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RESEARCH ARTICLE

Lipase Digestibility of the Oil Phase in a Water-in-oil-in-water Emulsion

Saehun Mun, Yongdoo Choi, and Yong-Ro Kim

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Abstract Hydrolysis of the oil phase in a water-in-oil-in-water (W/O/W) emulsion was studied using pancreatic lipase. The influence of the W/O volume fraction, lipase concentration, and concentration of emulsifiers used to prepare W/O/W emulsions on lipid digestion and release of encapsulated material were investigated. The extent of lipid digestion differed depending on the W/O volume and lipase concentration, and the amount of dye released was not dependent on the W/O content or lipase concentration. The concentration of emulsifiers did not affect the extent of digestion. The information obtained in this study will be useful for design of W/O/W emulsion formulations with controlled release profiles and lipid digestion.

Keywords: water-in-oil-in-water emulsion, digestion, lipase, release

Introduction

Numerous emulsion-based delivery systems have been developed to control digestion and release of bioactive components within the human gastric intestinal (GI) tract, including microemulsions, emulsions, solid lipid nanoparticles, filled hydrogel particles, and multiple emulsions (1-6). In this study, a multiple emulsion system consisting of water-in-oil-in-water (W/O/W) was monitored for lipid digestion and release of hydrophilic components included in the internal aqueous phase of W/O/W emulsions using *in vitro* digestion.

W/O/W emulsions are complex liquid dispersions in which oil globules containing small aqueous droplets are dispersed in an aqueous continuous phase (7). Interest has been growing in the development of W/O/W emulsions because of many potential benefits due to a double compartmental structure (8-12). In relation to the ability to entrap hydrophilic compounds, many previous studies have demonstrated that W/O/W emulsions are suitable materials for encapsulation of different active compounds, such as hormones, steroids, vitamins, and minerals (7,13-15). W/O/W emulsions can also protect hydrophilic bioactive substances included in the inner-water phase from degradation by pancreatic enzymes and deliver these substances to an absorption site (13-15). Insulin could be encapsulated within the internal water phase of W/O/W emulsions prepared with soybean oil or the medium-chain fatty acid triacylglycerol (MCT) as the oil phase, and the encapsulated insulin in a W/O/W emulsion was resistant to degradation by proteolytic enzymes (14). However, the digestibility of the oil phase of a W/O/W emulsion by lipase and how the hydrophilic components loaded in the internal aqueous phase are released within the GI tract during lipid digestion have not been well elucidated.

Therefore, the purpose this study was to examine hydrolysis by pancreatic lipase occurring in the oil phase of a W/O/W emulsion and to examine release of hydrophilic components loaded in the internal aqueous phase during hydrolysis. In particular, the influence of the W/O volume fraction, the lipase concentration, and the concentration of hydrophilic and hydrophobic emulsifiers used to prepare W/O/W emulsions on lipid digestion and release of encapsulated material were studied. W/O/W emulsions were prepared using polyglycerol polyricinoleate (PGPR)

Saehun Mun, Yong-Ro Kim (✉)
Center for Food and Bioconvergence, and Department of Biosystems and Biomaterials Science and Engineering, Seoul National University, Seoul 151-742, Korea
Tel: +82-2-880-4607; Fax: +82-2-873-2049
E-mail: yongro@snu.ac.kr

Yongdoo Choi
Molecular Imaging and Therapy Branch, National Cancer Center, Goyang, Gyeonggi 410-769, Korea

and whey protein isolate (WPI), and 1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt (PTSA) was used as a model hydrophilic substance.

Materials and Methods

Materials Whey protein isolate (WPI) (Product code 9500) and polyglycerol polyricinoleate (PGPR) CRS-75 were obtained from Protient Inc. (St. Paul, MN, USA) and Sakamoto Yakuhin Kogyo Co. Ltd. (Osaka, Japan), respectively. HCl and NaOH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium phosphate (dibasic and anhydrous) and sodium phosphate (monobasic, anhydrous) were purchased from Showa Chemical Co. (Tokyo, Japan). Soybean oil was purchased from a local supermarket and used without further purification. 1,3,6,8-Pyrenetetrasulfonic acid tetrasodium salt (PTSA) (CAS Registry No. 59572-10-0) was purchased from Fisher Scientific International L.L.C. (Hampton, NH, USA).

