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In Vitro Assessment of the Bioaccessibility of Fatty Acids and Tocopherol from Soybean Oil Body Emulsions Stabilized with ι-Carrageenan

Na-Na Wu,[†] Xu Huang,[†] Xiao-Quan Yang,[*,†] Jian Guo,[†] Shou-Wei Yin,[†] Xiu-Ting He,[†] Li-Juan Wang,[†,‡] Jian-Hua Zhu,[§] Jun-Ru Qi,[†] and Er-Li Zheng[†]

[†]Research and Development Center of Food Proteins, Department of Food Science and Technology, South China University of Technology (SCUT), Guangzhou 510640, People's Republic of China

[‡]College of Engineering and Technology, Shenyang Normal University, Shenyang 110034, People's Republic of China

[§]Yingdong Food Science and Technology College, Shaoguan College, Shaoguan 512005, People's Republic of China

ABSTRACT: The present investigation aimed to expand the knowledge of the in vitro bioaccessibility of fatty acids and tocopherol from natural soybean oil body emulsions stabilized with different concentrations of ι-carrageenan. Several physicochemical parameters including proteolysis of the interfacial layer, interfacial composition, and microstructure were evaluated with regard to their impact on the bioaccessibility of fatty acids and tocopherol. Results from simulated human digestion in vitro indicated that the bioaccessibility of total fatty acids and tocopherol decreased (62.7−8.3 and 59.7−19.4%, respectively) with the increasing concentration of ι-carrageenan. During the in vitro digestion procedure, ι-carrageenan affected physicochemical properties of the emulsions, thereby controlling the release of fatty acids and tocopherol. These results suggested that soybean oil body emulsions stabilized with ι-carrageenan could provide natural emulsions in foods that were digested at a relatively slow rate, the important physiological consequence of which might be increasing satiety.

KEYWORDS: *digestion bioaccessibility, fatty acid, tocopherol, soybean oil body emulsion, ι-carrageenan*

INTRODUCTION

Soybean seeds store triacylglycerols (TAGs) in intracellular organelles called oil bodies or oleosomes that are utilized as a source of energy during seed germination. Soybean oil bodies (about 0.2−0.5 μm in diameter) possess a structure consisting of a TAG core (94−98% of the dry weight) surrounded by a monolayer of phospholipids (0.6−2% w/w) containing embedded intrinsic oleosins (0.6−4% w/w).[1,2] These phospholipids and oleosins form a thickened surface layer that prevents coalescence of oil bodies.[3,4] Soybean oil bodies have three intrinsic oleosins: 24, 18, and 17 kDa.[5,6] The protein 34 kDa was initially considered soybean oil body oleosin because it could bind to the oil body strongly, but it was present in protein storage vacuoles and could be removed by the pH 11 extraction method.[5,6]

Soybean oil is a popular dietary source of lipids for human consumption and is a rich source of essential fatty acids and vitamin E (tocopherol), which may exert beneficial effects in humans including protection against cardiovascular disease and cancer.[7,8] Some authors have reported that oil bodies isolated from plant seeds are enriched in tocochromanols.[9,10] As already existing natural ingredients, oil bodies isolated from soybeans could be applied in aqueous media to form a natural emulsion that might represent a vehicle to deliver natural, minimally processed, pre-emulsified oil and other nutrients (e.g., vitamin E) into appropriate food systems.

The bioavailability of micronutrients from food sources has been more and more investigated to understand their functionality in human health. In vitro techniques to mimic the human digestive system are becoming increasingly popular to be used to evaluate the digestibility and bioaccessibility of a range of micronutrients, including vitamin E.[11,12] White et al.[13] investigated the sunflower oil body digestion in a simulated gastrointestinal tract and found that sunflower oleosins (19 and 20 kDa) were completely hydrolyzed by pepsin and broken up to more small molecules, and droplets coalescence occurred after incubation with simulated gastric conditions for 30 min. This might influence the bioavailability of vitamin E and fatty acids because micronutrients might be decomposed and lipids might be digested by gastric lipase in vivo conditions.[14]

Recently, some researchers designed the interfacial composition of oil-in-water emulsions to control the lipid digestion and the release of micronutrients, for example, encapsulation of emulsified lipids with chitosan to form thicken layers on the oil droplets.[15−20] The presence of polysaccharides might significantly saturate the protein-stabilized lipid droplets, thereby preventing the enzymes from coming in contact with the lipid hydrophobic core, and thus influencing the overall lipid digestion. This will help to rationally fabricate future foods with designed functional behaviors in the body, such as controlled release of lipid bioactive molecules. Such foods may have the potential benefits of fighting against obesity, cardiovascular diseases, and other food-linked health issues and/or of providing improved satiety responses. In our previous study, we found that ι-carrageenan was most effective

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at creating highly charged interfacial membranes to stabilize soybean oil bodies because of its most densely charged helical structure.[21] To date, only simple proteolytic and lipolytic assays have been used to assess the action of pepsin and lipase on natural oil bodies;[13,22,23] however, no studies have demonstrated the influence of polysaccharides concentration on the relative digestive bioaccessibility of micronutrients (vitamin E and fatty acids) from soybean oil bodies. It would be meaningful and put valuable insights for designing food structure with functional behaviors to test the influence of ι-carrageenan on the digestibility and bioaccessibility of bioactive components from soybean oil bodies during in vitro digestion.

This study was undertaken to examine the effect of different concentrations of ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) on the proteolysis of the interfacial layer, interfacial composition, and microstructures of soybean oil bodies before and after simulated gastric and duodenal digestion. In addition, these simulated digestive processes have been combined in sequence to compare the bioaccessibility of important nutritional components from soybean oil body emulsions stabilized with different concentrations of ι-carrageenan, for instance, fatty acids and tocopherol (vitamin E).

MATERIALS AND METHODS

Materials. Soybeans (variety: Beixiang) were obtained from Shenyang Lion King Industry and Trade Co. Ltd. (Shenyang, China). Food-grade ι-carrageenan samples were provided by TIC Gums Co. (Philadelphia, PA). The fluorescent dyes of Nile Blue A and Nile Red, pancreatin (porcine, 8 × USP specifications), lipase (from porcine pancreas type II, 100−400 units/mg of protein), sodiumdeoxycholate, and tocopherol (α-, β-, γ- and δ-) were all products of Sigma Chemical Co. (St. Louis, MO). Pepsin (DH232-1; activity, 1:3000) was purchased from Gen-View Scientific Inc. (Wilmington, DE). Other chemicals were analytical grade and purchased from Guangzhou Chemical Co. Ltd. (Guangzhou, China). Distilled and deionized water was used to prepare all solutions and emulsions.

