Comparative Proteomic Analysis of Changes in the Bovine Whey Proteome during the Transition from Colostrum to Milk

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ABSTRACT : Bovine whey protein expression patterns of colostrum are much different from that of milk. Moreover, bovine colostrum is an important source of protective, nutritional and developmental factors for the newborn. However, to our knowledge, no research has been performed to date using a comparative proteomic method on the changes in the bovine whey proteome during the transition from colostrum to milk. This study therefore separated whey protein of days 1, 3, 7 and 21 after calving using two dimension electrophoresis. Differentially expressed proteins at different collection times were identified using high-performance liquid chromatography in tandem with mass spectrometry (LC/MS) and validated by enzyme-linked immunosorbent assay (ELISA) in order to understand the developmental changes in the bovine whey proteome during the transition from colostrum to milk. The expression patterns of whey protein of days 1 and 3 post-partum were similar except that immunoglobulin G was down-regulated on day 3, and four proteins were found to be down-regulated on days 7 and 21 compared with day 1 after delivering, including immunoglobulin G, immunoglobulin M, albumin, and lactotransferrin, which are involved in immunity and molecule transport. The results of this study confirm the comparative proteomic method has the advantage over other methods such as ELISA and immunoassays in that it can simultaneously detect more differentially expressed proteins. In addition, the difference in composition of milk indicates a need for adjustment of the colostrum feeding regimen to ensure a protective immunological status for newborn calves. (**Key Words :** Bovine Milk Whey Protein, Colostrum, Milk, Two Dimension Electrophoresis, Mass Spectrometry)

INTRODUCTION

Bovine colostrum refers to the initial milk secreted by mammary glands during the first four days postpartum (Gopal and Gill, 2000). Bovine colostrum has a unique nutrient profile that differs substantially from milk, in which protein, fat, lactose, vitamins, and minerals are higher than milk (Kelly, 2003). In addition, it also has a distinct protein composition, which contains elevated levels of IgG, IgA and IgM, (Smolenski et al., 2007). For ruminant animals, proteins in colostrum are more valuable for neonate, as the transfer of passive immunity from the cow to the newborn only occurs through colostrum not by placenta. Nevertheless, no studies using a comparative proteomic approach on changes in the bovine whey proteome during the transition from colostrum to milk have been reported to date.

Proteomics is a relatively new field and one of the fastest-growing areas of biological research, thanks to its potential to simultaneously detect more differentially expressed proteins (Deborah Penque, 2009). Hogarth et al. (2004) utilized proteomics to analyze dairy cows with clinical mastitis, and found serotransferrin and albumin increased in concentration of whey, while α-lactalbumin and β-lactoglobulin decreased in concentration of whey. In addition, Reinhardt et al. (2008) also employed proteomics analyzing developmental changes in the milk fat globule membrane proteome during the transition from colostrum to milk. The objective of the present investigation was therefore to characterize the differentially expressed whey proteins, using 2-DE followed by LC/MS/MS, in order to understand the developmental changes in the bovine whey proteome during the transition from colostrum to milk.

MATERIALS AND METHODS

Animals and sample preparation

Fresh bovine colostrum and milk were collected from

eight primiparous healthy Chinese Holstein cows from commercial dairy herds located in northern China that were being fed a total mixed ration and milked three times daily. The colostrum samples were collected on day 1 and day 3 after calving and milk samples on day 7 and day 21 postpartum from each cow, respectively. The samples were immediately centrifuged (5,000×g, 15 min) at 4°C and the fat layer was carefully removed, making skim milk (Hogarth et al., 2004). The skim milk was then treated with sufficient acetic acid (30% v/v) to adjust the pH to 4.6 to precipitate the casein (Miera et al., 2008), yielding milk whey. The supernatant was mixed with protease inhibitors, complete Mini EDTA free (Roche Diagnostics, Mannheim, Germany) dissolved one tablet in 10 ml milk and stored at -80°C until further use. The protein content of all the samples was determined by Bradford assays using BSA (Sigma) as a standard (Seevaratnam et al., 2009).

