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Corn fiber gum and milk protein conjugates with improved emulsion stability☆

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ABSTRACT

Corn fiber gum (CFG), an alkaline hydrogen peroxide extract of the corn kernel milling by-product "corn fiber" was covalently conjugated with β-lactoglobulin (β-LG) and whey protein isolate (WPI). Covalent coupling of CFG to protein was achieved by dry heating reaction (Maillard-type) of CFG and protein mixtures (3:1) for up to 7 days at 75 °C and 79% relative humidity. The formation of the CFG and protein conjugate was confirmed by SDS–polyacrylamide gel electrophoresis. The conjugates as well as pure proteins and pure CFG were compared for their ability to stabilize oil-in-water (O/W) emulsions with 5% and 10% orange oil by turbidimetric and Dynamic Light Scattering (DLS) methods respectively. Measurements of turbidities, particles size distribution and average particle size in emulsions have shown that CFG–protein conjugates prepared by dry heating at 75 °C and 79% relative humidity for up to 2 days were more effective at stabilizing emulsions at room temperature than was CFG or protein alone.

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1. Introduction

It is well known that both polysaccharides and proteins play an important role in the formulation of food emulsions. Proteins are effective emulsifying agents and stabilizers of oil-in-water emulsions when they dissolve well in aqueous solution creating a suitable environment for steric and electrostatic stabilization (Akhtar & Dickinson, 2007). It has been well studied that protein and lipid present on hydrocolloids play very important roles in their emulsification properties (Yadav, Johnston, Hicks, & Nothnagel, 2006; Yadav & Nothnagel, 2007; Yadav, Moreau, & Hicks, 2007). The emulsifying behavior of proteins decreases if their solubility decreases due to aggregation or precipitation during food processing. Corn fiber gum (arabinoxylan, also called hemicellulose B) isolated from the fibrous portions (pericarp, tip cap, and endosperm cell wall fractions) of corn kernels by alkaline solution, often in the presence of hydrogen peroxide is an unique polysaccharide with low solution viscosity that has been proposed as a good stabilizer for oil-in-water emulsions (Whistler, 1993; Woo, 2001; Yadav et al., 2006; Yadav, Fishman, Chau, Johnston, & Hicks, 2007; Yadav, Johnston, & Hicks, 2007, Yadav, Johnston, Hotchkiss, & Hicks, 2007; Yadav, Johnston, & Hicks, 2008). As a result of recent studies it is now well-recognized that the solubility and functional properties of proteins can be improved by their conjugation to polysaccharides (Akhtar & Dickinson, 2003, 2007; Dunlap & Cote, 2005; Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005; Jimenez-Castano, Villamiel, & Lopez-Fandino, 2007).

Whey protein is the name for a collection of globular proteins such as β-lactoglobulin (~65%), α-lactalbumin (~25%), bovine serum albumin (~8%) and immunoglobulins (~1%). It is rich in essential amino acids which exhibit many functionalities and is used in a wide variety of food products (Foegeding, Davis, Doucet, & McGuffey, 2002). Whey protein isolates (WPI) and its main constituent β-lactoglobulin (β-LG) are good emulsifiers but their emulsifying properties change due to pH, salt and heating condition. β-LG has a good emulsifying ability in the alkaline pH range but it loses such ability in the acidic pH range (Shimizu, Saito, & Yamauchi, 1985). In general protein stabilized emulsions are destabilized in the presence of salt and on heating (Hattori & Takahashi, 1993). Many attempts were carried out to improve the emulsifying properties of protein by its conjugation with sugar. The emulsifying properties of β-LG are improved by its covalent conjugation with glucose (Waniska & Kinsella, 1988). The emulsion stability of ovalbumin is greatly enhanced by its conjugation with dextran and glucose (Kato, Sasaki, Furuta, & Kobayashi, 1990). But conjugation of protein with polysaccharides in comparison to mono- or disaccharides leads to a significant improvement in its emulsifying properties and heat stability (Akhtar & Dickinson, 2003; Dickinson & Semenova, 1992; Dunlap & Cote, 2005; Kato et al.,

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1990). The protein–polysaccharide conjugates are typically formed by a dry-heating reaction without any chemical catalyst at controlled humidity via Maillard-type conjugation (Oliver, Melton, & Stanley, 2006). The Maillard reaction starts with the condensation between the carbonyl group of a reducing end sugar and an unprotonated amino group (mainly ε-amino group of lysine residue in protein) leading to the Schiff base after releasing a molecule of water. The Schiff base subsequently undergoes an irreversible Amadori rearrangement to produce an open chain Amadori compound, 1-amino-1-deoxy-2-ketose. This Amadori compound does not undergo further rearrangement, if the starting reducing end sugar is a five carbon molecule (pentose). But if the starting reducing end sugar is a six carbon molecule (hexose), the oxygen atom from the C-6 hydroxyl group attacks the C-2 carbonyl group closing the ring to produce a cyclic 1-amino-1-deoxy-D-fructopyranose compound. Under various reaction conditions, Amadori compounds may undergo several irreversible degradation reactions, giving many colored and water insoluble poorly characterized compounds called melanoidins. Thus, the Maillard reaction needs to be performed under strictly controlled conditions to produce the desired carbohydrate–protein conjugate and to prevent later stage melanoidins formation (Oliver et al., 2006). The resulting high molecular weight glycoprotein conjugate is believed to enhance emulsion stability by combining the characteristic hydrophobic property of proteins to strongly adsorb at oil–water interfaces and the typical hydrophilic property of polysaccharide to dissolve it well in an aqueous medium (Dickinson & Galazka, 1991). Such CFG–protein conjugate can easily combine the excellent emulsification properties of the protein and the emulsion stabilizing effect of CFG making it very good emulsifier. The macromolecular conjugates make a thick layer around the oil droplets and stabilize them against creaming, flocculation and coalescence by steric repulsion of high molecular weight polysaccharides.

