Influence of Electrostatic Interactions on Behavior of Mixed Rice Glutelin and Alginate Systems: pH and Ionic Strength Effects

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Abstract

The influence of pH (2–7) and ionic strength (0 to 200 mM NaCl) on the properties of hydrolyzed rice glutelin (HRG), alginate, and their mixtures in aqueous solutions was investigated using turbidity, ζ -potential, and microscopy measurements. Soluble or insoluble complexes could be formed between HRG and alginate by manipulating solution conditions such as HRG:alginate ratio, pH, and ionic strength. Relatively small complexes that were relatively stable to aggregation and sedimentation over a wide range of pH conditions could be formed using a 3:1 HRG to alginate mass ratio. The addition of salt (NaCl) to solutions containing HRG-alginate complexes weakened the electrostatic attraction between them, which led to release of the protein. As a result, the protein molecules tended to interact with other protein molecules (rather than polysaccharide molecules) leading to the formation of precipitates and sediments. The electrostatic complexes formed in this study may be a useful means of extending the utilization of rice proteins as functional ingredients in food and beverage products.

Keywords: rice glutelin; alginate; electrostatic complex; turbidity; ionic strength

1. Introduction

Knowledge of the interactions between proteins and polysaccharides is of great interest in the food, agricultural, and personal care industries because of their ability to generate specific functional attributes in materials, such as texturization, stabilization, and encapsulation (Fioramonti, Perez, Aríngoli, Rubiolo, & Santiago, 2014; Li, Liu, et al., 2016; Razzak, Kim, & Chung, 2016). In aqueous solutions, proteins and polysaccharides may interact with each other through various types of attractive or repulsive forces, van der Waals, electrostatic, steric, hydrogen bonding, or hydrophobic. Depending on the nature of the interactions involved, the mixed system may exist as a miscible molecular solution, a colloidal dispersion of protein-polysaccharide complexes, or segregated phases (Bokkhim, Bansal, Grondahl, & Bhandari, 2015). The nature of any colloidal dispersion formed depends on the molecular characteristics of the biopolymers involved (charge density, molar mass, conformation, flexibility, and concentration), environmental conditions (pH and ionic strength), and the biopolymer mixing ratio (Souza & Garcia-Rojas, 2015; Stone & Nickerson, 2012; Stone, Teymurova, Chang, Cheung, & Nickerson, 2015; Yuan, Wan, Yang, & Yin, 2014). For electrically charged polysaccharides, electrostatic interactions play a particularly important role in the formation of protein-polysaccharide complexes. At pH values around and below its isoelectric point (pI), a protein carries positively charged groups that can interact with anionic polysaccharides by electrostatic attraction (Khalesi, Emadzadeh, Kadkhodaei, & Fang, 2015). The resulting electrostatic complexes formed can be used to create desirable functional attributes within food and beverage products. In this study, we examined the possibility of improving the solubility characteristics of a hydrolyzed rice protein by forming electrostatic complexes between it and an anionic polysaccharide.

Rice glutelin is the major storage protein in rice, and has been reported to have a high nutritive value, to lower cholesterol levels, and to be hypoallergenic (Du, et al., 2013). For this reason, there is considerable interest in using rice glutelin as a functional ingredient in the food industry. Nevertheless, its utilization in many food

and beverage products is currently limited due to the low water-solubility of the native protein. In a previous study, we used trypsin treatment to fabricate a partially hydrolyzed rice glutelin (HRG) ingredient that had a relatively high water-solubility under neutral pH conditions (Xu, et al., 2016). However, this HRG was still water-insoluble at pH values near its isoelectric point, which may restrict its application in many food and beverage products. We therefore examined the possibility of extending the range of pH conditions where HRG could be used by forming electrostatic complexes with polysaccharides. A previous study reported that the physical and oxidative stability of rice protein emulsions was improved by addition of xanthan gum, which was attributed to an increase in aqueous phase viscosity, as well as to an increase in the electrosteric repulsion between the droplets (Chen, et al., 2016). Another study reported that mixtures of deamidated rice protein and dextran underwent segregative phase separation through a thermodynamic incompatibility mechanism (Li, et al., 2010). More recently, the phase behavior of deamidated rice protein and κ -carrageenan/ dextran mixtures has been studied (Li, Liu, et al., 2016). These researchers suggested that the main driving forces for the observed phase separation in this system were depletion and electrostatic interactions. However, improving the physical stability of rice glutelin in aqueous solutions by addition of polysaccharides has rarely been reported.

