Proteolysis of milk fat globule membrane proteins during in vitro gastric digestion of milk

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ABSTRACT

The influence of gastric proteolysis on the physicochemical characteristics of milk fat globules and the proteins of the milk fat globule membrane (MFGM) in raw milk and cream was examined in vitro in simulated gastric fluid (SGF) containing various pepsin concentrations at pH 1.6 for up to 2 h. Apparent flocculation of the milk fat globules occurred in raw milk samples incubated in SGF containing pepsin, but no coalescence was observed in either raw milk samples or cream samples. The changes in the particle size of the fat globules as a result of the flocculation were dependent on the pepsin concentration. Correspondingly, the physical characteristics of the fat globules and the composition of the MFGM proteins in raw milk changed during incubation in SGF containing pepsin. The major MFGM proteins were hydrolyzed at different rates by the pepsin in the SGF; butyrophilin was more resistant than xanthine oxidase, PAS 6, or PAS 7. Peptides with various molecular weights, which altered with the time of incubation and the pepsin concentration, were present at the surfaces of the fat globules.

Key words: milk fat globule membrane (MFGM), simulated gastric fluid (SGF), pepsin, hydrolysis rate

INTRODUCTION

In its natural state, milk is a classical oil-in-water emulsion that consists of milk fat globules suspended in an aqueous phase that contains proteins, lactose, and salts. The diameter of the fat globules varies from 0.1 to 10 μm , and the globules are stabilized by the apical part of the plasma membrane, known as the milk fat globule membrane (MFGM; Mather, 2000; Ye et al., 2002). The MFGM is composed predominantly of phospholipids and proteins, and it is typically 8 to 10 nm thick. It has been speculated that the MFGM has pro-

found effects on the accessibility of triacylglycerols for lipase-catalyzed digestion (Patton and Keenan, 1975).

Several studies have attempted to understand the role of the composition of the interfacial layer and the size of the fat droplets in the ability of lipases to digest emulsified oil droplets in model emulsion systems and food and dairy products (Armand et al., 1992, 1999; Borel et al., 1994; Mun et al., 2007; Berton et al., 2009; Ye et al., 2010). However, in reality, lipid droplets must pass through the mouth and the stomach before reaching the small intestine, and some digestion and displacement of the adsorbed protein are likely to occur because of the presence of digestive enzymes and surface-active components in these regions of the gastrointestinal tract (Sarkar et al., 2009). Little information exists on the effect of the gastric environment and the presence of proteolytic enzymes on milk fat globules and the MFGM. For example, milk fat globules would be expected to undergo major changes in the stomach because of the possible action of pepsin on the proteins of the MFGM. The fat globules would mix with the gastric fluid, which contains different enzymes (pepsin and gastric lipase) and electrolytes (e.g., NaCl, CaCl₂), and would undergo a drastic change in pH (acidic environment: pH 1 to 3), while being exposed to shear because of the peristaltic movement in the stomach (Lindahl et al., 1997; Ekmekcioglu, 2002; Kalantzi et al., 2006).

In addition, the physicochemical behavior of fat droplets in gastric fluid and intestinal fluid is an area of interest, especially their morphology during gastric and intestinal digestion (Singh et al., 2009). Changes in the physicochemical properties of fat globules and dispersed hydrolytic products are important in understanding the digestion and absorption of lipids in milk products (Staggers et al., 1990).

The aim of this study was to unravel the physicochemical characteristics of milk fat globules and MFGM proteins during gastric proteolysis at low pH. Milk fat globules were subjected to biochemical conditions using an in vitro gastric model at pH 1.6 for up to 2 h. Changes in the size of the fat globules, the surface charge, and the composition of the MFGM proteins

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were examined using light scattering and SDS-PAGE analysis.

MATERIALS AND METHODS

Materials

Fresh raw milk without pasteurization was collected from the Massey University Dairy Farm, Palmerston North, New Zealand. Pepsin from porcine gastric mucosa (EC 3.4.23.1; catalog no. P7000, Sigma Chemical Co., St. Louis, MO) had an enzymatic activity of 800 to 2,500 units/mg of protein, as stated by the manufacturer. All other chemicals were obtained from BDH Chemicals (BDH Ltd., Poole, UK) unless otherwise specified. All solutions were prepared from analytical-grade chemicals. Milli-Q water (water purified by treatment with a Milli-Q apparatus, Millipore Corp., Bedford, MA) was used for the preparation of all solutions.