Preparation of W/O/W emulsions W/O/W emulsions were prepared using a 2-stage emulsification method (16). First, a water-in-oil (W/O) emulsion was prepared using sonication (VCX 750; Sonics & Materials Inc., Newtown, CT, USA) of a 20 wt% aqueous phase containing 0.2 wt% PTSA fluorescent dye and an 80 wt% oil phase containing PGPR (4, 8, and 12 wt%). W/O emulsions were prepared at 50°C. The aqueous phase was dispersed gradually into the oil phase under agitation using a magnetic stirrer and followed by sonication for 4 min at a frequency of 20 kHz, an amplitude of 40%, and a duty cycle of 0.5 s (VCX 750; Sonics & Materials Inc.). Second, W/O emulsions were sonicated with an aqueous WPI solution (1, 2, and 3 wt% for 10 wt% W/O, 5 mM phosphate buffer, pH 7) for 3 min at a frequency of 20 kHz, an amplitude of 40%, and a duty cycle of 0.5 s (VCX 750; Sonics & Materials Inc.).

Particle size measurement The mean size of water droplets stabilized using PGPR in W/O emulsions was determined in emulsions diluted using hexadecane based on dynamic light scattering with an ELS-8000 Electrophoretic Light Scattering Spectrophotometer (Otsuka Electronics Co., Ltd., Osaka, Japan). Particle sizes of W/O/W emulsions were measured using a particle size analyzer (Analysette-22 NanoTec; Fritsch, Idar-Oberstein, Germany). To avoid multiple scattering effects, emulsions were diluted with distilled water and stirred continuously throughout the measurement period to ensure emulsions were homogeneous.

Confocal laser scanning microscopy (CLSM) Emulsion samples for CLSM were prepared by dropping 20 μL of an emulsion on a glass slide and covering with a cover glass at room temperature. Then, confocal fluorescence images (excitation=405 nm, emission=420-480 nm) were obtained using a confocal laser scanning microscope (LSM 510 Meta; Carl Zeiss, Göttingen, Germany).

Determination of the encapsulation efficiency (EE) Highly water-soluble PTSA fluorescent dye was used as a model ingredient for encapsulation of active food supplements. The EE value of the dye in a W/O/W emulsion was defined as the percentage of dye retained within inner aqueous phase droplets following sonication of the W/O emulsion with the aqueous phase. A standard curve of fluorescence intensity versus PTSA concentration was prepared (17,18). A stock dye solution was prepared by dissolving 0.01% (w/v) PTSA in a buffer solution (5 mM phosphate buffer, pH 7). A standard curve was then created based on measurement of the fluorescence intensity of PTSA (excitation 374 nm and emission 404 nm) using a microplate reader (Safire2; Tecan, Männedorf, Switzerland). The dye concentrations in the external aqueous phases collected from W/O/W emulsions were then determined using the standard curve.

PTSA (0.2%) was dispersed in the aqueous phase that was used to prepare W/O emulsions, as described above. W/O/W emulsions were then prepared using sonication (VCX 750; Sonics & Materials, Inc.) of W/O emulsions and aqueous WPI solutions. Samples of W/O/W emulsions were ultracentrifuged (Himac CP100β; Hitachi, Tokyo, Japan) for 15 min at 50,310×*g* for separation into a creamed layer and a serum layer. An aliquot of the serum layer from each centrifuged sample was clarified using a syringe-driven filter unit (Millipore Corp., Bedford, MA, USA), and the fluorescence intensity of the dye was recorded using a microplate reader (Safire2; Tecan). This procedure was repeated for similar emulsions prepared without dye to obtain blank values, and these values were subsequently subtracted from dye counterpart values. The dye concentration present in the serum layer was determined using the previously constructed standard curve. The EE value was expressed as a percentage of PTSA that remained encapsulated within water droplets after homogenization:

$$E(\%)=\frac{M_i-M_e}{M_i}\times 100$$

where M_i is the mass of PTSA initially present in the internal water droplets in the W/O emulsion, and M_e is the mass of PTSA present in the external water phase in the W/O/W emulsion after sonication.

