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Turbidity and rheological properties of bovine serum albumin/pectin coacervates: Effect of salt concentration and initial protein/polysaccharide ratio

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ABSTRACT

The turbidity and rheological properties of bovine serum albumin (BSA)/pectin coacervates show correlations with sodium chloride concentration (C_{NaCl}) and initial protein/polysaccharide ratio (r). Increasing C_{NaCl} from 0.01 to 0.4 M shifts the critical pH ($pH_{\varphi 1}$), which designates as the critical pH for BSA/pectin coacervate formation, to lower values, and the storage modulus (G') values of BSA/pectin coacervates tend to be smaller, which can be explained by a salt screening effect in the BSA/pectin coacervates. Moreover, an increase of r from 1:1 to 10:1 favors the formation of BSA/pectin coacervates, as indicated by the increase in $pH_{\varphi 1}$ and the decrease in $pH_{\varphi 2}$. The values of G' increase simultaneously. With further increase of r to 20:1, the $pH_{\varphi 1}$ changes negligibly and G' values become much lower. These results reflect that a balance between the positive charges of BSA and the negative charges of pectin favors the formation of BSA/pectin coacervates with more compact network structures.

1. Introduction

Proteins and polysaccharides are the most widely used food ingredients in food systems, and these two biopolymers are often used simultaneously (Benichou, Aserin, & Garti, 2002; Schmitt & Turgeon, 2011). Proteins are good at producing small droplets, but have relatively poor stability to environmental stresses, including pH, salt, heating, and freezing. Polysaccharides, on the other hand, provide good stability to environmental stresses, but are relatively poor at producing small emulsion droplets or require high concentrations. Proteins and polysaccharides are present together in many kinds of food systems, and both macromolecules contribute to the structure, texture, and stability of food. The most interesting feature revealed by the formation of complexes and various kinds of particles is that their functional properties are potentially better than those of proteins and polysaccharides alone (Guzey, Kim, & McClements, 2004; Huang, Given, & Qian, 2009). Therefore, understanding the interactions of proteins and polysaccharides is of importance not only in making cost-efficient use of functional ingredients, but also in designing novel foods, controlling and improving structures of food ingredients and textural properties of fabricated foods.

The main attractive force between proteins and polysaccharides is the long-range electrostatic interaction, which can be affected by physicochemical parameters, such as pH, ionic strength, protein/polysaccharide ratio, polysaccharide linear charge density, protein surface charge density, and stiffness of the polysaccharide chain (Schmitt & Turgeon, 2011). The interactions between proteins and polysaccharides in solution have been extensively investigated and reviewed over the last 10 years (Benichou et al., 2002; de Kruif, Weinbreck, & de Vries, 2004; Dickinson, 2008; Doublier, Garnier, Renard, & Sanchez, 2000; Rodríguez Patino & Pilosof, 2011; Schmitt & Turgeon, 2011; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). The general picture for coacervation between protein and anionic polysaccharide is that the charge "patches" on the protein molecules cause the protein to interact with anionic polysaccharides to form soluble protein/polysaccharide complexes when pH > pI, initiated at the first critical pH (pH_c). At the second critical pH ($pH_{\varphi 1}$), abrupt increases in turbidity illustrate the continued aggregation of soluble complexes into insoluble protein/polysaccharide complexes due to charge neutralization (Cooper, Dubin, Kayitmazer, & Turksen, 2005; Kaibara, Okazaki, Bohidar, & Dubin, 2000; Weinbreck, de Vries, Schrooyen, & de Kruif, 2003). For carboxylic acid-based polysaccharides, such as pectin, when pH decreases to the third critical pH ($pH_{\varphi 2}$), insoluble complexes dissociate into soluble complexes, or even into uninteracted protein molecules and polysaccharide chains (Dickinson, 2008).

Generally, the addition of salt weakens the formation of protein/polysaccharide coacervates (de Kruif et al., 2004;

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Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2003; Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004), and the addition of excess salt may completely suppress the formation of protein/polysaccharide coacervates (Wang, Lee, Wang, & Huang, 2007). An increase in salt concentration (from 0 to 0.05 M) shrinks the pH window (between $pH_{\varphi 1}$ and $pH_{\varphi 2}$), where whey protein/gum arabic (WP/GA) complex coacervation takes place (Weinbreck, de Vries, et al., 2003). Above a critical NaCl concentration of 0.06 M, WP/GA complex coacervation is suppressed, and above 0.077 M NaCl, no coacervate phase is obtained because coacervation is inhibited (Weinbreck, Tromp, & de Kruif, 2004). The results are generally attributed to the addition of micro-ions in the mixture, which screen the charges of the polymers and decrease complex formation. However, in contrast to the salt screening effect, some authors noted that added salts can enhance the formation of protein/polysaccharide complexes at certain salt concentrations (Seyrek, Dubin, Tribet, & Gamble, 2003; Weinbreck, Nieuwenhuijse, et al., 2003). Laos, Brownsey, and Ring (2007) reported that the attractive interaction of both β-lactoglobulin (β-lg) and bovine serum albumin (BSA) with furcellaran becomes more favorable as the salt concentration increases to 0.03 M, and further increase in salt concentration from 0.05 M to 0.075 M reduce the strength of this interaction. In β-lg/pectin system with similar electrostatic nature, the addition of salt can either enhance or reduce the formation of β-lg/pectin complexes at specific salt concentrations of 0.1 M or 0.21 M, as shown by studies of turbidimetric titration, composition analysis, dynamic rheological measurements, and small-angle neutron scattering (Wang, Lee, et al., 2007; Wang, Li, Wang, Lal, & Huang, 2007; Wang, Wang, Ruengruglikit, & Huang, 2007). The initial protein/polysaccharide ratio is another important factor in protein/polysaccharide coacervation. The values of pH_c are independent of the initial protein/polysaccharide ratio, whereas $pH_{\varphi 1}$ values first increase and then stabilize with increasing initial protein/polysaccharide ratio to 30:1 for the coacervation between whey protein and λ-carrageenan (Weinbreck, Nieuwenhuijse, et al., 2004). Girard, Sanchez, Laneuville, Turgeon, and Gauthier (2004) found that β-lg/pectin complex amounts were higher at the 5:1 β-lg/pectin ratio than that at the 2:1 ratio. The higher initial protein/polysaccharide ratio often results in higher actual protein/polysaccharide ratio in the coacervates and favors the formation of stronger gel-like coacervates, which indicates that a more compact network structure is favorable for coacervation (Wang, Lee, et al., 2007; Weinbreck, Tromp, et al., 2004).

