterified citrus pectin (DM of 95.1%) with purified carrot PME at 30 °C for predetermined time periods. Pectins with a statistical distribution of methyl ester groups (SP) and various DM values namely 16.2% (SP16.2), 38.2% (SP38.2), 66.5% (SP66.5) and 77.7% (SP77.7) were produced chemically from highly methylesterified citrus pectin (DM of 95.1%) by titration with predetermined amounts of 0.1 M NaOH solution at 4 °C and a pH of 11. The detailed procedure for the preparation of BP and SP samples can be found in earlier work (Celus et al. 2017). After preparation, the resulting solutions were adjusted to pH 6, dialyzed (Spectra/Por®, MWCO = 12-14 kDa) for 48 h against demineralized water and subsequently lyophilized. Pectin from each batch was characterized in terms of DM using Fourier transform infra-red spectroscopy (FT-IR) (Shimadzu FTIR-8400S, Japan), as in Celus et al. (2017). The molar mass distribution profile of all samples did not reveal any depolymerisation as no shift in elution time was observed on HPSEC chromatograms. The average molar mass of the samples, as determined by HPSEC chromatography was 44.6 ± 3.5 kDa and varied between 41.1 kDa and 48.1 kDa whereas the mother pectin had a molar mass of 56.1 kDa (Celus et al., 2017). All reagents were of analytical reagent grade. Milli-Q ultrapure water was used in all experiments.

141 *2.1.2. Preparation of the protein and polysaccharide solutions and protein/polysaccharide*
142 *mixtures.* To prepare solutions of lys and pectin with the required concentrations, the weighed
143 amount of biopolymer sample was gradually added to an acetic acid-sodium acetate buffer (pH
144 5.1, ionic strength $I=0.01$) at 23 °C and stirred for 1 h. The resulting solutions were centrifuged at
145 50000g and 23 °C for 1 h to remove insoluble particles. The lys content in the stock solution was
146 determined by means of UV absorption using the extinction coefficient for highly purified lys
147 which is $2.64 \text{ ml mg}^{-1} \text{ cm}^{-1}$ at 281.5 nm (Aune, & Tanford, 1969). To study the effect of pH on
148 the complexation behavior, the pH of the complex mixtures was changed in a range from 5.0 to
149 11.0 by addition of 0.1–0.5 M NaOH. To study the effect of I on the complexation behavior, the I
150 of the complex mixtures was changed in a range from 0.01 to 0.5 by addition of 3.5 M NaCl.
151 Concentrations of pectin in the stock solutions were determined by drying at 104 °C to constant
152 weight. Solutions of the biopolymers were kept at least overnight in the fridge to allow for full
153 hydration of the molecules. To prepare mixed solutions of lys with pectin with the required
154 concentrations, weighed amounts of the pectin stock solution were added to the lys stock solution
155 at 23 °C.

156 **2.2. Methods**

157 *2.2.1. Turbidity measurements*

158 Turbidity values of the systems as a function of the pectin/lys weight ratio (q) were measured at
159 500 nm using a UNICCO SQ2800 UV/VIS spectrometer. The error of the turbidity measurements
160 was typically about 2%–3%. In the weight ratio range from 0.1 to 0.6 the errors were markedly
161 larger (6-8%). With increasing q , the CP behavior can be characterized by three transitions
162 identified as q_{onset} , q_{ϕ} , and q_{max} (Carlsson, et al., 2001). These correspond to respectively the
163 transition from the absence of CP to the formation of water soluble complexes, from water soluble
164 complexes to water insoluble complexes and their phase separation, and maximal CP. To obtain
165 accurate values for these transition points, additional characterizations were performed, as
166 described in detail in our recent work (Antonov et al. 2019).

167 *2.2.2. Light Scattering*

168 Determination of the intensity size distribution functions of pectin and lys solutions as well as
169 pectin/lys mixtures was performed by means of dynamic light scattering (DLS) with an
170 ALV/CGS-3 compact goniometer system (ALV GmbH, Germany). The measurement procedure
171 has been described in detail in our earlier work (Antonov et al, 2017). From these measurements,
172 the scattering intensity averaged hydrodynamic radius was determined, assuming a spherical shape
173 of the complexes.

174 *2.2.3. Bright light microscopy.*

Microscopy observations during CP have been performed, using an Olympus BX51W1 fixed stage microscope equipped with a high resolution CCD-camera, (1000x1000 pixels, C-8800-21, Hamamatsu), using different magnifications.

