KOREAN J. FOOD SCI. TECHNOL.
Vol. 21, No. 5, pp. 726~734 (1989)

Composition of Lipids Associated with Dense Coat-enriched Fractions of Bovine Milk Fat Globule Membrane

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Abstract

To analyze the components of dense coat fractions associated with fat globule membrane, The membrane was treated with various concentrations of Triton X-100, a non-ionic detergent, and the composition of lipids associated to the detergent insoluble material was analyzed. The amount of protein, phospholipid, cholesterol and ganglioside in milk fat globule membrane was reduced consistently with increasing concentrations of Triton X-100. Butyrophilin (band 12), xanthine oxidase (band 3) and band 16 as constituents of insoluble coat materials was revealed after electrophorisis on SDS-polyacrylamide gels. Phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol and sphingomyelin were identified as the major phospholipids of the coat materials without selective concentration relative to the original membranes. Percentages of total phospholipid were not changed by any of the treatments. Fatty acids of total lipid were myristate, palmitate, stearate (major saturated acids), oleate and linoleate (major unsaturated acids). Cholesterol contents on a protein basis were slightly reduced with increasing concentrations of Triton X-100. Cholesterol adhered to protein more tightly than other constituents. The contents of gangliosides was proportionally reduced with increasing concentration of Triton X-100.

Key words: milk fat globule membrane, protein, phospholipid, cholesterol, ganglioside

Introduction

The lipid composition of the fat globule membrane has been extensively studied.[1,2] Biological and morphological comparison of the milk fat globule membrane (MFGM) with plasma membranes from lactating bovine mammary gland has shown that these two membranes are nearly identical in distribution and composition of phospholipids and cholesterol esters.[3] It was also observed that fat globule membrane was derived from plasma membrane by rearrangement of membrane structure.

An intensely stained layer which is sandwiched between the internal face of the milk fat globule membrane and outer surface of the lipid droplet was observed by electron microscopy.[4] Dense coat material, 10 to 50 nm in thickness, remains associated with the membrane during isolation and extensively washing MFGM with low and high salt buffers and with Triton X-100 (non-ionic detergent). This coat material forms plaques of a finely filamentous texture. On electrophoresis in sodium dodecyl sulfate containing polyacrylamide gels the insoluble membrane has been shown to contain two major polypeptide bands which are band 3 (xanthine oxidase)[4] and band 12 (buthylophilin).[5]

It was shown that there were small amounts of phospholipids and gangliosides associated with the dense coat material of MFGM.[4] Keenan *et al.*[6] have analyzed fatty acids of coat material from bovine MFGM which had palmitate, stearate and oleate as major fatty acids.

As a continued study on dense coat-enriched fraction, composition of coat material associated lipid from bovine MFGM was determined. The

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analyses were centered on phospholipid/protein, polypeptides in protein, separation of polar lipids, quantitative distribution of phospholipids, fatty acids of total and polar lipids, cholesterol content and ganglioside content. The coat materials of bovine MFGM were prepared with 0.25, 0.50, 0.75 and 1.00% Triton X-100, a non-ionic detergent, to compared with untreated membrane.

This study may provide fundamental information for developing new dairy foods such as patient food, diet food and heath food.

