A Simple Procedure for the Preparation of Bovine Milk Fat Globule Membrane and a Comparison of Its Composition, Enzymatic Activities, and Electrophoretic Properties with Those Prepared by Other Methods

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A simple and rapid procedure for the preparation of milk fat globule membrane (MFGM) is proposed. The membrane fragments released from bovine milk fat globules were recovered as MFGM by acidification at pH 4.8 and centrifugation (AC-MFGM). The yield, gross compositions, enzymatic activities, and electrophoretic properties of the resultant MFGM were compared with those of MFGMs recovered by ultracentrifugation (UC-MFGM) and by salting out (SA-MFGM). The different methods for recovering MFGM had significant effects principally on the lipid content and protein composition of MFGM. Of the three MFGMs, AC-MFGM had a moderate lipid content, while UC-MFGM had the lowest and SA-MFGM the highest. High activities of marker enzymes for plasma membrane in AC- and UC-MFGM were retained but not in SA-MFGM. Glycoproteins PAS-6 and -7 were preferentially released from UC-MFGM. The pH was a factor in causing the release of these glycoproteins. The release of PAS-6 and -7 was also ascertained by the decrease in UC-MFGM of proteins extractable with 1 M KCl and 8 M urea. The yield of MFGM was influenced mostly by the smaller fat globules and releasable protein and, consequently, was low in UC-MFGM, moderate in AC-MFGM, and high in SA-MFGM. Acidification to the isoelectric point was the easiest method for recovering MFGM, and resultant AC-MFGM had advantages over both the UC- and SA-MFGMs.

The milk fat globule membrane (MFGM) that surrounds milk fat droplets is derived from the apical plasma membrane of secretory cells in the lactating mammary gland.1,2) MFGM tends to be a fairly pure form of plasma membrane and has merits as a model and source for biochemical studies.1) MFGM consists of protein, glycoprotein, enzymes, phospholipids, triacylglycerols, cholesterol, glycolipids, and other minor components. MFGM is rich in glycoproteins, which can be stained by periodic acid-Schiff reagent (PAS) and separated to the major 7 bands (PAS-1 to PAS-7) on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE).3−6) From a comparison of the electrophoretic profiles of MFGM polypeptides in the literature5−12) (see also refs. 3 and 4 for reviews), it is evident that there are differences in the relative amounts of major polypeptides. In particular, glycoproteins PAS-6 and -7 (=CB-7 and -8),5) corresponding to components 15 and 16 of Mather and Keenan,7) were major components as well as PAS-5 (=CB-5) in any MFGM prepared by salting out,5,10,11) but were less than PAS-5 in MFGM pelleted by ultracentrifugation.7,9) The compositional heterogeneity of the total MFGM in part has been due to different preparative methods.

The composition and properties of bovine MFGM are affected by the isolation method,
which involves four major steps: separation of the cream, removal of the skim milk component by washing the cream, release of the membrane by destabilizing the washed fat globules, and recovery of the membranes.\textsuperscript{4,12} The reported yields and compositions of each MFGM differ considerably, because different researchers have used different methods for isolating MFGM.\textsuperscript{4,13} However, little comparison in the composition and properties of MFGM prepared by different methods under the same conditions has been made. Of the four steps for isolating MFGM, the procedure for recovering the released membrane fragments might cause the quantitative differences of such MFGM polypeptides as PAS-6 and -7. Usually, the membrane fragments can be recovered by ultracentrifugation at neutral pH\textsuperscript{7,9,12\textendash}15 or by the addition of ammonium sulfate to salt out.\textsuperscript{10,16,17} The methods involving ultracentrifugation and salting out are time-consuming for preparing large amounts of MFGM and for removing salts by dialysis, respectively.

In this study, we propose a simple and rapid method for recovering the membrane fragments released from milk fat globules by lowering the pH to the isoelectric point. The yield, gross compositions, enzymatic activities, and electrophoretic properties of MFGM prepared by acidification will be compared with those of MFGM prepared by the two conventional methods of ultracentrifugation and salting out.

**Materials and Methods**

\textit{Materials.} Acrylamide and \textit{N},\textit{N}'-methylene-bis-acrylamide were from Seikagaku Kogyo (Tokyo, Japan). \textit{N}-Acetyleneuraminic acid, glucosamine, and galactosamine were from Nakarai Tesque (Kyoto, Japan). PMSF and molecular weight markers were from Sigma (St. Louis, U.S.A.). All other chemicals were of analytical grade, and the organic solvents were redistilled before use.

