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Influence of pH and ι-Carrageenan Concentration on Physicochemical Properties and Stability of β-Lactoglobulin-Stabilized Oil-in-Water Emulsions

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The influence of pH and ι-carrageenan concentration on the properties of β-lactoglobulin (β-Lg)-stabilized oil-in-water emulsions was investigated by measuring the particle charge, particle size distribution, and creaming stability. Emulsions containing droplets stabilized by β-Lg were produced by homogenization, and then, ι-carrageenan was added. At pH 3, the droplet charge did not change for ι-carrageenan concentrations ≤0.1 wt % but decreased rapidly at high concentrations, while the mean particle diameter increased slightly as the ι-carrageenan concentration was increased. These results suggest that the interaction between ι-carrageenan and β-Lg was weak at pH 3 probably because some sulfate groups were protonated ($pK_a = 2$). At pH 4 and pH 5, the droplet charge decreased dramatically as the ι-carrageenan concentration was increased from 0 to 0.15 wt %, but droplet aggregation and creaming occurred in the emulsions, indicating that interfacial complexes between ι-carrageenan and β-Lg could not stabilize the emulsions, probably due to bridging flocculation. At pH 6, the droplet charge in the primary emulsions was negative and became more negative as the ι-carrageenan concentration was increased. The mean particle diameter was relatively small at all ι-carrageenan concentrations, and emulsions were stable to creaming after 1 week of storage. We propose that carrageenan adsorbed to the droplet surfaces and increased the electrostatic repulsion between droplets. At pH 7 and pH 8, the droplet charge did not change as the ι-carrageenan concentration was increased, but these emulsions became unstable to creaming above a critical carrageenan concentration, which was attributed to depletion flocculation.

KEYWORDS: Emulsion; ι-carrageenan; β-lactoglobulin; flocculation; creaming

INTRODUCTION

Many food products can be categorized as oil-in-water (O/W) emulsions, which consist of small lipid droplets dispersed in an aqueous medium, e.g., milk, cream, beverages, dressings, dips, sauces, butters, and desserts (*1*−*3*). In addition, many natural and industrial materials also exist as this type of emulsion, including petrochemicals, cosmetics, and pharmaceuticals (*4*, *5*). Emulsion-based products are easily destabilized during processing and storage because they are thermodynamically unstable systems. Emulsion destabilization may occur through a variety of physicochemical processes, including gravitational separation, flocculation, coalescence, and Ostwald ripening (*2*). Many factors contribute to the destabilization of O/W emulsions during processing and storage, including the specific type of ingredients present, the way that the emulsion was produced, and the environmental conditions that it experiences.

Emulsifiers are commonly used in food emulsion systems to increase their short- and long-term kinetic stability (*3*, *6*). They are surface active ingredients that facilitate the production of small droplets during homogenization by lowering the interfacial tension and improve emulsion stability by forming protective membranes around droplets and/or by generating repulsive forces between droplets (*2*, *6*, *7*). A wide variety of different kinds of synthetic and natural emulsifiers are available for use in foods, including small molecule surfactants, phospholipids, proteins, and polysaccharides. There is a trend within the food industry to replace synthetic emulsifiers with more natural ones, such as proteins and polysaccharides. The most commonly used polysaccharides in food emulsions are gum arabic, modified starch, modified cellulose, galactomannans, and pectin (*1*). However, these molecules are not particularly surface active and/or have to be used in relatively large quantities to make stable emulsions. In addition, gum arabic is a fairly expensive ingredient whose quality can vary considerably from batch-to-batch, and so, there have been many suggestions in the literature for its replacement by other polymeric emulsifiers (*8*, *9*) and chemically modified polymers (*10*, *11*). Various milk proteins, such as β-lactoglobulin (β-Lg), α-lactalbumin, and casein, can be used in small quantities to prepare stable emulsions (*12*),

but the long-term stability of these protein-stabilized emulsions is highly sensitive to pH, ionic strength, and temperature (*13*, *14*). It should be noted that proteins and polysaccharides can also be used as thickening agents to improve emulsion stability via viscosity modification or gelation in the aqueous continuous phase (*15*).

