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Formation and functionality of whey protein isolate–(kappa-, iota-, and lambda-type) carrageenan electrostatic complexes

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ABSTRACT

The formation of electrostatic complexes between whey protein isolate (WPI) and (κ-, ι-, λ-type) carrageenan (CG) was investigated by turbidimetric measurements as a function of pH (1.5–7.0), biopolymer weight-mixing ratio (1:1–75:1 WPI:CG) and NaCl addition (0–500 mM) to better elucidate underlying mechanisms of interaction. Emulsion stabilizing effects of formed complexes was also studied to assess their potential as emulsifiers. Complex formation followed two pH-dependent structure-forming events associated with the formation of soluble (pH_c) and insoluble ($pH_{\phi 1}$) complexes. For both the WPI–κ-CG and WPI–ι-CG mixtures, pH_c and $pH_{\phi 1}$ occurred at pH 5.5 and 5.3, respectively, whereas in the WPI–λ-CG mixture values were slightly higher ($pH_c = 5.7$; $pH_{\phi 1} = 5.5$). In all mixtures, maximum turbidity was found to occur near pH 4.5, before declining at lower pHs. Biopolymer mixing ratios corresponding to maximum OD was found to occur at the 12:1 ratio for both the WPI–κ-CG and WPI–λ-CG mixtures, and 20:1 ratio for WPI–ι-CG mixture. The addition of NaCl disrupted complexation within WPI–κ-CG mixtures as levels were raised, whereas when ι-CG and λ-CG was present, complexation was enhanced up to a critical Na^+ concentration before declining. Adsorption of CG chains to the small WPI–WPI aggregates during complexation was proposed to be related to both the linear charge density and conformation of the CG molecules involved. Emulsion stability in the mixed systems (12:1 mixing ratio), regardless of the CG type (κ, ι, λ), was significantly higher than individual WPI solutions indicating enhanced ability to stabilize the oil-in-water interface.

1. Introduction

Protein–polysaccharide interactions play an important role in a wide range of food and biomaterial applications ranging from their structure controlling roles in food, to their use as controlled release vehicles, coatings and packaging (Fang, Li, Inoue, Lundin, & Appelqvist, 2006; Liu, Low, & Nickerson, 2009). Tailoring of such interactions is essential in order to maintain or improve ingredient functionality and/or product quality (Ye, 2008). Although there has been a significant amount of work in the literature relating to whey protein–polysaccharide interactions (Laneuville, Paquin, & Turgeon, 2000; Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2003, 2004; Weinbreck, Tromp, & de Kruif, 2004; Ye, 2008; Zaleska, Ring, & Tomasik, 2000), a fuller mechanistic understanding of the process and resulting impact on material functionality is warranted as it relates to the effect of pH, salt, mixing ratio and polysaccharide-type (Dickinson, 1998).

Whey protein isolates (WPI) primarily consist of β-lactoglobulin (~75%, Isoelectric point (pI) = 5.2) and alpha-lactalbumin proteins (pI = 4.1) (Weinbreck, Nieuwenhuijse, et al., 2004), and hence assumes a positive net charge below this pH. Carrageenan (CG) is a linear sulfated polysaccharide extracted from red seaweed (Rhodophyceae) (Millane, Chandrasekaran, Arnott, & Dea, 1998). Structurally, it comes in three major forms (κ, ι, and λ), all of which are comprised of partially sulfated repeating (1 → 3) linked β-D-galactose and (1 → 4) linked α-D-galactose disaccharide units, partially sulfated at position 2 and/or 6 and/or 3,6 anhydrided (Thanh et al., 2002). Kappa-, ι-, and λ-types contain one, two and three sulfate groups per disaccharide repeat unit, respectively, and typically have minor contaminants from other types within the material (Weinbreck, Nieuwenhuijse, et al., 2004).

Kappa- and ι-CG are known to undergo a thermally-induced disordered-ordered transition, where at elevated temperatures both chains exist as random coils with a larger amount of conformational entropy. Upon cooling, entropy is reduced and chains re-orient into a more ordered conformation (Rochas, Rinaudo, & Vincendon, 1980), which is believed to consist of either a double helix (Rees, Scott, & Williamson, 1970), aggregated mono-helices

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(Grasdalen & Smidsrød, 1981) or aggregated helical dimers (Rochas & Landry, 1987). Regardless of the nature of the ordered domain, the subsequent lateral association in the presence of specific-ions gives rise to larger aggregates in solution, and network junction zones under gelling conditions (Thanh et al., 2002). In the case of ι-CG, lateral associations (and network strength) is known to increase in the direction of Na^+, K^+ and Ca^{2+} ions (Te Nijenhuis, 1997). Calcium sensitivity relates to the ability to form intra- and inter-molecular bridges between sulfate groups of adjacent anhydro-D-galactose and D-galactose residues (Te Nijenhuis, 1997). Potassium ions form weaker bridging between the anhydro-bridge oxygen atom and the sulfate group of D-galactose residues. In contrast, κ-CG has greater sensitivity to K^+ than Ca^{2+}, forming intra- and inter-molecular bridges via an ionic bond between the K^+ ion and the sulfate group of D-galactose, and an electrostatic association between the K^+ ion and the anhydro-O-3, 6 ring of another D-galactose residue (Te Nijenhuis, 1997). In the case of λ-CG, no ordered conformation in solution is adopted due to the high linear charge density associated with the 3 sulfate groups per disaccharide repeat unit which creates a larger amount of electrostatic repulsive forces within and between chains (Rochas et al., 1980). As such, κ- and ι-type CG are typically used in gelling applications, whereas λ-type CG is used in thickening applications (Gu, Decker, & McClements, 2005).