In Vitro Gastric Digestion

Simulated gastric fluid (SGF) containing 2 g of NaCl and 7 mL of HCl, with or without the addition of 3.2 g of pepsin, diluted to 1 L, and pH adjusted to 1.2 using 1.0 M HCl was prepared (United States Pharmacopeial Convention, 1995). The in vitro gastric model consisted of a conical flask (250 mL) containing SGF maintained at 37°C with continuous shaking at 95 rpm/min in a temperature-controlled water bath (Lab-Line shaker bath, model LZ33070, Barnstead International, Dubuque, IA) to mimic the conditions in the stomach (Beysseriat et al., 2006). The pH and the temperature were continuously monitored and controlled.

Each milk sample was mixed with SGF in a 1:1 ratio and the mixture was incubated at 37°C for up to 2 h. Samples were taken periodically for characterization. The pH of the mixture was maintained at 1.6 using 1 M HCl.

In some experiments, raw milk was centrifuged at $1,900 \times g$ for 20 min. The cream layer was carefully removed and dispersed in water or diluted skim milk to the original weight of the raw milk. The dispersed cream samples were mixed with SGF in a 1:1 ratio and then incubated as described above.

Determination of Average Droplet Size of Fat Globules

A Malvern MasterSizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) was used to determine the average diameter (d_{32}) of the fat globules in milk samples. The relative refractive index (N); that is, the ratio of the refractive index of the fat globules

(1.456) to that of the dispersion medium (1.33), was 1.095. The absorbance value of the fat globules was 0.001. Droplet size measurements are reported as average diameters, d_{32} and d_{43} , with d_{32} being defined as $\Sigma n_i d_i^3 / \Sigma n_i d_i^2$, and d_{43} being defined as $\Sigma n_i d_i^4 / \Sigma n_i d_i^3$, where n_i is the number of particles with diameter d_i . Mean particle diameters were calculated as the average of duplicate measurements.

Measurement of ζ -Potential

The ζ -potential values of the milk-SGF mixture samples were measured using a laser Doppler velocimetry and phase analysis light scattering (M3-PALS) technique and a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd.). Samples were diluted to 500 times with Milli-Q water. The temperature of the cell was maintained at 20°C using a Peltier system. An individual ζ -potential measurement was calculated from the mean and standard deviation of at least 10 readings from an individual sample.

Determination of Microstructure

Samples (1 mL) were stained with 1.0% (wt/vol) Nile Blue and 1.0% (wt/vol) Fastgreen (fluorescent dye), placed on a concave confocal microscope slide (Sail; Sailing Medical-Lab Industries Co. Ltd., Suzhou, China), covered with a coverslip, and finally examined with a 63 $\times$ magnification lens using a confocal laser scanning microscope (Leica DM6000 B, Heidelberg, Germany). The images were recorded to observe the change in the microstructure of the samples during digestion.

Determination of MFGM Proteins

The time-dependent proteolysis of the MFGM was examined using an SDS-PAGE technique. The MFGM material was isolated from milk samples as described by Ye et al. (2004). Raw milk samples were centrifuged at $15,000 \times g$ for 20 min at 20°C in a temperature-controlled centrifuge (Sorvall RC5C, DuPont Co., Wilmington, DE). The top layer (cream) was removed from the centrifuge tube using a spatula. The cream was washed 10 times with Milli-Q water to remove protein material that was not bound to the fat globule membrane. The mixture was left at room temperature for 1 h, recentrifuged at $15,000 \times g$ for 20 min at 20°C, and then the top layer was collected. This washing step was repeated twice.

The cream samples were then treated with SDS buffer (0.5 M Tris, 2.0% SDS, 0.05% β -mercaptoethanol, pH 6.8) and heated to 95 to 100°C for 5 min. The samples were cooled to room temperature and were

loaded (10 mL) onto a precast gradient gel (10 to 20% Tri-Tricine/peptide, 1.0 mm, cat. no. 345-0067), which was purchased from Bio-Rad, New Zealand. The gel was run in a Mini-Protean system (Bio-Rad, Richmond, CA) at 200 V using a Bio-Rad power supply unit (model 1000/500, Bio-Rad). The gels were stained with Coomassie Blue R-250 [0.05% (wt/vol) in 25.0% (vol/vol) isopropanol, 10.0% (vol/vol) acetic acid] for 30 min. After destaining [10.0% (vol/vol) isopropanol, 10.0% (vol/vol) acetic acid], the gels were scanned using a dual media flat-bed scanner (Scanmaker i900, Microtek, Carson, CA) and the intensities of the protein bands were quantified using GelQuant (version 2.7) software. The percentage composition of each sample was determined by scanning the areas for each band, and expressing the individual peptides or the gradual reduction in protein peak as a fraction of the sum total of protein.