2.2.4. Fluorescent imaging

Fluorescent imaging was performed using a multi-beam confocal microscope (VisiTech, UK), equipped with an oil-immersion objective (20x, 0.85 NA, Olympus, Japan) using 532 nm and 642 nm as excitation wavelengths. Before imaging, pectin and lys were fluorescently labeled by storing a pectin solution containing Atto 647N dye (ATTO Tec. Germany) at 5°C during 3 days, whereas a lys solution containing Rhodamine B dye was kept under the same conditions. This labeling allowed to spectrally separate the signal from lys (red) and pectin (green). Image analysis was performed using ImageJ v1.43r software. More than 100 images were analyzed and representative ones are presented here. Using turbidity characterization it was verified that the fluorescent labelling has no significant effect on the complexation process.

2.2.5. Isothermal Titration Calorimetry (ITC)

Calorimetric experiments were performed using a TAM III isothermal calorimeter (TA Instruments). All solutions used for ITC were dialyzed against the buffer during 48 h at 4°C. The samples were degassed by sonicating the solutions for 15 minutes under vacuum. The system was equilibrated at 25°C while stirring at 120 rpm until the baseline shift was <50 nW/h. The pectin solution in the reaction cell (0.8 mL) was titrated with 13 successive 15-μL injections of lys solution. Each addition lasted 10 s with an interval of 20 min between consecutive injections. This long resting time between injections was necessary to reach thermodynamic equilibrium. This equilibrium was attained when no more energy was released or absorbed in the reaction cell. The heat of dilution from the blank titration of the lys into the buffer solution was subtracted from the raw data. Measurements were carried out two to five times, depending on the sample. The thermodynamic parameters, including the binding constant (K), enthalpy (ΔH), entropy (ΔS), and binding stoichiometry (n) (molar ratio monomeric lys/pectin), were calculated by curvefitting of the binding isotherms with a Levenberg-Marquardt nonlinear regression model. Thereto, an independent binding site interaction model with a single binding site was used (Freyer & Lewis, 2008, Brown 2009). This model includes a mass balance:

$$L_t = [L] + n \theta M_t \quad (1)$$

where L_t is the starting concentration of ligand L, $[L]$ is the free ligand concentration, M_t is the total macromolecule concentration, n is the binding stoichiometry and θ is the fraction of sites occupied by the ligand, whereby the amount of free and bound ligand depends on the association equilibrium constant K :

$$K = \frac{\theta}{(1-\theta)[L]} \quad (2)$$

Combining equations 1 and 2 gives:

$$\theta^2 - \theta \left[1 + \frac{L_t}{nM_t} + \frac{1}{nKM_t} \right] + \frac{L_t}{nM_t} = 0 \quad (3)$$

The total heat produced Q is described by Equation (4), with V_0 the initial volume of the sample cell:

$$Q = n\theta\Delta HM_t V_0 \quad (4)$$

Solving Equation (3) for θ and then substituting this into Equation (4) gives:

$$Q = \frac{nM_t\Delta HV_0}{2} \left[1 + \frac{L_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{L_t}{nM_t} + \frac{1}{nKM_t} \right)^2 - \frac{4L_t}{nM_t}} \right] \quad (5)$$

Equation (5) only applies to the liquid contained in volume V_0 . Therefore, after completing an injection, a correction must be made for the increase in volume. Equation (6) then accounts for the heat released, $\Delta Q(i)$, from the i^{th} injection, taking into account the increase in volume due to the injected volume:

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0} \left[\frac{Q(i) + Q(i-1)}{2} \right] - Q(i-1) \quad (6)$$

The NanoAnalyze data analysis software (TA instruments) was used to perform the data analysis.

Based on these data, the Gibbs free energy is calculated from the binding constant:

$$\Delta G = -RT \ln K \quad (7)$$

and subsequently the entropy change can be found from:

$$\Delta G = \Delta H - T\Delta S \quad (8)$$

2.2.6 Statistical analysis

DLS measurements of the intensity size distribution functions were carried out in duplicate and results are averages of five independent runs. Each turbidimetry curve was obtained by taking the average of four measurements. Calorimetric measurements were carried out two to five times, depending on the sample. The obtained results were analysed using the Origin 9 software (Microcal, USA).

3. Results and discussion

3.1 Determination of the main CP parameters (q_{onset} , q_ϕ , q^*_{ϕ} and q_{max}) via turbidimetry

First, turbidimetry was applied to determine the main CP parameters of mixtures containing lys and BP varying in DM. A series of more than 50 mixtures comprising lys and BP with various pectin/lys weight ratios (q) were prepared at pH 5.1 and ionic strength $I = 0.01$, and stirred for 120 min to reach the equilibrium state. The turbidimetry data of these systems at 500 nm (τ_{500}) as a function of q are presented in Figures 1 and 2. The thin dash dot lines in Figures 1 show the data

for the SP/lys system from our previous study (Antonov et al. 2019) for comparison. At first glance a comparison between the data obtained for BP16.9/lys, BP33.1/lys and BP68.2/lys systems and that for SP16.2/lys, SP38.2/lys and SP66.5/lys systems studied recently shows that replacing pectin with a statistical distribution of methyl ester groups with pectins with a blockwise distribution of methylester groups at the same DM values does not appreciably affect the turbidimetry results (Fig. 1).