Materials and Methods

Isolation of MFGM coat-enriched fraction

Milk fat globules were prepared according to Keenan *et al.*[7] The cream fraction from 5 gallons of fresh milk from Holstein cows was collected with a small separator, and washed three times with a total of 5 gallons of 0.9% saline. Washed cream was allowed to cool on ice in a Waring Blender for at least 20 minutes, and then blended with approximately 800 m*l* H_2O until butter was formed. The mixture was filtered through cheese cloth, saving the aqueous phase which contained MFGM. MFGM was obtained as an orange-brown pellet after centrifugation of the aqueous phase (40,000 × g) for 5 hours at 4 °C in a Type-19 rotor in a Beckman Model L3-50 ultracentrifuge. A suspension of MFGM in distilled water was mixed with an equal volume of 3.0 M KCl in 10 mM Tris (pH 8.0), and held for 1 hour on ice. It was then centrifuged in a Spinco SW 28.1 rotor (100,000 × g) for 1 hour at 2 °C. The pellet obtained was suspended in distilled water (5 m*l*) with ultrasound and with a Polytron. It was centrifuged again as above. Insoluble material (8 m*l*) was suspended in 2.0% Triton X-100 in 10mM Tris (pH 8.0) (8 m*l*), held for 1 hour on ice and insoluble material (coat fraction) was obtained by centrifugation as above. Protein content of all fractions was determined with the Folin phenol reagent[8] using bovine serum albumin as the standard.

For extraction of membranes in different concentrations of Triton X-100, the procedure was followed as above using the following amounts of Triton X-100, 0.25%, 0.50%, 0.75% and 1.00% in 10mM Tris (pH 8.0). No Triton X-100 was added to the control.

Characterization of MFGM coat-enriched fraction

Extraction and analysis of phospholipids

Analysis of phospholipids was according to the methods of Rouser,[9] and Keenan.[10] The native MFGM, KCl-washed MFGM and 2.0% Triton-washed MFGM. Samples (4 m*l*) of MFGM from the above treatments were stirred in 80 m*l* of chloroform-methanol (1:1, v/v). Combined filtrates were concentrated in a rotary vacuum evaporator. Five m*l* and 3 m*l* of chloroform-methanol (2:1, v/v) were added sequentially to dissolve the lipids, which were transferred to a screw-capped vial. After adding 0.2 volume (1.6 m*l*) of 0.9% KCl the chloroform-rich lower layer was collected and washed twice with 3 m*l* of methanol-water (1:1, v/v). Solvent was evaporated under a stream of nitrogen. Then 1 m*l* chloroform was added to the sample and 25 and 100 u*l* portions were taken for phosphorus assay.[9]

Two-dimensional thin-layer chromatography of the phospholipids was conducted on 250 um layers of silica gel HR (Supelco, Inc.). Thin-layer plates were heated in an oven at 100 °C for at least 30 minutes before use. An aliquot of the extract equivalent to 0.5 mg phospholipid was taken (20 ug phosphorus) and evaporated under nitrogen. Then 50 u*l* of chloroform was added and the sample was applied to the plate. Plates were developed in tank lined with solvent-saturated filter paper. The solvent systems used were chloroform, methanol, ammonium hydroxide, water (65:35:1:4, v/v/v/v) in the first dimension followed by chloroform, acetone, methanol, acetic acid, water (50:20:10:10:5, v/v/v/v/v) in the second dimension.[11] Positions of the lipids were revealed by spraying with H_2SO_4 in water (1:1, v/v) and heating in an oven at 120 °C for 30 minutes. Phos-

pholipid spots were outlined with a needle, scraped off with a razor blade, and analyzed for phosphorous content.[9]

Fatty acid analysis

Total lipid fatty acids were converted to their methyl esters.[12] Methyl esters were prepared from the samples treated with Triton X-100 and form controls by the following method. The samples in 100 u*l* chloroform were evaporated under nitrogen. Two m*l* boron triflouride-methanol were added to the vials and they were heated (80 °C) for 60 minutes. Saturated NaCl solution was added to the vials and methyl esters were extracted with hexane. Samples were then evaporated to the desired concentration under nitrogen before injection into the gas chromatograph.

Polar lipids were separated from neutral lipids by thin-layer chromatography on 500 u layers of silica gel G (Analtech, Inc.), One-dimensional separations were accomplished in the solvent system hexane: ethyl ether: acetic acid (85:15:1, v/v/v). After scraping from the plate (about 2 cm above and below the origin), the samples were treated as for total lipid.