Determination of PTSA release from digested W/O/W emulsions For calculation of the amount of dye (%) released from digested W/O/W emulsions, digested W/O/

W emulsions were centrifuged for 15 min at 10,000 rpm using an ultracentrifuge (CP100β; Hitachi) and the PTSA concentration in the outer-phase solution was determined.

***In vitro* digestion** A simulated intestine model was used to investigate the extent of lipid digestion and release of PTSA during digestion. This *in vitro* digestion model was a modification of a previous method (19). Thirty mL of emulsion was transferred into a glass beaker and incubated at 37.0°C for 10 min, then 5.0 mL of a bile extract solution (187.5 mg of bile extract dissolved in phosphate buffer, pH 7.0, 37.0°C) and 1.0 mL of a $CaCl_2$ solution (188 mM $CaCl_2$ in double-distilled water, 37.0°C) were added to the emulsion with stirring. The system was adjusted to pH 7.0 and then 1.5 mL of a freshly prepared lipase suspension (60 mg of lipase powder dispersed in a phosphate buffer, pH 7.0, 37.0°C) was added. Final concentrations of pancreatic lipase, the bile extract, and $CaCl_2$ in the reaction mixture were 1.6, 5 mg/mL and 5 mM, respectively. After reactions, 0.1 N NaOH was added with monitoring of the pH of the reaction cell and the volume of NaOH required to adjust the pH of the reaction cell to pH 7.0 was recorded and used to calculate the concentration of free fatty acids (FFAs) generated during lipolysis.

Statistical analysis Data were recorded for measurements of the release of PTSA from W/O/W emulsions prepared using different W/O volumes as mean±standard deviation (SD) and analyzed using SPSS for Windows (version 21.0) (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) followed by a Duncan's multiple range test were performed to identify statistical significance at $p < 0.05$.

Results and Discussion

Particle properties during digestion The ζ-potential of the W/O/W emulsion (PGPR 4 wt% for 20 wt% aqueous phase, WPI 1 wt% for 10 wt% W/O, EE=90%) was examined during digestion of the oil phase with the bile extract and lipase. The ζ-potential of the W/O/W emulsion stabilized using PGPR and WPI prior to digestion with pancreatic lipase was −53 mV (Fig. 1A). PGPR was used to prepare the primary W/O emulsion as a hydrophobic emulsifier and, hence, oil droplets of the W/O/W emulsion were primarily coated with WPI. The ζ-potentials of droplets stabilized using protein are usually highly negative at pH 7 because the isoelectric point of protein is considerably lower than the pH value (20). In the presence of bile extract, the negative charge of emulsion increased, suggesting that anionic part of bile extract adsorbed to the surfaces of emulsion droplets. This resultcould be attributed to the displacement of WPI molecules by bile salt and the penetration of bile salt between WPI molecules, or formation of an interfacial complex between bile salt and WPI molecules (19-23). When pancreatic lipase was participated in the digestion reaction, the negative charge of emulsion also increased. This result implied the existence of adsorbed pancreatic lipase on the surface of emulsion droplets. Results of this study followed a trend similar to results reported for digestion of oil droplets in O/W emulsions (20).

The mean droplet size of water droplets of the primary emulsion (W/O emulsion) was approximately 800 nm and, initially, the mean droplet size of W/O/W emulsions was 2.1 μm ($d_{3,2}$) or 15.6 μm ($d_{4,3}$) in the absence of either the bile extract or lipase. Change in droplet size during digestion