\textit{Preparation of the membrane fragments.} Fresh raw milk (30 kg) from Holstein cows of the university herd was warmed to 38°C and immediately separated into cream and skim milk by a stainless steel cream separator. The raw cream was washed three times with 3 volumes each time of deionized water at 37°C in a cream separator. After chilling to 4°C in an ice bath, the washed cream was diluted to about 30% fat content with chilled distilled water and churned in a metal churn. The buttermilk was collected. To butter granules melted at 40°C was added half the quantity of distilled water at 40°C, and the butter serum was recovered in a cream separator. The buttermilk and butter serum were combined and centrifuged for 15 min at 3,000 \textit{x} \textit{g}, the cream being removed by aspiration.\textsuperscript{16,17} The supernatant fraction, designated as the total released membrane fragments, was divided into three portions (each 1.0 kg containing 1.01 g of protein) for recovering MFGM in the next step (Fig. 1).

\textit{Collection of MFGM from the aqueous phase.} The MFGM was concentrated from the aqueous phase by acidification (method 1), in addition to using ultracentrifugation (method 2), and salting out (method 3) (Fig. 1).

\textit{Method 1.} The released membrane fragments were acidified to pH 4.8 with 1 N HCl, left for 30 min at room temperature, and centrifuged for 30 min at 40,000 \textit{x} \textit{g} and at 4°C. After removal of the floating cream layer by aspiration, the supernatant (AC-SUP) was recovered and adjusted to pH 6.8 with 1 N NaOH. To the precipitate (AC-MFGM) was added 300 ml of distilled water and the pH was adjusted to 6.8 with 1 N NaOH, with stirring at 4°C.

\textit{Method 2.} MFGM was concentrated from the membrane fragments by centrifugation for 60 min at 100,000 \textit{x} \textit{g} and at 4°C. After removal of the floating cream layer by aspiration, the supernatant (UC-SUP) was recovered and adjusted to pH 6.8 with 1 N NaOH. To the precipitate (UC-MFGM) was added 300 ml of distilled water and the pH was adjusted to 6.8 with 1 N NaOH, with stirring at 4°C.

\textit{Method 3.} To the membrane fragments was added solid ammonium sulfate at half saturation and, after stirring...
for 30 min at room temperature, the suspension was centrifuged for 30 min at 10,000 \( \times g \). The supernatant (SA-SUP) was collected by aspiration, and the residual floating material (SA-MFGM) was dispersed in 300 ml distilled water. Two fractions were separately dialyzed against distilled water at 4°C for about 3 days, changing this several times until there was a negative reaction with BaCl\(_2\). The SA-MFGM fraction thus obtained was further centrifuged for 30 min at 27,000 \( \times g \) to remove any cream from the SA-MFGM preparation.

Delipidation of the MFGM samples. Delipidation of the MFGM samples was done as described previously. Roughly, 11 volumes of a mixture of methanol–chloroform (1:2, v/v) were added and stirred overnight at 4°C. The aqueous layer containing apo-protein was treated twice with chloroform, and the residual solvent in the aqueous layer was removed by a vacuum rotary evaporator.

**Extraction of KCl-urea-soluble proteins.** To 200 ml of the delipidized MFGM suspension containing 500 mg of protein was added sodium chloride up to 0.02 M, the mixture being stirred for 30 min at room temperature and centrifuged at 25,000 \( \times g \) for 1 hr at 4°C. The precipitate was dispersed in 50 ml of 100 mM Tris-HCl buffer (pH 8.2) containing 8 M urea, 1 M KCl, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), and 0.02% sodium azide, then stirred at 4°C overnight and centrifuged at 40,000 \( \times g \) for 30 min at 4°C. The solubilized proteins in the supernatant were freeze-dried after exhaustive dialysis against distilled water at 4°C.

