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Peroxidase-mediated conjugation of corn fiber gum and bovine serum albumin to improve emulsifying properties

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ABSTRACT

The emulsifying properties of corn fiber gum (CFG), a naturally occurring polysaccharide–protein complex, was improved by kinetically controlled formation of hetero-covalent linkages with bovine serum albumin (BSA), using horseradish peroxidase (HRP). The formation of hetero-crosslinked CFG–BSA conjugates was confirmed using ultraviolet–visible and Fourier-transform infrared analyses. The optimum CFG–BSA conjugates were prepared at a CFG:BSA weight ratio of 10:1, and peroxidase:BSA weight ratio of 1:4000. Selected CFG–BSA conjugates were used to prepare oil-in-water emulsions; the emulsifying properties were better than those of emulsions stabilized with only CFG or BSA. Measurements of mean droplet sizes and zeta potentials showed that CFG–BSA-conjugate-stabilized emulsions were less susceptible to environmental stresses, such as pH changes, high K ionic strengths, and freeze–thaw treatments than CFG- or BSA-stabilized emulsions. These conjugates have potential applications as novel emulsifiers in food industry.

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1. Introduction

Proteins and polysaccharides are important macromolecules in food systems, and they are widely used to stabilize oil-in-water emulsions. There is particular interest in the use of polysaccharide–protein complexes instead of plain polysaccharides or proteins (Evans, Ratcliffe, & Williams, 2013). Polysaccharide–protein complexes could be formed through electrostatic interactions or covalent bonds (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). The newly formed complexes will exhibit combined functional properties of each component and their improved emulsifying abilities is receiving heated attention. (Galazaka, Dickinson, & Ledward, 2000; Li, Fang, Phillips, & Al-Assaf, 2013). Although electrostatic interactions are widely used, complexes formed by this method are susceptible to the variation of pH values and easily dislocated from each other. Compared with electrostatic interactions, covalent bonds could render the complexes more irreversible and stable to low pH and high ionic strength (Evans et al., 2013).

Covalent bonding between a polysaccharide and a protein is commonly achieved through Maillard reactions and enzymatic mediations. Maillard reactions usually take several days or weeks to conjugate the ε-amino groups in proteins and the reducing-end carbonyl groups in polysaccharides (Flanagan & Singh, 2006; Wong, Day, & Augustin, 2011). Enzymatic methods are more cost-effective than Maillard reactions, because milder conditions, lower quantities of reactants, and shorter reaction times are needed, they are less likely to produce toxic byproducts, and are acceptable to consumers (Poutanen, 1997).

Corn fiber gum is usually extracted from the byproducts of corn-milling processes, using the alkaline hydrogen method (Gaspar, Juhasz, Szengyel, & Reczey, 2005; Hespell, 1998). It has attracted increasing interest because of its potential use as a novel industrial emulsifier with certain health benefits, as shown by in vitro fermentation studies (Nino-Medina et al., 2009; Rose, Patterson, & Hamaker, 2010). It consists mainly of a β-(1→4)-xylan backbone with arabinofuranosyl substituents attached through O-2 and/or O-3 (Martinez-Lopez, Carvajal-Millan, Miki-Yoshida, et al., 2013; Martinez-Lopez, Carvajal-Millan, Rascon-chu, Marquez-Escalante, & Martinez-Robinson, 2013). In addition to the major arabinoxylan

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fraction, there are minor functional components, namely phenolic acids and proteins. These two fractions play important roles in the emulsifying properties and modification of CFG. Phenolic acids, namely ferulic acids, a small amount of p-coumaric acids and diferulic acids, are esterified to arabinosyl residues (Saulnier, Vigouroux, & Thibault, 1995). According to current studies, a small fraction of proteins may covalently bond, or physically precipitate, with polysaccharide fractions in CFG (Yadav, Johnston, Hotchkiss, & Hicks, 2007; Yadav, Nunez, & Hicks, 2011). This protein fraction anchors the CFG molecules at the oil–water interface, and carbohydrate fractions stretch into the aqueous phase, providing an electrosteric barrier (Randall, Phillips, & Williams, 1988); CFG can therefore stabilize oil-in-water emulsions, as can other naturally occurring complexes such as gum arabic and sugar beet pectin. However, not all CFGs have excellent emulsifying properties. The poor emulsifying properties of some CFGs are partly the result of insufficient protein contents (Yadav, Parris, Johnston, Onwulata, & Hicks, 2010).

In this study, we used horseradish peroxidase (HRP) to catalyze hetero-conjugation between CFG and bovine serum albumin (BSA), and used the hetero-crosslinked CFG–BSA conjugates to stabilize oil-in-water emulsions. HRP can use various aromatic components as substrates, e.g., aromatic phenols, phenolic acids, and amino acids (Veitch, 2004). It is therefore an ideal enzyme for achieving hetero-conjugation between phenolic acids in CFG and tyrosines in BSA. Previous studies showed that HRP can catalyze homo-conjugation of polysaccharides or hetero-conjugation between polysaccharides and open-chain proteins such as casein, or peptides (Boeriu et al., 2004; Martinez-Lopez, Carvajal-Millan, Rascon-chu, et al., 2013; Ng, Greenshields, & Waldron, 1997; Oudgenoeg et al., 2001). However, previous attempts to achieve oxidation between polysaccharides and compact proteins have been unsuccessful (Figueroa-Espinoza et al., 1999).

In this investigation, we selected BSA as a model protein, because it has a compact structure with tyrosine moieties mostly located on the outer surface (Zhao, Li, Carvajal, & Harris, 2009), so the tyrosyl residues are readily accessible to peroxidase oxidation to form dityrosines (Aeschbach, Amadoo, & Neukom, 1976; Chen et al., 2003). BSA also emulsifies oil-in-water emulsions, and has been used in the food industry for many years (Haque & Kinsella, 1988).

The objective of this study was to conjugate CFG with BSA. The emulsifying abilities of the conjugates were investigated, with the aim of broadening the range of CFG applications in the food industry.

2. Materials and methods

2.1. Materials

BSA (fraction V, M_w 68,000) was purchased from Roche (Nutley, NJ, USA). HRP was purchased from Sigma–Aldrich [St. Louis, MO, USA; 1000 U/mg solid, using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)]. Heat-resistant α-amylase was purchased from the Aladdin Industrial Corporation (Shanghai, China). CFG was extracted from corn fiber processed by wet-milling, using the procedure described in Section 2.3. p-Coumaric and ferulic acid standards were obtained from Sigma Chemical Co., St. Louis, MO. All other reagents were analytical grade. The wet-milled corn fiber was provided by the local corn-milling factory.