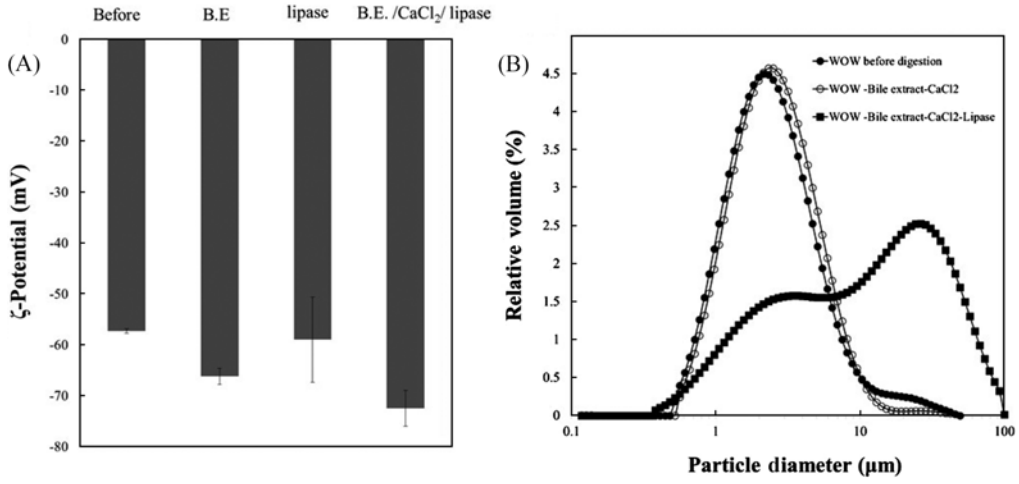

Fig. 1. (A) ζ-Potential (mV) and (B) particle size distribution of W/O/W emulsions before digestion and after W/O/W emulsions were subjected to a simulated small intestinal model. PGPR 4 wt% for 20 wt% aqueous phase and WPI 1 wt% for 10 wt% WO; B.E., bile extract

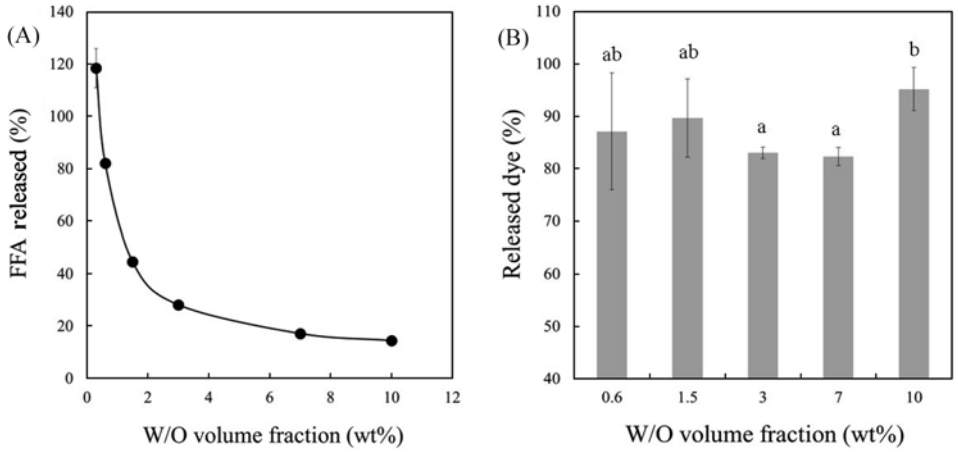

Fig. 2. (A) Influence of W/O fractions on the total amount of FFAs released and (B) the amount of dye released from W/O/W emulsions after 120 min of digestion. Different lower case letters on bars are significantly different based on Duncan's multiple test ($p<0.05$).

was also investigated (Fig. 1B). Many large droplets with a diameter >10 μm were observed in W/O/W emulsions before addition of the bile extract and lipase. The addition of the bile extract to these emulsions caused a decrease in the volume of large droplets (>10 μm), and the mean droplet size also decreased to 1.8 μm ($d_{3,2}$) and 4.2 μm ($d_{4,3}$), which indicated that the bile extract acted as an emulsifier, in addition to PGPR and WPI. Measurements of the æ-potential suggested that at least some of the surface-active components from the bile extract were adsorbed onto droplet surfaces since an increased negative charge was observed around W/O droplets.

The addition of pancreatic lipase to emulsions caused a substantial change in the droplet size distribution. Compared to addition of the bile extract alone, the number of small droplets (<10 μm) decreased, and the number of droplets between 10 and 100 μm increased with addition of lipase, indicating that coalescence might have occurred following lipase hydrolysis. Relatively low lipase, bile extract, and calcium concentrations were present in the simulated intestinal fluids, compared with concentrations used in a previous report that simulated the fed condition (20 mg/mL of bile and 2.4 mg/mL lipase) (19). Therefore, fewer enzyme molecules were available to catalyze hydrolysis of tri-acyglycerol, and fewer micelles were available to solubilize the FFAs formed, so FFAs may have tended to accumulate at the oil-water interface. FFAs and monoacylglycerols (MAGs) produced by lipid digestion are surface-active molecules that are likely to displace proteins from the lipid droplet surface during hydrolysis. These biological surfactants are relatively lipophilic (low hydrophilic-lipophilic balance (HLB) number] and ineffective for stabilization of O/W emulsions against coalescence (24), which may account for the observed increase in droplet size during hydrolysis.