**Chemical analyses.** Total solids were measured by the method of AOAC. All of the lipids were extracted by the method of Folch et al. and AOAC measurements followed. Protein was measured by the procedure of Markwell et al. A sample containing 1 M KCl and 8 M urea was treated by the procedure of Peterson et al., using 2% deoxycholate instead of 0.2%. Bovine serum albumin was used as the standard. Hexoses were measured by the method of Dubois et al., using a mixture of galactose and mannose (1:1) as the standard, and hexosamines by the method of Stewart-Tull, after hydrolyzing with 2 N HCl for 6 hr at 100°C and using a mixture of glucosamine and galactosamine (1:1) as the standard. Sialic acid was measured by the method of Warren, after hydrolyzing with 0.1 N sulfuric acid for 1 hr at 80°C and using N-acetylmuramic acid as the standard.

**Measurement of enzyme activities.** The activities of Na\(^+\), K\(^+\)-ATPase (EC 3.6.1.4), alkaline phosphatase (EC 3.1.3.1), \( \gamma \)-glutamyltranspeptidase (EC 2.3.2.2), phosphodiesterase I (EC 3.1.4.1), and 5'-nucleotidase (EC 3.1.3.5) were measured as described previously by Kanno et al. Electrophoresis. SDS-PAGE was done by the method of Laemmli, using 10% acrylamide gel in which the ratio of acrylamide and methylene-bis-acrylamide was 37:1 (w/w). A sample was solubilized with 10 mM Tris-HCl buffer (pH 6.8) containing 1% SDS, 1% 2-mercaptoethanol, and 2 mM EDTA by heating for 2 min in a boiling water bath. The amount of protein put on the gels was 100 \( \mu \)g for protein staining with CB, and 250 \( \mu \)g for carbohydrate staining with PAS reagent. The designation of the protein and glycoprotein bands of the MFGM samples is in accordance with Shimizu et al. The stained gels were scanned with an ISCO model UA-5 absorbance monitor with a linear transport system, the relative abundance of the CB- and PAS-positive polypeptides being calculated from the scanned relative peak areas.

**Results**

**Optimal pH for acidification**

The optimal pH for acidifying the released membrane fragments was identified by measuring the protein in a pellet precipitated at the indicated pH and at 40,000 \( \times g \) for 30 min (Fig. 2). The results show that most protein (97%) was precipitated between pH 4.75 and 4.85. For method 1, the released membrane fragments were recovered by acidifying to pH 4.80 and subsequent centrifugation.

**Gross composition**

The composition of the membrane fragments released from the fat globules is shown in Table I. From 10 kg of milk, about 3.6 g of membrane fragments was obtained. The composition of protein, lipid, and carbohydrate was 28.1, 64.6, and 3.3%, respectively, and they amounted to 96% of the total solids. The protein/lipid ratio...
Table I. CONTENT AND RECOVERY OF TOTAL SOLIDS, LIPIDS AND PROTEIN IN THREE MFGM PREPARATIONS AND IN THEIR SUPERNATANT FRACTIONS (SUP) RECOVERED FROM THE RELEASED MEMBRANE FRAGMENTS (RMF) BY THREE DIFFERENT METHODS

<table>
<thead>
<tr>
<th>MFGM</th>
<th>Total solids</th>
<th>Lipids</th>
<th>Protein</th>
<th>Carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MFGM SUP†</td>
<td>Total</td>
<td>MFGM SUP‡</td>
<td>Total</td>
</tr>
<tr>
<td>RMF* (g)</td>
<td>— — 3.59</td>
<td>— — 2.32</td>
<td>— — 1.01</td>
<td>— — 0.12</td>
</tr>
<tr>
<td>(SD)</td>
<td>±0.57</td>
<td>±0.42</td>
<td>±0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(%)</td>
<td>(100)</td>
<td>(64.6)</td>
<td>(28.1)</td>
<td>(3.0)</td>
</tr>
<tr>
<td>RMF**</td>
<td>— — 100</td>
<td>— — 100</td>
<td>— — 100</td>
<td>— — 100</td>
</tr>
<tr>
<td>Method 1 (AC)</td>
<td>73.5 ±0.9</td>
<td>77.3 ±3.6</td>
<td>92.2 ±1.1</td>
<td>63.7 ±1.1</td>
</tr>
<tr>
<td>Method 2 (UC)</td>
<td>43.9 ±3.9</td>
<td>27.5 ±3.1</td>
<td>79.2 ±3.3</td>
<td>59.6 ±1.3</td>
</tr>
<tr>
<td>Method 3 (SA)</td>
<td>80.3 ±2.4</td>
<td>84.4 ±4.5</td>
<td>94.3 ±1.3</td>
<td>50.0 ±2.9</td>
</tr>
</tbody>
</table>