Alginate (AL) is an anionic linear polysaccharide that is composed of (1,4)- β -D-mannuronic acid and (1,4)- α -L-guluronic acid units, which occurs naturally in brown marine algae (Razzak, et al., 2016). It is widely used in the food industry as a natural stabilizer, thickener, and gelling agent (Harnsilawat, Pongsawatmanit, & McClements, 2006; Razzak, et al., 2016). Alginate has been reported to form either soluble or insoluble complexes with various proteins in aqueous solutions, including pea protein (Klemmer, Waldner, Stone, Low, & Nickerson, 2012), β -lactoglobulin (Harnsilawat, et al., 2006), lactoferrin (Bokkhim, et al., 2015), whey protein (Fioramonti, et al., 2014) and fish gelatin (Razzak, et al., 2016). These studies reported that the formation of soluble or insoluble complexes between alginate and proteins was highly dependent on pH (Harnsilawat, et al., 2006), biopolymer ratio

(Klemmer, et al., 2012), and ionic strength (Razzak, et al., 2016). However, the behavior of HRG and alginate mixtures has not previously been reported.

In this study, we characterized the major factors affecting the interactions between HRG and alginate in aqueous solutions using turbidity, ζ -potential, and optical microscopy measurements. The formation of electrostatic complexes under different pH, ionic strength, and mass ratio conditions were studied. This study could be used to facilitate the design of rice protein-based food ingredients with more desirable functional properties.

2. Materials and Methods

2.1. Materials

Rice glutelin (93.42 wt%, dry basis) was provided by Golden Agriculture Biotech Company Limited (Jiangxi, China). Sodium chloride, alginate, sodium azide, trypsin and other chemicals were of analytical grade and were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). Corn oil was obtained from a commercial food supplier (Mazola, ACH Food Companies, Memphis, TN). Double-distilled water was used to prepare all solutions.

2.2 Preparation of hydrolyzed rice glutelin (HRG)

Hydrolyzed protein was prepared using trypsin according to a method described previously (Xu, et al., 2016). 2 g of rice glutelin was added to double-distilled water (30 mL) with constant stirring for 1 h. Trypsin was then added after the pH and temperature had been adjusted to the required values (pH=8, 50°C). After 4.2 min of hydrolysis, the dispersion was heated to 95 °C for 10 min to inactivate the trypsin, adjusted to pH 7.0 using HCl or NaOH, and then immediately cooled to room temperature by placing the test tube in water. The pH of the suspension was kept constant by addition of 2.5 M NaOH during the entire period of hydrolysis. The mixture was centrifuged (TGL-20B, Anting Scientific Instrument Factory, Shanghai, China) at 4800 g for 10 min and the supernatant was freeze-dried. The degree of hydrolysis (DH)

was analyzed using a pH-stat method described previously (Adler-Nissen, 1986). Finally, HRG with 2% degree of hydrolysis was obtained.

2.3 Preparation of biopolymer mixtures

Aqueous solutions of HRG (3 wt%) and alginate (2 wt%) were separately prepared by dispersing weighed amounts of the powdered ingredients in double-distilled water (containing 0.02 wt% sodium azide as an antimicrobial). Each solution was then adjusted pH to 7.00 using HCl or NaOH, and then stirred for at least 5 h at room temperature to ensure full dissolution. Mixed solutions containing HRG (0.3 wt%) and alginate (0-0.3 wt%) were prepared by mixing biopolymer and buffer solutions together at different volume ratios to obtain the required final concentrations. The resulting solutions were then stirred for at least 30 min to ensure complete dispersion and readjusted to pH 7.00 using 0.1M NaOH prior.