Stock phosphate stock buffer solution (PBS, pH 7.0, 50 mM) was prepared by adding sodium dihydrogen phosphate (50 mM) to disodium phosphate (50 mM), and then adjusting the pH to 6.0 using 1 M HCl. BSA and CFG solutions were prepared separately by dispersing a dry powder in PBS (pH 6.0), stirring at room temperature until completely dissolved, and storing at 4 °C overnight for complete hydration. Peroxidase solution (100 U/mL) was prepared using PBS (pH 6.0), stored at 4 °C, and used within 7 days. H_2O_2 solution (30%, v/v) was diluted with PBS (pH 6.0) to 0.06% (v/v); it was prepared freshly prior to the CFG–BSA conjugation experiments.

2.2. CFG isolation

Corn fiber and corn bran are byproducts from corn wet milling and dry milling processes, respectively. During the wet milling process, corn grains are soaked in warm water containing sulfur dioxide and corn fiber is collected in the step of wet sieving (Rose, Inglett, & Liu, 2009). In the investigation, corn fiber from wet-milling was dried in an oven and ground to 40 mesh. The ground fiber was de-oiled by adding hexane at a weight ratio of 1:7, and stirred for 2 h using a magnetic stirrer. The de-oiled fiber was mixed with distilled water at the weight ratio of 1:10 and boiled for 10 min. Heat-resistant α-amylase (3 mL/50 g) was added and the mixture was incubated in water at 40 °C for 2 h. The absence of starch was confirmed by staining the de-oiled and destarched corn fiber with I/I_2. CFGs were extracted from the de-oiled and destarched fiber using a modified alkaline hydrogen method (Yadav et al., 2007). The de-oiled and destarched corn fiber was stirred with an alkaline solution containing 2 meq/g fiber of NaOH and $Ca(OH)_2$, and boiled for 15 min. The reaction mixture was cooled to room temperature, and centrifuged at 8000 × *g* for 5 min. The supernatant was decanted from the residue, 30% H_2O_2 was added, the pH was adjusted to 11.5 with 6 mM NaOH, and the mixture was stirred at room temperature for 2 h. The pH of the alkaline H_2O_2 extract was adjusted to 4.0–4.5 with concentrated HCl and centrifuged at 8000 × *g* for 20 min. The supernatant was decanted and two volumes of ethanol were gradually added to it, with stirring. The supernatant–ethanol mixture was allowed to settle for 15 min; CFG was obtained as a white flocculent precipitate. The water–ethanol mixture was decanted, and the precipitate was transferred to plastic boxes to be lyophilized.

2.3. Compositional analysis

The isolated CFG samples were subjected to moisture, ash content, lipid, protein and phenolic acid analysis. Moisture and ash contents were determined using "AACC Approved Methods" 44-19 and 08-01, respectively (AACC International, 1995). The total protein content was measured using BCA protein assay reagent kit, which detects protein using bicinchoninic acid (BCA) (Smith et al., 1985). Bovine serum albumin (BSA) was used as the standard to construct a calibration curve. The total lipid content was measured using fat analyzer (ZF-06, Ruizheng Equipment Corporation, Shanghai). The total phenolic acid content was measured using UV spectroscopy (UVmini-1240, Shimadzu Corporation, Japan) at the wavelength of 325 nm. The total amount of phenolic acids was calculated from known standard curves of p-coumaric acids and ferulic acids and expressed as a sum of p-coumaric acids and ferulic acids ($y_1 = 12.246x - 0.2596$, $R^2 = 0.9979$; $y_2 = 8.523 - 0.3342$; x is absorbance, y_1 and y_2 is the concentration of ferulic acid and p-coumaric, respectively).

2.4. Optimum conditions for CFG–BSA conjugation using peroxidase

Oxidative crosslinking between CFG and BSA using a peroxidase/hydrogen system was performed using a modified version of the methods reported by Boeriu et al. (2004) and Oudgenoeg et al. (2001).

The optimum oxidation conditions for CFG–BSA conjugation were identified by performing the reaction at various weight ratios

with different amounts of HRP and H_2O_2. First, different amounts of peroxidase (0–100 μL, 0–10 U) plus different concentrations of H_2O_2 (0–100 μL, 0.006 wt%, 0.03 wt%, 0.06 wt%) were mixed with BSA solutions (1.0 mL, 0.2 wt%). Then CFG solutions (4.0 mL) were added in 10 aliquots (400 μL × 10), at time intervals of 5 min, over 1 h. Final reaction systems containing CFG and BSA mixtures at weight ratios of 0.25:1, 0.5:1, 1:1, 2:1, 4:1, 10:1 were obtained by preparing CFG solutions with six different concentrations, i.e., 0.0125%, 0.025%, 0.05%, 0.1%, 0.2%, and 0.5% (w/w). In control experiments (without peroxidase and H_2O_2), peroxidase and H_2O_2 solutions were substituted by the same amount of 50 mM PBS (pH 6.0). Then the mixtures were incubated in water at 35 °C for 4 h. The reactions were terminated by adding sodium azide solution (20 μL, 1.0 wt%). The reacted samples were concentrated in an oven at 40 °C, freeze-dried, and ground to solid powders for further analysis.

2.5. Characterization of CFG–BSA conjugates

2.5.1. Ultraviolet–visible (UV–vis) spectroscopy

All the reacted samples were first dispersed in distilled water and stirred to ensure the formation of homogeneous solutions at room temperature. The solutions were examined using UV spectroscopy (UVmini-1240, Shimadzu Corporation, Japan) in the wavelength range 400–200 nm at room temperature.

To select the reaction system that contained major hetero-conjugates, the absorbances of diphenolic acids, dityrosine, phenolic acids, and tyrosine were monitored separately. According to Oudgenoeg et al. (2001), dityrosine show maximum absorption values at 348 and 315 nm, respectively, and the tyrosine and ferulic acid maximum absorptions are at 280 and 325 nm, respectively. Quantitative data for the amount of reacted phenolic acids was obtained by comparison with the known standard curve of p-coumaric and ferulic acids as described in Section 2.3.

2.5.2. Fourier-transform infrared (FT-IR) spectroscopy

An appropriate reaction system was selected and examined using FT-IR spectroscopy (NEXUS 6700, Nicolet, USA), in the intermediate IR region (400–4000 cm^{-1}), with KBr.

