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Studies on self-assembly interactions of proteins and octenyl succinic anhydrate (OSA)-modified depolymerized waxy rice starch using rheological principles

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ABSTRACT: Dynamic tests (23 °C, pH ~ 7.0) yielding relaxation times, λ, as a function of frequency and polymer concentration were performed to assess self-assembly characteristics of biopolymers in aqueous solution. Reduction of λ-values (slope) up to a critical frequency value (CFV) helps characterize structure formation. Proteins and octenyl succinic anhydrate (OSA)-modified depolymerized waxy rice starch (DWxRc) show a well-defined λ-slope at all concentrations. Except for whey protein isolate (WPI, 0.184 g/L) and 7% OSA-modified DWxRc (1.84 g/L), the λ-values of the solutions are comparable ($P > 0.05$), indicating similar structures. Self-assembly interaction of α-lactalbumin (3.68 g/L) with OSA-modified polysaccharides is observed with 18.4 g/L of 7% OSA-modified DWxRc (CFV of 0.08 Hz), while WPI (3.68 g/L) exhibits self-assembly with all polysaccharides and concentrations. Transmission electron microscopy (TEM) of electrostatically precipitated proteins alone or in combination with OSA-modified polysaccharides confirms that $\lambda-$slope and CFV values relate to shape, size, and shear stability of the assembled structures. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43603.

KEYWORDS: biopolymers and renewable polymers; polysaccharides; proteins; self-assembly

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INTRODUCTION

High interest in the interaction between amphiphilic molecules, such as proteins (e.g., β-lactoglobulin and α-lactalbumin) and different types of surfactants (e.g., Tween 20 or sodium dodecyl-sulphate, SDS), has motivated studies on their potential application in the food industry. Proteins and polysaccharides are natural food materials, are considered GRAS (generally recognized as safe), widely available, and relatively inexpensive.[1] Similarly, polysaccharides such as starch and phytoglycogen have been hydrophobically modified by reaction with octenyl succinate anhydride (OSA) to become strongly surface active[2–4] and have been successfully handled to impart functional application as rheology modifiers, emulsion stabilizers, surface modifiers, encapsulation matrix for nanoparticles, and drug delivery vehicles.[5–8]

Whey proteins alone or in combination with polysaccharides have been extensively investigated for delivery of hydrophobic bioactive compounds.[1,9–12] Surface active molecules like proteins and octenyl succinic anhydrate (OSA)-modified polysaccharides possess both hydrophilic (polar, water-loving) and hydrophobic (nonpolar, lipid loving) properties. These amphiphilic molecules may form structures in aqueous solution by their spontaneous self-assembly.[13] These structures and the systems they form, instead of arising from strong covalent or ionic bonds, are influenced from weaker van der Waals, hydrophobic, hydrogen-bonding, and screened electrostatic interactions; thus, binding between pure or combination of different amphiphilic molecules not only depends on hydrophobic interactions but it is also mediated by ionic forces.[14] The surrounding characteristics of the medium, such as electrolyte concentration, pH, and temperature exert strong influence on these forces interactions[10] thus modifying the size and the shape of the structures.[13]

Currently, food technologists have a great number of options from different natural polymers as good alternatives with similar performance to synthetic ones. Consumer concerns on the use of synthetic materials have also increased the interest in the utilization of natural polymers for delivery of active agents. Different techniques have been used to study the interaction between amphiphilic molecules, such as surface tension, surface rheology,[15,16] fluorescent spectroscopy, circular dichroism spectroscopy,[17,18] differential scanning calorimetry,[19] and nuclear magnetic resonance.[20] However, these methods may be inaccurate and time consuming. A unique approach is to look at the viscoelastic properties of the polymeric solutions.

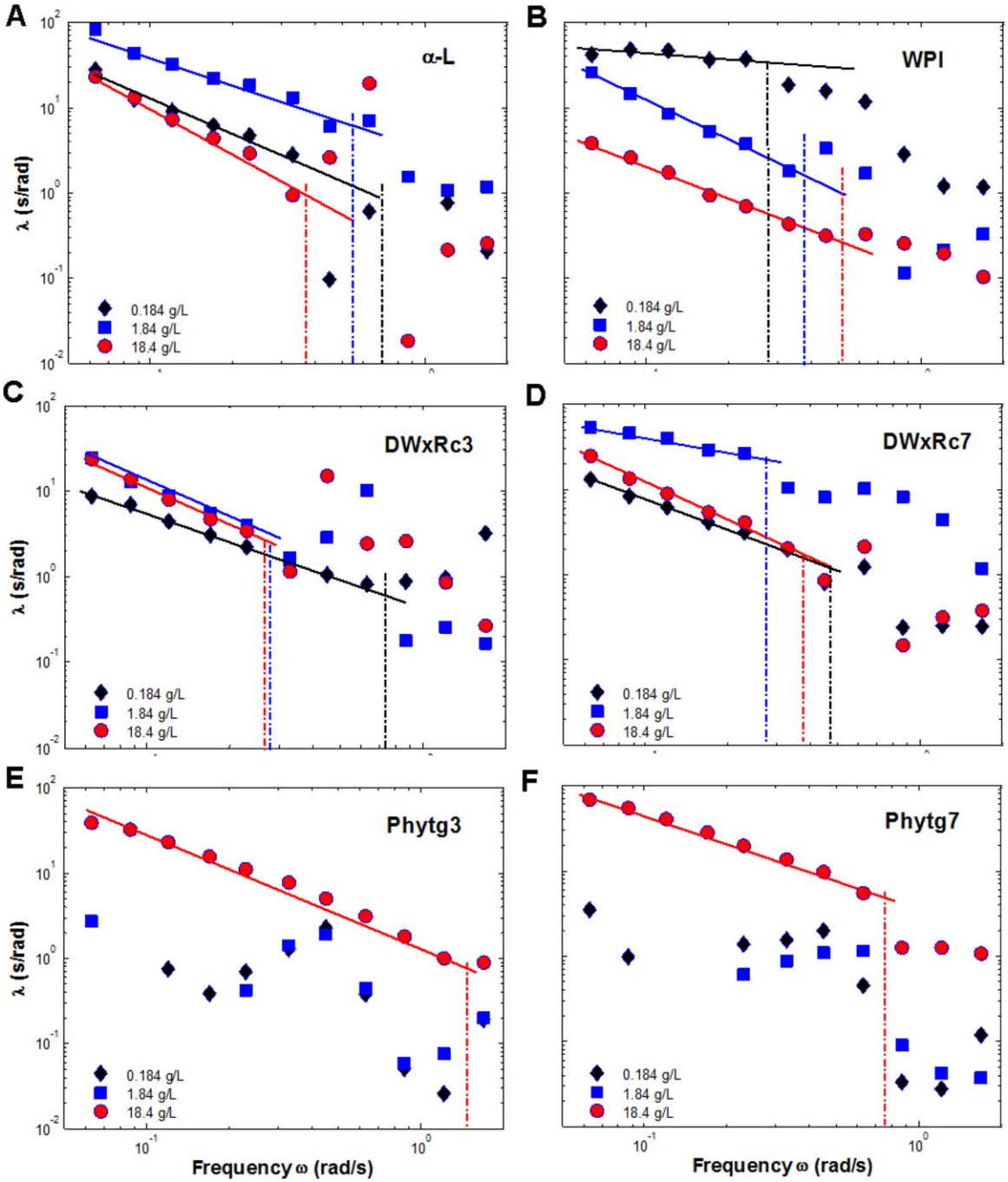

Figure 1. Relaxation time "λ" (solid lines) and critical frequency values (CFV, dashed lines) for different concentrations of α-lactalbumin (A), whey protein isolate (B), depolymerized 3% OSA-modified waxy rice (C), depolymerized 7% OSA-modified waxy rice (D), 3% OSA-modified phytoglycogen (E), and 7% OSA-modified phytoglycogen (F) evaluated by oscillatory frequency sweep test at 0.01 Pa and 23 °C. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