2.4 Turbidity measurements

The turbidity of dispersions containing HRG and alginate were measured with a UV-visible spectrophotometer at 600 nm (Ultrospec 3000 pro, Biochrom Ltd., Cambridge, UK) using plastic cuvettes (1 cm path length). Solution pH was changed by dropwise addition of HCl from 7.0 to 2.0. Dilution effects were minimized by using a gradient of HCl concentrations based on pH (0.05 M > pH 3.6, 0.5 M > pH 2.8, 1.0 M > pH 2.0) (J. Liu, Shim, Wang, & Reaney, 2015). The effect of ionic strength on HRG-alginate interactions was also measured by adding NaCl at a concentration of 0-200 mM while keeping the ratio of HRG to alginate constant at 3:1(w/w). Homogenous HRG and alginate solutions with corresponding concentrations were used as controls.

2.5 ζ -potential measurement

The ζ -potential of solutions at different pH (2-7) was measured using particle microelectrophoresis (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, UK). The solutions were measured without dilution. Duplicate measurements were performed at 25 °C on each sample.

2.6 Microscopy

Microscopic images of samples were measured using a light microscope (C1 80i Digital Eclipse, Nikon, Tokyo, Japan/Melville, NY, USA) with a 60× objective lens. Aliquots (10 µL) of sample were placed onto microscope slides and then carefully covered with a coverslip. The images were captured from at least four similar images.

2.7 Statistical analyses

Statistical analysis was carried out using an one-way ANOVA test using SPASS 19.0 (SPSS Inc., Chicago, IL). Significant differences among samples were taken as $p < 0.05$. Each experiment was performed in triplicate and the results are reported as means values $\pm$ standard deviation (SD).

3. Results and discussion

Electrostatic interactions between proteins and polysaccharides are related to the charge characteristics of each biopolymer, the biopolymer ratio, pH, and ionic strength (Fioramonti, et al., 2014; Rodríguez Patino & Pilosof, 2011). Consequently, we focused on the impact of these parameters on the formation of HRG-alginate complexes in the current study. Our main aim was to find system conditions where clear solutions or stable colloidal dispersions could be formed using the hydrolyzed rice protein, since these systems may be suitable for application in food and beverage products.

3.1 Effects of polysaccharide concentration on complex formation

Initially, the impact of polysaccharide-to-protein ratio on complex formation was examined by keeping the HRG level constant (0.3 wt%) and increasing the alginate level (0 to 0.25%). The pH dependence of the turbidity of the various biopolymer solutions was measured (Fig. 1a). The pure alginate solution remained transparent over the whole pH range studied (data not shown), indicating that the alginate molecules did not form complexes large enough to scatter light. This was presumably because of the strong electrostatic repulsion between the negatively charged alginate molecules

(Fig. 2). The turbidity of the pure HRG solution remained relatively low when the pH was reduced from 7 to 6, then it increased from pH 6 to 4, but then it decreased from pH 4 to 2 (Fig. 1a). In addition, digital photographs of the samples showed that they were transparent at pH 6 and 7, but contained white sediment at the bottom of the test tubes at pH 5 to 2 (Fig. 1b). These results confirm that the water-solubility of the hydrolyzed rice protein was relatively good around neutral pH, but poor at more acidic pH values.

For this reason, the impact of different levels of alginate on the solubility characteristics of the HRG was examined. The pH dependence of the turbidity and appearance of the mixed systems depended on alginate concentration (Fig. 1). It should be noted that the photographs of the samples show their overall appearance after settling, whereas the turbidity measurements were carried out after the samples were made homogeneous by inverting the test tubes. In general, as the pH was decreased from pH 7 to 2, the turbidity of the HRG-alginate solutions initially increased, then reached a maximum value, and then either decreased or remained fairly constant depending on alginate concentration. At pH 6 and 7, increasing the amount of alginate in the solutions caused a slight dose-dependent increase in turbidity, which may have been due to the slight turbidity of the alginate solutions or due to the formation of soluble complexes (Fig. 2). Soluble electrostatic complexes may form under conditions where both the protein and polysaccharide have negative charges due to the binding of anionic groups on the alginate molecules to cationic patches on the HRG surfaces (Razzak, et al., 2016). At most pH values, the addition of alginate suppressed the formation of white sediment at the bottom of the test tubes that was observed in the systems containing pure HRG (Fig. 1b). Instead, either a clear solution or a relatively stable cloudy colloidal dispersion was formed. These results suggest that it may be possible to incorporate hydrolyzed rice protein into aqueous-based products over a broad range of pH values when it is used in combination with an anionic polysaccharide. This result is in agreement with previous studies that have shown that the water-dispersibility of other types of proteins can be improved by forming electrostatic complexes with alginate (Zhang, Zhang, Decker, & McClements, 2015).