The corn fiber gum isolated from corn bran (a by-product of corn dry milling) contains very little protein and is an inferior emulsifier for oil-in-water emulsion systems compared to corn fiber gum isolated from corn fiber derived from the corn wet-milling process (Yadav, Johnston, Hotchkiss, et al., 2007). The aim of the present study was to determine the conditions to convert protein deficient CFG into covalently linked CFG–protein conjugates by a natural, food grade dry-heating reaction (Akhtar & Dickinson, 2003; Kato, 2002) for the purpose of making a superior emulsifier. Such natural dry heating reactions are common in many food materials and are also called non-enzymatic browning reactions. The study also aims to demonstrate the ability of such polysaccharide–protein conjugates to stabilize complex emulsions prepared at low pH and high ionic strength commonly used in many food products (Akhtar & Dickinson, 2003; Martínez, Riscardo, & Franco, 2007). For the purpose of increasing commercial potential, the conjugate emulsifier has been prepared from CFG, a low value by-product of corn milling and whey protein isolates, a by-product of cheese processing, respectively.

2. Materials and methods

2.1. Materials

The source of the corn "fiber" was corn bran produced by the corn dry milling process and kindly provided by Bunge (North America, Bunge Milling, Inc., St. Louis, MO). It was used for CFG preparation after removing oil by hexane extraction (Moreau, Powell, & Hicks, 1996) and starch by treating with termamyl α-amylase (Doner, Chau, Fishman, & Hicks, 1998). β-Lactoglobulin (β-LG) and whey proteins isolate, BiPro (WPI) were Davisco Food International, Inc. products. They had been produced from fresh sweet whey and concentrated and spray dried into a homogeneous, semi-hygroscopic, lactose free-powder. WPI contains ~65% of β-LG and is a by-product of cheese manufacturing from cow's milk. Sodium benzoate, sodium citrate, sodium nitrate and potassium bromide were purchased from Sigma–Aldrich (St. Louis, MO). Cold-pressed Valencia orange oil was supplied by Citrus and Allied Essences Ltd., Belcamp, MD. The Polytron homogenizer (Model PT 10/35) was purchased from Brinkmann, Westbury, NY and the EmulsiFlex-B3 high-pressure homogenizer was purchased from Avestin Inc. (Ottawa, Canada).

2.2. Isolation and purification of corn fiber gum

Corn fiber (bran) obtained from the dry milling industry contains fiber mostly from the pericarp portion of corn kernel and so is also called "corn pericarp fiber". CFG was isolated from de-oiled and de-starched corn bran according to the alkaline hydrogen peroxide technology (Doner et al., 1998). In brief, de-oiled and de-starched corn fiber was mechanically stirred in a mixture of 0.1 M NaOH and 0.05 M $Ca(OH)_2$ solution containing 1 meq each of them per gram of fiber in the extraction medium and boiled for 1 h. The residue obtained after centrifugation was resuspended in water, boiled for 5 min and centrifuged again. The combined supernatant was treated with H_2O_2 at pH 11.5 stirring at room temperature for 2 h and then pH was adjusted to 4.0–4.5 to precipitate hemicellulose A (Hemi. A). The supernatant was treated with two volumes of ethanol to precipitate hemicellulose B (Hemi. B), which is also called CFG. The precipitate was collected and dried in a vacuum oven at 50 °C.

The pericarp-derived CFG contains a low amount of nitrogen containing compound or peptide (~0.4%, Yadav, Johnston, Hotchkiss, et al., 2007) which was removed by dialyzing it against water using 12–14,000 MW cut-off dialysis tubing. The protein ($N \times 6.25$) content of CFG before (0.44%) and after dialysis (0.0%) was determined using an approved method of the "American Association of Cereal Chemists" (1995).

2.3. Preparation of CFG–protein conjugates

The dry-heating reactions of CFG and proteins were conducted at their 3:1 weight ratio at 75 °C and 79% relative humidity, conditions suitable for high molecular weight polysaccharide conjugation with protein (Akhtar & Dickinson, 2003, 2007; Dunlap & Cote, 2005). The preliminary conjugation reaction of CFG with each protein (β-LG and WPI) was done by incubating their mixtures for 4 days under the above-mentioned conditions. The covalent coupling of CFG and protein on dry heat treatment was monitored and confirmed by SDS–PAGE by visualizing the characteristic protein bands with Coomassie R350 dye (blue). This preliminary experiment showed a high degree of coupling between CFG and protein at 3:1 weight ratio. It is well known that β-LG has about 10% of lysine of the total amino acids (Block & Weiss, 1955; Stein & Moore, 1949), which provides a considerable number of accessible lysine ε-amino group (Gonzáles, Naranjo, Malec, & Vigo, 2003) to react with reducing end of CFG. After observing the preliminary experiment results, CFG and protein (β-LG or WPI) were mixed in a weight ratio of 3:1 and ground to bring them into good contact. The dry mixture was dissolved in deionized water and freeze-dried. The lyophilized product was further ground to make a white powder. The covalent coupling between the carbonyl group of arabinoxylan (CFG) reducing end and an available unprotected amino group of protein, mainly the ε-amino group of lysine residues or the N-terminal amino group was achieved by placing their lyophilized white powder mixture in a pre-heated desiccators at 75 °C at a relative humidity of 79% (saturated KBr solution). CFG, β-LG, and WPI were also incubated individually under the same conditions as con-

trols. Samples were taken out after 2 h, 1 day, 2 days, 3 days, 4 days and 7 days and their color change was noted. They were kept in a desiccator in the freezer until used. The resultant glycoprotein conjugates were analyzed by SDS–PAGE to confirm CFG–protein conjugation and their emulsifying properties were studied.

