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Developmental Changes in the Milk Fat Globule Membrane Proteome During the Transition from Colostrum to Milk[1]

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ABSTRACT

Shotgun proteomics, using amine-reactive isobaric tags (iTRAQ), was used to quantify protein changes in milk fat globule membranes (MFGM) that were isolated from d 1 colostrum and compared with MFGM from d 7 milk. Eight Holstein cows were randomly assigned to 2 groups of 4 cow sample pools for a simple replication of this proteomic analysis using iTRAQ. The iTRAQ labeled peptides from the experiment sample pools were fractionated by strong cation exchange chromatography followed by further fractionation on a microcapillary high performance liquid chromatograph connected to a nanospray-tandem mass spectrometer. Data analysis identified 138 bovine proteins in the MFGM with 26 proteins upregulated and 19 proteins downregulated in d 7 MFGM compared with colostrum MFGM. Mucin 1 and 15 were upregulated greater than 7-fold in MFGM from d 7 milk compared with colostrum MFGM. The tripartite complex of proteins of adipophilin, butyrophilin, and xanthine dehydrogenase were individually upregulated in d 7 MFGM 3.4-, 3.2-, and 2.6-fold, respectively, compared with colostrum MFGM. Additional proteins associated with various aspects of lipid transport synthesis and secretion such as acyl-CoA synthetase, lanosterol synthase, lysophosphatidic acid acyltransferase, and fatty acid binding protein were upregulated 2.6- to 5.1-fold in d 7 MFGM compared with colostrum MFGM. In contrast, apolipoproteins A1, C-III, E, and A-IV were downregulated 2.6- to 4.3-fold in d 7 MFGM compared with colostrum MFGM. These data demonstrate that quantitative shotgun proteomics has great potential to provide new insights into mammary development.
Key words: milk fat globule membrane, proteomics, mammary gland, mastitis

INTRODUCTION

Proteomics is a tool that will help identify proteins important to milk production and secretion. Identification of proteins associated with various aspects of milk production and secretion will provide a foundation for new research in lactation biology. Most of proteomic studies conducted thus far on mammary epithelial cells, organelles, membranes, and the secretion processes are focused on breast cancer, rodent lactation, or both (Wu et al., 2000a,b; Quaranta et al., 2001; Charlwood et al., 2002; Pucci-Minafra et al., 2002; Fortunato et al., 2003; Jacobs et al., 2004; Davies et al., 2006). Although these studies have advanced our understanding of mammary function and milk secretion, they may not address the unique aspects of milk secretion in dairy cattle. Recently, 2 papers on bovine mammary proteomics have appeared (Daniels et al., 2006; Reinhardt and Lippolis, 2006). The first paper examined mammary protein expression in growing virgin heifers (Daniels et al., 2006), and the second was a survey of proteins expressed in milk fat globule membranes (Reinhardt and Lippolis, 2006).

Our understanding of the molecular mechanisms critical to milk fat secretion is incomplete (Mather and Keenan, 1998a,b; Keenan, 2001). The scarcity of information is due in large part to the lack of cell lines that secrete milk and milk fat (Keenan, 2001). The MFGM is a rich source of membrane proteins, and proteomic analysis of these membranes has highlighted some of the possible signaling and secretory pathways used by the mammary gland (Reinhardt and Lippolis, 2006). Furthermore, the proteome of the MFGM provides additional insight into this membrane's cellular origin. The most widely accepted source of membrane for the MFGM is the apical membrane of the secretory cell (Mather and Keenan, 1998a; Keenan, 2001). Their conclusions are supported by biochemical, electron microscopy, and immunocytochemical evidence.

The major proteins in the MFGM have been identified using traditional biochemical approaches (Mather, 2000). These methods are slow, laborious, and address only one protein at a time. Proteomic and microarray approaches can identify gene and protein

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connections to a pathway that is not apparent or predictable from biochemical and genetic analysis of a biological system (Patterson and Aebersold, 2003). This approach has been applied widely to quantitative proteomics (Ross et al., 2004; DeSouza et al., 2005; Chen et al., 2006; Hu et al., 2006; Keshamouni et al., 2006; Lippolis et al., 2006) and overcomes many of the problems of 2-dimensional electrophoresis in the study of membrane proteins (Gu et al., 2003; Peirce et al., 2004; Lippolis et al., 2006).

We used a shotgun proteomics approach using amine-reactive isobaric tags (**iTRAQ**) to quantify protein changes in milk fat globule membranes (**MFGM**) that were isolated from d 1 colostrum compared with d 7 milk. Our objective was to examine how the transition from colostrum secretion to milk secretion changes protein expression in MFGM.