Some food polysaccharides have been shown to improve emulsion properties by forming an interfacial complex with an adsorbed protein layer after homogenization. Partial unfolding of globular proteins may make them more susceptible to interact with polysaccharides in oil droplet interfaces than in aqueous solutions (*16*). Evidence for bovine serum albumin (BSA) + dextran sulfate (*17*), BSA + sodium alginate (*18*), and BSA + ι-carrageenan interactions (*19*) at the oil−water interface have been provided by surface shear rheology, particle electrophoretic mobility, and surface tension measurements, respectively. The strength of protein−polysaccharide interactions at oil droplet interfaces depends on the distribution of ionizable groups on the surface of the protein, the ease of unfolding of the protein's native structure, and the backbone flexibility, charge distribution, and density on the polysaccharide (*17*−*20*). Strong attractive interactions between adsorbed proteins and polysaccharides produce a secondary layer of steric stabilizing polymer around droplets coated with protein in emulsions. However, the protein-coated droplets may be destabilized by bridging or depletion flocculation when charged polysaccharides are absorbing or nonabsorbing polysaccharides, respectively. Carrageenan induced bridging flocculation in BSA-stabilized emulsions at a certain range of ι-carrageenen concentrations at pH 6 (*19*). Protein-stabilized droplets are destabilized by depletion flocculation at relatively low concentrations of xanthan gum (*21*) but restabilized at high concentrations due to the formation of a gellike network of aggregated droplets (*22*).

Recently, we have utilized a technology that enabled us to combine the beneficial attributes of different kinds of emulsifiers to create emulsions with improved long-term stability. At pH 3, an anionic emulsifier (lecithin) was used to form a primary emulsion and then a cationic biopolymer (chitosan) was mixed with it to form a secondary emulsion coated with an emulsifier−biopolymer membrane. This secondary emulsion had a small mean droplet size and good long-term stability, after any flocs formed within it during the mixing process had been disrupted by the application of mechanical energy (*23*, *24*). The secondary emulsions had better stability than the primary emulsions against high calcium concentrations, lipid oxidation, thermal processing, and freeze−thaw cycling (*25*). In this study, we intend to prepare secondary emulsions using a similar approach but using a globular protein (β-Lg) as the emulsifier and a polysaccharide (ι-carrageenan) as the biopolymer. Carrageenans are commonly used as stabilizers, thickeners, and gelling agents in milk-based products (*26*). They have a strong electrolyte character due to their sulfate groups, and there are three major types of carrageenan, which mainly differ in the number of the sulfate groups in the polygalactose backbone (*27*). β-Lg will be used to produce a primary emulsion containing small oil droplets, and then, anionic ι-carrageenan will be added to the system to produce secondary emulsions containing droplets coated with protein−polysaccharide membranes. The specific objective of this study is to investigate the influence of pH and ι-carrageenan concentration on the stability of the primary and secondary emulsions by measuring particle size distribution, ζ-potential, and creaming, so as to determine the range of experimental conditions where ι-carrageenan can be used to improve emulsion stability by interfacial complexation.

MATERIALS AND METHODS

Materials. The food grade ι-carrageenan sample was kindly donated by FMC BioPolymer (Philadelphia, PA). The manufacturers reported that this sample was in almost pure sodium form with a low amount of contamination from other materials (<5%). The powdered β-Lg was obtained from Davisco Foods International (Lot no. JE 001-1-922, Le Sueur, MN). As stated by the suppliers, the β-Lg content of the powder determined by electrophoresis was 98% (the remainder was mostly globulins). Other chemicals used in this study were analytical grade and purchased from the Sigma Chemical Co. (St. Louis, MO). Double-distilled water was used to prepare all solutions and emulsions.

Solution Preparation. An emulsifier solution was prepared by dispersing 1 wt % β-Lg in 5 mM phosphate buffer (pH 7), containing 0.04 wt % NaN_3 (as an antibacterial agent) and stirring for 3 h to ensure complete dispersion. A ι-carrageenan solution was prepared by dispersing 0.3 wt % powdered ι-carrageenan in distilled water and stirring for 3 h to ensure complete dispersion. A series of ι-carrageenan solutions with different pH values (*3*−*8*) were then prepared by adding NaOH or HCl.

Emulsion Preparation. An O/W emulsion was prepared by homogenizing 10 wt % corn oil and 90 wt % aqueous emulsifier solution (1 wt % β-Lg in 5 mM phosphate buffer, pH 7) at room temperature. The oil and emulsifier solution was blended using a high-speed blender for 2 min (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) and passed through a two stage high-pressure value homogenizer three times at 5000 psi (LAB 1000, APV-Gaulin, Wilmington, MA). The pH of the emulsion was adjusted to values ranging from 3 to 8 ($\pm$0.1) using HCl or NaOH solutions. The emulsions were then diluted with different ratios of 0.3 wt % ι-carrageenan solution and 5 mM phosphate buffer (at the same pH as the emulsions) to form a secondary emulsion with a final composition of 5 wt % corn oil, 0.5 wt % β-Lg, and 0−0.15 wt % ι-carrageenan at pH values of 3−8. The primary and secondary emulsions were stored for 24 h at room temperature, and then, their electrical charge and mean particle diameter were measured after dilution with a buffer of the appropriate pH value. The creaming stability was measured after 1 week of storage at room temperature without further dilution of the emulsions.