Figure 1

An addition of even a small amount of a 0.04 wt% BP16.9 or BP33.1 solution in a 0.04 wt% lys solution results in the appearance of turbidity whereby the q values exceed the q_{onset} value. As usual, the turbidity measurements reveal five domains of q (Fig 1a', a'', Fig 1b', b'', Fig 2a', a'') corresponding to (I) the absence of CP, (II) soluble complex formation, (III) phase separation of an insoluble complex (Carlsson et al, 2001), (IV) suppression of phase separation, and (V) the absence of CP. The transition from region I to region II is denoted " q_{onset} ", the transition from region II to region III is denoted " q_{ϕ} ", the transition from region III to region IV is denoted " q^*_{ϕ} ", and the transition from region IV to region V is denoted " q_{set} ". The absence of CP at $q > q_{\text{set}}$ does not mean complete absence of interactions between the ion pairs of the protein and polysaccharide. Electrostatic complexation of proteins with polysaccharides includes at least two stages, first the formation of salt bonds between the amino groups of the protein and negatively charged (in the case of pectin) groups of the polysaccharide, and subsequently the aggregation of the complexes by secondary forces due to the decrease of the zeta potential of the system. At $q > q_{\text{set}}$ association and aggregation of the primary complex pairs is impossible due to the highly negative charge of the complex system.

The q_{ϕ} and q^*_{ϕ} values are a measure of the complex stability and characterize the phase boundary. The q_{onset} and q_{ϕ} values as a function of DM for BP/lys systems are presented in Figure 2. Similar to SP (Antonov et al, 2019) BP interacts with lys starting from an extremely low weight ratio of pectin in the mixtures namely $q_{\text{onset}} = 7 \cdot 10^{-4}$ for highly charged BP16.9 and ranges to $3 \cdot 10^{-3}$ for BP68. Water soluble complexes are formed in a narrow q range between $7 \cdot 10^{-4}$ and $1.4 \cdot 10^{-2}$ depending on the DM values, as shown in Figure 2.

Figure 2

The turbidimetry data clearly show that differences between systems with BP and SP exist only for mixtures with lowly charged polysaccharides (high DM). The main CP parameters including q_{Max} obtained for BP16.9/lys versus SP16.2/lys and BP33.1/lys versus SP38.2/lys are the same at corresponding DM values, as show in Figure 1. However the q_{Max} value for the BP68.2/lys system, which characterizes at first approximation the stoichiometric composition of the complexes, is appreciably higher (0.37) than that of the SP66.5/lys system. In other words, the stoichiometric composition for the former system is observed at a larger content of pectin (Fig. 1c"). Taking into account that the weight average molecular weight of Lys is 14.3 kDa (Rezwan, Meier, Gauckler, 2005) and that of pectin is 44.6 ± 3.5 kDa, we can roughly evaluate the “molar” ratio pectin/lys in the complex phases. A simple calculation shows that the lys/pectin ratio is approximately 8:1 mole lys/mole pectin for the BP68.2/lys system, which is less than that of the SP66.5/lys system (10:1) at almost the same DM value of functional groups. Moreover, for these lowly charged systems, liquid complex coacervates were observed for the SP/lys mixtures whereas the systems with BP resulted in water insoluble complex particles for all studied conditions.

3.2. Size of complex particles

DLS was applied to characterize the scattering intensity average size of the complex particles at different DM values. All measurements were performed at a constant total concentration of biopolymers (0.04 wt%) in a q range from 0.05 to 1.0. The scattering intensity size distribution functions of the BP/lys systems are presented in Figures 3 a-c. For comparison, the thin line shows the scattering intensity size distribution function of a SP/lys system at the corresponding DM values and for $q=q_{\text{Max}}$. The scattering intensity averaged hydrodynamic radius of all pectin particles was approximately 104-114 nm depending on the DM of pectin (Fig. 3a-c). Hence, the effect of the methylester distribution on the size of the pure pectin is negligible under the studied conditions. The average radius of the lys monomer was 1.9 nm (data not shown), in accordance with other known data (Parmar, & Muschol, 2009; Valstar, Brown, Almgren, 1999). Since the average size of pectin is much larger than that of lys, changes in the sizes of the complex particles can easily be monitored. For all studied systems the presence of even a small amount of pectin in the lys solution (at $q=0.05$), leads to a significant shift of the dominant peak to a larger average particle radius. The BP/lys particles are significantly larger than those of the SP/lys systems at the same DM value (Figure 3d). Hence, based on the DLS results one important difference in the CP of lys with SP and BP can be pinpointed. The complexes obtained with BP are more aggregated as compared to those obtained with SP at the same DM. This can be due to the higher capacity of BP to aggregate in the acidic pH range in the presence of monovalent ions which induce aggregation of stiffened BP chains. Such aggregation can arise from e.g. cooperative