2.4. SDS–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of samples was carried out on a Pharmacia Phast System (Piscataway, NJ) with a phast gel of 20% acrylamide. Dried samples were solubilized in 200 μL of protein solvent system (0.44 M Tris, 1 mM EDTA, 10% SDS, pH 8.0) plus 40 μL of 2-mercaptoethanol (2-ME), and the mixtures were heated at 100 °C for 10 min. Gels were stained for protein with 0.2% (w/v) Coomassie R350 dye. Molecular weight standards (Invitrogen Corp., Carlsbad, CA) and their corresponding molecular weights were as follows: myosin 188 kDa; phosphorylase 98 kDa; bovine serum albumin (BSA) 62 kDa; glutamic dehydrogenase 49 kDa; alcohol dehydrogenase 38 kDa; carbonic anhydrase 28 kDa; myoglobin red 17 kDa; lysozyme 14 kDa; aprotinin 6 kDa and insulin, B chain 3 kDa.

2.5. Emulsion preparation

CFG stock solution (10.53 mg CFG/g solution) containing 0.1% (w/w) sodium benzoate (a preservative) and 0.3% (w/w) citric acid was prepared for emulsification studies by slowly adding the calculated amount of CFG sample a little at a time with vigorous stirring to a solution of sodium benzoate and citric acid in water at room temperature and then gently stirring overnight to produce a hydrated, well dissolved and homogeneous solution. The samples for oil-in-water emulsions were prepared in triplicate for each sample in 1:5 emulsifier to oil weight ratio by taking 2.375 g of above CFG stock solution (25.00 mg CFG) and 5% (125 mg) of Valencia orange oil (total 2.5 g solution) in a glass vial. No weighting agent was added during emulsion preparation to avoid the effects of such agent on the emulsification process. The solution was vortexed and then homogenized using a polytron bench top homogenizer equipped with a 12 mm diameter head (Brinkmann, Switzerland, PT 10/35) at 20,000 rpm for 1 min. The above homogenized emulsion was passed through the EmulsiFlex-B3 high-pressure homogenizer (Avestin Inc., Canada) at 10,000 psi homogenization pressure 3 times to prepare the final emulsion concentrate.

2.6. Emulsion turbidity measurements

The emulsion concentrate was diluted 31.25× to 78.125 g in a 10.0% (w/w) sucrose solution containing 0.1% (w/w) sodium benzoate and 0.3% (w/w) citric acid and the emulsion stability (ES) evaluation was done by turbidity measurement (Pearce & Kinsella, 1978) with some modification as we explained in our previous article (Yadav, Johnston, Hotchkiss, et al., 2007). Observed turbidity was measured according to the following equation, $T = 2.303AD/l$, where T = turbidity in 1/cm, A = observed absorbance at 650 nm, D = dilution factor, and l = path length of the cuvette in cm. The emulsion stability was determined by absorbance (loss of turbidity) measurement of the emulsion at 650 nm using a UV-1700 Spectrophotometer (Shimadzu, Columbia, MA) against 10.0% sugar solution containing 0.1% sodium benzoate and 0.3% citric acid after 1 and 7 days of emulsion preparation.

2.7. Emulsion droplets distribution and size measurements

For oil droplet distribution measurements, emulsions were prepared in 0.2 M citrate buffer (pH 3.2). The aqueous citrate buffer (ionic strength 0.2 M) was prepared by mixing 0.2 M citric acid and 0.2 M sodium citrate to obtain pH 3.2 and 0.02% sodium benzoate was added as microbial agent. Corn fiber gum, proteins and their conjugates solutions were prepared by slowly adding their calculated amount, a little at a time with vigorous stirring to citrate buffer at room temperature (ca. 22 °C) and then gently stirring overnight to produce a hydrated homogeneous solution. The reported pH value refers to the sample solution pH value before emulsification. Oil-in-water emulsions (10 wt.% orange oil and 1% emulsifier in 0.2 M citrate buffer at pH 3.2) were prepared by first homogenization using a polytron bench top homogenizer and then homogenizing by passing through the EmulsiFlex-B3 high-pressure homogenizer at 10,000 psi as explained in Section 2.5. The emulsions were stored at room temperature for the duration of the experiment. At a given time interval, the emulsion was mixed and a 100 μL aliquot from it was diluted into 5 mL 0.1% SDS (Dunlap & Cote, 2005) in 0.2 M citrate buffer, pH 3.2 and characterized by Dynamic Light Scattering (DLS) method using a Nicomp 370 Submicron Particle Sizer (Particle Sizing System, Inc., Santa Barbara, CA). The instrument parameters were set as follows: control menu-channel width, auto set; liquid viscosity, 0.933 cP; liquid index of refraction, 1.333; intensity set point, 300 kHz. The emulsifying ability was assessed by the particles poly-dispersion and the value of the volume-weighted average droplet diameters. The average droplet size was measured at 0 time and after storage at room temperature for 3 h, 1 day and 7 days.

2.8. Statistical analysis

All reported data are an average of three with standard deviation. The data in Figs. 4 and 6 has been analyzed by performing analysis of variance (ANOVA) to determine if there were any significant effects due to CFG–protein conjugation on emulsions turbidity and particle size on various periods of heating and storage. Analysis of variance is a generalization of a t-test when there are more than two things to be compared. The comparison of means was performed using the Bonferroni least significant difference (LSD) method, which were different at the $p = 0.05$ significance level (Miller, 1981).

