Preparation, Characterization, and in Vitro Gastrointestinal Digestibility of Oil-in-Water Emulsion-Agar Gels

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Soybean oil-in-water (O/W) emulsion-agar gel samples were prepared and their digestibility evaluated by using an in vitro gastrointestinal digestion model. Emulsion-agar sols were obtained by mixing the prepared O/W emulsions with a 1.5 wt % agar solution at 60°C, and their subsequent cooling at 5°C for 1 h formed emulsion-agar gels. Their gel strength values increased with increasing degree of polymerization of the emulsifiers, and the relative gel strength increased in the case of droplets with an average diameter smaller than 700 nm. Flocculation and coalescence of the released emulsion droplets depended strongly on the emulsifier type; however, the emulsifier type hardly affected the ζ-potential of emulsion droplets released from the emulsion-agar gels during in vitro digestion. The total FFA content released from each emulsion towards the end of the digestion period was nearly twice that released from the emulsion-agar gel, indicating that gelation of the O/W emulsion may have delayed lipid hydrolysis.

**Key words:** oil-in-water emulsion; emulsion-agar gel; lipid hydrolysis; droplet diameter; in vitro digestion model

Many food products can be categorized as an emulsion gel; for example, fresh cheese, starch puddings, and various types of dairy dessert. Gels containing emulsion droplets have been subject to extensive study by food scientists. Emulsified fat strongly influences the texture and physical bonding during the ageing period.1–3) The effects of oil content and droplet size on the gel properties and rheological properties of emulsion gels have been studied in several systems. For example, McClements et al. have reported that the oil droplets may either act as a structural promoter or structural breaker depending on the interaction between the emulsifier adsorbed at the oil/water interface and the protein molecules in the gel network.5) Chen and Dickinson have demonstrated that a higher gelation temperature resulted in more rapid network formation, whereas a lower gelation temperature resulted in the development of stronger physical bonding during the ageing period.6) Wu et al. have investigated the effects of animal fat and vegetable oil at different lipid compositions on the rheological properties of myofibrillar protein gels.7) Their results have suggested the feasibility of totally or partially replacing animal fat by vegetable oil in the processing of cholesterol-reduced comminuted muscle foods.

The digestion of dietary lipids in adult humans begins in the stomach and is completed in the small intestine, where final absorption of the digestion products occurs.10,11) Lipolysis mainly occurs in the small intestine and a small proportion in the stomach.12) Lipids and their digestion products (e.g., free fatty acids (FFA), monoacylglycerols (MAG), cholesterol and phospholipids) are transported from the surface of the lipid droplets to the intestinal mucosa via mixed bile salt micelles and vesicles.11) Emulsifiers play important roles in producing many food products. Polyglycerol esters of fatty acids (PGEs) extracted from marine red algae, and is a mixture of agarose consisting of neutral polysaccharides with high gelling ability and agarpectin consisting of ionic polysaccharides with low gelling ability. The wide use of agar is based on its unique ability to form a strong gel in an aqueous solution.4) Other gelling agents such as polysaccharides5) and gelatin6) have also been widely used in emulsified products.