Depending on the biopolymer characteristics (e.g., size, concentration, mixing ratio, and type and number of reactive sites present) and solvent conditions (e.g., pH, temperature and the addition of salts), admixtures of proteins and polysaccharides will undergo either segregative or associative phase separation driven primarily by electrostatic forces between the two (Fang et al., 2006; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003; Ye, 2008). In segregative phase separation, biopolymers carry a similar net charge and experience electrostatic repulsive forces leading to the separation into both a protein-rich and polysaccharide-rich phase. If concentrations are sufficiently dilute, biopolymers will remain dispersed and co-soluble. In the case of associative phase separation (also known as complex coacervation), the two biopolymers are of opposing charges and experience electrostatic attractive forces leading to phase separation into both a biopolymer-rich (excluding solvent from the vicinity) and a solvent-rich phase (Laneuville et al., 2000; Turgeon et al., 2003; Ye, 2008). Typically, this occurs over a narrow pH range, at pHs < pI (isoelectric point) of the protein where the protein assumes a net positive charge, and at pHs > pKa of the reactive site along the polysaccharide backbone, in which the biopolymer remains negatively charged.

Experimentally, complex coacervation is associated with two structure-forming events during an acid pH titration: first, with the formation of a soluble complex (denoted as pH_c) described by the first detectable change in scattering intensity by turbidity or light scattering; and second, by the formation of insoluble complexes (denoted at $pH_{\phi 1}$) as macroscopic phase separation occurs, as evident by a significant increase in scattering intensity and the transition from a transparent solution to a turbid one (Liu et al., 2009). Kharenko et al. (1979) studied the formation of non-stoichiometric polyelectrolyte complexes involving polyelectrolytes of opposing charges to find soluble complexes formed when the proportion of ionized reactive sites was in a 3-fold excess than those taking part in salt bonds. The resulting structures carried a net charge allowing for greater water solubility. In-solubility of the formed complexes in water arises as the masking of reactive sites along the reacting polymers and the rise in hydrophobic properties for the coupled complexes both increase (Kharenko et al., 1979). Complexes continue to grow in size in a self-similar fractal manner, and number as pH declines further and the protein assumes a more positive charge through a nucleation and growth-type kinetic mechanism (Girard, Sanchez, Laneuville, Turgeon, & Gauthier, 2004; Sanchez, Mekhloufi, & Renard, 2006), until equimolar quantities between the two polymers is achieved, as evident by a maximum in scattering intensity (denoted as pH_{opt}) and an electrically neutral structure (Kharenko et al., 1979; Weinbreck et al., 2003; de Kruif, Weinbreck, & de Vries, 2004). Beyond this pH, scattering intensity declines until reaching the baseline (denoted as $pH_{\phi 2}$), as the reactive sites along the polysaccharide backbone become protonated leading to fewer biopolymer interactions and complete dissociation of structure. Depending on the strength of electrostatic interactions, structures formed may be either considered a coacervate or a precipitate. The former is reversible, contains a significant amount of entrapped mobile solvent, and typically involves the interaction of a protein with a weakly charged polysaccharide (de Kruif et al., 2004). In contrast, a precipitate structure is irreversible, contains less entrapped solvent, is more compacted relative to the coacervate, and typically involves a protein with a highly charged polysaccharide (de Kruif et al., 2004).

In general, globular proteins are typically considered effective emulsifiers at relatively low concentrations (<1%) but tend to be more prone to environmental stresses (e.g., pH, ionic strength, processing) relative to other types of surface active agents (Harnsilawat, Pongsawatmanit, & McClements, 2006). Information relating to the functionality of formed electrostatic complexes is somewhat limited, especially as it relates to WPI–CG systems. However, depending on the conditions employed, the use of protein–polysaccharide complexes for stabilizing oil-in-water emulsions has shown some potential relative to protein systems alone. Stabilization of the oil–water interface by the protein–polysaccharide absorbed layer has been previously reported under finite conditions in mixtures of β-lactoglobulin–pectin (Guzey, Kim, & McClements, 2004), sodium caseinate–dextran sulfate (Jourdain, Leser, Schmitt, Michel, & Dickinson, 2008), bovine serum albumin–κ-CG (Dickinson & Pawlowsky, 1998), pectin–sodium caseinate (Surh, Decker, & McClements, 2006) and β-lactoglobulin–CG (Gu et al., 2005). Depending on the concentration and the degree of interactions, polysaccharides can have either a positive or a negative effect on protein-stabilized emulsions. At low levels of polysaccharides, interactions may lead to bridging flocculation and instability, whereas at higher concentrations, polysaccharides may completely coat the surface of the protein-stabilized layer to improve stability via electrostatic complexation and steric stabilization (Dickinson & Pawlowsky, 1998; Harnsilawat et al., 2006).