attraction, or via entanglement of rigid structures (Ström, Schuster, MengGoh, 2014). The interactions between the blocks of the BP samples are more cooperative than those of SP. The solubility of the BP samples in our study was always less than the solubility of SP samples at the same DM values. However, for the pure pectins no significant size differences could be detected with DLS.

The dependence of the average sizes on q has an extremal character with the q value corresponding to the maximum particle size equal to or close to that determined by turbidimetry. At q values above q_{Max} ($q = 0.5-1.0$) the sizes of the complex particles gradually become similar to that of free pectin molecules. The evolution of the size of the complexes with q together with the evolution of turbidity with q relate to the degree of binding (yield) of lys in the water insoluble complex phase with pectin. At $q < q_{\text{Max}}$ the yield of lys in the complexes increases with increasing q . At $q > q_{\text{Max}}$ the yield of lys in the complex decreases with increasing q . At high q values, the secondary aggregation of complexes is hampered due to the highly charged nature of the formed complexes.

FIGURE 3

3.3. Morphology of complex BP/lys systems

Brightfield light microscopy images of complex BP68.2/lys particles at different q values and a total biopolymer concentration in the mixture of 0.04 wt% are presented in Figure 4. The microscopy images clearly demonstrate the increase of the average size of the complex particles when the content of pectin increased up to q_{Max} (Figure 4a-c). Separation of a complex BP68.2/lys system by centrifugation showed that the complex phase is a jelly-like soft material. In contrast to the BP/lys system the complex phases of the SP66.5/lys and SP77.7/lys systems are liquid and have a droplet morphology (Antonov et al, 2019). Hence, the microscopy images demonstrate that another main difference between the complex particles of the highly methoxylated BP/lys and SP/lys systems is the aggregation state (liquid or solid) of the complex phase.

Figure 4

3.4. Structure of the complex particles

Confocal Laser Scanning Microscopy (CLSM) was applied to monitor the distribution of biopolymers within the BP/lys complexes at different q values and the images are compared with those for SP/lys systems (Antonov et al, 2019) at the corresponding q values. Representative images are presented in Figures 5 and 6. For the BP16.9/lys system six characteristic compositions of the complex system were considered (Fig 5a-f) namely $q=0.05$, corresponding to an excess of lys, $q=q_{\max}$, corresponding to maximal complexation according to the turbidity analysis (Figs. 1), $q=0.5$ and 0.9 , and two compositions characterized by an excess of pectin in the complex system ($q=1.5$ and 2.0). At $q \leq q_{\max}$ the intensity signal from the positively charged labelled lys protein (red) is dominating (Fig. 5 a, b) whereas at $q > q_{\max}$ the intensity contribution from the labelled pectin (green) becomes dominant in the complex particles. Besides, the yellow signal corresponding to mutual compensation of both macro counterions is also present in most images. The results obtained for the BP16.9/lys systems show that the higher the content of pectin in the complex mixture the more the relative content of pectin within the complex particles. The structure of the BP33.1/lys system (data not presented) is quite similar to that of the BP16.9/lys system and SP16.2/lys system (Antonov et al, 2019). Since the composition of the complex particles for the BP16.9/lys and SP16.2/lys systems depends on the composition of the initial mixture, this implies that the reactivity of free pectin chains may be considered higher than that of pectin partially covered by lys (Antonov et al, 2019). The distribution of pectin and lys within the complex particles of the BP68.1/lys system differs from that of the BP16.9/lys as well as that from the SP66.5/lys system (Antonov et al, 2019). An increase of the BP68.1 content in the complex mixture (increase in q value) did not lead to an appreciable increase in the BP68.1 content within the complex particles (Fig. 6d). The later ones remain enriched in lys even at high q values. This indicates a difference in the mechanism of binding of lys with low methoxylated and highly methoxylated pectins similar to what we observed for SP/lys systems (Antonov et al, 2019). It is interesting to note that in the given system regions with complete compensation of the macro ions are very small (yellow color) even at $q_{\max}$ corresponding to maximum complexation according to turbidimetry (Fig. 1). A comparison of the images for the BP68.1/lys (Fig. 6d) and the SP66.5/lys system (Antonov et al, 2019) clearly shows that the degree of irregularity in the distribution of the biopolymers within the complex particles is much higher for the former system. This can be a result of the additional aggregation of lys during CP with BP. This stronger aggregation of these complexes may also be the reason for the absence of liquid coacervation in BP/lys systems at high DM values which was observed for SP66.5/lys and SP77.7/lys systems (Antonov et al, 2019).