Recovery and Purification of Soybean Oil Bodies. Oil bodies were physically isolated from a total homogenate of mature soybean seeds according to the method of Tzen et al.[24] with modifications. Soybean seeds were soaked for 20 h in distilled water (1:5 w/v) at 4−6 °C. Soaked beans were then suspended in 5 volumes of grinding buffer (50 mM Tris-HCl, pH 7.5, 0.4 M sucrose, and 0.5 M NaCl) at 4 °C and homogenized with a commercial food processor (JC-029, Crown, Guangzhou, China). The resulting slurry was filtered through three layers of cheesecloth, and the filtrate was centrifuged at 10000*g* (CR22G centrifuge, Hitachi Co., Japan) for 30 min. The crude oil bodies, which collected as a creamy pad at the top of the mixture, were carefully picked from the media and resuspended in 9 M urea (1:5 w/v) and then left on a rotary roller at 400 rpm for 10 min. The mixture was then centrifuged as above, and the creamy pad was isolated. This pad was then washed with buffer solution (50 mM Tris-HCl buffer, pH 7.5) for another three times to remove urea from the preparation.

Proximate Composition of Purified Soybean Oil Bodies. The moisture contents of soybean oil bodies were determined by oven drying.[25] Crude free fat contents of the samples were determined by the Soxhlet extractor system using hexane as the extraction solvent.[25] The nitrogen contents of the samples were determined with the Dumas combustion method[25] by using a rapid N cube Nitrogen Analyzer (Elementar Inc., Hanau, Germany). The 5.71 × N conversion factor was used to convert percentage of nitrogen to protein content. The data of fat and protein contents were reported on a wet weight basis (wb). All determinations were conducted in triplicate.

Emulsion Preparation. A ι-carrageenan solution (1.6% w/w, pH 7) was prepared by dispersing weighed amounts of the powdered material into sodium phosphate buffer solution (10 mM, pH 7) and heating at 70 °C for 20 min. Once the lipid contents of the purified oil body preparations were determined (see above), the oil body suspensions (20% w/w oil) were prepared by mixing 40 g (wet weight) of the creamy pad (oil bodies) with 56 g of sodium phosphate buffer solution (10 mM, pH 7) using a blender (RW20 digital, IKA, Germany). The initial soybean oil body−ι-carrageenan emulsions were prepared by mixing oil body suspensions (20% w/w oil), ι-carrageenan solution, and sodium phosphate buffer solution (10 mM, pH 7) to give a series of emulsions with the same oil concentration (10% w/w oil) but different ι-carrageenan concentrations (0, 0.1, 0.2, 0.4, and 0.8% w/w, respectively). The initial soybean oil body−ι-carrageenan suspensions were stirred for 2 h at room temperature and then diluted with different ratios of sodium phosphate buffer solution (10 mM, pH 7) and salt solution to make samples with the same oil body concentration (5% w/w oil), the same salt concentration (100 mM NaCl and 10 mM $CaCl_2$) but different ι-carrageenan concentrations (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively). These resulting emulsions were prepared in triplicate and stored at 4 °C for no longer than 12 h before use.

Pepsin and Lipase Digestion of Soybean Oil Body Emulsions Stabilized with ι-Carrageenan. The digestion procedure was a slight modification of that described by White et al.[13] The prepared soybean oil body emulsion stabilized with ι-carrageenan (20 mL; 5% w/w oil, 100 mM NaCl, and 10 mM $CaCl_2$) was adjusted to pH 2 using 6 M HCl, incubated in a shaking water bath (DF-101S, Yu-Hua Instrument Co. Ltd., Zhengzhou, China) at 37 °C for 10 min, and then mixed with pepsin solution (2 mL; 36 mg mL^{-1}, 0.1 M HCl). The mixture was incubated for 1 h at 37 °C under agitation (400 rpm) using a shaking water bath. Following the pepsin digestion, the pH of the media was then raised to 7.0 with the addition of 1 M $NaHCO_3$ (2.6 mL) before the addition of a pancreatin/bile salt mixture (2.4 mL; 100 mmol L^{-1} sodium deoxycholate, 5 mg mL^{-1} pancreatin, and 100 mmol L^{-1} sodium bicarbonate solution) and additional lipase solution (1 mL; 100 mg mL^{-1}). The preparation was then incubated for a further 2 h at 37 °C. Final concentrations of constituents in the preparation were 2.6 mg mL^{-1} pepsin, 3.6 mg mL^{-1} lipase, 8.6 mmol L^{-1} sodium deoxycholate, and 0.4 mg mL^{-1} pancreatin.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). After the pepsin digestion of soybean oil body emulsions stabilized with ι-carrageenan, each sample was withdrawn from the mixture at 60 min for SDS-PAGE analysis. Proteolysis was terminated by raising the pH of the mixture to 6.5 using 1 M $NaHCO_3$.[22] SDS-PAGE experiments were performed using the discontinuous buffer system at 5% stacking gel and 13% separating gel using Bio-Rad Mini-protein Tetra Electrophoresis System (Bio-Rad Laboratories Ltd., CA). Aliquots of initial soybean oil body emulsion (300 μL) and 300 μL of soybean oil body−ι-carrageenan emulsions after pepsin digestion were mixed with 300 μL of electrophoresis sample buffer (0.125 M Tris-HCl, pH 8.0), containing 1.0% w/v SDS, 0.05% w/v bromophenol blue, 30% v/v glycerol, and 5% v/v β-mercaptoethanol. The samples were then shaken using a vortex mixer (MS 3 basic, IKA, Germany) to disperse the samples homogeneously and boiled for 5 min. The resulting subnatant was loaded (10 μL) onto the gels, and the electrophoresis was then run at 20 mA in the stacking gel and at 40 mA in separating gel until the tracking dye reached the bottom of the gel. A control sample containing just the pepsin preparation was also run to aid possible identification of proteolytic bands. After each run, the gel was immediately dyed with Coomassie Blue R250 for 40 min and destained with a solution of acetic:-methanol:water (1:1:8 v/v/v). The band patterns were then photographed.