Viscoelastic characteristics of polymer dilutions have been established in the range of relatively low frequencies where viscoelastic properties are mainly influenced by motion rather than length of polymer chains.[21] The elastic response is understood as the deformation capability of the intermolecular interaction to overcome proportional stresses and strains without disrupting the integrity of the network structure, which is only complying within the limits of linear viscoelasticity.[21,22] Although the linear viscoelasticity characteristic of polymer melts (at high concentration) has been used to understand intrinsic properties, the application in real fluids to link to distinct molecular structures has been very difficult, particularly for amphiphilic biopolymers.

The solid-like behavior of polymer solutions has been associated with self-assembly and network formation between association of a hydrophobic polymer and a nonionic surfactant.[19] The viscoelastic behavior of polymeric nanocomposite materials may

be characterized by evaluating the interrelation between their elastic (G') and viscous (G'') moduli through their relaxation time "λ' spectrum during oscillatory testing.[23–26] The relaxation time "λ' is associated with the large scale motion (or changes) in the structure of the polymers. Studies have shown that use of the moduli is not sensitive enough to characterize molecular structure.[27,28] The proposed tests should provide critical information about the structure and the type of association presented by the different biopolymers. Hence, the purpose of this study was to evaluate the self-assembly characteristics of pure or a combination of OSA-modified polysaccharides and whey proteins in aqueous solution to better understand their intermolecular interactions since these materials could be used as carriers of bioactive compounds for food and pharmaceuticals.

EXPERIMENTAL

Materials

Waxy rice starch (Ingredion, Westchester, IL) was depolymerized with α-amylase-heat stable (Sigma-Aldrich Co., St. Louis, MO) to produce high dextrose equivalent (DE) values (DE = 24). The depolymerized rice starch (DWxRc) and phytoglycogen (Kewpie Corporation, Japan) were modified at different substitution levels (3 and 7%) with octenyl succinic anhydride (OSA, Dixie Chemical Co, Houston, TX).[29] Other materials included unmodified whey protein isolate (WPI, BIPRO, DaVisco Foods International, Eden Prairie, MN) and α-lactalbumin (DAVISCO), glucose standard, and other reagents and solvents (Sigma-Aldrich and VWR International, West Chester, PA).

Enzymatic Treatment of Waxy Starches

Waxy corn and rice starch samples (5 g, dry weight) were mixed with water to a 35% suspension by weight containing 200 ppm of $CaCl_2$. The pH of the mixture was adjusted to 5.9 with 0.1 *N* NaOH and α-amylase at 20 U per gram of starch was added into the mixture. The suspension was heat up on a hot plate at 100 °C for 20 min and then incubated in a shaking water bath (VWR International, West Chester, PA) set at 65 °C and 100 rpm for 360 min. The reaction was stopped by reducing the mix pH to 4.0 with 0.5 *N* HCl. Three volumes of ethanol were added to the mixture, followed by centrifugation at 3200 × g for 20 min (Allegra 25R centrifuge, Beckman Coulter, Fullerton, CA) and the precipitate solid were dried in a vacuum oven (Squared Lab Line Instruments, Melrose Park, IL) at 60 °C overnight.[29]

Total and Reducing Sugar Content

We followed the phenol-sulfuric acid assay method.[30] The amount of sugar present in the solution was determined by comparison with a calibration curve of D (+) glucose using a spectrophotometer (Thermo Scientific Genesys 10S UV–Vis, Waltham, MA). Quantification of reducing sugars was determined by the Somogyi-Nelson method[30] and the dextrose equivalent (DE) value was confirmed based on the following equation:

$$DE = \frac{Reducing\ sugar\ content\ \left(\frac{mg}{g}\right)}{Total\ sugar\ content\ \left(\frac{mg}{g}\right)} \quad (1)$$

Table I. Relaxation Time (λ) Slope and Critical Frequency Value (CFV) for Different Concentrations of Biopolymers Determined with Frequency Sweep Tests at 0.01 Pa and 23 °C

Polymer type	λ Slope	CFV (rad/s)	R^2
α-L			
0.184 g/L	-1.295 ± 0.501^{ab}	0.33	0.955
1.84 g/L	-1.132 ± 0.408^{ab}	0.63	0.961
18.4 g/L	-1.602 ± 0.187^{a}	0.33	0.996
WPI			
0.184 g/L	-0.1577 ± 0.321^{c}	0.23	0.935
1.84 g/L	-1.503 ± 0.229^{a}	0.33	0.993
18.4 g/L	-1.363 ± 0.199^{a}	0.45	0.993
DWxRc3			
0.184 g/L	-1.080 ± 0.172^{ab}	0.63	0.992
1.84 g/L	-1.353 ± 0.284^{a}	0.33	0.987
18.4 g/L	-1.499 ± 0.219^{a}	0.33	0.993
DWxRc7			
0.184 g/L	-1.091 ± 0.122^{ab}	0.33	0.996
1.84 g/L	-0.5727 ± 0.175^{bc}	0.23	0.972
18.4 g/L	-1.376 ± 0.275^{a}	0.45	0.988
Phytg3			
18.4 g/L	-0.9840 ± 0.219^{ab}	1.21	0.985
Phytg7			
18.4 g/L	-0.9637 ± 0.180^{ab}	0.63	0.989

Values represent mean ± standard deviation of three replicates per set of data.
[a–c] Means values with same letter are not significantly different ($P > 0.05$).
α-lactalbumin; WPI, whey protein isolate; DWxRc3 and DWxRc7, depolymerized (DE 24) waxy rice starch with 3% and 7% octenyl-succinate modification; Phytg3 and Phytg7, octenyl succinate-modified phytoglycogen (3 and 7%).

Preparation of OSA-Modified Polysaccharides

This procedure for preparation of OSA-modified polysaccharide is presented in a previous study,[29] which showed that, from the standpoint of physical properties, a higher degree of substitution (7%) does not necessarily improve the applicability of the biopolymers during storage due to instability. The percentage of OSA modification (% OSA) was calculated using a modified version of the method developed by the Joint FAO/WHO Expert Committee on Food Additives.[31]

Sample Preparation

Concentrated biopolymer solutions (184 g/L) were prepared in distilled water, vortexed for at least 30 s, stored at room temperature (~23 °C) for 6 h or until complete dissolution of the biopolymer. Afterwards, the solution was filtered with 0.2 μm nylon filters (VWR, West Chester, PA), then re-diluted in distilled water to different biopolymer concentrations (0.184–100.0 g/L) and subsequently stored at 4 °C overnight. The next day, the samples were allowed to warm up at room temperature (23 °C) for 1 h prior to performing the rheological tests.

