JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Interfacial Protein Cross-Linking on the in Vitro Digestibility of Emulsified Corn Oil by Pancreatic Lipase

SANDRA SANDRA, ERIC ANDREW DECKER, AND DAVID JULIAN MCCLEMENTS*

Biopolymers and Colloids Research Laboratory, Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003

The objective of this study was to investigate the influence of globular protein interfacial cross-linking on the in vitro digestibility of emulsified lipids by pancreatic lipase. 3% (wt/wt) corn oil-in-water emulsions stabilized by either lecithin or β-lactoglobulin were prepared (pH 7). A portion of the β-lactoglobulin stabilized emulsions was subjected to a heat treatment known to cross-link the adsorbed globular proteins (85 °C, 20 min). Pancreatic lipase and bile extract were then added to each emulsion at 37 °C (pH 7) and the evolution of the particle charge, particle size, appearance and free fatty acids released were measured over a period of 2 h. The rate and extent of lipid digestion did not differ greatly between lecithin and β-lactoglobulin stabilized emulsions, nor did it differ greatly for unheated (BLG-U) or heated (BLG-H) β-lactoglobulin stabilized emulsions. For example, the initial rate of lipid digestion was found to be 3.1, 3.4, and 2.3 mM fatty acids s^{-1} m^{-2} of lipid surface for droplets stabilized by BLG-U, BLG-H, and lecithin, respectively. Pancreatic lipase was able to adsorb to the droplet surfaces and access the emulsified lipids, regardless of the initial interfacial composition and the fact that some of the original emulsifier appeared to remain at the oil−water interface during digestion. These results help to explain why the human body is so efficient at digesting dietary triacylglycerols.

KEYWORDS: Emulsions; lipase; lipid digestion; globular proteins; interfacial cross-linking

INTRODUCTION

Lipids play a major role in the human diet, providing energy, essential nutrients and bioactive components (*1*). Consequently, it is important to understand the factors that impact their bioavailability. Lipids might be present in foods in a variety of different forms, including as bulk, structural, emulsified, or interfacial structures (*2–4*). In this study, we are primarily interested in the digestion of protein-coated lipid droplets, since these are present in many traditional foods (e.g., milk and cream) and they may be useful in nutraceutical delivery systems (*5, 6*). After ingestion emulsified lipids undergo a variety of compositional and structural changes as they pass through the human gastrointestinal (GI) tract (*4, 7*). In the mouth, an ingested emulsion is: mixed with saliva; changes its pH, ionic strength and temperature; is acted upon by digestive enzymes; interacts with the surfaces of the tongue, mouth and throat; and, experiences a complex flow/force profile (*8–14*). These factors may result in changes in lipid droplet size, aggregation state, and interfacial properties. In the stomach, the emulsion is: exposed to highly acidic conditions (pH 1−3); mixed with enzymes (e.g., proteases and gastric lipase) and surface active substances (e.g., phospholipids and proteins); and, experiences a complex flow/force profile (*7, 15–20*). Endogenous and exogenous surface active substances may displace the original surface active materials present at the oil−water interface. Gastric lipase initiates the digestion of emulsified lipids (*18, 20*), while proteases may digest proteins adsorbed to the droplet surfaces (*21–23*). Consequently, there may be further changes in the composition, structure, and interfacial properties of the lipid droplets. In the small intestine, the partially hydrolyzed and emulsified lipids are mixed with digestive juices containing bile salts, phospholipids, pancreatic lipase, colipase and bicarbonate (*20, 24*). As a result of mixing with these alkali digestive juices the pH of the emulsion in the small intestine moves close to neutral. Surface active substances present in the small intestine compete with surface active materials initially present at the lipid droplet surfaces, and may displace them. Pancreatic lipase may adsorb to the lipid droplet surfaces and hydrolyze the conversion of triglycerides and diglycerides into monoglycerides and free fatty acids (*25*), while proteases may carry out hydrolysis of any interfacial proteins (*23*). Consequently, there will be further changes in the composition, structure and interfacial properties of the lipid droplets (*21*). The lipid digestion products released from the droplets are eventually incorporated into bile salt/phospholipid micelles and vesicles that are transported to the enterocytes where they are absorbed (*26*).

In this study, we examine the impact of interfacial protein cross-linking on the lipase digestibility of lipid droplets coated by a model globular protein (β-lactoglobulin, β-Lg). In particular, we focused on the impact of heat treatment on the rate and extent of lipid digestion, since heat treatment is known to

* Author to whom correspondence should be addressed (e-mail mcclements@foodsci.umass.edu).

10.1021/jf800741w CCC: $40.75 © 2008 American Chemical Society
Published on Web 07/08/2008

promote interfacial cross-linking of this protein (*27, 28*). Previous studies have established that the composition and structure of the interfacial layer surrounding lipid droplets affects the rate of lipid digestion, as well as the release rate of any entrapped lipophilic constituents (*2, 16, 17, 19, 20, 29–36*). As mentioned earlier, there may be appreciable alterations in the composition and characteristics of the interfacial layer surrounding protein-coated lipid droplets as they pass through the human GI tract (*37*). The protein layer surrounding the lipid droplet could be desorbed (by surface active substances) or digested (by enzymes) prior to any lipid digestion occurring. Previous studies have shown that cross-linking adsorbed globular proteins by heating above their thermal denaturation temperature makes them more difficult to displace from lipid droplet surfaces by surface active substances (*27, 38*). We therefore hypothesize that cross-linked proteins should be more difficult to displace from lipid droplet surfaces by surface-active substances in digestive juices, which may impact the rate and extent of lipase digestibility. In addition, a cross-linked protein layer may also restrict access of digestive enzymes to emulsified lipids, which should also impact the rate and extent of lipase digestibility. This would have important consequences for formulating delivery systems to control lipid bioavailability (*5*), or for formulating foods that ensure high bioavailability of ingested lipids for individuals with compromised digestive systems (*24*). The conditions used in this study are designed to simulate the digestion of emulsified lipids in the small intestine. In reality, there may be substantial changes in interfacial composition and properties when an emulsion passes through the mouth and stomach, so future studies should also examine these factors.