The turbidity measurements and visual observations suggest that mixed systems containing different types of complexes could be formed depending on biopolymer ratio and pH. Soluble complexes tended to be formed at pH values well above the isoelectric point of the hydrolyzed rice protein leading to fairly clear solutions or only slightly turbid colloidal dispersions. Coacervate particles appeared to be formed at pH values around and slightly below the isoelectric point of the protein, leading to the generation of cloudy colloidal dispersions. Relatively large insoluble complexes (precipitates) were formed at pH values well below the isoelectric point leading to the formation of a sediment. Previous studies suggest that the formation of protein-polysaccharide complexes follows a number of sequential steps when the pH is reduced from well above to well below the protein's isoelectric point: (i) an *intimate mixture* of non-complexed protein and polysaccharide molecules is formed due to strong electrostatic repulsion; (ii) *soluble complexes* containing only a few biopolymer molecules are formed due to electrostatic attraction between positive patches on the protein molecules and negative groups on the polysaccharide molecules; (iii) *coacervates* are formed consisting of numerous protein and polysaccharide molecules loosely packed into relatively small colloidal particles; and, (iv) *precipitates* are formed consisting of many protein and polysaccharide molecules tightly packed into a separate phase leading to sedimentation; (v) an *intimate mixture* is again formed when the polysaccharide loses its negative charge well below the pK_a value of its charged groups (Li, et al., 2012; Weinbreck, De Vries, Schrooyen, & De Kruif, 2003). In our study, 0.3% HRG solutions containing 0.05 to 0.25 wt % alginate formed relatively stable colloidal suspensions from pH 3 to 7, but sediments were observed in many of the samples at pH 2 (Fig. 1b). Moreover, the 0.3% HRG solutions containing 0.1 wt% alginate remained relatively transparent or only slightly turbid across the entire pH range studied, indicating that they probably contained soluble complexes or small coacervate particles.

The pH dependence of the ζ -potential of HRG solutions containing different alginate concentrations was also determined (Fig. 2). The ζ -potential of pure HRG solutions (no alginate) changed from highly negative (-39.3 mV) at pH 7 to

moderately positive (+14.6 mV) at pH 2, with a point of zero charge around pH 4, which corresponds to the isoelectric point of this protein. The ζ -potential of pure alginate solutions went from strongly negative (-63.6 mV) at pH 7 to slightly negative (-5.54 mV) at pH 2. The lower magnitude of the negative charge on the alginate molecules at highly acidic conditions can be attributed to the fact that the carboxyl groups ($pK_a \approx 1.88$) along the alginate backbone became partially protonated at this pH (Klemmer, et al., 2012). All the HRG-alginate mixtures exhibited fairly similar trends in the ζ -potential *versus* pH profiles. The ζ -potential of the mixed systems was appreciably more negative than the pure HRG solutions, which suggested that electrostatic complexes were formed by the protein and polysaccharide molecules. The origin of this effect is electrostatic attraction between the carboxyl groups on the alginate and cationic groups on the protein surfaces, even at pH values above the isoelectric point where both the protein and polysaccharide have a net negative charge. A similar result has been reported for other combinations of globular proteins and anionic polysaccharides, such as wheat protein and pectin (Qiu, Zhao, & McClements, 2015) and pea protein and gum arabic (S. Liu, Low, & Nickerson, 2009). Interestingly, as the alginate concentration was increased, the ζ -potential of the mixed system approached the value of the pure alginate solution. This suggests that the protein molecules made little contribution to the overall ζ -potential signal of the mixed system, presumably because the high number of negative charges on the polysaccharide molecules dominated the positive charges on the surfaces of the protein molecules. A similar result has been reported for WPI-xanthan gum systems (Benichou, Aserin, Lutz, & Garti, 2007).