Rheological Evaluation

All tests were performed with a Haake RheoStress 6000 Rheometer (Thermo Fisher Scientific, Waltham, MA) using a cone and plate

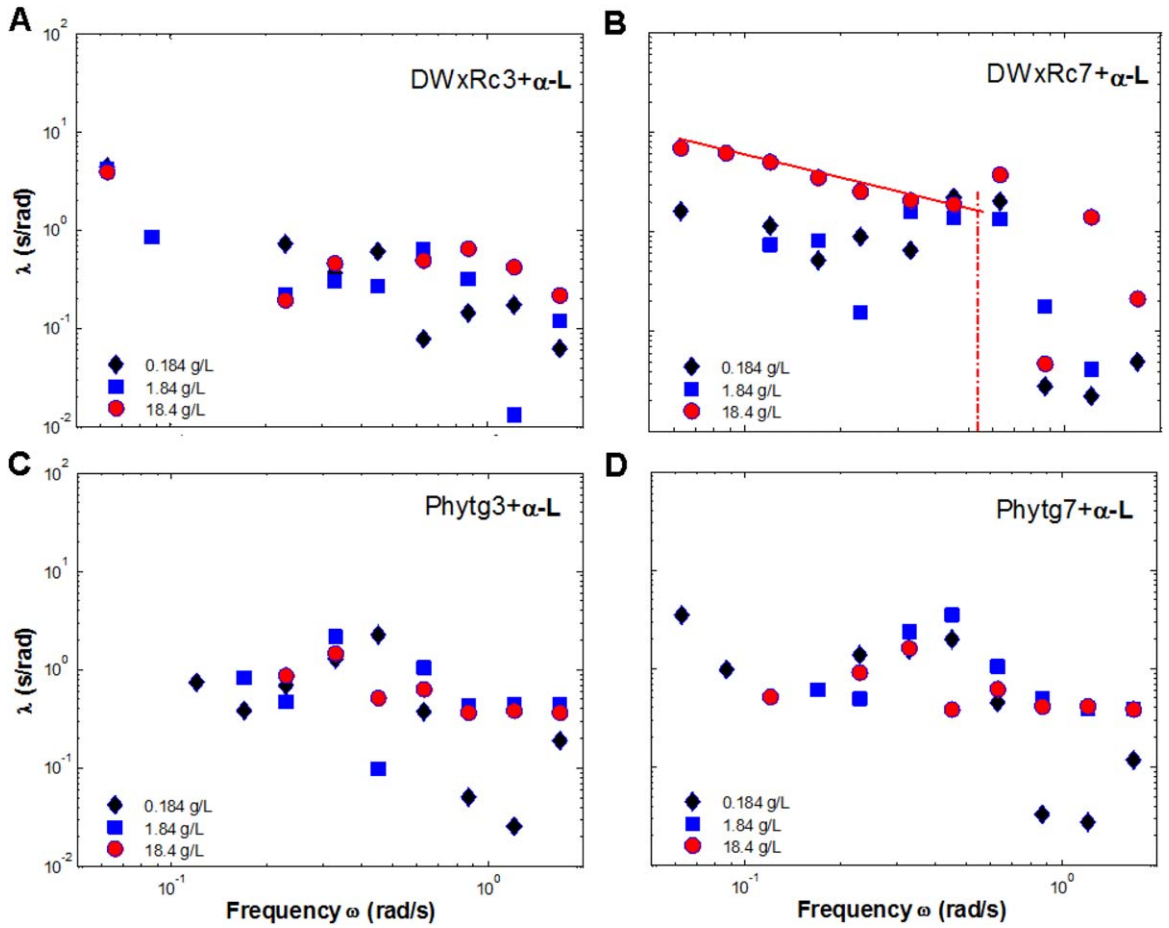

Figure 2. Comparison of hydrophobic interaction of 3.68 g/L α-lactalbumin with different concentrations of (A) 3% OSA-modified depolymerized waxy rice (DWxRc3), (B) 7% OSA-modified depolymerized waxy rice (DWxRc7), (C) 3% OSA-modified phytoglycogen (Phytg3), and (D) 7% OSA-modified phytoglycogen (Phytg7) by relaxation time (λ) values at 0.01 Pa and 23 °C of different aqueous concentrations. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

geometry, in which the rotating cone was 60 mm in diameter with an angle of 1°. The cone–plate geometry was chosen over the plate–plate system to ensure constant shear rate and better test reproducibility. About 1 mL of sample was carefully placed on the plate, and left to rest for 5 min for structure recovery. The temperature was maintained at 23 °C with the help of a Peltier TC 81 Thermo Haake system (Thermo Fisher Scientific, Waltham, MA). Analysis was carried out by triplicate on each sample after 1 day of preparation and storage at 4 °C as described above. Amplitude sweeps were performed at 1 Hz and 0.1 Hz at 23 °C to determine the linear viscoelastic region (LVR) of diluted samples of proteins as reference biopolymers. Dynamic oscillatory tests of all samples were performed at 0.01 Pa based on the LVR of the protein dilutions and the elastic (G′) and viscous (G″) moduli calculated.[22]

The likelihood of self-assembly structures of pure α-lactalbumin or WPI and in combination with OSA-modified polysaccharides was assessed using the relationship between G' and G'' as a function of the angular frequency, ω, known as the relaxation time "λ" associated with large scale motion (or changes) in the structure of the polymers.[22,28]

According to Maxwell fluid element dynamic analysis, the elastic (G′) and viscous (G'') moduli could be written as a function of frequency ω (rad/s) and relaxation time "λ" (s/rad) as follows[22]:

$$G' = \frac{G\omega^2\lambda^2}{1+(\omega\lambda)^2} \tag{2}$$

$$G' = \frac{G\omega\lambda}{1+(\omega\lambda)^2} \tag{3}$$

where G represents the spring modulus from the Maxwell's model. The interrelation between G' and G'' is determined by dividing these two equations:

$$\frac{G'}{G''} = \omega\lambda \tag{4}$$

$$\lambda = \frac{G'}{G''\omega} \tag{5}$$

The values of λ have shown to be sensitive to the frequency oscillatory sweep and polymeric nanocomposite concentration.[28] Moreover, the linear decreasing values of λ (slope) have been associated with structure formation as:

