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

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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: $<37.9^{\circ}\text{C}$) 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 $\text{SCC} < 9.4 \times 10^4 \pm 8.0 \times 10^4$ 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

A)**Samples: NF, F1, and F2** **Samples: F3, F4, and F5** **B)**

Figure 1. Experimental design of sampling, pooling, and fractionation. Panel A presents sampling and pooling for nonfractionated milk (NF) and the following fractions: cell and fat free (F1), cell pellet (F2), acidification to whey (F3), filtration to whey (F4), and centrifugation to whey (F5). Panel B presents the process of fractionation of bovine milk into the 5 fractions. By applying low-speed centrifugation on the whole NF collected immediately after milking, the 2 first fractions (F1 and F2) were produced. The NF was further used for whey preparation by different methods, including acidification (F3), 100-kDa UF (F4), or high-speed centrifugation (F5). The top fat layer (gray/orange) was not analyzed. Two replicates of all fractions were produced, enclosed in a square box, and were subjected to 2-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS) analysis. Color version available in the online PDF.

the processing of milk samples in our study, although the fraction number differs between the 2 studies. The processed results on milk are reported in Supplemental Tables S1 and S2 (available online at <http://dx.doi.org/10.3168/jds.2013-7106>).

Protein Determination and 2D-LC-MS/MS Analysis

The protein concentration was determined in milk before pooling the milk samples (on a volume basis or protein weight basis) and in NF and fractions before the proteome analysis (Nissen et al., 2012). A detailed description of the 2D-LC-MS/MS analysis performed on the NF and the fractions F1 to F5, including protein precipitation, digestion, sterile filtration and peptide elution, is also given in Nissen et al. (2012). A QSTAR Elite mass spectrometer (Applied Biosystems Inc., Foster City, CA) was used for the final 2D-LC-MS/MS analysis.

Database Search, Spectral Counts, and Gene Ontology Analysis

The data files from the 2D-LC-MS/MS analysis were searched with the UniProt *Bos taurus* database (<http://www.uniprot.org/taxonomy/9913>) as described by Nissen et al. (2012). Briefly, protein sequences for the target database were obtained from the UniProtKB (Wu et al., 2006) database (release 14.8/56.8; <http://www.uniprot.org/help/uniprotkb>), with only *Bos taurus* selected for matching. To evaluate and increase the confidence of the protein identification, a target-decoy database search was applied (Elias and Gygi, 2007; Elias and Gygi, 2010). The construction of the decoy part of the database: the search of the raw data files with the ProteinPilot 1.0 software (Applied Biosystems Inc.) and data modification are described in Nissen et al. (2012). Proteins were accepted according to a protein score of 1.3, equivalent to 95% confidence. Protein matches were considered valid if the protein was detected in both replicates (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2013-7106>). For protein names reported as a short abbreviation, a BLAST search (<http://www.uniprot.org>; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was performed (Altschul et al., 1997). The resulting alternative name was determined on the basis of strong homology to a better characterized protein, having a highly similar amino acid sequence. The alternative name was added to the result file (Table 2 and Supplemental Tables S1 and S2; <http://dx.doi.org/10.3168/jds.2013-7106>) in parentheses. For each replicate, repeatability was calculated as $100 \times (\text{number of proteins detected in each replicate} / \text{number of proteins common between the 2 replicates})$

and reported as the average of the 2 replicates' repeatability. Also, for each replicate, a false discovery rate (**FDR**) was calculated as $\text{FDR} = 100 \times [\text{false positive} / (\text{false positive} + \text{true positive})]$ for each fractionation technique and reported as the average of the 2 replicates' FDR.

A semiquantitative analysis was performed for selected proteins (see Supportive Protein Analysis section) by counting of the ProteinPilot winner peptide spectra. The total number of winner spectra for proteins detected in both replicates was reported by adding the 2-replicate number of winner spectra. For proteins detected in a single replicate, the number of winner spectra in the 1 replicate was reported. Finally, a Gene Ontology (**GO**; <http://www.geneontology.org/>) analysis of the proteins was performed. To avoid exaggerated detailed annotation, a slimmed set of the GO annotation terms was used, using the Protein Information Resource (**PIR**; Wu et al., 2004) with the PIR GO Slim, iProClass database version 4.10, and AmiGO web service from GO (version 1.8; Ashburner et al., 2000).