BSA is a model globular protein with a well-known structure. One BSA molecule contains 583 amino acids in a single polypeptide chain with a molecular weight of about 66 kDa. The anionic water-soluble polysaccharide examined in the present work is pectin, a natural polysaccharide extracted from plant cell walls widely known as a gelling and thickening agent, and a stabilizer for food, cosmetics, and pharmaceutical products (Thakur, Singh, Handa, & Rao, 1997). Pectin has traditionally been used to achieve jams or marmalades with jelly-like consistency, and stabilize acidic protein drinks, such as yogurt. Among more than 100 globular protein-polysaccharide mixtures studied, only BSA and pectin (with 0–100% degree of esterification) are thermodynamically compatible due to the formation of soluble interbiopolymer complexes (Burova et al., 1999). Pectin is identified by two parameters: the degree of methyl-esterification (DM) of the carboxyl group and the distribution of these methyl-esters along the pectin backbone. Based on its degree of esterification, pectin is divided into low methyl-esterified pectin (LM), DM < 50%, and high methyl-esterified pectin (HM), DM > 50%. Further characterization of pectin can be based on its degree of blockiness (DB), which is a measure for the size of the non-methyl-esterified blocks of GalA along the pectin backbone. A high DB value means that the methyl-esters are distributed in a blockwise manner; while a low DB value means a random distribution. Pectin with high total charge density leads to a relatively larger amount of protein molecules bound on pectin chains. Therefore, protein molecules will screen the charges of pectin to a large extent (Wang, Li, et al., 2007). Furthermore, pectin with the same DM but different DB value or local charge density, shows different behavior in its complexation with β-lg. This difference in local charge density has an effect on the sensitivity of complex formation between β-lg and pectin for ionic strength. The blockwise distributed pectin with high local charge density allows the formation of complexes at a higher ionic strength than the random distributed pectin having a low charge density. LM pectin has a higher local charge density and forms complexes with β-lg even at high ionic strengths (Sperber, Schols, Cohen Stuart, Norde, & Voragen, 2009). In the current research, the BSA/pectin coacervates are prepared at various NaCl concentrations and different initial protein/polysaccharide ratios. The aim of current study is to investigate the effects of added salt and the initial protein/polysaccharide ratio on the turbidity and rheological properties of BSA/pectin coacervates. In addition, schematic pictures of the microstructures of BSA/pectin coacervates achieved at various initial protein/polysaccharide ratios are also provided.

2. Materials and methods

2.1. Materials

BSA (A-7030, lyophilized powder, ≥98% pure by gel electrophoresis and essentially fatty acid free) was purchased from Sigma Chemical Co. and used without further purification. According to the manufacturer, the molecular weight of the BSA was about 66 kDa. GRINDSTED® Pectin LC 950 (413008), a powder manufactured from citrus peel, was provided by Danisco A/S, Denmark. It is a LM pectin with approximately 31% degree of esterification. It was further purified by dialysis (Spectra/Por dialysis membrane with a molecular weight cutoff equal of 12,000) against 0.005 M EDTA at pH 6.4 for 2 days followed by freeze-drying. The average molecular weight (M_w) of the purified pectin as determined by gel permeation chromatography was approximately 7.0×10^5 (relative to protein standards), and the polydispersity (M_w/M_n) was 1.2. Since the pectin molecules used in current study has been dialyzed, they contained negligible amount of metal ions. Sodium chloride (NaCl, purity >99%), standard sodium hydroxide (NaOH, 0.5 M), and standard hydrochloric acid (HCl, 0.5 M) were purchased from Fisher Scientific (Pittsburgh, PA) and used as received. Milli-Q water (18.3 Ω) was used in all experiments.

2.2. Sample preparation

For the turbidimetric titrations, the final concentration of pectin was fixed at 0.1 wt%, and the final concentration of BSA was from 0.1 wt% to 2 wt%. The same total volume but different ratios of BSA and pectin stock solutions were mixed together with initial BSA/pectin ratios varied from 1:1 to 20:1. The BSA/pectin ratios of 1:1 and 20:1 were defined as 1 part BSA to 1 part pectin and 20 parts BSA to 1 part pectin, respectively. The BSA/pectin mixtures were prepared by first adjusting the pH to 7.0 under magnetic stirring.

Samples prepared for rheological measurements had two differences compared with those for turbidimetric titrations. First, the final pectin concentration was fixed at 0.5 wt% for all BSA/pectin mixtures because large amounts of coacervates were needed for the rheological measurements. Second, the pH of BSA/pectin mixtures was adjusted to 3.0 by 0.5 M standard HCl solution. Following acidification, the coacervates were collected after through centrifugation at 3000 rpm (≈2500 × g) for 30 min at 25 °C, followed by

the removal of supernatants. The yields of BSA/pectin coacervates varied from 3% to 7%.

2.3. Turbidimetric titrations

The pH-dependence of turbidity was measured using a Brinkman PC 910 colorimeter equipped with a 1-cm path length optical probe and a 420-nm filter. The colorimeter was calibrated to read 100% transmittance (T) with Milli-Q water. Turbidity was defined as $100 - T\%$. The solutions were filtered with 25 mm 0.45-μm Whatman GD/X syringe filters before turbidimetric titrations. 0.5 M HCl solution was used to adjust the pH of the mixed solutions containing BSA and pectin, or the pure pectin and BSA solutions. After each small droplet of HCl was added, the turbidity value was collected, and the pH was monitored with a Thomas Scientific pH meter (Model 8025) calibrated with two buffers of pH 4 and 7. All titrations were carried out with magnetic stirring, and the time interval between measurements was fixed at 1 min. All measurements were conducted at 25 °C and repeated four times.

2.4. Rheological measurements

Dynamic rheological measurements were performed using Advanced Rheometric Expansion System (ARES, TA Instruments, New Castle, USA) on a strain-controlled rheometer with parallel plate geometries (25 or 50 mm in diameter). The BSA/pectin coacervate samples were loaded onto the plate for 10 min to allow the stresses to relax and the samples to reach thermal equilibrium. The storage modulus (G') and the loss modulus (G'') were measured while the frequency was varied from 0.1 to 100 rad/s. Strain sweep tests were preliminarily carried out to determine the proper conditions of ARES operation. All measurements were conducted at 25 °C and repeated four times.