$$slope = \left[\frac{d\log\lambda}{d\log\omega}\right] \tag{6}$$

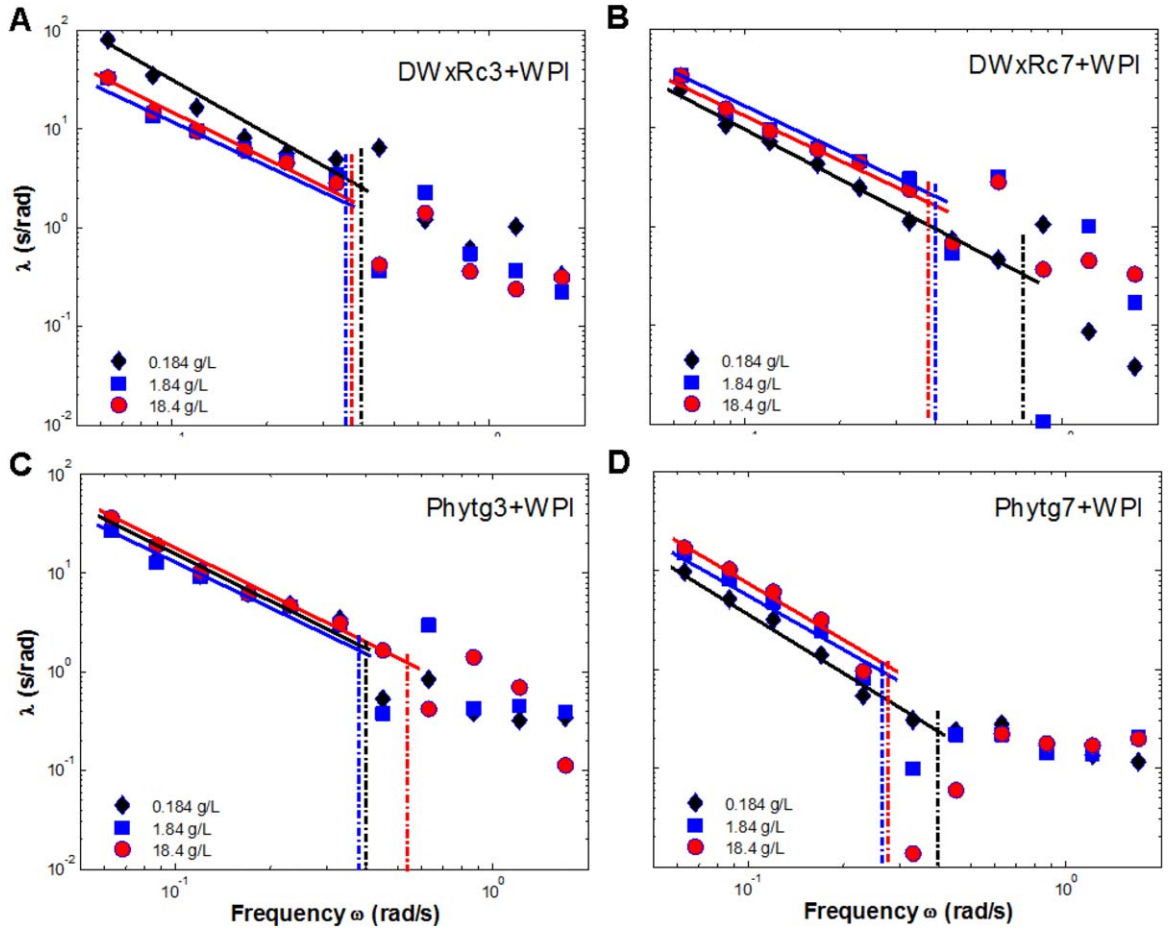

Figure 3. Comparison of hydrophobic interaction of 3.68 g/L whey protein isolate with different concentrations of (A) 3% OSA-modified depolymerized waxy rice (DWxRc3), (B) 7% OSA-modified depolymerized waxy rice (DWxRc7), (C) 3% OSA-modified phytoglycogen (Phytg3), and (D) 7% OSA-modified phytoglycogen (Phytg7) by relaxation time (λ) values at 0.01 Pa and 23 °C of different aqueous concentrations. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

Changes in λ slope have been related to structure changes for combinations between polyamide-6 and polymeric nanocomposites.[28] An early study on the same combination of polyamide-6 and polymeric nanocomposites showed that the structural changes at a critical frequency have also been associated with an apparent break of the disk-like (or platelet) structure at higher frequencies.[28]

Particle Synthesis by Electrostatic Precipitation of Biopolymers and Morphology

Particle synthesis of protein alone or in combination with OSA-modified polysaccharide (ratio 1:1 to achieve a total amount of biopolymer of 184 g/L in aqueous solution) were obtained by acid precipitation (pH 4.5) of the complexes structure. The aqueous solution was vortexed for 30 s and allowed to rest at 4 °C for 16 h, when the particle was precipitated by addition of citrate buffer (1 *M*) at pH 2.6 to achieve the isoelectric point of the proteins involved in the particle complex (~pH 4.5). After 30 min, the solution was centrifuged (Allegra 25R centrifuge, Beckman Coulter, Fullerton, CA) at 4 °C for 20 min, and the liquid supernatant was eliminated and subsequently dried in a vacuum oven (Squared Lab Line Instruments, Melrose Park, IL) at 60 °C overnight.[29]

Aqueous suspensions of electrostatically precipitated self-assembly structures were examined using a FEI Morgagni Transmission Electron Microscope (TEM) (FEI Company, Hillsboro, OR) located at the School of Veterinary Medicine and Biomedical Sciences of Texas A&M University (College Station, TX). Samples (~10 μL) were placed on 300 mesh copper grids and stained with a 2% (w/v) uranyl acetate aqueous stain (Electron Microscopy Sciences, Hatfield, PA) to provide contrast under magnification. Excess liquid on the mesh was removed with filter paper and the grid was allowed to dry before viewing under 50,000–100,000 times magnification. Observations were performed at 80 kV.

Statistical Analysis

All experiments were replicated three times and the results were reported as average. Statistical analysis software (IBM SPSS Statistics, Version 14, IBM Corporation, Armonk, NY) was used to perform analysis of covariance (ANCOVA) in a general linear model adjustment of multiple comparisons with Bonferroni's test in order to compare relaxation times "λ" slope calculated from dynamic oscillatory test as indication of self-assembly structure by pure or combination of biopolymers (whey protein isolate, α-lactalbumin, 3% and 7% OSA-modified

Table II. Relaxation Time (λ) Slope and Critical Frequency Value (CFV) for Different Combinations of Biopolymers Determined with Frequency Sweep Tests at 0.01 Pa and 23 °C

Polymer type	λ slope	CFV (rad/s)	R^2
α-L 3.688 g/L +			
DWxRc7-18.4 g/L	-0.7087 ± 0.158^{c}	0.45	0.973
WPI 3.68 g/L +			
DWxRc3-0.184 g/L	-2.394 ± 0.337^{a}	0.33	0.996
DWxRc3-1.84 g/L	-1.88 ± 0.639^{ab}	0.33	0.964
DWxRc3-18.4 g/L	-1.863 ± 0.495^{ab}	0.33	0.979
WPI 3.68 g/L +			
DWxRc7-0.184 g/L	-1.963 ± 0.371^{a}	0.63	0.988
DWxRc7-1.84 g/L	-1.942 ± 0.589^{a}	0.33	0.972
DWxRc7-18.4 g/L	-1.923 ± 0.375^{a}	0.33	0.988
WPI 3.68 g/L +			
Phytg3-0.184 g/L	-1.955 ± 0.725^{a}	0.33	0.961
Phytg3-1.84 g/L	-1.584 ± 0.483^{ab}	0.33	0.969
Phytg3-18.4 g/L	-1.767 ± 0.209^{ab}	0.45	0.995
WPI 3.68 g/L +			
Phytg7-0.184 g/L	-1.871 ± 0.189^{a}	0.45	0.997
Phytg7-1.84 g/L	-1.826 ± 0.218^{a}	0.23	0.997
Phytg7-18.4 g/L	-1.694 ± 0.312^{ab}	0.23	0.992

Values represent mean ± standard deviation of three replicates per set of data.
[a–c] Means values with same letter are not significantly different ($P > 0.05$).
α-lactalbumin; WPI, whey protein isolate; DWxRc3 and DWxRc7, depolymerized (DE 24) waxy rice starch with 3% and 7% octenyl-succinate modification; Phytg3 and Phytg7, octenyl succinate-modified phytoglycogen (3 and 7%).

depolymerized waxy rice starch (DWxRc), and phytoglycogen). Significance was predetermined at $P < 0.05$.

