Vol.54, n. 4: pp. 761-768, July-August 2011
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Proteomic Analysis of Whey from Bovine Colostrum and Mature Milk

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ABSTRACT

The aim of this study was to standardize a methodology to obtain two-dimensional (2D) maps of whey proteins from bovine colostrum and mature milk, using two-dimensional electrophoresis, in order to identify the minor proteins by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS). A total of 38 proteins were identified, 20 spots in the colostrum whey and 18 in the mature milk whey; 5 of them were identified for the first time.

Key words: bovine whey proteins, colostrum proteins, low-abundance proteins, two-dimensional electrophoresis, mass spectrometry, matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS)

INTRODUCTION

Milk is essential for the growth and development of newborn mammals. It has bioactive properties that facilitate the transition from the intra-uterine to the extra-uterine condition, stimulating the development of the brain, digestive tract, and immune system (Grosvenor et al., 2007). According to the stage of lactation, the milk is classified as colostrum, produced up to 72 h after the birth on average; or mature milk, secreted after 72 h from the birth (Sgarbieri, 2004).

Mature milk and colostrum proteins can be divided into two main groups: caseins and whey proteins, which represent 80 and 20% of the total proteins from in milk, respectively. Bovine whey in particular contains more than 200 different proteins, with β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), and immunoglobulins as the major constituents (Sgarbieri, 2004). Knowledge of the identity and function of the minor protein constituents of bovine milk whey is still incomplete, although many of these proteins may have biological properties that contribute to the health benefits of whey (Fong et al., 2008; Rusu et al., 2009). Bulk bovine whey has been used in the production of nutraceuticals (Rusu et al., 2009) and as raw material for several other products (Revillion et al., 2003; Mariotti et al., 2008).

The presence of minor proteins have been described in both bovine milk and colostrum by 2-D-gel-based proteomic methods (O'Donnell, Holland, Deeth, and Alewood, 2004; Yamada et al., 2002). Among them, gelsolin and chitinase 3-like 1 were described only in bovine colostrum

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(Yamada et al., 2002). Gelsolin is necessary for rapid motile responses in cell types involved in stress responses, inflammation and wound healing. Chitinase-3 like 1, known as cartilage glycoprotein-39 in humans, is detected in chondrocytes and synovial cells, as well as lung, heart, infant brain and placenta.

Approaches such as immunoabsorption (Murakami et al., 1998; Palmer et al., 2006; Yamada et al., 2002), solution isoelectric focusing (IEF) (Zuo and Speicher, 2002), affinity tagging (Holland et al., 2006; Conte-Junior et al., 2006), and semi-coupled anion- and cation-exchange chromatography (Fong et al., 2008) have been used to remove the dominant proteins and increase the relative abundance of the minor proteins, to aid proteomic analysis of complex samples such as bovine milk.

Proteomic methodology has been used in the study and characterization of proteins from milk and dairy products (Yamada et al., 2002; Manso et al., 2005; Fong et al., 2008). However, the large amount of immunoglobulins in bovine whey is particularly troublesome for the proteomic analysis of the minor proteins (Yamada et al., 2002). Immunoglobulins are located in a region of the 2D-gel where most of the minor proteins, such as lactoferrin, transferrin, plasmin, lipoprotein lipase, alkaline phosphatase, and others yet to be identified are found (Fong et al., 2008).

Several methods can be used to identify new proteins in complex samples. Among them, the gel-based method has been the method of choice for decades, and is still widely practiced. Two-dimensional electrophoresis method is commonly employed in quantitative proteomics (Wu et al., 2006). Mass spectrometry (MS) has increasingly become the method of choice for analysis of complex protein samples. MALDI TOF/TOF MS is robust, sensitive and relatively inexpensive, and so have produced much of the proteomics data reported in the literature (Aebersold and Mann, 2003).