3.2 Aggregation characteristics of mixed biopolymer systems

In this section, the formation of protein-polysaccharide complexes was characterized in more detail for HRG (0.3%) in the absence or presence of the optimum amount of alginate (0.1%) established in the previous section. Again, complex formation was monitored by measuring changes in turbidity *versus* pH using an acid titration (Fig. 3).

In the absence of alginate, the pure HRG solution remained relatively transparent from pH 7 to 6, exhibited a steep increase in turbidity from pH 5.8 to 3.9, and then exhibited a decrease in turbidity from pH 3.8 to 2. The turbidity peak observed around pH 3.9 corresponded to the point of zero charge observed in the ζ -potential measurements (Fig. 2). This suggests that the maximum amount of protein aggregation occurred when the protein had the lowest net charge, which can be attributed to the reduction in electrostatic repulsion between the protein molecules. The turbidity decreased as the pH was raised above or lowered below this value because the increase in electrostatic repulsion between the protein molecules partially inhibited their aggregation. The optical microscopy images clearly showed that there were large aggregates within the protein solutions around the isoelectric point of the protein (Fig. 3, inset).

The presence of alginate (0.1%) in the HRG solution repressed the large increase in turbidity normally observed around the protein's isoelectric point, and there was no evidence of large aggregates in the microscopy images (Fig. 3). The turbidity of the mixed protein-polysaccharide solution was relatively low at pH 7.0 due to the relatively strong electrostatic repulsion between the anionic alginate and HRG molecules. In this case, the two biopolymers probably formed a mixture of intermingled molecules or small soluble complexes. As the pH was decreased, the turbidity began to increase slightly around pH 5.8 suggesting the formation of soluble protein-polysaccharide complexes. When the pH was reduced further, there was a steeper increase in turbidity near pH 3.6, which is probably due to association of the soluble complexes leading to the formation of coacervates. These coacervates remained dispersed in the aqueous phase as a colloidal suspension that visibly appeared turbid. The stability of the coacervate suspension to coalescence can be attributed to the relatively strong electrostatic repulsion between the coacervate particles due to their relatively high negative charge (Fig. 2). Similar results have been reported in other studies of electrostatic complexation between proteins and anionic polysaccharides, such as casein and alginate (Zhang, et al., 2015), pea protein and alginate (Klemmer, et al., 2012), and napin protein and carboxylated alginate (Stone, et al., 2014).

Complexation of HRG and alginate is mainly driven by electrostatic interactions. Consequently, the addition of salts to the solutions would be expected to alter the nature of the complexes formed by screening any attractive or repulsive electrostatic interactions in the system. For this reason, the impact of NaCl addition on the properties of HRG solutions (0.3%) in the absence and presence of alginate (0.1%) was investigated.

3.3 Effect of NaCl on the properties of HRG solutions

The effect of ionic strength on the turbidity-pH profiles of pure HRG solutions was measured (Fig. 4a). All the samples exhibited the same general trend as the pH was decreased from 7 to 2: first the turbidity increased and then decreased. Nevertheless, there were some noticeable differences between the turbidity-pH profiles depending on salt level. The turbidity of the HRG solutions increased in the presence of NaCl at pH values somewhat above (pH 6.2 to 5.2) and somewhat below (pH 3.4 to 2.0) the protein's isoelectric point. This suggested, that the addition of salt may have screened the electrostatic repulsion between the protein molecules, thereby widening the range of pH values where isoelectric precipitation occurred. Indeed, protein precipitation and sedimentation was observed at a higher pH value (pH 5.5) in the presence of NaCl than in its absence (Fig. 4b). Conversely, there was a pronounced decrease in turbidity around the isoelectric point of the protein, *i.e.*, from pH 5.2 to 3.4 (Fig. 4a). This effect may have been due to a weakening of the electrostatic attraction between positive and negative patches on neighboring protein surfaces leading to smaller insoluble protein particles (Stone, et al., 2012).