RESULTS AND DISCUSSION

Dynamic Oscillatory Sweep Test: Elastic (G′) and Viscous (G″) Moduli Relationship

The linear viscoelastic region for the different aqueous solutions of proteins was set at 0.01 Pa (data not shown). To understand the mechanisms influencing the solid-like and liquid-like behavior of biopolymer solutions, a relationship of the elastic (G′) and viscous (G″) moduli as a function of the angular frequency was established [eqs. (2–6)]. The values of λ were sensitive to the level of frequency and polymer concentration (Figure 1). The λ-value decreased as the frequency increased up to a critical frequency value (CFV), where the λ-value changed from a linear decreasing trend (slope) to a more scattered distribution. Proteins, 3% and 7% OSA-modified depolymerized waxy rice (DWxRc3 and DWxRc7), showed a defined slope at all polymer concentration levels [Figure 1(A–D)] while dilutions of OSA-modified phytoglycogen only showed a defined slope at the highest concentration evaluated in this study (18.4 g/L) [Figure 1(E,F)].

The solid-like dynamic behavior may be associated with structures that present difficult rotational movement. This assumption is based on previous studies that show the connection between symmetrical structure geometry of anisotropic structures and rheological characteristics, which are influenced by the characteristic period of structure rotational motility.[30] Hence, similar structure geometry would present similar rotational motility and similar relaxation time ("λ" slope value). For example, rods and ellipsoids (e.g., platelets and disks) have been easy to differentiate based on their rotational motility,[32–34] that have been related to their geometric aspect ratio. The ratio of the largest to the smallest dimension seems to be more realistic to define different structures.[32] Therefore, the aspect ratio for a rod could be defined as r is the length/diameter $\gg 1$, for sheet-type structure r is the length/thickness $\gg 1$, for disk or platelets as r is the diameter/thickness > 1, and spheroids r $\sim$1.

Since we did not have access to a cryogenic electron microscope to compare structures at low dilution concentrations, we evaluated the dynamic response (G′ and G″ as a function of frequency) to confirm the solid-like behavior of the samples. The solid-like behavior of solutions of 0.184 g/L WPI, 1.84 g/L α-lactalbumin, 7% OSA-modified WxRc, and 3% and 7% OSA-modified phytoglycogen (data no shown) was influenced by the low rotational motility offered by the types of structure. For instance, β-lactoglobulin, the main constituent of WPI, has been observed to exist mainly as noncovalently associated dimer structures at room temperature in the pH range of 2.0–3.7 and 5.2–9.0[32]; therefore, the dimer structure must present a motility similar to a hinge-like structure with the geometric aspect ratio r is the length/thickness $\gg 1$. Likewise, a geometric aspect ratio structure much greater than 1.0 must be assembled for the other biopolymer concentrations with solid-like behavior.

The critical frequency value (CFV) for proteins and OSA substituted DWxRc was between 0.23 and 0.63 rad/s (Table I), depending on polymer type and concentration. However, the CFV for OSA-modified phytoglycogen at 18.4 g/L was 0.63 and 1.21 rad/s for 7% and 3% OSA-modified phytoglycogen, respectively (Table I). This result suggests that higher concentration of phytoglycogen particles are required to provide sufficient OSA ester groups and impart van der Waals, and hydrophobic attraction among particles in order to hold a spontaneous assembly and overcome the steric repulsion of the phytoglycogen granule (approximately 40 nm)[29]; therefore, the higher the CFV, the larger size of the structure. In consequence, 3% OSA-modified may form the largest structures.

An analysis of covariance in a general linear model with adjustment of multiple comparisons with Bonferroni test was used to compare relaxation time (λ) slopes among the different concentrations of biopolymers (Table I). The frequency sweep test was considered as covariance because this variable was not directly tested but had a direct influence on the dependent variable, λ (Figure 1). The biopolymer concentration was evaluated as independent variable and considered as fixed factor for the covariance analysis. In general, with the exception of 0.184 g/L WPI and 1.84 g/L DWxRc7, there were no significant differences ($P > 0.05$) among all slopes of α-lactalbumin, DWxRc3, 18.4 g/L Phytg3, and Phytg7 (Table I), which suggest the formation of similar geometric structures.

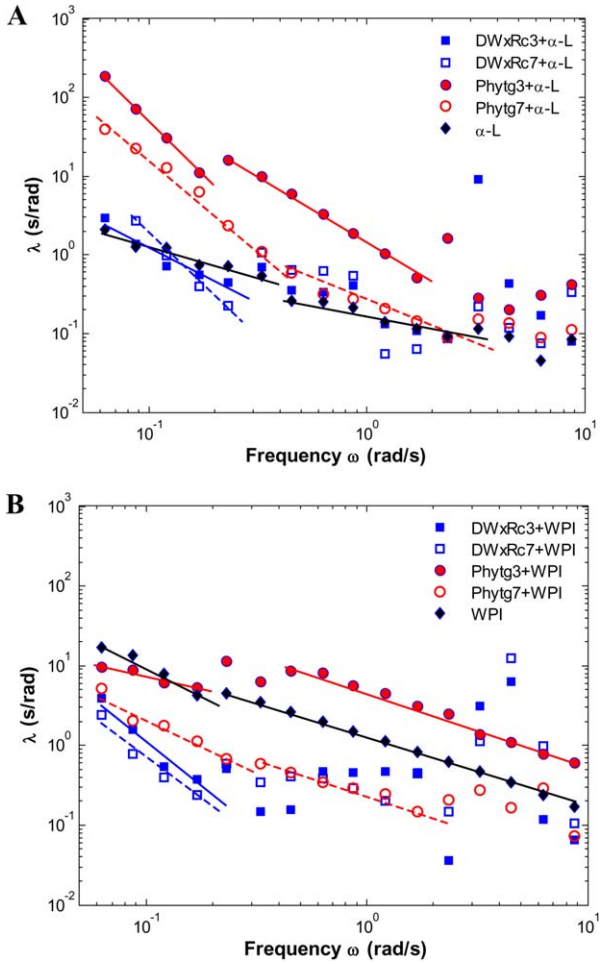

Figure 4. Relaxation time (λ) curves for different combinations of biopolymers at 1:1 weight ratio with a total concentration of 184 g/L in aqueous solutions (oscillatory sweep test at 0.01 Pa and 23 °C). [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