* The numerals in RMF indicate the amount (g) obtained from 10 kg of different lot of raw milk (n=3, mean ± S.D.).
** The numerals in methods 1 to 3 indicate the content and recovery, which are expressed as a percentage of the amounts for RMF (n=3, mean ± S.D.). The recovery of MFGM to total RMF shows the yield of MFGM prepared by each method.
† Top cream layer was not taken into account.

was 0.44, the total membrane fragments being rich in lipid, compared with published values of MFGM (0.59 to 1.72).4)

The composition of MFGM preparation recovered from the released membrane fragments by method 1 is also shown in Table I in comparison with those of the MFGM prepared by the other two methods. The three methods chosen for recovering the released membrane fragments significantly affected the yield and composition of the resultant MFGM. The contents of total solid, lipid and protein were very low in the UC-MFGM prepared by method 2. These contents of AC-MFGM was higher than those of UC-MFGM, but lower than those of SA-MFGM. Accordingly, the mean yield was 2.6 g for AC-MFGM, 1.6 g for UC-MFGM, and 2.9 g for SA-MFGM from 10 kg of milk.

The amounts of total solids, lipid, and protein in the supernatant were significantly higher in UC-SUP than in AC-SUP and SA-SUP (Table I). Their total values in the preparations decreased in the total solids and lipid, since the smaller fat globules floating as the top cream layer during centrifugation were not included in the supernatant. Especially, the recovery of the preparations in method 2 was lower than those of the other preparations. The ratio of protein/lipid increased to 1.25 for UC-MFGM, while that of SA-MFGM (0.49), and AC-MFGM (0.52) was not as improved as the released membrane fragments (0.44). About 20% of the total protein was released from UC-MFGM to UC-SUP, while the protein released from SA- and AC-MFGM was less.

The carbohydrate recovered in MFGM was 50 to 60%. Between 30 and 34% of the total carbohydrate was released from UC- and AC-MFGMs to UC- and AC-SUP. The carbohydrate content in the preparation by method 3 was low (55%), 50% of which was in SA-MFGM and only 5% in SA-SUP.

Carbohydrate composition
The results of a further analysis of the carbohydrate are shown in Table II. The composition of the carbohydrate was also different among the three MFGM samples. Most of the carbohydrate in the released membrane fragments consisted of hexoses...
Table II. Carbohydrate Composition of Three MFGM Preparations and Their Supernatant Fractions (SUP) Prepared from the Released Membrane Fragments (RMF) by Three Different Methods

<table>
<thead>
<tr>
<th></th>
<th>Hexoses</th>
<th>Hexosamines</th>
<th>Sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MFGM</td>
<td>SUP*</td>
<td>Total</td>
</tr>
<tr>
<td>RMF* (mg)</td>
<td>—</td>
<td>—</td>
<td>75.0 ± 5.6</td>
</tr>
<tr>
<td>RMF**</td>
<td>—</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>Method 1 (AC)</td>
<td>46.7 ± 1.1</td>
<td>44.6 ± 8.0</td>
<td>91.3 ± 7.1</td>
</tr>
<tr>
<td>Method 2 (UC)</td>
<td>54.2 ± 6.5</td>
<td>43.8 ± 6.3</td>
<td>98.0 ± 1.3</td>
</tr>
<tr>
<td>Method 3 (SA)</td>
<td>37.4 ± 4.7</td>
<td>2.7 ± 0.5</td>
<td>40.1 ± 4.9</td>
</tr>
</tbody>
</table>

* The numerals in RMF indicate the amount (g) obtained from 10kg of different lot of raw milk (n=3, mean ± S.D.).
** The numerals in methods 1 to 3 indicate the percentage amounts of RMF, and the values for “Total” in each saccharide show the recovery (n=3, mean ± S.D.).
† Top cream layer was not included.

(62%), 37 to 54% of which was recovered in each MFGM, and the residual hexoses were contained in the supernatant. The amount of hexoses in SA-SUP was only 3% of the total hexoses. On the other hand, the amounts of hexosamines and sialic acid, which are concerned with the oligosaccharide chain of glycoprotein, were less in UC-MFGM and more in UC-SUP, suggesting the selective release of glycoprotein. The results of the hexose content by method 3 suggests that the lactose was trapped in the MFGM and eliminated by dialysis, which permitted small molecules to leak out. The other two methods retained the whole materials except for the top cream layer, which was removed intentionally. The acidification method (AC-MFGM) retained most proteins, hexosamine, and sialic acid.

