Application of Phospholipids Extracted from Bovine Milk to the Reconstitution of Cream Using Butter Oil

Susumu Miura[a,*], Miyako Tanaka[b], Asuka Suzuki[b], and Kaoru Sato[a]

[a]Technology and Research Institute and [b]Product Planning Department, Snow Brand Milk Products, Saitama, 350-1165, Japan

ABSTRACT: Phospholipid (PL) extracted from bovine milk was tested for its emulsifying properties in conjunction with the reconstitution of cream using butter oil. PL from bovine milk dispersed in the oil phase was found to stabilize the cream, whereas PL extracted from soybean oil was found to solidify the cream. Different PL species purified from bovine milk PL were tested for their emulsifying properties. PC from bovine milk dispersed in butter oil was shown to stabilize the cream, whereas PE and sphingomyelin had no such effects. PC from soybean oil also was found to have emulsifying abilities. It was suggested that PC stabilized the reconstituted cream, regardless of its origin.

Paper no. J10637 in *JAOCS 81*, 97–100 (January 2004).

KEY WORDS: Bovine milk, buttermilk powder, butter oil, cream, oil-in-water (O/W) emulsion, phosphatidylcholine, phospholipids, solidification, stability.

Bovine milk contains approximately 4% fat in the form of globules that are surrounded by a thin membrane called the milk fat globule membrane (MFGM), which consists of a complex mixture of lipids, proteins, enzymes, and other components. Approximately half of MFGM is composed of lipids, of which approximately 69% is TAG, 27% is phospholipids (PL), 3% is cholesterol, and the rest is other minor components such as glycolipids (1). A liter of bovine milk contains 0.3–0.4 g of PL, or 1% of total lipids, most of which exist in MFGM. PL in bovine milk can be further subdivided into PC (34.5%), PE (31.8%), sphingomyelin (SPM) (25.2%), PI (4.7%), PS (3.1%), and lyso-PL (0.7%) (2).

Biological functions of PL in bovine milk have come to the attention of researchers in the last decade, and the role of bovine milk PL in lipid metabolism, cell proliferation, and maturing of intestinal cells in infants have been reported elsewhere (3–7). However, owing to the technological difficulties in extracting PL from bovine milk, and the high price created by the technology, extensive use of PL from bovine milk has been limited to pharmaceutical and cosmetic applications.

PL extracted from bovine milk are commercially available in technical quantities (8). Malmsten *et al.* (8) studied the liposome and emulsion-forming properties of SPM from milk and reported that SPM from milk has physicochemical properties similar to saturated PC. They concluded that it may be advantageous to use SPM both for the stability and the biological acceptance of the formulation, especially in the case of oral administration of drugs intended to be absorbed in the small intestine and for cosmetic applications.

*To whom correspondence should be addressed at Technology and Research Institute, Snow Brand Milk Products, 1-1-2 Minamidai, Kawagoe, Saitama, 350-1165, Japan. E-mail: YRB00152@nifty.ne.jp

Other workers have concentrated on the physicochemical properties of PL extracted from soybeans, since PL from soybeans have long been commercially available for use as emulsifiers in the food industry. Rydhag and Wilton (9) analyzed PL compositions and emulsifying properties of commercially available soybean lecithins and showed that the presence of PE and PI enhanced the incorporation of water and the swelling of the lamellar liquid crystalline phase of PC, thus stabilizing oil-in-water (O/W) emulsions. Boode *et al.* (10) studied the behavior of natural cream and recombined cream, made from milk fat, skimmed milk, water, and soybean lecithin, during and after temperature fluctuation. They found that the behavior of natural cream and recombined cream appeared to be the same, being stable at 5°C, but both exhibited considerable thickening when they were subjected to temperatures at which most of the fat melted and subsequently cooled again. Oortwijn and Walstra (11) also studied recombined cream made of skimmed milk and butter fat in which PL were dissolved and showed that thickening (rebodying) took place when the recombined cream was subjected to temperature cycling. They also found that recombined cream without PL added to the fat could not be rebodied.

All the above studies suggest that the presence of lecithin as emulsifier at the surface of the fat globule is essential for stabilization of the O/W emulsion.