Particle Size Measurements. The prepared emulsions were diluted to a droplet concentration of approximately 0.006 wt % using buffer solution to avoid multiple scattering effects prior to analysis. The particle size distribution of the emulsions was then measured by laser light scattering (Mastersizer X, Malvern Instruments Ltd., Malvern, U.K.). This instrument finds the particle size distribution that gives the best fit between the experimental measurements and the predictions made using light scattering theory (i.e., Mie theory). A refractive index ratio of 1.08 was used by the instrument to calculate the particle size distributions. Measurements are reported as the volume−surface mean diameter: $d_{32} = \sum n_i\, d_i^3 / \sum n_i\, d_i^2$, where n_i is the number of droplets of diameter d_i (*2*). The particle size measurements are reported as the average and standard deviation of measurements made on two freshly prepared samples, with two readings made per sample.

ζ-Potential Measurements. The prepared emulsions were diluted to a droplet concentration of approximately 0.04 wt % using buffer solution prior to analysis. Diluted emulsions were then injected into the measurement chamber of a particle electrophoresis instrument (ZEM 5003, Zetamaster, Malvern Instruments, Worcs., U.K.), and the ζ-potential was determined by measuring the direction and velocity that the droplets moved in the applied electric field. The ζ-potential measurements are reported as the average and standard deviation of measurements made on two freshly prepared samples, with five readings made per sample.

Creaming Stability Measurements. Ten grams of emulsion was transferred into a test tube (internal diameter, 15 mm; height, 125 mm), tightly sealed with a plastic cap, and then stored at room temperature. After storage, a number of emulsions separated into an opaque layer (Op) at the top, a turbid (Tu) layer in the middle, and/or a transparent (Tr) layer at the bottom. We defined the serum layer to be the sum of the turbid and transparent layers. The total height of the emulsion (H_E) and the height of the serum layer (H_S) were measured. The extent of creaming was characterized by a creaming index = 100 × (H_S/H_E).

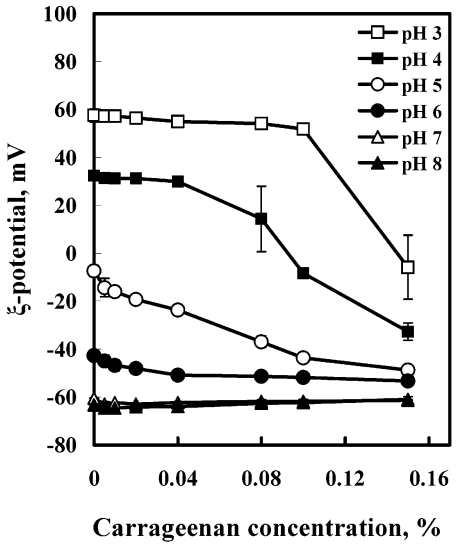

Figure 1. Dependence of particle electrical charge (ζ-potential) on pH and ι-carrageenan concentration for secondary emulsions (5 wt % corn oil, 0.5 wt % β-Lg, and 5 mM phosphate buffer).

The creaming index provided indirect information about the extent of droplet aggregation in an emulsion.

Optical Microscopy. The microstructure of selected emulsions was determined using optical microscopy (Nikon microscope Eclipse E400, Nikon Corporation, Japan). Emulsions were gently agitated in a glass test tube before analysis to ensure that they were homogeneous. A drop of emulsion was then placed on a microscope slide, covered by a cover slip, and observed at a magnification of 400×. An image of the emulsion was acquired using digital image-processing software (Micro Video Instruments Inc., Avon, MA) and stored on a personal computer.

Statistical Analysis. Experiments were performed twice using freshly prepared samples. Averages and standard deviations were calculated from these duplicate measurements.