Enzyme activity
Several enzymes have been routinely detected in bovine MFGM.6,10,26,31,32) Alkaline phosphatase, 5'-nucleotidase, Na+ K+-AT-Pase, phosphodiesterase I, and γ-glutamyl-transpeptidase are marker enzymes common to MFGM and the plasma membrane in lactating bovine mammary gland.26,32) The enzymatic activities in the three MFGMs and their supernatants are shown in Table III with their recoveries. The specific activities of the enzymes in UC-MFGM and AC-MFGM were close to those in the total MFGM, except that alkaline phosphatase was low in AC-MFGM and high in UC-MFGM, and γ-glutamyl-transpeptidase was high in AC-MFGM and UC-MFGM. However, the specific activities of SA-MFGM, which were lower than the other MFGMs, show inactivation of the enzymes during the prolonged dialysis of MFGM concentrated by method 3. SA-MFGM is thus unsuitable for enzymatic study. The enzyme activities of the supernatants were less than those of the corresponding MFGM.

The total activity shows that most enzymes were retained in MFGM and was less than 5% in the supernatants. However, the high total activity in UC-SUP (16%) indicates that enzymes were also released from UC-MFGM by ultracentrifugation at neutral pH.

Electrophoretic properties
Figure 3 shows SDS-PAGE patterns of MFGM and its supernatant prepared by the
Table III. Specific Activities (A), Total Activities (*), and Recovery (B) of Marker Enzymes of MFGM Preparations and Their Supernatant Fractions

<table>
<thead>
<tr>
<th></th>
<th>5'-Nucleotidase</th>
<th>Na&lt;sup&gt;+&lt;/sup&gt;, K&lt;sup&gt;+&lt;/sup&gt;-ATPase</th>
<th>Alkaline phosphatase</th>
<th>Phosphodiesterase I</th>
<th>γ-Glutamyl-transpeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>RMF</td>
<td>79.5</td>
<td>8.4*</td>
<td>0.53</td>
<td>0.06*</td>
<td>51.2</td>
</tr>
<tr>
<td>AC-MFGM</td>
<td>85.6</td>
<td>99.1</td>
<td>0.60</td>
<td>104.2</td>
<td>15.8</td>
</tr>
<tr>
<td>UC-MFGM</td>
<td>70.5</td>
<td>72.7</td>
<td>0.78</td>
<td>120.7</td>
<td>108.9</td>
</tr>
<tr>
<td>SA-MFGM</td>
<td>42.6</td>
<td>49.3</td>
<td>0.17</td>
<td>29.5</td>
<td>0.6</td>
</tr>
<tr>
<td>AC-SUP</td>
<td>26.5</td>
<td>2.3</td>
<td>0.13</td>
<td>1.7</td>
<td>18.5</td>
</tr>
<tr>
<td>UC-SUP</td>
<td>50.0</td>
<td>12.0</td>
<td>0.05</td>
<td>1.8</td>
<td>29.6</td>
</tr>
<tr>
<td>SA-SUP</td>
<td>12.7</td>
<td>0.6</td>
<td>0.18</td>
<td>1.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Specific activity (A) is expressed as \( \times 10^{-2} \mu\text{mol}/\text{mg protein/min} \).
* Total activity is expressed as \( \mu\text{mol}/\text{total protein/min} \). Recovery (B) is calculated as % of the total activity for the released membrane fragments (RMF). Data are the means of triplicate assays.

Fig. 3. SDS-PAGE Patterns of MFGM Preparations and Their Supernatants Prepared from the Released Membrane Fragments (RMF) by Three Different Methods.

A and B were stained with Coomassie brilliant blue R-250 (CB) and periodic acid-Schiff reagent (PAS), respectively. Lane 1, RMF; lane 2, AC-MFGM; lane 3, UC-MFGM; lane 4, SA-MFGM; lane 5, AC-SUP; lane 6, UC-SUP; and lane 7, SA-SUP. Molecular weight markers from the top to the bottom are myosin, β-galactosidase, phosphorylase, bovine serum albumin, ovalbumin, and carbonic anhydrase.