The aim of this study was to standardize a methodology to obtain two-dimensional (2D) maps of whey proteins from bovine colostrum and mature milk, using two-dimensional electrophoresis in order to identify minor proteins by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS).

MATERIAL AND METHODS

Collection of bovine milk and preparation of whey

The samples of colostrum (collected up to 48 h post-partum) and mature milk (collected after 72 h post-partum) were from the females derived from crosses between Holstein and Jersey breeds (n=10). The cows were fed only with grass. The samples were acidified with 1N acetic acid, and caseins were collected by centrifugation (16,500*g* for 30 min at 4ºC). The crude whey (10 mL) was dialyzed against 2 L of 10mM tris-HCl, pH 6.7, for 48 h using a dialysis membrane with a molecular weight cut off of 7 kDa.

Removal of major whey proteins

To remove the major proteins from the colostrum and mature milk whey, two procedures were tested: removal of Bovine Serum Albumin (BSA) and IgG using the Albumin and IgG removal kit (GE Healthcare Life Sciences, UK); and removal of immunoglobulins with the Vivaspin 500 ultrafiltration cartridge (GE Healthcare Life Sciences, UK) with a membrane of molecular-weigh cut-off of 100 kDa, according to the manufacturer's instructions.

Removal of interfering substances

For the removal of low-molecular-weight interfering substances two different methods were used: the Clean Up Kit, used according to the manufacturer's instructions (GE Healthcare Life Sciences, UK), and the precipitation with 10% of trichloroacetic acid (TCA). The TCA-treated samples were incubated overnight at 4°C and the precipitated proteins were collected by centrifugation at 10,000*g* for 30 min at 4°C. The pellets were re-suspended in 90% acetone and the suspensions were incubated at 4°C for 15 min and the proteins were collected by centrifugation (10,000*g* for 30 min at 4°C). The supernatant was discarded and the pellet was dissolved in rehydration buffer (Golinelli et al., 2008).

Protein measurement

Protein concentration was estimated by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Treated samples were analyzed by 2D-PAGE on a Multiphor II system using an immobilized pH gradient (IPG) strip with an immobilized linear gradient of pH (4 to 7) in the first dimension, followed by separation on a polyacrylamide gradient gel (12 to 14%) with sodium dodecyl sulfate. The molecular weight (M_r) and isoelectric point (pI) of each protein spot were calculated using protein standards (Bio-Rad). Proteins were stained with colloidal Coomassie Brilliant Blue G-250. Two-dimensional gels were analyzed by Topspot 2.0 software (Algorithmus, Prehm, Berlin), previously used by Müller et al. (1996).

Treatment of protein spots and identification by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS)

Protein spots were excised from the 2D-PAGE and digested with trypsin (20 ng/μL). The resulting peptides were concentrated and desalted using Zip Tip C18 (Millipore, Bedford, MA, USA), and mixed with alpha-cyano-4-hydroxycinnamic acid to be analyzed by MALDI TOF/TOF MS (Applied Biosystems 4700 Proteomics Analyzer). Selected peptides were fragmented in the second dimension, and the proteins were identified by mass searches in the Swiss Prot Database (Swiss Institute of Bioinformatics) using the MS/MS ion search software Mascot (Matrix Science - http://mas8.lnls.br/mascot).

RESULTS AND DISCUSSION

Removal of major whey proteins

Treatment with the Albumin and IgG removal kit was ineffective in removing IgG and BSA from the bovine colostrum whey, as judged by 2D-PAGE (Fig. 1). Immunoglobulin G light and heavy chains, 25 and 50 kDa, respectively, were still present in the treated sample (circled in Fig. 1). BSA (66.2 kDa) was also present, but it did not interfere with the observation of minor proteins in the 2D-gels, probably because of its low abundance compared with other, major whey proteins. The albumin and IgG removal kit contains agarose-immobilized anti-IgG and serum albumin antibodies against human proteins. The failure of these antibodies to capture BSA and IgGs present in bovine milk whey could be ascribed to a lack of cross-reactivity of the antibodies to the bovine proteins.

