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Effects of salt and protein concentrations on the association and dissociation of ovalbumin-pectin complexes

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ABSTRACT

Formation of ovalbumin-pectin coacervate complexes was analyzed in various NaCl concentrations and with various protein:polysaccharide ratios by isothermal titration calorimetry (ITC), by measuring zeta (ζ)-potentials, and by X-ray diffraction. The titration curve of a 1:1 ovalbumin:pectin coacervate complex formed in 0.01 M NaCl displayed a region containing insoluble complexes, a region of considerable complex formation, and a region of complex dissociation. Changes in protein concentrations led to shifts in the region of insoluble complex formation (at the isoelectric point). At an ovalbumin:pectin ratio of 8:1, complex formation was suppressed. At NaCl concentrations of 0.05 and 0.1 M, ovalbumin self-aggregation increased. When NaCl concentrations increased from 0.1 to 0.4 M, complex dissociation was suppressed. X-ray diffraction of the ovalbumin-pectin coacervate complex showed a partially defined crystalline region from 27 to 20° suggesting that the structure of the complex is more organized than the individual amorphous polymers. Finally, this study addressed the effect of ovalbumin self-aggregates on ovalbumin-pectin complex formation.

1. Introduction

Technical applications involving complexes formed with polyelectrolytes and oppositely charged colloids have received considerable attention since the early 20th century (Hartley, 1948). The term "complex" includes soluble complexes that involve liquid–liquid phase separations and coacervate complexes that involve solid–liquid separations and precipitations (Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2003). Polymer and protein complexes have been used in the development of controlled-release proteins and protein-based drugs (Malmsten, Bysell, & Hansson, 2010), to encapsulate bioactive compounds, cosmetic additives, flavorings, and living cells (Katona, Sovilj, & Petrovic, 2010; Leclercq, Milo, & Reineccius, 2010), to separate proteins (Hansen & Chang, 1968; Montilla et al., 2007), and to define biofilm structure (Duan, Jiang, & Zhao, 2011; Kuorwel, Cran, Sonneveld, Miltz, & Bigger, 2011).

Factors affecting complex formation and associative phase separations can be classified as intrinsic or extrinsic. Extrinsic factors include the macromolecules in the mixture, pH, ionic strength, amount of total solids, the rate of acidification, and the shear rate during acidification. When the pH is increased and is close to the p*I* (also known as the critical pH, pH_c), turbidity values of soluble complexes formed by non-covalent bonds increase slightly. When the pH is lowered, soluble complexes grow in size and number until a critical point, $pH_{\varphi 1}$. At $pH_{\varphi 1}$, insoluble complexes form and macroscopic separation occurs (Jones & McClements, 2010). When complexes have an overall neutral charge (zeta (ζ)- potential equals 0), the pH is ideal, pH_{ot} and complex formation is optimal. Reducing pH ($pH_{\varphi 2}$) leads to dissociation of protein and polysaccharide molecules due to positive protonation (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003).

Intrinsic factors include the characteristics of the polymer molecules, such as molecular weight, charge density, chain flexibility, and concentrations of the polymers (Kruif, Weinbreck, & Vries, 2004; Schmitt & Turgeon, 2011). The ratio of protein and polysaccharide in a mixture affects charge balance, thereby altering

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complex formation. The protein:polysaccharide ratio for maximum complex formation at a given pH must be determined (Tainaka, 1980; Turgeon, Schmitt, & Sanchez, 2007). When the ionic strength is high, the charges on the proteins and polysaccharides are screened, the electrostatic interactions are reduced, and complex formation is inhibited (Weinbreck, de Vries, et al., 2003; Ye & Singh, 2006). At low ionic strength, the effects on the protein and polysaccharide charges are small and do not impede electrostatic interactions and complex formation.

Although many biopolymer coacervation studies have focused on interactions between milk proteins (e.g. bovine serum albumin, milk protein isolate and β-lactoglobulin) (Li et al., 2012; Schmitt, Kolodziejczyk, & Leser, 2005; Weinbreck, de Vries, et al., 2003) and vegetable proteins (e.g. soy protein and pea protein) (Huang, Sun, Xiao, & Yang, 2012; Liu, Low, & Ickerson, 2009) with anionic polysaccharides (e.g. acacia, alginate, pectin) (Espinosa-Andrews, Baéz-González, Cruz-Sosa, & Vernon-Carter, 2007; Harnsilawat, Pongsawatmanit, & McClements, 2006; Sperber, Schols, Stuart, Norde, & Voragen, 2009), some have focused on egg proteins (Al-Hakkak & Al-Hakkak, 2010; Kudryashova & de Jongh, 2008; Mleko et al., 2010; Niu et al., 2014; Souza, Garcia Rojas, Melo, Gaspar, & Lins, 2013). Approximately 65% of egg white protein is ovalbumin. Ovalbumin has been used extensively in food technology because of its emulsifying and stabilizing properties (Sim & Nakai, 1994).

In addition, ovalbumin has been found to have antimutagenic and anticarcinogenic (Vis, Plinck, Alink, & van Boekel, 1998), immunomodulatory (Goldberg, Shrikant, & Mescher, 2003) and antioxidant (protection of linoleic acid and docosahexaenoic acid) properties (Nara, Miyashita, & Ota, 1995).