Supportive Protein Analysis

Seven different commercial ELISA kits were used to verify and quantify the actual protein concentration in NF (Nissen et al., 2012). The proteins and sample dilutions were total bovine immunoglobulins (total Ig): 1:10 (Bio-X Diagnostics S.P.R.L., Jemelle, Belgium); bovine lactoferrin (**LF**): 1:2,000 (Bethyl Laboratories Inc., Montgomery, TX); multispecies LPS-binding protein (**LBP**): 1:500 (Hycult Biotech, Uden, the Netherlands); multispecies milk amyloid A (**MAA**): 1:50 (Tridelta Development Ltd., Maynooth, Ireland); bovine haptoglobin (**HP**): 1:20 (Immunological Laboratory Inc., Newberg, OR); human transforming growth factor β 1 (**TGF- β 1**): 1:7.8 (R&D Systems Inc., Minneapolis, MN); and human transforming growth factor β 2 (**TGF- β 2**): 1:7.8 (R&D Systems Inc.). All ELISA were analyzed according to the manufacturers' instructions and included an in-house positive control sample with a high and low concentration of the specific proteins. Analysis was performed in duplicate on fraction NF and all coefficients of variation were below 8.6% for controls as well as samples, except for TGF β 2, which had a coefficient of variation of 19.6%.

RESULTS

The total protein results are presented in Supplemental Tables S1 and S2 (<http://dx.doi.org/10.3168/jds.2013-7106>). Supplemental Table S1 presents the 376 proteins found in both replicates in either NF or each of the fractions F1 to F5. Supplemental Table S2 presents

an additional 366 proteins detected once, but not in both replicates in either NF or each of the fractions F1 to F5. The proteins presented in Supplemental Table S2 should, therefore, be regarded as tentative, except for the 91 proteins in Supplemental Table S2 that had already been confirmed in both replicates in Supplemental Table S1.

Ranking of Milk Fractionation Techniques

Considering all samples, an initial number of 635 proteins were detected in NF and the 5 fractions (F1–F5; Table 1, row 1). The dominating protein groups [caseins (α 1, α 2, β 1, and κ), β -LG, and the d-10 milk less-abundant immunoglobulins (IgG1, IgA, IgM, and IgG2)] could not be entirely removed by any of the fractionation techniques applied. In the final protein result files, the dominant casein and immunoglobulin proteins and the common contaminant proteins keratin and trypsin were removed. This resulted in a reduction of detected protein as presented in Table 1 (row 2). Filtering for redundancy in the list of identified proteins reduced the number of distinct milk proteins to 376. In terms of number of proteins detected (rows 1 and 2), the efficiency of the fractionation techniques was F2

> F5 > NF > F3 $\geq$ F1 > F4. Rows 3 and 4 in Table 1 present the number of proteins gained (F2 > F5 > F4 > F1 = F3) or lost (F4 > F1 $\geq$ F3 $\geq$ F2 > F5) by each fractionation technique when compared with NF. Further, Table 1 (rows 6–11) presents the results on proteins detected in the single replicates (Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2013-7106>). Of the fluid-phase fractions, high-speed centrifugation to whey (F5) performed the best in every respect. The entire set of proteins detected in the fluid phase is presented in Table 2. As expected, the nonfluid-phase cell pellet fraction (F2) contained the largest number of proteins exclusively detected in any fraction. For clarity, F2 was not included in Table 2 and the proteins detected in it are only presented in Supplemental Tables S1 and S2 (<http://dx.doi.org/10.3168/jds.2013-7106>). As can be seen from Table 2, all individual fractionation techniques added to the final protein results, and each fractionation technique resulted in detection of a unique subset of proteins.

