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Changes in bovine milk fat globule membrane proteins caused by heat procedures using a label-free proteomic approach

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ABSTRACT

The milk fat globule membrane (MFGM) plays an important role in stabilizing fat in the aqueous phase, and the components of this membrane are involved in multiple biological functions. Here, we investigated changes in the protein composition of the MFGM fraction between raw and heated whole milk using a label free proteomic approach. In total, 612 MFGM-related proteins were identified in all groups. Compared with raw milk, the number of proteins that were not identified in the MFGM fraction was increased from pasteurized milk to ultra-high-temperature milk, whereas the number of milk proteins incorporated into the MFGM fraction was similar among heated groups. The abundances of several milk proteins (β-lactoglobulin and β-casein) were increased in the heated milk groups in a temperature-dependent manner. From our functional analysis, proteins that were not identified in the MFGM fraction of heated milk were mainly associated with multiple biological functions. These findings provided novel insights into the effects of heat procedures on MFGM protein components and their potential physiological functions, thereby yielding data on the appropriate heating procedures to use for raw milk.

1. Introduction

Milk fat exists as fat globules in milk surrounded by an inner phospholipid monolayer and a complex lipid bilayer, called the milk fat globule membrane (MFGM) (Dewettinck et al. 2008). Previous studies have demonstrated that the MFGM plays a crucial role in stabilizing lipids in the aqueous phase and exhibits multiple biological functions (Dewettinck et al. 2008; Zanabria, Tellez, Griffiths, & Corredig 2014). The structure and components of the MFGM have been widely explored, and the protein components of bovine MFGM have been characterized by proteomic analyses (Affolter, Grass, Vanrobaeys, Casado, & Kussmann 2010; Fong & Norris 2009; Lu et al. 2011).

In modern dairy technology, heat treatment is used to reduce bacterial loads and increase the shelf life of milk products. Raw milk is processed by pasteurization and has a shelf-life of about 2 weeks; in contrast, milk processed by ultra-high temperature (UHT) and shelf-life of approximately 6 months (Gallier et al. 2013). Milk is a heat-stable system; however, changes in the physicochemical characteristics of milk are induced by heat processing (Sakkas, Moutafi, Moschopoulou, & Moatsou 2014). In particular, heat treatment has a major impact on the MFGM and disruptions in the MFGM occur during direct UHT processing (Fox, Uniacke-Lowe, Mcsweeney, & O'Mahony 2015). In terms of the structure of the MFGM, several previous studies have indicated that homogenization and thermal treatments result in decreased size and increased surface area of milk fat globules (Lee & Sherbon 2002; Sharma, Oey, & Everett 2015). With regard to the components of the MFGM, several major milk proteins, such as, β-lactoglobulin and β-casein, were identified in the MFGM fraction of heated whole milk (Houlihan, Goddard, & Nottingham 1992; Lee & Sherbon 2002). In particular, MFGM/β-lactoglobulin complexes were observed in heated milk by differential scanning calorimetry (Sharma, Oey, & Everett 2016). Moreover, previous studies have indicated that the MFGM fraction and milk proteins interact in whole milk via intermolecular disulfide bonds and surface hydrophobic interactions

Abbreviations: ALDH2, mitochondrial aldehyde dehydrogenase; BTN1A1, butyrophilin subfamily 1 member A1; FABP3, heart fatty acid-binding protein; LBP, lipopolysaccharide-binding protein; MFGM, milk fat globule membrane; PCA, principal component analysis; PCs, principal components; PVDF, polyvinylidine difluoride membrane; TBST, 0.1 M Tris pH 7.4, 0.15 M NaCl, and 0.1% Tween 20; THY1, thy-1 membrane glycoprotein; UHT, ultra-high-temperature; UP, ultra-pasteurization of milk; XDH, xanthine dehydrogenase/oxidase

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(Corredig & Dalgleish 1996; Sharma et al. 2016; Ye, Singh, Oldfield, & Anema 2004). Notably, the contents and types of proteins interacting with the MFGM fraction are associated with the intensity of heat treatment (Sharma et al. 2016; Ye et al. 2004). In addition to the milk proteins coupled with MFGM fraction, proteins originating from the MFGM fraction, such as lactadherin were downregulated (Lee & Sherbon 2002). These results suggested that protein components of the MFGM were altered, due to incorporation of milk proteins into the MFGM fraction and that the original components were decreased in the membrane by heat treatment.

With the development of analytical techniques, proteomics approaches have been used to investigate heat-induced changes in the whey protein components of milk (Zhang et al. 2016). In particular, the heat-induced Maillard reaction commonly occurs between lactose and milk proteins, and lactulosyl residues are the most predominant modifications of β-lactoglobulin and α-lactalbumin (Jing & Kitts 2002; Meltretter, Seeber, Humeny, Becker, & Pischetsrieder 2007). Shotgun proteomic analysis of advanced glycation end-products and the composition of fat globules from raw, pasteurized, UHT, and infant powdered milk found a total of 310 non-redundant lactosylation sites in 56 MFGM proteins, and significant lactosylation of proteins was observed in milk after UHT sterilization (Arena, Renzone, Novi, & Scaloni 2011; Renzone, Arena, & Scaloni 2015). Heat-induced changes in the structure and composition of the MFGM fraction have been implicated in the biological functions of dariy products (Ye et al. 2004; Zanabria et al. 2014). Although, these results provided useful information on changes in the protein components of the MFGM fraction and their potential functions due to heat treatments, changes in MFGM proteome profiles associated with the degree of heating have not been reported.

