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Changes in the repertoire of bovine milk proteins during mammary involution

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ABSTRACT

Changes of abundance that occur in the repertoire of low abundance milk proteins after cessation of milk removal have not been characterised. Skimmed milk and whey from cows sampled at day 0 and either day 3 or day 8 after drying off were subjected to three untargeted proteomics techniques; 2-D gel electrophoresis, GeLC-MS, and dimethyl isotopic labelling of tryptic peptides. The changes observed included 45 fragments of abundant milk proteins and 36 host-defence proteins, suggesting activation of proteolysis and inflammation. The findings form a basis for adding value to dairy production.
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1. Introduction

Continued production of milk by the mammary gland in a lactating mammal requires regular removal of milk by the suckling young or by machine milking. Accumulation of milk in the mammary gland results in physiological changes that serve to shut down milk production and initiate remodelling of the gland tissue to its non-lactating state, a process known as mammary involution. In dairy cows, mammary involution occurs over a period of three to four weeks after drying off, during which time the udder gradually loses its capacity to produce milk upon resumption of milking [1]. The concentration of leukocytes in bovine milk/mammary secretion increases substantially in the first seven days after drying off [2]. In addition, increased expression of inflammation- and oxidative stress-associated genes in the mammary gland has been observed as soon as 1–2 days after drying off [3]. These data suggest that substantial physiological changes occur in the udder during the first week of involution.

Drying off is known to result in changes in the composition and functionality of the secretion retained in the udder cisterns. Transfer of blood proteins into the alveolar lumen occurs in the first few days after drying off, resulting in increased levels of serum albumin and immunoglobulins in the milk [4,5]. These changes are thought to occur through either facilitated transport [6] or loss of integrity of the tight junctions between the mammary epithelial cells [7]. Also, the relative abundance of the major milk proteins is altered during this time [8,9]. However despite these observations, changes in the physiological status of milk during involution or alterations in the repertoire of low abundance milk proteins during this time have not been well characterised to date.

Proteomics technologies have been used to characterise the repertoire of low abundance proteins in bovine milk, revealing the presence of at least 95 proteins in initial investigations [10] to as many as 2971 proteins when extreme fractionation and enrichment approaches are used [11]. Proteomics approaches provide an opportunity to investigate in unprecedented detail the changes in milk protein composition that accompany physiological responses, thereby providing insight into the functionality of milk. Such studies have revealed the presence of many proteins with useful bioactivities, particularly those associated with host-defence [12].

The physiological response to drying off described above is similar to the response of the mammary gland during inflammation of the udder (mastitis), such as occurs during intra-mammary infection. Mastitis in dairy cows is associated with a lowered rate of milk production, an increase in leukocytes in the milk, and loss of tight junction integrity [7,13]. Several proteomics studies have revealed extensive changes in the repertoire of low abundance

Abbreviations: BCA, bicinchoninic acid assay; DML, dimethyl labelling; EmPAI, exponentially modified protein abundance index; FTMS, fourier transform mass spectrometry; GeLC, gel electrophoresis-LC-MS/MS.

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milk proteins with mammary infection, including proteins with antimicrobial, pathogen-recognition and immunomodulatory activities [14,15]. We therefore hypothesize that drying off is accompanied by similar changes among the low abundance proteins, possibly presenting an alternative means of maximising yields of host-defence-associated bioactive proteins. To test this hypothesis, we used three different proteomics technologies to characterise changes in the low abundance proteins in skimmed milk and whey at day 3 and day 8 after drying off. The results reveal changes in the abundance of many proteins, including those with potentially useful bioactive properties.

2. Materials and methods

2.1. Collection of milk samples

Raw milk samples were collected from 12 pregnant pasture-fed Friesian–Holstein dairy cows at a single dairy farm, located in the Waikato region of New Zealand, during late lactation. Their ages ranged from 4 to 9 years of age. All were milked twice daily until drying off. The cows were selected based on somatic cell count less than 200,000 cells/ml in milk from each of their quarters taken from a morning milking at both one week and two weeks prior to the experiment. Average SCC was 72,000/ml (SD = 35,000). The cows were randomly split into two groups of six. The first group was sampled on day 0 (D03), the last day of milking, and day 3 after drying off (D3) and the second group sampled on day 0 (D08), the last day of milking, and day 8 after drying off (D8). Milk was obtained from each quarter and an equal volume of milk from the four quarters of the six cows in each group was pooled. The milk samples were centrifuged at 1500 × *g* for 20 min and the fat layer was removed before storage at −20 °C (skimmed milk). An aliquot of the skimmed milk was centrifuged at 100,000 × *g* for 60 min at 30 °C to pellet the casein micelles, and the clear supernatant (whey) was removed and stored at −20 °C. The protein concentration of all the samples was estimated with a Coomassie blue binding assay based on that of Bradford [16] using a commercial reagent (Bio Rad, Hercules, CA, USA). Subsamples of pooled skimmed milk and whey were lyophilised for quantitative proteomics analysis. 2-D gel electrophoresis, GeLC and quantitative proteomics were performed on pooled samples per condition as a means of screening the secretions for candidate protein responses that could be consistent among individual animals. An equal volume of skimmed milk or whey from each of the six cows within a condition was pooled. A pooled milk sample of all 12 cows on the last day of milking (so a combination of D03 and D08) will be referred to as D0. Western blot and ELISA was performed on the individual samples per cow, by mixing equal volumes of milk from each quarter.