Methyl esters were analyzed by gas chromatography in a Hewlett-Packard Model 5730A equipped with flame ionization detectors. The column was packed with 10% SP-2330 on 100/120 mesh Supelcoport (Supelco, Inc., two meter column) and was operated isothermally at 200 °C. Peak areas were calculated with a Model 3390A Intergrator (Hewlett-Packard).

Cholesterol analysis

Sample obtained as for total phospholipid were evaporated. Chloroform (3 m*l*) was pipetted to each sample and cold acetic anhydride-sulfuric acid reagent (4:1, v/v) was added. Sample were held at room temperature for 15 minutes to develop the blue-green color and absorbance was measured at 625 nm in a Gilford Model 250 Spectrophotometer.[13]

Ganglioside analysis

Gangliosides were recovered from methanol-chloroform supernatants by partitioning and dialysis.[14,15] Ganglioside sialic acid was released by hyrolysis with 0.05 M H_2SO_4 for 1 hour at 80 °C and measured by the thiobarbituric acid method with N-acetylneuraminic acid as the standard.

Electrophoresis

Polypeptides were treated with SDS and separated by electrophoresis in 9% polyacrylamide slab gels containing SDS.[16]

Results and Discussion

For comparative purposes coat fractions of milk fat globule membrane (MFGM) were prepared by extraction with high salt buffer (3.0 M KCl) and Triton X-100 (2.0%) and phospholipids, one of the major membrane lipid classes, were measured by the methods of Rouser *et al.*[9] and Keenan *et al.*[10] Table 1 shows that after washing original MFGM with potassium chloride (3.0 M) most phospholipid still remained (98%) but treating it with 2.0% Triton X-100, a non-ionic detergent, resulted in remarkable reduction of phospholipid (86%). In measureing phospholipid per mg protein of MFGM (Table 1) all of phospholipid remained in treatment with high salt buffer but only 37% of phospholipid remained in the non-

Table 1. Comparison of high salt buffer with Triton X-100 (2.0%) for extraction of phospholipid from MFGM

Treatment	Phospholipid in sample	Phospholipid / mg protein
	ug / ml sample	ug / mg protein
Original MFGM	6143.25	510.66
3.0 M KCl	6045.88 (98%)	632.08 (123%)
2.0% Triton X-100	841.88 (14%)	192.65 (37%)

Samples were treated as described in the text. Phospholipid was determined by phosphorus x 25. The values were the average of two samples.

Table 2. Protein and phospholipid removal from milk fat globule membrane by Triton X-100 extraction

% Triton X-100	Removed protein	Removed phospholipid	Remaining phospholipid
	mg/original MFGM protein[a]	mg/original MFGM PL[b]	ug/mg protein[c]
0.25	17.06 (79.9%)	14.11 (73.7%)	1,177 (131.2%)
0.50	17.10 (80.1%)	16.97 (88.6%)	513 (57.2%)
0.75	18.28 (85.7%)	17.82 (93.0%)	433 (48.3%)
1.00	18.35 (85.9%)	18.10 (94.5%)	350 (39.0%)

Isolated milk fat globule membrane was treated with 0.25%, 0.50%, 0.75% and 1.00% Triton X-100. MFGM protein was measured by the method of Lowry *et al.* (1951). Phospholipids were determined as lipid phosphorus x 25. values are the means of at least five determinations.

a) Original MFGM protein average was 21.34 mg.

b) Original MFGM phospholipid was 19,147 ug.

c) original MFGM phospholipid per mg protein was 897 ug.

MFGM; Milk fat globule membrane.

PL; Phospholipid.

ionic detergent treated MFGM. These results suggested that integral proteins interact extensively with the hydrocarbon chains of membrane lipids so they can be released only by agents that complete for these non-polar interactions.