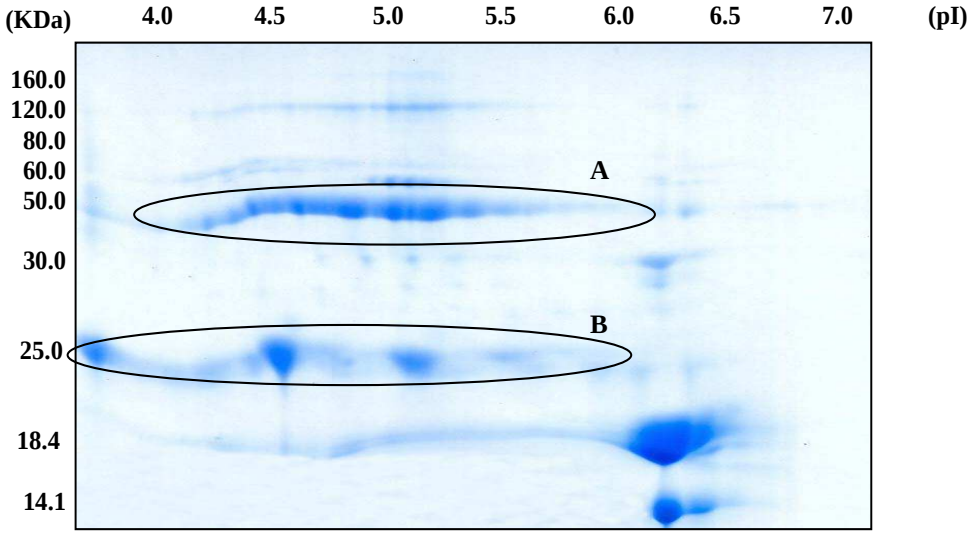

Figure 1 - Two-dimensional electrophoresis of bovine colostrum whey proteins (1mg) after dialysis and treatment with the Albumin and IgG Removal Kit. Major proteins with ca 50 kDa (A) and 25 kDa (B). The gel was stained with colloidal Coomassie Brilliant Blue G-250.

As expected, the treatment of colostrum whey with Vivaspin 500 removed most of the IgG and IgA (Fig. 2), because the regions corresponding to proteins of molecular weights of 25 and 50 kDa, marked with circles, were less burdened (Fig. 1 and 2). Several spots, possibly of minor proteins that had been masked by the presence of immunoglobulin chains, became well resolved. On the other hand, spots containing α-lactalbumin (14.1 kDa), β-lactoglobulin (18.4 kDa), and other major proteins remained on the gel (bottom left in Fig. 2), but they did not interfere with the analysis of minor proteins (Golinelli, 2009). Furthermore, a good correlation was observed between the present data (Mr and pI) and the data from other studies (Yamada et al., 2002; Fong et al., 2008), indicating that no degradation and/or artifacts were introduced during the removal method and 2D-PAGE.

