J. Dairy Sci. 96:7854–7866
http://dx.doi.org/10.3168/jds.2013-7106
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Expanding the bovine milk proteome through extensive fractionation

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ABSTRACT

Bovine milk is an agricultural product of tremendous value worldwide. It contains proteins, fat, lactose, vitamins, and minerals. It provides nutrition and immunological protection (e.g., in the gastrointestinal tract) to the newborn and young calf. It also forms an important part of human nutrition. The repertoire of proteins in milk (i.e., its proteome) is vast and complex. The milk proteome can be described in detail by mass spectrometry-based proteomics. However, the high concentration of dominating proteins in milk reduces mass spectrometry detection sensitivity and limits detection of low abundant proteins. Further, the general health and udder health of the dairy cows delivering the milk may influence the composition of the milk proteome. To gain a more exhaustive and true picture of the milk proteome, we performed an extensive pre-analysis fractionation of raw composite milk collected from documented healthy cows in early lactation. Four simple and industrially applicable techniques exploring the physical and chemical properties of milk, including acidification, filtration, and centrifugation, were used for separation of the proteins. This resulted in 5 different fractions, whose content of proteins were compared with the proteins of nonfractionated milk using 2-dimensional liquid chromatography tandem mass spectrometry analysis. To validate the proteome analysis, spectral counts and ELISA were performed on 7 proteins using the ELISA for estimation of the detection sensitivity limit of the 2-dimensional liquid chromatography tandem mass spectrometry analysis. Each fractionation technique resulted in identification of a unique subset of proteins. However, high-speed centrifugation of milk to whey was by far the best method to achieve high and repeatable proteome coverage. The total number of milk proteins initially detected in nonfractionated milk and the fractions were 635 in 2 replicates. Removal of dominant proteins and filtering for redundancy across the different fractions reduced the number to 376 unique proteins in 2 replicates. In addition, 366 proteins were detected by this process in 1 replicate. Hence, by applying different fractionation techniques to milk, we expanded the milk proteome. The milk proteome map may serve as a reference for scientists working in the dairy sector.
Key words: *Bos taurus*, proteomics, fractionation technique, milk protein

INTRODUCTION

Bovine milk is an agricultural product with tremendous economic importance worldwide and is the primary source of nutrition for the newborn and young calf. In wildlife, ruminant offspring may suckle their dam for many months (Sidibé-Anago et al., 2008) and in some cases, the entire lactation period from calving up to the dam's next calving. The cow's lactation period can be divided into the following: colostrum (until 2 d postpartum), transitional milk (from d 3 to 6), early-lactation milk (from d 7 to 105), mid-lactation milk (from d 106 to 210), and late-lactation milk (from d 211 to 315; Gurmessa and Melaku, 2012). On modern dairy farms, the calf's milk feeding period is limited to the first 8 wk of the calf's life (Quigley et al., 2006). Early milk has a content of bioactive proteins that, for example, provides local immunological protection in the gastrointestinal tract of the calf. The quality of the milk fed to the calf is speculated to influence its future milk potential as heifer calves (Khan et al., 2011).

In addition to calf feeding, the exploitation of dairy cow milk naturally forms an important component in human nutrition in its raw form, as skim milk, UHT milk, cream, cheese, or as an ingredient in many more dietary products (Haug et al., 2007; Nagpal et al., 2012). In the dairy processing industry, fractionation of bovine milk is an important production process in obtaining pools of specific milk proteins having either specific physical-chemical properties, nutritional, or biological importance. Industrially produced fractions often range from containing just a few types of milk proteins (e.g., lactoferrin, lactoperoxidase, and lysozyme; Stelwagen

Received June 7, 2013.
Accepted September 2, 2013.
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et al., 2009) to a more complex composition of powders containing many different proteins (i.e., whole milk powder, whey powder, and casein fraction) and formulations aimed at substituting human milk to neonatal humans (Koopman et al., 1985; Nagpal et al., 2012).