3. Results and discussion

3.1. CFG–protein conjugates

The SDS–PAGE pattern of CFG, β-LG, WPI and their conjugates from 2 h, 1 day, 2 days, 3 days, 4 days and 7 days of dry-heating are shown in Fig. 1. After 2 h incubation, the formation of high molecular weight component was not seen (Fig. 1A). But a single band of high molecular weight conjugate appeared near the boundary between the stacking and separating gels in the lanes containing 1 day incubated CFG-β-LG and CFG-WPI mixtures in addition to the unreacted protein bands (Fig. 1B, lanes 2 and 3). The characteristic bands of starting protein in 1 day incubated CFG–protein conjugation mixture have not disappeared completely indicating that all protein has not reacted to CFG under the reaction conditions employed. As the conjugation reaction proceeded for a longer than 1 day incubation, a gradual decrease in the protein bands with increasing incubation time (2 days, 3 days, 4 days and 7 days, Fig. 1C–F) was seen. It is clear from the gel pattern that 7 days dry-heating was required for disappearance of all protein (β-LG or WPI) bands (lanes 2 and 3, Fig. 1F) indicating completion of conjugation reaction. But when the dry-heating reaction was continued for more than 2 days, the resulting conjugation products became less soluble in aqueous medium indicating that more than 2 days heating was not desirable. Good solubility of CFG–protein conjugates in water is important for their application in making emulsions. The

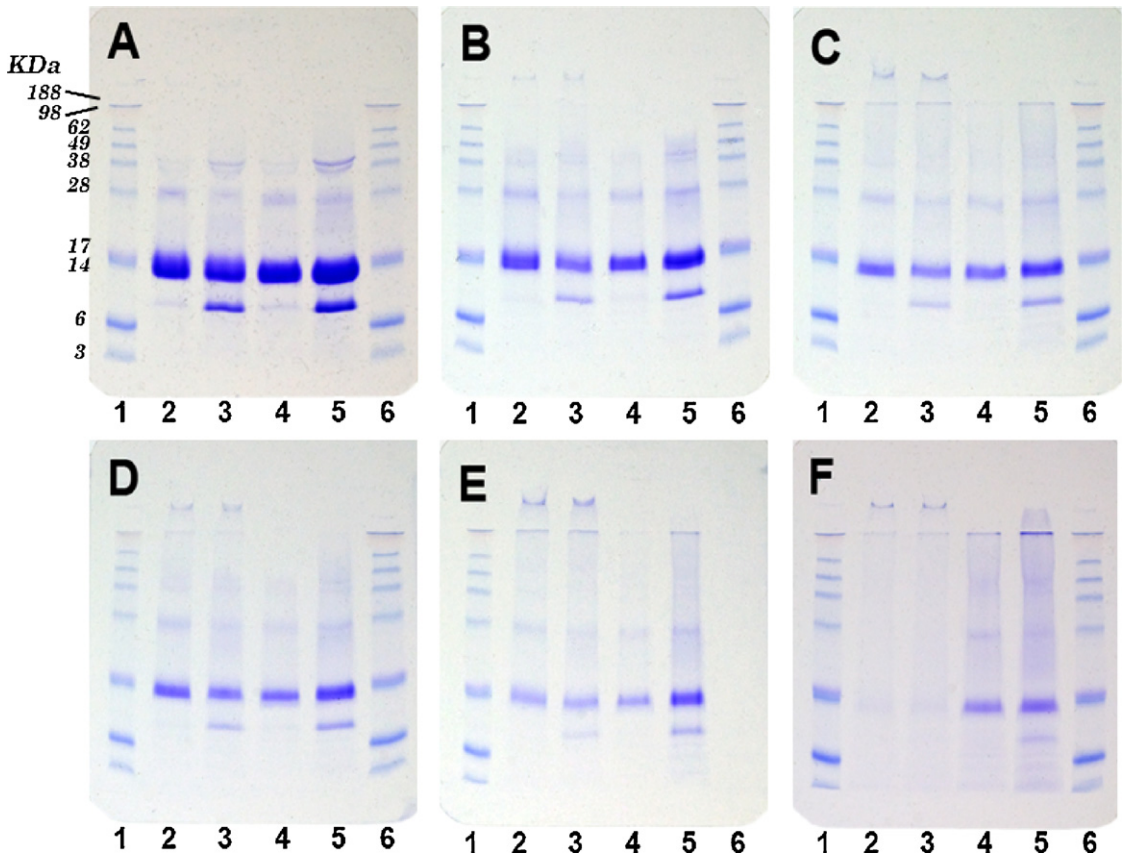

Fig. 1. SDS–polyacrylamide gel electrophoresis of β-LG, WPI and their conjugates with CFG. Lane 1, protein molecular weight standards; lane 2, β-LG-CFG conjugate; lane 3, WPI-CFG conjugate; lane 4, β-LG; lane 5, WPI and lane 6, protein molecular weight standards. (A) 2 h, (B) 1 day, (C) 2 days, (D) 3 days, (E) 4 days and (F) 7 days of dry-heating as described in Section 2.

main reason for their low solubility in water may be due to some irreversible degradation reactions of the intermediate "Amadori compounds" on dry heating for longer than 2 days as explained in detail in Section 1. Thus after finding the right reaction conditions (time and temperature), the dry-heating reaction was conducted on a large scale, 2 g sample (1.5 g CFG and 0.5 g protein) for 1 day at 75 °C and 79% relative humidity to make enough CFG–protein conjugates for the study of their functional properties. On visualizing the SDS–PAGE (Fig. 2) for large scale 1 day heated reaction mixture, the formation of higher molecular weight conjugate was confirmed by Coomassie blue (Fig. 2, lanes 3 and 4). The gel pattern definitely confirms that β-LG or WPI complexed with CFG forming conjugates of higher molecular weight.