Electrical Charge and Particle Size Measurements. The electrical charges (ζ-potential) of the emulsions before and after digestion with pepsin and lipase were calculated through measuring the electrophoretic mobility of the droplets using a capillary electrophoresis cell (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, United Kingdom). The emulsions were diluted to a droplet concentration of approximately 0.05% w/v using sodium phosphate buffer solution (at the same pH as the emulsions) prior to analysis. The particle size distributions of the emulsions before and

after digestion with pepsin and lipase were evaluated by a laser light scattering technique using a Malvern Mastersizer 2000 unit (Malvern Instruments), and the emulsions were diluted with distilled water for approximately 1000 times before each test. Particle sizes are reported as the surface-weighted ($d_{32} = \Sigma n_i d_i^3/\Sigma n_i d_i^2$) mean particle diameter, where n_i is the number of droplets of diameter d_i. The electrical charge and particle size measurements were carried out at 25 °C, and the data were the average values and standard deviations of three replications performed with three individually prepared samples.

Confocal Laser Scanning Microscopy (CLSM). CLSM images of soybean oil body emulsions stabilized with ι-carrageenan before and after digestion with pepsin and lipase were observed on a Leica TCS SP5 Confocal Laser Scanning Microscope (Leica Microsystems Inc., Heidelberg, Germany), equipped with an inverted microscope (Model Leica DMI6000) and a 20 × HC PL APO/0.70NA oil immersion objective lens. The various emulsion samples were stained with an appropriate amount of 0.1% w/v Nile Blue A and 0.01% w/v Nile Red (fluorescent dye) in isopropyl alcohol (40 μL of the mixed dye solution per mL sample). The stained emulsions were then placed on concave confocal microscope slides (Sail; Sailing Medical-Lab Industries Co. Ltd., Suzhou, China), covered with glycerol-coated coverslips, and examined with a 100 magnification lens and an argon/krypton laser having an excitation line of 488 nm and a Helium Neon laser (He/Ne) with excitation at 633 nm. The reported images were recorded at a penetration depth of 15 μm. This depth avoided artifacts that occurred close to the glass slide and resulted in representative images. The oil phases or particles are usually green, while the proteins are red.

Analysis of Fatty Acids and Vitamin E. After digestion with pepsin and lipase solution, micelles were separated from soybean oil body emulsions stabilized with ι-carrageenan by ultracentrifugation according to the method of White et al.[13] Aliquots (1 mL) of each emulsions after digestion were transferred to ultracentrifuge tubes, placed in a rotor (S140AT-0239, Hitachi, Japan), and then centrifuged at 165000g at 4 °C for 95 min (Micro Ultracentrifuge CS15ONX, Hitachi, Japan). The aqueous fraction was collected from the centrifuge tube using an 18 gauge needle fitted to a 1 mL syringe and then filtered through a cellulose acetate membrane (Satorious Company, Germany) with a pore size of 0.22 μm. The filtrate was stored at −80 °C prior to use.

Fatty acids were extracted from the micellar fractions (1 mL) using chloroform/methanol as Bligh and Dyer[26] to achieve a relative concentration of chloroform/methanol/water of 1:2:0.8 during monophasic extraction and 1:1:0.9 during biphasic separation. Extracted free fatty acids were then derivatized using methanolic sulfuric acid (1%) at 50 °C.[27] The fatty acyl groups of the oil and the oil bodies, extracted from soybean seeds, were also derivatized to allow fatty acid compositional analysis. Identification and quantification of fatty acid methyl esters (FAMEs) were carried out using GC-MS (Agilent 6890N GC and 5975 inert Mass Selective Detector; 70 eV ionization energy) equipped with a DB-23 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent Co., United States). The temperature of the oven was kept at 50 °C, increased to 140 °C at a rate of 10 °C/min, and then increased to 220 °C at a rate of 5 °C/min. The injector temperature was kept at 250 °C. The carrier gas was helium with a flow rate of 1.0 mL/min. FAMEs were identified by comparison of retention times and mass spectral data against authentic standards and the NIST/LIBTX library. The FAMEs content was quantified by total correction peak areas.

Vitamin E in emulsions and micellar fractions was recovered as described previously for carotenoids.[28] Aliquots (500 μL) of the samples were extracted three times with 1 mL of acetone. Distilled water (3 mL) was added to the pooled acetone extracts before the samples were re-extracted into 2 mL of hexane three times. The pooled hexane extract was evaporated under a stream of nitrogen. Dried samples were reconstituted in 500 μL of mobile phase (methanol) and analyzed by reversed-phase high-performance liquid chromatography (Waters 1525 liquid system, United States) equipped with a Waters C_{18} column (150 mm × 3.9 mm, 5 μm) and a Waters 2487 ultraviolet detector. Samples (20 μL) were injected with a run time of 20 min at 25 °C. The eluent was operated at a flow rate of 1 mL min^{-1}, and the absorbance of the eluent was monitored at 280 nm. Identification and quantification were made using standards of α-, β-, γ-, and δ-tocopherol.

Calculations and Statistics. The bioaccessibility of fatty acids and vitamin E was defined as a percentage release: [total substrate released into the micellar phase/total available substrate] × 100. All experiments were carried out in triplicate using freshly prepared samples. An analysis of variance (ANOVA) of the data was performed using the SAS system (version 9.0, SAS Institute Inc., Cary, NC), and Duncan's multiple-range test with a confidence interval of 95% was used to compare the means. Values are expressed as means ± standard deviations (SDs).

RESULTS AND DISCUSSION

Purified Oil Body Composition. The soybean oil body preparation (purified) contained 44.2 ± 0.5% moisture, 48.2 ± 0.3% fat (wb), and 6.6 ± 0.2% protein (wb). The soybean oil bodies, ι-carrageenan, and saline solutions were prepared to provide soybean oil body−ι-carrageenan emulsions with the same oil content (5% w/w) but different ι-carrageenan concentrations (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) for further studies.

