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The association of low-molecular-weight hydrophobic compounds with native casein micelles in bovine milk

M. Cheema,* M. S. Mohan,* S. R. Campagna,† J. L. Jurat-Fuentes,‡ and F. M. Harte*[1]
*Department of Food Science, Pennsylvania State University, University Park 16802
†Department of Chemistry, and
‡Department of Entomology and Plant Pathology, University of Tennessee, Knoxville 37996

ABSTRACT

The agreed biological function of the casein micelles in milk is to carry minerals (calcium, magnesium, and phosphorus) from mother to young along with amino acids for growth and development. Recently, native and modified casein micelles were used as encapsulating and delivery agents for various hydrophobic low-molecular-weight probes. The ability of modified casein micelles to bind certain probes may derive from the binding affinity of native casein micelles. Hence, a study with milk from single cows was conducted to further elucidate the association of hydrophobic molecules into native casein micelles and further understand their biological function. Hydrophobic and hydrophilic extraction followed by ultraperformance liquid chromatography-high resolution mass spectrometry analysis were performed over protein fractions obtained from size exclusion fractionation of raw skim milk. Hydrophobic compounds, including phosphatidylcholine, lyso-phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, showed strong association exclusively to casein micelles as compared with whey proteins, whereas hydrophilic compounds did not display any preference for their association among milk proteins. Further analysis using liquid chromatography-tandem mass spectrometry detected 42 compounds associated solely with the casein-micelles fraction. Mass fragments in tandem mass spectrometry identified 4 of these compounds as phosphatidylcholine with fatty acid composition of 16:0/18:1, 14:0/16:0, 16:0/16:0, and 18:1/18:0. These results support that transporting low-molecular-weight hydrophobic molecules is also a biological function of the casein micelles in milk.
Key words: casein micelle, liquid chromatography-mass spectrometry, binding, hydrophobicity, sphingomyelin

INTRODUCTION

Bovine milk is a good source of protein comprising *ca* 3.6% of the total composition of the milk. About 80% of the total protein content in bovine milk consist of 4 types of casein, which are α_{s1}-, α_{s2}-, β-, and κ-CN in the approximate ratio of 4:1:3.5:1.5 (by weight). Caseins are phosphoproteins that exist as colloidal aggregates known as casein micelles (Horne, 1998; De Kruif and Holt, 2003; Dalgleish, 2011). Although the casein micelle structure is still under investigation, certain models have been proposed (De Kruif and Holt, 2003; Horne, 2006). The current consensus is that proteins in casein micelles are stabilized by various hydrophobic and electrostatic interactions, whereas calcium phosphate clusters bind to the phosphoseryl residues found on α_s- and β-CN. The localization of κ-CN to the surface of the casein micelle to act as negatively charged hairs plays an important role in stabilizing adjacent casein micelles against aggregation. Recent electron microscopy observations suggest a sponge-like structure with native casein micelles containing internal serum-filled channels and voids that allow a dynamic interaction between the micelles and their surroundings (Trejo et al., 2011).

The agreed biological function of the casein micelle is to transport minerals (calcium, phosphorous) as well as amino acids from mother to newborns for their growth and development (De Kruif and Holt, 2003). This hypothesis is supported by the fact that milk is supersaturated with calcium, and phospho-serine residues are conserved in mammalian species (Oftedal, 2013).

Evidence exists in the literature for the use of casein micelles as delivery agents for hydrophobic molecules. For instance, reformed casein micelles formed by exposing commercially available skim milk to high-pressure homogenization were employed to encapsulate hydrophobic, antimicrobial triclosan (Roach and Harte, 2008). Reassembled casein micelles formed by treatment with potassium citrate bound hydrophobic vitamin D_2 (Semo et al., 2007). Alternatively, native casein micelles separated from raw skim milk by ul-

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[1]Corresponding author: fede@psu.edu

tracentrifugation were used as encapsulating agents for carrying and delivering curcumin, a natural spice with potential therapeutic properties, to cancer cells (Sahu et al., 2008). The formation of the complex between bovine casein micelles and curcumin was attributed to hydrophobic interactions. Our previous work conducted on native casein micelles in ultrapasteurized skim milk purified by size-exclusion chromatography (**SEC**) provides further evidence for the natural ability of casein to associate with hydrophobic vitamin A as compared with whey proteins (Mohan et al., 2013). It was also found that the amount of hydrophobic phospholipids associated with casein proteins was higher as compared with the whole milk (Cerbulis and Zittle, 1965).