Two dimension electrophoresis

A volume of each whey sample that would contain 300 μg of total whey protein based on the BSA results was diluted in rehydration solution (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10% (v/v) iso-propanol, 5% (v/v) glycerol, 2.5% (w/v) DTT and 2% (v/v) Pharmalytes (pH 3-10)), to reach a final volume of 350 μl mixed and then applied to each electrophoresis strip. The first-dimensional isoelectric focusing was conducted using a flat bed electrophoresis system (protein IEF CELL, BIO-RAD) in precast IPG strips (17 cm, pH 3-10NL BIO-RAD) at 20°C for 17 h to a total of about 80,000 Vh. Upon completion of the first dimension, reduction was performed with dithiothreitol (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 0.2 g DTT) at room temperature for 12 min. A second equilibration step was carried out for 12 min in similar solution, with the exception that DTT was replaced by 2.5% w/v iodoacetamide. Two steps mentioned above were done with gentle shaking. The second dimension of SDS-PAGE was carried out on 12.0% homogeneous running gels. The program was as follows: 1 W for 1 h, 2 W for 1 h, followed by 48 W, until the dye front reached the end of the gel. During this procedure the circulating water temperature was maintained at 12°C.

Gels staining and image analysis

After 2-DE procedure was performed, the gels were soaked in a fixing solution containing 40% methanol and 10% acetic acid for 30 min or overnight, then rinsed in millipore water three times for a total of 60 min. The gels were placed in a Coomassie brilliant blue G-250 solution consisting of 0.12% G-250, 10% $(NH_4)_2SO_4$, 10% H_3PO_4, 20% methanol (Candiano et al., 2004). All steps were done with gentle shaking. When protein spots became visible, the gels were scanned using Umax scanner (PowerLook

2100XL). The images captured were subjected to background subtraction and automatically matching using a ImageMaster 2-DE platinum 6.0 software package (Bio-Rad).

In-gel digestion

Differentially expressed protein spots from whey in day 1, day 3, day 7 and day 21 after calving were excised as about 1-3 mm^2 pieces using a scalpel, rinsed in milipore water, and destained with a solution containing 50% (v/v) acetonitrile and 50 mM ammonium bicarbonate (pH 7.8) for three times (a total of 3 h). The gel pieces were then dried under vacuum and rehydrated with modified trypsin (Promega, cat. # V5111) in 50 mM ammonium bicarbonate (pH 7.8). After 20 min, 20 μl of 50 mM ammonium bicarbonate (pH 7.8) was added and the mixture was incubated overnight at 37°C while being continuously shaked. Then the peptides were extracted, first with 20 μl of 25 mM ammonium bicarbonate (pH 7.8), twice more with 20 μl of a solution containing 0.25% (v/v) TFA and 50% (v/v) acetonitrile, finally with 20-100 μl acetonitrile. The collected extracts were dried and resuspended in 25 μl 50% (v/v) acetonitrile/5% (v/v) formic acid for LC ion trap MS analysis.

LC/MS/MS

The peptide mass was assayed on an ion trap mass spectrometer (LCQ Deca XP, Thermo Finnigan) equipped with a Surveyor HPLC system (Thermo). The peptide mixture was diluted to approximate 0.07-0.1 mg/ml and 20 μl of sample was injected onto a BioBasic C-18 column (150×0.18 mm, Thermo) with a flow rate of 120 μl/min. The column temperature was maintained at 30°C. Liquid chromatography column was equilibriumed using millipore water/0.1% formic acid and then was carried out gradient elution by acetonitrile/0.1% formic acid at flow rate of 200 μl/min. HPLC columns were connected to the electrospray interface of the mass spectrometer without splitting. The LCQ mass spectrometer was operated with the capillary temperature at 250°C, sheath gas at 80 (arbitrary units) and the auxiliary gas at 20 (arbitrary units). The electrospray voltage was set to 4.5 kV, the capillary voltage at 22 V, and the tube lens offset at -6 V. In the full scan mode, ions were collected in three micro-scanning with a maximum ion injection time of 200 ms, MS spectra for all samples were measured with an overall mass/charge (m/z) range of 400 to 2,000, MS/MS was carried out in data-dependent mode. Peptides were characterized using SEQUEST software (Bioworks 2.0, Thermo Finnigan), which used the tandem mass spectra of peptide ions to search against the publicly available NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov). The protein identification

criteria were based on Delta CN ($\geq$0.1) and Xcorr (one charge$\geq$1.9, two charges$\geq$2.2, three charges$\geq$3.75).