MATERIALS AND METHODS

Materials. Mazola corn oil (ACH Food Companies, Inc., Memphis, TN) was obtained from the local grocery store and used without further purification. BioPURE β-lactoglobulin (β-Lg) was provided by Davisco Foods International, Inc. (Le Sueur, MN). A 0.62% (w/w) β-Lg solution was prepared in 10 mM phosphate buffer at pH 7. The solution was stirred for at least 2 h at ambient temperature (25 ± 3 °C) and then kept overnight in the refrigerator prior to use. Lecithin (Ultralec F deoiled lecithin) was supplied by ADM (Decatur, IA). A 0.62% (w/w) lecithin dispersion was made in 10 mM sodium phosphate buffer at pH 7 and stirred at ambient temperature (25 ± 3 °C) overnight prior to use. The pH of the emulsifier solution was adjusted to pH 7, if needed, prior to use. Porcine pancreatic lipase type II (batch 115K0681, EC 232-619-9) and porcine bile extract (batch 106K0097, EC 232-369-0) were obtained from Sigma Aldrich (St. Louis, MO). All analytical grade chemicals were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma Aldrich (St. Louis, MO).

Emulsion Preparation. 3% (w/w) corn oil and 97% (w/w) of emulsifier solution (0.62% w/w in aqueous phase) were mixed using a high speed blender (Tissue Tearer, model 985370-395, BioSpec Products, Inc., Bartlesville, OK) for 2 min. This coarse emulsion was then homogenized for 3 passes (2700 psi first stage, 300 psi second stage) at room temperature (25 ± 3 °C) using a two-stage homogenizer (APV, Lake Mills, WI). The final temperature of the emulsion did not exceed 30 °C (as determined by dipping a thermometer in the emulsion that exited the homogenizer). The pH of the emulsion was adjusted to 7.0, if it had changed by more than 0.1 units from the initial value in the emulsifier solution. The β-Lg stabilized emulsion was divided into two portions that received different heat treatments: *unheated emulsions* (BLG-U) were held at ambient temperature (25 ± 3 °C); *heat-treated emulsions* (BLG-H) were subjected to heat treatment at 85 °C (±2 °C) for 20 min in a water bath. Previous studies showed that β-Lg thermally denatured when held at this temperature (*39, 40*). About 10 mL of the emulsions were placed into test tubes. The time to reach the heating temperature was between 110 and 150 s. After heating, the emulsions were quickly cooled down in an ice−water bath. The emulsions were kept in a refrigerator (4 °C) until used within the in vitro digestion model (from 1 to 3 days). Results are reported as the average and standard error of measurements on three batches of emulsion prepared on different days.

In Vitro Pancreatic Lipase Digestion. The emulsion, bile extract solution, pancreatic lipase solution, and pancreatic lipase/bile extract solutions were incubated at 37 (±2) °C for at least 10 min prior to use. To 30 mL emulsion, 7.5 mL of solution containing 187.5 mg bile extract and/or 300 mg pancreatic lipase in phosphate buffer (10 mM, pH 7) was added, and then stirred for 30 s. The final concentrations of the bile extract and pancreatic lipase were 5 mg/mL and 8 mg/mL, respectively. The pH of the solution was adjusted to 7.0, if required. The samples were then incubated at 37 (±2) °C in an incubated shaker (Innova 4080, New Brunswick Scientific, Edison, NJ) for 2 h at 95 rpm to mimic the intestinal digestion process (*2*). Subsamples were taken periodically during this incubation time for analysis. At the end of the 2 h digestion, the pH of the samples were 6.81 (±0.04), 6.68 (±0.05), and 6.69 (±0.01) for unheated β-Lg, heated β-Lg, and lecithin emulsions, respectively. The samples were then stored for another 22 h (total 24 h digestion) to highlight any differences between the emulsions, and then centrifuged at 2500 g for 10 min (Fisher Scientific Centrific, centrifuge model 225A, Suwanee, GA).

Optical Microscopy. Samples were periodically taken during the 2 h digestion period, and a drop was placed on a glass microscope slide and covered by a coverslip. The microstructure of the emulsion was observed using optical microscopy (Nikon Eclipse 80i, Nikon Instrument Inc., Japan), with a 50× objective lens. The samples were held at 37 (±1) °C using a temperature controlled microscope stage (LTS 120 stage, PE94 Temperature Controller, Linkam Scientific Instruments Ltd., Surrey, U.K.). Images were acquired using a digital camera (QImaging Retiga 2000R Fast1394 camera, QImaging, Surrey, Canada) and analyzed using a software program (NIS-Elements Imaging Software BR 2.30, Nikon Instrument Inc.).

ζ-Potential Measurements. The ζ-potential of the lipid droplets in the emulsions was measured periodically during the 2 h digestion. In addition, the ζ-potential of a subsample collected after 2 h of digestion and then adjusted to pH 3 was measured (to ascertain whether protein was still adsorbed to the lipid droplet surfaces). Approximately 5−6 drops of the sample was added to 50 mL phosphate buffer (pH 7, 10 mM) during the digestion process, or to 50 mL phosphate buffer (pH 3, 10 mM) at the end of the 2 h digestion process. About 5−6 mL of diluted emulsion was then injected into the measurement chamber of a commercial particle electrophoresis instrument (Zetamaster, Malvern Instruments, Westborough, MA). Each individual measurement was determined from the average of five readings of the same sample at room temperature (25 ± 3 °C).

Static Light Scattering (SLS). The mean particle diameter ($d_{4,3}$ and $d_{3,2}$) and particle size distribution (PSD) of samples were measured using a static light scattering instrument (Mastersizer S, Malvern Instruments). A presentation code of **3NAD** was used in the analysis of the light scattering data, which corresponded to an oil phase refractive index of 1.47 and a water phase refractive index of 1.33. A few drops of sample were dispersed in approximately 125 mL phosphate buffer (10 mM, pH 7) in the sample chamber with agitation until approximately 11−14% obscuration was obtained. Measurements were done in duplicates at ambient temperature.

Free Fatty Acid (FFA) Release. Lipase activity was determined by measuring the liberated free fatty acids (FFA) using a previously described titration method (*2*) with some modifications. Briefly, subsamples were taken at certain times during the 2 h digestion period. To 5 mL sample was added 10 mL of 95% ethanol (to inactivate the lipase) containing 3 drops of 1% phenolphthalein indicator, which was then titrated with 0.05 N NaOH to end point (pink color). A standard curve was prepared using oleic acid (0, 50, 100, 150, and 200 mM), and this was used to calculate the free fatty acid concentration of the samples. The free fatty acids liberated from the samples due to lipase activity were determined by subtracting the FFA concentration measured in the samples before digestion from that measured after digestion. The calculation of % FFA released was done by assuming that the average molecular mass of corn oil triacylglycerides is 800 g/mol and that 1 mol of triacylglyceride would be converted to 2 mol of free fatty acids and 1 mol of monoglyceride after 100% digestion. Measurements were done in duplicates.