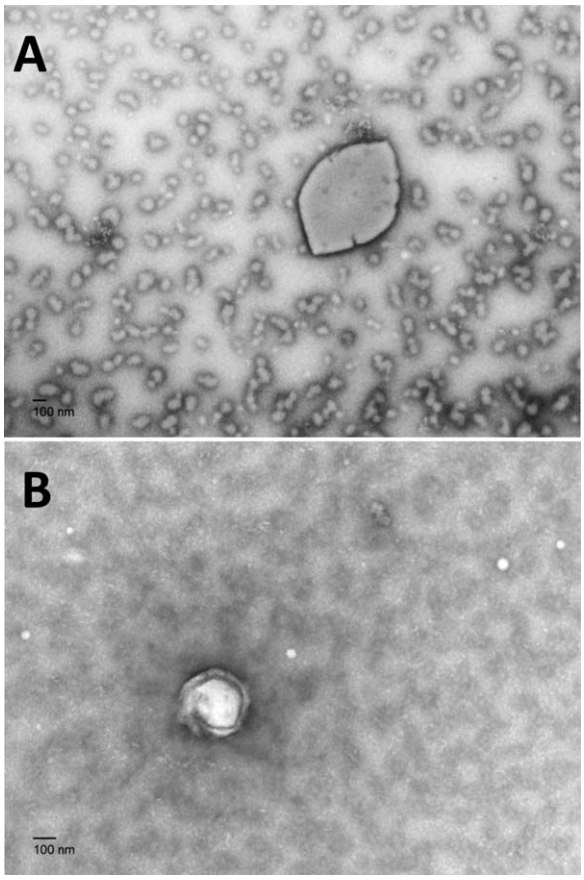

Figure 5. Micrographs of electrostatically precipitated particles formed at 1:1 weight ratio to a total concentration of 184 g/L for different combinations of α-lactalbumin (α-L) with (A) DWxRc3, (B) DWxRc7, (C) Phytg3, and (D) Phytg7. Images were taken at 50,000× magnification.

Relaxation Time Curve Slopes of Diluted Combinations of Biopolymer Solutions

The characteristic λ-slope obtained from the increment in the frequency sweep was only observed in a combination of 18.4 g/L of DWxRc7 and 3.68 g/L of α-lactalbumin with a CFV of 0.5 rad/s (Figure 2). The other combinations of 3.68 g/L α-lactalbumin and OSA-modified polysaccharides showed scattered λ values at all angular frequencies (Figure 2). These results indicate that no self-association occurs in those combinations.

The hydrophilic characteristic of the polysaccharides is attributed to the polarity of hydroxyl groups (–OH) which impart its hydrophilicity forming hydrogen bonds with water[33–38]; therefore, different polysaccharides such as OSA-modified phytoglycogen showed ionic characteristic within a wide range of acidic pH.[33–36] The presence of unfavorable repulsive interactions between α-lactalbumin and most of the OSA-modified polysaccharides evaluated in this study, suggest a high probability of ionic repulsive forces and mutual exclusion (steric effects) of each amphiphilic biopolymer in the solution. On the other hand, a defined λ slope was observed for all combinations of 3.68 g/L WPI at different concentrations (0.184, 1.84, and 18.4 g/L) of OSA-modified polysaccharides (Figure 3). A low CFV of 0.33 rad/s was obtained for the combination of WPI and the different concentrations of DWxRc3. However, higher CFV at 0.63 rad/s and 0.45 rad/s were obtained in combination with 0.184 g/L DWxRc7 and Phytg7, respectively (Table II). This result indicates that larger hydrophobic interactions of WPI occur with higher level of OSA modification at lower polysaccharide concentration. Instead, a high CFV of 0.45 rad/s was obtained for the combination of WPI with the higher concentration of Phytg3 (Table II), suggesting that WPI needs more molecules to associate when the percentage of OSA modification is lower. Then again, WPI seems to create stronger interactions at lower concentration (0.184 g/L) of 7% OSA-modified phytoglycogen, with a higher CFV of 0.45 rad/s compared to 0.23 rad/s CFV at higher concentration of 7% OSA-modified phytoglycogen (Table II). A degree of competition in the self-association of WPI with higher OSA-modified polysaccharides could produce less stable structures as the concentrations of OSA-modification in phytoglycogen increase. It seems that the type of protein and polysaccharide structure have a strong influence in their molecular interactions.

Table II presents the results of the analysis of covariance in a general linear model comparing the relaxation time (λ) slopes among all the combinations of 3.68 g/L concentration of

Table III. Relaxation Time (λ) Slope and Critical Frequency Value (CFV) for α-Lactalbumin and WPI Alone or in Combinations (1:1) with Different OSA-Modified Polysaccharides, Evaluated with Oscillatory Sweep Test at 0.01 Pa and 23 °C

Polymer mixture	λ slope	CFV (rad/s)	R^2
α-L (low freq.)	-0.906 ± 0.265^{c}	0.33	0.957
α-L (medium freq.)	-0.647 ± 0.221^{cd}	2.34	0.913
α-L + DWxRc3	-1.627 ± 0.758^{bc}	0.23	0.888
α-L + DWxRc7	-2.245 ± 0.576^{a}	0.23	0.955
α-L + Phytg3 (low freq.)	-3.174 ± 0.278^{a}	0.17	0.961
α-L + Phytg3 (medium freq.)	-1.643 ± 0.513^{bc}	1.69	0.996
α-L + Phytg7 (low freq.)	-2.088 ± 0.585^{b}	0.63	0.996
α-L + Phytg7 (medium freq.)	-2.176 ± 0.6036^{ab}	2.34	0.964
WPI (low freq.)	-1.894 ± 1.04^{b}	0.17	0.997
WPI (medium freq.)	-0.808 ± 0.06^{c}	23.4	0.998
WPI + DWxRc3	-2.324 ± 0.69^{ab}	0.17	0.997
WPI + DWxRc7	-2.362 ± 0.99^{ab}	0.17	0.992
WPI + Phytg3 (low freq.)	-1.461 ± 0.60^{b}	0.17	0.919
WPI + Phytg3 (medium freq.)	-0.852 ± 0.30^{c}	32.5	0.969
WPI + Phytg7 (low freq.)	-1.42 ± 1.45^{b}	1.69	0.929
WPI + Phytg7 (medium freq.)	-0.68 ± 0.17^{c}	12.1	0.988

Values represent mean ± standard deviation of three replicates per set of data.
[a–c] Means values with same letter are not significantly different ($P > 0.05$).
Total biopolymers concentration in aqueous solution 184 g/L.
α-lactalbumin; WPI, whey protein isolate; DWxRc3 and DWxRc7, depolymerized (DE 24) waxy rice starch with 3% and 7% octenyl-succinate modification; Phytg3 and Phytg7, octenyl succinate-modified phytoglycogen (3 and 7%).

α-lactalbumin or WPI with different concentrations of OSA-modified polysaccharides influenced by an increment in the oscillatory frequency. The λ slope of the combination of 3.68 g/L of α-lactalbumin with 18.4 g/L DWxRc7 was significantly different ($P < 0.05$) from the combination of 3.68 g/L WPI with the different concentrations of OSA-modified polysaccharides (0.184, 1.84, and 18.4 g/L). No significant differences ($P > 0.05$) were observed among the λ slopes from combinations of 3.68 g/L of WPI and the different concentrations of polysaccharides, regardless the percentage of OSA modification, which suggests the formation of similar structures in all these different combinations of OSA-modified polysaccharide and WPI. However, the stability of the structure, denoted by the CFV, seems to be influenced by the percentage of OSA modification. These results suggest that the type of protein has a strong influence in the intermolecular associations with OSA-modified polysaccharides, and the percentage of OSA modification in polysaccharides influences –to a certain extent – the amount of molecules interacting with the proteins (e.g., WPI); consequently, more molecules of lower percentage of OSA-modified polysaccharides are needed to self-assemble with protein molecules, thus forming larger size structures.