The kinetics data were obtained from the quantification of PAGE, in which the protein bands in PAGE were scanned in a Geldoc (Bio-Rad, Universal Hood II, Hercules, CA).

Statistical Analysis

The samples were prepared in duplicate, and all tests were replicated twice. Experimental data were evaluated by running ANOVA tests, which determine whether any significant differences existed among the means at a 95.0% confidence level. These analyses were performed using the Minitab 14 (Minitab Pty Ltd., State College, PA) for Windows package. Treatment means were considered to be significantly different at $P \leq 0.05$.

RESULTS AND DISCUSSION

The fat globules in raw milk are coated by natural MFGM, which is composed predominantly of phospholipids and MFGM proteins (Mather, 2000). The average size (d_{32}) of the milk fat globules in the raw milk samples was approximately 3.10 μm . Figure 1 shows the changes in this average size during incubation for up to 90 min in SGF containing a range of pepsin concentrations. After an initial lag phase, d_{32} increased with an increase in incubation time. The lag time and the rate of increase were dependent on the concentration of pepsin. For example, at 12 min, a marked increase was observed in the size of the fat globules in the samples containing pepsin at 1.6 mg/mL, but no increase occurred in the samples containing pepsin at 0.1 or 0.4 mg/mL.

Figure 2 shows an example of the particle size distributions of milk fat globules in the SGF with pepsin (0.4

mg/mL) at different incubation times. A small peak appeared at large size area when milk was adjusted to pH 1.6, indicating the flocculation of the small part of fat globules. However, no change in the distribution occurred when the system containing pepsin was incubated at incubation time less than 21 min. At longer times (>20 min), the fat globule size peak with remaining monomodal distribution shifted toward to a large range. No further changes in the distribution of fat globules were observed after 60 min incubation.

Surface charge reflects the surface components of fat globules. The ζ -potentials of the fat globules in the raw milk after different incubation times are shown in Figure 3. When the milk samples were mixed with SGF, the ζ -potentials of the fat globules changed to positive values (approximately +8 mV) because the pH of the mixtures (pH 1.6) was considerably lower than the isoelectric point of milk proteins. However, no significant changes occurred in the ζ -potential values of the raw milk samples even at a pepsin concentration of 1.6 mg/mL after incubation for 90 min.

Figure 4A shows the protein patterns of MFGM material isolated from raw milk samples that were incubated in SGF containing pepsin at 0.1 mg/mL for up to 30 min, as determined by SDS-PAGE under reducing conditions. In the samples containing no pepsin, all major MFGM proteins were detected, but the intensity of xanthine oxidase appeared to be relatively less in the MFGM of milk mixed with SGF (no pepsin; pH 1.6) than in the MFGM of raw milk at neutral pH (Figure 4). The major MFGM protein bands ap-

Figure 1. Average particle size (d_{32}) of the fat globules in raw milk samples after incubation in simulated gastric fluid (SGF) containing pepsin at 0.1 mg/mL ($\diamond$), 0.4 mg/mL ($\square$), 0.8 mg/mL ($\blacktriangle$), and 1.6 mg/mL ($\circ$) as a function of time. Each data point is the average $\pm$ SE of 2 determinations on 3 separate samples.

Figure 2. Size distributions of milk fat globules in raw milk samples after incubation in simulated gastric fluid (SGF) containing 0.4 mg/mL pepsin at different time.

peared to decrease in intensity at different rates when the milk samples were incubated for various times in SGF containing pepsin. Xanthine oxidase decreased markedly, had almost disappeared after incubation for 5 min with pepsin at 0.1 mg/mL (Figure 4A), and had disappeared completely after incubation for 0.5 min with pepsin at 1.6 mg/mL (Figure 4B). Compared with xanthine oxidase, the decrease in the intensity of the butyrophilin band with incubation time was more gradual. The butyrophilin band decreased gradually in the sample containing pepsin at 0.1 mg/mL, whereas it disappeared at 20 min in the sample containing pepsin at 1.6 mg/mL. Both the PAS 6 band and the PAS 7 band appeared to decrease faster than the butyrophilin band in the presence of pepsin, and PAS 7 was more sensitive than PAS 6.