In the present study, complex formation of WPI with (κ-, ι- and λ-type) CG was investigated as a function of pH, biopolymer weight-mixing ratio and NaCl content to better discern mechanisms for structure formation, and to compare differences and similarities between the main CG types. The stabilizing effects of formed complexes in oil-in-water emulsions were also evaluated, to show potential for use of the formed complexes as an emulsifier. Previous complexation work on WPI–CG mixtures focused on an aggregate-free WPI solution (i.e., removal of denatured proteins) and the λ-CG type only, in which they investigated the effect of pH, mixing ratio and salt content on complex formation (Weinbreck, Nieuwenhuijse, et al., 2004). The authors reported complexation was promoted in the presence of Na^+ up to 45 mM NaCl before additional Na^+ led to a disruption of biopolymer interactions; that the mixing ratio corresponding to maximum turbidity was 30:1 for the WPI–λ-carrageenan, above which reactive sites on the CG molecule became saturated; and that precipitation ensued above $pH_{\phi 1}$.

2. Materials and methods

2.1. Materials

Whey protein isolate powder (BiPRO JE 061-7-440) used in this study was kindly donated by Davisco Foods International., Inc

(Le Sueur, MN, USA), whereas all CG types were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Chemical analysis of the WPI found it to be comprised of 89.78% protein (%N × 6.38), 0.10% lipid, 4.92% moisture, 2.06% ash (includes: 0.08% Ca^{2+}, 0.01% Mg^{2+}, 0.02% K^{+}, 0.66% Na^{+}), and 3.13% carbohydrate. The commercial WPI product is considered to have ~95% of its protein in its native state. In the case of the CG materials, κ-type was comprised of 66.50% carbohydrate, 10.65% moisture and 22.86% ash (includes: 2.4% Ca^{2+}, 0.16% Mg^{2+}, 5.4% K^{+}, 0.49% Na^{+}); ι-type comprised of 64.40% carbohydrate, 10.82% moisture and 24.97% ash (includes: 3.4% Ca^{2+}, 0.18% Mg^{2+}, 3.2% K^{+}, 1.2% Na^{+}); and λ-type contained 63.79% carbohydrate, 12.26% moisture and 23.95% ash (includes: 3.0% Ca^{2+}, 0.83% Mg^{2+}, 2.4% K^{+}, 1.3% Na^{+}). Kappa-, ι- and λ-type CG had molecular weights of ~154 kDa, ~250 kDa and ~250 kDa, respectively (information provided by supplier). Protein and lipid levels for all CG materials were assumed negligible. Chemical analyses of all materials were performed according to the Association of Official Analytical Chemists Methods 925.10, 923.03, 920.87 and 920.85 for moisture, ash, crude protein and lipid (%wet weight basis), respectively. Carbohydrate content was determined based on percent differential from 100%. Biopolymer concentrations used in this study reflect the protein (WPI) or carbohydrate (κ-, ι,- and λ-CGs) content rather than powder weight. Flaxseed oil was kindly donated by Bioriginal Food and Science Corporation (Saskatoon, SK). All chemicals used in this study were reagent grade, and purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada).

2.2. Turbidimetric analysis

Turbidimetric acid pH titrations of individual and mixed WPI and CG systems were performed using modified methods of Liu et al. (2009) to identify critical structure-forming events (pH_c, $pH_{\phi 1}$ and pH_{opt}). The effect of pH (7.00–1.50) on complex formation was initially investigated for a 15:1 WPI–CG weight-mixing ratio at room temperature (21–23 °C) and a total biopolymer concentration of 0.25% (w/w). The 15:1 WPI–CG mixing ratio was selected to build upon previous work of Weinbreck, Nieuwenhuijse, et al. (2004) who investigated the effect of pH and salt on complexation between WPI and λ-CG. In the present study, WPI–CG mixtures (15:1) were prepared by dispersing the respective powders in Milli-Q water and stirring at 500 rpm for 1 h at room temperature using a mechanical stirrer at the appropriate concentration and ratio. Optical density (OD) readings were recorded during the acid titration using a UV/Vis spectrophotometer (Thermo Scientific, Madison, WI) at 600 nm using plastic cuvettes (1 cm path length). Solution pH was lowered by dropwise addition of HCl from pH 7.00 to 1.50. Diluting effects were kept to a minimum using a gradient of HCl concentrations based on pH (0.5 M > pH 5.1; 1.0 M > pH 3.3; 2.0 M > pH 1.5). The critical pH values (pH_c, $pH_{\phi 1}$, and pH_{opt}) measured in this study corresponded to conditions where minimal HCl addition occurred. For instance, within the pH range (6.0–4.0) corresponding to pH_c, $pH_{\phi 1}$, and pH_{opt}, the rise in conductivity (~175 μS/cm) and level of dilution (<0.5%) were considered to be insignificant. The rise in conductivity measured in this study is associated with the addition of acidulants and the release of counterions during complexation. Individual WPI and CG solutions were run as controls at their respective concentrations used in the mixed systems (i.e., 0.235% w/w (WPI) and 0.015% w/w (CG) – 15:1 WPI–CG ratio). Critical pHs corresponding to structure-forming events (pH_c and $pH_{\phi 1}$) were determined as the intersection between two curve tangents as described by Weinbreck et al. (2003) and Liu et al. (2009), whereas pH_{opt} corresponded to the maximum optical density at 600 nm.