The slow reaction between CFG and protein is probably due to the high molecular weight and high branched structure of CFG (~290 kDa, Yadav, Fishman, et al, 2007), which is too bulky for rapid binding to the protein molecule. A high temperature (75 °C) heating for more than 2 days is undesirable as it causes some structural rearrangement affecting the solubility and functionality of the conjugates. The possible changes could also be an unfolding of the molecules and functional groups orientation or a heat induced aggregation as well as random coil formation and a loss of solubility. Both β-LG and WPI were also incubated separately along with their mixtures with CFG to test any change in their functionality due to heating. For other systems (e.g. whey protein-dextran, milk protein-pectin, lysozyme or casein-dextran), the formation of a covalent linkage on dry-heating of protein–polysaccharide mixtures has been demonstrated and verified by using SDS–PAGE analysis (Aminlari, Ramezani, & Jadidi, 2005; Dunlap & Cote, 2005; Einhorn-Stoll et al., 2005; Jimenez-Castano et al., 2007).

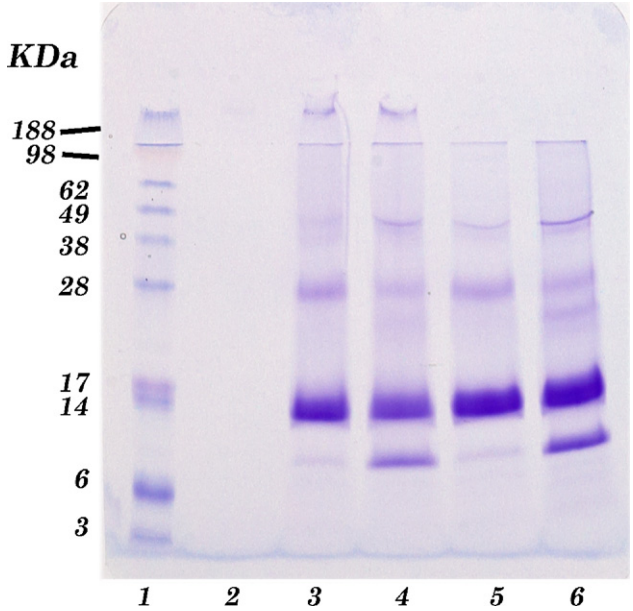

Fig. 2. SDS–polyacrylamide gel electrophoresis of 1 day dry-heated β-LG, WPI and their conjugates with CFG (large scale preparation). Lane 1, protein molecular weight standards; lane 2, CFG; lane 3, β-LG-CFG conjugate; lane 4, WPI-CFG conjugate; lane 5, β-LG; and lane 6, WPI.

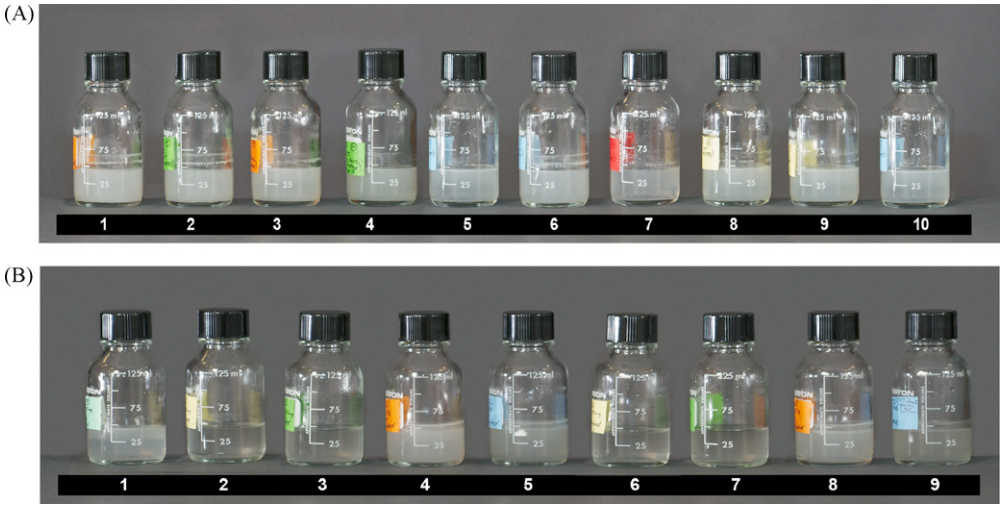

Fig. 3. Bottles containing emulsions (5% orange oil and 1% emulsifier, w/w) at room temperature showing emulsion breakage after 1 week. The bottles are labeled as follows: (A) 1 Day dry-heated samples: (1) CFG (2) β-LG (3) WPI (4) CFG-β-LG conjugate and (5) CFG-WPI conjugate; 2 days dry-heated samples (6) CFG (7) β-LG (8) WPI (9) CFG-β-LG conjugate and (10) CFG-WPI conjugate. (B) 4 Days dry-heated samples (1) CFG (2) β-LG (3) WPI (4) CFG-β-LG conjugate and (5) CFG-WPI conjugate; 7 days dry-heated samples (6) β-LG (7) WPI (8) CFG-β-LG conjugate and (9) CFG-WPI conjugate. During emulsion breakage, the small oil droplets form aggregates and float at the top making the lower part of the emulsion clear. The breakdown of the emulsion is slow in the bottles containing 1 and 2 days dry-heated conjugates. The breakdown of the emulsion is the fastest in the bottles containing 4 and 7 days dry-heated β-LG and WPI but moderate with their 4 and 7 days dry-heated conjugates with CFG.