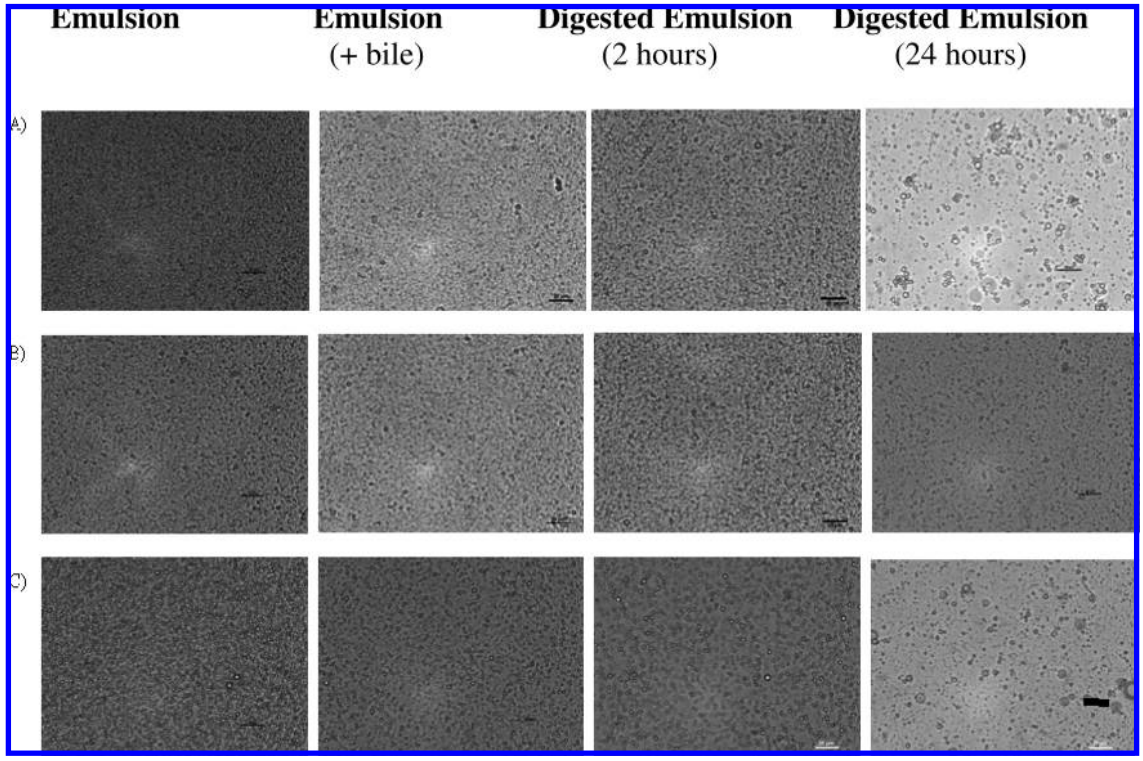

Figure 1. Optical microscopy images of emulsions before and during digestion (objective lens: 50 ×): (**a**) unheated β-Lg emulsionl (**b**) heated β-Lg emulsionl (**c**) lecithin emulsion. Scale bar represents 20 μm.

Opacity Measurements. The opacity of emulsion samples was determined using a laser backscattering method (Turbiscan Classic MA2000, Formulaction, l'Union, France). Ten milliliters of emulsion was mixed with either 2.5 mL bile extract or bile extract/lipase or buffer solution and put in test tubes. The samples were incubated at 37 °C during digestion with constant swirling (95 rpm). Prior to measurements samples were vortexed to make them homogeneous and then placed in the measurement chamber of the vertical scanning laser instrument (Turbisan Classic MA2000, SCI-TEC Inc., Sandy Hook, CT), and the backscattering was measured periodically (about every 30 min) at a height of 35 mm during 2 h digestion.

Statistical Analysis. ANOVA and Tukey-HSD analysis were done by SPSS v15.0 (SPSS Inc., Chicago, IL) using 95% confidence level.

RESULTS AND DISCUSSION

Emulsion Microstructure. The change in the microstructure of the three emulsions during the digestion process was measured (**Figure 1**). Initially, the emulsion stabilized by lecithin contained appreciably larger oil droplets than the ones stabilized by β-Lg, which indicated that the globular protein was better at facilitating the formation and/or preventing the aggregation of lipid droplets during homogenization. Previous studies showed that β-Lg is effective at forming small droplets during homogenization that are stable to aggregation at neutral pH and low salt concentrations (*41, 42*). The addition of bile extract to the emulsions used in this study did not cause any observable changes in their microstructure. The microstructure of the emulsions did not change appreciably during the first 2 h of digestion for all emulsions, however, appreciable changes were observed after 24 h digestion (**Figure 1**). The unheated β-Lg emulsion showed coalescence and flocculation, while the heated β-Lg emulsion showed a much lower extent of coalescence. The lecithin emulsion also showed some increase in lipid droplet size after 24 h digestion, which can be attributed to droplet coalescence. On the other hand, the lecithin emulsion containing no lipase and bile extract remained relatively stable for 24 h storage at 37 °C (results not shown). It is interesting to note that the lipid droplets did not completely disappear after the digestion period, which suggested either (i) the lipid phase was not fully digested or (ii) there was insufficient bile salts and phospholipids present to solubilize all the lipid digestion products formed (i.e., mono- and diacylglycerols and free fatty acids). Previous studies showed that lipid digestion is inhibited when the digestion products are not removed from the droplet surfaces (*24*), which may at least partially account for the fact that not all the emulsified lipid was digested.