As a quality control measure, the repeatability and FDR were calculated for all fractionation techniques. The repeatability was calculated on the basis of 2 technical replicates (Figure 1) and resulted in an average repeatability of $74.3 \pm 4.9\%$. Fraction F5 achieved the

Table 1. An overview of the number of milk proteins detected by the 2-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS) analysis in the nonfractionated whole milk sample (NF) and the 5 different milk fractions (F1–F5) in 2 and 1 replicates considering all fractions¹

Number of protein detections	Milk fraction ²					
	NF	F1	F2	F3	F4	F5
Proteins detected in both replicates ³						
Initially detected number of proteins (n = 635)	65	47	327	47	37	112
Without dominant and contaminant proteins ⁴ (n = 533; unique proteins: n = 376 ⁵)	49 ⁶	30 ⁶	310	31 ⁶	26 ⁶	87 ⁶
Detected in the specific fraction compared with NF	—	5	283	5	8	50
Detected in NF compared with the specific fraction	—	24	22	23	31	12
Proteins detected in 1 replicate ⁷						
Without dominant and contaminant proteins ⁴ (n = 561; unique proteins: n = 457; 366 additional proteins compared with 2 replicates detected ⁸)	59	40	354	32	23	53
Detected in the specific fraction compared with NF	—	19	340	17	17	41
Detected in NF compared with the specific fraction	—	38	45	44	53	47
Repeated proteins (also detected in replicates) ⁹	42	33	32	25	17	17
Nonrepeated proteins (only single-replicate detection) ¹⁰	17	7	322	7	6	36

¹The table further presents the number of proteins gained (rows 4 and 8) or lost (rows 5 and 9) by a given fractionation technique, when compared with NF. The number of proteins detected in 1 replicate (rows 6–11) should be regarded as tentative.

²F1 = cell- and fat-free fraction; F2 = cell pellet fraction; F3 = acidification to whey fraction; F4 = filtration to whey fraction; F5 = centrifugation to whey fraction.

³Proteins detected in both replicates of each milk fraction.

⁴Removal of the dominant proteins casein and immunoglobulin and the contaminant proteins keratin and trypsin reduced the number of detected proteins.

⁵The complete protein list is presented in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2013-7106>).

⁶Proteins detected in the milk fluid-phase fractions are presented in Table 2.

⁷Proteins only based on single-replicate detection in each milk fraction.

⁸The complete protein list is presented in Supplemental Table S2 (<http://dx.doi.org/10.3168/jds.2013-7106>).

⁹Proteins that had previously been detected by 2-replicate detection.

¹⁰Proteins that had not previously been detected by 2-replicate detection.

Table 2. Bovine proteins (n = 103) detected in whole milk and the different milk fluid fractions by the 2-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS) analysis in both replicates (Supplemental Table S1, <http://dx.doi.org/10.3168/jds.2013-7106>)¹