Rheology is a widely used technique in the food industry for new product development and design.3) The effects of oil content and droplet size on the gel properties and rheological properties of emulsion gels have been studied in several systems. For example, McClements et al. have reported that the oil droplets may either act as a structural promoter or structural breaker depending on the interaction between the emulsifier adsorbed at the oil/water interface and the protein molecules in the gel network.5) Chen and Dickinson have demonstrated that a higher gelation temperature resulted in more rapid network formation, whereas a lower gelation temperature resulted in the development of stronger physical bonding during the ageing period.6) Wu et al. have investigated the effects of animal fat and vegetable oil at different lipid compositions on the rheological properties of myofibrillar protein gels.7) Their results have suggested the feasibility of totally or partially replacing animal fat by vegetable oil in the processing of cholesterol-reduced comminuted muscle foods.
are one group of the commonly used emulsifiers in foods (e.g., margarine and dessert), together with other products. Many different types of PGEs are also widely used in cosmetics and toiletries. Yin et al. have investigated the influence of the polysorbate monolaurate (ML) and polyglycerol monoooleate (MO) series as non-ionic emulsifiers on the physicochemical properties and stability of β-carotene O/W emulsions during digestion in simulated gastric fluid. Those authors reported that the higher polymerization degree of the glycerol in PGEs led to the preparation of a β-carotene emulsion with smaller particle size and exhibited improved resistance against gastric digestion. We have reported that the stability of a submicron emulsion against droplet flocculation and coalescence during in vitro gastrointestinal digestion was strongly dependent on the polymerization degree of glycerol in the ML series. We have also demonstrated that lipid conversion depended on the oil-to-lipase ratio in the reaction mixture, rather than on the emulsifier composition.

Although several studies on the physicochemical properties of gels containing oil droplets have been reported, they have basically used protein-based gels. To the best of our knowledge, no previous studies comparing the properties of gels containing oil droplets have been reported, they have basically used protein-based gels. To design new foods to control the bioavailability of active compounds.

**Materials and Methods**

**Materials.** Refined soybean oil and reagent grade agar powder (400–600 g/cm² gel strength at 1.5%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The following PGEs were supplied by Sakamoto Yakuhin Kogyo Co. (Osaka, Japan): ML310 (tetraglycerol monolaurate, polymerization degree (n) = 2), ML500 (hexaglycerol monolaurate, n = 4), and ML750 (decaglycerol monolaurate, n = 8). Milli-Q water with a resistivity of 18 MΩ cm was used as the medium for preparing all the aqueous solutions. The bile extract and pancreatic lipase (from porcine pancreas, type II) containing both lipase and co-lipase were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Hydrochloric acid (HCl), sodium bicarbonate (NaHCO₃), sodium hydroxide (NaOH), disodium hydrogen phosphate dodecahydrate (sodium phosphate, dibasic; Na₂HPO₄·12H₂O), sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O) and phenolphthalein (C₂₃H₃₄O₆) were purchased from Wako Pure Chemical Industries.

**Preparation of the solutions.** The phosphate buffer (5 mmol/L, pH 7.0) was prepared by dissolving Na₂HPO₄·12H₂O and NaH₂PO₄·2H₂O in Milli-Q water. Each emulsifying solution was prepared by dissolving 2.0 wt% PGE in the phosphate buffer and then passing through a hydrophilic polycarbonate filter with a pore size of 5 μm (Isopore® membrane filter, type TMTP, Merck Millipore, Billerica, USA). A 0.9-mol/L NaHCO₃ solution and 1-mol/L NaOH solution were separately prepared by dissolving the respective powdered material in Milli-Q water. The pepsin, bile extract and pancreatic lipase solutions were prepared by dissolving the materials in the phosphate buffer to respective final concentrations of 3.2 mg/mL, 25 mg/mL and 8 mg/mL.

**Preparation of the emulsion-agar gels.** The preparation scheme for O/W emulsion-agar gel is presented in Fig. 1. An O/W emulsion was prepared by using refined soybean oil as the dispersed phase and a phosphate buffer solution containing an emulsifier as the continuous phase. A pre-mixture of the dispersed phase of 20 wt% was homogenized with a Polytron PT3000 device (Kinematica, Lucerne, Switzerland) at 5,000 rpm for 5 min to produce a coarse O/W emulsion. This O/W emulsion was immediately subjected to high-pressure homogenization with an M-110EH Microfluidizer Processor (Microfluidics Co., Newton, USA) in a single pass at 10, 50 or 100 MPa. The pH value of the prepared O/W emulsion was 7.0.