There were no qualitative differences between the PAGE profiles of the polypeptides in the three MFGMs. However, the intensity of CB-7 and -8 (=PAS-6 and -7) in UC-MFGM was apparently weaker than those in the other MFGMs (Fig. 3, lane 3). On the other hand, the major bands from all the supernatants were CB-1, -5 (=PAS-5), -7 and -8. In UC-SUP, the bands of CB-7+8 (=PAS-6+7) were stained most densely with both CB and PAS (Fig. 3, lanes 6).

The relative abundance of polypeptides and glycoproteins stained with PAS or CB was estimated by scanning (Table IV). The ratio of PAS-6+7 to all the glycoproteins of UC-MFGM was about 13%, which was significantly lower than that (18 to 20%) of SA- and AC-MFGM. On the contrary, PAS-6+7 contained 43% of the glycoproteins of UC-SUP, and it was much higher than those of the other supernatants (16 to 21%). CB-7+8 (=PAS-6+7) in UC-SUP amounted to about 61% of all polypeptides stained by CB, while being 25 to 27% in the other supernatant. These
Simple Method for Preparing MFGM

**Table IV. Relative Abundance of PAS- and CB-Positive Polypeptides on the SDS-PAGE Patterns of Three MFGM Preparations and Their Supernatant Fractions Recovered from the Released Membrane Fragments (RMF)**

<table>
<thead>
<tr>
<th>Glycoproteins*</th>
<th>RMF</th>
<th>AC-MFGM</th>
<th>UC-MFGM</th>
<th>SA-MFGM</th>
<th>AC-SUP</th>
<th>UC-SUP</th>
<th>SA-SUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS-1</td>
<td>18±5</td>
<td>21±3</td>
<td>19±3</td>
<td>21±3</td>
<td>22±2</td>
<td>24±2</td>
<td>23±2</td>
</tr>
<tr>
<td>PAS-2</td>
<td>8±2</td>
<td>10±&lt;1</td>
<td>8±3</td>
<td>8±1</td>
<td>10±1</td>
<td>7±1</td>
<td>11±1</td>
</tr>
<tr>
<td>PAS-3</td>
<td>11±1</td>
<td>11±&lt;1</td>
<td>12±3</td>
<td>10±1</td>
<td>13±2</td>
<td>9±&lt;1</td>
<td>12±2</td>
</tr>
<tr>
<td>PAS-4</td>
<td>19±3</td>
<td>17±1</td>
<td>19±1</td>
<td>17±1</td>
<td>15±4</td>
<td>14±4</td>
<td>20±5</td>
</tr>
<tr>
<td>PAS-5</td>
<td>24±3</td>
<td>21±4</td>
<td>28±4</td>
<td>26±4</td>
<td>19±5</td>
<td>4±2</td>
<td>19±6</td>
</tr>
<tr>
<td>PAS-6+7</td>
<td>19±3</td>
<td>20±&lt;1</td>
<td>13±1</td>
<td>18±1</td>
<td>21±&lt;1</td>
<td>43±&lt;1</td>
<td>16±&lt;1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polypeptides*</th>
<th>RMF</th>
<th>AC-MFGM</th>
<th>UC-MFGM</th>
<th>SA-MFGM</th>
<th>AC-SUP</th>
<th>UC-SUP</th>
<th>SA-SUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB-1</td>
<td>21±3</td>
<td>19±2</td>
<td>22±3</td>
<td>18±2</td>
<td>24±1</td>
<td>17±3</td>
<td>20±1</td>
</tr>
<tr>
<td>CB-5</td>
<td>32±1</td>
<td>33±1</td>
<td>37±4</td>
<td>32±1</td>
<td>27±2</td>
<td>7±2</td>
<td>27±3</td>
</tr>
<tr>
<td>CB-7+8</td>
<td>25±3</td>
<td>30±1</td>
<td>21±4</td>
<td>28±1</td>
<td>27±3</td>
<td>61±3</td>
<td>25±3</td>
</tr>
<tr>
<td>Others</td>
<td>22±5</td>
<td>19±2</td>
<td>21±2</td>
<td>22±2</td>
<td>22±4</td>
<td>15±2</td>
<td>28±3</td>
</tr>
</tbody>
</table>

* Values are shown as a percentage of the sum of the respective peak areas from PAS-1 to PAS-6+7 for glycoproteins, and of the total peak area for all polypeptides (n=3, mean±SD).