3. Results and discussion

3.1. pH-induced phase separation for the BSA/pectin complexes

The turbidity arises mainly from the change in mass and size of aggregates in the solution, so the changes in turbidity are believed to be the result of the formation and dissociation of protein/polysaccharide coacervates, similar to other protein/polymer systems (Xia, Dubin, & Dautzenberg, 1993). The initial pH of the mixture is set to 7.0. The addition of 0.5 M HCl slowly decreases the pH to 1.0 in approximately 2 h. Fig. 1 shows the typical turbidimetric titration curve ($100 - T\%$) versus pH for the BSA/pectin mixture at $r = 5{:}1$ in 0.1 M NaCl solution. Primary soluble protein/polysaccharide complexes are usually formed before insoluble protein/polysaccharide complexes (Wang, Wang, et al., 2007). Based on previous studies of polyelectrolyte/protein complexation (Laos et al., 2007; Seyrek et al., 2003), soluble complexes between protein and polysaccharide are observed at pH values above the pI. The point of soluble complex formation, initiated at pH_c, is viewed as the point of incipient binding of pectin and BSA, and cannot be clearly determined from the nearly constant turbidity prior to $pH_{\varphi 1}$ in the current pectin and BSA mixtures (Wang, Wang, et al., 2007). As the point of soluble complex formation is not the focus of the present paper, we focus instead on the effects of C_{NaCl} and r on the coacervates formation (at $pH_{\varphi 1}$) and dissociation (at $pH_{\varphi 2}$) in the BSA and pectin mixtures. The phase diagram indicates that the pI of BSA is a critical pH that causes the sudden change in turbidity. At pH 4.7, which is considered as the global phase separation point ($pH_{\varphi 1}$) of pectin–BSA coacervates, the solution turbidity starts to increase dramatically, and then reaches a plateau. This stage is followed by a large decrease in turbidity with decreasing pH, ending at another critical value $pH_{\varphi 2}$. No $pH_{\varphi 2}$ is observed in the pH titration

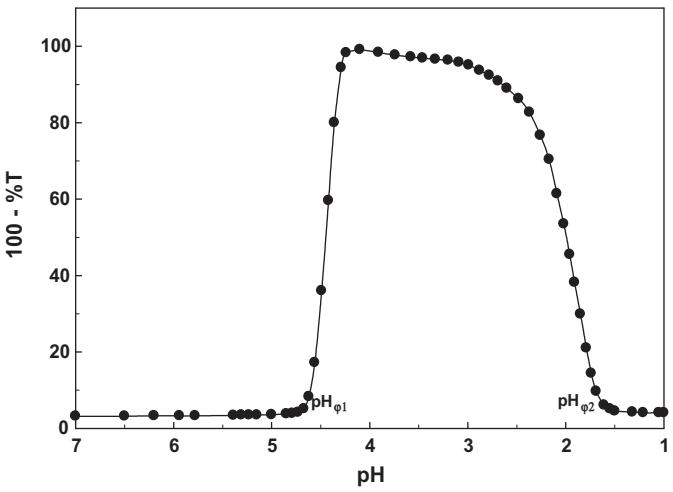

Fig. 1. The turbidity ($100 - T\%$) of the BSA/pectin mixture as a function of pH at NaCl concentration (C_{NaCl}) = 0.1 M and BSA/pectin ratio (r) = 5:1.

curve of κ-carrageenan–BSA, even at pH values as low as 1.0 (Lee, 2008). This finding suggests that $pH_{\varphi 2}$ is mainly affected by the pK_a of the polysaccharides used. For pectin, when the pH decreases to values below its pK_a due to the low charges of pectin chains, as well as the repulsion between the positively charged proteins, the protein (e.g., BSA)/pectin coacervates may dissociate into soluble complexes, or even into uninteracted protein molecules and polysaccharide chains (Dickinson, 2008).

3.2. Rheological properties of BSA/pectin complexes

Although no chemical reaction occurs between salt ions and polysaccharide, changes in the viscosity, elasticity, fluidity, and stability of the food system may occur upon the addition of salt. In addition, the pH and the protein/polysaccharide ratio influence the rheological properties of such systems (Magnin, Lefebvre, Chornet, & Dumitriu, 2004; Turgeon et al., 2003; Weinbreck, Nieuwenhuijse, et al., 2004). Fig. 2a and b shows typical results of small deformation oscillatory measurements of BSA/pectin coacervates and 2 wt% pure pectin solution at 0.1 M C_{NaCl}, respectively. In Fig. 2a, a gel-like behavior is observed for the BSA/pectin coacervate. In contrast, the pure pectin solution exhibits the typical viscoelastic behavior of a polymer solution, and has much smaller G' and G'' values compared with BSA/pectin coacervates at similar pectin concentrations. Furthermore, the pure pectin solution is less elastic. As G' and G'' are frequency-dependent, and G' is significantly higher than G'', the BSA/pectin coacervates have highly interconnected gel-like network structures with mainly elastic behavior. The complex viscosity decreases nearly linearly with frequency, showing the general shear-thinning phenomenon, as depicted in Fig. 2b. Therefore, the elastic behavior of the BSA/pectin coacervates should originate mainly from the electrostatic interactions between the protein and the polysaccharide chains (Bastos et al., 2010), which are consistent with the behaviors of β-lg/pectin coacervates reported previously (Wang, Lee, et al., 2007). Many complex foods are composed of proteins, polysaccharides, or their mixtures. Intermolecular or intramolecular binding of polysaccharides with water affects the dynamic properties of final food products, which become more complicated in the presence of salt, especially when the charged functional groups are present in the polysaccharide chains. Our dynamic rheological results of BSA/pectin coacervates agree with the rheological properties of the simple coacervates formed by gelatin (Mohanty & Bohidar, 2005). Nevertheless, in reported frequency sweep experiments of