The effect of biopolymer mixing ratio (WPI–CG 1:1–75:1) was also investigated by dispersing the respective powders at the appropriate concentration (0.25% w/w) and mixing ratio, followed by adjusting the pH to 4.50 with 0.5 N HCl. Optical density was then measured at 600 nm as previously described. The effect of NaCl (0–500 mM) on complexation was also studied using a similar sample preparation, except studies were only performed for the 12:1 WPI–CG mixing ratio. NaCl was added to the mixture after pH adjustment, and allowed to stir for 5 min before measuring OD. All turbidity measurements were prepared in triplicate, and reported as the mean value ± one standard deviation.

2.3. Electrophoretic mobility

Electrophoretic mobility (U_E) (a particles velocity in an electric field) for mixed WPI–CG solutions (12:1 WPI–CG mixing ratio, 0.25% w/w total biopolymer concentration) was investigated as a function of pH (7.00–1.50) using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA). Individual WPI and CG solutions were also prepared as controls, at the same protein or polysaccharide concentration used in the mixed systems. Solutions were acidified by dropwise addition of HCl, using the same concentration gradient as in Section 2.2, and measured at every 0.5 pH increments between pH 7.00 and 1.50. Samples were prepared as previously described. All measurements were preformed at room temperature (21–23 °C). By applying the Henry equation the electrophoretic mobility can be used to calculate the zeta potential (ς), which gives an estimate of the surface charge on the biopolymer:

$$U_E = \frac{2\varepsilon \times \zeta \times f(\kappa\alpha)}{3\eta} \quad (1)$$

where η is the dispersion viscosity, ε is the permittivity, and $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α) and the Debye length (κ). Using the Smoluchowski approximation $f(\kappa\alpha)$ equaled 1.5. All measurements were made in triplicate, and reported as the mean value ± one standard deviation.

2.4. Emulsion stability

The emulsion stability (%ES) of an oil-in-water (50/50) emulsion was investigated according to a modified method of Liu, Elmer, Low, and Nickerson (2010) using flaxseed oil and a 1.00% (w/w) WPI–CG mixture (12:1 mixing ratio, pH 4.50) as a non-aqueous and aqueous phase, respectively. Individual WPI and CG aqueous solutions served as controls, prepared at equivalent concentrations and conditions as found in the mixed systems. WPI and CG solutions (1.00% w/w) were prepared as previously described and adjusted to pH 4.50 with the addition of 1.0 N HCl. Aliquots (2 mL) of each solution (WPI + CG = 4 mL) were transferred into a 50 mL plastic centrifuge tube, followed by the addition of 4 mL of flaxseed oil. The mixture was then homogenized using an Omni Macro Homogenizer (Omni International, Inc., Marietta, GA) equipped with a 20 mm diameter saw tooth generating probe, at 7,200 rpm for 5 min. Immediately following homogenization, emulsions were transferred into individual 10 mL graduated cylinders (inner diameter = 10.80 mm; height = 100.24 mm; as measured by a digital caliper) and allowed to separate for 24 h. All measurements were performed in triplicate. The %ES was determined using Eq. (2), were V_B and V_A are the volume of the aqueous (or serum) layer before emulsification (4.0 mL) and after 24 h of drainage, respectively.

$$\%ES = \frac{V_B - V_A}{V_B} \times 100\% \quad (2)$$

2.5. Statistics

A one-way analysis of variance (ANOVA) with a Scheffe Post-Hoc test was used to identify mean differences in a) critical pH values (pH_c and $pH_{\phi 1}$) between WPI–κ-CG, WPI–ι-CG and WPI–λ-CG mixtures; and b) %ES between the mixed WPI–CG and individual WPI systems. Statistical analysis was performed using Systat Software (SPSS Inc., Ver. 10, 2000, Chicago, IL).

3. Results and discussion

3.1. Complex formation

3.1.1. Effect of pH

Complex formation within WPI–CG (κ-, ι-, λ-types) mixtures were investigated by changes in OD during a pH acid titration, initially for a 15:1 protein:polysaccharide-mixing ratio in the absence of added salt, as shown in Fig. 1. In the case of individual WPI solutions, a rapid rise in OD was observed at pH ~5.40 associated with the initial formation of WPI–WPI aggregates as solution pH approached its pI value. Optical density reached a maximum at pH 4.80 (OD 0.440), before declining in magnitude to the baseline at pH 2.80. Individual κ-, ι-, λ-type CG solutions showed no OD over the entire pH range (data not shown in Fig. 1). The addition of CG to the WPI solutions caused significant changes to the turbidity spectrums, as the result of the formation of soluble and insoluble electrostatic complexes. Changes in pH alters the ionization of charged groups on the WPI, enabling the two biopolymers to become attracted when oppositely charged at pH < pI of WPI, and pH > pKa of the charges sites on the CG backbone (Weinbreck, Nieuwenhuijse, et al., 2004). Soluble (pH_c) and insoluble ($pH_{\phi 1}$) complexes for WPI–κ-CG mixtures were found to occur at pH 5.51 ± 0.06 and pH 5.30 ± 0.05, respectively. Due to the dilute nature of the solution (0.25% w/w), biopolymers were assumed to be co-soluble at pHs > pH_c (Fig. 1). At $pH_{\phi 1}$, solutions changed from being transparent to cloudy. Complex formation involving WPI–ι-CG mixtures ($pH_c = 5.51 \pm 0.02$; and $pH_{\phi 1} = 5.36 \pm 0.01$) gave similar results as with the WPI–κ-CG mixture ($p > 0.05$), whereas critical structure-forming events within the WPI–λ-CG mixture ($pH_c = 5.67 \pm 0.06$; and $pH_{\phi 1} = 5.49 \pm 0.04$) occurred at slightly higher pHs relative to κ- and ι-types ($p \leq 0.05$) (Fig. 1).