Proteolysis of the Interfacial Layer. Figure 1 shows the profile of the protein molecular masses (SDS-PAGE) in

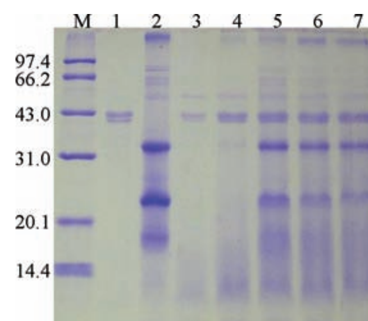

Figure 1. SDS-PAGE of soybean oil body emulsions stabilized with ι-carrageenan after digestion with pepsin solution. Track M, relative molecular mass markers, in decreasing molecular masses of 97.4, 66.2, 43.0, 31.0, 20.1, and 14.4 kDa. Track 1, pepsin; track 2, purified soybean oil bodies; tracks 3−7, soybean oil body emulsions with different concentrations of ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) after digestion with pepsin solution (36 mg mL^{-1}, 0.1 M HCl) at 37 °C for 60 min.

purified soybean oil bodies and soybean oil body emulsions stabilized with different concentrations of ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) after incubation with pepsin. After the soybean oil bodies were purified with Tris-HCl buffer solution, four proteins with molecular masses of 17, 18, 24, and 34 kDa were obtained in soybean oil bodies (track 2, Figure 1). Although the extraction method of soybean oil bodies could not remove the protein of molecular mass 34 kDa,[5] it removed much of the background proteins (β-conglycinin, glycinin, etc.) and was available for our study. The loss of bands at 17, 18, 24, and 34 kDa and the increase in bands below 14.4 kDa (tracks 3 and 4, Figure 1) suggested that pepsin digestion of soybean oil body emulsions stabilized with 0 and 0.05% w/w ι-carrageenan resulted in complete hydrolysis of oleosins within 60 min in the simulated gastric phase. Moreover, for soybean oil body emulsions stabilized with 0.1, 0.2, and 0.4% w/w ι-carrageenan (tracks 5−7, Figure 1), protein bands at 17 and 18 kDa became more dim, and there

was an increase in bands below 20.1 kDa following incubation with pepsin. Comparison with proteins in the pepsin preparation itself (track 1, Figure 1) indicated that these were genuine fragment products of pepsin catalysis. Pepsin hydrolyzes peptide bonds on the N terminus side of aromatic residues.[29] Therefore, the N-terminal region of 17 kDa oleosin[30] was prior to digested, whereas protein bands at 24 and 34 kDa still existed (tracks 5−7, Figure 1). The pepsin action on the soybean oleosins is unclear; thus, further work would be required to elucidate the breakdown of oleosins and the actual profile of oleosin degradation during pepsin digestion. The enzymolysis degree of oleosins at an oil−water interface in soybean oil body emulsions decreased with the increasing concentrations of ι-carrageenan, suggesting that ι-carrageenan might play an important role on resisting the pepsin digestion. This might be because ι-carrageenan, which had the most densely charged helical structure, was most effective at creating highly charged and sterically hindered interfacial membranes.[21]

Interfacial Composition Changes. ζ-Potential measurements were conducted to investigate the changes in the interfacial composition of emulsion droplets during the in vitro digestions. The results for each digestive periods of soybean oil body emulsions stabilized with ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) studied are shown in Figure 2. The

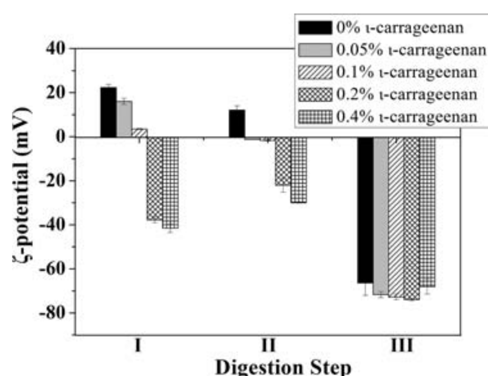

Figure 2. ζ-Potentials of soybean oil body emulsions stabilized with different concentrations of ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) as they pass through an in vitro digestion model: (I) before digestion, (II) after digestion with pepsin solution (36 mg mL^{-1}, 0.1 M HCl) at 37 °C for 60 min, (III) after digestion with pancreatin/bile salt mixture (100 mmol L^{-1} sodium deoxycholate, 5 mg mL^{-1} pancreatin, and 100 mmol L^{-1} sodium bicarbonate solution) and additional lipase solution (100 mg mL^{-1}) at 37 °C for 120 min.

electrical charge (ζ-potential) on the lipid droplets in the initial emulsions (pH 2) depended on the concentration of ι-carrageenan used to stabilize them (Figure 2). The ζ-potential of the initial soybean oil body emulsion (pH 2) was around +23 mV, whereas the ζ-potential of the initial emulsions stabilized with 0.05, 0.1, 0.2, and 0.4% w/w concentrations of ι-carrageenan were around +16, +3.5, −38, and −41 mV, respectively. With the increasing concentration of ι-carrageenan, the positive electrical charges on the soybean oil body emulsion droplets were gradually neutralized by the negative electrical charges of ι-carrageenan molecules; therefore, the ζ-potential of the initial emulsions decreased to around zero and then became negative. After digestion with pepsin solution, the ζ-potential of the emulsions stabilized with different concentrations of ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) were around +12, −1.3, −1.7, −22, and −30 mV, respectively (Figure 2). The magnitude of the ζ-potential of the emulsions became lower than those of the initial emulsions; particularly, the ζ-potential of the emulsions stabilized with 0.05 and 0.1% w/w ι-carrageenan changed from positive to negative. As a result of proteolysis of the interfacial protein layer, the peptides generated were not strong enough to provide emulsion stability; thus, coalescence might occur.[14] If there were no change in interfacial composition, the lipid droplets of the emulsions stabilized with 0.05 and 0.1% w/w ι-carrageenan would be positively charged in the simulated stomach conditions because their pH was well below their pI values around pH 4.[21,31] When the emulsions moved from the simulated stomach to duodenal environment (around pH 7), they all became negatively charged, and the final charges of the emulsions stabilized with ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) were stable between −66 and −74 mV regardless of different concentrations of ι-carrageenan (Figure 2). The observed changes in electrical charges on the droplets might be attributed to a number of factors, including changes in solution conditions (pH and ionic strength), adsorption of charged peptides generated from the pepsin digestion periods, or the existence of negatively charged ι-carrageenan. There was no significant difference of the ζ-potential for the emulsions stabilized with different concentrations of ι-carrageenan after digestion with lipase ($p < 0.05$). This might be attributed to the fact that the soybean oil body droplets after digestion with lipase were completely charged by negative charges at pH 7 and not available to interact with anionic carrageenan molecules as discussed in our previous study.[21]

Structural Changes. The microstructures of the emulsions were recorded using laser diffraction measurements (Figures 3 and 4) and confocal fluorescence microscopy (Figure 5) as they passed through each stage of the in vitro digestion model.