Database searching and protein identification

ImageMaster 2-DE platinum 6.0 software package (Bio-Rad) was used to compare positions and relative intensities of individual spots. Spots of protein were detected to be differentially expressed with changes in the stain density and intensity of twofold or more. Protein spots detected as differential expression protein were identified by peptide sequencing with the search programs of SEQUEST. The searching criteria for acceptable matches was better than 95% confidence and peptides sequence matching score greater than 30, which was considered as the unambiguous protein identification.

Determination of lactotransferrin by sandwich ELISA

Quantitative determination of lactotransferrin in colostrum and in milk whey was performed using the bovine lactotransferrin ELISA Quantitation Kit (Bethyl Laboratories) according to the manufacturer's instructions. The milk whey from day 1, day 3, day 7 and day 21 after delivery were diluted to 1:6,000, 1:6,000, 1:2,000 and

1:2,000, respectively, and a standard curve was generated. The final absorbance of the samples was measured at 450 nm, using an ELISA plate-reader (Infinite F 200; Tecan, Männedorf, Switzerland). The results are summarized in Figure 2.

Statistical analysis

The difference of lactotransferrin concentration in whey was analyzed by ANOVA model of SAS system (version 9.0, SAS Institute, Inc., Cary, NC) that included day in milk and cow. Significance was declared at $p < 0.05$.

RESULTS

Bovine milk whey proteins were separated by 2-DE using 17 cm strips with non-linear IPG in pH range of 3-10 in the first dimension. The expression patterns of bovine whey proteome during the transition from colostrum to milk showed apparently different. Marked protein spots in day 1 and day 3 after calving (Figure 1A and B) were up-regulated when compared with day 7 and day 21 after delivery (Figure 1C and D). Milk whey protein expression patterns of day 1 and day 3 after calving were similar
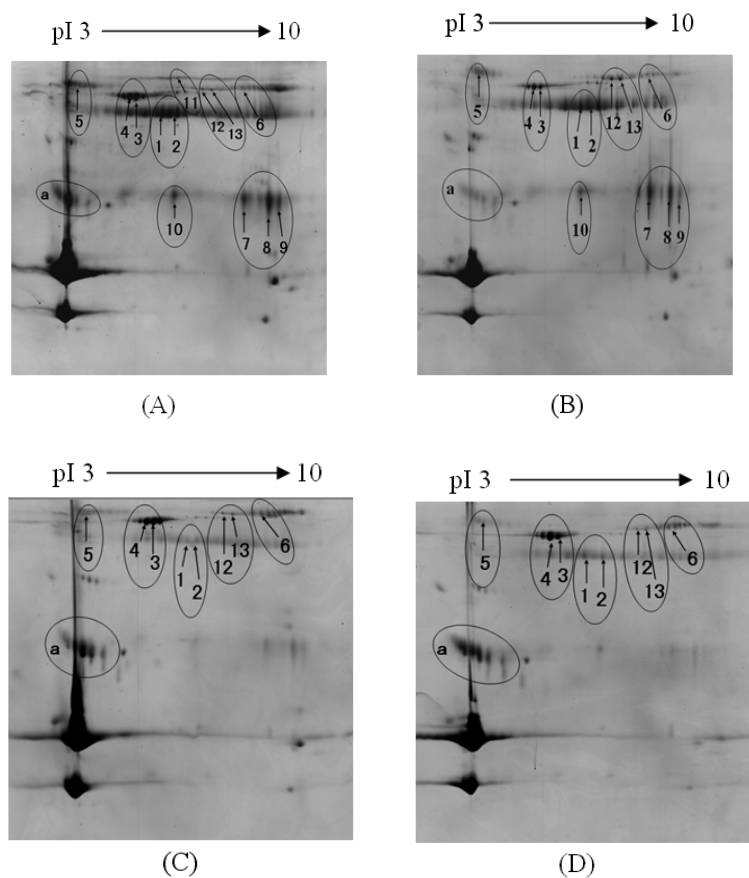