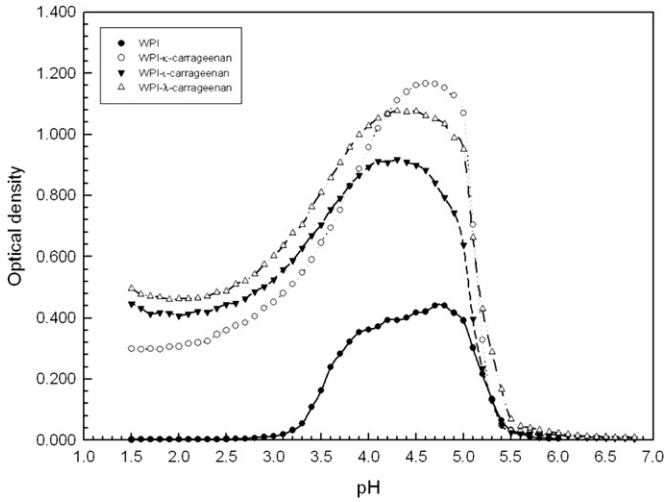

Fig. 1. Optical density for individual (WPI) and mixed (WPI–κ-CG, WPI–ι-CG, and WPI–λ-CG) biopolymer solutions at a 15:1 mixing ratio as a function of pH. Data represent the mean ± one standard deviation ($n = 3$).

Weinbreck, Nieuwenhuijse, et al. (2004) reported insoluble complexes ($pH_{\phi 1}$) formed in WPI–λ-CG over the pH range of 5.2–5.5 depending on the NaCl level added to the system using a WPI-aggregate-free material. Typically, electrostatic attraction between biopolymers occurs when the biopolymers have opposing net charges (i.e., pH < pI), however previous studies have shown that interactions with highly charged polyelectrolytes (e.g., CG) have been known to occur at pH > pI due to association with positively charged patches on the protein's surface and the presence of charge fluctuations on the protein's surface near its pI (de Kruif et al., 2004; Fang et al., 2006; Weinbreck, Nieuwenhuijse, et al., 2004). In the present study, all CG types initiated complex formation at pHs > pI when biopolymers were of similar net charge. However, λ-CG did so at a higher pH than the κ- and ι-types, most likely due to the presence of a greater number of sulfate groups available for binding (i.e., having three per disaccharide repeat unit versus two (ι) or one (κ)) with the WPI aggregates.

At pHs below $pH_{\phi 1}$, OD values in the WPI–κ-CG mixture continued to rise until reaching a maximum value of ~1.160 between pHs 4.50 and 4.80 (pH_{opt}), before declining (Fig. 1). For WPI–ι-CG and WPI–λ-CG mixtures, maximum OD occurred between pHs 4.00–4.50 (OD value = ~0.920) and pHs 4.20–4.50 (OD value = ~1.080), respectively (Fig. 1). At pH_{opt}, biopolymer interactions are considered to be greatest, where opposing charges cancel each other out to give a net neutral surface charge (Liu et al., 2009). In the present study, it was presumed that complexes formed between CG chains and small WPI–WPI aggregates, rather than individual proteins, as evident by an overlapping individual WPI turbidity curve with the mixed systems (Fig. 1). Under the experimental conditions (21–23 °C), κ-CG and ι-CG are presumed to be present in their ordered state (i.e., as double helices, aggregated mono-helices or aggregated helical dimers) with some level of lateral associations between neighboring CG ordered domains; mediated by residual ions present in solution (i.e., Ca^{2+} in the case of ι-CG and K^+ in the case of κ-CG). In contrast, λ-CG is thought to remain in its disordered random coil conformation. The magnitude of OD occurring between the mixtures seems to be related to both linear charge density and conformation of the CG molecule (Gu et al., 2005). Effects related to CG size are thought to be negligible since chains were of similar size (154–250 kDa). Typically, it would be expected that as the linear charge density on the CG increases (κ < ι < λ), the magnitude of OD should decline, as the greater amount of unbound or free sulfate groups would act to inhibit WPI–WPI aggregation due to electrostatic repulsion between CG molecules and shift structure-forming events (i.e., pH_c, $pH_{\phi 1}$ and pH_{opt}) to lower pH. However the experimental results show that the WPI–λ-CG mixture has a greater OD than the WPI–ι-CG mixture (Fig. 1). At ambient temperatures both κ-CG and ι-CG are in their ordered conformation (Gu et al., 2005). Complexation of WPI with κ-CG appears to be stronger (leading to higher OD values) than with ι-CG most likely due to a reduced number of unbound sulfate groups present. In contrast, λ-CG remains in a random coil conformation due to presence of 3 sulfate groups per disaccharide unit that restricts folding into a more order conformation (Gu et al., 2005), and hence behaves differently from the other two CG types in terms of adsorption to the proteins in solution. At pH < pH_{opt}, complexes for all types began to break down, however only a partial disassociation was observed, thought to be attributed to: a) the disassociation of WPI–WPI aggregates (as evident by a lack of scattering at pHs < 2.80 in the individual WPI system (Fig. 1); and b) remaining electrostatic interactions due to the presence of the highly charged sulfated polysaccharide.