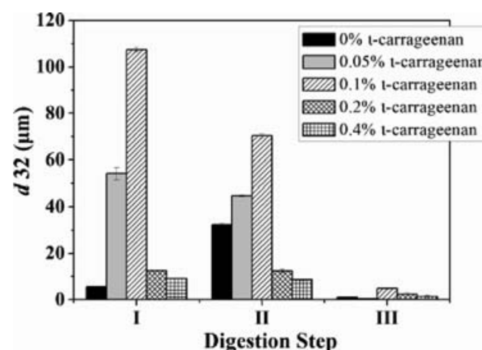

Figure 3. Mean particle diameters (d_{32}) of soybean oil body emulsions stabilized with different concentrations of ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) as they pass through an in vitro digestion model: (I) before digestion, (II) after digestion with pepsin solution (36 mg mL^{-1}, 0.1 M HCl) at 37 °C for 60 min, and (III) after digestion with pancreatin/bile salt mixture (100 mmol L^{-1} sodium deoxycholate, 5 mg mL^{-1} pancreatin, and 100 mmol L^{-1} sodium bicarbonate solution) and additional lipase solution (100 mg mL^{-1}) at 37 °C for 120 min.

Initially, the soybean oil body emulsions stabilized with ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) contained relatively large lipid droplets with a wide range of different diameters (around 6−108 μm). With the increasing concentration of ι-carrageenan, the mean diameters of the emulsions increased and then became smaller (Figures 3 and

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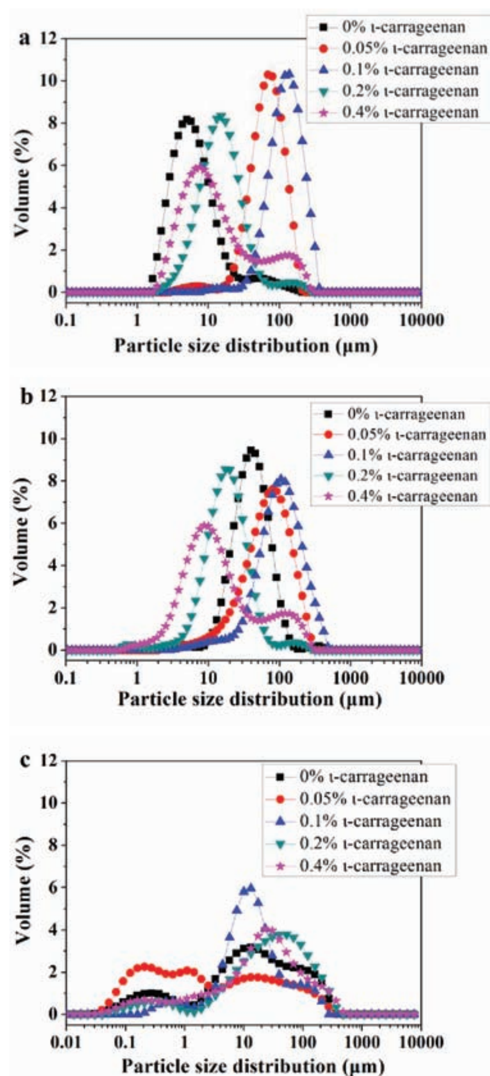

Figure 4. Particle size distribution of soybean oil body emulsions stabilized with different concentrations of *ι*-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) as they pass through an in vitro digestion model: (a) before digestion, (b) after digestion with pepsin solution (36 mg mL^{-1}, 0.1 M HCl) at 37 °C for 60 min, and (c) after digestion with pancreatin/bile salt mixture (100 mmol L^{-1} sodium deoxycholate, 5 mg mL^{-1} pancreatin, and 100 mmol L^{-1} sodium bicarbonate solution) and additional lipase solution (100 mg mL^{-1}) at 37 °C for 120 min.

4). Obvious flocculations or coalescences were observed in the images of CLSM for the initial emulsions stabilized with 0.05 and 0.1% w/w *ι*-carrageenan (Figure 5). These might be because of the interaction of the electrical charges between oil body droplets and *ι*-carrageenan molecules as we pointed out previously. Furthermore, the existence of 100 mM NaCl and 10 mM $CaCl_2$ might also affect the integrity of soybean oil body droplets at a low concentration of *ι*-carrageenan, because of the electrostatic screening and ion-binding effects.[32,33]

Following pepsin incubation for 1 h, the mean diameter of soybean oil body emulsions stabilized without *ι*-carrageenan increased from around 5.5 μm to around 32 μm, and the mean diameters of the emulsions stabilized with 0.05 and 0.1% w/w *ι*-carrageenan decreased from around 54 and 108 to 45 and 70 μm, respectively, but no changes were recorded for the emulsions stabilized with 0.2 and 0.4% w/w *ι*-carrageenan (Figures 3 and 4). There were larger oil droplets in the CLSM images for the emulsions stabilized with 0, 0.05, 0.1, and 0.2% w/w *ι*-carrageenan, but little large oil droplets were detected in the CLSM observations for the emulsion stabilized with 0.4% w/w *ι*-carrageenan (Figure 5). Oleosin has three structural regions: an amphipathic N-terminal region, a central hydrophobic antiparallel β-strand domain, and an amphipathic C-terminal domain of variable length. The central domain is embedded into the TAGs matrix, while amphipathic N and C termini are located on the oil body surface.[2−4] It is likely that the protruding part of the oleosin molecule, which provides a strengthened layer on the surface, is susceptible to enzymatic cleavage and once digested leads to the weakening and consequential coalescence of oil bodies. Therefore, two or more oil body droplets coalesced together at the *ι*-carrageenan concentrations of 0, 0.05, 0.1, and 0.2% w/w, but little coalescences occurred in the oil body emulsion stabilized with 0.4% w/w *ι*-carrageenan during the pepsin digestion process (Figure 5), which showed that the effect of *ι*-carrageenan on resisting the pepsin digestion was weaker at low concentrations (0, 0.05, 0.1, and 0.2% w/w) than that at 0.4% w/w *ι*-carrageenan. This suggested that electrical repulsion and steric hindrance of *ι*-carrageenan molecules at high concentration might resist pepsin approach to the substrate position on the surface of oil body droplets as we discussed previously (Figures 1 and 6); however, pepsin molecules were prone to attach to a protruding part of oleosins on the surface of oil body droplets at 0% w/w or low concentrations of *ι*-carrageenan; then, the oleosins were hydrolyzed, the stabilities of oil bodies were destroyed, and coalescences occurred (Figure 6). That was because both oleosins and phospholipids (PLs) were required to stabilize the oil bodies and that oleosins prevented oil bodies from coalescing by providing steric hindrance.[3,34] The hydrolysis of oleosins and coalescences might influence the following lipase incubation and the release of fatty acids and tocopherol to the micelles. The results of CLSM images were inconsistent with the mean diameters for the emulsions stabilized with 0.05, 0.1, and 0.2% w/w *ι*-carrageenan before and after pepsin digestion, that was, the mean diameters for the emulsions stabilized with 0.05, 0.1, and 0.2% w/w *ι*-carrageenan became smaller or changeless, but larger oil droplets were still observed after pepsin digestion (Figures 3−5). It should be noted that the mean particle diameters determined by light scattering on the highly aggregated and/or coalesced emulsions should only be used to provide a qualitative indication of droplet aggregation, rather than as quantitative values. This was because sample dilution and stirring might influence the size and structure of any particles present in the systems.[35]