Figure 1. 2-DE colloidal Coomassie stained gel of bovine milk whey proteins separated using pH 3-10 IPG strips and 12% SDS-PAGE. Numbered spots were identified using LC/MS/MS and are listed in Table 1. (A) show 2-DE pattern of d 1 after calving, (B) show 2-DE pattern of d 3 after calving, (C) show 2-DE pattern of d 7 after calving, and (D) show 2-DE pattern of d 21 after calving.

except IgG down-regulated in day 3 and those of day 7 and day 21 after calving were identical. Representative gels from the different collection time were shown in Figure 1A, B, C and D. The identification results of the numbered protein spots by LC/MS/MS were shown in Table 1, mainly containing IgG (spot 1, 2), albumin (spot 3, 4), IgM (spot 5), lactotransferrin (spot 6) anti-testosterone antibody (spot 7, 8, 9) and VI1a protein (spot 10). Moreover, the 2-DE gel patterns showed the presence of several proteins as multiple spots tandem (spots 7, 8, 9) and the presence of such

clusters of spots with similar Mr but different pI values may be indicative of posttranslational modifications, such as phosphorylation or glycosylation.

To validate the differentially expressed protein further, we performed sandwich ELISA on cow samples using antibody to lactotransferrin. The results demonstrated that there was no difference in lactotransferrin concentration between day 1 and day 3. On day 7, the lactotransferrin concentration declined greatly, and down-regulated 3.60- and 3.06-fold compared with day 1 and day 3. Compared

Table 1. Differentially expressed proteins in the bovine whey between colostrum and milk

Spot	Protein name	Accession no.	Sequence	Protein score
1, 2	IgG heavy chain precursor	gi:108750	IQHQDWTGGKEFK CKVHNEGLPAPIVR EPQVYVLAPPQEELSK YGTTPPQLDADSSYFLYSK FSWFVDDVEVNTATTKPR	230
3, 4	Albumin	gi:30794280	DAFLGSFLYEYSR KVPQVSTPTLVEVSR HLVDEPQNLIK RHPEYAVSVLLR SLHTLFGDELCK VHKECCHGDLLECADDRADLAK LCVLHEKTPVSEKVTK LFTFHADICTLPDTEK LSQKFPKAEFVEVTK LKECCDKPLLEK HLVDEPQNLIKQNCDQFEK	320
5	IgM heavy chain constant region	gi:2232299	IHGFDLAAINLQR VGPLLACLLGR AEVLSPVVSVFVPPR YAASSYLSLTSSDWK	210
6	Lactotransferrin	gi:506	LRPVAAEIYGTK GSNFQLDQLQGR FFSASCVPCIDR NLLFNDNTECLAK DLLFKDSALGFLR SPPSVTLFPPSTEELNGNK	310
7, 8, 9	Anti-testosterone antibody	gi:432627	GSYSCEVTHEGSTVTK SPPSVTLFPPSTEELNGNK SKGSYSCEVTHEGSTVTK YAASSYLSLTSSDWK	230
10	VI1a protein	gi:86438072	GSYSCEVTHDGSTVTK SKGSYSCEVTHDGSTVTK	170
11	IgG heavy chain precursor	gi:108750	FSWFVDDVEVNTATTKPR VHNEGLPAPIVR	240
12, 13	Polymeric immunoglobulin receptor	gi:32189338	SSVTFDCSLGPEVANVPK GGSVTVSCPYNPK TVTINCPFTR NGGACNVVINTLGK CTTLISSEGYVSDDYVGR WISTVELK TIQDCFQVVDSTGYVSNSYK CTTLISSEGYVSDDYVGR IVSVPKDNGVFSVHITSLR	350

with day 7, there was no significant decrease on day 21 (Figure 2A). The lactotransferrin concentrations determined using the sandwich ELISA procedure were consistent with those determined using LC/MS/MS (Figure 2B).