CFG and BSA mixtures without peroxidase and H_2O_2 were used as control groups. Plain CFG and BSA were also tested. To better illustrate hetero-conjugation between BSA and CFG, the CFG–CFG conjugation was investigated separately, using the procedure described in Section 2.4. The concentrations of the CFG and peroxidase solutions were determined using the same method as for the selected reaction system. The BSA solution was substituted by the same amount of PBS.

The samples were analyzed in the solid state to avoid the effects of strong water absorption, in particular in the amide I band region.

2.6. Emulsion preparation

CFG–BSA conjugate solutions (2 wt%) were prepared by mixing with distilled water, stirring for at least 2 h, and storing at 4 °C overnight to achieve complete hydration. A coarse emulsion was prepared by homogenizing 10% (w/w) soybean oil with 90% (w/w) CFG–BSA conjugate solution in a high-speed blender (20,000 rpm; T25basic, IKA, Germany) for 1 min. A fine emulsion was prepared by passing the coarse emulsion through a high-pressure valve homogenizer (NS1001L Panda2K, GEA Niro Soavi, Italy). The emulsion formula was selected based on preliminary studies. The emulsion was stored at 4 °C.

2.7. Emulsion stability under various environmental stresses

Emulsions with different KCl concentrations and pH values were prepared according to the method reported by Chanamai and McClements (2002). The emulsions were diluted to 10% (v/v) with solutions containing different concentrations of KCl, ranging from 0 to 300 mM. Then the diluted emulsions were adjusted to different pH values (4.0, 5.0, 6.0, 7.0, and 8.0) with 1% (w/v) NaOH or 1% (w/v) HCl solution. The final emulsions contained 1% (w/w) soybean oil, 0.2% (w/w) CFG–BSA conjugate, and 0–300 mM KCl. The emulsions were transferred to plastic test tubes and stored at room temperature for 24 h prior to further analysis. For freeze–thaw studies, the emulsions were prepared in the absence and in the presence of KCl (0–50 mM) and then subjected to one freeze–thaw cycle, consisting of 24 h at −20 °C in a freezer, followed by complete thawing at room temperature. The emulsion stability was evaluated by zeta potential and mean particle size analysis.

2.8. Emulsion evaluation

2.8.1. Zeta potential measurements

The zeta potential values of the diluted emulsions were measured using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). The analyzer detects the electrophoretic mobility of particles and calculates the zeta potential from the measured velocity, using the Smoluchowski equation. The emulsions were diluted 10 times prior to measurements. The measurements were conducted at 25 °C, and results are reported as the average of two separate injections, with 30 readings per injection.

2.8.2. Particle size analysis

The droplet distributions and mean particle sizes of the emulsions were determined using a laser diffraction particle size analyzer with a small volume mode (LS 320, Beckman Coulter, Inc., FL, USA). The results are reported as $d_{3,4}$, as an average of two measurements.

2.9. Statistical analysis

All the experiments were performed at least twice. Origin 8.0 and Excel 2013 was used for graph plotting and data processing. OMNIC 7.0 was used for IR peak analysis. Different samples were subjected to ANOVA analysis using SPSS at probability levels of 0.05 ($p < 0.05$).

3. Results and discussions

3.1. Compositional analysis

The compositional analysis results of CFG samples is given in Table 1. The protein content of our CFG samples was much lower than those industrial grade CFG. The total amount of phenolic acids is a rough estimate by measuring p-coumaric and ferulic acids. It is relatively high, compared with the data (300–800 μg/g) shown in present works (Yadav et al., 2007).

3.2. Optimum conjugation conditions

BSA was first mixed with peroxidase and then CFG solutions were added. This was because tyrosines are poorer substrates for HRP than phenolic acids are, therefore kinetic control was used in our study, i.e., a large excess of BSA over CFG was used to produce equal amounts of radicals (Boeriu et al., 2004; Oudgenoeg et al., 2001). Hetero-conjugation can occur through phenolic acids in CFG and tyrosines in BSA, or tyrosines in proteins originally present in CFG and in BSA. Homo-conjugates, i.e., CFG–CFG or BSA–BSA, can

Table 1
Compositional analysis of CFG and industrial grade CFG. (The compositional analysis data of industrial CFG was according to the report of Yadav et al., 2007). ND represents the compositions have not been detected yet).

Samples	Moisture (wt%)	Ash (wt%)	Protein (wt%)	Phenolic acid (μg/g)	Total lipid (wt%)
CFG	14.16	3.07	2.21	1732	0.06
Industrial CFG	10.73	16.97	5.25	ND	ND

be formed via phenolic acid–phenolic acid and tyrosine–tyrosine linkages.

In order to select a proper H_2O_2 concentration level, each group was mixed with different concentrations of H_2O_2 and the absorption changes were measured at 325 nm. The UV absorption of the group with the CFG to BSA weight ratio of 10:1 was shown in Fig. 1. When concentration of H_2O_2 changed from 0.006% to 0.06%, there was no significant difference between their absorption. Similar results were observed in other groups. Because a higher H_2O_2 concentration may lead to inactivation of HRP, the concentration of H_2O_2 was set at the level of 0.006% (Zaidel, Arnous, Holck, & Meyer, 2011). According to Fig. 2, the concentration of peroxidase in the final reaction system was set as1 μg/mL (50 μL peroxidase, 5 U). Because the addition of 50 μL of peroxidase and 50 μL of H_2O_2 (0.006%) was sufficient to promote complete conjugation; the addition of higher concentration of peroxidase did not cause further declines in the UV absorbance intensities. Moreover, this concentration level could ensure that increases in viscosity and apparent degradative losses were low (Geissmann & Neukom, 1973).

As the results in Fig. 2A–C show, the reacted amounts of tyrosine and phenolic acids varied for groups with different CFG and BSA weight ratios. There was no increase in the absorbance at 318 nm for all the experimental groups (data not shown), which indicated that large amount of dityrosines were not formed, and BSA homo-crosslinking was weak. For groups 0.25:1, 0.5:1, and 1:1, there was no significant decline in the amount of tyrosine (280 nm absorbance), as well as the amount of phenolic acids (325 nm absorbance); this indicated that the enzymatic reaction process did not work effectively on these groups. For groups 1:2, 1:4, and 1:10, the amounts of tyrosine and phenolic acids both decreased significantly, so there was both hetero- and homo-conjugation (CFG–BSA and CFG–CFG) in these groups. The absorbance at 348 nm, i.e., for diphenolic acids, was the lowest in group 1:10, indicating that CFG–BSA conjugation was maximum in this reaction system. The reacted amount of phenolic acids in group 1:10 was quantified according to known standards. The initial amount was 34 μg/g and 16.5 μg/g when the reaction was completed. The reacted degree was about 50%, lower than the oxidative cross-linking of plain corn bran hemicellulose (up to 80%), which may be due to the structure difference of different CFG samples (Ng et al., 1997). This reaction system, which had a CFG to BSA weight ratio of 10:1 and 5 U of peroxidase, was selected for further analysis.