Figure 5

Figure 6

371

372 3.5. Effect of ionic strength and pH

373 Figure 7a presents τ_{500} values of complex pectin/lys systems as a function of the pH obtained for
374 BP with various DM values. All plots were determined at q values corresponding to q_{Max} for each
375 system at $I=0.01$. The results obtained and their comparison with those determined for SP/lys
376 systems at corresponding conditions (Antonov et al, 2019) show at least one principal difference
377 in their behavior. The minimal pH value at which τ_{500} becomes zero (pH_{set}), indicating the absence
378 of CP, is not dependent on the DM value in BP/lys systems, i.e on the overall charge of the pectin
379 molecules, whereas in SP/lys systems the pH_{set} value decreases with increasing DM.

380 These facts allow to state that the main factor determining CP in BP/lys systems in the alkaline pH
381 range is the aggregation of pectin and the formed complex particles, steered by secondary forces,
382 whereas in SP/lys systems the overall charge of pectin is the driving force determining CP. In the
383 pH range from 5 to 9.5 the positive charge of lys decreases monotonically (Bharti, 2014) and the
384 carboxyl groups of pectin are completely ionized (Kohn, 1973). Therefore decreasing τ_{500} values
385 with increasing pH values from 5 to 9.5 were expected. However, strong aggregation of the
386 complex particles under the given conditions extends the stability of the complexes to higher pH
387 values. The origin of the forces responsible for the strong aggregation in BP/lys systems, especially
388 in the alkaline range is not quite clear.

389 **Figure 7**

390

391 The effect of I on the τ_{500} values of the BP/lys systems at different DM values is demonstrated in
392 Figure 7b. One common feature of systems containing either BP or SP (Antonov et al, 2019) is
393 that the minimal I value at which τ_{500} becomes zero (I_{set}), indicating the absence of CP, is the same
394 ($I_{\text{set}}=0.1$) for the highly charged (DM=16%) SP and BP. For the highly charged BP16.9 and BP33.1
395 the dependence of τ_{500} on I has a nonmonotonic character displaying a maximum in CP at $I \approx 0.06$.
396 It is well known that ionic strength and the presence of counterions strongly modulate the
397 affinity between macromolecules. Manning (1969) and Seyrek, Dubin, Tribet, & Gamble (2003)
398 have shown that the binding of polyanions to a positive domain results in a combination of
399 short-range attractive interactions coupled with longer range repulsive interactions due to the
400 presence of charged patches on the protein. Since lys is known to exhibit a multipolar charge
401 distribution (Van de Weert, Andersen, & Frokjaer, 2004) both interactions are expected in the
402 pectin/lys mixtures. Hence, at low ionic strength, when the Debye length $\kappa^{-1} \approx 0.3/\sqrt{I}$ (nm) is

large, the effect of salt is mainly to screen the repulsions and thus to strengthen association. At higher ionic strength values, corresponding to a small Debye length, addition of salt weakens the attractions thereby suppressing CP. For the SP/lys systems the maximum in CP occurred around $I = 0.03$ (Antonov et al., 2019), which corresponds to a Debye length of 1.7 nm, a value comparable to the size of the lysozyme molecule. The behavior of the BP16.9/lys and BP33.1/lys systems was a little different because the maximum in CP is observed at a higher ionic strength namely $I=0.06$. A possible reason for the small shift of $I_{\max}$ from 0.03 to 0.06 for the BP16.9/lys and BP33.1/lys systems can be the more pronounced screening of the repulsions due to a stronger aggregation in BP/lys systems as compared to SP/lys systems. This behaviour is thus in correspondence with the formation of much larger complex particles as compared to the particles obtained on the basis of SP (Figure 2).

In addition, similar to the pH_{set} values (Fig. 7a) the results presented in Fig. 7b and their comparison with those determined for SP/lys systems at corresponding conditions (Antonov et al, 2019) show that the I_{set} value in BP/lys systems is not dependent on DM, i.e on the overall charge of the pectin molecules, whereas in SP/lys systems the I_{set} values decrease significantly with the increase of DM. These observations allow to state that the main factor determining the I_{set} values in SP/lys systems is the overall charge of pectin, whereas in BP/lys systems the I_{set} values are determined by a combination of the secondary aggregation forces and, to a lesser degree, the overall charge of pectin.