The particle size distribution and mean particle diameter of the emulsions was initially measured without the addition of lipase and/or bile extract to show the ability for the emulsifiers to produce small lipid droplets. Initially, all of the emulsions had a monomodal particle size distribution, i.e., there was a single peak in the particle size distribution (**Figure 2**). The initial volume-weighted ($d_{4,3}$) mean particle diameters of unheated β-Lg, heated β-Lg, and lecithin emulsions were 0.47 (±0.01), 0.46 (±0.01), 0.75 (±0.02) μm, respectively. The initial surface-weighted ($d_{3,2}$) mean particle diameters of the same emulsions were 0.30 (±0.01), 0.30 (±0.02), and 0.36 (±0.01) μm, respectively. These results again showed that the globular proteins were capable of forming emulsions with smaller initial droplet sizes, which can be attributed to their ability to facilitate droplet disruption within the homogenizer and prevent subsequent droplet aggregation (*6*). The heat-treatment of β-Lg emulsions after homogenization did not promote droplet aggregation as shown by the similar mean particle diameter between heated and unheated β-Lg emulsions. This supports previous studies that have shown that β-Lg emulsions are stable to heating at neutral pH, provided they are heated in the absence of high levels of salt (*41*). On the other hand, previous studies have shown that these heating conditions are sufficient to cause appreciable interfacial protein cross-linking through disulfide bond formation (*27, 43*). These differences in interfacial cross-linking might be expected to (1) impact the adsorption of bile

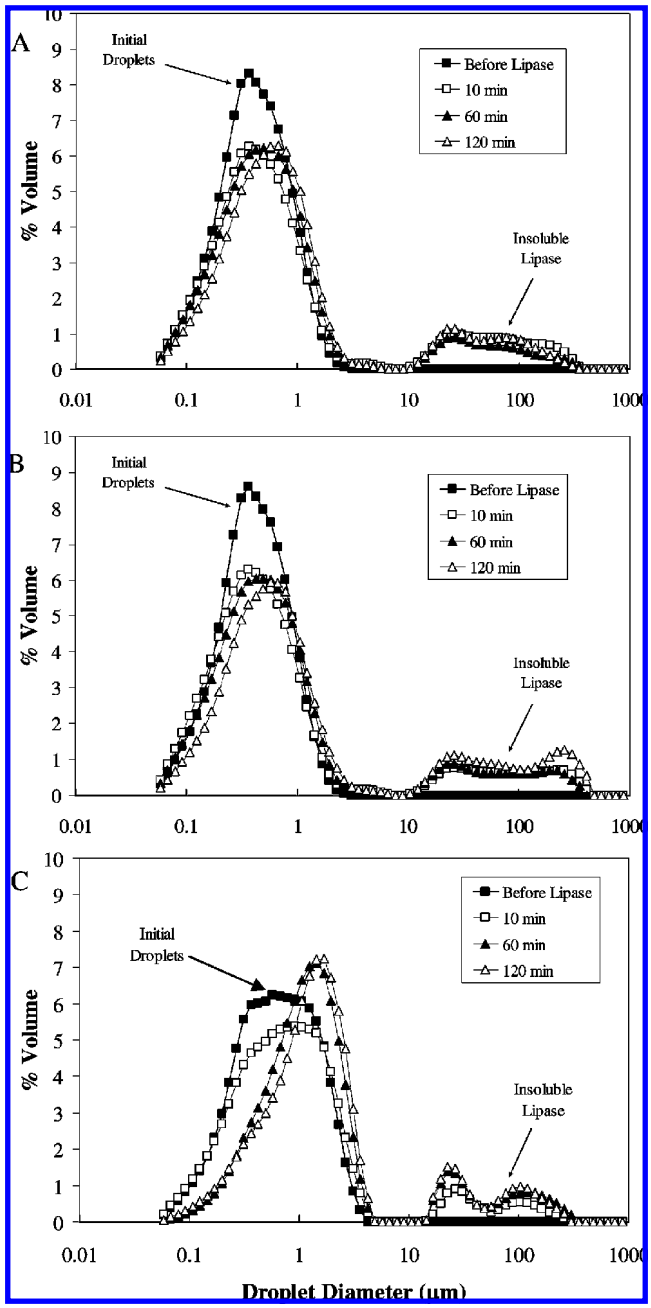

Figure 2. Particle size distributions of corn oil-in-water emulsions with different emulsifiers as a function of pancreatic lipase digestion time: (**a**) unheated β-Lg emulsion; (**b**) heated β-Lg emulsion; (**c**) lecithin emulsion. The "before lipase" emulsion contained bile extract, but no lipase. The times shown are the incubation periods in the digestion model.

salts and lipase to the lipid droplets surfaces, and (2) to impact the access of lipase to the emulsified lipids.

With the addition of bile extract, the $d_{4,3}$ values of unheated β-Lg, heated β-Lg, and lecithin emulsions were 0.47 ($\pm$0.01), 0.48 ($\pm$0.01), 0.75 ($\pm$0.00) μm, respectively. The $d_{3,2}$ values of the same emulsions were 0.30 ($\pm$0.02), 0.32 ($\pm$0.01), 0.36 ($\pm$0.01) μm, respectively. All three emulsions initially had a monomodal particle size distribution before and after bile extract addition. These results indicated that the addition of bile extract alone did not cause an appreciable alteration in the particle size distribution of the three types of emulsion i.e., it did not promote droplet flocculation or coalescence. On the other hand, when lipase extract was added to the emulsions a second peak of large particles (d = 10−1000 μm) was observed in the particle size

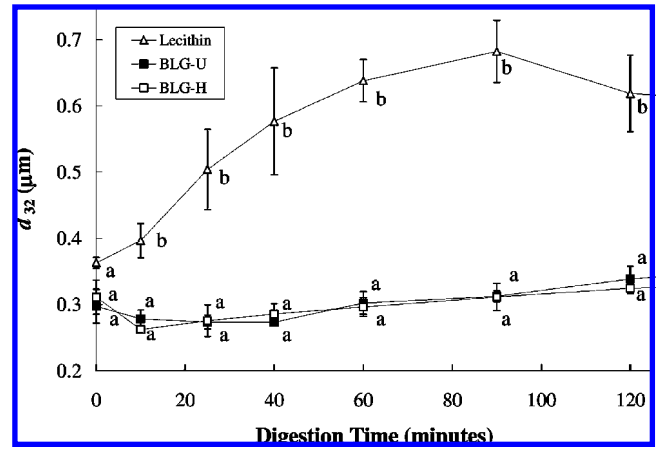

Figure 3. Surface-weighted mean particle diameters ($d_{3,2}$) of corn oil-in-water emulsions with different emulsifiers as a function of pancreatic lipase digestion. The lower case letters indicate samples that were significantly different ($p < 0.05$) at the same measurement time.

distribution profiles (**Figure 2**). This peak can be attributed to the fact that the lipase extract contained some insoluble particulate matter that also scattered light—the lipase extract solution had a cloudy appearance. During digestion there were alterations in the particle size distribution of the emulsions, which could be attributed to physicochemical changes resulting from lipid droplet digestion and/or aggregation (**Figure 2**). One would expect a decrease in droplet size if lipid digestion products (free fatty acids and monoacylglycerols) left the droplets and became incorporated within bile salt/phospholipid micelles. On the other hand, one would expect an increase in droplet size if coalescence occurred due to displacement or digestion of the emulsifiers originally stabilizing the droplets. Our results showed that there was a gradual shift of the peak corresponding to the lipid droplets to larger droplet sizes during the digestion period, which suggested that there was some coalescence in the emulsions (**Figure 2**).