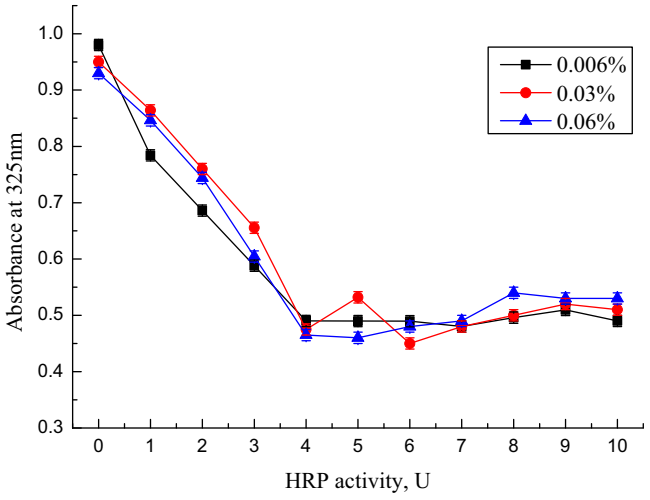


Fig. 1. Influence of the concentration of H_2O_2 (0.006%, 0.03%, 0.06%) on the enzymatic reaction process, measured at the CFG to BSA weight ratio of 10:1 under various HRP quantity (0–10 U) using UV spectroscopy at 325 nm.

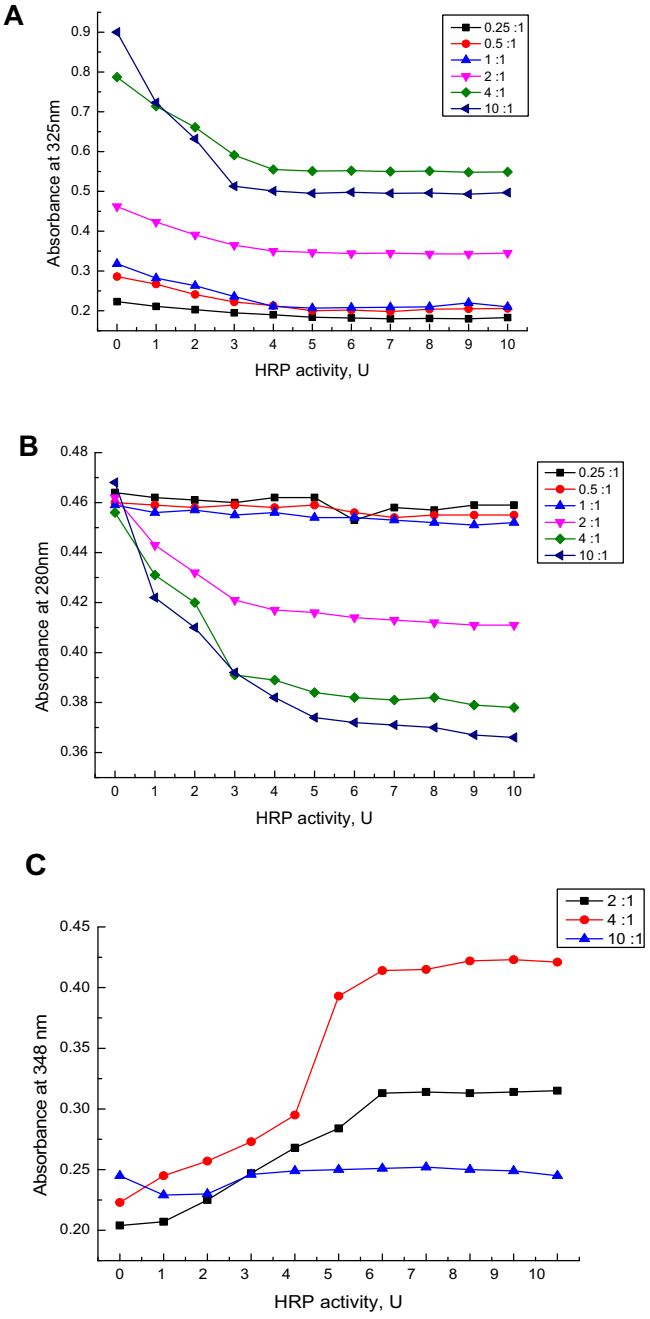


Fig. 2. Influence of the amounts of HRP (0–10 U) and the CFG to BSA weight ratios (0.25:1–10:1) on the enzymatic reaction process, measured by UV spectroscopy at 325 nm (A), 280 nm (B) and 348 nm (C), respectively.

3.3. FT-IR spectroscopy

FT-IR spectroscopy was used to confirm hetero-conjugation between CFG and BSA. BSA has bands for C=O stretching (amide I), C—N stretching and N—H bending of primary amine groups (amide II), and C—N bending (amide III) at 1650, 1545, and 1387 cm^{-1}, respectively. The linear and branched (1→4)-β-xylan structure in CFG showed a strong band at 1047 cm^{-1}, and a weak absorbance at around 903 cm^{-1}, in the anomeric region (Boeriu et al., 2004). Although chemical analysis showed minor protein and phenolic acid contents, these components did give resolved bands in the IR spectra (Kacurakova et al., 2000).

IR analysis of the mixture without peroxidase showed a new band at 1545 cm^{-1}, indicating possible interactions between BSA and CFG; this could be because the C=O and N—H groups of BSA are involved in hydrogen-bond formation or electrostatic interactions with the hydroxyl groups of CFG (Secundo & Guerrieri, 2005). The C=O band at 1633 cm^{-1} in CFG shifted to 1653 cm^{-1} in CFG–BSA mixtures in the absence of peroxidase, but in CFG–BSA mixtures in the presence of peroxidase, the band shifted to 1644 cm^{-1}, with four new peaks appearing at 1302, 942, 871, and 667 cm^{-1}, respectively.