3.6. Thermodynamic parameters of the binding

To shed more light on the differences in the complexation behaviour between BP/lys and SP/lys, ITC characterizations were performed for both systems. The raw data from the ITC measurements of the SP16.2/lys system are presented in the inset Figure 8a'. The peak areas correspond to the energies released by the cell containing pectin at each lys injection. The binding isotherm of this system is determined as the integrated area of the heat peaks plotted against the molar ratio of the components (Figure 8a). By determining the thermodynamic parameters of the CP of proteins or polysaccharides with a ligand, it is possible to establish whether CP is favored by enthalpy or entropy. Because all the injections throughout the analysis released energy, the CP between lys and SP (Figs. 8a,c,e) and lys and BP (Figs. 8b, d, f) is exothermic. The binding isotherms of all the pectin/lys systems have one inflection point. It is interesting to compare the CP of the pectin/lys systems with that of pectin/ β -lactoglobulin systems studied before (Girard et al, 2003a). The binding isotherms of the later system have two inflection points and they reveal

the formation of soluble intrapolymer complexes (C1) further followed by their aggregation in interpolymer complexes (C2). For that reason, the authors used a “2-site model” interpreted as a “2- step model”. In the first step, the amount of β -lg complexed with pectin decreased with each injection until pectin was saturated. Then, the second step began with a plateau where the same amount of β -lg molecules interact in each injection. Pectin became gradually saturated with β -lg until no more energy was released. Girard et al (2003a) assumed that the existence of two steps involved in the pectin/ β -lactoglobulin CP process is in agreement with the Tainaka theory (Tainaka, 1979,1980), which states that the complexes are formed in two steps passing from the molecular to the aggregated state. However, the presence of just one inflection point in the binding isotherms in our systems does not mean that the CP process in the given case is in disagreement with the Tainaka theory. The presence of one inflection point in the binding isotherm is rather a more typical phenomenon for ITC measurements of complex protein-polysaccharide systems (Harnsilawat, Pongsawatmanit, McClements, 2006; Schmitt, da Silva, Bovay, Rami-Shojaei, Frossard, Kolodziejczyk, Leser, 2005; de Souza, Bai, Gonçalves, M. Bastos, 2009). It can be explained by the insignificant concentration range corresponding to the formation of water soluble complexes in these systems (see Fig. 1), or in other words, the absence of a clear isotherm (C1). Moreover, the nonspecific nature of the interactions and the fact that the carboxyl group is the only interacting group on the pectin, would further result in a one-step process.

FIGURE 8

Curvefitting of the binding isotherms was used to determine the enthalpic (ΔH) and entropic (ΔS) contributions of the CP, the binding constant K , and the stoichiometry n of the complexes at 23°C and pH 5.1 (Figure 9 a-d). The binding constant K (M^{-1}) as a function of DM and the stoichiometry values n (mole lys/mole pectin) as a function of DM for all systems are presented in Figures 9a and 9b respectively. The binding constant of the low methoxylated pectin is more than 50 times higher as compared with that of high methoxylated pectin ($11.8 \cdot 10^{-6} M^{-1}$ and $2.2 \cdot 10^{-7}$ for SP16.2/lys and SP77.7/lys systems respectively). For pectin/ β -lg systems, the binding constants of the first step were not statistically different, and for the second step this parameter for low methoxylated pectin/ β -lg was about 8 times higher than for high methoxylated pectin/ β -lg systems. The effect of DM with pectin/lys systems is thus much more pronounced than that observed for low methoxylated SP/ β -lg and high methoxylated SP/ β -lg systems (Girard et al, 2003a). These differences between the different systems suggest that minor differences in the origin of the forces determining CP in these systems affect the binding constants. Interactions between β -lg and pectin occurred through ionic and hydrogen bonds, whereas hydrophobic interactions were negligible

(Girard, Turgeon, Gauthier, 2002). However, in our case hydrophobic interactions may also play an appreciable role together with Coulomb forces. As compared to the effect of DM, the pattern of methylester distribution has a more limited effect on the binding constant. Fig. 9a shows that for all DM values the binding constant for the systems including SP is slightly higher as compared with that of BP. The differences are however very limited, especially when compared to the results of Celus et al. (2018) for binding of pectin with Zn^{2+} , where the binding constants of the BP at low DM were a factor of 30 higher than those of SP. This is possibly caused by the larger size of lys as compared to Zn^{2+} , which makes the local pattern of ester distribution less relevant for the binding strength. Saturation of the SP16.2 and BP16.9 with lys was reached at lys/pectin molar ratios of about 17:1 and 15:1 mole/mole respectively, which are ratios similar to those determined by turbidimetric titration (16:1 and 15:1). These stoichiometric values decreased monotonically with increasing DM values. Corresponding values for SP66.5 and BP68.1 determined by ITC were 8:1 and 4.5:1 respectively.