The effect of ionic strength on the ζ -potential-pH profile of HRG solutions was also measured (Fig. 5). All the samples exhibited fairly similar general trends in the pH dependence of the ζ -potential, going from negative at high pH to positive at low pH. Nevertheless, there were appreciable differences in the magnitudes of the ζ -potential depending on salt level. In the absence of NaCl, the ζ -potential of HRG changed from negative (-39.3 mV) to positive (+14.6 mV) when pH was adjusted from 7 to 2, with a point of zero charge near pH 3.8. As the salt concentration was

increased, the magnitude of the ζ -potential at both low and high pH values decreased, which can be attributed to electrostatic screening effects. On the other hand, the point of zero charge was not strongly affected by the NaCl concentration, which suggests that specific salt ions (Na^+ or Cl^-) did not preferentially bind to the surfaces of the protein molecules.

3.4 Effect of NaCl on the properties of HRG-alginate solutions

The effect of ionic strength on the turbidity and appearance of HRG-alginate solutions was also determined as a function of pH (Fig. 6), since salt ions would be expected to influence the attractive and repulsive electrostatic interactions between the two types of biopolymer (Hadian, et al., 2016; Schmitt, Aberkane, Sanchez, Phillips, & Williams, 2009). Interestingly, salt addition had a much larger impact on the pH dependence of the turbidity and appearance of the mixed systems than for the pure HRG systems. In a broad pH range around the isoelectric point of the protein (pH 3.5 to 6.5), increasing the NaCl level in the mixed systems caused an appreciable increase in turbidity (Fig. 6a) and led to the formation of visible sediments in many systems (Fig. 6b). In addition, the pH dependence of the turbidity and appearance of the mixed systems became more similar to those of the pure protein systems in the presence of salt. These results suggest that the addition of even low levels of salt (50 mM NaCl) weakened the electrostatic attraction between the protein and polysaccharide molecules, thereby allowing the protein molecules to interact with each other rather than with the anionic polysaccharide molecules. Similar effects have been reported for other protein-anionic polysaccharide combinations, such as gelatin-alginate (Razzak, et al., 2016) and whey protein-carrageenan (Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004). As mentioned earlier, the pH range where visible precipitation and sedimentation occurred in the mixed systems increased in the presence of salt (Fig. 6b), which suggests that the electrostatic complexes would not remain intact in foods that had relatively high salt contents.

The effect of NaCl concentration on the ζ -potential-pH profile of the HRG-alginate mixed was also measured (Fig. 7). All the mixed systems had a net

negative charge at all pH values studied, but the magnitude of the ζ -potential decreased from pH 7 to 2. The relatively steep decrease in the ζ -potential from pH 4 to 2 observed in all systems can be attributed to protonation of the carboxyl groups on the HRG and alginate molecules around their pK_a values. The magnitude of the ζ -potential in the mixed systems decreased over the entire pH range as the NaCl concentration was increased, which can be partly attributed to electrostatic screening effects (Guzey & McClements, 2006; Li, Li, Shen, Niu, & Fu, 2016). In addition, it is likely that the electrostatic attraction between the protein and polysaccharide molecules was weakened at higher salt levels, which promoted the release of some of the protein molecules from the complexes thereby altering the electrical properties of the system.

4. Conclusions

The present study demonstrated that the range of pH values where hydrolyzed rice protein could be successfully incorporated into aqueous solutions could be extended by addition of alginate. This effect was attributed to the formation of an electrostatic HRG-alginate complex, which inhibited isoelectric precipitation of the protein. A protein-to-polysaccharide mass ratio of 3:1 was found to produce either clear solutions, stable turbid colloidal suspensions, or unstable precipitates depending on solution pH. The addition of NaCl to these systems weakened the electrostatic interactions in the system, which released some of the protein from the complexes and led to sedimentation. The native rice glutelin would not have same phenomenon because it has low solubility even under neutral conditions and limited protein molecules could interact with alginate when pH changed from 7 to lower. These results may be useful for the design and formulation of food or beverage products fortified with rice proteins.

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Fig.1a

Fig.1b

Fig.2

Fig.3

Fig.4a

Fig.4b

Fig.5

Fig.6a

Fig.6b

Fig.7

“Influence of Electrostatic Interactions on Behavior of Mixed Rice Glutelin and Alginate Systems: pH and Ionic Strength Effects”

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Graphical Abstract