A 1.5 wt% agar solution was prepared by stirring agar powder and Milli-Q water at room temperature for 10 min, before autoclaving at 100 °C for 10 min. After autoclaving, the agar solution and prepared O/W emulsion were kept separately in a water bath at 60 °C for 5 min. The emulsion-agar sol was prepared by mixing equal volumes of the agar solution and O/W emulsion at 60 °C. The emulsion-agar gel was formed by cooling the emulsion-agar sol at 5 °C for 1 h.

**Gel strength measurements.** The gel strength of each emulsion-agar gel was measured with a TPU-2C texture profile unit (Yamaden Co., Tokyo, Japan), using a plunger with a diameter of 40 mm. The emulsion-agar gel was removed from a 60-mm plastic Petri dish and cut into 12.5-mm-thick cylinders by a cork borer with an internal diameter.
of 12 mm. The emulsion-agar gel strength \(S_g\) is defined as the load on a sample required to compress it to 20% of its original height at a crosshead speed of 1 mm/s. The small change in cross-section of the emulsion-agar gel during its compression was neglected. It was also assumed that stress relaxation and surface friction did not appreciably affect the measurements. The results for each emulsion-agar gel are reported as the relative gel strength indicated in eq. (1):

\[
R = \frac{S_a}{S_g} \quad (1)
\]

where \(R\) is the relative gel strength, \(S_a\) is the strength of the O/W emulsion-agar gel and \(S_g\) is the strength of the agar gel containing the emulsifier.8)

In vitro gastrointestinal digestion model. An in vitro digestion model that simulates gastric and small intestinal environments was used in this work, with the following slight modifications to the procedures previously described by Yin et al.13) and Mun et al.15)

Stage I (gastric digestion, pH 1.8): Twenty grams of the emulsion-agar gel sample was manually sheared into fragments having a size of approximately 5 mm and mixed with 40 g of a pepsin solution (3.2 mg/mL). The pH value was adjusted to 1.8 with 1 mol/L of HCl. The resulting mixture was transferred to a clean bottle and incubated at 37 °C for 2 h in a Personal-11 water bath (Taitec, Saitama, Japan) while shaking at 115 strokes/min.

Stage II (small intestinal digestion, pH 6.8): The pH value of the acidified emulsion-agar gel sample was raised to 6.8 by adding 0.9 mol/L of NaHCO\(_3\). Forty grams of a mixture containing a bile extract (25 mg/mL) and pancreatic lipase (8 mg/mL) in a 5 mmol/L phosphate buffer solution was then added to the emulsion-agar gel sample. The mixture was held at 37 °C while shaking at 115 strokes/min for 4 to 14 h.

Pancreatic lipase-catalyzed hydrolysis of the emulsion-agar gel. The amount of FFA hydrolyzed from the emulsified lipids due to pancreatic lipase activity was monitored by titration as previously described by Mun et al.15) After incubation, acetone was added to the reaction mixture to terminate the lipolysis. Several drops of phenolphthalein were added to the emulsion or emulsion-agar gel, and the FFA content was then determined by end-point titration with 0.01 mol/L of NaOH. The amount of FFA released from the sample was determined by using a standard curve constructed by the same titration method to measure FFA for emulsionsamples with a known concentration of added oleic acid (0–1000 μmol). The lipase activity is expressed as μmol of FFA released from 1 mL of the emulsion sample.

Droplet diameter and ζ-potential measurements. The mean droplet diameter and droplet size distribution of each sample was measured by using an LS 13320 laser diffraction particle size analyzer provided with polarization intensity differential scattering (PIDS) technology (Beckman Coulter, Brea, USA). The mean droplet diameter is expressed as the Sauter mean diameter (\(d_{3,2}\)),

\[
d_{3,2} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}
\]

where \(n_i\) is the number of drops of diameter \(d_i\). The \(d_{3,2}\) value of each emulsion-agar sol was measured after mixing with a 1.5 wt% agar solution. The \(d_{3,2}\) value of each emulsion-agar gel was measured after being melted in a water bath at 90 °C for 5–10 min. After each digestion stage, the mean diameter of the released droplets was measured by mixing the emulsion-agar gel sample with digestion juice, after passing through no. 5A filter paper with a pore size of >10 μm (Advantec Toyo Kaisha, Tokyo, Japan). The ζ-potential was measured with a Zetasizer Nano ZS instrument, after the sample had been stored for 24 h at room temperature, by a combination of laser doppler velocimetry and phase analysis light scattering (M3-PALS; Malvern Instruments, Worcestershire, UK). All measurements are reported as the average and standard deviation of measurements made on at least two freshly prepared samples.