FIGURE 9

Despite the moderate effects of the local pattern of charge distribution on the binding constants characterizing the binding of SP and BP with lys, significant differences in the morphology of the formed complexes (Figures 4, 5 and 6) as well as their sensitivity to pH and I (Figure 7) were observed. Hence, differences in complex formation cannot directly be related to differences in binding strength. First, it should be noted that the formed complexes may contain both lys that underwent actual binding as well as lys that got entrapped in the complexes, whereas the ITC measurements only allow to characterize the first type. Figures 9c and 9d clearly show that under all conditions, the enthalpy contribution dominates over the entropy contribution. The enthalpy and entropy changes remain almost the same when replacing SP16.2 with SP38.2 and BP16.9 with BP33.1, which indicates the absence of significant structural changes in the lys molecule upon complexation (Fig. 9c,d). However, when exchanging SP16.2 with SP77.7, the enthalpy change significantly reduces namely from -33.5 kJ/mol to 10.9 kJ/mol or from 8.01 kcal/mol to -2.6 kcal/mol. This change is much more pronounced than in the systems with lactoglobulin (which was from 14 kcal/mol to 10 kcal/mol) (Girard et al, 2003). When comparing BP and SP, it becomes clear that mainly at high DM values, where other than electrostatic forces come into play, the relative contributions of entropy and enthalpy to the interaction process are rather different. At high DM, the entropy gain only becomes a significant contribution in the interaction energy for SP. The increasing disorder resulting in the entropy change can result from the release of bound water during complexation. However, as this entropy change significantly increases with DM while the complexation with lys reduces, this can indicate the presence of more pronounced

contributions of hydrophobic forces, which are generally entropy-driven (Ross and Subramanian 1981). In conclusion, although the ITC measurements reveal differences in the binding behavior of lys with either SP or BP, further research is required to elucidate the precise relations between the molecular level polyelectrolyte binding behaviour and the macroscopic complexation behavior.

4. Conclusions

The CP of lys with pectin varying in local charge density has both common and specific features. Both SP and BP form water-soluble complexes in a very small composition range (q between $7 \cdot 10^{-3}$ and $3 \cdot 10^{-2}$) depending on DM, and water insoluble complexes at q above $7 \cdot 10^{-3}$ -0.015 and less than 1.5-3.8 depending on DM. Optical microscopy shows that pectin with a blockwise distribution of charges forms very large complex particles at all DM values studied, whereas pectin with a statistical distribution of charges forms considerably smaller complex particles, the average size of which is strongly dependent on DM. Separation of the complex pectin/lys systems by centrifugation and isolation of the complex phase showed that pectin with a blockwise charge distribution forms a gel-like complex phase at all DM values studied. Moreover, in contrast to pectin with a statistical charge distribution, pectin with a blockwise charge distribution does not form liquid coacervates at high DM values.

The critical I_{set} values above which complexes do not form is about the same for both types of pectin (0.10-0.11). According to De Vries (2004) and De Vries et al. (2003) this indicates there is only a small difference in the strength of CP of lys with SP and BP. It is interesting that the critical I_{set} and pH_{set} values of BP/lys systems, above which complexes did not form, are insensitive to DM, whereas they decrease significantly for SP/lys systems with the increase of DM. The effect of I on the CP of lys with pectin has a nonmonotonic character displaying a maximum at $I=0.03$ for the SP/lys system and $I=0.06$ for the BP/lys system. This ionic strength corresponds to a Debye length of approximately 1.7 nm, a value comparable to the size of the lys molecule.

The distribution of BP and SP within the complex particles has an irregular character and it is different for high DM values. The binding constant and the stoichiometry ratio of the systems with SP are slightly higher than those of BP at the same DM, and these values decrease sharply (50 times) with the increase of DM. Negative changes in enthalpy of BP/lys system with the increase of DM from 16 till 68% (change of ΔH from -41 kJ/mol to -58 kJ/mol) give evidence of the formation of a large quantity of hydrogen bonds or Van der Waals interactions during CP.