For the CFG–BSA mixtures containing peroxidase and H_2O_2, new peaks appeared at 942, 871, and 667 cm^{-1}, which indicated formation of new linkages with aromatic rings. The typical external plane bending vibration of hydrogen atoms on aromatic rings are in the range 650–900 cm^{-1}, therefore the results show that CFG may react intermolecularly with BSA to form C—O—C via phenolic acids or proteins (Selinheimo, Lampila, Mattinen, & Buchert, 2008). A new peak at 1302 cm^{-1} was also observed; this might indicate formation of C—O—C by tyrosines in proteins and phenolic acids in CFG (see Fig. 3).

Unlike the CFG–BSA conjugates, the CFG–CFG conjugates showed no new peaks, but the band at 903 cm^{-1} disappeared, and the peak at 1387 cm^{-1} became more intense. This suggests that the four new peaks in the spectra of the CFG–BSA mixtures can be attributed to intermolecular crosslinking between CFG and BSA.

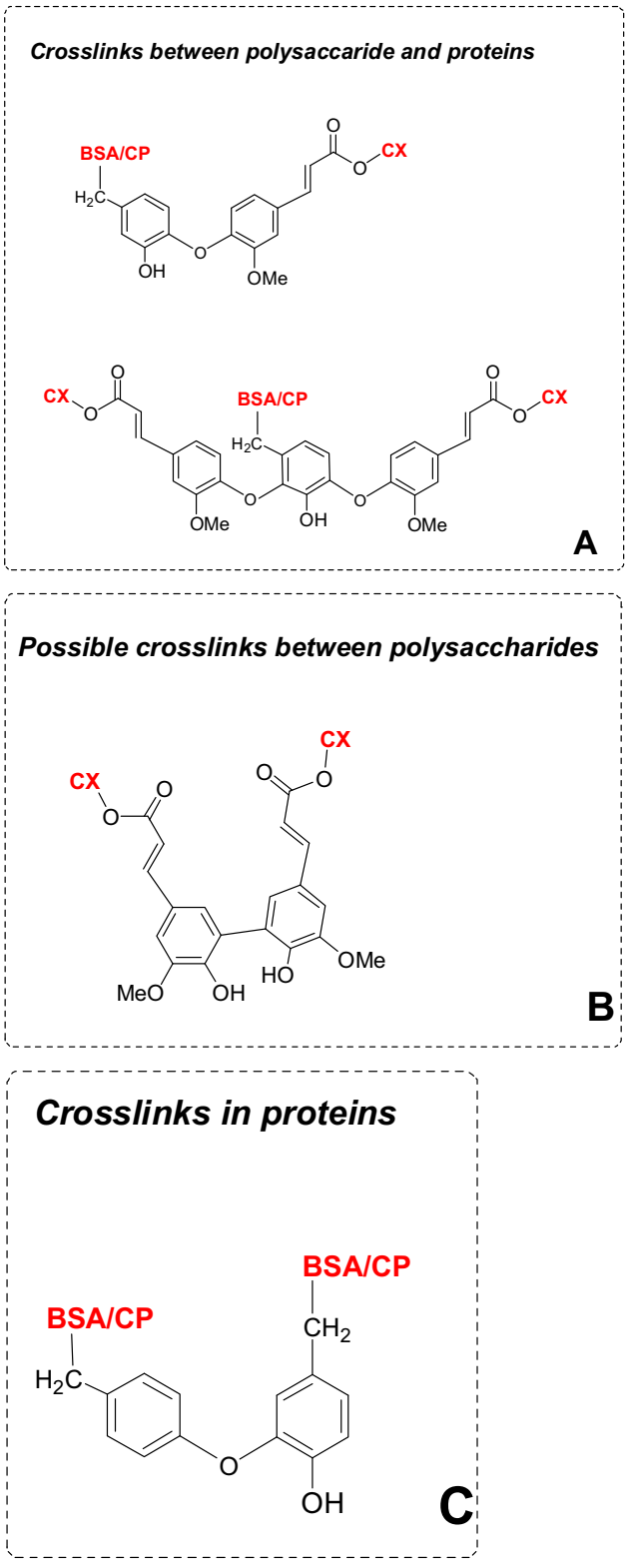
The UV–vis and FT-IR results therefore show that CFG and BSA at a weight ratio of 10:1 form CFG–BSA intermolecular conjugates. The linkage between CFG and BSA is shown in Scheme 1A. However, intramolecular CFG–CFG and BSA–BSA conjugations cannot be completely ruled out. CFG–CFG and BSA–BSA are shown in Scheme 1B and C. The proteins that are originally present in CFG may also be involved in the reaction system.

3.4. Emulsion evaluation

3.4.1. Influence of pH and ionic strength on emulsion stability

The purpose of these experiments was to examine the influence of KCl (0–300 mM) and pH on the stability of emulsions containing CFG–BSA-conjugate-coated, CFG–CFG-conjugate-coated, CFG-coated, and BSA-coated droplets. The emulsion prepared using CFG–BSA conjugates was diluted before further analysis to evaluate the potential applications of these conjugates in food industry.

The droplet size of the CFG–BSA-stabilized emulsion (~0.99 μm) was smaller than those of the emulsions stabilized with plain BSA (1.32–2.73 μm), CFG (2.71–3.04 μm) or CFG–CFG-conjugates (1.69–1.89 μm). This could be because proteins have high binding affinities and surface activities (Dickinson, 2009) therefore conjugated BSA chains could anchor more CFG molecules at the oil–water interface (Dickinson, 2012). It is therefore expected that the CFG–BSA conjugates would have higher surface activity than plain CFG, and would form smaller droplets. The droplet size of the CFG–CFG-conjugate-stabilized emulsions was smaller than those stabilized with plain CFG, which was in accordance with the works of other researchers (Jung & Wicker, 2012; Zaidel, Chronakis, & Meyer, 2013).



Scheme 1. Possible links in CFG–BSA conjugates (CP represents proteins in CFG and CX represents arabinoxylan chains in CFG).

