J. Dairy Sci. 97:5964–5974
http://dx.doi.org/10.3168/jds.2014-7951

Lateral lipid organization of the bovine milk fat globule membrane is revealed by washing processes

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ABSTRACT

Evidence for the asymmetric distribution of phospholipids in the milk fat globule membrane (MFGM) was obtained by applying 3 washing processes using aqueous solutions with different degrees of stringency (mild, intermediate, and intensive) to milk fat globule (MFG) surfaces in simulated milk ultrafiltrate buffer. We detected no change in the amount of cholesterol after the mild washing process; however, intensive washing yielded a relative enrichment of surface cholesterol with concomitant damage to the outer bilayer of the MFGM. This finding supports the hypothesis of repartitioning of cholesterol on MFG surfaces during mechanical treatments. An updated model system of lipid organization was developed according to the results of relative depletion of individual phospholipids, as analyzed by HPLC.
Key words: milk fat globule membrane, washing process, lipid organization, cholesterol

INTRODUCTION

The milk fat globule (**MFG**) is enveloped and stabilized by a bioactive, multi-component layer referred to as the milk fat globule membrane (**MFGM**) with a thickness of around 10 to 20 nm (Walstra, 1999). This membrane regulates the physicochemical and nutritional functions of MFG (Michalski and Januel, 2006; Argov et al., 2008; Dewettinck et al., 2008). According to physiological secretion mechanisms of the MFG, the MFGM is constructed of phospholipids (**PL**), cholesterol, and membrane proteins (Keenan and Mather, 2006; MacGibbon and Taylor, 2006); however, to date, the composition and structure of the MFGM are not conclusively known.

Food is a complex arrangement of multiple components that are digested and absorbed though a dynamic and interrelated process, rather than as individual components, highlighting the importance of food microstructure in delivering health benefits. For instance, food structure may be designed to control digestion, release, and absorption of lipophilic food components (McClements et al., 2008). The biofunctionality of the MFGM is not simply a sum of those properties from individual molecules but is affected by the specific organization and structure of the PL, cholesterol, and membrane proteins. Although current models show differences in the organization of lipids and proteins (Michalski et al., 2002; Lopez et al., 2011; Mather, 2011), it is generally agreed that the MFG inner core (triacylglycerides) is coated with a trilayer MFGM system consisting of a primary monolayer of proteins and lipids and an outer PL bilayer with associated glycocalyx. Mather (2011) presented a topology of the major bovine MFGM proteins, where the location and specific interactions within the MFGM were shown. Lopez and coworkers (2011) examined MFGM lipid organization and suggested co-existence and phase separation between tightly packed liquid-ordered domains ($\boldsymbol{L}_{\mathbf{o}}$, also known as lipid rafts in biological membranes) enriched in sphingomyelin (**SM**) and cholesterol, and liquid-disordered domains ($\boldsymbol{L}_{\mathbf{d}}$) enriched in other glycerolphospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine, with an asymmetric distribution of L_{o} in the outer leaflet of the outer PL bilayer.

Separation of MFG and subsequent washing to remove non-membrane-associated proteins is the first step to extract the MFGM (Mather, 2000). Methods have been reported utilizing different centrifugal forces, centrifugation times, and washing buffers (Huang and Kuksis, 1967; Patton and Huston, 1986; Ye et al., 2002; Le et al., 2009). It is widely accepted that mechanical treatments such as centrifugation induce depletion of MFGM materials from MFG (Walstra et al., 2006); therefore, it is reasonable to question whether the MFGM compositional data generated from different isolation methods can be compared.

Protein structural changes in the MFGM may be induced by centrifugal washing processes (Zheng et al., 2013), which can cause irreparable damage to the

Received January 15, 2014.
Accepted June 27, 2014.
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outer phospholipid layer of the MFGM and depletion of MFGM components. In this study, the relative compositional changes in lipids were measured after centrifugal washing processes. From these measurements, we proposed an updated MFGM structural model of lipid organization.