In the present study, PL extracted from bovine milk and from soybeans are compared for their emulsifying properties in O/W emulsions using butter oil as the fat source. Of particular interest are the emulsifying properties of various PL species and the identification of an effective way to add PL to obtain stable O/W emulsions, which are vital factors in using PL from bovine milk as emulsifiers commercially.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise stated, all chemicals were of reagent grade and were purchased from Wako Pure Chemicals (Tokyo, Japan).

Butter oil was obtained by melting 100 g of commercial butter (unsalted), holding it at 70°C for 15 min, and then centrifuging the melt at 3,500 × *g* for 5 min. The top layer was collected, held at 70°C for a further 10 min, and centrifuged again at 3,500 × *g* for 5 min before the top layer was collected. The butter oil obtained was checked for its purity by TLC following the method of Christie (12) and was found to be free of PL and other polar lipids.

PL concentrate extracted from buttermilk by using ultrafiltration and acetone extraction was obtained in our laboratory based on the method reported by Ohba *et al.* (13); the resultant concentrate contained 80% PL. PC, PE, and SPM were purified from

TABLE 1
Compositions of PL Concentrate and SB Lecithin[a]

Component	PL concentrate (%)	SB lecithin (%)
PL	80	95
PC	30	30.7
PE	15	26.6
SPM	28	—
PI	0.3	16.9
PS	3.2	—
PA	—	8.4
Lyso-PL	—	2.1
Others	3.5	10.3
Neutral lipids	10	5
Protein	5	—
Moisture	5	—

[a]SPM, sphingomyelin; SB, soybean; PL, phospholipid.

this PL concentrate, as described below. Buttermilk powder (BMP) was obtained from Snow Brand Milk Products (Tokyo, Japan).

Soybean lecithin (SB lecithin) containing over 95% PL was obtained from Nissin Oil (Tokyo, Japan). Compositions of PL concentrate and SB lecithin are shown in Table 1.

PC, PE, lyso-PC, and lyso-PE from porcine liver, and SPM and lyso-SPM from beef brain were all of 99% purity and were purchased from Funakoshi (Tokyo, Japan). PC from soybeans also was of 99% purity and was purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Purification of PL species from PL concentrate. PC, PE, and SPM species were purified from PL concentrate as follows. PL concentrate (150 g) was dissolved in 4.5 L of a methanol/chloroform (1:2,vol/vol) mixture. Insoluble matter was removed by passing this mixture through a filter with pore size of 0.5 μm (Sunprep V-FH; Millipore, Bedford, MA). The solvent was removed from the mixture by using a rotary evaporator, and the residue was dissolved again in 150 mL of a chloroform/methanol (8:2, vol/vol) mixture. The sample was then applied onto a column of Iatrobeads (200–300 mesh; Iatron Laboratories Inc., Tokyo, Japan) with dimensions of 11 cm diameter by 60 cm length, which had been pre-equilibrated with chloroform. Five liters of chloroform/methanol (7:3, vol/vol) and then 25 L of chloroform/methanol (6:4, vol/vol) were passed through the column to obtain PC and PE. Finally, SPM was obtained by passing a chloroform/methanol (5:5) mixture through the column. Eluents were collected in 1-L flasks, and each 1-L fraction was checked for eluted PL species by TLC following the method of Christie (12). Finally, the solvents in which the PL species obtained were dissolved were removed by a rotary evaporator, and the PL were checked for purity by HPLC using a Spherisorb SI 60 column (5 μm, 4.6 mm i.d. × 250 mm; Senshu Kagaku, Tokyo, Japan) equipped with an ELSD (Varex II; Varex, Burtonsville, MD). The column temperature was 30°C. The elution solvent used was a linear gradient of hexane/isopropanol/water (6:8:0.75 to 6:8:1.4) at a flow rate of 1.0 mL/min (14,15).

Preparation of model cream. The compositions of model creams are shown in Table 2. The oil-to-water ratio was kept at 40:56 (w/w) in all compositions. PL concentrate and SB lecithin were each added at 1 part of the total weight, and BMP was added at 3 parts of the total weight (Creams A and A′). In the case of purified PL species, they were added at 1:3 (i.e., 0.3 parts) of the weight of PL concentrate, since PL concentrate contains approximately 30% PC, PE, and SPM (Cream B). Creams C and D were designed to test the emulsifying properties of PL and BMP in the absence of each other.