Protein name ²	UniProtKB ID ³	Milk fraction ⁴				
		NF	F1	F3	F4	F5
78-kDa glucose-regulated protein	Q0VCX2					X
A2M protein (alpha 2 macroglobulin)	A8E647					X
Actin, cytoplasmic 1, 2	P60712	X			X	X
Adipophilin	Q9TUM6	X	X			X
Adenosine diphosphate (ADP)-ribosylation factor 1, 2, 3	P84080					X
Albumin (ALB) protein (serum albumin)	B0JYQ0			X		
Alpha-1-acid glycoprotein	Q3SZR3	X	X	X		X
Alpha-1-antiproteinase	P34955			X	X	X
Alpha-1B-glycoprotein	Q2KJF1	X		X		X
Alpha-2-HS-glycoprotein	P12763		X	X		X
Alpha-lactalbumin	P00711		X		X	
Amyloid protein A (milk amyloid A)	Q8SQ28	X				
Angiogenin-1	P10152	X		X		
Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Q3SZH5					X
Annexin A5	P81287	X				
Apolipoprotein A-I	P15497	X				X
Apolipoprotein A-IV	Q32PJ2	X	X			X
Apolipoprotein E	Q03247	X				X
Beta-1,4-galactosyltransferase 1	P08037	X	X	X		X
Beta-2-microglobulin	P01888	X	X	X	X	X
Beta-lactoglobulin	P02754	X	X	X	X	X
Butyrophilin subfamily 1 member A1	P18892	X	X	X		X
Carboxypeptidase B2	Q2KIG3					X
Cathepsin Z	P05689					X
CD5L protein	A6QNW7					X
Cellular repressor of E1A-stimulated genes 1	Q148D9	X	X		X	X
Clusterin	P17697	X		X		X
Complement C3	Q2UVX4	X	X	X		X
Complement factor B	P81187					X
Complement factor I	Q32PI4					X
CP protein (ceruloplasmin)	Q32P72					X
Cystatin E/M	Q5DPW9					X
Cystatin-C	P01035					X
Endopin 2, 2B	Q3SZQ8	X				X
Endopin 2C	Q32T06					X
Epididymal secretory protein E1	P79345					X
Factor XIIa inhibitor	P50448	X				X
Fatty acid synthase	Q71SP7	X			X	
Fatty acid-binding protein, heart	P10790	X		X	X	X
FGG protein (fibrinogen gamma-β chain)	Q3SZZ9		X			X
Fibrinogen α chain	P02672	X	X	X		X
Fibrinogen β chain	P02676					X
Fibroblast growth factor-binding protein 1	Q9MZ06	X	X	X	X	X
Fibronectin	P07589					X
Folate receptor α	P02702	X	X	X	X	X
G protein-coupled receptor, family C, group 5, member B	Q1JPD9	X				
Glycoprotein 2 (zymogen granule membrane)	Q0IIA4	X	X	X	X	X
Glycosylation-dependent cell adhesion molecule 1	P80195	X	X	X	X	X
Heat shock cognate 71-kDa protein	P19120	X				
Hemoglobin subunit α	P01966				X	
Hemoglobin subunit β	P02070				X	X
Hemopexin	Q3SZV7					X
Hibernation protein 20-like	Q2KIX7					X
Hibernation-associated plasma protein HP-20-like	Q2KIT0		X			X
Inter-α-trypsin inhibitor heavy chain H4	Q3T052					X
Isocitrate dehydrogenase [NADP], cytoplasmic	Q9XSG3	X				X
Kininogen-1, 2	P01044					X
Lactadherin	Q95114	X	X	X		X
Lactoperoxidase	P80025	X	X	X	X	X
Lactotransferrin (lactoferrin)	P24627	X	X	X		X
Leucine-rich α-2-glycoprotein 1	Q2KIF2					X
Lipopolysaccharide-binding protein	Q2TBI0					X

Continued

Table 2 (Continued). Bovine proteins (n = 103) detected in whole milk and the different milk fluid fractions by the 2-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS) analysis in both replicates (Supplemental Table S1, <http://dx.doi.org/10.3168/jds.2013-7106>)¹