Statistical analysis. All measurements were made in triplicate. An analysis of variance (ANOVA), using a single-factor analysis, was conducted to analyze the effect of different emulsifiers on the \(d_{3,2}\) value of emulsions, emulsion-agar sols, and droplets released from emulsion-agar gels at a confidence level of 95% \((p < 0.05)\). A least significant difference (LSD) test with a 95% confidence level was used to compare differences between the treatments (three types of emulsifier). The LSD value was calculated by using Statistix 8.1 software.16)

Results

Changes in droplet diameter and droplet size distribution during gelation of the emulsion

Figure 2a–c show \(d_{3,2}\) values for the prepared O/W emulsions, emulsion-agar sols, and emulsion-agar gels. The \(d_{3,2}\) values for the prepared O/W emulsions were 394 ± 2.2 nm for the ML310-stabilized emulsion, 377 ± 2.5 nm for ML500, and 376 ± 4.6 nm for ML750. After mixing with a 1.5 wt% agar solution, the \(d_{3,2}\) values of the emulsion-agar sols changed to 477 ± 5.0 nm for ML310, 368 ± 12.0 nm for ML500, and 364 ± 12.7 nm for ML750. After melting the emulsion-agar gels in a water bath at 90 °C for 5–10 min, there was a decrease in the \(d_{3,2}\) values of the released droplets for ML500 and ML750.
Effects of Emulsifier Type on the Droplet Size Distributions

Figure 3 shows the droplet size distributions for the prepared O/W emulsions, emulsion-agar gels and emulsion-agar sols. It can be seen in Fig. 3a that the volume percentage of ML310-stabilized droplets larger than 200 nm increased after gelation in the ML500- and ML750-stabilized emulsions. While emulsions containing droplets in the micrometer to sub-micron size range are known to be unstable, micro-emulsions, which generally incorporate small droplets with a size range of 5–200 nm and are known to have very low oil/water interfacial tension, are reportedly thermodynamically stable. The authors presume that, in this case, a micro-emulsion may also have been formed during emulsification, even though within a minor volume ratio. Furthermore, after melting at 90 °C, thermodynamically stable micro-emulsion droplets and unstable droplets may have co-existed, thereby leading to an overall decrease in the mean droplet diameter.

Fig. 3. Effect of Emulsifier Type on the Droplet Size Distributions of the Emulsions, Emulsion-Agar Sols, and Emulsion-Agar Gels: ML310 (a), ML500 (b), ML750 (c).

Physical properties of droplets released from the O/W emulsion-agar gels during in vitro gastrointestinal digestion