MATERIALS AND METHODS

Materials

Bovine raw milk samples were collected from the same Jersey cow in good health and in mid lactation (autumn) on a local farm (Port Chalmers, New Zealand). We sampled milk from one cow to avoid the problem of mixing milk from cows at different stages of lactation; to avoid the need for stirring and refrigerated storage of pooled milk, which would alter the MFG surface; and to avoid possible inconsistent pooling of milk from several cows. The cow was pasture-fed and vacuum-milked over 6 consecutive sampling days. No extra mechanical or thermal treatments were applied to the fresh raw milk before experiments. Simulated milk ultrafiltrate (**SMUF**) at pH 6.5 (Jenness and Koops, 1962) was prepared and used as a buffer and washing solution. The following phospholipid standards were obtained: 3-*sn*-phosphatidylethanolamine (**PE**) and L-α phosphatidylcholine (**PC**), both from bovine brain; L-α-phosphatidylinositol (**PI**) from bovine liver; 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (**PS**) from bovine brain (Sigma-Aldrich, St. Louis, MO); and SM from bovine milk (Avanti Polar Lipids, Alabaster, AL). All other chemicals used were analytical grade and obtained from Merck (Darmstadt, Germany).

Isolation of MFG

Three centrifugal isolation methods: (1) 3,000 × *g*, 5 min, 3 washes (**M1**); (2) 3,750 × *g*, 15 min, 1 wash (**M2**); and (3) 15,000 × *g*, 20 min, 3 washes (**M3**) were used for collecting and washing MFG from raw milk according to previously published work (Patton and Huston, 1986; Ye et al., 2002; Gallier et al., 2010b) respectively, with minor modifications. Methods M1 and M3 were carried out by washing centrifuged raw milk 3 times in SMUF using either the M1 or M3 centrifugation process. The cream obtained from this initial centrifugation of raw milk was resuspended in 10 volumes of SMUF and left at ambient temperature for 1 h, and then centrifuged again (using either the M1 or M3 process, as appropriate) to remove non-MFGM proteins from the MFG surface. This is referred to as the first wash (M1-1 and M3-3), and was repeated a further 2 times using M1 or M3 to obtain the second and third washed MFG suspensions (M1-2, M1-3; M3-2, M3-3). The M2 process (Patton and Huston, 1986) used a sucrose density gradient separation as a single wash process that was applied as a comparison method. In this method, 15 mL of sucrose-treated raw milk (containing 5% wt/vol sucrose) was layered under 30 mL of SMUF buffer in centrifugation tubes, and washed MFG were collected after M2 centrifugation. A control MFG suspension sample from M2 was collected by direct centrifugation of sucrose-treated raw milk. The temperature of the SMUF buffer was 30°C and centrifugation temperature was 25°C for all experiments. Raw milk and MFG fraction samples, which were collected after each centrifugation process, were stored at −80°C before further analysis.

Size Distribution and Assessment of Specific Surface Area of MFG

Volume-weighted mean diameter (d_{43}) and surface area-weighted mean diameter (d_{32}) were determined by laser diffraction using a Horiba particle analyzer LA-950 (Horiba, Irvine, CA). The refractive index of MFG was taken as 1.460 at 655 nm and 1.470 at 405 nm (Michalski et al., 2001; Lopez et al., 2011). Resuspended MFG were diluted in SMUF at pH 6.5 to yield a suspension with a similar fat content to that of the original milk. The diluted MFG solutions or milk were mixed with 35 m*M* EDTA:NaOH containing 2% (wt/vol) SDS buffer solution (final pH 7.0) in a 1:1 (vol/vol) ratio before loading into the particle size analyzer flow cell. The transparency of the sample cell for both light sources (655 and 405 nm) was around 90%. Measurements were carried out in triplicate. Specific surface area (**SSA**) of MFG was calculated from the surface area-weighted mean diameter (d_{32}): $SSA = 6\varphi/d_{32}$, where φ is the volume fraction of milk fat (Huppertz and Kelly, 2006).

Total Lipid Extraction and Quantification

A Folch extraction (Folch et al., 1957; Hundrieser et al., 1984) was carried out to extract total lipids from milk and MFG fraction samples for subsequent PL extraction and total fat content determination. Samples were mixed with chloroform:methanol (2:1, vol/vol) solvent in a 1:8 (vol/vol) ratio for milk or 1:20 (wt/vol) for MFG samples, respectively. The mixtures were washed with 0.2 volumes of 0.9% (wt/vol) NaCl solution to increase total lipid recovery. The chloroform-enriched lipid fraction phase was collected and the solvents were dried by rotary evaporation (or by a gentle nitrogen stream when the volume of solvent was not more than 3 mL). All extractions were carried out in triplicate.