The mean diameters of the emulsions stabilized with *ι*-carrageenan decreased to 0.45−4.9 μm when the emulsions moved from the simulated stomach to duodenal conditions, and the largest change in mean droplet size occurred (Figures 3 and 5). Moreover, the shifts from monomodal or bimodal distribution to more apparent peaks were recorded for the emulsions stabilized with *ι*-carrageenan when digested with pepsin, and the volume distribution intensities of the emulsions after incubation with duodenal conditions were much lower than initial emulsions and pepsin digested emulsions (Figure 4). These might be attributed to the digestion of the soybean lipids by lipase and the incorporation of the lipid digestion products within mixed micelles and vesicles. The mean diameters of the emulsions stabilized with 0.1, 0.2, and 0.4% w/w *ι*-carrageenan were 4.91, 2.26, and 1.46 μm, respectively,

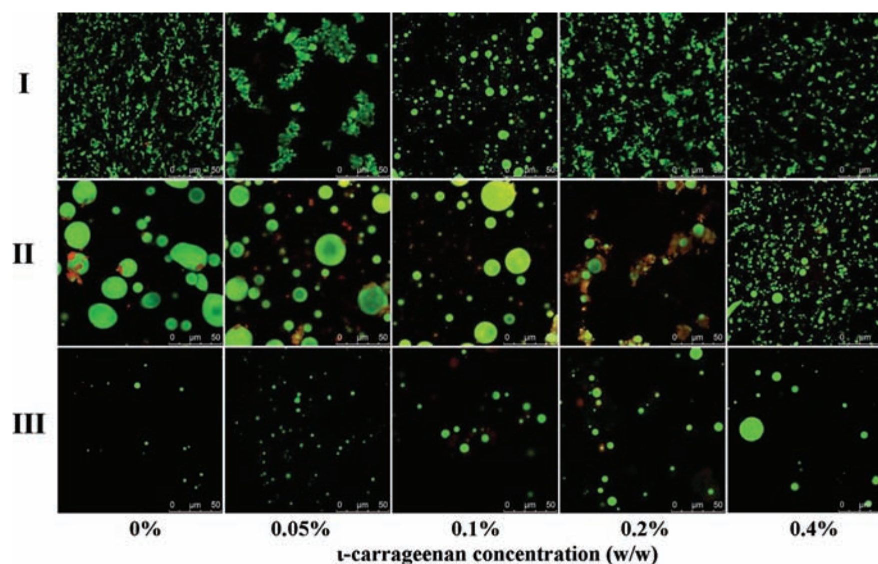

Figure 5. Representative confocal images of soybean oil body emulsions stabilized with different concentrations of ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) as they pass through an in vitro digestion model: (I) before digestion, (II) after digestion with pepsin solution (36 mg mL^{-1}, 0.1 M HCl) at 37 °C for 60 min, and (III) after digestion with pancreatin/bile salt mixture (100 mmol L^{-1} sodium deoxycholate, 5 mg mL^{-1} pancreatin, and 100 mmol L^{-1} sodium bicarbonate solution) and additional lipase solution (100 mg mL^{-1}) at 37 °C for 120 min.

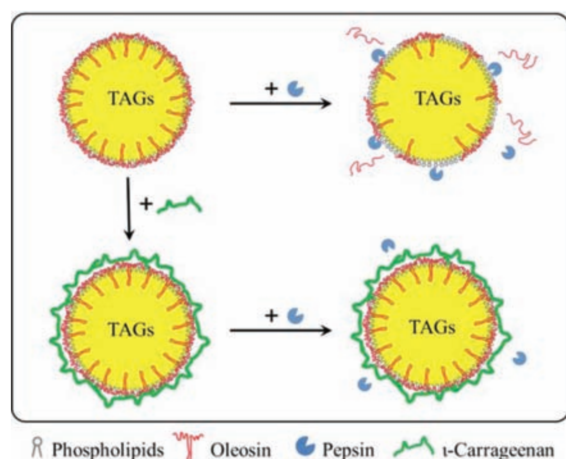

Figure 6. Schematic representation of soybean oil body emulsions stabilized with and without ι-carrageenan resulting from pepsin addition.

slightly larger than the emulsions stabilized with 0 and 0.05% w/w ι-carrageenan (1.06 and 1.45 μm, respectively) after digestion with lipase (Figures 3−5). This might be because the ability of carrageenan at high concentration could increase the attraction between droplets through a steric-exclusion (osmotic) effect, thereby inducing depletion flocculation,[32,36] whereas bridging flocculation occurred in protein-stabilized emulsions when charged polysaccharides were mixed at low concentration.[32,37] Both depletion flocculation and bridging flocculation might have an effect on lipase digestion, but the effects of depletion flocculation were stronger than those of bridging flocculation in our study.