535

536 **Highlights (for review)**

537 Blockwise distribution of methoxyl groups enhances pectin/lysozyme complex aggregation.

538 Phase transition compositions depend weakly on methoxyl group distribution.

539 Critical ionic strength of complexation at low DM is independent of methoxyl group distribution.

540 Critical pH and ionic strength of complexation with blockwise pectin are insensitive to DM.

541 Binding constant is larger with statistical methoxyl group distribution.

542

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546

547 **References**

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Captions for Figures

Figure 1. The turbidity values at 500 nm (τ_{500}) as a function of q for BP16.9/lys (a, a', a''), BP33.2/lys (b, b', b'') and BP68.2/lys (c, c', c'', c''') mixtures at $I=0.01$, pH 5.1 and 23°C. In the insets (a', a'', b', b'', c', c'', c''') q_{onset} and q_{set} indicate the transitions between no complex formation and formation of soluble complexes whereas q_{ϕ} and q_{ϕ}^* indicate the transitions between formation of soluble and insoluble complexes. $C_{\text{tot}}^M = 0.04$ wt%. As a reference the results for SP16.2/lys and SP38.2/lys systems are added.

Figure 2. (a) The q_{onset} value of BP/lys systems as a function of DM and q_{ϕ} value of BP/lys systems as a function of DM. (b) The q_{Max} value of BP/lys systems as a function of DM, and $1/q_{\text{max}}$ value of BP/lys systems as a function of DM, $C_{\text{tot}}^M = 0.04$ wt%, $I=0.01$, pH 5.1 and 23°C. Error bars indicate the standard deviation and are smaller than the symbol size in Figure 2a.

Figure 3. The intensity size distribution function for BP16.9/lys (a), BP33.1/lys (b) and BP68.2/lys (c) mixtures. The arrow indicates the direction of change of the average size of the particles from $q=0.05$ to higher q values. d) Average radius of complex particles at q_{Max} as a function of DM values for SP/lys and BP/lys systems, $C_{\text{Tot}}^M=0.04$ wt%, $C_{\text{Pect}}^o=C_{\text{Lys}}^o=0.04$ wt%, pH 5.1, $I=0.01$ and 23°C.

Figure 4. Morphological changes in BP68.2/lys systems as a function of the weight ratio q . $C_{\text{Tot}}^M = C_{\text{pectin}}^o = C_{\text{lys}}^o = 0.04$ wt%, pH 5.1, $I=0.01$, 23°C, (a) $q=0.10$, (b) $q=0.20$, (c) $q=0.35$, (d) $q=0.51$. Full length of image is 120 μm .

Figure 5. Confocal microscopy images of the BP16.9/lys mixtures at different q values for $C_{\text{tot}}^{\text{M}} = 0.2\text{wt}\%$. a) $q=0.05$, b) $q=0.25$, c) $q=0.5$, d) $q=0.9$, e) $q=1.5$, f) $q=2.0$ for $C_{\text{tot}}^{\text{M}} = 0.2\text{wt}\%$, pH 5.1, $I=0.01$ and 23°C . Full length of images is 410 μm .

Figure 6. Confocal microscopy images of the BP68.2/lys mixtures at different q values for $C_{\text{tot}}^{\text{M}} = 0.2\text{wt}\%$, pH 5.1, $I=0.01$ and 23°C . a) $q=0.30$, b) $q=0.9$, c) $q=1.5$, d) $q=2.0$. Full length of images is 136 μm .

Figure 7. (a) The turbidity values at 500 nm (τ_{500}) as a function of pH for BP16.9/lys, BP33.2/lys, and BP68.2/lys systems, $C_{\text{Tot}}^{\text{M}} = C_{\text{Pect}}^{\text{o}} = C_{\text{Lys}}^{\text{o}} = 0.04 \text{ wt}\%$, $I=0.01$ and 23°C . (b) The turbidity values at 500 nm (τ_{500}) as a function of the ionic strength I for BP16.9/lys, BP33.2/lys, and BP68.2/lys systems, $C_{\text{Tot}}^{\text{M}} = C_{\text{Pect}}^{\text{o}} = C_{\text{lys}}^{\text{o}} = 0.04 \text{ wt}\%$, pH 5.1 and 23°C . The inset (b') presents I_{set} as a function of DM.

Figure 8. (a,c,e) Heat released in each injection versus the ratio between total ligand concentration and total protein concentration for SP16.2/lys (a), SP38.2/lys (c), and SP66.5/lys systems (e). (a') A characteristic titration experiment of the SP16.2/lys system. (b,d,f) Heat released in each injection versus the ratio between total ligand concentration and total protein concentration for BP16.9/lys (b), BP33.2/lys (d), and BP68.2/lys systems (f).

Figure 9. (a) The binding constants of the SP/lys and BP/lys systems as a function of DM and (b) stoichiometry values of the SP/lys and BP/lys systems as a function of DM, (c) the enthalpy of binding as a function of DM. The concentrations of lys and highly charged pectin were 1.833 mM and 0.0128 mM respectively, and the concentration of lys and low charged pectin were 1.3747 mM and 0.0192 mM.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9