Effect of the W/O volume fraction on *in vitro* lipid digestion and release of PTSA from W/O/W emulsions

The effect of the W/O volume fraction (oil phase of the W/O/W emulsion) on the extent of lipid digestion was investigated using a simulated intestine model. Different amounts of W/O/W emulsions stabilized using PGPR and WPI (PGPR 4 wt% for 20 wt% aqueous phase, WPI 1 wt% for 10 wt% W/O, $d_{3,2}$=2.1 μm, ζ-potential= −53 mV and EE=90%) were used for a digestion reaction to achieve final W/O contents of 0.3 wt%, 0.6 wt%, 1.5 wt%, 3.0 wt%, 7.0 wt%, and 10 wt% and a final pancreatic lipase concentration was 1.6 mg/mL. The amount of FFA released after 120 min of digestion for all emulsions is shown in Fig. 2A.

The extent of lipid digestion decreased as the W/O volume fraction increased. The absolute amount of FFAs released could have been higher, and the percentage of total fatty acids released that were originally present within the droplets was less for emulsions with a high lipid content (19). This effect may be attributable to several physicochemical mechanisms, including, 1) a decrease in the amount of lipase per unit surface area of oil droplet as the lipid concentration increased, 2) insufficient amount of bile salts to solubilize all the digestion products, and 3) insufficient amount of calcium to remove FFAs from the surfaces of droplets at high lipid concentrations (19,25). Removal of FFAs from lipid droplet surfaces during digestion using solubilization in bile salts or precipitation by calcium ions is known to play an important role in promotion of full hydrolysis of triglycerides (26,27).

The amount of dye released (%) following lipid digestion of W/O/W emulsions containing different W/O volume fractions using a simulated small intestinal model is shown in Fig. 2B. After digestion for 120 min, over 80% of the

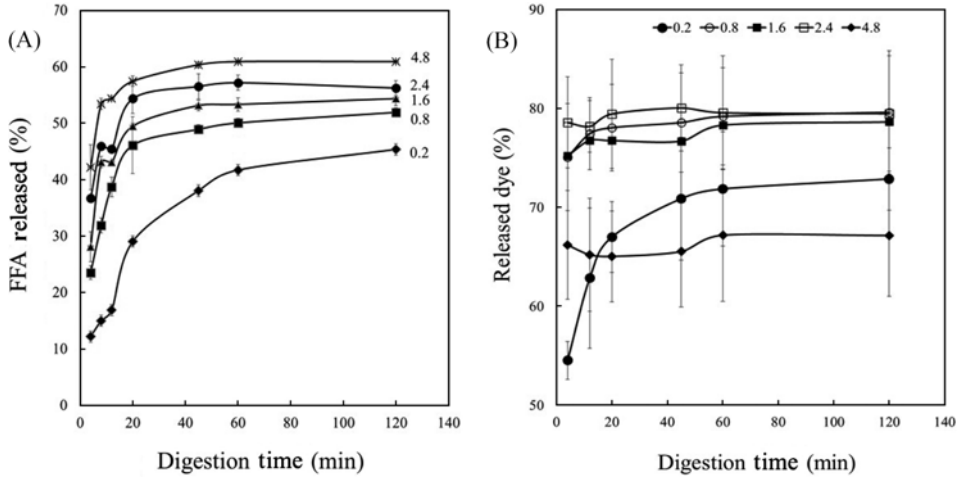

Fig. 3. (A) Influence of lipase concentrations (mg/mL) on the amount of FFAs released and (B) the amount of dye released from W/O/W emulsions during digestion.

PTSA incorporated into the inner aqueous phase of W/O/W emulsions was released. Even though the extent of lipid digestion differed depending on the W/O volume and ranged from ~10 to 100, the amount of dye released was not dependent on the W/O content, indicating that control of the W/O volume fraction in the W/O/W emulsion was not a major factor for control of PTSA release under the experimental conditions.