Preparation of the model creams was carried out by following the method of Sugimoto *et al.* (16) with minor changes. Briefly, PL were added to 2 g of butter oil and were dispersed at 60°C with constant agitation. BMP was added to 2.8 g of water and was dissolved at 60°C. Both the oil phase and the aqueous phase were heated to 60°C and mixed together at 60°C, then prehomogenized by using an ultradisperser (Ultra-Turrax T25; Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 5 min. This mixture was further homogenized by sonication for 2 min using a sonicator (UR-20P; Tomy Seiko Co., Tokyo, Japan). The O/W emulsion obtained was immediately cooled to 0°C in ice water for 12 h, after which the emulsions were kept at 5°C. All of these processes were carried out in a 15-mL plastic tube with screw cap.

Evaluation of cream solidification. The state of the cream after storage at 0°C for 12 h was classified into two categories based on visual observation: liquid (L) and solid (S) state. A laser diffraction particle size analyzer (SALD-2000; Shimadzu, Kyoto, Japan) was employed for measuring the particle size distribution of the emulsion droplets.

RESULTS AND DISCUSSION

Emulsifying properties of BMP and PL concentrate. A model cream with BMP in the aqueous phase and PL concentrate in the oil phase was prepared as described in the Experimental

TABLE 2
Compositions of Model Creams on a Mass Basis, and the State[a] of the Creams After Thermal Treatment

	Cream A (parts)	Cream A′ (parts)	Cream B (parts)	Cream C (parts)	Cream D (parts)
Butter oil	40	40	40	40	40
Water	56	56	56	56	56
PL concentrate	1	—	—	1	—
SB lecithin		1			
PL species	—	—	0.3	—	—
BMP	3	3	3	—	3
State of cream	L	S	—	S	S

[a]L, liquid state; S, solid state; BMP, buttermilk powder; for other abbreviation see Table 1.

Procedures section (Cream A). At the same time, a model cream without BMP in the aqueous phase (Cream C) and a model cream without PL concentrate in the oil phase were prepared (Cream D).

After being stored at 0°C for 12 h, Cream A remained liquid, whereas Creams C and D solidified. Cream A remained liquid even after 1 wk at 5°C. This result suggests that the presence of both PL and proteins is necessary to obtain a stable O/W emulsion.

Emulsifying properties of PL concentrate and SB lecithin. Model creams (Creams A and A′ in Table 2) were prepared with butter oil as the fat source. PL concentrate and SB lecithin were each added to the oil phase. At the end of 12 h of storage at 0°C, the addition of PL concentrate to butter oil was found to give a stable O/W emulsion, whereas the addition of SB lecithin to butter oil did not prevent solidification of the emulsion. Figure 1 shows the particle size distributions of the O/W emulsions with PL concentrate and SB Lecithin. The particle size distribution was narrow in the emulsion that remained in the liquid state, with an average particle diameter of 2.3 μm. On the other hand, the emulsion with SB lecithin showed a broad particle size distribution, suggesting that coagulation of oil droplets in the emulsion led to the solidification of the cream.

When both the PL and the oil used in preparation of the emulsion originated from bovine milk, a stable O/W emulsion was obtained. It is not clear why different emulsifying properties were observed when PL concentrate and SB lecithin were each added to butter oil. One factor that may influence the emulsion properties of PL is the difference in the composition of PL species between the PL concentrate and the SB lecithin. According to Table 1, the most common PL species in the PL concentrate and SB lecithin were PC and PE, although the content of PE was higher in SB lecithin. Another factor may be the difference in the FA composition of the PL. On the basis of the hypothesis that PL species play an important role in the stabilization of the O/W emulsion with butter oil as fat source, purification of PL species from PL concentrate was carried out to evaluate the emulsifying properties of each PL species in milk.