RESULTS AND DISCUSSION

Influence of pH and ι-Carrageenan Concentration on Properties of Primary and Secondary Emulsions. *Droplet Charge*. The ζ-potential of the droplets in the primary emulsion changed from +60 to −63 mV as the pH was increased from 3 to 8 (**Figure 1**) because the net electrical charge of adsorbed β-Lg on the surface of the oil droplets changed from positive to negative as the pH was increased from below to above the protein's isoelectric point (IEP = 5.2). The net droplet charge of the primary emulsion was around zero at pH 5, which was close to the IEP of the protein. The droplet charge of the secondary emulsion was different from that of the primary emulsion at all pH values except pH 7 and pH 8. The trends of droplet charge vs ι-carrageenan concentration were different for the emulsions at different pH values. At pH 3, the droplet charge of the secondary emulsions did not change at ι-carrageenan concentrations ≤0.1 wt % but became highly negative at 0.15 wt % ι-carrageenan. This result suggests that ι-carrageenan molecules did not adsorb strongly to the surface of β-Lg-coated droplets at this pH, presumably because the interaction between ι-carrageenan molecules and β-Lg was weak. The highly negative ζ-potential measured at 0.15 wt % carrageenan may have been because the light scattering by the anionic polysaccharide molecules dominated that from the cationic emulsion droplets. The reason that only weak interactions occurred between ι-carrageenan molecules and oil droplets coated with β-Lg at pH 3 is probably because some of the sulfate groups on the ι-carrageenan were protonated at this pH, since the pK_a value of the anionic sulfate groups on ι-carrageenan is around pH 2 (*28*). The strength of the electrostatic interactions between proteins and polysaccharides is dependent on the sign, number, and distribution of ionizable groups on the molecules at specific pH values (*20*, *29*). The charged β-Lg is unable to bind strongly with protonated sulfate groups attached to the ι-carrageenan molecules at low pH. Hence, the ι-carrageenan molecules may not have been bound to the droplet surfaces or they may have been only weakly bound and were displaced when the emulsions were diluted for the ζ-potential measurements.

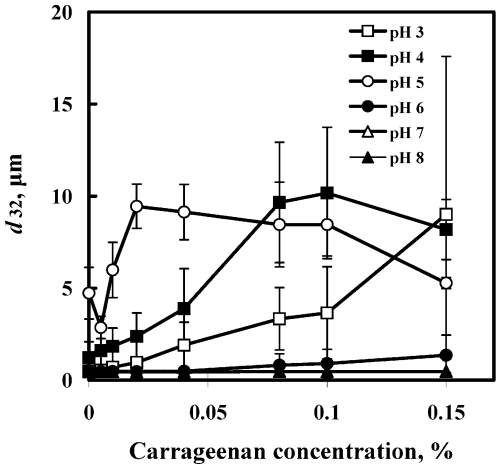

Figure 2. Dependence of mean particle diameter (d_{32}) on pH and ι-carrageenan concentration for secondary emulsions (5 wt % corn oil, 0.5 wt % β-Lg, and 5 mM phosphate buffer).

At pH 4, the droplet charge of the secondary emulsions decreased gradually as the ι-carrageenan concentration was increased from 0 to 0.04 wt %, had zero net charge around 0.1 wt % ι-carrageenan, and then became increasingly negatively charged at higher ι-carrageenan concentrations. These changes suggested that ι-carrageenan adsorbed on the surface of β-Lg-coated droplets due to their opposite net charges. At pH 5 and pH 6, the droplet charge of the primary emulsions was negative and became more negative when the ι-carrageenan concentration was increased from 0 to 0.15 wt % in the secondary emulsions. The increased negative charge on the droplets at pH 5 and pH 6 suggested that there was an electrostatic interaction between ι-carrageenan molecules and oil droplets coated with β-Lg, even though they had the same net negative charge. This result can be attributed to an electrostatic attraction between anionic ι-carrageenan and exposed cationic amino acid residues on the β-Lg molecules. At pH 7 and pH 8, the ζ-potential of the droplets in the secondary emulsions did not change as the ι-carrageenan concentration was increased, suggesting that there was no electrostatic attraction between ι-carrageenan molecules and β-Lg-coated droplets. This can be attributed to the fact that β-Lg-coated oil droplets were completely negatively charged at these pH values and not available to interact with anionic ι-carrageenan molecules.

Droplet Aggregation. The mean particle diameters of the primary emulsions (i.e., 0 wt % carrageenan in **Figure 2**) were relatively small ($d_{32} \approx 0.5$ μm) at all pH values except pH 5, where the particles were relatively large ($d_{32} \approx 5$ μm). The larger particle diameter observed at pH 5 suggests that droplet aggregation occurred close to the IEP of the protein (IEP = 5.2). Extensive droplet aggregation was observed in the primary emulsions around pH 5 because the β-Lg-coated droplets have a low net electrical charge near the IEP of the protein, thus reducing the electrostatic repulsion between the droplets (*14*).

