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The association of lipophilic phospholipids with native bovine casein micelles in skim milk: Effect of lactation stage and casein micelle size

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ABSTRACT

A known biological role of casein micelles is to transport calcium from mother to young and provide amino acids for growth and development. Previous reports demonstrated that modified casein micelles can be used to transport and deliver hydrophobic probes. In this study, the distribution of lipid-soluble phospholipids, including sphingomyelins (SM) and phosphatidylcholines (PC), was quantified in whole raw milk, skim raw milk, and casein micelles of various sizes during early, mid, and late lactation stages. Low-pressure size exclusion chromatography was used to separate casein micelles by size, followed by hydrophobic extraction and liquid chromatography–mass spectrometry for the quantification of PC and SM. Results showed that the SM d18:1/23:0, d18:1/22:0, d18:1/16:0, d16:1/22:0, d16:1/23:0, and d18:1/24:0 and the PC 16:0/18:1, 18:0/18:2, and 16:0/16:0 were dominating candidates appearing in maximum concentration in whole raw milk obtained from late lactation, with 21 to 50% of total SM and 16 to 35% of total PC appearing in skim milk. Of the total SM and PC found in skim milk, 35 to 46% of SM and 22 to 29% of PC were associated with the casein micelle fraction. The highest concentrations of SM d18:1/22:0 (341 ± 17 μg/g of casein protein) and PC 16:0/18:1 (180 ± 20 μg/g of casein protein) were found to be associated with the largest casein micelles (diameter = 149 nm) isolated in milk from late lactation, followed by a decrease in concentration as the casein micelle size decreased.

Key words: casein micelle, lipophilic, phospholipid, sphingomyelin, phosphatidylcholine

INTRODUCTION

Bovine milk contains approximately 3.3% (wt/wt) protein, and casein proteins account for approximately 80% of the total protein content in bovine milk. Casein proteins exist as colloidal particles known as casein micelles, comprising 4 major casein proteins: α_{S1}-, α_{S2}-, β-, and κ-casein in an approximate ratio of 4:1:3.5:1.5 (Farrell et al., 2004). Although the casein micelle structure is still under investigation, it is known that κ-casein is predominantly present on the surface of the casein micelle, imparting stability against micellar aggregation by electrostatic and steric repulsion, whereas α_{S1}-, α_{S2}-, and β-caseins are predominantly present inside the core of the casein micelle (Dalgleish, 2011; de Kruif et al., 2012; Holt et al., 2013). Modified casein micelles were used to encapsulate and deliver several hydrophobic probes, including vitamin A (Mohan et al., 2013), vitamin D (Semo et al., 2007), curcumin (Sahu et al., 2008), triclosan (Roach et al., 2009), and docosahexaenoic acid (Zimet et al., 2011). It is not known whether the ability of modified casein micelles to associate with hydrophobic probes is derived from their native preference to bind hydrophobic compounds in milk.

Lipophilic phospholipids (**PL**), including sphingomyelins (**SM**) and phosphatidylcholines (**PC**), are a major source of choline and are important for neurological development in infants (Tanaka et al., 2013). Choline is needed for infant organ growth and membrane biosynthesis, therefore rendering PL as significant ingredients in infant formula (Holmes-McNary et al., 1996; Caudill, 2010). Sphingomyelins and PC account for approximately 30 and 34% of total phospholipids in whole bovine milk, respectively. Although polar phospholipids are known components of the milk fat globule membrane (**MFGM**), approximately 60% of the total phospholipids have been found in skim milk after removal of fat, with PC and SM constituting approximately 31 and 29% of total PL content, respectively (Christie et al., 1987). Zeisel et al. (2003) found 0.71 mg of SM/100

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g of commercial skim milk versus 0.65 mg of SM/100 g of whole milk and 0.75 mg of PC/100 g of skim milk versus 0.61 mg of PC/100 g of whole milk despite their low solubility in water. Various factors may explain these differences. For instance, it was demonstrated that the concentration of individual PC in whole milk changes with lactation stage (Artegoitia et al., 2014).