Overall, all MFGM protein bands had disappeared from the PAGE patterns after 15 min of incubation with pepsin at 1.6 mg/mL, indicating that all native MFGM proteins had been hydrolyzed by pepsin. Correspondingly, some peptides with low molecular weight were produced during incubation (Figure 4). For example, a peptide band with molecular weight of approximately 40 Da was observed after 0.5 min and the intensity of this band increased with increasing incubation time with pepsin at 0.1 mg/mL (Figure 4A). However, the intensity of the band decreased with incubation time at a high concentration of pepsin (1.6 mg/mL, Figure 4B), indicating that this peptide was further hydrolyzed by pepsin to form smaller peptides at high concentrations of pepsin and at long incubation times.

Kinetic data for the major MFGM proteins hydrolyzed by pepsin are shown in Figure 5. At low pepsin concentrations, the hydrolysis rates of the major proteins were different, in that xanthine oxidase and PAS 7 were hydrolyzed more rapidly and their amounts decreased to a low level at longer incubation times. When the concentration of pepsin was increased, the difference in the hydrolysis rates among the MFGM proteins became small. The amounts of intact protein decreased to approximately 10% at 15 min and were close to zero at 30 min.

The microstructures of the milk fat globules in raw milk as a function of the incubation time in SGF containing pepsin (1.6 mg/mL) were observed using confocal laser scanning microscopy (Figure 6). After incubation for 1.5 min, the fat globules remained uniform with even size distributions; no flocculation was observed. However, the fat globules flocculated at 10 min and this flocculation was enhanced at longer incubation times (≥ 30 min). The confocal micrographs showed that the fat globules were linked and aggregated through some protein-peptide material within the globules (Figure 6).

Flocculation of oil droplets during incubation in SGF containing pepsin was also observed in protein-stabilized model emulsion systems in an earlier study (Sarkar et al., 2009). They reported that changes in particle size, ζ -potential, and microstructure were observed in β -LG-stabilized emulsions (1.0 wt-% protein and 20.0 wt-% soy oil) under simulated gastric conditions with pepsin as a function of incubation time. Initially,

Figure 3. ζ -Potential of the fat globules in raw milk samples after incubation in simulated gastric fluid (SGF) containing pepsin at 1.6 mg/mL as a function of time. Each data point is the average $\pm$ SE of 2 determinations on 3 separate samples.

Figure 4. Sodium dodecyl sulfate-PAGE patterns of the milk fat globule membrane proteins and peptides obtained from the cream material of raw milk samples incubated in simulated gastric fluid (SGF) containing pepsin at 0.1 mg/mL (A) and 1.6 mg/mL (B) as a function of time. RM = raw milk.

β -LG formed a stable anionic emulsion at pH 7, but the emulsion underwent extensive droplet flocculation. It was considered that the peptides that remained at the interfaces of the oil droplets were unable to create strong interfacial layers with sufficient electrostatic repulsion and steric barriers to prevent the flocculation of droplets. In addition, the presence of relatively high concentrations of salt in the SGF further enhanced the process of flocculation.

Fat globules in milk are stabilized by a complex layer of phospholipids and proteins. Similar to the flocculation observed in protein-stabilized emulsions (Sarkar et al., 2009), the flocculation of the fat globules during incubation in SGF may have arisen from hydrolysis

of charged MFGM proteins, resulting in a decrease in electrostatic repulsion. However, the ζ -potential data (Figure 3) did not support this possibility, as it did not change significantly during digestion.

The casein micelles in the serum phase also aggregate with a reduction in pH and hydrolysis by pepsin (data not shown). These aggregated casein micelles may be involved in linking the fat globules together or the fat globules may be physically entrapped in the casein aggregate network. Confocal micrographs of the incubated raw milk confirmed the presence of protein material between the fat globules (Figure 6). To examine this further, cream obtained from the raw milk by centrifugation was dispersed in water or in diluted

Figure 5. Rate of pepsin hydrolysis of the major milk fat globule membrane (MFGM) proteins in simulated gastric fluid (SGF) containing pepsin at 0.1 mg/mL (a), 0.4 mg/mL (b), 0.8 mg/mL (c), and 1.6 mg/mL (d). XO = xanthine oxidase; BTN = butyrophilin.

skim milk (skim milk:water = 1:1). The sizes of the fat globules in the cream samples were determined during incubation in SGF containing pepsin (Figure 7). It is interesting that the fat globule size of the cream dispersed in water did not change during incubation. However, the fat globule size increased during incubation when the cream was dispersed in diluted skim milk, although the extent of the increase was less than that in raw milk. This result indicates that the protein in the serum phase was responsible for the aggregation of the fat globules during incubation in SGF containing pepsin. It appears that a low pH combined with a low level of hydrolysis caused aggregation of the casein micelles in the serum, which led to apparent flocculation of the fat globules.