There was extensive droplet aggregation in the secondary emulsions at pH values below and around the IEP of the protein

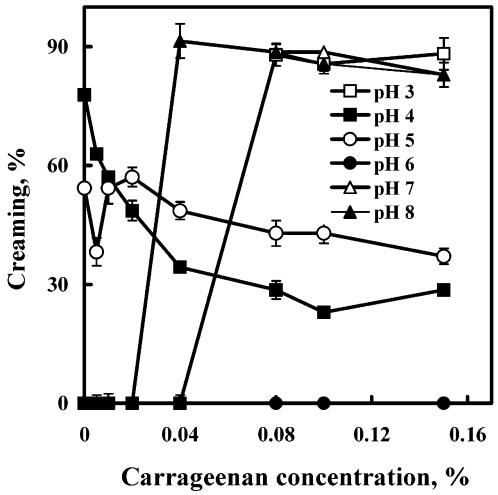

Figure 3. Dependence of emulsion creaming stability on pH and ι-carrageenan concentration for secondary emulsions after 1 week of storage (5 wt % corn oil, 0.5 wt % β-Lg, and 5 mM phosphate buffer).

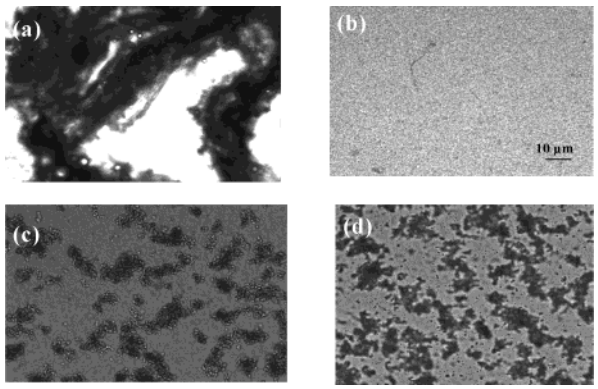

Figure 4. Photomicrographs of emulsions stabilized by β-Lg–ι-carrageenan membranes (5 wt % corn oil, 0.5 wt % β-Lg, 0.08% ι-carrageenan, and 5 mM phosphate buffer) at pH 3 (**a**), 6 (**b**), 7 (**c**), and 8 (**d**).

(pH 3−5). At pH 3, the particle diameters in the emulsion increased gradually as the ι-carrageenan concentration was increased, with the maximum amount of droplet aggregation occurring at 0.15 wt % ι-carrageenan. At pH 4, the mean particle diameter increased greatly at ι-carrageenan concentrations $\leq$0.08 wt %, with the maximum amount of droplet aggregation occurring at ι-carrageenan concentrations around 0.08−0.1 wt %, which corresponded to conditions where charge neutralization of the droplets occurred (**Figure 1**). At pH 3 and pH 4, the mean particle diameters at high ι-carrageenan concentrations ($\geq$0.08 wt % ι-carrageenan concentrations) had large standard deviations between samples measurements. Particle size distribution measurements indicated that there was a mixture of small and large droplets in the secondary emulsions at high ι-carrageenan concentrations. The small particles were probably emulsion droplets that had been restabilized by excess ι-carrageenan molecules completely covering their surfaces (*19*). The large particles appeared to be transparent and were big enough to observe by eye and optical microscope (**Figure 4a**). We postulated that these large particles were the result of aggregation of nonadsorbed β-Lg and ι-carrageenan at high ι-carrageenan concentrations. This aggregation was correlated to a rapid and large decrease in particle charge at 0.15 wt % ι-carrageenan (**Figure 1**). It is therefore possible that the high negative particle charge measured at high carrageenan concentrations at pH 3 was due to the protein−polysaccharide complex, rather than the emulsion droplets.

At pH 5, the particle diameter of the secondary emulsion increased rapidly with increasing ι-carrageenan concentrations up to 0.02 wt % but decreased gradually as the ι-carrageenan concentration was increased higher. Extensive droplet aggregation was observed in the secondary emulsions at pH values below and around the IEP of the protein because the anionic ι-carrageenan molecules could act as bridges between the cationic droplet surfaces. Bridging flocculation occurs in protein-stabilized emulsions when charged polysaccharides are present at relatively low concentrations (*16*, *21*, *29*).