3.2. Emulsion stability measurements

3.2.1. Turbidimetric method

The diluted emulsions prepared with dry-heated CFG, β-LG, WPI and their conjugates (bottles 1–5 for 1 day and 6–10 for 2 days heated samples) are shown in Fig. 3A. There was no remarkably visible difference in the cloudiness between 1 and 2 days heated samples showing that up to 2 days heating does not have any negative effect on the emulsion cloudiness. But when the diluted emulsions were made with 4 and 7 days heated CFG, proteins and their conjugates, both proteins (β-LG and WPI) lose their emulsions stabilizing properties (bottles 2–3 for 4 days and 6–7 for 7 days heated proteins, Fig. 3B). CFG also loses some of its cloudy emulsions stabilizing ability on heating for 4 days (bottle 1, Fig. 3B) but not as much as 4 and 7 days heated proteins. The CFG and protein conjugates (bottles 4–5 for 4 days and 8–9 for 7 days heated

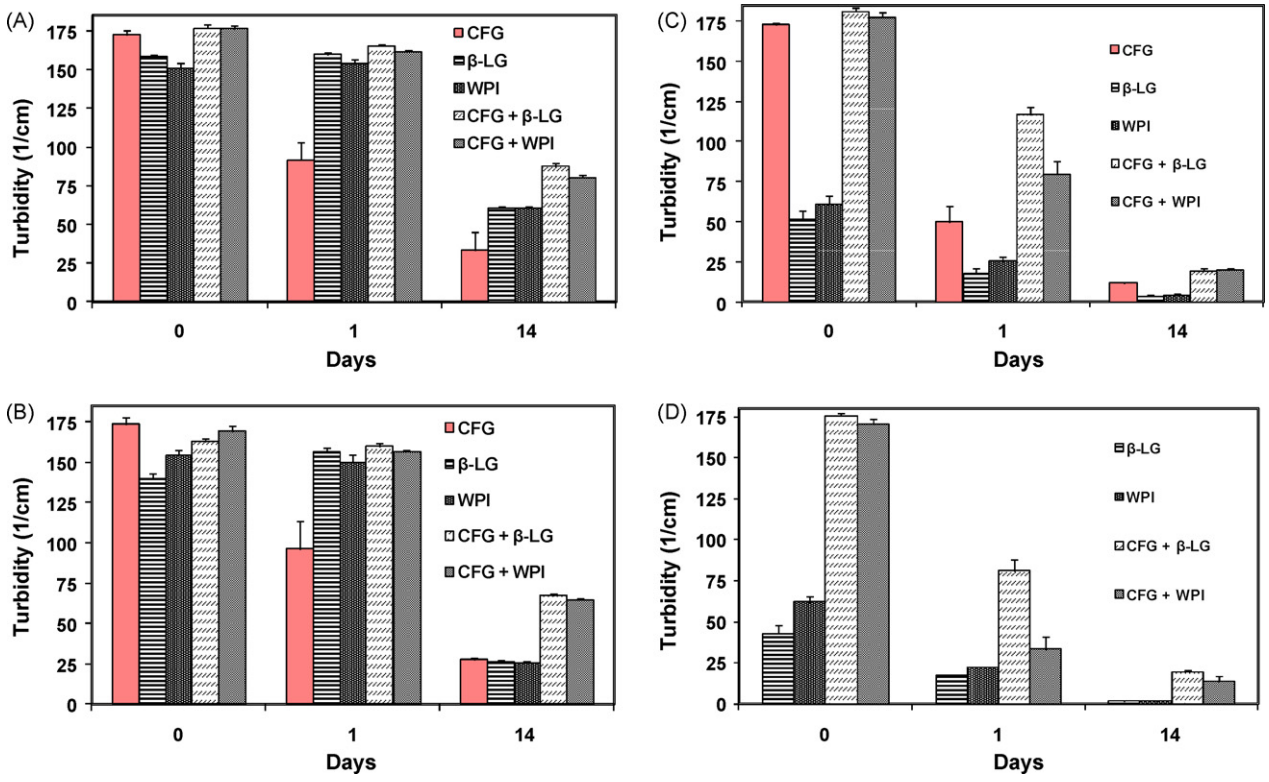

Fig. 4. Comparison of emulsion stabilities (turbidities) of orange oil (5% oil and 1% emulsifier, w/w) at room temperature stabilized with (A) 1, (B) 2, (C) 4, and (D) 7 days dry-heated CFG, β-LG, WPI and their conjugates. Turbidities were measured by taking an aliquot from the middle of the diluted emulsion immediately after making emulsions and after 1 and 14 days. Higher turbidity is an indication of greater emulsion stability. Data are an average of three trials ± standard deviation.

conjugates, Fig. 3B) lose some of their emulsions stabilizing abilities in comparison to their 1 and 2 days heated conjugates (bottles 4–5 for 1 day and 9–10 for 2 days heated conjugates, Fig. 3A) but they have better emulsion stabilizing capacity than the unconjugated CFG or protein alone. This visual observation clearly indicates that CFG–protein conjugates make more stable cloudy emulsions than the individual unconjugated CFG or protein when they are incubated in dry condition. When the dry-heating reaction is continued for more than 2 days, probably they rearrange causing changes in their structures and functional properties. During the sample preparation for emulsification studies, it was noticed that 4 and 7 days heated proteins and their CFG conjugates were less soluble in water than 1 or 2 days heated respective samples.