Despite increasing evidence, the affinity of native casein micelles toward hydrophobic low-molecular-weight molecules has not yet been investigated thoroughly, most likely because of the belief that the milk fat globule is the appropriate system to transport hydrophobic compounds from mother to young. In addressing this knowledge gap, the objective of this study was to investigate and compare the capacity of casein micelles relative to other significant proteins in raw bovine milk (i.e., BSA, α-LA, β-LG) to associate with hydrophobic and hydrophilic compounds. Milk protein fractions purified by SEC were analyzed by ultraperformance liquid chromatography-high-resolution mass spectrometry (**UPLC-HRMS**) to compare the association of hydrophobic and hydrophilic compounds with each major milk protein fraction (i.e., casein micelle, BSA, and whey protein). Further analysis using liquid chromatography-tandem mass spectrometry (**LC-MS/MS**) was performed to identify the hydrophobic compounds associated exclusively with casein micelles by analyzing the corresponding mass fragments of parent ions. This study provides detailed information on associations between the casein micelle and hydrophobic molecules. Understanding fundamental structure-function properties of the casein micelle in milk is key to the development of future applications, including encapsulation and delivery systems.

MATERIALS AND METHODS

Milk Sample Collection and Preparation

Raw milk samples were collected from 6 Holstein cows of first parity at mid-lactation stage from the University of Tennessee Dairy Research and Education Center. The raw milk samples were collected directly from each cow in the milking apparatus without any mixing followed by transportation in ice and storage at 4°C. The fat removal from the raw milk was done by centrifugation at 4°C (6,414 × g for 20 min) followed by storage at 4°C. Tangential flow ultrafiltration was employed to generate protein-free serum (**PFS**) from the raw milk of corresponding cow using a 3KDa MWCO cellulose filter (PLBC Prep scale TFF Cartridge, Millipore, Billerica, MA). Protein-free serum was preserved in 0.07% NaN_3 to prevent microbial growth and was stored at 4°C until SEC was performed to separate the proteins in the milk sample.

SEC

Casein micelle, BSA, β-LG, and α-LA fractions from raw milk were separated by SEC using a Superdex column (Superdex 200 prep grade, GE Life Sciences, Piscataway, NJ) connected to an AKTA fast-performance liquid chromatography unit (GE Life Sciences). The raw skim milk samples were loaded on the column and run using ice-cold PFS generated from the milk samples of the corresponding cow as mobile phase. The use of PFS during protein separation ensured that proteins were kept in their native state. The proteins were eluted using a 1.0 mL/min flow rate for 2 column volumes (240 mL total) and collecting 4-mL fractions. Protein elution was monitored using absorbance with a UV detector at 280 nm wavelength. Fractions were collected based on elution peaks corresponding to casein micelles, BSA, and β-LG and α-LA (based on SDS-PAGE analysis, see below), yielding 4 samples along with PFS as control. These samples were freeze-dried and stored at −40°C until liquid chromatography-mass spectrometry analysis.

SDS-PAGE

Sodium dodecyl sulfate-PAGE was performed on all 4 SEC fractions (CN, BSA, β-LG and α-LG, PFS) to verify the purity of the samples and also to confirm that PFS did not contain proteins (Figure 1). Electrophoresis was run under denaturing and reducing conditions. The sample loading buffer was prepared using 0.5 M Tris-HCl at pH 6.8, glycerol, 10% (wt/vol) SDS, β-mercaptoethanol, and 0.5% (wt/vol) bromophenol blue in water (Fisher Scientific, Fair Lawn, NJ). The samples were suspended in loading buffer and heat denatured for 5 min at 95°C before electrophoresis in 15% acrylamide SDS-PAGE ready gels (Bio-Rad, Hercules, CA). Sample volumes of 15 μL were loaded into each well, and electrophoresis was run at constant 200 V for 35 min. After electrophoretic separation, proteins were detected in gels using Coomassie blue staining.

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