Release of Fatty Acids and Tocopherol from Soybean Oil Body Emulsions Stabilized with ι-Carrageenan during Simulated Digestion. The bioaccessibility of fatty acids in soybean oil body emulsions stabilized with different concentrations of ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) has been shown in Table 1. The five major fatty acids consistently present in the soy oil of the various emulsions (mainly as TAGs >98%) were palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid with the relative proportions around 15, 5, 19, 51, and 10%, respectively (Table 2). All further calculations were carried out on the basis of only these fatty acids. The total concentration of fatty acids was 450 mg per assay. Following ultracentrifugation recovery of the micellar fraction after simulated digestion and analysis of free fatty acids present in micelles, the bioaccessibility of total and individual fatty acids was calculated (Table 1). The transfer of fatty acids from emulsion droplets to the micellar fraction

Table 1. Bioaccessibility of Fatty Acids during Simulated Digestion of Soybean Oil Body Emulsions Stabilized with ι-Carrageenan (n = 3 ± SD)

emulsion	free fatty acid content in the micellar phase after digestion (mg)					total per assay (mg)	bioaccessibility[a] (%)
	palmitic (16:0)	stearic (18:0)	oleic (18:1)	linoleic (18:2)	linolenic (18:3)		
0% ι-carrageenan	19.8 ± 1.3 a	6.8 ± 0.3 a	65.0 ± 3.7 a	164.8 ± 10.5 a	25.8 ± 1.7 a	282.3 ± 17.4 a	62.7 ± 3.9 a
0.05% ι-carrageenan	11.5 ± 1.6 b	4.3 ± 0.5 b	40.6 ± 5.8 b	101.2 ± 15.0 b	15.6 ± 2.1 b	173.2 ± 24.9 b	38.5 ± 5.5 b
0.1% ι-carrageenan	5.1 ± 0.6 c	1.8 ± 0.2 c	31.5 ± 3.6 bc	80.2 ± 9.3 bc	11.8 ± 1.4 c	130.3 ± 15.1 c	29.0 ± 3.4 c
0.2% ι-carrageenan	7.1 ± 0.2 c	2.4 ± 0.1 c	28.1 ± 1.0 c	71.4 ± 2.9 c	10.9 ± 0.5 c	119.9 ± 4.6 c	26.6 ± 1.0 c
0.4% ι-carrageenan	2.1 ± 0.1 d	0.7 ± 0.0 d	8.5 ± 2.5 d	22.5 ± 6.2 d	3.4 ± 0.9 d	37.2 ± 9.7 d	8.3 ± 2.2 d

[a]Calculated on the basis of each emulsion containing 450 mg of total fatty acid per assay. Different letters in the same column following each figure indicate significant differences (P < 0.05, ANOVA).

Table 2. Fatty Acid Composition of the Oil before Digestion and Free Fatty Acids Composition in the Micellar Phase after Digestion ($n = 3 \pm$ SD)

soy oil and emulsion	free fatty acid composition in parent oil and the micellar phase after digestion[a] (rel mol %)				
	palmitic (16:0)	stearic (18:0)	oleic (18:1)	linoleic (18:2)	linolenic (18:3)
soy oil	14.8 ± 2.8 a	5.2 ± 1.0 a	19.1 ± 3.4 a	51.2 ± 9.3 a	9.8 ± 1.8 a
0% ι-carrageenan	7.0 ± 0.5 b	2.4 ± 0.1 bc	23.0 ± 1.3 a	58.4 ± 3.7 a	9.2 ± 0.6 a
0.05% ι-carrageenan	6.6 ± 0.9 b	2.5 ± 0.3 b	23.5 ± 3.4 a	58.5 ± 8.7 a	9.0 ± 1.2 a
0.1% ι-carrageenan	3.9 ± 0.4 b	1.4 ± 0.1 c	24.2 ± 2.8 a	61.5 ± 7.1 a	9.1 ± 1.1 a
0.2% ι-carrageenan	5.9 ± 0.2 b	2.0 ± 0.1 bc	23.5 ± 0.8 a	59.6 ± 2.4 a	9.1 ± 0.4 a
0.4% ι-carrageenan	5.5 ± 0.2 b	1.9 ± 0.0 bc	22.8 ± 6.6 a	60.5 ± 6.8 a	9.2 ± 2.5 a

[a]Values for free fatty acid composition in the micellar phase after digestion are calculated from the results shown in Table 1 from triplicate samples. Different letters in the same column following each figure indicate significant differences ($P < 0.05$, ANOVA).

was greatest in the soybean oil body emulsions stabilized without ι-carrageenan (62.7 ± 3.9%), then followed by the emulsions stabilized with 0.05, 0.1, and 0.2% w/w ι-carrageenan (38.5 ± 5.5, 29.0 ± 3.4, and 26.6 ± 1.0%, respectively), which were all greater ($P < 0.05$) than for the emulsions stabilized with 0.4% w/w ι-carrageenan (8.3 ± 2.2%). Examination of the bioaccessibility of individual fatty acids indicated that palmitic acid and stearic acid were transferred to a less extent than oleic, linoleic, and linolenic acid for all of the emulsions stabilized with ι-carrageenan, and these were significant ($P < 0.05$) for all of the soybean oil body emulsions stabilized with different concentrations of ι-carrageenan (Table 2).

The tocopherol concentrations in soybean oil body emulsions (5% w/w oil) stabilized with different concentrations of ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively) are shown in Table 3. The concentration of tocopherol in initial soybean oil body emulsions was obtained (725.5 ± 16.9 μg) as calculated from standard curves. Following simulated gastric and then duodenal digestion and measurement of tocopherol in micellar fractions, the bioaccessibility (percent of tocopherol transfer from emulsion to micellar fraction) for the emulsions stabilized with different concentrations of ι-carrageenan was calculated and followed the order 0% ι-carrageenan (59.7 ± 2.2%) > 0.05% ι-carrageenan (44.6 ± 1.0%) > 0.1% ι-carrageenan (43.3 ± 1.3%) > 0.2% ι-carrageenan (24.1 ± 1.7%) > 0.4% ι-carrageenan (19.4 ± 1.2%).

Table 3. Bioaccessibility of Tocopherol during Simulated Digestion of Soybean Oil Body Emulsions Stabilized with ι-Carrageenan[a] ($n = 3 \pm$ SD)

emulsion	released tocopherol (μg)[b]	bioaccessibility[c] (%)
0% ι-carrageenan	433.3 ± 16.1 a	59.7 ± 2.2 a
0.05% ι-carrageenan	323.6 ± 7.4 b	44.6 ± 1.0 b
0.1% ι-carrageenan	313.8 ± 9.3 b	43.3 ± 1.3 b
0.2% ι-carrageenan	174.7 ± 12.0 c	24.1 ± 1.7 c
0.4% ι-carrageenan	140.9 ± 8.5 d	19.4 ± 1.2 d

[a]Total available tocopherol per assay in the initial soybean oil body emulsion (5% w/w oil) was 725.5 ± 16.9 μg. [b]Total tocopherol per assay released into micellar phase. [c]Percent of total tocopherol per assay released into micellar phase. Different letters in the same column following each figure indicate significant differences ($P < 0.05$, ANOVA).