Protein name ²	UniProtKB ID ³	Milk fraction ⁴				
		NF	F1	F3	F4	F5
Lipoprotein lipase	P11151	X	X			X
Lithostathine	P23132				X	X
Monocyte differentiation antigen CD14	Q95122					X
Mucin-15	Q8MI01	X	X	X	X	X
Myristoylated alanine-rich C-kinase substrate	P12624					X
Nucleobindin-1	Q0IHH5	X	X	X		X
Nucleobindin-2	Q0P569					X
Nucleotide exchange factor SIL1	Q32KV6					X
Peptidyl-prolyl <i>cis-trans</i> isomerase B	P80311					X
Peptidyl-prolyl <i>cis-trans</i> isomerase FKBP1A	P18203					X
Pigment epithelium-derived factor	Q95121					X
Plasminogen	P06868	X				
Platelet glycoprotein 4	P26201		X	X		X
Polymeric immunoglobulin receptor	P81265	X	X	X	X	X
Proactivator polypeptide	P26779					X
Prostaglandin-H2 D-isomerase	O02853				X	X
Protein OS-9	Q3MHX6	X				X
Protein S100-A9	P28783					X
Prothrombin	P00735	X				
QSOX1 protein (sulfhydryl oxidase 1 isoform a)	A6QQA8	X				
Ras-related protein Rab-18	Q0IIG8					X
Retinol-binding protein 4	P18902	X				
Rho guanosine diphosphate (GDP)-dissociation inhibitor 1	P19803					X
Ribonuclease pancreatic	P61823				X	X
Secretoglobulin, family 1D, member 2	A0JNP2	X				
Serotransferrin	Q29443	X	X	X	X	X
Serpin A3-1 (similar to endopin-1A)	Q9TTE1			X		X
SERPINA3-5 (similar to endopin-2C)	A2I7N1					X
SERPINA3-6 (similar to endopin-2B)	A2I7N2					X
Serum albumin	P02769	X	X	X	X	X
Sodium-dependent phosphate transport protein 2B	Q27960					X
Stromal cell-derived factor 4	Q3ZBZ1	X				X
Superoxide dismutase [Cu-Zn]	P00442				X	
Syndecan-2	Q58DD4					X
Tetranectin	Q2KIS7					X
TGOLN2 protein (<i>trans</i> -Golgi network isoform 6)	A6QQ20					X
TOMM22 protein (mitochondrial import receptor subunit homolog)	A6QPI6					X
Ubiquitin	P62990	X				
Vitamin D-binding protein	Q3MHN5	X	X	X	X	X
Xanthine dehydrogenase/oxidase	P80457	X	X	X	X	X
Zinc- α -2-glycoprotein	Q3ZCH5	X		X	X	X

¹For clarity, the results presented in the table do not include the cell pellet fraction (F2), which contained 310 proteins. Tentative proteins detected in 1 replicate are presented in Supplemental Table S2 (<http://dx.doi.org/10.3168/jds.2013-7106>).

²Proteins names reported as a short abbreviation also includes an alternative name in parentheses, obtained by the BLAST search.

³UniProtKB ID = protein accession number in the UniProtKB database (<http://www.uniprot.org/help/uniprotkb>).

⁴NF = nonfractionated whole milk; F1 = cell- and fat-free fraction; F3 = acidification to whey fraction; F4 = filtration to whey fraction; F5 = centrifugation to whey fraction.

maximal repeatability (82.9%) of all fractions. The FDR analysis was calculated on the results from the combined target/decoy database search and resulted in an average FDR of $0.1 \pm 0.1\%$.

Effect of Fractionation on Protein Detection

To clarify the effect of fractionation, an analysis of the intersection and complement of a reduced set of the different milk fractions was performed. The 3 groups

consisted of group I: whole milk (NF and F1), group II: whey (F3, F4, and F5), and group III: cell pellet (F2) presented in the Venn diagram in Figure 2. It can clearly be seen that the extensive fractionation applied in this study had a strong effect on the proteome coverage. Only 54 proteins could be identified by 2D-LC-MS/MS analyses of the fractions of the whole milk group (group I), of which 6 proteins were exclusively detected in this group. In contrast, fractionation to whey (group II) and cell pellet (group III) allowed for a larger num-

ber of proteins to be detected and contributed with 41 (group II) and 273 unique proteins (group III). All of the groups shared a substantial part of their detected proteins with proteins detected in the other 2 groups.

ELISA Measurements and 2D-LC-MS/MS Spectral Counts

Validation of the 2D-LC-MS/MS identified proteins was performed with ELISA on 7 proteins in NF (Figure 3A). The ELISA results were correlated with the spectral counts from the 2D-LC-MS/MS results for proteins detected in both replicates and in single replicates in NF and in all the fractions. In the results from the spectral search against the UniProt database (Table 2 and Supplemental Tables S1 and S2; <http://dx.doi.org/10.3168/jds.2013-7106>), MAA is listed as amyloid protein A and lactoferrin is listed as lactotransferrin. The ELISA detected 6 of the 7 selected proteins in NF, which were ranked as follows: total Ig > LF > LBP > HP > TGF- β 2 > TGF- β 1, based on their concentration in NF. The selected proteins covered a large part of the dynamic range spanning from total Ig (0.36 mg/mL) to MAA (lower detection limit reported by manufacturer: 0.44 μ g/mL) that was undetectable by ELISA.