Purification of PL species from PL concentrate. Table 3 shows the elution pattern of PE, PC, and SPM species, as monitored by TLC. PE eluted when the chloroform/methanol ratio was changed to 6:4 (fractions 6 to 10), followed closely by elution of PC (fractions 11 to 25). PC was further eluted when the chloroform/methanol ratio was changed to 5:5, although the elution of both PC and SPM was observed from fractions 52 to 61. Elution of SPM by itself was observed from fractions 62 to 93. Fractions 6 to 10 were combined to obtain PE, 12 to 25 and 42 to 51 were mixed to obtain PC, and 62 to 93 were combined to obtain SPM species. Solvents from each mixture were removed by using a rotary evaporator, and the purity of each mixture was analyzed by HPLC. At the same time, the retention time of each PL species obtained was compared with PL standards. HPLC chromatograms of the PE, PC, and SPM fractions revealed that PE and PC were free of any other species. However, the HPLC chromatogram of SPM showed two peaks, although the elution pattern on TLC showed no indication of the presence of a second band. The retention time of the larger peak was the same as that for the standard SPM from beef brain, and the area percentage of the peak was 86%. The retention time of the smaller peak was compared to the retention times of standard lyso-PC, lyso-PE, and lyso-SPM; the smaller peak was not lyso-PE, lyso-PC, or lyso-SPM. The identification of the smaller peak is yet to be clarified. To evaluate the emulsifying properties of PL species, the SPM obtained with 86% purity was used.

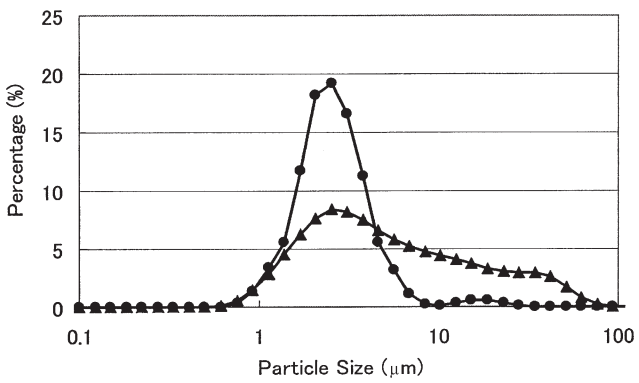

FIG. 1. Particle size distribution of model cream: (●) Phospholipid concentrate dispersed in butter oil; (▲) soybean lecithin dispersed in butter oil.

Emulsifying properties of PE, PC, and SPM purified from milk and of PC from soybeans. A model cream (Cream B in Table 2) was prepared by dissolving PE, PC, and SPM species purified from milk in either butter oil or water. Table 4 shows the state of each cream after 12 h at 0°C.

PC dispersed in the oil phase stabilized the O/W emulsion, whereas the addition of PE or SPM in the oil phase did not prevent the solidification of the emulsion. To confirm the emulsifying properties of PC, PC purified from bovine milk and PC from soybean were each dissolved in butter oil, and cream was prepared as described in the Experimental Procedures section. In either case, the O/W emulsion remained in the liquid state after 12 h at 0°C. Average particle size distributions of O/W emulsions were determined to be 2.3 μm for both emulsions. These results suggest that PC itself has a stabilizing effect on the O/W emulsion regardless of its origin, and this ability may be hampered by its combination with other PL components, as was exhibited in the case of SB lecithin dispersed in butter oil. These observations may be due to structural differences between PC, PE, and SPM. PC has a choline headgroup, whereas PE has an ethanolamine headgroup. PC and SPM, on the other hand, have the same choline headgroup but a different backbone structure; PC has a glycerol backbone structure, whereas SPM has a sphingosine backbone structure. These differences may have led to the different abilities of these PL species to stabilize the O/W emulsion.

Table 4 shows that the addition of PL species to the water phase before homogenization did not prevent the solidification of the cream. With respect to PC, this result suggests that the presence of PC in the oil phase before homogenization, that is to say, before the oil/water interface of the fat globules is formed, is necessary for PC to be able organize itself at the oil/water interface with polar headgroups protruding into the aqueous phase. Otherwise, the presence of BMP and PC in the aqueous phase

TABLE 3
Elution Pattern of PL Species[a,b]

Solvent	C/M = 7:3					C/M = 6:4																			
Fr. no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
PE						X	X	X	X	X	X														
PC											X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
SPM																									

Solvent	C/M = 6:4					C/M = 5:5																			
Fr. no.	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
PE																									
PC																	X	X	X	X	X	X	X	X	X
SPM																									

Solvent	C/M = 5:5																								
Fr. no.	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
PE																									
PC	X	X	X	X	X	X	X	X	X	X	X														
SPM		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Solvent	C/M = 5:5																			C/M = 4:6					
Fr. no.	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
PE																									
PC																									
SPM	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X							

[a]C/M, ratio of chloroform to methanol used in elution of PL from the column of Iatrobeads; for details see the Experimental Procedures section. Fr. no., fraction number; for other abbreviation see Table 1.
[b]The letter "X" indicates the presence of the PL in question in that fraction number of the elution scheme.

may promote the formation of PC/protein aggregates, which may prevent PC molecules from organizing at the oil/water interface and lead to the destabilization of the emulsion.