Effect of the lipase concentration on *in vitro* lipid digestion and release of PTSA during digestion of W/O/W emulsions The concentration of pancreatic lipase in the human small intestine depends on many factors, including the individual, the time of day, and the amount and type of food consumed (19). The influence of the lipase concentration on lipid digestion of W/O/W emulsions was investigated. W/O/W emulsions prepared using a 1 wt% W/O volume fraction were used with an initial mean droplet size of 2.31 μm ($d_{3,2}$). The final compositions of lipids, bile extract, and calcium were 240 mg, 5 mg/mL, and 5 mM $CaCl_2$, respectively. As the lipase concentration used in the digestion reaction increased, the initial digestion rate and the amount of FFAs released after 120 min of digestion increased. At 0.2 mg/mL of lipase concentration, FFAs released slowly, and a large fraction of oil remained undigested, compared with the lipids digestion at higher lipase concentration.

At lipase concentrations above 0.8 mg/mL, initial rate of lipid digestion increased rapidly within 20 min, then leveled off. However, at these lipase concentrations (0.8 mg/mL), all of the oil within droplets was not fully digested and undigested oil remained within droplets. Lipase has the ability to adsorb to the lipid droplet surface, hence it competes with other surface-active components existing on the surface of lipid droplets during lipid digestion in small intestine (19). At low lipase concentrations, insufficient lipase may be lost in the competition with WPI and/or bile to take over the surface of lipid droplets, resulting in insufficient lipid hydrolysis.

Changes in the amount of dye released (%) during lipid digestion of W/O/W emulsions with different lipase concentrations are shown in Fig. 3B. At a relatively low lipase concentration (0.2 mg/mL), the amount of dye released rapidly increased for 20 min, then slowly increased, achieving a 72% dye release after 120 min of digestion. At intermediate lipase concentrations of 0.8, 1.6, and 2.4 mg/mL, most of the dye was immediately released once digestion was initiated, and the amount of dye released ranged from 70 to 80%. An initial period occurred during which the rate of FFAs release increased rapidly for the first 20 min, then leveled off at these intermediate concentrations (Fig. 3A). However, when dye was incorporated in the inner aqueous phase of the W/O/W emulsion, most of the dye was released at the initiation of digestion.

The amount of dye released was least when W/O/W emulsions were digested using a relatively high lipase concentration (4.8 mg/mL), and the amount of dye released was lower than for emulsions digested using 0.2 mg/mL of lipase. The amount of released FFA (%) was highest when emulsions were digested using 4.8 mg/mL of lipase (Fig. 3A). Thus, patterns of dye release obtained during digestion did not match patterns of FFAs (%) release.

W/O/W emulsions digested using a relatively high lipase concentration probably resulted in more FFAs released. Although the amount of FFAs released increased, the amounts of calcium and the bile extract, which can precipitate and solubilize FFAs, were fixed in the reaction vessel. Therefore, calcium and the bile extract were less effective at precipitating and solubilizing the FFAs that accumulated at the oil-water interface during digestion. In this situation, the dye released likely binds with FFAs that