Relaxation Time Slopes of Combinations of Concentrated Biopolymer Solutions at a 1:1 Weight Ratio

All solutions of pure protein or in combination with OSA-modified polysaccharides (total concentration of 184 g/L) showed a decreasing (linear) trend on the λ values (slope) as the frequency increased (Figure 4). This result suggests the formation of some type of structure for all combinations of protein (92 g/L) with OSA-modified polysaccharides (92 g/L).

The combinations of α-lactalbumin or WPI with DWxRc at both percentages of OSA modification (3 and 7%) had smaller CFV. However, pure proteins and in combination with 3% or 7% of OSA-modified phytoglycogen, showed two distinct slopes, with the one observed at medium frequency (0.31–6.2 rad/s) having higher CFV. perhaps, a higher concentration of OSA-modified phytoglycogen (>18.4 g/L) is required to provide sufficient OSA ester groups and impart van der Waals, and hydrophobic attraction among particles in order to hold a spontaneous assembly and overcome the steric repulsion of the bigger phytoglycogen particles (approx. 40 nm); therefore, the higher the CFV (~32.5 rad/s), a more stable structure was formed. The higher λ values observed in Figure 5 also relate to a solid-like dynamic response, because solid-like dynamic behavior is associated with structures that show difficult rotational movement, which have a high geometric aspect ratio.[32]

Table III presents the results of the analysis of covariance in a general linear model comparing relaxation time (λ) slopes among solutions of α-lactalbumin or WPI alone and in combination (1:1) with OSA-modified polysaccharides influenced by an increment in the oscillatory frequency. Two types of structures were formed by α-lactalbumin alone (-0.906 ± 0.265 and -0.647 ± 0.221) were not significantly different ($P > 0.05$), as well as between the structures formed in combination with 3% OSA-modified WxRc (-1.627 ± 0.758) and 3% OSA-modified phytoglycogen (-1.643 ± 0.513). However, significant differences were found in combinations of α-lactalbumin alone compared to the combination with DWxRc7 (-2.245 ± 0.576), and Phytg3 (-3.174 ± 0.27885) and with Phytg7 (-2.088 ± 0.585,

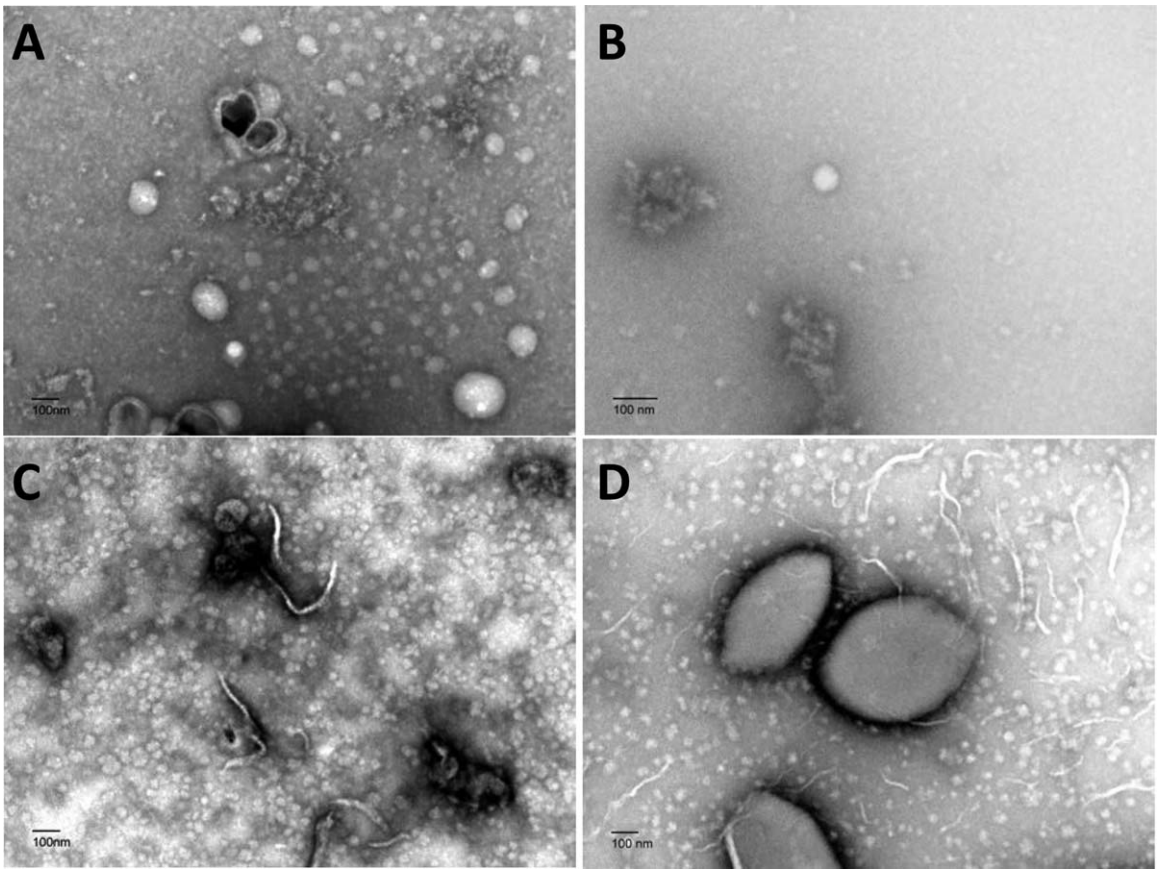

Figure 6. Micrographs of electrostatically precipitated particles formed at a concentration of 184 g/L (A) α-lactalbumin (α-L) and (B) Whey protein isolate (WPI). Images were taken at 50,000× magnification.

-2.176 ± 0.6036). It is possible that these biopolymer combinations formed two very distinct structures.

Based on the analysis of covariance, the two λ-slopes for WPI alone and in combination with 3% and 7% OSA-modified phytoglycogen were significantly different ($P < 0.05$). Therefore, two distinct structures may be formed in these systems. On the other hand, no significant differences ($P > 0.05$) were found between the structures formed by WPI alone (-1.8494 ± 1.04) compared to those formed in combination with DWxRc3 (-2.324 ± 0.69), DWxRc7 (-2.362 ± 0.99) Phytg3 (-1.461 ± 0.60), and Phytg7 (-1.42 ± 1.45), which suggests formation of similar structures.

Microstructure Formation by Combinations of Biopolymers at Ratio 1:1

Although the particle synthesis was made at a different pH from the rheology studies, the precipitated structures showed significant differences which could be influenced by the protein and polysaccharides interaction in the aqueous solution.