MATERIALS AND METHODS

Animals and Milk Fat Membrane Preparation

Colostrum or milk was collected from 8 Holstein cows at parturition and d 7 of lactation. The colostrum or milk from each collection was brought immediately to the lab and centrifuged at 10,000 × *g* for 15 min at 4°C. The floating milk fat pellet was removed, mixed with 10 volumes of ice-cold phosphate buffered saline (pH 7) + complete protease inhibitor cocktail from Boehringer Mannheim (Indianapolis, IN), and centrifuged at 10,000 × *g* for 15 min. This washing step was repeated 3 times until the supernatant was clear (Reinhardt and Lippolis, 2006).

The MFGM were prepared from the washed milk fat as previously described (Reinhardt et al., 2000; Prapong et al., 2005; Reinhardt and Lippolis, 2006). Washed milk fat from colostrum or milk was diluted in 10 volumes of buffer A, which contained Tris-HCL (10 m*M*), $MgCl_2$ (2 m*M*), phenylmethylsulfonyl fluoride (0.1 m*M*), EDTA (1 m*M*), 4 μg/mL of aprotinin, and 4 μg/mL of leupeptin at pH 7.5. The sample was homogenized using a Polytron PT-10 homogenizer (Brinkman Instruments, Boston, MA) running at 12,000 rpm. Each homogenization step was for 12 s with 30 s of sample cooling between each homogenization run. A total of three 12-s homogenizations were performed on the sample. The homogenate was mixed with an equal volume of buffer B (buffer A plus 300 m*M* KCl) and centrifuged at 100,000 × *g* for 1 h. The supernatant was discarded and the membrane pellet was resuspended in buffer C (buffer A plus 150 m*M* KCl) (Reinhardt et al., 2000). The resuspended membrane preparation was centrifuged at 100,000 × *g* for 1 h. The supernatant was discarded and MFGM pellet was resuspended in buffer A. Protein concentration was determined using the BioRad Protein Assay Kit using a BSA standard. The MFGM were stored at –70°C until needed.

Extraction of Extrinsic Proteins to Concentrate MFGM Intrinsic Proteins

The MFGM prepared as described above were pelleted by centrifugation at 100,000 × *g* for 1 h. All procedures were done at 4°C. The membrane pellet was resuspended in a small amount of 300 m*M* sucrose, 10 m*M* Tris-HCL at pH 7.5. This suspension was diluted with ice-cold 0.1 *M* sodium carbonate (pH 11.5) to a protein concentration 0.01 mg/mL (Fujiki et al., 1982; Reinhardt and Lippolis, 2006). The sample was incubated on ice for 1 h to extract extrinsic proteins and then centrifuged at 100,000 × *g* for 1 h through a cushion of 300 m*M* sucrose (10% of the tube volume). The MFGM intrinsic protein pellet was resuspended in buffer A. Protein concentration was determined, and the extracted MFGM were stored at –70°C until needed. This procedure enriched the MFGM preparation for intrinsic proteins by reducing but not eliminating extrinsic protein content.

Sample Preparation for Mass Spectroscopy Analysis

Samples were randomized, and 4 sample pools were created (Figure 1). One hundred micrograms of protein from each sample pool was dried and processed as follows. Membrane proteins from the 4 samples were each resuspended in 50 μL of 25 m*M* triethyl-ammonium bicarbonate (pH 8) in 1.5-mL microcentrifuge tubes. After adding cap locks, the proteins were thermally denatured at 90°C for 20 min as described (Park and Russell, 2000, 2001). The samples were then cooled on ice for 10 min and then dried in a vacuum centrifuge. For trypsin digestion, 25 μL of proteomic-grade trypsin (20 μg/mL in 25 m*M* triethyl-ammonium bicarbonate) was added to each sample. We added acetonitrile (**ACN**) so that the solution was 30% ACN (Russell et al., 2001). This ACN/trypsin solution was incubated at 37°C overnight. The next day the samples were cooled to room temperature and then dried in a vacuum centrifuge. The samples were stored dry at –20°C until used.

Each sample was then labeled using the iTRAQ kit for amine-modifying labeling reagents for multiplexed relative and absolute protein quantitation (Applied Biosystems, Foster City, CA). See Figure 1 for the sample analysis work flow. The iTRAQ-labeled peptides were dried and resuspended in 300 μL of 20 m*M* formic acid and 20% ACN). Samples were run on a