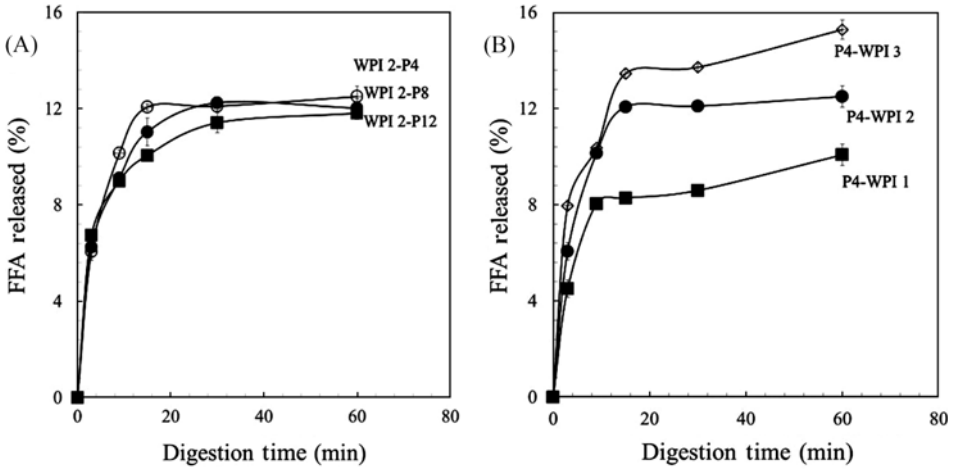

Fig. 4. (A) Influence of the PGPR concentration and (B) WPI concentration on the total amount of FFAs released after 120 min of digestion of W/O/W emulsions. WPI 2-P4 indicate that W/O/W emulsions were prepared using 4% PGPR and 2% WPI; P4-WPI 3 indicate that W/O/W emulsions were prepared using 4% PGPR and 3% WPI.

accumulate at the oil-water interface. However, further study is needed.

Effects of hydrophilic and hydrophobic emulsifier concentrations on *in vitro* lipid digestion in W/O/W emulsions The properties of interfacial layer coating lipid droplets are one of the important factors to determine the stability of lipid droplets against disruption and coalescence occurring during lipid digestion. Furthermore, the properties of interfacial layer may affect the hydrolysis of lipid droplets by lipases in small intestine (3).

Two kinds of emulsifiers were used to prepare W/O/W emulsions (unlike O/W emulsions). In addition to the hydrophilic emulsifier, which coats the surface of oil droplets, hydrophobic emulsifiers that stabilize water droplets within oil droplets were also used. W/O/W emulsions were prepared with a 10 wt% W/O volume fraction, and with WPI 2 wt% and PGPR 4 wt%, 8, and 12 wt% to investigate the effects of the PGPR concentration used to prepare W/O emulsions. W/O/W emulsions were also prepared using PGPR 4 wt% and WPI 1, 2, and 3 wt% to investigate the effects of the WPI concentration. The PGPR concentration did not affect the extent of lipid digestion in W/O/W emulsions (Fig. 4).

W/O/W emulsions prepared using relatively high levels of WPI displayed an increased extent of hydrolysis. The mean droplet size of W/O/W emulsions prepared using different WPI and PGPR concentrations was investigated (Table 1). The mean droplet sizes ($d_{4,3}$ and $d_{3,2}$) of W/O/W emulsions prepared using different WPI concentrations were not significantly ($p>0.05$) different among treatments. Therefore, the reason that W/O/W emulsions prepared using a relatively high level of protein resulted in a relatively high level of FFAs being released after digestion might have been due to a high level of protein that contributed to the calculated amount of FFAs released.

Table 1. Mean droplet sizes ($d_{3,2}$ and $d_{4,3}$) of W/O/W emulsions prepared using different WPI and PGPR concentrations

(unit: μm)

	$d_{3,2}$	$d_{4,3}$
WOW-P4-WPI-1[1)]	2.52±0.74	18.54±19.50
WOW-P4-WPI-2	2.77±0.01	17.12±1.21
WOW-P4-WPI-3	2.59±0.41	21.62±15.00
WOW-WPI2-P4	2.62±0.25	14.31±4.93
WOW-WPI2-P8	2.49±0.16	16.71±10.30
WOW-WPI2-P12	4.40±1.57	72.32±58.30

[1)]W/O/W-P4-WPI-1 indicates W/O/W emulsions prepared using 4% PGPR and 1% WPI.

Observation of changes in microstructure during digestion of W/O/W emulsions using confocal microscopy Structural changes that occurred during digestion were investigated using confocal microscopy. W/O/W emulsions containing a 10% W/O fraction and a 1% of WPI were used. The original emulsion contained blue-colored W/O/W droplets, indicating that the dye remained within the internal aqueous phase (Fig. 5). When the bile extract was added to emulsions, the microstructure remained unchanged and dye remained inside the internal aqueous phase. Addition of lipase to emulsions, either alone or in combination with the bile extract, caused changes in microstructures. The structure of fat droplets hydrolyzed using lipase was different between emulsions in the absence and presence of the bile extract. With addition of lipase alone, both large coalesced droplets and small fat droplets were observed. However, in the presence of the bile extract, only coalesced fat droplets were seen. Confocal microscopy clearly showed that extensive droplet coalescence occurred and that the droplet shape was