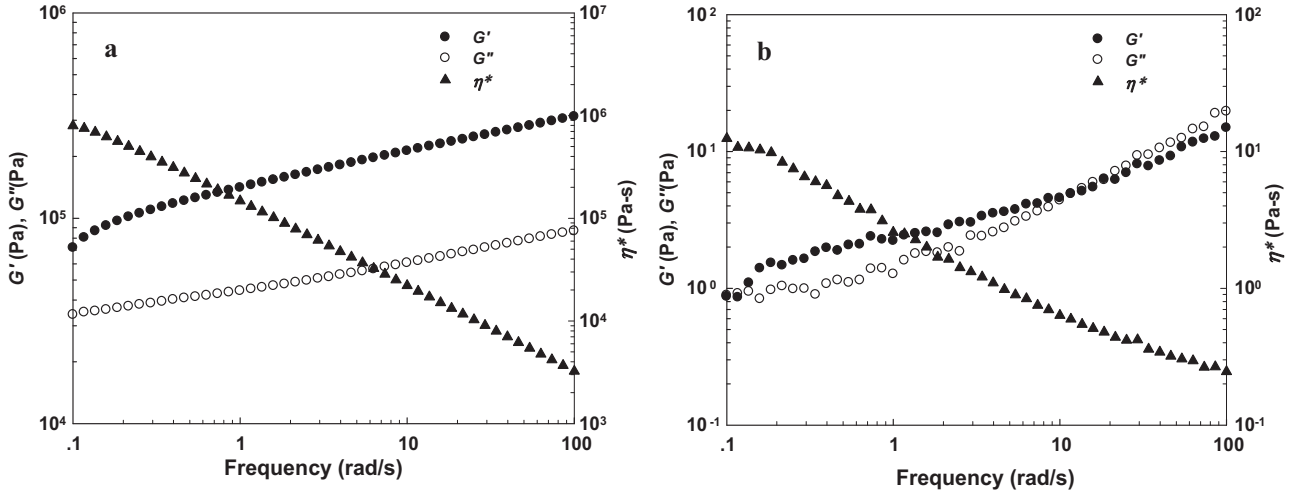

Fig. 2. (a) The complex viscosity η^*, the storage modulus G', and the loss modulus G'' versus the angular frequency for the BSA/pectin coacervates prepared at NaCl concentration (C_{NaCl}) = 0.1 M and BSA/pectin ratio (r) = 5:1; (b) The complex viscosity η^*, the storage modulus G', and the loss modulus G'' versus the angular frequency for 2 wt% pectin solution at NaCl concentration (C_{NaCl}) = 0.1 M.

WP/GA coacervates (Weinbreck, Wientjes, Nieuwenhuijse, Robijn, & de Kruif, 2004), the values of G'' are often 3–7 times higher than the values of G', showing the highly viscous character of WP/GA coacervates. Coacervates of BSA with synthetic polyelectrolyte poly(diallyldimethylammonium chloride) also have a viscous nature, with G'' larger than G' at high frequency ranges (Bohidar, Dubin, Majhi, Tribet, & Jaeger, 2005). The different viscoelastic properties observed in the different systems reflect the characteristics of the protein/polymer pairs and their distinct coacervate structures. The gel-like structure of the BSA/pectin coacervates implies that the BSA molecules and the pectin chains form a more strongly entangled network.

3.3. Effect of salt concentration

Fig. 3a shows the effect of C_{NaCl} on the turbidity of the mixtures of BSA and pectin. The turbidities for all the turbidimetric titration curves are constant and very small until the pH reaches a critical value designated as $pH_{\varphi 1}$, after which the turbidities rapidly increase and reach a plateau at all C_{NaCl}. Nevertheless, the turbidities change differently in different salt concentration regions when the pH was further decreased. When $C_{NaCl} \leq 0.2$ M, the plateau is followed by a large decrease in turbidities with pH decreasing to $pH_{\varphi 2}$. When C_{NaCl} reaches 0.4 M, after the plateau, the decrease in turbidity is much smaller and $pH_{\varphi 2}$ is not observed. The effect of salt concentration on $pH_{\varphi 1}$ and $pH_{\varphi 2}$ values is summarized in Fig. 3b. The values of $pH_{\varphi 1}$ shift to smaller values with increasing C_{NaCl}, and $pH_{\varphi 2}$ does not show dependence on C_{NaCl} except at 0.4 M NaCl. Thus, increasing C_{NaCl} reduces the range of $pH_{\varphi 1}$–$pH_{\varphi 2}$ for BSA/pectin coacervates, which is in agreement with the previous work of Weinbreck et al. in whey protein/gum arabic (WP/GA) complexes (Weinbreck, de Vries, et al., 2003). The salt-dependent change in $pH_{\varphi 1}$ is a well-known phenomenon. Weinbreck, de Vries, et al. (2003) highlighted the strong effect of salt concentration on complex formation because the addition of NaCl causes a decrease in $pH_{\varphi 1}$. The added micro-ions can screen charges on the protein and the polysaccharide, so the more BSA carries positive charges for interacting with the negatively charged pectin, the weaker the tendency for BSA and pectin to form complexes. In Fig. 3b, $pH_{\varphi 2}$ cannot be determined from the turbidity curve for a C_{NaCl} of 0.4 M. The disappearance of $pH_{\varphi 2}$ may be due to the unique character of pectin of self-aggregation. Pectin molecules aggregate when their pH is lowered, and an increase in the salt concentration enhances this aggregation (Yoo, Fishman, Savary, & Hotchkiss, 2003). Fig. 3c shows the formation of pectin aggregates from the increase in turbidity for pure 0.1 wt% pectin in NaCl solution (at pH value about 1.6), which is similar to the pH window for the dissociation of BSA/pectin coacervates. BSA does not show self-aggregation even at the highest C_{NaCl} (0.4 M) solution in our experiment (data not shown). Comparing Figs. 3a and c, the observed turbidity curves in the mixtures of BSA and pectin can be attributed to both the formation of BSA/pectin complexes and pectin aggregates, especially at higher salt concentrations. Therefore, the competition between self-aggregation of pectin and BSA/pectin coacervation must be considered.

Physicochemical parameters, which affect either protein–polysaccharide interactions or polysaccharide–polysaccharide aggregation, have an impact on the rheological properties of BSA/pectin coacervates. In general, the presence of salt in the solution weakens the interaction, and hinders the formation of protein/polysaccharide coacervates (de Kruif et al., 2004; Weinbreck, Nieuwenhuijse, et al., 2003; Weinbreck, Nieuwenhuijse, et al., 2004; Weinbreck, Tromp, et al., 2004). Dynamic rheological measurements were carried out on the BSA/pectin coacervates, and all the coacervates show higher G' values than G'' values. This finding suggests the formation of a gel-like structure. The results of G' as a function of frequency for the BSA/pectin coacervates prepared at r = 5:1 with C_{NaCl} varying from 0.01 to 0.4 M are plotted in Fig. 4a. To clearly illustrate the salt effect on the values of G', the variation of G' values at 0.1 rad/s frequency as a function of C_{NaCl} is given in Fig. 4b. From Fig. 4, the values of G' are inversely proportional to C_{NaCl}, mainly because the coacervate network is weakened by the added salt. Complex formation is driven by the increase of entropy due to the expulsion of small ions from the double layers around the individual polyelectrolyte chains, while in the case of weak polyelectrolytes, the polyelectrolyte is able to increase the charge of the polyelectrolyte groups, which implies a further decrease of the free energy (Biesheuvel & Cohen Stuart, 2004). A large amount of salt hinders the release of small ions, thus suppressing the driving force for complexation (Espinosa-Andrews, Báez-González, Cruz-Sosa, & Vernon-Carter, 2007), and effectively reducing the available sites for interactions between both macromolecules. Therefore, higher salt concentrations lead to a more watery coacervate structure as the total biopolymer concentrations in the protein/polysaccharide coacervates decrease monotonically and the water concentration