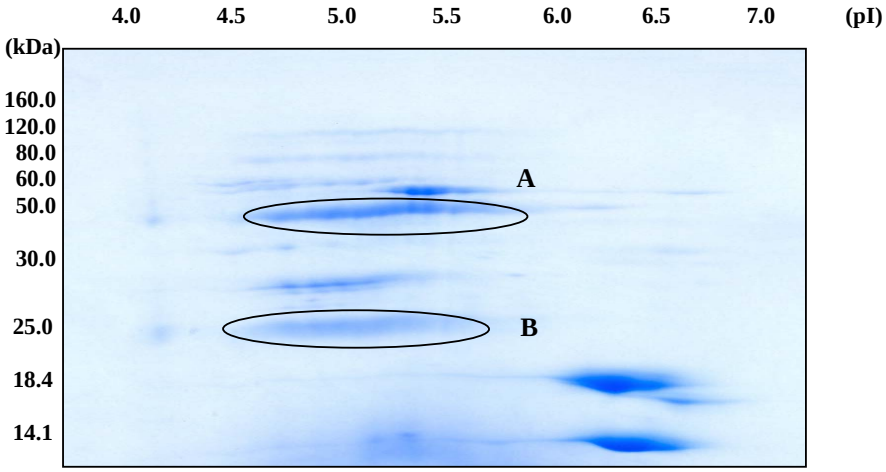

Figure 2 - Two-dimensional electrophoresis of bovine colostrum whey proteins (1mg) after dialysis and treatment with Vivaspin 500 (100 kDa). Remaining major proteins with *ca* 50 kDa (A) and 25 kDa (B). The gel was stained with colloidal Coomassie Brilliant Blue G-250.

The bovine colostrum and mature milk whey contain salts and other substances that also can interfere in the resolution of protein spots in 2D electrophoresis. Initially, the colostrum whey was dialyzed and treated with the clean-up kit, but the treatment by this procedure did not work to prepare the sample adequately for analysis by 2-DE. However, when the samples were dialyzed and treated by precipitation with TCA (10% w/v), the two-dimensional map was produced with better resolution and clearest background compared with clean-up kit treatment (data not shown).

Two-dimensional electrophoresis of proteins from colostrum and bovine mature milk whey

Figures 3 and 4 showed gels made in triplicate from whey free from major proteins and interfering substances obtained from the colostrum and mature milk samples, respectively. Whey proteins from colostrum and mature milk were found spread around the 2D-PAGE, but most of them were localized in the gel region, corresponding to a pI range from 4.5 to 5.5, as previously reported by Fong et al. (2008). Although not all the major proteins were eliminated by the procedure, the pattern obtained was clearly enriched in minor proteins, which increased in relative abundance when major proteins were removed. Notably, no great differences were observed among the patterns of protein spots from each set of gel replicates (data not shown). However, comparison of the 2D-PAGE map using the spot graphs routine of Topspot 2.0 software revealed quantitative and qualitative differences between spots from the different lactation phases. During the first week of lactation, colostrum, proteomic map was more complex. There was more diversity of proteins or their isoforms (Fig. 3 and 4). The concentrations of some low-abundance proteins were higher in the bovine colostrum than in mature milk whey, in agreement with the results of Yamada et al. (2002), which used immunoabsorption to remove

the major proteins and increase the relative abundance of minor proteins, and in accordance with the expected variation in the major protein composition of the whey with nutrition, season, disease state, and stage of lactation (Feagan, 1979; Heck et al., 2009).