Certainly, these casein proteins involved in flocculation of fat globules were no longer intact proteins as they were hydrolyzed to peptides by pepsin (Figure 4). When the incubation time was long (e.g., ~24 h), the fat globule size decreased and almost returned to that in the original raw milk (data not shown), indicating that the large peptide or material involved in joining the fat globules had been extensively hydrolyzed to small peptides and hence the flocculated fat globules disappeared.

The ζ -potentials of the fat globules in the cream (in a water solution) after different incubation times were similar to those of the fat globules in the raw milk (Figure 8). In SGF (pH 1.6), the ζ -potential was approximately 8 mV and did not change significantly in

Figure 6. Confocal micrographs of the fat globules in raw milk after incubation in simulated gastric fluid (SGF) containing pepsin at 1.6 mg/mL at different times. Red or orange (gray) color represents fat; green (black) color represents protein. Color version available in the online PDF.

the SGF containing pepsin at 1.6 mg/mL after incubation for up to 90 min.

Individual fat globules surrounded by intact membrane were observed using confocal microscopy in the cream samples dispersed in water and SGF (pH 1.6; Figure 9, panels A and B). For the cream sample incubated for 10 min, most of the fat globules were still individual, without flocculation. The MFGM did not appear to be intact; some pieces of membrane were observed on the interfaces of the fat globules (Figure 9C), indicating hydrolysis of the membrane proteins by pepsin.

It is interesting to note that no coalesced, large fat globules were observed in either the raw milk samples

or the cream samples after incubation in SGF (Figures 6 and 9), even though strong flocculation occurred in the raw milk samples after a short incubation time. This indicates that the increase in the size of the fat globules during incubation in the SGF determined by light scattering was due to flocculation, rather than coalescence, of the fat globules. It also suggests that the surface layer of the fat globules was still able to prevent their coalescence even after extensive hydrolysis of the membrane proteins. It was shown by SDS-PAGE that the membrane or surface layer on the fat globules consisted mainly of peptides after incubation for 30 min (Figure 4). This may indicate that the peptides hydrolyzed from the membrane proteins did not move

Figure 7. Average particle size (d_{32}) of the fat globules in raw milk (RM; $\blacklozenge$), cream dispersed in diluted skim milk (skim milk:water = 1:1, DRM; $\square$), and cream dispersed in water (RMC; $\blacktriangle$) after incubation in simulated gastric fluid (SGF) containing pepsin at 1.6 mg/mL as a function of time. Each data point is the average $\pm$ SE of 2 determinations on 3 separate samples.

into the aqueous phase but remained at the surface of the fat globules. These peptides formed a new surface layer on the fat globules in the flocculated particles, which prevented coalescence of the fat globules. Alternatively, the phospholipid in the MFGM may be able to prevent the coalescence of fat globules in the absence of membrane protein.

Figure 8. ζ -Potential of the fat globules in cream dispersed in water after incubation in simulated gastric fluid (SGF) containing pepsin at 1.6 mg/mL as a function of time. Each data point is the average $\pm$ SE of 2 determinations on 3 separate samples.

Figure 9. Confocal micrographs of the fat globules in cream samples: dispersed in water (A), in simulated gastric fluid (SGF) at pH 1.6 (B), and after incubation in SGF containing pepsin at 1.6 mg/mL for 10 min (C). Red or orange (gray) color represents fat; green (black) color represents protein (white pieces on the surface of fat globules are membrane proteins). Color version available in the online PDF.

CONCLUSIONS

The composition and properties of the MFGM protein layer in raw milk changed when the milk passed through gastric fluid containing pepsin. The major MFGM proteins were hydrolyzed by pepsin in the SGF at different rates; butyrophilin was more resistant than xanthine oxidase, PAS 6, and PAS 7 to hydrolysis by pepsin. Peptides with various molecular weights were present at the surface of the fat globules; the molecular weights of the peptides also changed with the time of incubation and the concentration of pepsin. The fat globules flocculated to form large particles during incubation in SGF. The aggregation of casein micelles in the serum phase, induced by a combination of low pH and hydrolysis by pepsin, may have been responsible for flocculation of the fat globules through the linking of the fat globules together or by simply trapping the fat globules in the casein aggregates. However, the fat globules in these large flocculated particles were still intact. This may be attributed to the peptides proteolyzed from the MFGM and serum protein, or phospholipid in the MFGM remaining at the surface of the fat globules to prevent their coalescence. The findings in the present study provide useful information for understanding the effect of the surface of fat globules on the digestion of lipids in dairy products.

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