In order to highlight the changes occurring in the lipid droplets we calculated the mean particle diameter of only those particles with $d < 10$ μm, so as to avoid the contribution from the insoluble lipase extract (**Figure 3**). These results indicated that there was little change in the mean particle size of the protein-stabilized lipid droplets during the 2 h digestion period, but that appreciable growth of the lecithin-stabilized lipid droplets occurred (**Figure 3**). These results suggest that the droplets initially coated by lecithin were more unstable to aggregation than the ones initially coated by protein. A rough indication of the change in lipid droplet concentration during digestion was obtained from backscattering measurements (**Figure 4**). These results suggest that there was little change in the concentration of lipid droplets present in the protein-stabilized emulsions, but that there was an appreciable decrease in the concentration of droplets in the lecithin-stabilized emulsions. Nevertheless, this decrease may also be partially due to the increase in droplet size that occurred during digestion, which would also impact the light scattering properties of the droplets (*44*). These results supported the particle size distribution measurements, which showed that there was little change in the relative volume of lipid droplets within the emulsions during digestion (**Figure 2**). One reason that the lecithin-coated droplets may be more susceptible to dissolution than the protein-coated droplets is that lecithin molecules can be incorporated within the mixed micelles and vesicles that normally solubilize lipid digestion products (*36*). Hence, it might be possible to solubilize more of the digestion products in emulsions containing lecithin, than in ones containing protein.

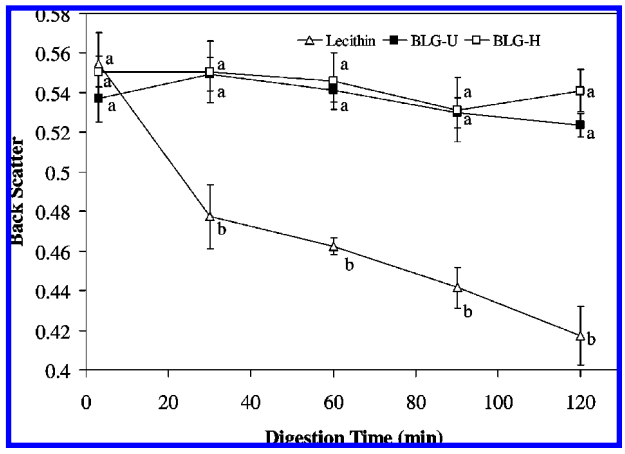

Figure 4. Change in backscattering from corn oil-in-water emulsions with different emulsifiers as a function of pancreatic lipase digestion time.

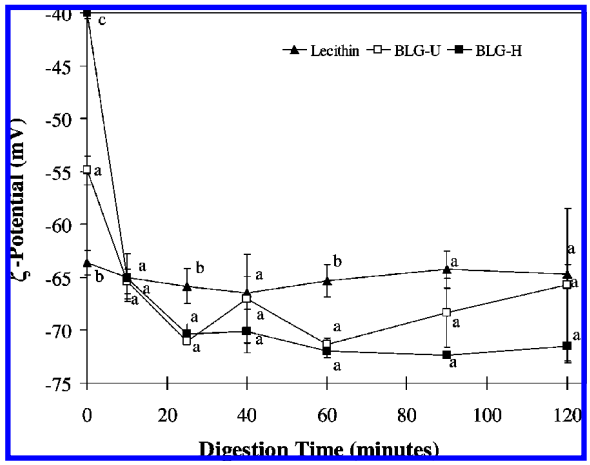

Figure 5. Electrical charge (ζ-potential) within corn oil-in-water emulsions stabilized by different emulsifiers as a function of pancreatic lipase digestion time.

Interfacial Composition. Some insights into the changes in interfacial composition during the digestion process were obtained using ζ-potential measurements (**Figure 5**). In the absence of bile or lipase, the ζ-potentials of the lipid droplets in the unheated β-Lg, heated β-Lg, and lecithin emulsions (pH 7) were −48.9 (±0.4), −41.3 (±0.3), and −53.7 (±0.9) mV, respectively. When bile extract was added, there was an appreciable change in the measured ζ-potential values: ζ = −54.9 (±1.4), −40.0 (±0.6), −63.6 (±0.7) mV, for unheated β-Lg, heated β-Lg, and lecithin emulsions. These changes in ζ-potential suggest that there was some alteration in the composition of the lipid droplet surfaces induced by the addition of bile extract. For the heated β-Lg emulsion, the change in ζ-potential was relatively small ($\Delta\zeta = +1.3 \pm 0.7$ mV), which might be an indication that bile salts were unable to absorb to the lipid droplet surfaces and/or displace cross-linked proteins. On the other hand, the ζ-potential on the lipid droplets in the unheated β-Lg ($\Delta\zeta = -6.0 \pm 1.5$ mV) and lecithin ($\Delta\zeta = -9.9 \pm 1.1$ mV) emulsions became much more negative after bile was added, which suggested that bile was able to adsorb to the droplet surfaces and possibly displace some of the original emulsifier. The ζ-potential of bile-stabilized lipid droplets in emulsions prepared using bile extract as an emulsifier were found to be −85.7 ± 1.1 mV at pH 7, which suggested that bile was unable to completely displace the original emulsifiers from the lipid droplet surfaces at the levels used.

Table 1. ζ-Potential Measurements of Oil-in-Water Emulsions Containing Lipid Droplets Initially Coated by Lecithin or β-Lactoglobulin (Unheated or Heated) at pH 3[a]

	ζ-potential (mV)		
sample	lecithin	BLG-U	BLG-H
emulsion	−39.7 ± 1.2 a	+38.7 ± 2.3 b	+47.1 ± 1.1 c
emulsion + bile	−44.9 ± 1.0 a	+27.3 ± 3.0 b	+29.3 ± 2.3 b
emulsion + bile + lipase*	−19.8 ± 0.6 a	+13.6 ± 0.8 b	+8.4 ± 3.9 b

[a] The emulsions containing lipase were measured after 2 h of digestion. Different letters in a row indicate significant difference at the 95% confidence level.