The TEM micrographs show a variety of particle shapes and sizes formed by α-lactalbumin and WPI alone (Figure 6) or in combination with OSA-modified polysaccharides (Figures 5 and 7) (total concentration = 184 g/L). The shape and size of the particles were affected by the type of amphiphilic biopolymer interaction previously characterized by the dynamic oscillatory test. Each solution of protein, alone or in combination with OSA-modified polysaccharides, formed one or more structures, which is in agreement with the relaxation time "λ" slope values determined using rheological methods (Figure 4). Particles formed by α-lactalbumin were small (~100 nm) and big oblate ellipsoids (~1000 nm) (Figure 5A), similar to those observed for combination of α-lactalbumin and 7% OSA-modified phytoglycogen (Figure 5D).

The geometric aspect ratio of ellipsoids, defined as the ratio of the major and minor axes ($r = a_1/a_2$), drastically affects their rotational motion.[22] An interesting observation is the presence of a fiber-type structure (~1000 nm) along with spheroid particles formed in the combination of α-lactalbumin with 3% (~300 nm) and 7% OSA-modified phytoglycogen (~1000 nm) [Figure 5(C,D)]. It is possible that the solid-like behavior characterized for this combination of biopolymers was influenced by the low rotational motion offered by this type of structure with a geometric aspect ratio much greater than one. Similarly, small (<100 nm) and big (200–400 nm) spheroid structures were observed on the combination of WPI with 3% and 7% OSA-modified phytoglycogen [Figure 7(C,D)] with a geometric aspect ratio of one.[32]

The combination of WPI with 3% OSA-modified phytoglycogen (Figure 7C) presented a cluster of particles (~200 nm) besides small spheres, possibly formed by aggregation of WPI alone. On the other hand, combination of WPI with 7% OSA-modified phytoglycogen presented big disk-type particles of approximately 400 nm (aspect ratio $r > 1$) besides small spheres

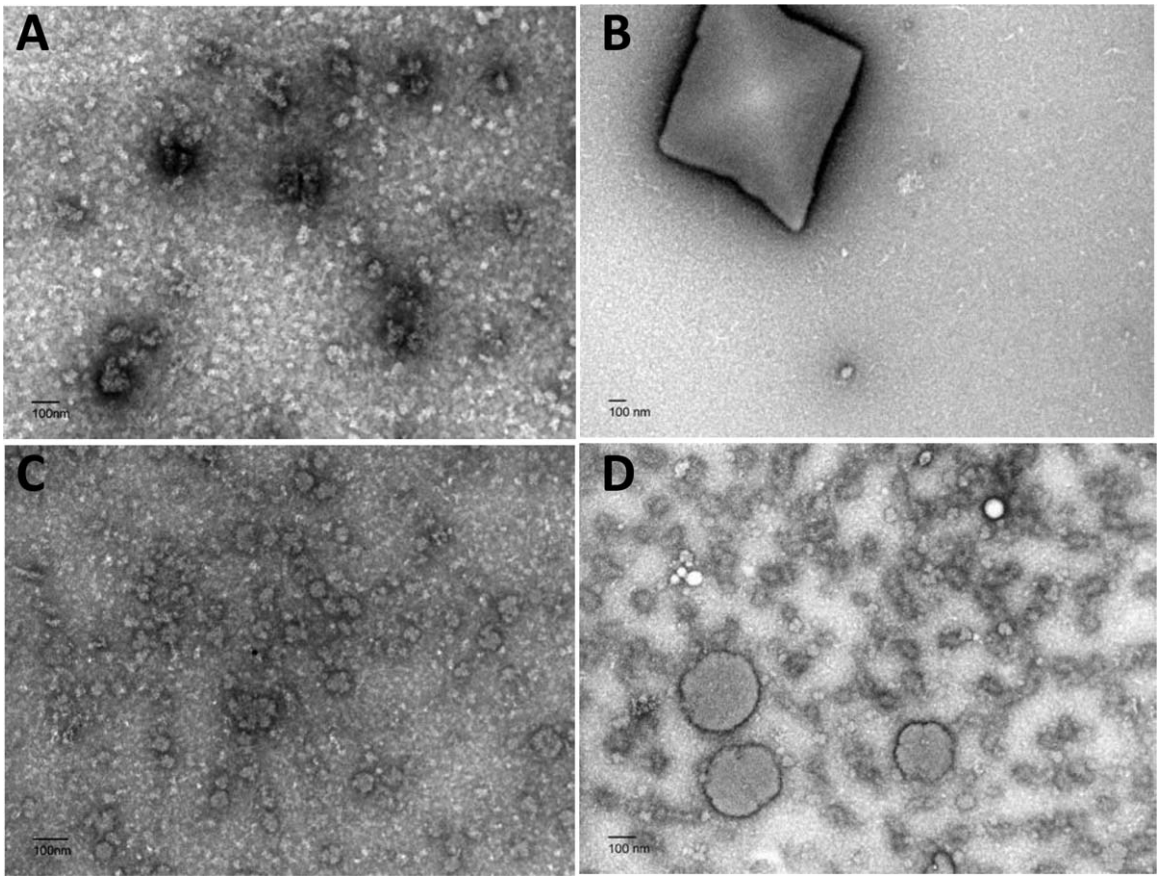

Figure 7. Micrographs of electrostatically precipitated particles formed at 1:1 weight ratio to a total concentration of 184 g/L for different combinations of whey protein isolate (WPI) with (A) DWxRc3, (B) DWxRc7, (C) Phytg3, and (D) Phytg7. Images were taken at 50,000× magnification.

(~100 nm), possibly formed by aggregation of WPI alone (Figure 7D). Unlike the spherical structures (200–300 nm) formed by WPI alone (Figure 6B), or the combination of WPI and α-lactalbumin with 3% OSA-modified DWxRc (Figures 7A and 5A, respectively), the combination of WPI and 7% OSA-modified DWxRc formed a rhomboid type structure of approximately 1000 nm (Figure 7B), with similar rotational motion as platelets, due to their similar geometric aspect ratio ($r > 1$).

CONCLUSIONS

Rheological characterization of biopolymers in aqueous solution using small amplitude dynamic oscillatory tests at 0.01 Pa, 23 °C, and neutral pH (~7.0) is suitable method to describe the intermolecular association of amphiphilic biopolymers.

The presence of a "λ" slope at a critical frequency value (CFV) can be correlated to the type and size of structure formation The relaxation time "λ" value ($G'/G''^{*}\omega$), was sensitive to type, concentration, and combination of biopolymers.

The "λ" slope and critical frequency value are useful tools to evaluate the geometric aspect ratio and size of anisotropic particles formed from electrostatic precipitation of proteins alone or in combination with OSA-polysaccharides.

The type of protein under evaluation has a strong influence in the intermolecular associations with OSA-modified polysaccharides, and the percentage of OSA modification in polysaccharides influences, to a certain extent, the amount of molecules interacting with the proteins.

The rheological analysis for intermolecular interactions between biopolymers provides a better understanding of their properties and structure formation for practical applications as natural carriers of bioactive compounds. This result opens the possibility for research to evaluate the effect of shear rate, temperature, and ionic strength on the intermolecular affinity of different amphiphilic compounds to design aforetime and engineer particles of diverse size and shapes.

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