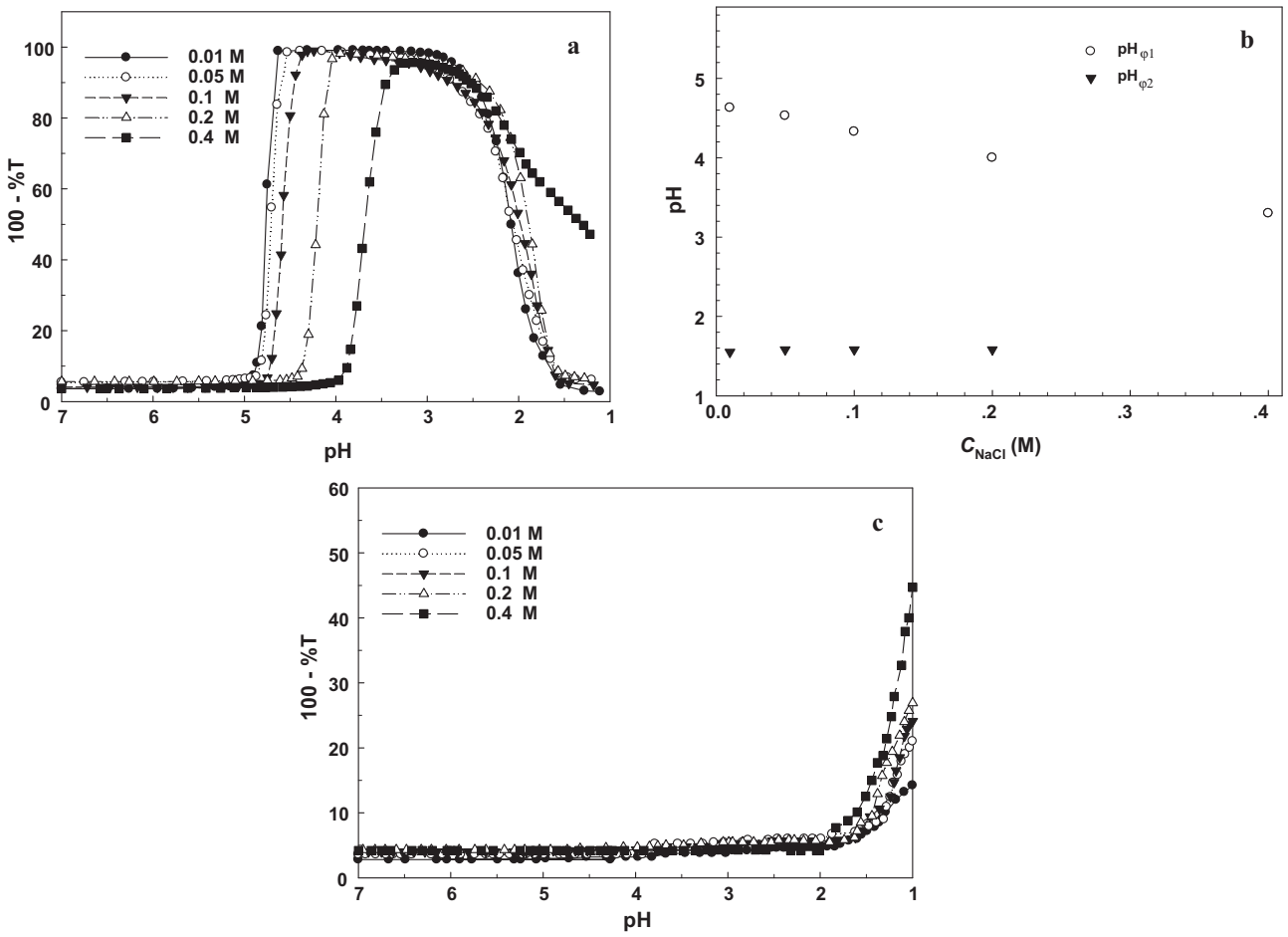

Fig. 3. (a) The turbidity ($100 - T\%$) of the BSA/pectin mixtures as a function of pH at BSA/pectin ratio (r) = 5:1 and various NaCl concentration (C_{NaCl}) values; (b) the variation of $pH_{\varphi 1}$ and $pH_{\varphi 2}$ as a function of NaCl concentration (C_{NaCl}); (c) the turbidimetric titration curves of a 0.1 wt% pectin solution at various NaCl concentration (C_{NaCl}) values.

increases conversely with the increase of salt concentration (Wang, Lee, et al., 2007), which is ascribed to the screening of the electrostatic interaction between the protein and the polysaccharide (Weinbreck, Tromp, et al., 2004).

In agreement with the Veis–Aranyi model (Veis & Aranyi, 1960), several experimental results (Cousin, Gummel, Ung, & Boue, 2005) suggest that complex coacervation occurs in two steps. First, the oppositely charged protein and polysaccharide form neutral aggregates, and then these aggregates rearrange to form coacervates. The first step is mainly driven by electrostatic interactions between the protein and polysaccharide. The second process of rearrangement of the protein/polysaccharide complexes into coacervates may