The droplet size distribution of the released droplets is shown in Fig. 5. In stage I (gastric digestion), the $d_{3,2}$ values for the released droplets markedly increased from 482 ± 6.8 nm to 1060 ± 23.9 nm for ML310, from 266 ± 12.2 nm to 442 ± 14.2 nm for ML500, and from 240 ± 20.0 nm to 311 ± 5.1 nm for ML750. There was thus a slight change in the droplet size distribution for ML750, whereas significant increases ($p < 0.05$) in the cases of ML500 and ML310 were apparent during digestion, respectively resulting in either a broad or bimodal size distribution (Fig. 5). It is concluded that
the stabilization capacity of ML750 was better than that of ML310 or ML500. In stage II (small intestinal digestion), the addition of pancreatic lipase and the bile extract to the emulsion-agar gels caused slight changes in the \(d_{3,2}\) values of the released droplets in the presence of ML500 and ML750 (data not shown). Their size distributions were similar to those obtained after stage I. A peak of the ML-stabilized droplets larger than 1000 nm appeared after 14 h of stage II (Fig. 5c). Since each series of emulsifier used in this study had the same type of fatty acid (lauric acid) as the hydrophobic part, these differences were mainly attributed to a variation in the degree of glycerol polymerization, and in the amount of hydroxyl groups existing in the hydrophilic part of the emulsifier. The molecular size of the emulsifier itself could also have affected its adsorption to the interfacial film which might have influenced the effective thickness of the layering structure. Emulsifiers with a higher degree of polymerization have an increased size of their hydrophilic part and acquire more hydroxyl groups, thus improving the hydration properties at the interface of the emulsion droplets. It is therefore reasonable to assume that an emulsifying agent having a higher degree of polymerization could form a thicker or stronger interfacial film which would be beneficial for enhancing the stability of the emulsion formed.\(^{13}\)

\[ \zeta \] -potential measurements were conducted to examine the interaction of pancreatic lipase and the bile extract with the droplets released from the O/W emulsion-agar gels. The \(\zeta\)-potential of the released droplets was \(-40.2 \pm 1.2\) mV for ML310, \(-47.5 \pm 1.1\) mV for ML500 and \(-48.6 \pm 1.1\) mV for ML750 (Fig. 6).

**Lipid hydrolysis during in vitro gastrointestinal digestion**

The lipolysis of O/W emulsion droplets or droplets released from the O/W emulsion-agar gel samples was investigated by *in vitro* gastrointestinal digestion. During stage I, lipid hydrolysis was negligible for both the emulsions and emulsion-agar gels, regardless of the emulsifier type (Fig. 7). During stage II, however, there was a sharp increase in the content of
hydrolyzed FFA in both the emulsion droplets and droplets released during the first 1 h for ML500 and ML750, after which the FFA content slowly increased (Fig. 7b and c). We also observed a gradual increase in the content of hydrolyzed FFA in both the emulsion droplets and droplets released for ML310 (Fig. 7a). After 14 h, the contents of FFA from the emulsion droplets were 14.7 \( \text{mmol/mL} \) for ML310, 16.0 \( \text{mmol/mL} \) for ML500 and 13.3 \( \text{mmol/mL} \) for ML750. On the other hand, the contents of FFA from the released droplets were 10.2 \( \text{mmol/mL} \) for ML310, 7.2 \( \text{mmol/mL} \) for ML500 and 6.7 \( \text{mmol/mL} \) for ML750. The surface area can increase as droplets diameter decreases. In this case, the diameter of the ML500- and ML750-stabilized oil droplets was smaller than that of the ML310-stabilized droplets (Figs. 2 and 3). It is considered that the volume of FFA released from the ML500- and ML750-stabilized oil droplets was more than that from the ML310-stabilized droplets. On the other hand, the ML310-stabilized oil droplets had weaker gel strength than the ML500- and ML750-stabilized droplets after gelation. It suggested that the oil droplets could be easily release from the ML310-stabilized emulsion-agar gel because it could break easily. As a result, after finishing digestion, more total FFA was released from the ML310-stabilized emulsion-agar gel than from the others (Fig. 7a–c).