The stabilities of diluted emulsions prepared with CFG, β-LG, WPI and their conjugates heated for 1, 2 and 4 days are shown in Fig. 4A–C respectively while Fig. 4D shows emulsions stability of only β-LG, WPI, CFG-β-LG conjugate and CFG-WPI conjugate heated for 7 days. In this experiment, higher turbidity is an indication of a better emulsion stability. The emulsions prepared with 1 day and 2 days heated proteins and their conjugates do not look too much different at 1 day but the emulsion stabilities of CFG conjugates with both β-LG and WPI look superior than the unconjugated CFG or protein after 14 days (Fig. 4A and B). After 14 days of storage at room temperature the turbidities of conjugates are above 50 while the turbidities of individual CFG or protein are dropping down to almost 25. Though the turbidities of 4 days and 7 days heated conjugates look high for 1 day, it drops down close to 10 or below after 14 days (Fig. 4C and D) showing that longer than two days dry heating for conjugation makes some modification in their structures affecting their emulsifying properties.

3.2.2. Droplet distribution and size measurements

First the particles size distribution in emulsions made with varying concentration of CFG was measured to determine the optimum concentration of emulsifier needed for doing the comparative study of emulsion stabilities of CFG, proteins and their conjugates. The particles size distribution in volume percent of emulsions prepared with 0.2%, 0.5%, 1.0%, 1.5% and 2.0% CFG immediately after making emulsions (0 time) and storing them at room temperature for 1 day is shown in Fig. 5. At 0 time, emulsions made with CFG concentration 0.5–2% shows similar monomodal particle distribution giving maximum particles distribution around 1 μm. But emulsions made with 0.2% CFG were quite unstable showing a shift in particles distribution curve more toward higher diameter (Fig. 5A). So the particles size distribution of emulsions made only with 0.5–2.0% were further studied to assess their stabilities on storage at room temperature (Fig. 5B). The 0.5% CFG stabilized emulsions have relatively large particle size than 1–2% CFG stabilized emulsions and it also shows a bimodal size distribution after 1 day storage. The emulsions prepared with 1–2% CFG show similar curves but decreasing average particle size on increase of concentration after 1-day storage period. The 1% CFG stabilized emulsion has relatively larger particle size than 1.5% and 2% CFG and so there is scope for improvement in its emulsion stability on its conjugation with protein. So 1% emulsifier (CFG, proteins or their conjugates) concentration was chosen for comparing their emulsions stability. There is an increase in particle size of emulsions during storage at room temperature for 1 day (Fig. 5B) shifting distribution curve toward higher droplet diameter. This shift may have been due to individual droplet growth (Ostward ripening) or droplet aggregation (flocculation or coalescence).

The comparative average emulsions particle diameter of CFG, β-LG, WPI and their conjugates immediately after making emulsions and after storage at room temperature up to a week at pH 3.2 is presented in Fig. 6. The CFG and both proteins showed poor emulsion stability under the acidic conditions in terms of the retention of

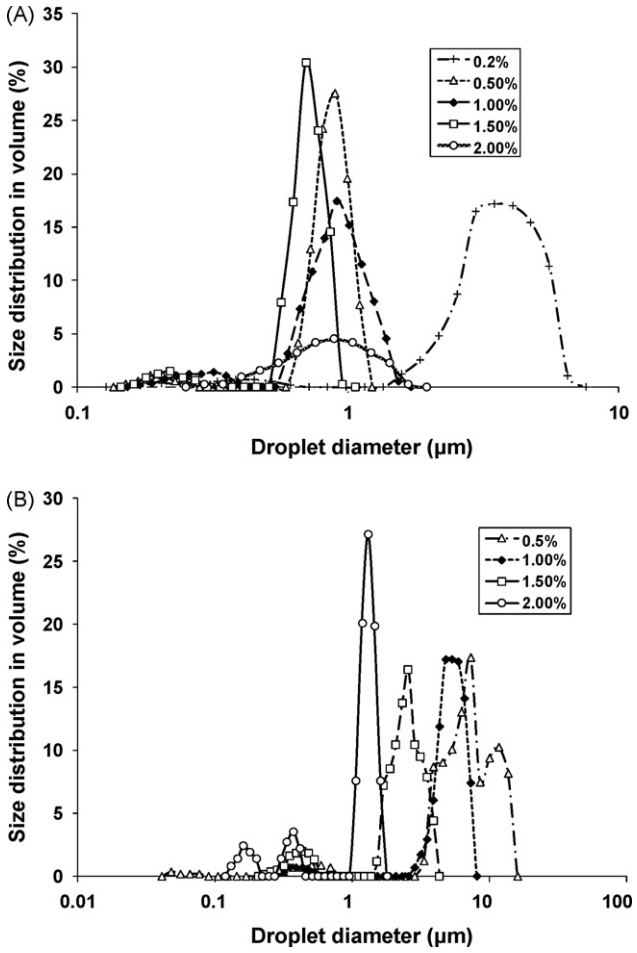

Fig. 5. Droplet distribution curves of emulsions (10% orange oil and 1% emulsifier in 0.2 M citrate buffer, pH 3.2) determined by dynamic light scattering (in volume, %) at room temperature as a function of CFG concentration (0.2–2.0%). (A) Immediately after making emulsions and (B) 1 day storage at room temperature. Droplets size distribution was measured after diluting emulsions 100 times in 0.1% SDS and 0.2 M citrate buffer, pH 3.2. Data are an average of three trials ± standard deviation.