At pH 6, the mean particle diameters of the secondary emulsions were small over all ι-carrageenan concentrations. According to the ζ-potential data, ι-carrageenan interacted with droplets coated with β-Lg even though they had the same net negative charge (**Figure 1**). The droplets are stable to aggregation at this pH probably because anionic sulfate groups on ι-carrageenan bind to cationic groups on β-Lg, thereby increasing the overall negative charge on the droplets. Thus, the increase in droplet charge would strengthen the electrostatic repulsion between the droplets and prevent droplet aggregation. Particle size measurements indicated no evidence of droplet aggregation at pH 7 and pH 8 as the ι-carrageenan concentration was increased (**Figure 2**). We will discuss this point further in the next section.

Creaming Stability. The creaming stability of the secondary emulsions stabilized by β-Lg−ι-carrageenan membranes was determined by manually measuring the height of the boundaries separating the different layers formed after 1 week of storage (**Figure 3**). Different layers were observed in the emulsion: an opaque white layer at the top, a turbid layer at the middle or bottom, and/or a transparent layer at the bottom. The opaque layer probably consisted primarily of flocculated droplets that creamed rapidly, whereas the turbid layer probably consisted of nonflocculated droplets that creamed more slowly (*30*). The height of each of these layers was measured and expressed as a percentage of the height of the whole emulsion (**Table 1**). At pH 3, emulsions became unstable to creaming at a ι-carrageenan concentration $\geq$0.08 wt % after 1 week of storage. We observed transparent particles in the serum layer at pH 3, which was attributed to the formation of insoluble β-Lg−ι-carrageenan complexes that were denser than water. These particles could clearly be seen using an optical microscope (**Figure 4**). At pH 4 and pH 5, the primary emulsions were very unstable to creaming (**Table 1**) because the pH was near the IEP of β-Lg. The creaming stability of the secondary emulsion at pH 4 and pH 5 improved with increasing ι-carrageenan concentration (**Figure 3**), which was probably due to the increase in viscosity of the continuous phase (*15*) and/or bridging flocculation (*19*, *21*, *29*, *30*) by the polysaccharides, leading to the formation of a network of aggregated droplets that retards creaming. At pH 6, emulsions were very stable to creaming at all ι-carrageenan concentrations after 1 week of storage (**Figure 3**). It is likely that charged ι-carrageenan molecules interact with exposed cationic amino acid residues (*31*−*34*) to create relatively thick and highly charged surfaces, thus reducing interactions between droplets through electrostatic repulsion. Optical microscopy showed that the droplets remained small in the emulsion at pH 6 (**Figure 4b**) but that extensive flocculation occurred at pH 7 and pH 8 (**Figure 4c**,**d**, respectively). It is interesting to note that we did not observe extensive depletion flocculation in the pH 6 emulsions, but we did in the pH 7 and pH 8 emulsions (**Figure 4**). This may have been because a significant fraction

Table 1. Appearance of O/W Emulsion Stabilized by β-Lg and ι-Carageenan after 1 Week[a]

		carrageenan concentration (%)							
pH		0	0.005	0.01	0.02	0.04	0.08	0.1	0.15
3	Op	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	12.1 ± 2.1	14.3 ± 0.5	94.3 ± 2.1
	Tu	0	0	0	0	0	87.9 ± 1.4 (+++)	85.7 ± 1.3 (+++)	88.2 ± 1.9 (+++)
	Tr	0	0	0	0	0	0	0	5.7 ± 0
4	Op	22.2 ± 1.3	37.1 ± 1.7	42.9 ± 2.5	51.4 ± 0.6	65.7 ± 2.6	71.4 ± 2.1	77.1 ± 2.5	71.4 ± 2.7
	Tu	63.9 ± 1.4	62.9 ± 2.1 (+)	0	0	0	0	0	0
	Tr	13.9 ± 0.9	0	57.1 ± 2.1	48.6 ± 1.2	34.3 ± 1.9	28.6 ± 2.7	22.9 ± 2.5	28.6 ± 2.1
5	Op	45.7 ± 1.2	51.4 ± 2.3	45.7 ± 1.5	42.9 ± 2.3	51.4 ± 2.7	57.1 ± 1.6	57.1 ± 1.9	62.9 ± 1.7
	Tu	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Tr	54.3 ± 2.3	38.2 ± 1.9	54.3 ± 1.7	57.1 ± 2.1	48.6 ± 3.1	42.9 ± 1.2	42.9 ± 1.6	37.1 ± 1.5
6	Op	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Tu	0	0	0	0	0	0	0	0
	Tr	0	0	0	0	0	0	0	0
7	Op	100 ± 0	100 ± 0	100 ± 0	100 ± 0	8.6 ± 1.7	11.4 ± 2.1	11.4 ± 1.6	17.1 ± 2.3
	Tu	0	0	0	0	91.4 ± 2.1 (+++)	88.6 ± 2.4 (++)	88.6 ± 1.2 (+)	82.9 ± 2.5 (+)
	Tr	0	0	0	0	0	0	0	0
8	Op	100 ± 0	100 ± 0	100 ± 0	100 ± 0	8.6 ± 0.4	11.4 ± 2.1	14.3 ± 0.3	17.1 ± 1.4
	Tu	0	0	0	0	91.4 ± 0.2 (+++)	88.6 ± 1.9 (++)	85.7 ± 0.7 (+)	82.9 ± 1.8 (+)
	Tr	0	0	0	0	0	0	0	0