DISCUSSION

In this study, significant differences in 2-DE patterns of the low-abundance bovine milk proteins were observed when we analyzed samples of colostrums and milk by 2-DE in combination with mass spectrometry. Our results show that 4 low- abundance proteins including IgG, IgM, albumin and lactotransferrin are greater in bovine colostrum than that in milk. Of these 4 differentially expressed proteins, the concentration of IgG decreases fastest in the first 3 day after calving, which is in agreement with the result of Yamada et al. (2002). The other 3 proteins low dramatically at day 7 postpartum.

Several proteins were down-regulated in the whey at day 7 and day 21 compared with day 1 and day 3 after calving, among which was albumin. It has a Mr of 69.2 kDa and pI of 5.82 and is known to be a component of milk at low concentration (Auldist and Hubble, 1998). The increase of albumin in colostrum whey presumably results from increased leakage from the circulating blood. Albumin plays a critical role in the transport of small molecule. For instance, it is known that albumin is the transport vehicle for free fatty acids released from adipocytes (Evans, 2002). In addition, the albumin level is one of several clinical parameters of the status of general health. There is a high correlation found between low albumin levels and the incidence of morbidity and mortality in hospitalized patients (Doweiko and Nompleggi, 1991).

Theoretically, the majority of proteins of bovine milk, such as casein, α-lactoalbumin and β-lactoglobulin are synthesized *de novo* in the mammary gland from amino acids derived from the blood. But the levels of IgG and IgM are similar to, if not identical with, the proteins of blood serum, which suggests that the IgG and IgM in bovine milk whey are largely derived from blood serum, with transport across mammary alveolar cells mediated by a active transfer mechanism (Jones et al., 2004). French researchers (Levieux and Ollier, 1999) reported that active concentration of IgG in mammary gland secretions begins to rise several weeks prior to parturition and ceases at or near calving. The principal immunoglobulin in bovine colostrum is IgG (Mehra et al., 2006). IgG accounts for approximately 85% to 90% of the immunoglobulins in colostral whey. Ingestion and absorption of IgG via colostrum are very important to the survival and health of the neonates. Even though gut absorption of immunoglobulins stops by 36 h postpartum, they still can function partially in the gut lumen to control bacterial populations during preweaning (Godden, 2008). Anti-testosterone antibody protein (spots 7, 8, 9) and Ⅶ1a protein (spots 10) are also present higher level in colostrum whey than that in the milk whey, which are IgG light chain and can associate with any of IgG heavy chains to form a tetramer which has immune function.

Lactotransferrin (LTF, Mr 77.7 kDa, pI 6.8) is one of the differentially expressed proteins. Lactotransferrin, an iron binding glycoprotein, plays a crucial role in the transport of iron from sites of absorption and storage to iron-requiring cells. It is synthesized mainly in the liver and located in blood (Beutler et al., 2000). Lactotransferrin is thought to come from blood and its concentration is higher in bovine colostum than in milk. It promotes the growth of fibroblasts and intestinal epithelial cells and plays a role in gut
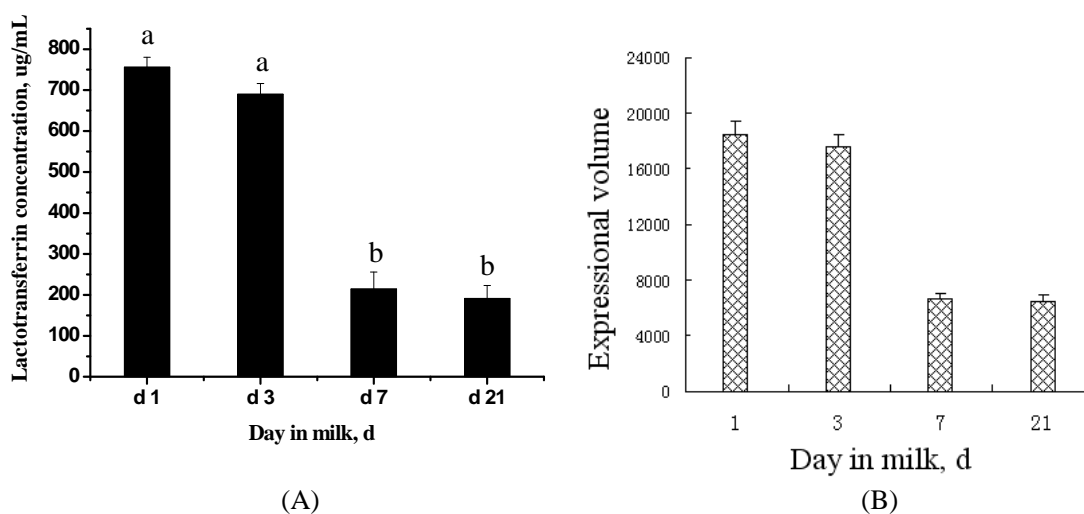