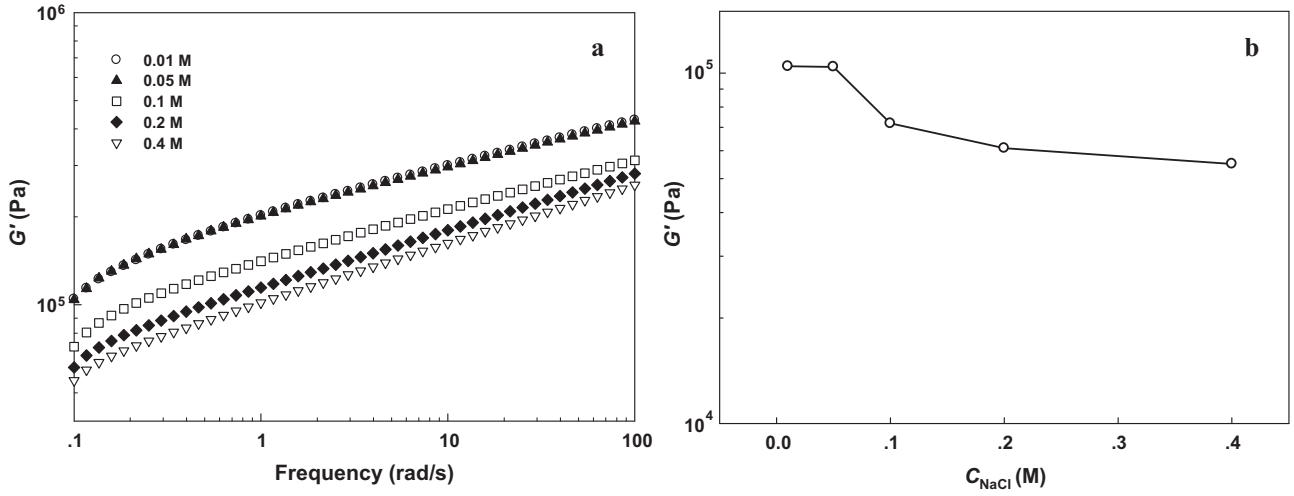

Fig. 4. (a) The storage modulus G' curves for the BSA/pectin coacervates prepared at BSA/pectin ratio (r) = 5:1 and various NaCl concentration (C_{NaCl}) values; (b) the variation of the storage modulus G' values at 0.1 rad/s frequency as a function of NaCl concentration (C_{NaCl}).

involve non-electrostatic interactions, such as hydrogen bonding, hydrophobic interactions, or van der Waals forces. If only electrostatic interactions are taken into account, the addition of salt can weaken the interaction strength between the protein and polysaccharide, causing the protein/polysaccharide coacervates to more likely dissociate into soluble protein/polysaccharide complexes. Non-electrostatic attractions are expected to promote protein-polysaccharide association and facilitate protein/polysaccharide cluster formation (Carlsson, Linse, & Malmsten, 2001; Carlsson, Malmsten, & Linse, 2003). Due to the low degree of pectin esterification (31% esterified pectin was used), hydrogen bonding may give a minor contribution to coacervate formation, since higher-esterified pectin favors the formation of complexes between pectin and protein (Girard, Turgeon, & Gauthier, 2003b). Girard, Turgeon, and Gauthier (2002) demonstrated that the interactions in the β-lg/pectin system are mainly caused by electrostatic forces, and, to a lesser extent, hydrogen bonding. Hydrophobic interactions between pectin chains can be neglected due to the hydrophilic backbone of pectin. Therefore, hydrogen bonding and van der Waals forces as minor interactions may be considered to coexist with electrostatic interaction during BSA/pectin coacervation, as suggested by the slight decrease in G' values with increasing salt concentration.

3.4. Effect of BSA to pectin ratio

The effect of biopolymer mixing ratio is critical in controlling the charge balance within mixed systems (Ye, 2008). The development of a structure within the homogenous and mixed BSA/pectin systems was investigated as a function of r by turbidimetric analysis during a base titration. In Fig. 5a, the titration curves of turbidity ($100 - T\%$) from the turbidimetric titration versus pH for the BSA/pectin mixtures as a function of r illustrate that the turbidities increase as r increases from 1:1 to 3:1. The turbidities then rapidly increase and reach a plateau as r increases from 3:1 to 20:1. The higher turbidity results from the larger amount of aggregates that form due to the larger number of positively charged BSA molecules available to neutralize pectin negative charges. The number of protein molecules available per pectin chain is important in electrostatic complex formation (Girard, Turgeon, & Gauthier, 2003a). pH_φ is the state boundary between the soluble complexes, and is related to charge neutralization in the protein-polysaccharide complex, which implies that variations in the ratio between the protein and polysaccharide affect pH_φ (Cooper et al., 2005; Weinbreck, de Vries, et al., 2003). The dependence of $pH_{\varphi 1}$ and $pH_{\varphi 2}$ values on the BSA to pectin ratio is depicted in Fig. 5b, which indicates the variance of $pH_{\varphi 1}$ and $pH_{\varphi 2}$ on r. An increase in r of up to 10:1 shifts the $pH_{\varphi 1}$ to higher pH values. When there is more BSA in the solution, more BSA binds to the pectin, resulting in charge neutralization at a higher pH, which is in contrast to the salt screening effect. Similar $pH_{\varphi 1}$ shifts have been reported by Weinbreck, Nieuwenhuijse, et al. (2003) and Elmer, Karaca, Low, and Nickerson (2011). This finding reflects the abundance of protein molecules available per polysaccharide chain. The values of $pH_{\varphi 2}$ tend to have smaller pH values when r increases from 1:1 to 10:1. The smaller $pH_{\varphi 2}$ indicates that the BSA/pectin coacervates are more difficult to dissociate at higher r, which is in accordance with the foregoing discussion about r effects. pH_φ remains stable at $r = 10{:}1$, indicating that saturation of BSA has probably taken place, in agreement with the previously reported findings by Klassen, Elmer, and Nickerson (2011), Liu, Low, and Nickerson (2009), Weinbreck, de Vries, et al. (2003), and Weinbreck, Nieuwenhuijse, et al. (2003, 2004). The authors attribute this behavior to the increased amount of protein molecules available per polysaccharide chain for binding. Fig. 5c indicates the onset of BSA aggregation from the increase in turbidity for pure 0.2 wt% BSA solution, suggesting the occurrence of BSA aggregation at around pH 4.9. This result indicates that BSA undergoes self-aggregation at 0.2 wt%, although this concentration is a slightly lower than the findings in other reports (Cao et al., 2008).

Dynamic rheological measurements were also completed to investigate the influence of r on the structure of the BSA/pectin coacervates. Fig. 6a shows the values of G' as a function of frequency for BSA/pectin coacervates prepared at various r. Fig. 6b gives the variation of G' values at 0.1 rad/s frequency as a function of r. When r reaches 10:1, the maximum yield of the coacervates is reached, and the maximum shear modulus is observed. The high G' values indicate a stronger intermolecular BSA/pectin network, possibly due to interactions between protein–protein and protein–polysaccharide molecules. This phenomenon is also observed in coacervation of whey protein and λ-carrageenan due to the occurrence of charge neutralization between both types of macromolecules (Weinbreck, Nieuwenhuijse, et al., 2004). Therefore, the positive charges in the BSA and the negative charges in the pectin chains are nearly balanced at $r = 10{:}1$. Wang, Lee, et al. (2007) found significant correlations between the rheological properties and the composition of the β-lg/pectin coacervates. The increase in r favors the formation of stronger gel-like coacervates. However, further increases in r to 20:1 cause excess amounts of BSA molecules to actually decrease the G' value of the coacervates.