The results from the 2D-LC-MS/MS spectral counts of the 7 proteins in the NF and 5 different fractions are presented in Figure 3B. In comparison to the ELISA, only 3 proteins were detected by the proteome analysis of the NF. Based on their spectral counts, their ranking was as follows: total Ig > LF > MAA. None of the fractions could detect all 7 proteins. Based on their number of spectral counts in the 5 fractions (F1–F5), the proteins were ranked as follows: total Ig > LF > HP > LBP > MAA. However, fractionation into differ-

ent fractions changes the concentrations of the different proteins. None of the fractionation techniques were able to accommodate detection of the cytokines TGF- β 2 or TGF- β 1 by the 2D-LC-MS/MS analysis, which had an ELISA concentration of 7.2 and 1.3 ng/mL in NF, respectively. The cell pellet fraction (F2) had 5 of the 7 investigated proteins detected with relatively high spectral counts, and had the lowest count of total Ig. Of the fluid-phase fraction, high-speed centrifugation to whey (F5) resulted in the highest spectral count for all detected proteins, except MAA, as well as the highest number of protein detections, when also considering single replicates. In NF, MAA was successfully detected in both replicates. Filtration to whey (F4) had the poorest protein detection, with 1 of 7 proteins being detected and this protein being total Ig.

GO Analysis of Protein Participation in Biological Processes

To obtain an overview of the biological process (BP) that the fluid-phase proteins (NF, F1, F3, F4, and F5) participated in, a GO analysis was performed (Figure 4). The initial PIR analysis resulted in 462 annotations. For clarity, these were slimmed with AmiGO GO Slimmer to a higher level annotation within the BP category. The proteins in the milk fluid phase were divided into the 22 BP categories, ranging from categories of 10% down to >0.5% of the total annotations (Figure 4). Based on the presented GO annotations, it is clear that the proteins present in the bovine milk fluid phase participate in a wide variety of BP. Of the applied fractionation technique, no single technique had a strong preference for isolation of proteins within a specific GO annotation group (data not presented).

DISCUSSION

In this study, extensive measures were taken to ensure that only raw, composite milk samples were collected from healthy cows with udders free of mastitis pathogens and low SCC. The primary aim was to characterize the bovine milk proteome, the secondary to illustrate the effect on protein/peptide detection when applying different fractionation techniques before the 2D-LC-MS/MS analysis, and the third aim was to map the peptides and proteins to their fraction(s).

The bovine milk proteome was successfully characterized detecting 376 unique proteins in 2 replicates, and an additional 366 unique proteins in 1 replicate (tentative proteins). Performing a GO analysis on the 103 fluid-phase proteins showed that these proteins contained 462 annotations, which were divided into the 22 BP top-level terms. The finding of more than 742

Figure 2. Venn diagram displaying the effect of fractionation on milk protein detection based on the milk proteins detected in the duplicates ($n = 376$). A reduced set of groups was defined, consisting of group I: proteins detected in nonfractionated milk (NF) and the cell- and fat-free fraction (F1); group II: milk whey proteins detected in fractions produced by acidification (F3), filtration (F4), or centrifugation (F5); and group III: proteins detected in the milk cell pellet (F2). Color version available in the online PDF.

Figure 3. (A) Validation and quantification performed by ELISA on 7 selected bovine proteins analyzed in nonfractionated whole milk (NF). The bars indicate the ELISA-determined concentration in NF. The results were correlated with the spectral counts from the 2-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS) analysis in NF and the 5 different milk fractions. (B) Spectral count analysis for the 7 proteins in NF and 5 fractions (F1–F5) indicates the total number of winner peptide spectra in NF and each fraction. MAA = milk amyloid A (i.e., amyloid protein A); TGF-b1 = transforming growth factor β 1; TGF-b2 = transforming growth factor β 2; HP = haptoglobin; LBP = LPS-binding protein; LF = lactoferrin (i.e., lactotransferrin); total Ig = total immunoglobulin; F1 = cell and fat free; F2 = cell pellet; F3 = acidification to whey; F4 = filtration to whey; F5 = centrifugation to whey. The detection limit of the MAA ELISA kit for milk was 0.44 μ g/mL. Color version available in the online PDF.

unique proteins and 22 BP top-level terms is in accordance with the earlier studies on less characterized milk (Smolenski et al., 2007; Hettinga et al., 2011; Le et al., 2011) or processed milk (D'Amato et al., 2009).