average droplet size during one week storage. CFG-β-LG and CFG-WPI conjugates produced emulsions droplets of smaller size (less than 1 μm) in comparison to unconjugated CFG, β-LG and WPI which produced average droplets of more than 6 μm. The average droplet sizes of CFG, β-LG, and WPI increased steadily from ~1 to 6 μm, 1 to 10 μm and 1.5–10 μm respectively during one week storage. However, emulsification with CFG-β-LG and CFG-WPI conjugates generated smaller droplets (<1 μm) than CFG or protein alone and retained the droplet diameter about 1 μm on extended storage for one week. The maintenance of a constant low droplet size during extended storage of emulsions indicates that both conjugates are very efficient in making stable emulsions under the conditions employed. There was no visible creamy layer separation over the storage period of one week in the emulsions prepared with conjugates unlike emulsions of CFG or protein alone, which showed creamy layer separation in a few days (visual observation). It has been clearly seen that both conjugates were very effective emulsifiers in stabilizing orange oil-in-water emulsions under this acidic condition in the presence of 0.2 M sodium citrate. As mentioned by Dickinson (2009) and Dickinson and Galazka (1991) for other polysaccharides proteins conjugates, the improved emulsion stabilizing ability of CFG–protein complexes might be due to the increased steric and electrostatic repulsion between the bulky hydrophilic polysaccharide (CFG) moiety around the tiny oil droplets, which protect them against flocculation and coalescence.

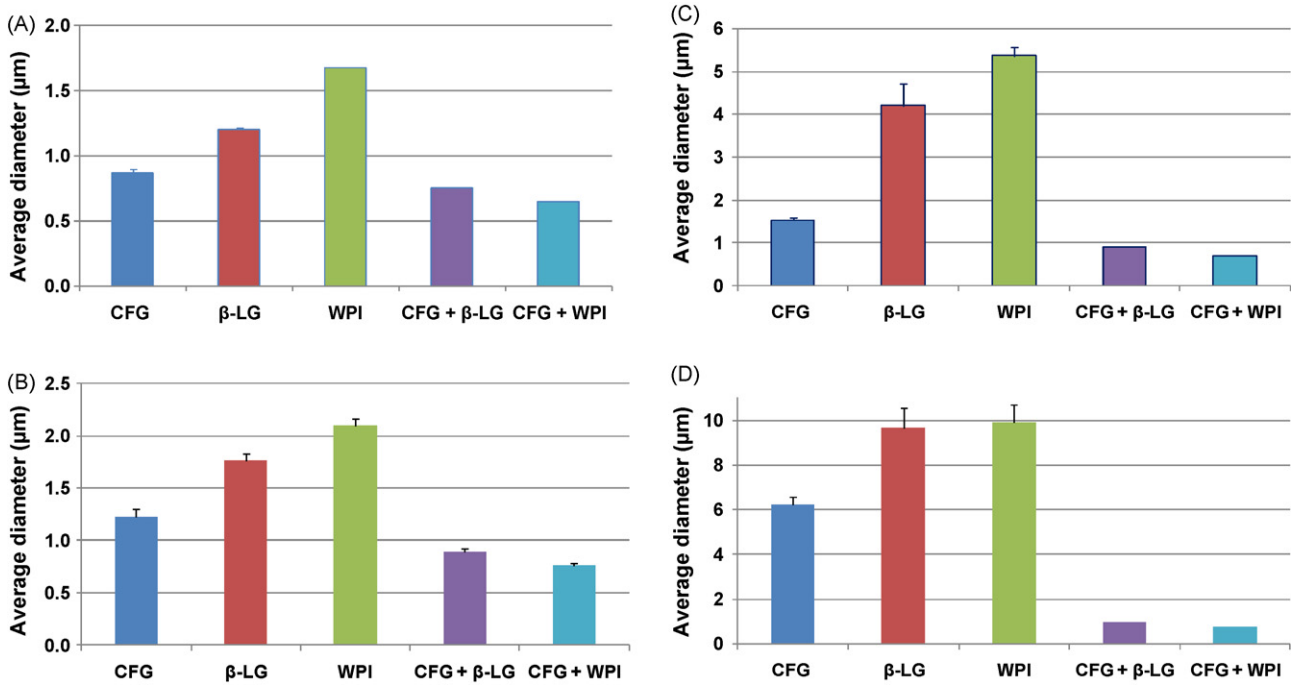

Fig. 6. Comparison of the volume-weighted average droplet diameter of emulsions (10% orange oil and 1% emulsifier w/w, in 0.2 M citrate buffer, pH 3.2) stabilized with 1 day dry-heated CFG, β-LG, WPI, and their conjugates (A) immediately after making emulsions and after storage at room temperature for (B) 3 h, (C) 1 day and (D) 1 week. Data are an average of three trials ± standard deviation.

4. Conclusions

It has been demonstrated that CFG–protein complexes prepared by dry-heated Maillard reaction are capable of producing fine emulsions with a better stability than either CFG or protein alone under high salt concentration and acidic condition. The solubility of proteins improves greatly on conjugation with CFG (data not shown), which may be largely responsible for the improved emulsifying properties of CFG-β-LG and CFG-WPI conjugates in comparison to CFG, β-LG or WPI alone. The improved emulsion stabilizing capacity of CFG–protein conjugate is largely attributed to the adsorption of the hydrophobic protein moiety of the conjugate at the oil–water interface acting as an anchor and stabilizing the oil droplets by steric repulsion of big hydrophilic CFG molecules extended into the aqueous phase.

It has also been found that only one to two days dry-heating at 75 °C and 79% relative humidity was required for conjugate formation. The continuation of dry-heating reaction for longer than two days under the given conditions produced higher percent of conjugates but they were less soluble which decreased their emulsion stabilizing capacities. The glycosylation of protein with CFG under a control condition may also increase its applications due to its increased heat stability at low pH and high salt concentration.

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