In this study, the in vitro techniques were used to measure the bioaccessibility of fatty acids and tocopherol from soybean oil body emulsions stabilized with ι-carrageenan (0, 0.05, 0.1, 0.2, and 0.4% w/w, respectively). The transfer of these fatty acids (palmitic, stearic, oleic, linoleic, and linolenic acid), in total or individually from emulsions to micelles, was significantly greater in soybean oil body emulsion without ι-carrageenan as compared to the emulsions stabilized with ι-carrageenan (0.05, 0.1, 0.2, and 0.4% w/w, respectively). The lower micellar incorporation of fatty acids from oil bodies stabilized with ι-carrageenan witnessed in this study was likely a result of reduced action of pepsin and lipase on the intact oil bodies (Figures 1 and 3−5) as we discussed previously, which was a prerequisite for the transfer of free fatty acids to micelles. In all five emulsions, the relative bioaccessibility of palmitic and stearic fatty acids was less than that of oleic, linoleic, and linolenic fatty acid (Table 2). This was contrary to the results from sunflower oil bodies.[13] Perhaps soybean oil bodies have a nonrandom distribution of TAG molecules, with higher melting point molecules enriched in the internal core of oil bodies that were not beneficial for lipase enzymolysis. The other possible reason was that saturated fatty acids (palmitic and stearic acid) with direct carbon chain on the TAGs molecules,[38] which might interact strongly with ι-carrageenan, were not prone to be acted by lipase. In the simulated intestinal fluids, carrageenen may also alter lipid digestibility by binding to calcium ions that are required to remove long chain fatty acids from lipid droplet surfaces during the in vitro digestion.[39] Therefore, the relative content of palmitic and stearic fatty acids in the micelles generally decreased with the increasing concentration of ι-carrageenan (Table 2). However, sunflower oil bodies without ι-carrageenan were beneficial for the action of 1,3-specific lipase contained in the pancreatin, and unsaturated fatty acids were predominant at the sn-2 position of the TAG molecules.[13] The existence of ι-carrageenan in soybean oil body emulsions might reduce the saturated fatty acid intake, which was helpful to keep healthy for humans because saturated fatty acid could increase the cholesterol concentrations.[40] The percentage transfer of vitamin E from the emulsions to the micellar phase was highest ($P < 0.05$) for the emulsions without ι-carrageenan, followed by the emulsions stabilized with 0.05, 0.1, and 0.2% w/w ι-carrageenan, and was significantly lower for the emulsions stabilized with 0.4% w/w ι-carrageenan. These results were generally in agreement with those of micellar incorporation of fatty acids and supported the notion that oil bodies stabilized with ι-carrageenan (0.05, 0.1, 0.2, and 0.4% w/w, respectively) were not digested as rapidly as the emulsions without ι-carrageenan under the conditions used in this investigation. The bioaccessibility of vitamin E was highly dependent on the food matrix due to different locations within the matrix, different physiochemical properties, the influence of associated compounds, and the surrounding layer of oil droplets.[11]

The concentrations of fatty acids and tocopherol in the micelles decreased with the increasing concentrations of ι-carrageenan (Tables 1 and 3). As we discussed previously, different concentrations of ι-carrageenan had an effect on pepsin and lipase digestion, thereby affecting interfacial layer, microstructure, and stability of oil body emulsions. Furthermore, ι-carrageenan in soybean oil body emulsions might simultaneously influence the release of fatty acids and tocopherol to the micelles. There were some factors for decreasing pepsin and lipase activity, which accounted for the release and utilization of lipophilic components in oil body emulsions. The phospholipid/protein coat of oil bodies was responsible for reducing pepsin and lipase activity, and oil bodies behaved like other proteolipid organelles, such as milk fat globules, when they were considered as a substrate for lipolytic activity.[41] On the other hand, when the emulsions were digested under conditions representing digestion in the human stomach, the breakdown of oleosins and the fragment molecules would cause a decrease in the available surface area for lipase activity, both in the stomach by gastric lipase and once the emulsions have been transferred from the stomach into the intestine. Most significantly, the steric hindrance of ι-carrageenan in the emulsions during the simulated digestion might prevent pepsin and lipase from combing with oleosins and lipids of soybean oil bodies, thus reducing the activity of enzymes (Figure 6). Carrageenan may also slow down lipid digestion rate by binding to calcium ions in the simulated intestinal fluids as we pointed out previously, and the calcium ions are necessary to remove long chain fatty acids from lipid droplet surfaces.[39] These might result in a slower release of lipids and associated lipophilic compounds, for example, vitamin E from the emulsion into the micellar phase of the gut digestion. Indeed, other authors also have reported that the interfacial composition of emulsions would have an effect on the rate of enzymatic hydrolysis.[18] The physicochemical properties of the oil−water interface could affect the adsorption of the enzyme and the catalytic cleavage of fatty acids from the triglyceride molecules.[42]

If all of the experimental results were considered together and held true in vivo, it would suggest that soybean oil bodies stabilized with ι-carrageenan were not as rapidly digested as the emulsions without ι-carrageenan. ι-Carrageenan in soybean oil body emulsions might form a protective layer around the oil body droplets and that it promoted extensive droplet flocculation, both of which inhibited the ability of pepsin and lipase to interact with the soybean oil body droplets. Potentially, this would lead to a reduced rate of absorption of lipids and associated lipophilic compounds, which required micellar incorporation prior to intestinal absorption. The slower rate of digestion of lipids affected gastrointestinal tract physiology and might result in increasing satiety.[43,44] Therefore, emulsion properties that promoted slower digestion were of fundamental and applied interest and could be effectively employed to help reduce calorie intake and utilize lipophilic micronutrients.

AUTHOR INFORMATION

Corresponding Author

*Tel: +86-20-87114262. Fax: +86-20-87114263. E-mail: fexqyang@163.com or fexqyang@scut.edu.cn.

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