In this study, we aimed to characterize changes in MFGM proteins after heat treatment of whole milk and compared these results with those of raw milk using a label free proteomic approach. Protein profiles were used to distinguish protein properties in different types of heated whole milk and to evaluate the MFGM protein components related to heat treatment.

2. Materials and methods

2.1. Chemicals and reagents

High-performance liquid chromatography-grade acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany). NH_4HCO_3, iodoacetamide, urea, and polyvinylidine difluoride (PVDF) membranes were supplied by Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade trypsin was supplied by Promega (Madison, WI, USA). Sodium dodecyl sulfate (SDS), dithiothreitol, and Tris-base were purchased from Amersco (Solon, OH, USA). Bicinchoninic acid assays were purchased from Beyotime Biotechnology (Shanghai, China). Ultrafiltration filters were purchased from Sartorius (10-kDa cutoff; Germany). MilliQ water used in solution was obtained from Milli-Q Ultrapure Water Systems (Merck).

2.2. Sample treatment

Raw milk was collected (approximately 3 × 50 L) from a dairy farm in the Beijing area and then transferred to the laboratory within 2 h. Milk was processed using a homogenizer at 20 Mpa (APV 1000; APV Homogenisers, Albertslund, Denmark) and then transferred to a tubular heat exchanger unit (FT74 UHT/HTST processing system; Armfield Technical Education Co. Ltd., Ringwood, UK). According to the manufacturer's instructions, milk samples were subjected to one heat treatment cycle, including heating at 85 °C for 15 s (pasteurized milk), 125 °C for 4 s (ultra pasteurized [UP] milk), 138 °C for 4 s, and 145 °C for 4 s (UHT milk). The heat treatment circle was repeated three times. For each cycle, 1000 mL heated milk was collected approximately midway through the procedure. Thus, three samples of raw milk without homogenization and heat treatment and three samples from each type of heated milk were collected and stored at −80 °C for subsequent sample preparation.

2.3. Protein sample preparation

After thawing of samples, 15 whole milk samples from raw, pasteurized, UP, and two UHT milks (three samples from each group) were centrifuged for 15 min at 3000 ×*g* and 4 °C. The fat layer was collected with a spoon. The recovered milk fat was mixed with 0.01 M phosphate-buffered saline (PBS, pH 7.4) and incubated at 39 °C. The resuspended mixture was centrifuged at 3000 ×*g* for 30 min. The fat layer was recovered, and washed three times with PBS, and then with MilliQ water to remove residual caseins and whey proteins. Finally, milk fat globules were recovered.

One volume of fat globules was added to two volumes of lysis buffer (50 mM Tris-HCl [pH 7.4], 4% SDS) and incubated at 39 °C for 1 h with periodic vortexing. Subsequently, the mixtures were incubated at 95 °C in a water bath for 5 min. After the samples were cooled, the mixtures were centrifuged at 12,000 ×*g* for 15 min to remove the fat and pellet as the top and bottom layers, respectively. The middle layer containing MFGM proteins was collected and centrifuged again to remove the residual fat layer (Yang et al. 2015). The aliquots were then transferred into tubes and stored at −80 °C until further analysis.

2.4. Protein digestion

Aliquots of the MFGM proteins were thawed, and 200 μL sample was mixed with 1 mL acetone and stored at −20 °C for 20 h. The mixture was centrifuged at 14,000 ×*g* for 40 min. Protein pellets were dissolved in 100 μL of 5 M urea, and protein concentrations were determined using bicinchoninic acid assays (Beyotime Biotechnology) (Weng et al. 2016). Sixty micrograms of proteins were reduced, alkylated, and digested as described in a previous study (Yang et al. 2015). Briefly, the protein sample were loaded onto the filter (10-kDa cutoff; Sartorius), reduced with 10 mM dithiothreitol solution, and then washed twice with 8 M urea and 150 mM Tris-HCl buffer at pH 8.0. Subsequently, the sample was alkylated with 50 mM iodoacetamide solution at room temperature for 30 min in the dark and then washed twice as described above. Finally, the protein sample was digested with sequencing-grade trypsin buffer at 37 °C for 16–18 h. Tryptic peptides were collected by centrifugation, desalted using a C18 solid phase extraction column (Sep-pack Cartridges), and then dried.

2.5. Liquid chromatography-tandem mass spectrometry analysis

Dried peptides were dissolved in a solution of 0.1% (*v*/v) formic acid. The peptide mixture was separated and identified using an EASY-nLC 1000 system coupled with Q-Exactive mass spectrometry (ThermoFisher Scientific, San Jose, CA, USA). The chromatography column was equilibrated for 10 min with 95% (*v*/v) buffer A consisting of 0.1% (v/v) formic acid in MilliQ water. Tryptic peptides were loaded onto the trap column (20 mm × 100 μm, 5 μm) by an autosampler and separated on a reverse-phase C18 analytical chromatographic column (150 mm × 75 μm, 3 μm; Column Technology Inc., Fremont, CA, USA) with buffer B consisting of 90% (*v*/v) acetonitrile in buffer A at a flow rate of 300 nL/min. Peptides were eluted from 4% to 35% (*v*/v) buffer B for 103 min and from 35% to 90% (*v*/v) buffer B for 5 min, held at 90% (*v*/v) buffer B for 4 min, and then held at 2% (*v*/v) in buffer B for 7.5 min.

The Q-Exactive was used in the positive ion mode with a range of 300–1800 mass/charge (*m*/*z*). The resolving power for the survey scan was set as 70,000, and that for the MS/MS scans was set as 17,500 at *m*/*z* 200. The top 20 most abundant precursor ions selected from the survey scan were fragmented via higher energy collisional dissociation with 30.0% collision energies to acquire MS/MS data. The maximum