[a] Op, opaque layer; Tu (turbid) + Tr (transparent), serum layer; +, slightly turbid; ++, intermediately turbid; +++, strongly turbid.

Table 2. Appearance of Mixtures of β-Lg and ι-Carrageenan in Buffer System after 1 Week at pH 6, 7, and 8 and Tr = 100, Tu = 0, and P = 0 at All ι-Carrageenan Concentrations[a]

		carrageenan concentration (%)							
pH		0	0.005	0.01	0.02	0.04	0.08	0.1	0.15
3	Tr	100 ± 0	0	0	0	0	64.6 ± 1.3	46.2 ± 1.9	40 ± 0.7
	Tu	0	100 ± 0 (+)	100 ± 0 (++)	92.3 ± 2.4 (+++)	84.6 ± 3 (+++)	0	0	0
	P	0	0	0	7.7	15.4	35.4 ± 1.5	53.8 ± 3.2	60 ± 1.6
4	Tr	95.4 ± 0.2	90.8 ± 1.1	84.6 ± 1.5	73.8 ± 0.6	69.2 ± 1.8	58.5 ± 0.3	53.8 ± 1.2	50.8 ± 2.4
	Tu	0	0	0	0	0	0	0	0
	P	4.6	9.2	15.4	26.2 ± 2.4	30.8 ± 1.8	41.5 ± 2.5	46.2 ± 3.5	49.2 ± 0.3
5	Tr	95.4 ± 1.3	90.9 ± 2.5	87.1 ± 2.1	86.2 ± 0.4	70.8 ± 2.1	0	0	0
	Tu	0	0	0	0	0	80 ± 0.3 (+++)	23.1 ± 1.1	28.3 ± 0.4
	P	4.6 ± 1.3	9.1 ± 2.3	12.7 ± 0.5	13.8 ± 2.1	29.2 ± 0.3	20 ± 0.2	76.9 ± 1.4	71.7 ± 1.0

[a] Tr, transparent; Tu, turbid; P, precipitated layers in the test tubes; +, slightly turbid; ++, intermediately turbid; +++, strongly turbid.

of the carrageenan added to the emulsions was at the droplet surface and therefore not free to induce a depletion attraction or because the increased repulsive interactions between the droplets were sufficient to overcome the depletion attraction.

At pH 7 and pH 8, the emulsions were stable to creaming at low ι-carrageenan concentrations $\leq$0.02 wt % after 1 week of storage but exhibited extensive creaming at higher ι-carrageenan concentrations. The height of the serum layers decreased slightly, and the serum layers became less turbid at ι-carrageenan concentrations higher than 0.04 wt % (**Table 1**). Particle size measurements did not show any droplet aggregation in the emulsion at pH 7 and pH 8, but observation of the emulsions by optical microscopy indicated that the droplets were highly flocculated (**Figure 4**). This suggests that the flocs formed in the emulsions were easily disrupted by dilution, which is characteristic of depletion flocculation (*30*). No change of droplet charge was observed after mixing ι-carrageenan into the β-Lg-stabilized emulsions at pH 7 and pH 8 (**Figure 1**), which suggests that there was no electrostatic attraction between ι-carrageenan molecules and β-Lg-coated droplets.