3.1.2. Effect of biopolymer mixing ratio

The effect of mixing ratio (1:1–75:1 WPI–CG) on the maximum OD for all biopolymer mixtures was investigated at a constant pH

(4.50, near the pH_{opt} for all mixtures), whereas individual WPI solutions served as a control at corresponding concentrations (Fig. 2). Turbidity was found to increase in individual WPI solutions up to a concentration of 0.23% (w/w) (corresponding to the 10:1 mixing ratio) before reaching a plateau, indicating the absence of further increases in size and/or number of aggregates present in solution above this concentration (at ratios > 10:1) (Fig. 2). In the case of both WPI–κ-CG and WPI–λ-CG mixtures, OD values increased to a maximum at the 12:1 mixing ratio. Afterward, those mixtures with the κ-type declined steadily, and those with the λ-type reached a small plateau until a 30:1 mixing ratio, before declining as ratios increased (Fig. 2). Mixtures of WPI–ι-CG showed maximum OD values at a 20:1 mixing ratio ($p \leq 0.05$), which then remained constant as ratios increased ($p > 0.05$) (Fig. 2). At the maximum OD (i.e., 12:1 for κ-/λ-types; 20:1 for ι-type), small WPI–WPI aggregates become saturated with CG chains. The higher ratio needed for saturation of WPI–WPI aggregates in the WPI–ι-CG mixture may imply the charge density on the ι-CG ordered domain is greater than on the κ-CG ordered domain or the λ-CG random coil, and therefore associates with a greater number of WPI proteins. This hypothesis would also explain why WPI mixed with ι-CG has a lower OD than WPI mixed with the other CG types (Fig. 1). Surface charge measurements of individual WPI solutions over a pH range (7.00–1.50) found the pI to occur at pH 4.90 (zeta potential = 0 mV), where at pHs > pI WPI assumed a net negative charge and at pH < pI, WPI assumed a net positive charge (Fig. 3). In contrast, all CG types carried a highly negative charge ranging from ~−40 mV to −70 mV throughout the entire study (Fig. 3). Net neutrality (zeta potential = 0 mV) for biopolymer complexes were similar (12:1 mixing ratio), found to occur at pHs between ~4.30 and 4.50 (Fig. 3), which was close to pH_{opt} for each mixture.

3.1.3. Effect of NaCl addition

The addition of NaCl (0–500 mM) to individual and mixed (12:1 ratio) biopolymer solutions at pH 4.50 showed significant sample dependent changes, as the presence of ions acts to screen charged sites between the two approaching biopolymers. Individual WPI solutions showed only a marginal decrease in OD values with increasing levels of NaCl, possibly due to a salting in effect. WPI–κ-CG mixtures showed a rapid decline in OD values with added NaCl up to 300 mM, above which the system resembled that of the control (Fig. 4). Findings suggest that the presence of Na^+ and Cl^- acts to screen the electric double layer on both the WPI and κ-CG molecules, disrupting the electrostatic attractive forces between the two. Above 300 mM NaCl, no WPI–κ-CG complexes were presumed to exist. A similar decrease in complexation was reported by Fang et al. (2006) in gelatin–κ-CG mixtures with the addition of NaCl due to electrostatic screening of charges. In contrast, OD values for both WPI–ι-CG and λ-CG mixtures were relatively constant at levels between 0 and 100 mM NaCl, and then increased to a second plateau between 150 and 300 mM, prior to declining rapidly until reaching 500 mM NaCl where OD readings resembled that of the WPI control (Fig. 4). Differences in NaCl tolerance between the ι-/λ- and the κ-types reflects differences in the polysaccharide backbone in terms of the number of sulfated reactive sites present. Weinbreck, Nieuwenhuijse, et al. (2004) reported WPI (aggregate-free)–λ-CG mixtures experienced a slight increase in $pH_{\phi 1}$ to higher pHs as NaCl levels increased from 0 to 45 mM, then shifted back to lower pHs at higher NaCl levels. The authors reasoned that the enhanced solubility at low salt concentrations was related to charge compensation of the formed complexes. At pHs < pI, WPI assumes a net positive charge and begins to bind to the λ-CG molecule. However, due to the high linear charge density on the CG backbone, complex neutrality is not achieved until a much lower pH due to a spatial packing issue associated with the WPI molecules. As such, Na^+ ions in solution are available to screen charge sites on the backbone to effectively promote complexation up to a point, above which the presence of ions begins to interfere with complexation. In the present study, this was evident for both the ι- and λ-types, where complexation was enhanced up to 300 mM NaCl, versus the κ-type which experiences an immediate reduction in complex formation (i.e.,