Bovine milk has previously been examined by mass spectrometry-based proteomics. Some of the first proteomic studies applied a modest number of fractionation techniques and primarily or exclusively focused on a unique subcategory such as milk fat globule membranes (**MFGM**; Reinhardt and Lippolis, 2006, 2008; Smolenski et al., 2007). Subsequent studies have applied combinatorial peptide ligand library (D'Amato et al., 2009), enhanced ion-exchange chromatography (Le et al., 2011), or other approaches (Hettinga et al., 2011; Senda et al., 2011; Han et al., 2012). In some cases, the studies were conducted on commercial milk products, and others on milk sampled from dairy cows that had not been thoroughly screened for health status before the milk collection. We have previously shown how fractionation techniques applied before the 2-dimensional liquid chromatography tandem mass spectrometry (**2D-LC-MS/MS**) analysis can be used to expand the bovine colostrum proteome (Nissen et al., 2012). Separation of milk into different fractions can be accomplished by various methods. By applying low-speed centrifugation, milk can be separated into 3 visible layers: a minor fat layer, a major fluid phase, and a pellet containing leukocytes and mammary epithelial cells (Le Jan, 1996). The fluid phase contains the water-soluble components, including the high-abundant proteins: caseins, β-LG, and the slightly less abundant immunoglobulin. The casein forms micelles that are composed of casein and calcium ions and have a variable diameter, which in milk is roughly ≤200 nm (Tsioulpas et al., 2007a,b). This allows for further separation by high-speed centrifugation, microfiltration, or acid precipitation, from which whey is obtained containing the remaining free proteins. In addition, UF can be used to separate monomer immunoglobulins, which have an average molecular weight of 160 to 180 kDa (Korhonen et al., 2000; Mix et al., 2006) from proteins with a lower molecular weight, which will pass the filter and remain in the whey phase.

In this study, we aimed to expand on the results from our previous fractionation and shotgun proteomic study of colostrum (Nissen et al., 2012). The performance, in terms of protein detection yield, of the physical and chemical fractionation techniques was evaluated on early lactation milk collected from Danish Holstein-Friesian cows. Further, the aim was to use these fractionation techniques to produce a comprehensive proteome map of raw composite milk sampled from healthy dairy cows in early lactation.

MATERIALS AND METHODS

Animal Health and Milk Sampling

Composite milk was collected on d 10 postpartum from 4 Danish Holstein-Friesian cows in second lactation. The cows had been dried off 5 to 7 wk before calving. Each cow was milked twice per day at 0700 and 1600 h. Udder health and body temperature (d 10: <37.9°C) was confirmed in the period from parturition to d 10 (milk sampling). To score udder inflammation from foremilk strippings, a semiquantitative cowside test, the California mastitis test (**CMT**; Kruuse A/S, Marslev, Denmark), was used (Pyörälä, 2003). The milk was collected immediately after calving and was accepted if the CMT score was <3. On d 10 (milk sampling), milk was accepted if the CMT score was <2. Bacteriological analyses were done on foremilk samples according to National Mastitis Council guidelines on the first 6 milkings and on d 10 and tested negative for major bacterial pathogens (NMC, 1999). Further, milk samples were collected at the first 6 milkings postpartum and on d 10 and analyzed for SCC in a diagnostic dairy laboratory (Eurofins Steins Laboratory, Holstebro, Denmark). All collected milk samples on d 10 for 2D-LC-MS/MS analysis had SCC $<9.4 \times 10^4 \pm 8.0 \times 10^4$ cells/mL. Hence, the cows were considered to have a healthy udder. Milk composite samples (representing the entire milk collected during milking) were collected from the 4 cows. The composite sample from each cow was pooled in the laboratory and used for the proteome analysis (Figure 1).The process of milk sampling and pooling is illustrated in Figure 1A.

Fractionation of Raw Milk

Fractionation of the composite milk is illustrated in Figure 1B. In total, 6 different milk samples were obtained; for each of the cow samples, nonfractionated whole milk (**NF**), cell- and fat-free fraction (**F1**), and the cell pellet fraction (**F2**) were obtained and a pool was made from the 4 cows in replicates. Further, an additional pool of milk from the 4 cows was constructed. This pool was divided into 2 and was used for preparation of the 3 different whey fractionations: acidification (**F3**), filtration (**F4**), and centrifugation (**F5**). The process resulted in 2 replicates of each fraction (NF and F1–F5) and each fraction replicate was then analyzed once by 2D-LC-MS/MS. The 4 different fractionation techniques thus resulted in 5 different fractions (F1–F5) in addition to the NF. In total, this resulted in 12 samples to be analyzed. A detailed description of the 4 fractionation techniques is presented in Nissen et al. (2012), where these were used to process bovine colostrum. The exact same procedures were used for

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