Figure 2. (A) Changes of lactotransferrin concentration in bovine whey. [a,b]Different letters indicate a significant difference. (B) Densitometric analysis of spot 6 (lactotransferrin) on the 2-DE maps.

immunity (Rawal et al., 2008). Recently, Lactotransferrin and its receptor have been tested to diminish tumour cells by employing the receptor to attract antibodies. As studies continue and additional information is collected on LTF, possibly life protecting roles in the host defence systems are being discovered through the molecular biology and protein biochemistry investigations (Macedo and de Sousa, 2008). Lactotransferrin has been reported to protect lymphohemopoietic and hepatic cells from Fas-mediated cell death (Lesnikov et al., 2001; Lesnikov et al., 2004). Furthermore, Lactotransferrin is a negative acute-phase protein that is down-regulated in inflammatory conditions such as diabetes (Koc et al., 2003).

An attempt to remove caseins, which comprises approximately 80% of the total protein content, by precipitation pretreatment with acetic acid, is only partially successful, as several spots are considered as casein with Mr in the region of 25 kDa and pI of 4.3-4.9 compared with the corresponding milk whey protein patterns performed by Hogarth et al. (2004), which indicates that the process had not removed all the casein proteins present (Figure 1 area a). Therefore, there is still the possibility that minor proteins of similar pI and Mr to casein, are also present in the casein precipitate, but cannot be seen on the gel map owing to the major abundance of the casein spots. In addition, utilization of staining methods of super sensitivity than colloidal Coomassie, such as EYPRO-Ruby or silver would then allow detection of minor abundance proteins without interference from the major milk proteins, which will overcome the problem of low rate of the spots cut out of the gels being successfully identified. Furthermore, the success rate for protein identification is likely to be limited by the incompleteness of bovine protein databases. Because there is no B. taurus specific taxonomy in the database used in this study.

Bovine colostrum has much higher amounts of immunoglobulins, growth factors, cytokines, and nucleosides than are found in milk (Kelly, 2003). Colostrum provides the calf with antibodies and nutrition that will aid in the fortification of the immune system. Management and feeding of high-quality colostrum can reduce calf mortality, strengthen immunity, and increase animal life span (Quigley and Drewry, 1998).

A very unique advantage of the method of 2-DE in combination with mass spectrometry over other methods such as ELISA and immunoassays is that it can simultaneously detect more differentially expressed proteins (O'Donnell et al., 2004). Furthermore, the observation from the sandwich ELISA result of lactotransferrin has confirmed that the method of 2-DE combined with LC/MS/MS is capable of identifying differences of the whey proteins in each lactation state.

CONCLUSIONS

The results of the present study confirm the comparative proteomic method has the advantage over other methods such as ELISA and immunoassays that it can simultaneously detect more differentially expressed proteins. In addition, the difference in composition of milk during the transition from colostrum to milk indicates a need for adjustment of the colostrum feeding regimen to ensure a protective immunological status for newborn calves.

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