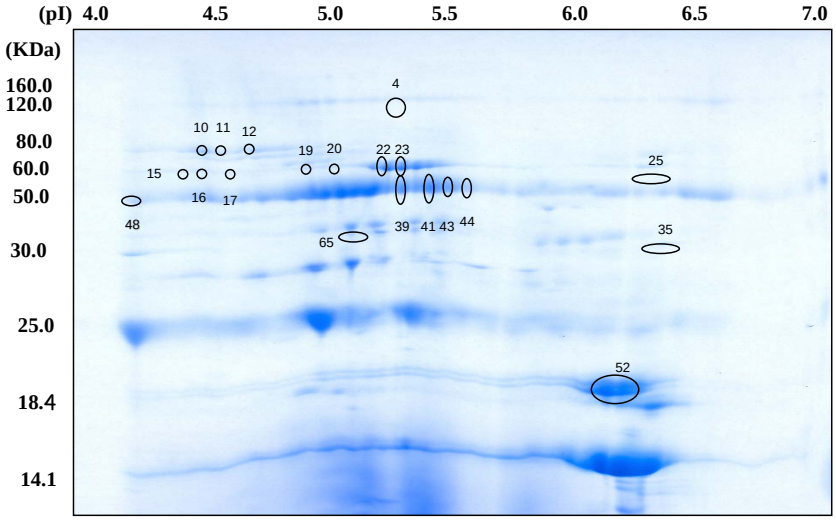

Figure 3 - Two-dimensional electrophoresis pattern of bovine colostrum whey proteins (2 mg) after dialysis, treated with Vivaspin 500 (100kDa) and precipitated by 10% TCA. Samples were subjected to a horizontal 2D system (first dimension, immobilized pH linear gradient, pH 4-7; second dimension, 12-14% SDS-PAGE). Proteins were observed by colloidal Coomassie Brilliant Blue G-250 staining. The indicated proteins, spots marked with different numbers, were identified by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS) after 2D-PAGE.

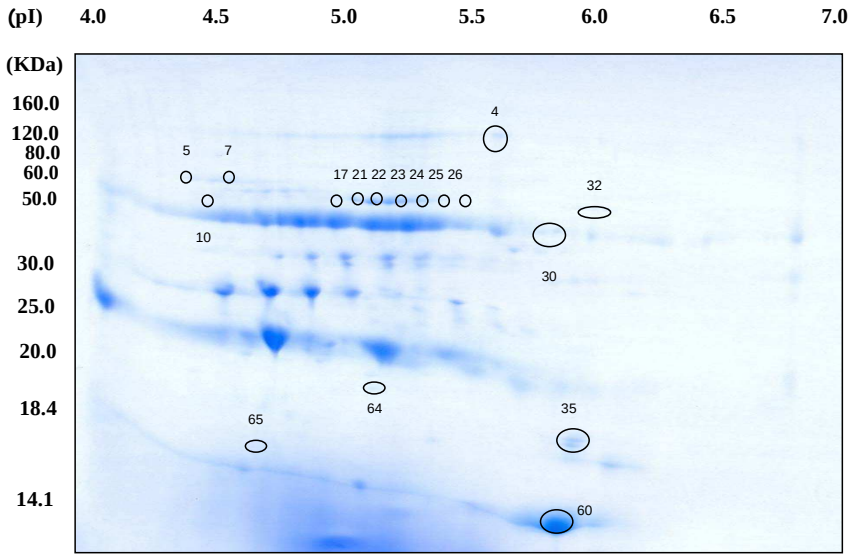

Figure 4 - Two-dimensional electrophoresis pattern of bovine mature milk whey proteins (4 mg) after dialysis, treated with Vivaspin 500 (100kDa) and precipitated by 10% TCA. Samples were subjected to a horizontal 2D system (first dimension, immobilized pH linear gradient, pH 4-7; second dimension, 12-14% SDS-PAGE). Proteins were observed by colloidal Coomassie Brilliant Blue G-250 staining. The indicated proteins, spots marked with different numbers, were identified by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS) after 2D-PAGE.

Identification of bovine whey minor proteins by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS)

Using the procedure described above, 159 spots were detected in bovine colostrum whey fractionated by 2D-PAGE (Fig. 3), 20 of them were identified with high reliability by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS) (circled in 3). Similarly, the analysis of 2D-PAGE of bovine mature milk whey proteins (Fig. 4) revealed the presence of 154 spots, 17 of them were also identified with high reliability by MALDI TOF/TOF MS (enclosed by the circles). Some of the identified proteins are reported for the first time in bovine whey in this study (Table 1). However, a number of them have been observed previously in both bovine milk and colostrum whey by 2D-PAGE-based proteomic methods (Yamada et al., 2002; O'Donnell et al., 2004; Fong et al., 2008). Alpha-1β-glycoprotein (spot #25 in Fig. 3 and spot #32 in Fig. 4) and Zinc-alpha-2-glycoprotein (spot #35 in Fig. 3) in the bovine mature milk whey were also identified by Fong et al (2008), in accordance with the present study. Nevertheless, the Alpha-1β-glycoprotein was found in the samples of colostrum and mature milk whey, whereas the Zinc-alpha-2-glycoprotein was recognized only in the bovine colostrum whey.