TABLE 4
State[a] of Cream with PL Species Dispersed in Oil and Aqueous Phases After 12 h at 0°C

	Oil phase	Aqueous phase
PC	L	S
PE	S	S
SPM	S	S

[a]For abbreviations see Tables 1 and 2.

REFERENCES

1. Kanno, C., Recent Studies on Milk Fat Globule Membrane with Special Reference to the Constituent Proteins, *Jpn. J. Zootech. Sci. 51*:75–88 (1980) (in Japanese).
2. Morrison W.R., The Distribution of Phospholipids in Some Mammalian Milks, *Lipids 3*:101–103 (1968).
3. Sasaki, H., H. Kume, A. Nemoto, S. Narisawa, and N. Takahashi, Ethanolamine Modulates the Rate of Rat Hepatocyte Proliferation *in vitro* and *in vivo*, *Proc. Natl. Acad. Sci. USA 94*:7320–7325 (1997).
4. Imaizumi, K., K. Mawatari, M. Murata, I. Ikeda, and M. Sugano, The Contrasting Effect of Dietary Phosphatidylethanolamine and Phosphatidylcholine on Serum Lipoproteins and Liver Lipids in Rats, *J. Nutr. 113*:2403–2411 (1983).
5. Simon, K., and D. Toomre, Lipid Rafts and Signal Transduction, *Nature Rev. 1*: 31–39 (2000).
6. Brown, D.A., and E. London, Structure and Function of Sphingolipid- and Cholesterol-rich Membrane Rafts, *J. Biol. Chem. 275*:17221–17224 (2000).
7. Motouri, M., H. Matsuyama, J. Yamamura, M. Tanaka, S. Aoe, T. Iwanaga, and H. Kawakami, Milk Sphingomyelin Accelerates Enzymatic and Morphological Maturation of the Intestine in Artificially Reared Rats, *J. Pediatr. Gastroenterol. Nutr. 36*: 241–247 (2003).
8. Malmsten M., B. Bergenstahl, L. Nyberg, and G. Odham, Sphingomyelin from Milk—Characterization of Liquid Crystalline, Liposome and Emulsion Properties, *J. Am. Oil Chem. Soc. 71*: 1021–1026 (1994).
9. Rydhag L., and I. Wilton, The Function of Phospholipids of Soybean Lecithin in Emulsions, *Ibid. 58*:830–837 (1981).
10. Boode, K., C. Bisperink, and P. Walstra, Destabilization of O/W Emulsions Containing Fat Crystals by Temperature Cycling, *Colloids Surf. 61*:55–74 (1991).
11. Oortwijn, H., and P. Walstra, The Membrane of Recombined Fat Globules. 4. Effects on Properties of the Recombined Milks, *Neth. Milk Dairy J. 36*:279–290 (1982).
12. Christie, W.W., *HPLC and Lipids: A Practical Guide*, Pergamon Press, Oxford, 1987, pp. 102–103.
13. Ohba, T., Y. Matsuoka, K. Takahashi, K. Ahiko, and S. Kurosawa, Powder Having High Content of Complex Lipid Derived from Milk, Its Production and Use, Japan Patent 3103218 (1993).
14. Geurts van Kessel, W.S.M., W.M.A. Hax, R.A. Demel, and J. de Gier, High-Performance Liquid Chromatographic Separation and Direct Ultraviolet Detection of Phospholipids, *Biochim. Biophys. Acta 486*:524–530 (1977).
15. Hax, W.M.A., and W.S.M. Geurts van Kessel, High-Performance Liquid Chromatographic Separation and Photometric Detection of Phospholipids, *J. Chromatogr. 142*:735–741 (1977).
16. Sugimoto, T., T. Mori, J. Mano, T. Mutoh, Y. Shiinoki, and Y. Matsumura, Effects of Fat Crystallization on the Behavior of Proteins and Lipids at Oil Droplet Surfaces, *J. Am. Oil Chem. Soc. 78*:183–188 (2001).

[Received May 7, 2003; accepted September 24, 2003]