Protein and phospholipid removal from MFGM by different concentrations of Triton X-100 is shown in Table 2. Proteins removed from original MFGM were 79.7%, 80.1%, 85.7% and 85.9% for 0.25%, 0.50%, 0.75% and 1.00% Triton X-100 extraction, respectively. Removed phospholipids per original MFGM phospholipid were 73.7%, 88.6%, 93.0% and 94.5% for 0.25%, 0.50%, 0.75% and 1.00% Triton X-100 extraction, respectively. These results show a nearby proportional release of protein and phospholipid as expected. Similar results were previously obtained by Freudenstein *et al.*[4]

Electrophoresis in the presence of sodium dodecyl sulfate on 9% polyacrylamide gels was employed to determine if there was any selective concentration of polypeptides in the Triton-insoluble fraction of MFGM. As shown Fig. 1, band 1 (a), band 3 (b), band 12 (c), band 16 (d), band 18 (e) and band 20 (f) were observed in

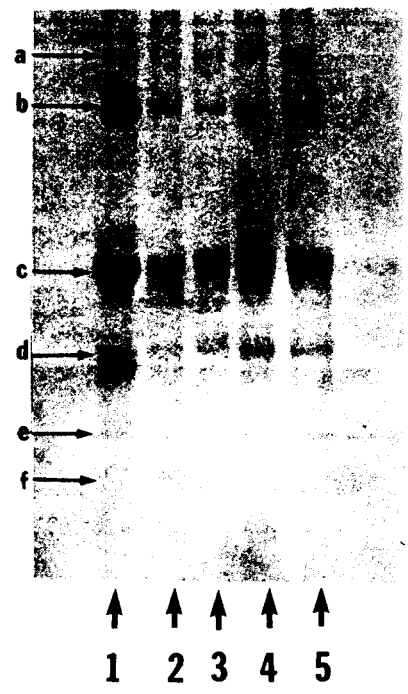

Fig. 1. Selective removal of proteins from MFGM by Triton X-100 extraction as seen after electrophoresis on SDS polyacrylamide (9%) gels.

1, untreated MFGM as a control; 2, 3, 4, and 5; pelleted residue fractions after treatment with Triton X-100 at 0.25%, 0.50%, 0.75% and 1.00%, respectively. Arrows along lane 1 lettered a through f denote MFGM polypeptide bands 1, 3, 12, 16, 18 and 20, respectively. The gel was stained with Coomassie blue

0.25%, 0.50%, 0.75% and 1.00% Triton X-100 extracted MFGM. Band 3, band 12 and band 16 were major components in both untreated and Triton X-100 treated preparations. These were no significant differences among major polypeptides on exposure to various concentrations of Triton X-100. These results suggested that retained polypeptides may be "intrinsic" or structural components of the membranes. Band 3 and 12 were previously identified as xanthine oxidase (mol. wt. 155,000)[4] and as butyrophilin (mol. wt. 67,000).[5] It was suggested that xanthine oxidase and butyrophilin were major components of the dense coat material of MFGM.

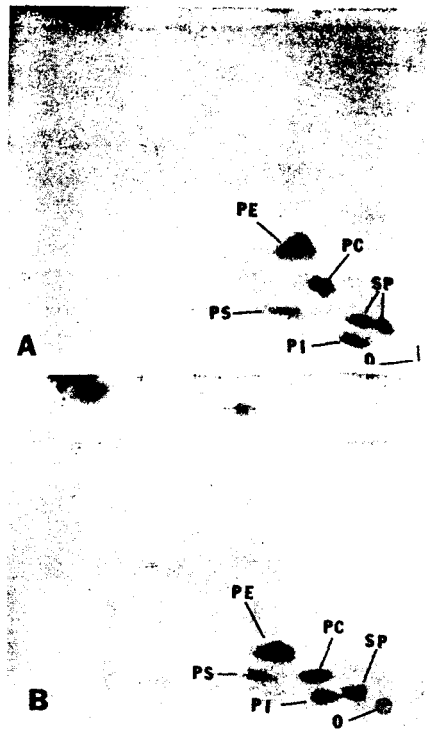

Fig. 2. Two dimensional thin-layer chromatographic separation of polar lipids from MFGM (A) and Triton X-100 (0.25%) washed MFGM (B).
Approximately 0.5 mg of phospholipid was applied to plates. The chromatograms were developed in the horizontal direction in chloroform-methanol-ammonium hydroxide-water (65:35:1:4, by vol.) and, after drying, in the vertical direction in chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by vol.). Spots were visualized by charring with 50% H_2SO_4 at 120°C. O, origin; SP, sphingomyelin; PC, phosphatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine.