Influence of pH and ι-Carrageenan Concentration on Interactions of β-Lg and ι-Carrageenan in Aqueous Solution. To further understand the interaction of ι-carrageenan molecules with droplets coated by β-Lg in emulsions, we also examined interactions between ι-carrageenan and β-Lg in buffer solutions (i.e., in the absence of oil droplets). A series of solutions were prepared that contained a final concentration of 0.5 wt % β-Lg and various ι-carrageenan concentrations (0−0.15 wt %) and pH values (3−8). The prepared samples (10 mL) were placed in test tubes, and the heights of the boundaries separating the transparent, turbid, and precipitated layers in the test tubes after 1 week of storage were measured manually. The data were expressed as a percentage of the total height of the samples in the test tubes (**Table 2**). β-Lg solutions were transparent at pH values of 3, 6, 7, and 8 but were turbid at pH 4 and pH 5, indicating some protein precipitation. At pH 3, the mixtures of β-Lg and ι-carrageenan in buffer solution produced a turbid layer at ι-carrageenan concentrations $\leq$0.01 wt %, turbid/precipitated layers at ι-carrageenan concentrations between 0.02 and 0.04 wt %, and clear/precipitated layers at higher ι-carrageenan concentrations. At pH 4 and pH 5, precipitate formation in β-Lg solutions increased when the ι-carrageenan concentration was increased from 0 to 0.15 wt %, while β-Lg solutions at neutral pH values (6, 7, and 8) did not show any turbid and precipitated layers in the presence or absence of ι-carrageenan. This change (from soluble to precipitation) at pH 3 and pH 4 indicates that electrostatic interactions existed between β-Lg and ι-carrageenan due to the net opposite charges of the two polymers at those pH values. During complexation, the net charge of the anionic ι-carrageenan decreased by gradual attachment of cationic protein molecules, resulting in reduced solubility and hydrophilicity of the resultant complex. The maximum complexation yield usually occurs at pH values where the polymers carry equal and opposite charges (*35*). At a high concentration of ι-carra-

geenan, precipitation and/or gelation may occur due to the unbalance of charge density of β-Lg and ι-carrageenan (*36*). The degree of precipitation at pH 5 was less than that at pH 4, especially at ι-carrageenan concentrations between 0.02 and 0.08 wt %. This is probably because there are less positive charges on the protein molecules to bind and neutralize the negative charge on the carrageenan molecules.

There is no strong electrostatic attraction between β-Lg and ι-carrageenan at pH values of 6, 7, and 8 because they both have net negative charges. However, soluble complexes may be produced even though both polymers carry a net negative charge at pH values above the IEP of the protein. In this case, the electrostatic interactions involve the anionic ι-carrageenan interacting with positively charged local patches on β-Lg (*32*−*34*). This local electrostatic attraction at pH values above the IEP of β-Lg may increase the net negative charge on the β-Lg and enhance protein−protein electrostatic repulsion at low carrageenan concentrations and electrostatic repulsion between ι-carrageenan and ι-carrageenan at high carrageenan concentrations. At low ionic strength, sulfated hydrocolloids have relatively high charge densities and form fairly strong reversible complexes with protein even at neutral and alkali pH values (*31*, *36*). This fact means that the occurrence of soluble complexes between sulfated anionic polymers (e.g., carrageenan) and proteins at pH values above their IEP is possible.

We could not directly compare the data from aqueous solutions and emulsions because the structure of β-Lg is different in the two systems, (e.g., native form in aqueous solution and surface-denatured form in emulsions), and the interaction between proteins and polysaccharides is closely dependent on the structures of the proteins (*31*, *36*). Interactions between ι-carrageenan molecules and β-Lg-coated droplets at pH 3 were weak due to the IEP of sulfate groups on ι-carrageenan, while those at pH 4 and pH 5 were stronger than at pH 3 due to their strong net opposite charge. It seemed that the protein−polysaccharides complexes formed at pH 3−5 could not be used to improve emulsion stability. However, adsorbed complexes on the surface of β-Lg-coated droplets at pH 6 are soluble and can stabilize emulsions. Other studies have shown that several insoluble and soluble complexes of protein and polysaccharide can be produced at pH values above the IEP of the protein and be used to stabilize emulsion, e.g., whey proteins and carboxymethyl cellulose (*37*), gelatin and alginate (*38*).

In conclusion, the aim of this study was to examine the influence of pH and ι-carrageenan concentration on the stability of β-Lg-stabilized O/W emulsions, so as to identify conditions where improved emulsion stability could be obtained. There were electrostatic attractions between ι-carrageenan and β-Lg in emulsions at pH values of 3, 4, and 5, but extensive droplet aggregation and creaming occurred, probably due to the unbalance of opposite charge density between the two biopolymers. Addition of ι-carrageenan to the emulsions at pH 6 led to droplets coated by an interfacial protein−polysaccharide complex that improved emulsion stability. This study has helped to identify optimum conditions for preparation of secondary emulsions without the need for mechanical agitation to disrupt flocs.

In future studies, we intend to examine other factors that may be useful for improving the stability and properties of secondary emulsions, including the application of mechanical agitation, changing the emulsifier and biopolymer type, and altering the ionic composition of the aqueous phase.

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