The effects of KCl on emulsions stabilized with CFG–BSA conjugates, CFG, BSA and CFG–CFG conjugates at pH 4.0 and 7.0 are shown in Fig. 4. At pH 4.0, the approximate isoelectric point of BSA, there was significant droplet growth in the BSA-stabilized

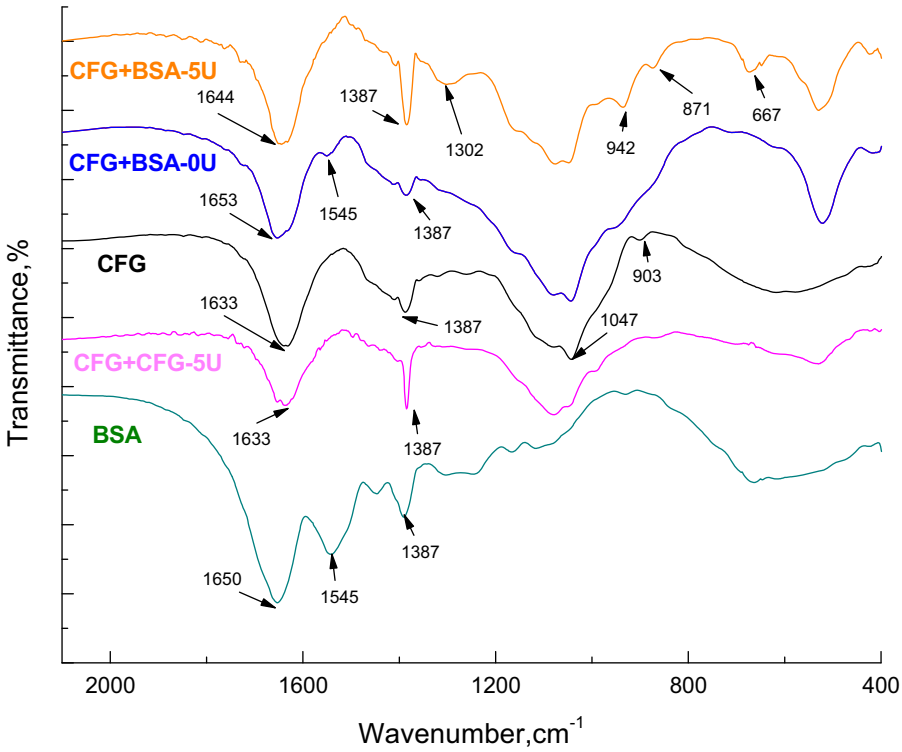


Fig. 3. FTIR analysis of CFG-BSA (10:1) with 0 U, 5 U peroxidase; plain CFG and BSA; CFG-CFG with 5 U peroxidase.

emulsion, but at pH 7.0, the droplet size remained relatively stable. This could be because BSA stabilizes emulsions mainly through electrostatic interactions. When the pH of the aqueous bulk was around the isoelectric point of BSA, the charges were screened, so the aqueous bulk tended to be neutral, and electrostatic interactions were weak. The BSA-stabilized emulsion was more susceptible to increasing ionic strength (McClements, 2004). A higher KCl concentration leads to bridging or depletion of BSA with little net charge, which promotes coalescence of oil droplets (Rangsansarid & Fukada, 2007). At pH 4.0 and 7.0, the mean droplet sizes of CFG–BSA-conjugate-stabilized, CFG-stabilized and CFG–CFG-conjugate emulsions were stable over the KCl concentration range 0–300 mM. CFG stabilizes emulsions mainly through steric interactions, similar to other polysaccharide–protein complex hydrocolloids (Randall et al., 1988). CFG–CFG-conjugates have shown better emulsifying properties probably due to thicker layer that developed at the oil–water interface (Jung & Wicker, 2012). For the CFG–BSA conjugates, not only a thicker layer, but also a combination of steric interactions in CFG and electrostatic interactions in BSA is present. Therefore, CFG–BSA conjugates have shown

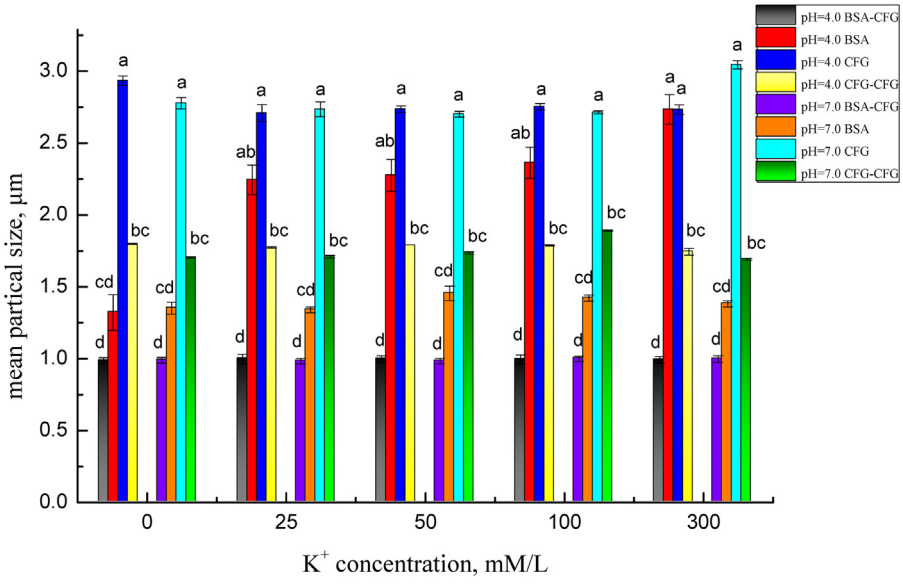


Fig. 4. Mean particle size of emulsions containing [CFG–BSA conjugates]-coated, [BSA]-coated, [CFG]-coated and [CFG–CFG conjugates]-coated droplets under potassium concentrations ranging from 0 to 300 mM at pH 4.0 and 7.0, respectively. Different letters (a, b, c, d) indicate statistically significant difference between emulsions stabilized with different emulsifiers, i.e., CFG–BSA (CFG, BSA, CFG–CFG) under different pH and K^+ concentration ($P < 0.05$).