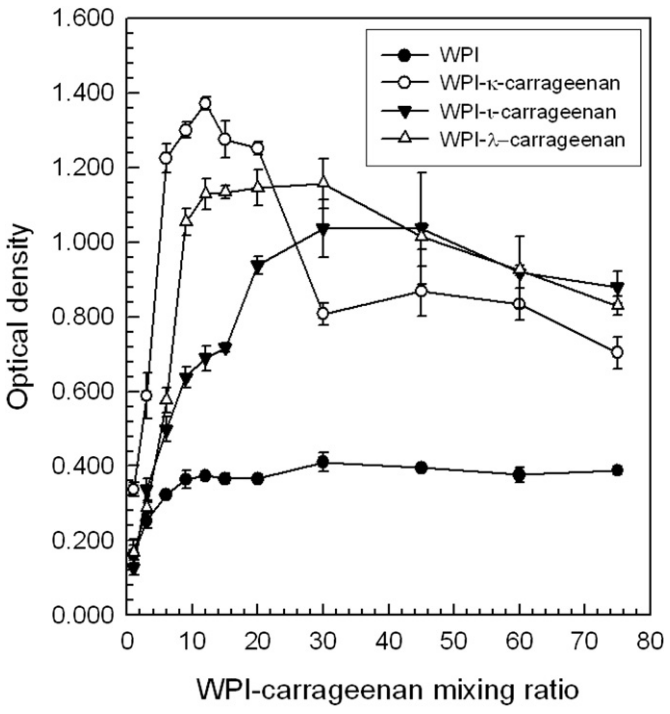

Fig. 2. Optical density for individual (WPI) and mixed (WPI–κ-CG, WPI–ι-CG, and WPI–λ-CG) biopolymer solutions at pH 4.50 as a function of mixing ratio. Data represent the mean ± one standard deviation ($n = 3$).

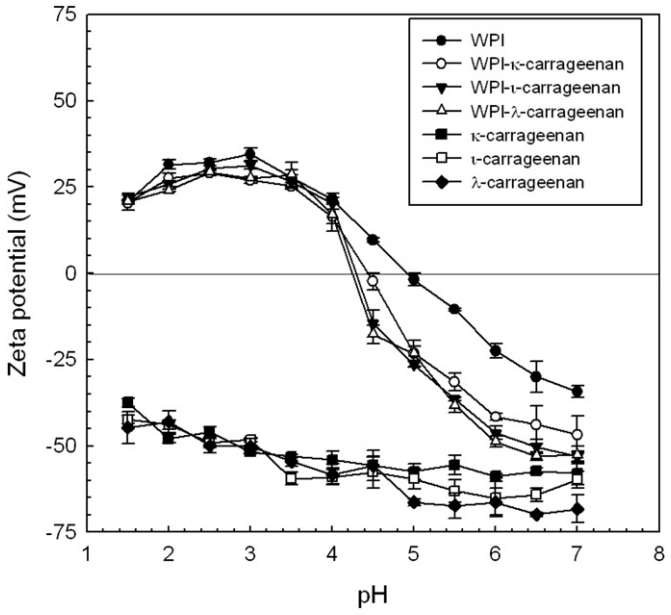

Fig. 3. Surface charge (zeta potential, mV) of individual (WPI – 0.23% w/w; κ-, ι-, λ-CG – 0.02% w/w) and mixed (WPI–κ-CG, WPI–ι-CG, and WPI–λ-CG) biopolymer solutions (12:1 mixing ratio, 0.25% w/w) as a function of pH and in the absence of added NaCl. Data represent the mean ± one standard deviation ($n = 3$).

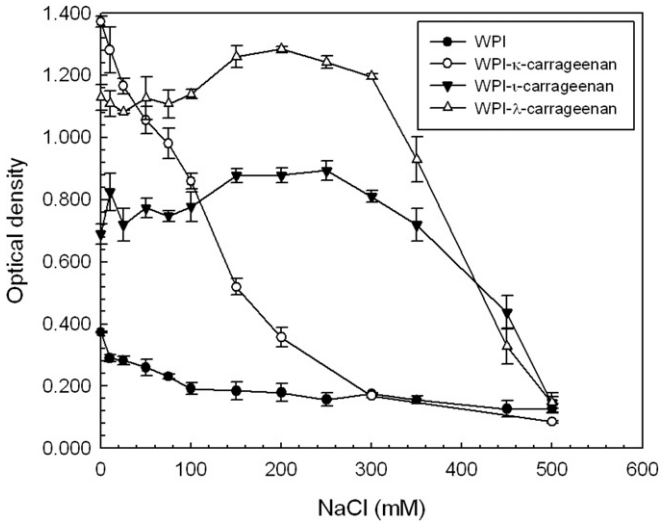

Fig. 4. Optical density for individual (WPI) and mixed (WPI–κ-CG, WPI–ι-CG, and WPI–λ-CG, 12:1 ratio) biopolymer solutions at pH 4.50 as a function of NaCl concentration. Data represent the mean ± one standard deviation ($n = 3$).