**Discussion**

According to the literature, the emulsifier type can affect the droplet size by reducing the interfacial tension between the oil and aqueous phases, thereby leading to a smaller droplet size. McClements et al. have reported that whey protein isolate (WPI) gels containing small emulsion droplets (<1 \( \mu \text{m} \)) maintained a relatively higher gel strength, whereas WPI gels containing larger droplets (>1 \( \mu \text{m} \)) maintained similar or slightly lower strength. They suggested that the droplets were larger than the pore size of the gels and disrupted the threedimensional gel network. PGEs with higher degree of polymerization resulted in high R values. Previous studies have shown that the type of emulsifier used to stabilize the droplets could affect the gel strength. The preceding results have suggested that the stability of the droplets released during *in vitro* gastrointestinal digestion was dependent on the type of emulsifier used, this being similar to the *in vitro* digestibility of O/W emulsion droplets. McClements has reported a minimum \( \zeta \)-potential of ±30 mV was required to maintain a physically stable dispersion stabilized by an emulsifier. This indicated that the released droplets were
adsorption of OH-phenomenon could be attributed to the preferential emulsifiers. Previous studies have proposed that this a negative charge, even though PGEs were non-ionic of the released droplets stabilized by each emulsifier had almost stable against coalescence and flocculation. All of the released droplets stabilized by each emulsifier had a negative charge, even though PGEs were non-ionic emulsifiers. Previous studies have proposed that this phenomenon could be attributed to the preferential adsorption of OH- ions to the hydrophilic head group of the surfactant.19) The electro-statistical properties of oil droplets stabilized by non-ionic surfactants seem to be dominated by those of the bare droplets, but are modified somewhat by the presence of the interfacial layer of adsorbed surfactant molecules.18,19) The droplets released during stage I had a negative ζ-potential of around −50 mV, indicating that mixing the prepared emulsion-agar gel with simulated gastric fluid had little influence on the surface charge of the released droplets. The droplets released during stage II had a highly negative ζ-potential of <−100 mV independent of the emulsifier type, this probably being due to the adsorption of some anionic ions (e.g., free fatty acid) to the droplet surface. Our previous study has also suggested that the change in ζ-potential during in vitro gastrointestinal digestion was independent of the emulsifier type used.12) The lipase molecules may have been adsorbed to the oil-water interface and displaced some surface-active molecules, either in the absence or presence of bile salts. The addition of bile salts could promote the adsorption of lipase to the oil-water interface, increasing its activity.20,21)

Fave et al. have reported that a small portion of lipid hydrolysis occurs in the stomach, and the remainder in the small intestine.12) Ivanova et al. have reported that bile salts could displace emulsifier molecules adsorbed to an oil-water interface and promote lipase activity.21) The FFA contents were similar among the emulsifiers used at the end of stage II. Our previous work has indicated that lipid conversion depended on the ratio of oil to lipase in the reaction mixture, rather than on the emulsifier structure.14) The emulsion-agar gel samples having a size of approximately 5 mm were sheared. The authors presume that oil droplets may have been released more easily from the surface of small agar fragments and subsequently digested. On the other hand, the digestive juice may also have penetrated the agar network, so hydrolyzing the oil droplets. In this case, the permeation speed may have been low, hence released FFA was likely to be entrapped by the agar network. This could presumably have been the reason for the small volume of FFA released from the agar network.

Oil hydrolysis was more intense in case of droplets released from the gel network. Furthermore, the FFA release speed from the agar network was delayed upon gelation. Towards the end of digestion, the total FFA content from the emulsion droplets was around twice that from the released droplets, suggesting that gelation of the emulsions could slow down the lipolysis reaction.

Conclusions

Emulsion-agar gels containing soybean oil droplets as small as 400 nm were successfully obtained by using PGE-stabilized O/W emulsions and an agar solution. The results indicate that the stability for emulsion gelation was dependent on the type of PGE used. An increased degree of polymerization of PGEs can decrease the droplet diameter and increase the gel strength. The in vitro gastrointestinal digestion experiments indicate that the stability of the droplets released from an emulsion-agar gel was dependent on the type of PGE used. Based on the FFA contents released from the emulsion and the emulsion release during in vitro gastrointestinal digestion, it is reasonable to assume that gelation of the emulsion can delay lipid digestion. The present research provides a better understanding of the gelation of emulsion can affect lipid digestibility using in vitro gastrointestinal digestion model. It may give an important contribution to the design of foods that can control the bioavailability of active compounds, as well as the extent of lipids hydrolysis.

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