Whether the fractionation of raw composite milk in our study led to the discovery of new unique milk proteins or proteins unique to milk in early lactation remains to be shown.

Figure 4. Gene Ontology (GO; <http://www.geneontology.org/>) annotation using the biological process category of the milk proteins detected in the fluid phase ($n = 103$) in any of the 5 milk fluid-phase fractions produced. Color version available in the online PDF.

In any proteomic study it is important to obtain thorough proteome coverage for the samples analyzed. However, body fluids such as milk contain dominant proteins, which cause the current state of 2D-LC-MS/MS-based technologies to detect only a fractional part of the sample's proteome (Surinova et al., 2011). In our study, the proteome coverage of bovine milk was increased by exploiting different fractionation techniques to reduce the complexity of the milk sample. Unfortunately, fractionation of a sample can often result in adding technical variation to the analysis, which reduces repeatability (Surinova et al., 2011). In our study, the repeatability ranged from 69.7% to about 82.9%. This is in line with what is normally observed in shotgun proteomics using 2D-LC-MS/MS technology (Tabb et al., 2010). In the present study, reducing the complexity of the milk samples (e.g., by removal of the dominant casein proteins by fractionation) resulted in lower variation between replicates and better repeatability. This was particularly true in the fraction obtained by high-speed centrifugation to whey (F5). Here, we also clearly observed that this fractionation

technique resulted in the largest number of proteins being detected in any fluid-phase fraction. A similar phenomenon of improved repeatability has previously been described in fractionation of human serum and has been attributed to improved chromatography because of the depletion of abundant serum proteins (Whiteaker et al., 2007). Fractionation of bovine colostrum by similar fractionation techniques, including high-speed centrifugation, also resulted in improved detectability and repeatability of the proteome (Nissen et al., 2012).

In the current study, results from proteomic analysis of the milk fat layer were not included, nor was MFGM prepared from it. The MFGM contains many different proteins and has previously been thoroughly described (Reinhardt and Lippolis, 2006, 2008; Hettinga et al., 2011). In the preliminary experiments, analysis of the crude milk fat layer fraction resulted in a very low number of proteins detected. However, prior freezing of our milk sample (-20°C for >1 wk) and subsequent analysis of the crude fat layer revealed a larger number of proteins, most likely originating from cellular lysis of the milk cells (data not presented). Hence, when

analyzing fresh milk preparation of MFGM, an initial freezing of the milk samples is necessary for detection of proteins from the raw milk fat layer.

The extensive fractionation of milk into different fractions by various techniques improved the detection of proteins. Only 6 proteins were exclusively detected in the whole milk group (NF and F1), whereas both the whey and cell group contributed with numerous proteins. This finding is in accordance with our former study on bovine colostrum (Nissen et al., 2012). A large number of the proteins detected in the fluid phase were also found in the cell pellet (F2). It has previously been shown that cell-derived proteins in milk originate from viable cells by secretion or from lysis of dead cells (Reinhardt et al., 2012). However, the proteins detected in the fluid phase (NF, F1, F3, F4, and F5) and also in the cellular fraction (F2) could equally well be due to specific or unspecific binding of the proteins to the cells in milk. Further studies will be necessary to clarify on this matter.

As an auxiliary quality control of the 2D-LC-MS/MS analysis and the udder health status of the cows, several ELISA were performed on NF. Seven selected proteins were analyzed, including the 2 indicators of udder inflammation and mastitis, HP and MAA (Pyörälä, 2003). Haptoglobin had a concentration of 70.3 ng/mL and MAA was undetectable by the ELISA. The low concentrations of the acute-phase proteins in milk combined with the clinical recordings and microbiological analysis ruled out the presence of mastitis and approved the udder health of the cows (Pyörälä, 2003; Gerardi et al., 2009). Hence, in contrast to recent studies (D'Amato et al., 2009; Hettinga et al., 2011; Le et al., 2011) that used commercial milk or raw milk of uncategorized or undocumented quality, our proteome analysis was conducted on raw composite milk from clinically and paraclinically verified healthy cows.