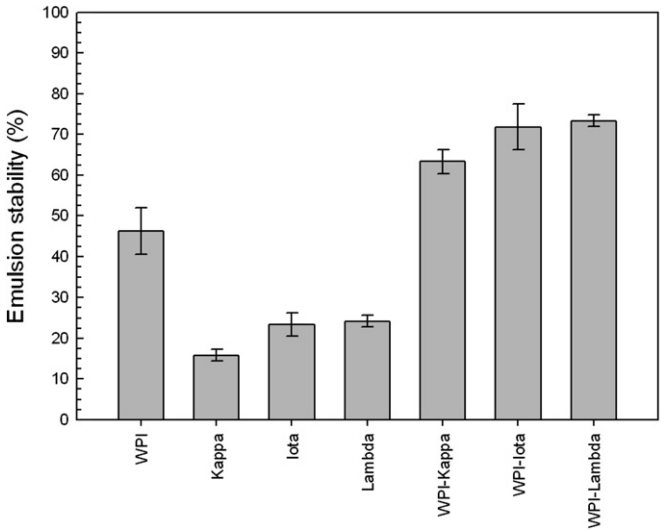

Fig. 5. Emulsion stability for individual (WPI (0.92% w/w), and κ-, ι-, λ-CG (0.08% w/w)) and mixed (WPI–κ-CG, WPI–ι-CG, and WPI–λ-CG (1.00% w/w)) biopolymer stabilized emulsions prepared at pH 4.50 and at a 50/50 aqueous/oil ratio using flaxseed oil. Data represent the mean ± one standard deviation ($n = 3$).

maximum OD) with the addition of salt (Fig. 4). Differences between this critical NaCl content between the present study (300 mM) and that of Weinbreck, Nieuwenhuijse, et al. (2004) (45 mM) for WPI–λ-CG mixtures reflect the nature of the initial WPI material used (i.e., aggregate versus aggregate-free). The presence of aggregates is presumed to reduce the number of positively charged sites spatially available for interacting with the CG chains, leading to an increased number of free sulfate groups on the CG in solution. In contrast, an aggregate-free system at an equivalent concentration would have a greater number of positively charged sites on the WPI exposed, which in turn would lead to less excess free sulfate groups on the CG in solution and lower Na^+ tolerances.

3.2. Coacervate stabilization of oil-in-water emulsions

The emulsifying properties of WPI–CG complexes (12:1 mixing ratio) were investigated within a 50/50 oil-in-water emulsion. Emulsion stability values (%ES) for individual WPI solutions (46.25 ± 5.73) were substantially higher than found for all CG types (κ, ι, λ) which range between ~16 and 24% (Fig. 5). However, comparisons between the controls should be made with caution since CG concentrations were much lower than WPI. This difference could result in differing %ES due to different bulk phase viscosities or loading at the interface. In contrast, %ES in the mixed WPI–CG complexes regardless of the CG type (κ, ι, λ) was significantly higher than the WPI solution ($p < 0.05$) indicating enhanced ability to stabilize the oil-in-water interface. The difference in total biopolymer concentration between the mixed systems (1.00% w/w) and the WPI control (0.92% w/w) was assumed to have negligible effects on the findings. %ES between the WPI and CG systems were considered to be similar ($p > 0.05$) (Fig. 5). Enhanced stability in the mixed systems is hypothesized to be caused by a saturation of the oil–water interface with a viscoelastic film of electrostatically stabilized complexes; steric stabilization from polysaccharide tails oriented in the aqueous phase; and electrostatic repulsion between neighboring droplets of similar charge.

Gu et al. (2005) investigated the effect of pH and, CG type and concentration on the stabilities of β-lactoglobulin stabilized oil-in-water emulsions. At pH 3, β-lactoglobulin–CG (κ, ι, λ) emulsions were stable at CG ≤ 0.08% w/w, but became unstable at higher levels due to bridging flocculation and creaming. At pH 6, β-lactoglobulin–ι-CG led to improved emulsion stability, whereas the addition of κ-CG and λ-CG were unstable to creaming above a critical concentration due to depletion flocculation. Harnsilawat et al. (2006) reported electrostatic complexes improved the stability of β-lactoglobulin-stabilized oil-in-water emulsions when polysaccharides were added (i.e., alginate, CG and gum Arabic), once the concentration of polysaccharide was sufficient to saturate the protein-coated droplets; with alginate and CG being most effective. Jourdain et al. (2008) investigated emulsion stability containing sodium caseinate and dextran sulfate (DS), and found stability to be related to the DS concentration, pH and the emulsification procedure used, where under some conditions stability was enhanced through steric stabilization, and in others the presence of DS lead to instability via bridging flocculation.

4. Conclusions

The formation of complexes between WPI and (κ-, ι-, λ- type) CG is largely driven by electrostatic attractive forces between biopolymers of opposing net charge, with secondary stabilization most likely from hydrogen bonding, van der Waals forces and hydrophobic interactions. However, since sulfated CG carries a strong negative charge, initial associative phase behavior began at pH > pI due to the presence of positively charged patches along the protein's surface. A trend that was most pronounced in the presence of λ-CG due to the presence of 3 sulfide groups per repeating disaccharide units. Complexation conditions were found to be greatest near pH 4.50 for all mixtures, however mixing ratios corresponding to the highest OD were found to be at 12:1 for both the WPI–κ-CG and WPI–λ-CG mixtures, and 20:1 for WPI–ι-CG mixture. The addition of NaCl disrupted complexation within WPI–κ-CG as levels were increased, whereas when ι-CG and λ-CG were present, complexation initially was enhanced up to a critical Na^+ concentration, and then declined. Complexes formed showed potential as an oil-in-water emulsion stabilizer, showing enhanced emulsion stability relative to individual WPI or CG systems. Furthermore, the pH-sensitivity of the WPI–CG mixtures could be tailored for controlled release applications, where a WPI–CG

coating could be stable at pH 4.5, and then undergo material degradation (and controlled release) at higher pHs.

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