Among the proteins identified for the first time in the colostrums, IGHG1 protein, was uncharacterized protein C1 or f158, and VI1a protein (Table 1). The uncharacterized protein C1 or f158 observed in this study (spot #48 in Table 1) was found only in colostrum. Further characterization would be needed to identify this protein. On the other hand, among the 17 identified proteins in mature milk whey, cAMP-responsive element-binding protein-like 2 and vitamin D-binding protein had not been found previously (Table 1).

Table 1 - Low-abundance proteins of whey from bovine colostrum and mature milk identified by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry

Spot	Protein Name	Accession Number	Protein Score C.I.%	Protein Score	Function
C39	IGHG1 protein	A6QL91_BOVIN	99.99	85	Function not found
C43	IGHG1 protein	A6QL91_BOVIN	100	181	Function not found
C44	IGHG1 protein	A6QL91_BOVIN	100	224	Function not found
C48	Uncharacterized protein C1orf158	CA158_HUMAN	83.13	56	Function not found
C65	VI1a protein	Q2KIF5_BOVIN	99.97	82	Function not found
M4	cAMP-responsive element-binding protein-like 2	CRBL2_BOVIN	85.14	49	May play a regulatory role in the cell cycle
M30	Vitamin D-binding protein	VTDB_BOVIN	72.65	54	Multifunctional protein found in plasma, ascitic fluid, cerebrospinal fluid, and urine and on the surface of many cell types. In plasma, it carries the vitamin D sterols and prevents polymerization of actin by binding its monomers

These proteins were observed for the first time in bovine colostrum and mature milk
C39, C43, C44, C48 and C65 are spots from colostrum
M4 and M30 are spots from mature milk

Yamada et al. (2002) found changes in the composition of minor milk whey proteins during the lactation, which was related to their functions. For example, α1-acid glycoprotein, which is found in high concentrations in colostrum immediately after parturition, dramatically decreases in absolute and relative amounts during the early stages of lactation (Ceciliani et al., 2005). In contrast, α-lactoglobulin and β-lactoglobulin are relatively stable throughout the lactation period (Levieux et al., 1999). Determination of mammary-gland protein expression should allow

some differentiation between those proteins expressed and secreted by mammary tissue, and those derived adventitiously from blood or somatic cells. It may be that the proteins that are manifest in whey in low copy numbers, in fact represent the somatic (or even epidermal) cell proteome rather than the mammary-expressed proteome (Fong et al., 2008). The origin and role of these minor proteins occurring at such low levels in milk remain to be determined, but are challenging. The completion of the identification of the proteome constituents of the colostrum and mature milk whey may reveal new biofunctional properties of the milk that can stimulate the development of the newborn, such as the vitamin D-binding protein in the whey of mature milk (spot #30 in Table 1).

CONCLUSIONS

A consistent technique was developed for the removal of major whey proteins and the subsequent analysis of minor whey proteins by 2D-PAGE. Ultrafiltration of samples from bovine colostrum and mature milk whey with Vivaspin 500 eliminated mainly imunoglobulins and promoted and consequently increased the number of minor proteins present in the sample. The other major whey proteins, which remained in the sample, did not interfere with the observation of the minor proteins in 2D-PAGE because they were placed in different gel regions.

The study for the first time reported the IGHG1 protein, uncharacterized protein C1 or f158 and Vl1a protein in bovine colostrum whey. Furthermore, cAMP-responsive element-binding protein-like 2 and vitamin D-binding protein were found for the first time in the bovine mature milk whey. The identification of all the proteins found in 2D maps derived from the colostrum and mature milk whey could reveal new biofunctional properties in the milk.

ACKNOWLEDGEMENTS

To FAPERJ for a master's degree fellowship granted to the student Luciana Pacheco Golinelli.

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Received: April 05, 2010;
Revised: September 14, 2010;
Accepted: May 17, 2011.