After the addition of lipase and bile extract, the ζ-potential for all three emulsions became more negative than when bile extract alone was added. For example, 10 min after the lipase and bile extract were added the ζ-potentials of all three emulsions were approximately −65 mV (**Figure 5**). This increase in negative charge suggested that there was a change in the lipid droplet surfaces, possibly due to adsorption of the lipase, but also possibly due to the release of anionic free fatty acids (FFA) that remained at the oil−water interface. The ζ-potential remained highly negative through the two hour digestion period, which may be attributed to the fact that most of the FFA released remained within the lipid droplets (see below).

Additional information about interfacial composition at the end of the 2 h digestion period was obtained by adjusting the emulsions to pH 3 (**Table 1**). Emulsions stabilized by β-lactoglobulin should be positively charged at this pH, whereas those stabilized by bile salts should be negatively charged. For comparison, we also measured the ζ-potential values of emulsions stabilized by the same emulsifiers at pH 3 in the absence or presence of bile extract (**Table 1**). The ζ-potential of lipid droplets coated by bile extract was also measured at pH 3 as −24.1 (±1.1) mV. The fact that the droplets are positively charged at pH 3 in the emulsions initially stabilized by β-lactoglobulin in the presence of bile extract or bile extract plus lipase suggested that some of the protein remains adsorbed to the lipid droplet surfaces. In other words, the bile salts do not completely displace the proteins from the oil−water interface. We used a bile salt concentration of approximately 5 mM in this study, which is close to the reported levels found in the small intestine during fasting (2.0−6.4 mM), but is low compared to the levels found after digestion of a meal (6.8−16.2 mM) (*36*). We therefore postulate that there may not have been sufficient bile salts present to completely displace all of the original emulsifier molecules from the lipid droplet surfaces.

It should be noted that there may have been additional enzymes in the lipase extract used in this study that may have digested the adsorbed lecithin (phospholipases) or β-lactoglobulin (proteases) coating the lipid droplets. These enzymes may have facilitated the displacement of lecithin and proteins from the oil−water interface.

Lipase Activity. The influence of protein cross-linking on the digestibility of lipid droplets was determined by monitoring the formation of free fatty acids (FFA) during lipase digestion (**Figure 6**). The initial rate of FFA formation in the emulsions containing lipid droplets initially coated by lecithin was less than that of the emulsions containing lipid droplets initially coated by β-lactoglobulin. This phenomenon can be attributed to the fact that the lecithin-coated droplets were larger than the protein-coated droplets, and hence the surface area of lipid exposed to lipase was less (*33*). Alternatively, it may have been due to differences in interfacial composition that altered the ability of the lipase to interact with the emulsified lipids. After 2 h of digestion approximately the same amount of free fatty acids was released from all three emulsions.

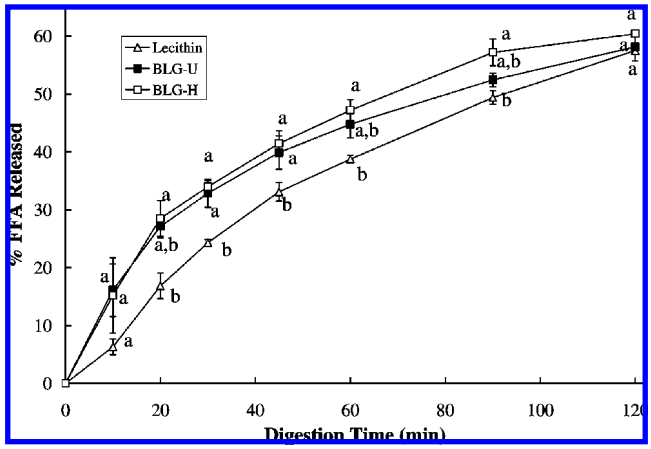

Figure 6. Free fatty acid release from corn oil-in-water emulsions stabilized with different emulsifiers as a function of pancreatic lipase digestion time.

We also found that there was little difference in the initial rates or the final amounts of free fatty acids released from the heated or unheated emulsions containing protein-coated lipid droplets (**Figure 6**). In other words, the lipase appeared to be able to access and digest the triacylglycerols encapsulated within the lipid droplets regardless of whether the globular proteins at the oil−water interface were cross-linked or not. Finally, there was a fairly similar decrease in solution pH after 2 h digestion for the three different systems, which can be attributed to similar levels of free fatty acid formation: 6.81 (±0.04), 6.68 (±0.05), and 6.69 (±0.01) for unheated β-Lg, heated β-Lg, and lecithin emulsions, respectively.

The initial rate of lipid digestion (k_S) was calculated as the free fatty acids produced per unit time per unit surface area of lipid: $k_S = k_i d_{32}/6\varphi V_E$, where k_i is the initial slope of a plot of free fatty acids released over time, V_E is the total volume of the emulsion, d_{32} is the initial mean particle diameter, and φ_i is the initial volume fraction of the lipid droplets. We calculated values of 3.1, 3.4, and 2.3 mM s^{-1} m^{-2} for the emulsions stabilized by BLG-U, BLG-H, and lecithin, respectively. These calculations again show that there is little difference between the rate of lipid digestion in the cross-linked (heated) and non-cross-linked (unheated) protein coated droplets.

After 24 h, the emulsion samples were centrifuged, which resulted in the formation of a number of visibly distinct layers. The lecithin emulsions separated into a transparent oil layer at the top, a turbid serum layer in the middle, and a precipitated pellet at the bottom. The unheated β-Lg emulsions separated into a transparent oil layer at the top, a transparent aqueous phase in the middle, and a precipitated pellet at the bottom. The heated β-Lg emulsions separated into an opaque cream layer at the top, a turbid aqueous phase in the middle, and a precipitated pellet at the bottom. These measurements indicated that the lipid droplets initially stabilized by lecithin or unheated β-Lg were more prone to coalescence, than the ones initially stabilized by heated β-Lg. Previous studies of the digestion of oil-in-water emulsions have shown that the upper oil layer contains undigested triacylglycerols and their digestion products, while the transparent or turbid serum layer contains mixed micelles and vesicles (*45*). Presumably, the precipitated pellet found in this study contained the insoluble matter from the pancreatic lipase.

The fact that relatively high amounts of fatty acids were formed (>50%), even though there was little change in lipid droplet concentration in the emulsions (**Figures 2** and **4**), suggested that fatty acids and other digestion products remained within the droplets during the digestion process. Presumably, the extent of lipid digestion may have been greater if there were more bile salts present in the aqueous phase to solubilize the digestion products formed.