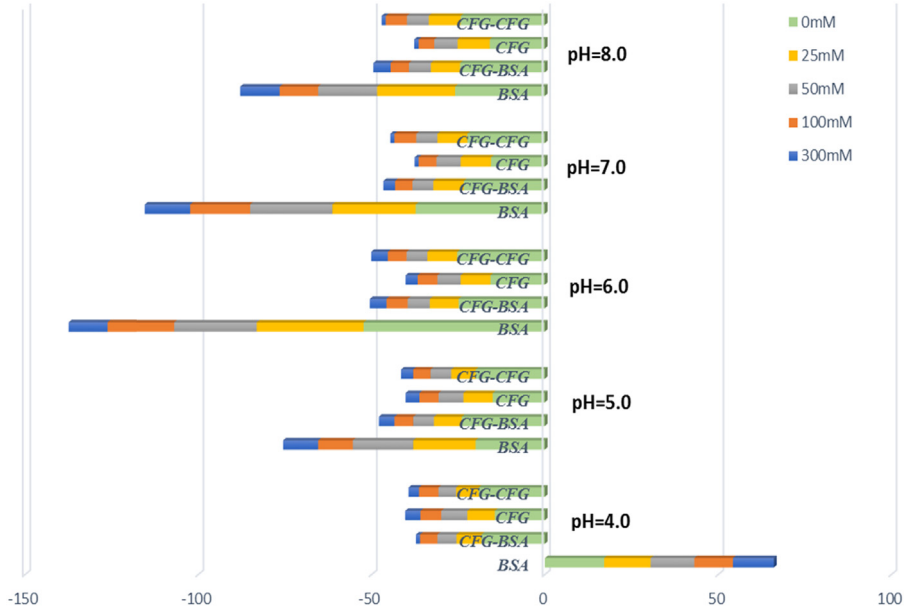


Fig. 5. zeta potential of emulsions containing [CFG–BSA conjugates]-coated, [CFG]-coated and [BSA]-coated droplets at the pH ranging from 4.0 to 8.0 and potassium concentrations ranging from 0 to 300 mM.

better emulsifying properties than CFG and CFG–CFG-conjugates have. The arabinoxylan chains in the aqueous solution create a strong steric barrier against flocculation and coalescence. Furthermore, the CFG–BSA conjugates are more highly charged than plain CFG, as shown by the zeta potential measurements.

The effects of pH on emulsions stabilized with CFG–BSA conjugates, CFG, BSA or CFG-CFG conjugates in the presence of K^+ (300 mM) were also investigated. The mean droplet sizes of the emulsions stabilized with CFG–BSA conjugates (~0.995 μm) and CFG (~2.934 μm) were stable at different pH values. The mean droplet size of the emulsion stabilized with BSA increased at its isoelectric point, from 1.670 μm at pH 5.0 to 2.734 μm at pH 4.0. These phenomena could be explained by a mechanism whereby the CFG–BSA conjugates stabilize the emulsion through electrosteric interactions.

3.4.2. *Influence of pH and ionic strength on zeta potential*

The effects of pH on the zeta potentials at different KCl concentrations of emulsions containing CFG–BSA-conjugate-coated, CFG-coated, BSA-coated and CFG–CFG-conjugate-coated droplets are shown in Fig. 5 and Table 2.

In the absence of KCl, the CFG–BSA conjugates, CFG and CFG–CFG-conjugates all remained negatively charged at all pH values, probably because of the carboxyl residues in CFG (Chanamai & McClements, 2002). The zeta potential values were relatively low at all pH values. This indicates that the CFG–BSA conjugates and CFG stabilize emulsions through electrosteric rather than purely electrostatic interactions; similar behaviors have been observed for other hydrocolloids containing polysaccharide–protein complexes (Acedo-Carrillo et al., 2006). In the absence of KCl, the zeta potential of the emulsion stabilized with CFG–BSA conjugates was higher than that of the CFG-stabilized stabilized emulsion at pH 5–8. This is probably caused by the conjugated BSA moiety. At pH 4.0, below the isoelectric point (~4.7) of BSA, droplets stabilized with BSA were positively charged, and when the pH was increased, the droplets became negatively charged, because of the carboxyl groups in BSA. The negatively charged carboxyl groups in BSA could therefore cause the CFG–BSA conjugates to have a higher zeta potential than CFG (Wooster & Augustin, 2006). In the absence of KCl, the zeta potential of CFG–CFG-coated emulsions was more negative than that of CFG. It might be caused by the exposure of more carboxyl residues in the oxidation system. In the presence of KCl, the zeta potential of CFG–CFG-coated emulsions dropped significantly, similar to CFG-coated emulsions.

In the presence of KCl, when the K^+ concentration was increased from 0 mM to 25 mM, the CFG- and CFG–BSA-conjugate-stabilized droplets became significantly less negatively charged. This was probably caused by electrostatic screening and ion binding. However, the K^+ concentration did not have much influence on the mean droplet size of the emulsion stabilized with CFG–BSA conjugates, suggesting that steric interactions are predominant in these emulsions.

3.4.3. *Influence of freezing and ionic strength on emulsion stability*

The mean droplet sizes and distributions of the emulsions containing CFG–BSA-conjugate-coated, CFG-coated, and BSA-coated droplets after one freeze–thaw cycle (−20 °C) at K^+ concentrations

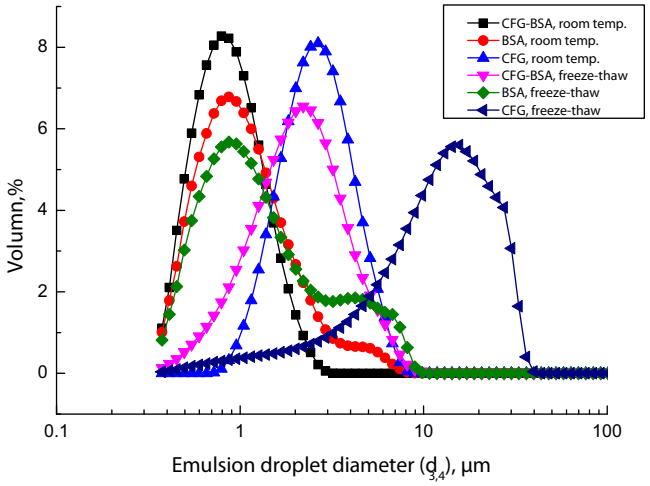


Fig. 6. Droplet distribution of emulsions containing [CFG–BSA conjugates]-coated, [CFG]-coated and [BSA]-coated droplets after one freeze–thaw cycle (−20 °C) and stored at room temperature in the absence of potassium chloride at pH 4.0.

Table 2
zeta potential of emulsions containing [CFG–BSA conjugates]-coated, [CFG]-coated and [BSA]-coated droplets at the pH ranging from 4.0 to 8.0 and potassium concentrations ranging from 0 to 300 mM.