The results of the turbidimetric titration and dynamic rheological measurements suggest that the protein/polysaccharide coacervates can be considered to be a network of polysaccharide chains generated from the aggregation of protein/polysaccharide complexes, where bound proteins are taken as linkers. When the coacervates dissociate at $pH_{\varphi 2}$, the electrostatic repulsive and van der Waals attractive potentials are expected to play important roles. Furthermore, a higher protein/polysaccharide ratio means that more protein molecules are available for binding with the polysaccharide chains. Protein/polysaccharide coacervates have higher amounts of protein molecules in the coacervate phase (Wang, Lee, et al., 2007; Weinbreck, Tromp, et al., 2004). More protein molecules bound to the polysaccharide chains may cause higher electro-neutrality in the polysaccharide chains. Consequently, a higher protein/polysaccharide ratio will cause the protein/polysaccharide complexes to aggregate more tightly to form protein/polysaccharide coacervates. The value of G' is relatively higher at $r = 10{:}1$, which suggests that the density of the positive charges on the BSA is approximately equal to that of the negative charges on pectin, and their interaction is almost maximized.

3.5. Schematic pictures of the microstructure of the BSA/pectin coacervates at different r

Based on the turbidimetric titration and rheological measurements of the BSA/pectin coacervates, schematic pictures of the microstructures of the BSA/pectin coacervates prepared at 0.1 M NaCl and different r are presented in Fig. 7. Structure A denotes the microstructure of the BSA/pectin coacervates at $r = 1{:}1$. In this case, because of the lower BSA amount, the electrostatic interactions between the BSA molecules and the pectin chains are relatively weak, and the amounts of the protein molecules and the pectin chains are relatively small in the coacervates. Most BSA molecules are separately bound onto the network of the pectin chains. The increase in r from 1:1 to 10:1 causes relatively larger amounts of BSA molecules and pectin chains to exist in the BSA/pectin coacervates. The results from the turbidimetric titration and dynamic rheological measurements demonstrate that an increase in r from 1:1 to 10:1 leads to the formation of tighter coacervate networks with higher turbidities and G' values. This case is illustrated in Structure B, which corresponds to the BSA/pectin

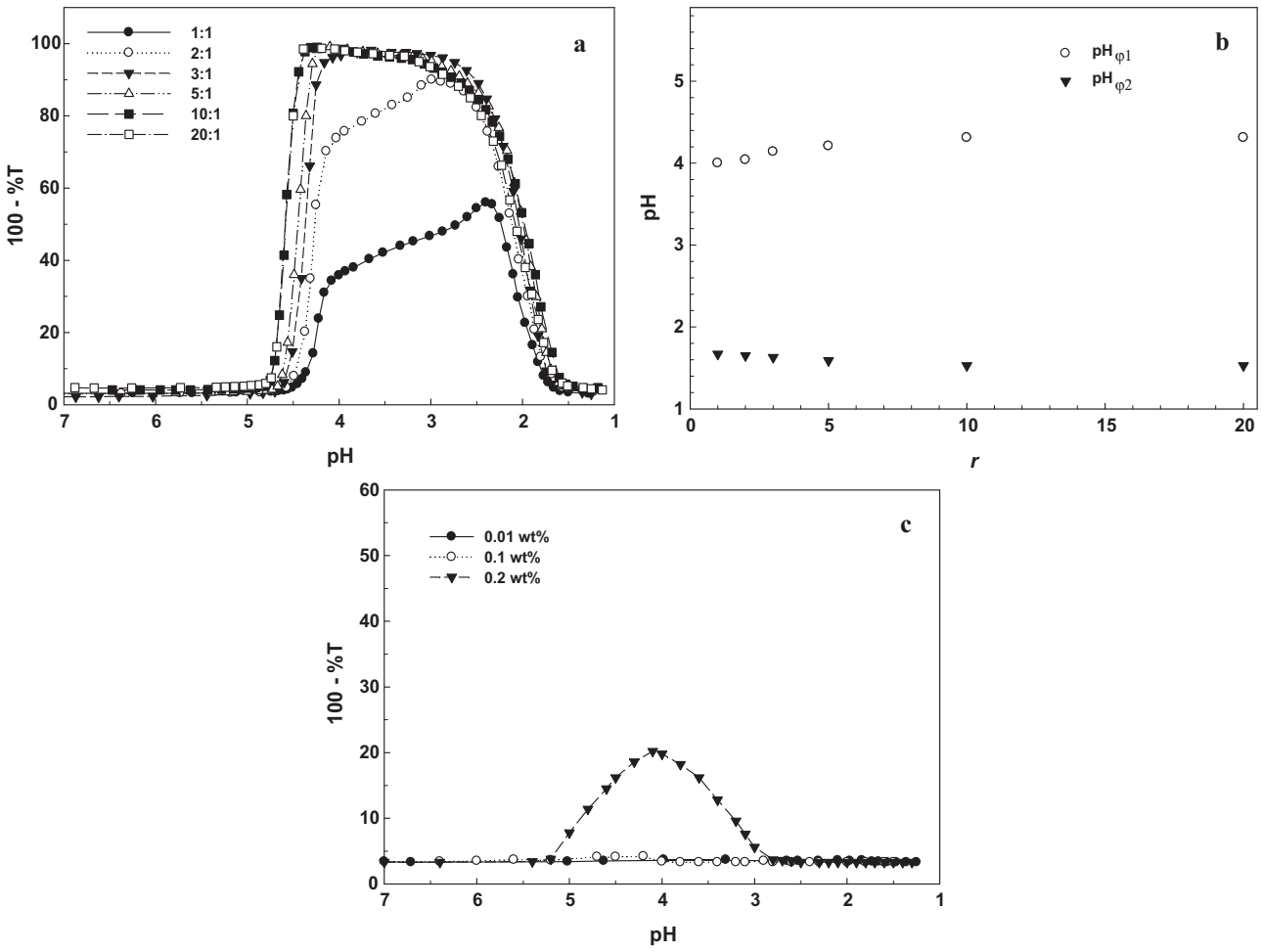

Fig. 5. (a) The turbidity ($100 - T\%$) of the BSA/pectin mixtures at NaCl concentration (C_{NaCl}) = 0.1 M and various initial BSA/pectin ratios (r); (b) the variation of $pH_{\varphi 1}$ and $pH_{\varphi 2}$ as a function of initial BSA/pectin ratio (r); (c) the turbidimetric titration curves of the BSA solution at NaCl concentration (C_{NaCl}) = 0.1 M with various BSA concentrations.

coacervates at r = 10:1. Structure C corresponds to the structure of the BSA/pectin coacervates at r = 20:1. In this case, excess amounts of BSA promote the formation of BSA aggregates with larger sizes and weaken the binding between BSA and pectin, as suggested by the lower G' value at r = 20:1. The proposed microstructure of the BSA/pectin coacervates and the mesh size of the BSA aggregation shown in Fig. 7 are hypothetical. To obtain a more definite picture of the coacervates formed, it would be necessary to carry out detailed structural analyses using an array of experimental techniques.