Conclusions. This study has provided some interesting new insights into the impact of globular protein interfacial cross-linking on the ability of pancreatic lipase to access and digest emulsified lipids under conditions simulating the human small intestine. We found that the rate and extent of fatty acids released from emulsified lipids did not depend strongly on protein cross-linking. Our results suggested that lipase could adsorb to the droplet surfaces and gain access to the emulsified triacylglycerols regardless of the nature of the initial interfacial layer surrounding the droplets. Nevertheless, further studies are required using analytical techniques that directly measure the adsorption of lipase to the lipid droplet surfaces to support this hypothesis. Our experiments also showed that emulsified triacylglycerols can be digested by lipase, even when the digestion products are not released into the surrounding aqueous phase and solubilized by bile salt micelles. It should be noted that our experiments were carried out at relatively low bile salt concentrations, which should have accentuated the impact of the interfacial layer on the rate of lipid digestion. Hence, the fact that we did not observe an appreciable effect of protein cross-linking on lipid digestion in this study suggests that an effect would not be observed at higher bile salt concentrations.

In future studies, it would be useful to examine the impact of bile salt concentration on the rate and extent of lipid digestion since the levels used in this study were closer to those found in the fasting state of humans rather than in the postprandial state. In addition, it would be useful to use a more realistic in vitro digestion model that examined the influence of simulated mouth, stomach and small intestine conditions on lipid droplet properties and digestion. Particularly, it would be important to investigate the role of proteases in the stomach and small intestine that can digest the adsorbed protein layer, since these may alter the accessibility of the lipase to the lipids. Finally, our results support the observation that the human body is highly efficient at digesting lipids from a range of different sources that have different initial interfacial compositions. Nevertheless, animal and human feeding studies using lipid droplets coated by either cross-linked or non-cross-linked proteins would be needed to support this hypothesis.

LITERATURE CITED

(1) McClements, D. J.; Decker, E. A. , Lipids. In *Food Chemistry*, 4th ed.; Damodaran, S., Parkin, K. L., Fennema, O. R., Eds.; CRC Press: Boca Raton, FL, 2007; pp 155−216.

(2) Mun, S.; Decker, E. A.; McClements, D. J. Influence of emulsifier type on in vitro digestibility of lipid droplets by pancreatic lipase. *Food Res. Int.* **2007**, *40*, 770–781.

(3) Mun, S.; Decker, E. A.; Park, Y.; Weiss, J.; McClements, D. J. Influence of interfacial composition on in vitro digestibility of emulsified lipids: potential mechanism for chitosan's ability to inhibit fat digestion. *Food Biophys.* **2006**, *1*, 21–29.

(4) McClements, D. J.; Decker, E. A.; Park, Y. Physicochemical and structural aspects of lipid digestion. In *Understanding and Controlling the Microstructure of Complex Foods*; McClements, D. J., Ed.; CRC Press: Boca Raton, FL, 2007; pp 483−503.

(5) McClements, D. J.; Decker, E. A.; Weiss, J. Emulsion-based delivery systems for lipophilioc bioactive components. *J. Food Sci.* **2007**, *72*, R109–R124.

(6) McClements, D. J. *Food Emulsions: Principles, Practice, and Techniques*, 2nd ed.; CRC Press: Boca Raton, FL, 2005.

(7) Johnson, L. R. *Gastrointestinal Physiology*, 6th ed.; Mosby: St. Louis, MO, 2001.