Two dimensional thin-layer chromatographic separation of polar lipid from original MFGM and Triton X-100 treated MFGM is shown in Fig. 2. Both of the samples revealed comparatively identical results. Sphingomyelin, phosphatidyl choline and phosphatidyl ethanolamine were observed to be the major constituents. Less amounts of phosphatidyl serine and phosphatidyl inositol were also present. This same quantitative distribution has been observed in bovine milk and mammary tissue[11] and in milk fat globule membrane and plasma membrane.[3] These results indicated that there was no selective retention of any individual phospholipid in insoluble dense coat material of MFGM. In this experiment sphingomyelin in MFGM (Figure 2, A) was partially separated into two spots, probably because of differences in fatty acid and sphingosine chain lengths.

For quantitative analysis of Triton X-100 washed MFGM phospholipid, two dimensional thin-layer chromatographic separation[11] and analysis of phospholipid[9] were employed (Table 3). Surprisingly, the percentages of each phospholipid were similar comparing original MFGM with coat-enriched residual MFGM in phosphatidyl choline, phosphatidyl inositol and sphingomyelin. However, a tendency toward enrichment of phosphatidyl ethanolamine was evident in insoluble coat fractions[4] and phosphatidyl serine was released by increasing concentrations of Trition X-100.

Table 3. Distribution of phospholipids in bovine MFGM and coat-enriched fractions from MFGM prepared with Triton X-100

Component	% of total phospholipid				
	MFGM	Triton X-100 washed MFGM (%)			
		0.25	0.50	0.75	1.00
SP	22.23±7.06	22.21±2.73	17.76±5.95	16.61±8.65	24.59±6.24
PC	28.97±5.01	25.88±1.37	26.80±3.92	28.02±4.75	24.13±5.31
PS	2.07±0.72	0.70±0.46	1.37±0.38	1.04±0.89	0.64±0.40
PI	10.16±2.67	9.24±2.29	12.44±1.34	10.75±3.35	11.74±2.91
PE	35.42±4.85	41.22±1.84	41.45±3.74	42.51±4.95	38.93±4.50

Phospholipid spots on thin-layer plates were scraped off and analyzed for phosphorus content. Results are means ± S.D. for at least 5 samples. SP, sphingomyelin; PC, phosphatidyl choline; PS, phosphatidyl serine, PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine.

Table 4. Fatty acid composition of total lipid from MFGM and coat-enriched fractions from MFGM prepared with Triton X-100

Fatty Acid	Composition (%)				
	MFGM	Triton X-100 washed MFGM (%)			
		0.25	0.50	0.75	1.00
10:0	1.5	0.5	0.4	1.3	1.0
12:0	2.1	1.6	1.9	3.3	2.3
14:0	7.7	5.7	7.1	7.2	6.4
14:1	0.6	0.6	0.7	0.9	0.8
15:0	0.9	1.9	0.6	1.0	0.5
16:0	26.3	21.0	20.7	23.2	22.4
16:1	2.3	3.5	3.8	3.9	3.8
17:0	0.8	1.0	0.7	1.2	1.7
18.0	11.7	10.5	10.2	12.9	12.9
18:1	33.6	31.6	33.4	31.7	32.4
18:2	6.6	8.8	7.2	7.7	8.6
18:3	1.3	2.7	2.5	1.5	2.6
unknown	1.5	2.3	3.2	3.0	2.8
unknown	1.8	2.0	2.5	1.3	1.8
20:4	1.3	6.3	5.1	trace	trace

Methyl esters of total lipids were prepared and analyzed by gas liquid chromatography. All values are averages of triplicate determinations. Data are given as weight percentage of total fatty acid methyl esters. Number before colon gives number of carbon atoms; number after colon gives number of double bons.