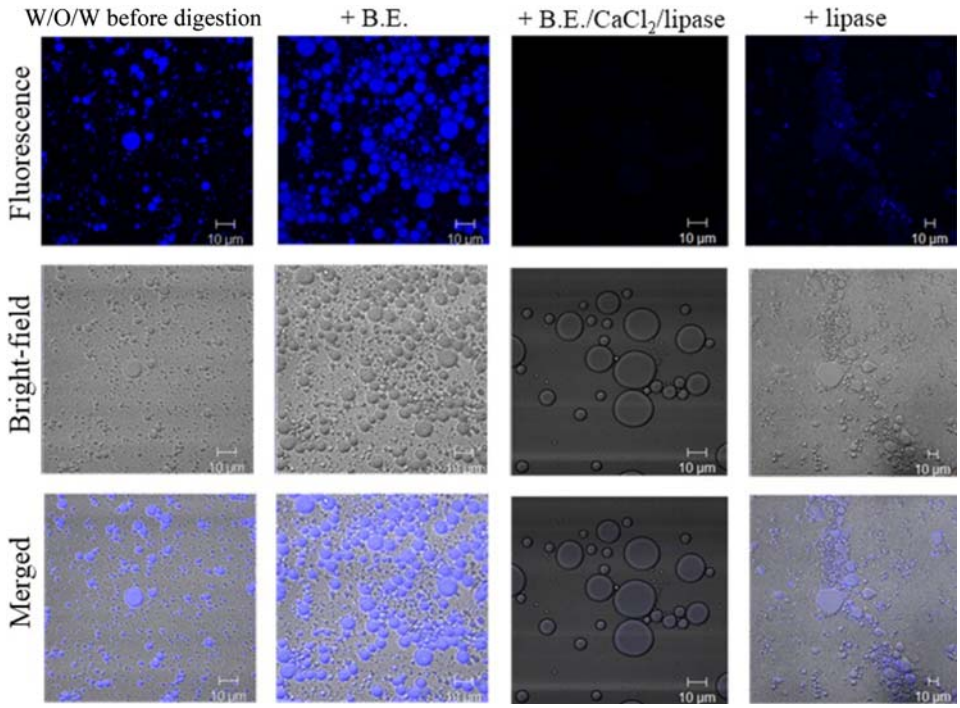

Fig. 5. Confocal laser scanning microscope images of W/O/W emulsions before and after digestion. B.E. indicates emulsions containing the bile extract; B.E./$CaCl_2$/lipase indicates emulsions containing the bile extract, $CaCl_2$, and lipase.

spherical for lipids hydrolyzed using lipase and the bile extract. Conversely, the droplet shape for lipids hydrolyzed using lipase alone was irregular, and droplets stuck together and/or exhibited surfaces marked with intensified blue colored rods.

Coalesced fat droplets observed in lipids digested using the bile extract might have occurred because fewer enzyme molecules were available to catalyze hydrolysis of triacyglycerol, and fewer micelles were available to solubilize the FFAs that were formed.

Reasons for differences in structure between hydrolyzed fat droplets in the absence and presence of the bile extract were not clear, but there are 2 possible reasons. First, the bile extract enhanced the solubility of lipolysis products in the aqueous phase via formation of different vesicles that were soluble in water, thereby removing the lipolysis products from the surface of fat droplets. However, in the absence of the bile extract, lipolysis products accumulated on the surface of fat droplets, hydrolysis was limited, and small droplets of a size similar to the original fat droplets remained and were detected. Second, in the absence of the bile extract, lipolysis products that accumulated on the surface of fat droplets might have formed complexes with released dye, revealing an intensified blue colored rod. Further research is needed to explore these mechanisms.

This study should be helpful for design of W/O/W formulations with controlled release profiles and lipid digestion. In order to illuminate the overall mechanism of W/O/W emulsion behavior during digestion, further studies based on *in vitro* methods with sequential use of digestive enzymes (amylase, proteases, lipase) under physiological conditions with consideration of pre-processing in the mouth and stomach are needed.

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Disclosure The authors declare no conflict of interest.

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