Pectin is a natural polysaccharide present in nearly all land plants and is responsible for the structural properties of fruits and vegetables (Li et al., 2012; Ru, Wang, Lee, Ding, & Huang, 2012). Pectins are characterized according to their degree of esterification (DE) or their degree of methoxylation (DM). Those with DMs greater than 50% (more than half of the carboxyl groups are methyl esters) are called pectin high methoxyl (HM), and those with less than 50% DM are called pectin low methoxyl (LM). In both cases, the remaining carboxyl groups are free acids (−COOH) and salts ($-COO^{-}Na^{+}$) (Lopes da Silva & Rao, 2010; Sperber et al., 2009).

The purpose of this study was to evaluate the effects of varying of pH, NaCl concentrations and protein: polysaccharide ratios on the formation of ovalbumin-pectin coacervate complexes.

2. Materials and methods

2.1. Materials

Ovalbumin (Ova; purity > 90%; 4.4×10^4 Da) and pectin high methoxyl (Pec; GM > 69%; 2.2×10^5 Da) were obtained from Sigma Chemicals (St. Louis, USA). Sodium chloride (NaCl, purity > 99%), hydrochloric acid (HCl, 0.5 M), and sodium hydroxide (NaOH, 0.5 M) were purchased from VETEC® Ltda, (Rio de Janeiro, Brazil). The water used was ultrapure with a conductivity of 0.05 μS/cm ± 0.01 (Gehaka-Master P&D − Brazil). Stock solutions of pectin (0.1% w/w) and ovalbumin (0.1–1% w/w) were prepared by gently stirring the powders in deionized water for 6 h at room temperature (25 °C).

2.2. Formation of complexes

2.2.1. Preparation of complexes

The concentration of pectin used was 0.1% w/w and the concentration of ovalbumin used varied from 0.1 to 1% w/w. Five ratios of Ova:Pec (1:1, 2:1, 3:1, 5:1, 10:1) were evaluated. To determine the effect of NaCl on complex formation, Ova:Pec complexes were formed in five concentrations of NaCl (0.01 M, 0.05 M, 0.1 M, 0.2 M, 0.4 M). The Ova:Pec mixtures were stirred and adjusted to pH 9.0.

2.2.2. Turbidimetric measurements

pH-dependent turbidity was measured at a wavelength of 400 nm using a spectrophotometer (Biochrom mod. LIBRA S12, England) calibrated with ultrapure water to 100% transmittance (T). Turbidity was defined as $100 - \%T$. With the aid of a magnetic stirrer (Nova Técnica, NT 101, Brazil) and a pH meter (Tecnopon, mPA-210, Brazil) the pH of the solutions were adjusted (9.0–1.0) with HCl, 0.5 M. Measurements of complexes and the solutions with biopolymers isolates were made at room temperature (25 °C ± 1), and each sample was measured four times at 1 min intervals.

2.3. Zeta − potential

A Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK) was used to determine zeta (ζ)-potentials. Pectin and ovalbumin stock solutions and Ova:Pec complexes formed in 0.01 M NaCl were diluted to 0.01% w/w and transferred to an MPT-2 autotitrator (Malvern Instruments, Worcestershire, UK) that adjusted pH using 0.5 M NaOH, 0.25 M HCl and 0.025 M HCl solutions. pH was varied from 9.0 to 1.0 by 0.5 unit increments with a confidence interval of ±0.1 unit. ζ-potentials were calculated using the Smoluschwski mathematical model. The Stokes–Einstein equation was used to calculate the average size of the particles. Complexes with different Ova:Pec ratios were diluted as described above and the pH was adjusted to 3.55. Each experiment was performed three times and sample readings were done in triplicate at 25 °C.

2.3.1. X-ray diffraction

X-ray diffractometer (XRD 6000, Shimadzu, Japan) was performed to determine whether polymers alone and in complex were crystalline or amorphous. The biopolymers pectin, Ovalbumin and Ova:Pec coacervates complexes were analyzed in triplicate using cokα radiation (λ = 1,7889 Å), scan angle of 2θ range of 15–60°, with a steep angle of $2°/min^{-1}$ and working conditions of 40 kV and 30 mA.

3. Results and discussion

3.1. Effect of pH

When Ova:Pec coacervate complexes were formed at varying pHs, we found three distinct regions of turbidity (A, B, C; Fig. 1) the transition regions ($pH_{\varphi 1}$, pH_{ot} and $pH_{\varphi 2}$) were determined by the intersections of the two tangents. In region A, the turbidimetric titration analysis (Klassen, Elmer, & Nickerson, 2011; Weinbreck, de Vries, et al., 2003) and dynamic light scattering analysis (Sperber et al., 2009) have showing that polysaccharide-protein complexes form soluble complexes through non-covalent attractions between polymers when the pH is near the isoelectric point of the protein (the pI of ovoalbumin is ~ 4.9), and this complex formation (referred to as pH_c) increases turbidity slightly however, we did not find significant differences in turbidity when titrating between pH 9.0 and 5.18. The absence of pH_c behavior observed in region A may be due to higher deprotonation because the pH of the system was above the pKa of pectin (pKa 3.5), and this deprotonation may have reduced the non-covalent attractions between the biopolymers. The absence of pH_c was also described by Ru et al. (2012) in a system containing 5:1 BSA (pI 4.7) and pectin (pKa 3.5) in 0.1 M NaCl; when titrating these complexes from pH 7–1, they observed