2.2. 2-D gel electrophoresis and sample analysis

A 500 μg portion of the pooled samples from each of the conditions (D03, D3, D08, D8 for skimmed milk and whey) was subjected to 2-D gel electrophoresis (2-DE), each in duplicate, following a previously described method [10]. Samples were focused on 18 cm pI 3.5–11 NL IPG strips (Amersham, Uppsala, Sweden), separated in the second dimension using 14% (w/v) SDS-PAGE gels and then stained with colloidal Coomassie G-250. The resulting patterns of stained spots were captured using a GS-800-calibrated densitometer. Image analysis was performed using the PDQuest v8.0.1 software package (Bio Rad, Hercules, CA, USA), which produced integrated spot intensities ("volumes") for each of the selected spots. These volumes were normalised for unequal loading or staining among the 2-DE gels using the LOESS local regression model that is built into the PDQuest software package.

2.3. Protein identification

Selected proteins detected as spots on the 2-DE gels were subjected to in-gel trypsin digestion as previously described [15]. Peptides were extracted using pieces ofEmpore™ C18 membrane (Supelco, Bellefonte, PA, USA) and prepared on an MTP AnchorChip 600/384 TF target plate as described previously [17]. Peptide mass fingerprinting (PMF, based on MS data) and peptide fragment fingerprinting (PFF, based on the MS/MS data from some of the peptides located in the PMF and fragmented by LIFT-TOF) was performed on an Autoflex III TOF/TOF mass spectrometer (Bruker, Billerica, MA, USA) with FlexControl 3.0 data acquisition software (Bruker). Peptide identification was performed by MASCOT (Matrix Science, London, UK) against the NCBI non-redundant database, restricted to *Bos taurus* (release date, December, 2014). Peptide and fragment mass tolerances were set to 50 ppm for precursor ions and 0.4 Da for fragment ions, respectively. Carbamidomethylation was selected as a fixed modification, whereas oxidation on methionine was selected as a variable modification. A MOWSE score producing a probability value of less than 0.05 was considered to be a positive identification.

2.4. Gel electrophoresis-LC MS/MS (GeLC)

A 50 μg portion of the D0 as well as the D3 and D8 sample pools were resolved by 1D SDS-PAGE using 12.5% (w/v) Bis–Tris pre-cast gels (Bio Rad, Hercules, CA, USA) and then stained with colloidal Coomassie G-250. Each lane was cut into 10 slices. The resulting 60 slices were then destined, the disulphide bonds reduced with dithiothreitol, the resulting free cysteines alkylated with iodoacetamide, the modified proteins digested *in situ* with trypsin, and the resulting peptides extracted from the gel pieces as previously described [10]. A 10 μL portion of each of the 60 peptide extracts was loaded onto a C18 precolumn (Varian Microsorb 300 μm i.d., 5 μm particles, 300 Å pore size) at a flow rate of 8 μL/min. The precolumn was then connected with the analytical column (Microsorb C18, 20 cm, 75 μm i.d., 5 μm particles, 300 Å pore size), and eluted at a flow rate of 150 nL/min, with a gradient from 2% to 55% solvent B in 50 min. Solvent A was HPLC-grade H_2O (Fisher Scientific, USA) with 0.2% (v/v) formic acid, and solvent B was LC-MS-grade acetonitrile containing 0.2% (v/v) formic acid. The column outlet was directly connected by a nano-electrospray source to a Q-STAR Pulsar *i* mass spectrometer (Applied Biosystems, USA) which was programmed to acquire tandem mass spectrometry (MS/MS) traces of 1+, 2+, 3+, 4+ and 5+ ions. The MASCOT software package (Matrix Science, UK) was used to extract peak lists from the data files. The top three precursor ions were selected and dynamic exclusion was enabled for 30 s after a repeat count of 2. The peak lists from all m/z segments of each sample were combined and imported into the ProteinScape v2.1 software package (Bruker) resulting in the collection of 55366 spectra. The data was used to query the NCBI non-redundant database (release date, January, 2012) restricted to *Bos taurus* taxonomy using MASCOT. Search parameters for peptide and fragment mass tolerances were set to 100 ppm for MS and 0.6 Da for MS/MS, respectively, with allowance made for one missed tryptic cleavage. Modification of cysteine through carbamidomethylation, was selected as a fixed modification, while oxidation on methionine, deamination of asparagine and glutamine, and phosphorylation of serine and tyrosine were selected as variable modifications. A significance threshold of less than 0.05 was selected and all searches were conducted using the peptide-decoy option selected within ProteinScape. This produced an average false discovery rate of 1.4%. The exponentially modified protein abundance index (EmPAI value), calculated by the MASCOT

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