(8) Vingerhoeds, M. H.; Blijdenstein, T. B. J.; Zoet, F. D.; van Aken, G. A. Emulsion flocculation induced by saliva and mucin. *Food Hydrocolloids* **2005**, *19*, 915–922.
(9) Malone, M. E.; Appelqvist, I. A. M.; Norton, I. T. Oral behaviour of food hydrocolloids and emulsions. Part 1. Lubrication and deposition considerations. *Food Hydrocolloids* **2003**, *17*, 763–773.
(10) Malone, M. E.; Appelqvist, I. A. M.; Norton, I. T. Oral behaviour of food hydrocolloids and emulsions. Part 2. Taste and aroma release. *Food Hydrocolloids* **2003**, *17*, 775–784.
(11) Blijdenstein, T. B. J.; van der Linden, E.; van Vliet, T.; van Aken, G. A. Scaling behavior of delayed demixing, rheology, and microstructure of emulsions flocculated by depletion and bridging. *Langmuir* **2004**, *20*, 11321–11328.
(12) de Hoog, E. H. A.; Prinz, J. F.; Huntjens, L.; Dresselhuis, D. M.; van Aken, G. A. Lubrication of oral surfaces by food emulsions: the importance of surface characteristics. *J. Food Sci.* **2006**, *71*, E337–E341.
(13) Silletti, E.; Vingerhoeds, M. H.; Norde, W.; Van Aken, G. A. The role of electrostatics in saliva-induced emulsion flocculation. *Food Hydrocolloids* **2007**, *21*, 596–606.
(14) Gwartney, E. A.; Larick, D. K.; Foegeding, E. A. Sensory texture and mechanical properties of stranded and particulate whey protein emulsion gels. *J. Food Sci.* **2004**, *69*, S333–S339.
(15) Armand, M.; Hamosh, M.; Dipalma, J. S.; Gallagher, J.; Benjamin, S. B.; Philpott, J. R.; Lairon, D.; Hamosh, P. Dietary-fat modulates gastric lipase activity in healthy humans. *Am. J. Clin. Nutr.* **1995**, *62*, 74–80.
(16) Armand, M.; Pasquier, B.; Andre, M.; Borel, P.; Senft, M.; Peyrot, J.; Salducci, J.; Portugal, H.; Jaussan, V.; Lairon, D. Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. *Am. J. Clin. Nutr.* **1999**, *70*, 1096–1106.
(17) Armand, M.; Pasquier, B.; Borel, P.; Andre, M.; Senft, M.; Peyrot, J.; Salducci, J.; Lairon, D. Emulsion and absorption of lipids: The importance of physicochemical properties. *Ol. Corps Gras Lipides* **1997**, *4*, 178–185.
(18) Bauer, E.; Jakob, S.; Mosenthin, R. Principles of physiology of lipid digestion. *Asian−Aust. J. Anim. Sci.* **2005**, *18*, 282–295.
(19) Armand, M. Lipases and lipolysis in the human digestive tract: where do we stand. *Curr. Opin. Clin. Nutr. Metab. Care* **2007**, *10*, 156–164.
(20) Fave, G.; Coste, T. C.; Armand, M. Physicochemical properties of lipids: new strategies to manage fatty acid bioavailability. *Cell. Mol. Biol.* **2004**, *50*, 815–831.
(21) Chen, L. Y.; Remondetto, G. E.; Subirade, M. Food protein-based materials as nutraceutical delivery systems. *Trends Food Sci. Technol.* **2006**, *17*, 272–283.
(22) Leaver, J.; Dalgleish, D. G. Variations in the binding of β-casein to oil−water interfaces detected by trypsin-catalyzed hydrolysis. *J. Colloid Interface Sci.* **1992**, *149*, 49–55.
(23) Dalgleish, D. G. Conformations and structures of milk proteins adsorbed to oil−water interfaces. *Food Res. Int.* **1996**, *29*, 541–547.
(24) Pafumi, Y.; Lairon, D.; de la Porte, P. L.; Juhel, C.; Storch, J.; Hamosh, M.; Armand, M. Mechanisms of inhibition of triacylglycerol hydrolysis by human gastric lipase. *J. Biol. Chem.* **2002**, *277*, 28070–28079.
(25) Lowe, M. The triglyceride lipases of the pancreas. *J. Lipid Res.* **2002**, *43*, 2007–2016.
(26) Tso, P. Overview of digestion and absorption. In *Biochemical and Physiological Aspects of Human Nutrition*; Crissinger, K. D.; Stipanuk, M. H., Eds.; W. B. Saunders: Philadelphia, PA, 2000; pp 75−90.
(27) Monahan, F. J.; McClements, D. J.; German, J. B. Disulfide-mediated polymerization reactions and physical properties of heated WPI-stabilized emulsions. *J. Food Sci.* **1996**, *61*, 504–509.
(28) Monahan, F. J.; McClements, D. J.; Kinsella, J. E. Polymerization of whey proteins in whey protein-stabilized emulsions. *J. Agric. Food Chem.* **1993**, *41*, 1826–1829.
(29) Park, G. Y.; Mun, S.; Park, Y.; Rhee, S.; Decker, E. A.; Weiss, J.; McClements, D. J.; Park, Y. Influence of encapsulation of emulsified lipids with chitosan on their in vivo digestibility. *Food Chem.* **2007**, *104*, 761–767.
(30) Mun, S.; Decker, E. A.; Park, Y.; Weiss, J.; McClements, D. J. Influence of interfacial composition on in vitro digestibility of emulsified lipids: potential mechanism for chitosan's ability to inhibit fat digestion. *Food Biophys.* **2006**, *1*, 21–29.
(31) Beysseriat, M.; Decker, E. A.; McClements, D. J. Preliminary study of the influence of dietary fiber on the properties of oil-in-water emulsions passing through an in vitro human digestion model. *Food Hydrocolloids* **2006**, *20*, 800–809.
(32) Armand, M.; Borel, P.; Dubois, C.; Senft, M.; Peyrot, J.; Salducci, J.; Lafont, H.; Lairon, D. Characterization of emulsions and lipolysis of dietary lipids in the human stomach. *Am. J. Physiol.* **1994**, *266*, G372–G381.
(33) Armand, M.; Borel, P.; Ythier, P.; Dutot, G.; Melin, C.; Senft, M.; Lafont, H.; Lairon, D. Effects of droplet size, triacylglycerol composition, and calcium on the hydrolysis of complex emulsions by pancreatic lipase—an in vitro study. *J. Nutr. Biochem.* **1992**, *3*, 333–341.
(34) Armand, M.; Hamosh, M.; Mehta, N. R.; Angelus, P. A.; Philpott, J. R.; Henderson, T. R.; Dwyer, N. K.; Lairon, D.; Hamosh, P. Effect of human milk or formula on gastric function and fat digestion in the premature infant. *Pediatr. Res.* **1996**, *40*, 429–437.
(35) Muller, R. H.; Ruhl, D.; Runge, S. A. Biodegradation of solid lipid nanoparticles as a function of lipase incubation time. *Int. J. Pharm.* **1996**, *144*, 115–121.
(36) Porter, C. J. H.; Trevaskis, N. L.; Charman, W. N. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. *Nat. Rev. Drug Discovery* **2007**, *6*, 231–248.
(37) McClements, D. J.; Decker, E. A.; Park, Y. Physicochemical and structural aspects of lipid digestion. In *Understanding and Controlling the Microstructure of Complex Foods*; McClements, D. J., Ed.; Woodhouse Publishing: Cambridge, U.K., 2007; pp 483−502.
(38) Dickinson, E.; Hong, S. T. Surface coverage of β-lactoglobulin at the oil−water interface —influence of protein heat-treatment and various emulsifiers. *J. Agric. Food Chem.* **1994**, *42*, 1602–1606.
(39) Hoffmann, M. A. M.; vanMil, P. Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulfide bonds. *J. Agric. Food Chem.* **1997**, *45*, 2942–2948.
(40) Fang, Y.; Dalgleish, D. G. Conformation of β-lactoglobulin studied by FTIR: effect of pH, temperature, and adsorption to the oil−water interface. *J. Colloid Interface Sci.* **1997**, *196*, 292–298.
(41) Kim, H. J.; Decker, E. A.; McClements, D. J. Role of postadsorption conformation changes of β-lactoglobulin on its ability to stabilize oil droplets against flocculation during heating at neutral pH. *Langmuir* **2002**, *18*, 7577–7583.
(42) Kim, H. J.; Decker, E. A.; McClements, D. J. Influence of free protein on flocculation stability of β-lactoglobulin stabilized oil-in-water emulsions at neutral pH and ambient temperature. *Langmuir* **2004**, *20*, 10394–10398.
(43) Kim, D. A.; Cornec, M.; Narsimhan, G. Effect of thermal treatment on interfacial properties of β-lactoglobulin. *J. Colloid Interface Sci.* **2005**, *285*, 100–109.
(44) McClements, D. J. Theoretical prediction of emulsion color. *Adv. Colloid Interface Sci.* **2002**, *97*, 63–89.
(45) Hernell, O.; Staggers, J. E.; Carey, M. C. Physical−chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase-analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human-beings. *Biochemistry* **1990**, *29*, 2041–2056.

Received for review March 10, 2008. Revised manuscript received May 21, 2008. Accepted June 10, 2008.

JF800741W