Table 5. Fatty acid composition of polar lipids from MFGM and coatenriched fractions from MFGM prepared with Triton X-100

Fatty Acid	Composition (%)				
	MFGM	Triton X-100 washed MFGM (%)			
		0.25	0.50	0.75	1.00
10:0	0.1	trace	trace	trace	trace
12:0	0.6	1.0	1.0	2.7	trace
14:0	4.2	3.7	3.5	4.9	1.6
14:1	0.5	0.6	0.7	1.8	1.9
15:0	1.0	0.8	0.6	0.9	trace
16:0	19.3	20.5	17.6	18.8	20.3
16:1	2.4	3.0	3.0	9.0	11.8
17:0	0.6	trace	trace	trace	trace
18:0	13.2	14.0	18.9	22.6	25.7
18:1	42.4	40.5	41.5	39.3	33.0
18:2	11.7	12.7	13.2	trace	5.7
18:3	0.7	2.0	trace	trace	trace
unknown	1.4	1.3	trace	trace	trace
unknown	2.0	trace	trace	trace	trace

Methyl esters were obtained from polar lipids and were analyzed by gas liquid chromatography as described in methods. All values are averages of triplicate determinations. Data are given as weight percentage of total fatty acid methyl esters. Number before colon gives number of carbon atoms; number after colon gives number of double bons.

Data on the fatty acid composition of total lipid from MFGM and coat-enriched fractions from MFGM treated with Triton X-100 are in Table 4. Myristate, palmitate and stearate were the major saturated acids of original MFGM and Triton X-100 treated MFGM. 10:10, 12:0, 15:0 and 17:0 were also present in measurable amounts. Fatty acids of chain length less than 10 carbon were detected in trace amounts. Oleate and linoleate were present as major unsaturated acids of MFGM and non-ionic detergent treated MFGM. 14:1, 16:1, 18:3 and 20:4 were observed as minor constituents in both MFGM and detergent-insoluble fractions. Two unknown fatty acids were contained in both MFGMs. Palmitate as percentage of the total fatty acid was reduced from original MFGM (26%) to Triton X-100 treated MFGM (22%), but conversly linoleate, linolenate and an unknown acid were increased in percentage of total fatty acids. Strangely, in 0.25% and 0.50% Triton X-100 washed MFGM arachidonate was increased (6.3% and 5.1% from 1.3%) but in 0.75% and 1.00% Triton X-100 washed MFGM arachidonate was detected in only trace amounts. Results of major fatty acids were nearly identical with previous work.[3,19]

Fatty acid composition of total polar lipids from original MFGM and coat-enriched fractions from MFGM is shown in Table 5. Palmitate and stearate were present as major saturated acids in original MFGM and Triton X-100 washed MFGM. Laurate, myristate, 15:0, 17:0 and linoleate were minor constituents in both preparations. Fatty acids of chain length less than 12 carbons were detected in trace amounts in both preparations. Palmitoleate, oleate and linoleate were the major unsaturated acids of untreated MFGM and Triton X-100 treated MFGM and 14:1 and li-

Table 6. Cholesterol content of Triton X-100 washed MFGM

	MFGM	Triton X-100 (%) washed MFGM			
		0.25	0.50	0.75	1.00
Cholesterol/protein (μg/mg)	66.6	78.4	65.1	54.0	42.6
Removed cholesterol/ Original MFGM protein (μg/mg)		46.1	52.4	56.3	58.7

Cholesterol was extracted with chloroform-methanol, recovered and determined as described in the text. Original MFGM protein was 23.2 mg. 0.25, 0.50, 0.75 and 1.00% Triton washed MFGM protein levels were 6.11, 5.10, 4.54 and 4.38 mg, respectively. Results are average of 5 samples.