The ELISA results supported the 2D-LC-MS/MS findings to a certain extent. Five of the 7 proteins quantified by the ELISA were detected either in a milk fraction or NF in 2 replicates by the 2D-LC-MS/MS analysis. Furthermore, the low milk concentration of the 2 cytokines TGF- β 1 and TGF- β 2 could serve as an indirect measure of the 2D-LC-MS/MS analysis detection sensitivity. However, none of these cytokines were detectable by the 2D-LC-MS/MS analysis, which, on the basis of LBP, could suggest a detection limit of proteins higher than 21.3 μ g/mL in the fluid phase. Milk amyloid A, which was undetectable by ELISA, was identified as the isoform serum amyloid A3 (SAA3) in NF (both replicates) and F5 (1 replicate) by 2D-LC-MS/MS. The relatively high detection limit of the MAA ELISA may have prevented quantification of the MAA protein in the milk fractions. Hence, based on the

ELISA results, it was not possible to clearly estimate a protein detection limit. The failure in further detection of the low-abundant proteins by the 2D-LC-MS/MS analysis is likely to be attributed to inadequate removal of the dominant proteins by the fractionation techniques and the sensitivity of the mass spectrometry instrument.

In this study, no effort was made to quantify proteins using, for example, the iTRAQ technique (Danielsen et al. 2007; Reinhardt and Lippolis, 2008). The qualitative data set generated in this study did, however, allow for label-free semiquantitative analysis, which can be used to determine the relative concentration among the proteins. To compensate for the very large dynamic range in milk, we chose to apply spectral counting. The ELISA performed on NF was compared with the 2D-LC-MS/MS spectral analysis of the 7 proteins in NF as well as in the 5 fractions. The spectral count of NF showed relatively poor correlation with the ELISA results of NF in terms of number of detected proteins and ranking of the proteins based on their spectral counts. Although the overall picture was the same for the high-abundant proteins, the large dynamic range in milk could not be accommodated by the spectral analysis, but was fully covered by the ELISA.

The fractionation techniques applied in this study can be even further improved by refinement or by combining them, or both. The supplemental tables (<http://dx.doi.org/10.3168/jds.2013-7106>), presenting the proteins detected in bovine milk, may serve as a reference for scientists and knowledge about the proteins can be used for further characterization of, for example, milk and mastitis markers and milk proteins in other species than *Bos taurus*. Knowledge about the proteins in each fraction may also be useful for scientists working on the isolation of protein fractions in bovine milk by processing equipment in the dairy industry. Peptides that were robustly observed in milk by our 2D-LC-MS/MS analysis have already been used for developing targeted selected reaction monitoring analysis for bovine milk (Bislev et al., 2012a,b), highlighting the value of thorough shotgun proteome mapping studies for further exploitation of biomarker candidates and methods. Finally, the presented milk proteome is currently used for mapping the unique complementary proteins as well as common proteins in colostrum and milk.

CONCLUSIONS

By applying a selection of fractionation techniques, the proteome coverage of bovine milk was successfully expanded. As with bovine colostrum, high-speed centrifugation proved to be the single-most-recommendable fractionation technique for detection of proteins

in the fluid phase of bovine milk. Fractionation by this technique resulted in coverage of the largest dynamic range, highest repeatability, detection of the majority of the proteins detected in NF, and added low-abundant proteins to the reported milk proteome. However, each milk fraction resulted in the detection of a unique subset of proteins and contributed with new insight on how milk proteins can be isolated. The ELISA validation supported the proteome results to a certain extent, although we were unable to estimate a clear-cut 2D-LC-MS/MS detection limit based on the ELISA findings. The results confirm that a combination of fractionation techniques is an advantage for obtaining sufficient proteome coverage of milk.

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