The hypothesis driving the current study was that the open structure of the native casein micelle allows for the association of small PL to micellar hydrophobic regions. In our previous study, we showed that highly lipophilic PC and SM (log $P > 10$; Log P is logarithm of the partition between octanol and water) were found to be associated with casein micelles in bovine skim raw milk (Cheema et al., 2015). However, the partitioning of individual PC and SM into casein micelles and the effect of casein micelle size were not previously studied. The relative ratio of each casein protein changes with micelle size, with comparatively smaller micelles containing higher κ-casein and lower β-casein relative content and α_{S1}- and α_{S2}-casein remaining constant (Dalgleish et al., 1989). Hydrophobic domains in β-caseins are known to play a role in interacting with hydrophobic compounds, and smaller casein micelles are expected to have fewer sites available for binding hydrophobic compounds, partially due to decreasing β-casein content.

The objective of this study was to demonstrate that the casein micelles are reservoirs for SM and PC in commercial skim milk. Additionally, we sought to determine whether micellar size and stage of lactation influenced the association of SM and PC with casein micelles.

MATERIALS AND METHODS

Milk Sample Collection and Preparation

Raw milk samples were collected from a total of 9 Holstein cows, 3 cows per lactation stage (early, mid, and late), from The Pennsylvania State University Research and Teaching Dairy Center (University Park). The raw milk samples were collected from first-parity cows with low SCC ($<5 \times 10^{-4}$ SCC/mL) and average BW of 580 ± 30 kg in early lactation (DIM = 21), 607 ± 70 kg in mid lactation (DIM = 91), and 630 ± 35 kg in late lactation (DIM = 217). The milk was transported on ice and stored at 4°C until analysis. The skim milk samples were generated by removing fat using centrifugation at 20°C (6,414 × g for 20 min) and stored at 4°C until fractionation of casein micelles using size exclusion low-pressure chromatography (**SEC**).

SEC

Samples of skim raw milk (5 mL) were subjected to fractionation into casein micelles of varying sizes by using a XK 26/100 (mm/cm) column packed with Sephacryl medium (S-1000, GE Life Sciences, Piscataway, NJ) connected to an AKTA 150 fast performance chromatography unit (GE Life Sciences). The mobile phase consisted of 20 mM imidazole buffer, 10 mM calcium chloride, and 0.07% sodium azide (NaN_3; Fisher Scientific, Fair Lawn, NJ) with pH adjusted to 6.8. The buffer was empirically shown to maintain the integrity of the casein micelles in milk's physiological pH of 6.6 to 6.8. The column was equilibrated for 2 column volumes (956 mL). The proteins were eluted at a flow rate of 1.0 mL/min for 1.5 column volumes (717 mL) by collecting 30 mL of each fraction. The protein elution was monitored at a 280-nm wavelength. Five fractions of casein micelles of varying sizes were collected and verified by SDS-PAGE. A total of 200 μL of volume of each fraction was saved for particle size analysis and casein protein content analysis. The remaining fractions were freeze-dried and stored at −20°C until extractions were performed.

SDS-PAGE

The 5 SEC fractions ($2A_1$, $2A_2$, $2A_3$, $2B_1$, and $2B_2$; see Figure 1) were freeze-dried and subjected to SDS-PAGE to confirm the presence of casein proteins. All reagents and precast gels for SDS-PAGE gel electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA). The SDS-PAGE electrophoresis of all 5 casein fractions was performed under denaturing and reducing conditions. The loading buffer was prepared using 0.5 M Tris-HCl (pH 6.8), glycerol, 10% (wt/vol) SDS, β-mercaptoethanol, and 0.5% (wt/vol) bromophenol blue in water. The freeze-dried samples (5 mg) were suspended in 500 μL of loading buffer followed by heating to 95°C for 5 min before electrophoresis in 15% polyacrylamide SDS-PAGE ready gels. The gel electrophoresis was performed by loading 15 μL of sample in each well. The gels were run at a constant 200 V for 35 min. The protein bands were silver stained following the manufacturer's protocol (Silver stain plus, Bio-Rad Laboratories).

Particle Size Analysis

The particle size of the 5 fractions collected from SEC was determined by dynamic light scattering using a Viscotek 802 DLS instrument equipped with Omni-