nolenate were observed as minor unsaturated acids in both. Two unknown fatty acids were present in trace amounts. 14:1, palmitoleate and stearate were consistently increasedin percentage of total fatty acid in accordance with increasing concentrations of Triton X-100. Oleate and linoleate were increased in 0.25 and 0.50% Triton X-100 washed MFGM but reduced in increased concentrations of Triton X-100. Clearly, in 1.00% Triton washed MFGM, many fatty acids were present in only trace amounts. Results of the polar lipid composition study were similar to those of an earlier study.[20]

Cholesterol contents of original MFGM and Triton X-100 washed MFGM are in Table 6. Cholesterol was calculated on the basis of insoluble protein and as the amount removed based on original MFGM protein. Cholesterol contents were 66.6 ug/mg protein in original MFGM and were 78.4, 65.1, 54.0 and 42.6 ug/mg protein in 0.25%, 0.50%, 0.75% and 1.00% Triton X-100 treated MFGM, respectively. Removed cholesterol contents were 46.1, 52.4, 56.3 and 58.7 ug/mg original MFGM protein in 0.25%, 0.50%, 0.75% and 1.00% Triton X-100 washed MFGM, respectively. Cholesterol was proportionally removed in increasing amounts as concentration of Triton X-100 was increased. McCarthy and Headon[21] reported that cholesterol content increased as concentration of fat in milk increased. This result suggested that cholesterol remains more in non-polar area of the bilayer membrane, and with mainly polypeptide band 3 and 12, which remain in the dense coat fraction in MFGM.

Table 7. Ganglioside content of MFGM and coat-enriched fractions of MFGM prepared with Triton X-100

Triton X-100 (%)	Ganglioside sialic acid (nmoles/mg protein)
none	4.1
0.25	3.2 (78%)
0.50	2.6 (63%)
0.75	1.9 (46%)
1.00	1.3 (32%)

Ganglioside contents of coat-enriched fractions of MFGM prepared with Triton X-100 are in Table 7. Untreated MFGM contained 4.1 nmoles ganglioside sialic acid per mg protein. The amounts of ganglioside sialic acid were 3.2, 2.6, 1.9 and 1.3 nmoles per mg protein in MFGM washed with 0.25, 0.50, 0.75 and 1.00% Triton X-100 solution, respectively. In original MFGM the ganglioside content agreed with previous work (3.7 to 8.9 nmoles ganglioside sialic acid per mg protein)[22] and (5.78 ± 2.07 nmoles of ganglioside sialic acid per mg protein).[15] In this study at least 32% of the ganglioside sialic acid of untreated MFGM remained in the coat fraction of MFGM.

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(Received Sep. 18, 1989)

우유 지방구막의 고밀도 표피에 결합된 지질의 조성

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우유 지방구막의 고밀도 표피에 결합된 지질의 조성을 분석하기 위하여 지방구막의 고밀도 표피부분을 여러 농도의 비이온성 세제 Triton X-100으로 처리하였고 세제에 용해되지 않는 물질, 즉 지방과 결합된 성분을 분석하였다. 유지방구막의 단백질, 인지질, 콜레스테롤과 ganglioside의 양은 Triton X-100의 농도가 증가함에 따라 감소하였다. 불용성 표피물질로서 butyrophilin(band 12), xanthine oxidase(band 3)와 band 16이 SDS-polyacrylamide gel을 이용한 전기영동에서 나타났다. Phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol 및 spingomyeline 함량은 처리되지 않은 원래의 막의 것과 큰 차이가 있어 표피물질의 주요 인지질로 규명되었다. 전체 지질에서 지방산은 myristate, palmitate, stearate(주요 포화지방산), oleate, linoleate(주요 불포화지방산)이었다. 단백질에 결합된 콜레스테롤은 다른 성분에서보다 단백질에 더 견고하게 부착되어 있었다. Ganglioside의 함량은 Triton X-100의 농도가 증가함에 따라 비례 감소하였다.