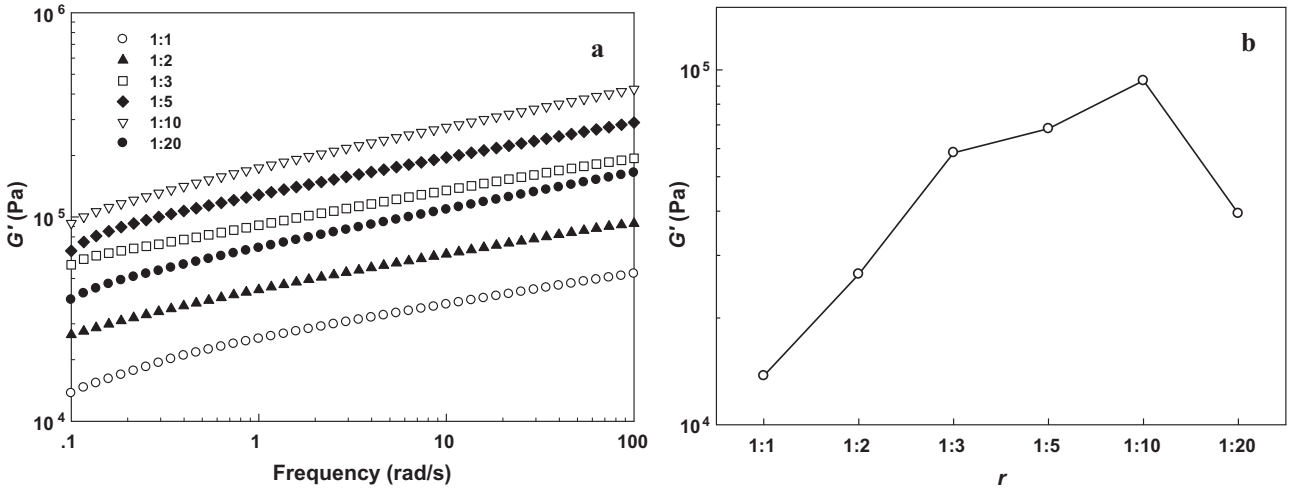

Fig. 6. (a) The storage modulus G' curves for the BSA/pectin coacervates prepared at NaCl concentration (C_{NaCl}) = 0.1 M and various initial BSA/pectin ratios (r); (b) the variation of the storage modulus G' values at 0.1 rad/s frequency as a function of initial BSA/pectin ratio (r).

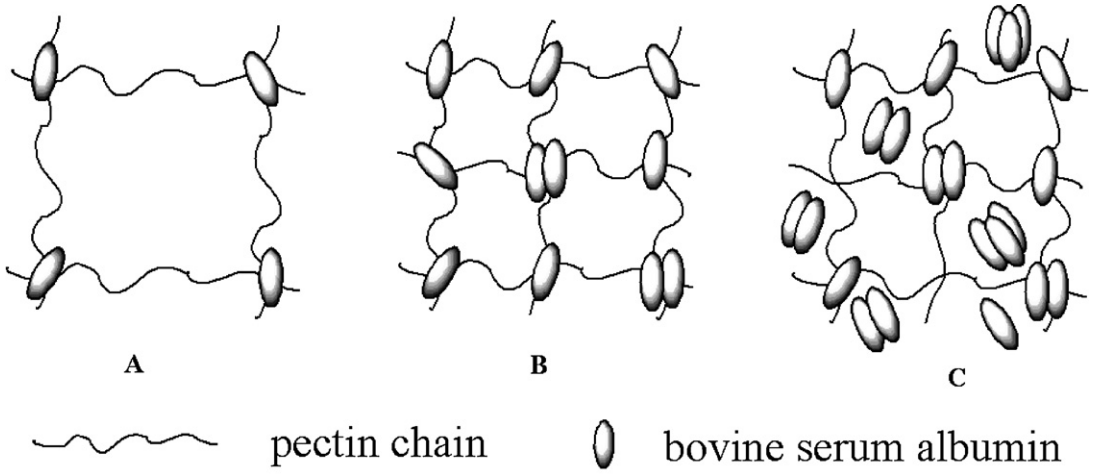

Fig. 7. The schematic of the possible microstructures of the BSA/pectin coacervates prepared at NaCl concentration (C_{NaCl}) = 0.1 M and various initial protein/polysaccharide ratios. (A) BSA/pectin ratios (r) = 1:1; (B) BSA/pectin ratios (r) = 10:1; (C) BSA/pectin ratios (r) = 20:1. *Note*: The microstructure of the BSA/pectin coacervates and the mesh size of the BSA aggregation shown are hypothetical, and the BSA molecules are not really to be taken as located on a square lattice, which is cross-linked by pectin chains.

4. Conclusion

We carried out turbidimetric titrations and dynamic rheological studies on the coacervation between BSA and pectin, and found that the BSA/pectin coacervates are dependent on C_{NaCl} and r. Similar to the commonly accepted monotonic salt-reducing effect, the added salt hinders coacervate formation and weakens the elasticity of BSA/pectin coacervates. Generally, the increase in r promotes BSA/pectin coacervation. An increase in r from 1:1 to 10:1 leads to the formation of tighter coacervate networks. The maximum shear modulus is observed at r = 10:1, which suggests that the density of the positive charge on BSA is approximately equal to that of the negative charges on pectin, and their interaction is almost at a maximum. However, with further increases in r to 20:1, the elasticity of the BSA/pectin coacervates decreases. These results show that a balance between the positive and negative charges of the biopolymers favors the formation of BSA/pectin coacervates with more compact network structures.

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