Emulsifier	pH	K+ concentration, mM				
		0	25	50	100	300
CFG-BSA	4.0	−18.3 ± 1.1	−7.2 ± 0.1	−5.5 ± 0.3	−5.0 ± 0.8	−1.2 ± 0.9
	5.0	−24.0 ± 0.1	−8.0 ± 0.2	−6.0 ± 0.2	−5.4 ± 0.1	−4.5 ± 0.1
	6.0	−24.9 ± 1.3	−8.3 ± 0.6	−6.4 ± 0.6	−6.1 ± 0.2	−4.8 ± 1.4
	7.0	−23.4 ± 0.3	−8.8 ± 1.2	−6.0 ± 0.1	−5.0 ± 0.3	−3.4 ± 1.7
	8.0	−24.5 ± 1.5	−8.4 ± 0.2	−6.3 ± 0.1	−5.3 ± 0.4	−5.0 ± 0.9
BSA	4.0	17.1 ± 1.0	13.4 ± 1.7	12.6 ± 0.7	11.2 ± 0.6	11.8 ± 1.8
	5.0	−20.0 ± 0.2	−18.0 ± 0.1	−17.4 ± 0.2	−10.0 ± 0.2	−10.1 ± 0.1
	6.0	−52.3 ± 1.8	−30.8 ± 0.8	−23.8 ± 1.6	−19.3 ± 0.5	−11.2 ± 2.3
	7.0	−37.3 ± 0.1	−24.0 ± 0.4	−23.7 ± 0.8	−17.3 ± 1.1	−13.1 ± 2.1
	8.0	−25.9 ± 1.3	−22.5 ± 1.7	−17.0 ± 0.5	−11.1 ± 0.1	−11.4 ± 2.2
CFG	4.0	−14.4 ± 0.4	−8.0 ± 0.5	−7.5 ± 0.3	−6.0 ± 0.4	−4.4 ± 0.2
	5.0	−15.0 ± 0.2	−8.5 ± 0.2	−7.2 ± 0.2	−5.5 ± 0.1	−4.0 ± 0.1
	6.0	−15.7 ± 0.2	−8.6 ± 0.2	−6.7 ± 1.2	−5.7 ± 1.0	−3.5 ± 0.3
	7.0	−15.5 ± 0.7	−8.8 ± 0.3	−7.0 ± 0.1	−5.1 ± 0.5	−1.2 ± 0.1
	8.0	−15.9 ± 0.1	−9.3 ± 1.0	−6.7 ± 0.4	−4.5 ± 0.2	−1.3 ± 0.1
CFG-CFG	4.0	−19.1 ± 0.6	−6.5 ± 0.1	−5.1 ± 1.0	−5.6 ± 0.5	−3.2 ± 0.1
	5.0	−20.1 ± 0.1	−7.0 ± 0.1	−6.0 ± 0.1	−5.0 ± 0.1	−3.5 ± 0.1
	6.0	−25.3 ± 1.3	−8.6 ± 0.3	−6.0 ± 0.8	−5.4 ± 0.8	−4.8 ± 1.7
	7.0	−22.4 ± 0.1	−8.6 ± 0.7	−6.1 ± 0.5	−6.3 ± 0.5	−1.2 ± 0.1
	8.0	−24.3 ± 0.6	−9.2 ± 1.5	−6.3 ± 0.1	−6.1 ± 0.1	−1.2 ± 0.3

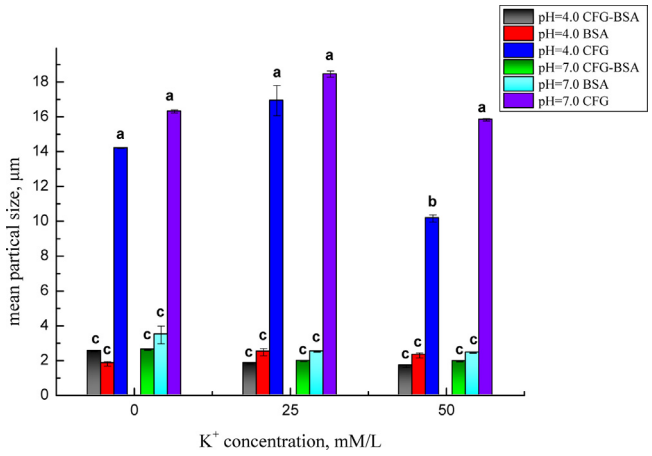


Fig. 7. Mean droplet size of emulsions containing [CFG–BSA conjugates]-coated, [CFG]-coated and [BSA]-coated droplets after one freeze–thaw cycle (−20 °C) under potassium concentrations ranging from 0 to 50 mM at pH 4.0 and 7.0, respectively. Different letters (a, b, c) indicate statistically significant difference between emulsions stabilized with different emulsifiers, i.e., CFG–BSA (CFG, BSA) under different pH and K^+ concentration ($P < 0.05$).

of 0–50 mM at pH 4.0 and 7.0 are shown in Figs. 6 and 7. All the emulsions were unstable under freeze–thaw cycling, and the mean droplet sizes increased. Fig. 6 shows that the droplet distributions of the emulsions stabilized with BSA and CFG had bimodal peaks, indicating that flocculation and coalescence could occur in BSA- and CFG-stabilized emulsions. But the droplet distribution of emulsions stabilized with CF–BSA conjugates remain stable after freeze–thaw treatment.

Fig. 7 shows that the mean droplet sizes of emulsions stabilized with CFG–BSA conjugates and BSA were relatively stable, whereas the droplet size of the emulsion stabilized with CFG increased significantly. This can be explained by two mechanisms. First, proteins are more stable than carbohydrates under freezing conditions, probably because of the relatively thick interfacial layer and its ability to increase the force separating the droplets from expanding ice (Ghosh, Cramp, & Coupland, 2005). The covalently bonded BSA fraction makes the CFG–BSA layer thicker than that of CFG alone, so it is harder for water or oil crystals to rupture the interface (Aoki, Decker, & McClements, 2005). Secondly, as stated above, the CFG–BSA conjugates were more resistant to high ionic strength; so, when water crystallizes and the ionic strength increases, CFG–BSA can still form sufficient steric interactions around the oil droplets.

4. Conclusions

Polysaccharide–protein conjugation using peroxidase is easily achieved. The conjugates formed have promising applications in the food industry. In this study, CFG was covalently bonded to a globular protein, i.e., BSA, using peroxidase, at a high CFG to BSA weight ratio. The conjugates were used to prepare oil-in-water emulsions and were found to have better emulsifying abilities than CFG or BSA alone over a wide pH range, at high ionic strength, and in freeze–thaw treatments. Future studies will focus on increasing the yields of intermolecular CFG–BSA conjugates and broadening their applications in the food industry.

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