# Notice the high value of PAS-6+7 (=CB-7+8) in UC-SUP compared with SA- and AC-SUP.

**Fig. 4. SDS-PAGE Patterns of Supernatants Obtained by Centrifuging the Released Membrane Fragments at pH 4.8, 6.8, and 11.0.**

A and B were stained with CB and PAS, respectively. C is illustration of B. Lane 1, pH 4.8; lane 2, pH 6.8; and lane 3, pH 11.0.

Influence of pH on the release of PAS-6 and -7

To examine the influence of pH on the release of PAS-6+7 during the collection of MFGM, the pH of the released membrane fragments was adjusted to 4.0, 4.8, 6.0, 6.8, 8.0, 9.0, 10.0, and 11.0 before each MFGM sample was recovered by ultracentrifugation (100,000 x g, 30 min). Figure 4 shows SDS-PAGE patterns of the supernatant released from MFGM at pH 4.8, 6.8, and 11.0. The released proteins amounts correspond to 11% of the total MFGM proteins. The results show that part of CB-7 and -8 was preferentially released from the total MFGM by method 2, but not much in the precipitated MFGM (SA- and AC-MFGM). The selective release of glycoproteins of CB-7+8 (=PAS-6+7) might cause the higher contents of protein and carbohydrate of UC-SUP (Table I).
were predominantly PAS-6+7 (=CB-7+8) in the supernatant at each pH, the amount of PAS-6+7 released increasing with increasing pH. In addition, PAS-1 and -2 were released at neutral and alkaline pHs. The proportion of released protein to the total MFGM protein estimated by scanning was about 2% at pHs below 4.8, 16% at pH 6.0-6.8, 22% at pH 8.0, 25% at pH 9.0-10.0, and 28% at pH 11.0. Acidification (method 1) effectively inhibited proteins from releasing.

Extraction of KCl-urea-soluble proteins

Glycoproteins CB-7 and -8 can be extracted selectively from MFGM by KCl.30) The proteins extracted from the apo-MFGM of three MFGMs with a mixture of 1 M KCl and 8 M urea were analyzed by SDS-PAGE (Fig. 5). The major polypeptides in the KCl-urea-soluble fraction obtained from the membrane fragments and UC-MFGM were CB-7 and -8 (=PAS-6 and -7) with both protein and carbohydrate staining (Fig. 5, lanes 2 and 5). Similar results were obtained from SA-MFGM and AC-MFGM (data not shown). The ratio of extracted protein to the total protein was about 21% for AC-MFGM and SA-MFGM, and 15% for UC-MFGM. This indicates that part of the proteins extractable with KCl and urea had already been released from UC-MFGM into the supernatant (Table I and Fig. 3, lanes 6).

Discussion

Factors affecting the composition and properties of MFGM are the washing process of the cream, destabilization of the fat globules, and concentration of the released membrane fragments. In this study, we paid particular attention to the method for recovering the released membrane fragments and to the properties of the resultant MFGM preparations. An acidification to pH 4.8 (method 1) was adapted for concentrating the released membrane fragments, in addition to ultracentrifugation (method 2) and salting out (method 3). Three MFGM preparations were recovered from the same released membrane fragments by the three methods.

In the first, the total protein and lipid contents of the released membrane fragments were within the values reviewed,1-4) although variation is evident in the values for MFGM reported by several groups. It would be difficult to make a satisfactory comparison of the properties of MFGM from this study and published papers, because of the variety of conditions used in washing the cream, destabilizing the fat globules, and subsequently
concentrating the released membrane materials, in addition to the state of the milk before separation (e.g., cooling and aging).4)

The three different methods for recovering the released membrane fragments resulted in differences of composition, enzymatic activities, and electrophoretic properties among the resultant MFGMs. Of three MFGMs, UC-MFGM had a much lower lipid content than SA-MFGM and AC-MFGM. The lipid/protein ratio of UC-MFGM was 0.80, which is very close to that of the plasma membrane (0.61 to 0.77) of lactating bovine mammary secretory cells.33) The difference of lipid content among the three MFGMs seems to have depended on the smaller fat globules, which ranged from 0.1 to 11µm in globular size distribution.34) By method 2, the lipid content of UC-MFGM was less, since the smaller fat globules, which had not been destabilized, could be effectively removed from UC-MFGM as the top cream layer. In contrast, by method 1 (AC-MFGM) and method 3 (SA-MFGM), the smaller fat globules were coprecipitated with MFGM. A higher ratio of lipid/protein in these MFGM causes not only triacylglycerols, which are included in MFGM during the destabilization of milk fat globules,3) but also smaller fat globules, which could not be removed by whole precipitation methods (methods 1 and 3). Consequently, the yield of membrane was higher in AC- and SA-MFGM than in UC-MFGM.

The destabilized membrane fragments have easily releasable CB-7 and -8 glycoproteins. By method 2, part of these glycoproteins was preferentially released from UC-MFGM (Fig. 3). The protein content was 10% less in AC- and SA-SUP, but 3 to 5 times more in UC-SUP (Table I). The SDS-PAGE pattern of UC-MFGM is similar to that of MFGM in published papers.12,13) Glycoproteins CB-7 and -8 are peripheral proteins and associated loosely with other constituent proteins of the membrane by a weak interaction.35) The pH value was also a factor affecting the release of CB-7 and -8 (Fig. 4), which were not improved by 0.25M sucrose in 10mM Tris–HCl buffer (pH 7.5)7) instead of the water used here (data not shown). The results of extraction with KCl and urea, furthermore, indicate that part of the proteins extractable with KCl and urea had already been released from UC-MFGM (Fig. 5). By method 1 (AC-MFGM) and method 3 (SA-MFGM), these releasable proteins could be recovered together with other membrane fragments. The low yield of UC-MFGM was caused by the release of about 20% of the protein in addition to thoroughly removing the smaller fat globules.

A comparative study by Basch et al. of washing cream with different buffers has pointed out that, as the salt concentration in the buffer was decreased, such milk proteins as caseins were not washed out, and bands B3 and B4, referred to as PAS-6 and PAS-7, were strongly released.12) Method 5 in those results corresponds to our method 2. A comparison of the electrophoretic pattern between both MFGM proteins showed two very different points: their preparation contained a lot of casein and much less PAS-6 and PAS-7 than our preparation. Some detailed differences between the two preparation methods existed. Basch et al. using PMSF, centrifuged to wash the cream, and chilled the washed cream at 5°C overnight in their preparation.12) The use of a cream separator for washing the cream in this study was more effective than centrifugation. The aging of washed cream at 5°C overnight caused the release of many PAS-6 and PAS-7 glycoproteins (data not shown). It is unlikely that these glycoproteins are released by the action of indigenous proteinase in the absence of PMSF during aging at 5°C.

The isoelectric point of the whole MFGM materials is unknown. Electrofocusing pH values of each major MFGM protein have ranged from 3.5 to 7.6.6) The optimal value of pH 4.80 for acidification may correspond to the isoelectric point of whole MFGM. Isoelectric precipitation at pH 3.9 to 4.0, which was used for concentrating MFGM by Palmer and Samuelsson36) and Jenness and Palmer,37) has not subsequently been used for concentrating the released MFGM, being replaced by the
wide use of centrifugation since Brunner and Thompson.\textsuperscript{15) Method 1 was improved by combining acidification to the isoelectric point at pH 4.8 with centrifugation at a lower speed, and the resultant AC-MFGM had some advantages over both UC-MFGM and SA-MFGM with respect to the yield, enzymatic activity, protein, and carbohydrate retention, etc. Furthermore, method 1 was easiest for recovering MFGM from the released membrane fragments, while method 2 needed much more time for precipitation when there were large sample volumes, and method 3 also required a long time for removing the ammonium sulfate. The releasable CB-7 and -8 could also be pelleted with MFGM by method 1. Although AC-MFGM was neutralized with 1 n NaOH for further analysis of composition, it may be more desirable to disperse with an appropriate basal reagent such as trishydroxymethylamethane, especially for an enzymatic analysis. In addition, an alternative procedure combining method 1 and 2, i.e., to ultracentrifuge again AC-MFGM recovered by method 1 at neutral pH by method 2, offers either a lower lipid content of MFGM by the removal of top cream layer alone or separation of the selectively released CB-7 and -8 glycoproteins by partition.

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References