Identification of Bovine Pregnancy-Specific Whey Proteins using Two-Dimensional Gel Electrophoresis

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ABSTRACT

The early diagnosis of bovine pregnancy is an essential component of successful reproductive planning on farms, because lack of bovine pregnancy over the long term results in reproductive failure and low milk yield-the latter of which is a special concern on dairy farms. This study was designed to identify early pregnancy-specific whey proteins in bovine, by comparing milk samples collected from cattle during pregnancy (Days 30 and 50) and from non-pregnant cattle. In this study, differentially expressed proteins in five pregnant and five non-pregnant Holstein dairy cattle were investigated and compared, using proteomics analysis. The first dimension was applied to a pH 3.0~10.0 strip, by loading a 2-mg milk protein sample. After the second-dimension separation was performed, the gels were stained with colloidal Coomassie brilliant blue. The stained gels were scanned and the images were analyzed, to detect variations in protein spots between non-pregnant and pregnant cattle milk protein spots, using ImageMaster; this was followed by analysis with MALDI TOF-MS. Analysis of the 2-DE gel image resulted in a total of approximately 500~600 protein spots, of which 12 spots were differentially expressed, six spots were up-regulated, and four spots were down-regulated; two spots were identified as pregnancy-specific proteins. These proteins were identified as lactoferrin, NADH dehydrogenase subunit 2, albumin, serum albumin precursor and transferrin. Our results via 2-D PAGE analysis revealed composite profiles of several milk proteins related to early bovine pregnancy, implying the possible use of these milk proteins in the early detection of bovine pregnancy.

(Key words : Dairy cattle, Whey proteins, 2-D gel electrophoresis, Mass spectrometry)

INTRODUCTION

Milk secretion is a defining characteristic of mammals. Milk is the only source of nourishment for the newborn mammal, and it serves as an important means of transferring pathogenic immunity from the mother to the offspring. Mammalian milk contains various soluble and indiscernible components that provide essential nutrients and immunological proteins to the newborn animal. The major proteins found in milk include immunoglobulins, alpha lactalbumin, casoxins, glycoproteins, and lysozyme (Lonnerdal, 1985); bovine milk, in particular, contains four major milk proteins (α_{S1}-, α_{S2}-, β-, and κ-caseins) (Farrell *et al.*, 2004; Smolenski *et al.*, 2007). The major whey proteins are β-lactoglobulin and α-lactalbumin, and there are various kinds of minor proteins such as lactoferrin and lactoperoxidase (Bertram, 2008).

Because of the economic relevance of animal production, special attention is being paid to early pregnancy detection in cattle, with investigations of proteins in both the tissues and cells of pregnant animals (Talamo *et al.*, 2003; Bouley *et al.*, 2004; Jalili *et al.*, 2004) or those animals' biological fluids (Wait *et al.*, 2002; Talamo *et al.*, 2003; Lippolis *et al.*, 2005). In early pregnancy in mammals, special signals are produced during contact of the fertilized embryo with the uterus; they consist mainly of hormones and proteins that change the maternal physiology, to maintain the pregnancy and promote developments in the fetoplacental unit that are required for a successful pregnancy. Several pregnancy-related proteins were identified, including pregnancy-specific protein B (PSP-B), pregnancy-associated glycoprotein (PAG), prolactin-related protein (PRP), placenta lactogen (PL), and pregnancy serum protein 60 (PSP60).

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One protein that is secreted continuously for the duration of the pregnancy is placenta lactogen (PL), which is detectable in trophoblast tissues (Arima *et al.*, 1983; Schuler *et al.*, 1988; Sasser *et al.*, 1989). Finally, the expression of prolactin-related protein (PRP) gene by placental binucleate cells was detected into bovine peripheral blood (Scott *et al.*, 1992).

Currently, 2-D gel electrophoresis (2-DE), in collaboration with MALDI-TOF mass spectrometry, is a frequently applied technique that isolates composite cellular or biological fluid proteomes (Görg *et al.*, 2000). Several reports address the proteomics of the bovine reproductive system (Ali *et al.*, 2004; Jobim et al., 2004). Milk components have been studied extensively (Séverin *et al.*, 2005), as have proteomic analyses of milk or whey protein (Galvani *et al.*, 2001; Yamada *et al.*, 2002; Lindmark-Mansson *et al.*, 2005; Manso *et al.*, 2005). However, to the best of our knowledge, a direct comparison of milk proteins between pregnant and non-pregnant cattle has not been reported.

In this experiment, differential protein patterns in milk between pregnant and non-pregnant dairy cattle were investigated, using a proteomics approach that utilizes 2-DE and MALDI-TOF MS, to identify whey proteins expressed in the course of pregnancy.

MATERIALS AND METHODS

Whey Samples

Bovine whey proteomics were analyzed using the milk of five pregnant Holstein dairy animals at Day 35 after artificial insemination (AI) and five non-pregnant animals. Whey protein was obtained via the centrifugation of milk samples at 3,000 g, at 4℃ for 20 min, following the lowering of the pH to 4.6 to remove casein proteins. The whey proteins were concentrated by centrifugation at 10,000 g and then stored at −70℃ until later use.

2-D Gel Electrophoresis

Precast 18 cm IPG strips with a pH 3~10 range were obtained from Amersham Biosciences. Two milligrams of preparative protein samples was used for isoelectric focusing (IEF). The samples were mixed with modified rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2.5% DTT, 10% isopropanol, 5% glycerol, 2% v/v IPG buffer; pH 3~10) for a total volume of 450 μl (Görg *et al.*, 2000). A mixture of samples was loaded onto an IPG strip (pH 3~10; 180×3×0.5 mm). The strip was allowed to rehydrate overnight in a swelling tray.

Following rehydration, the first dimension, IEF, was performed using an Amersham Pharmacia Multiphor II IEF unit. Automatic isoelectric focusing was carried out at 1.5×10^5 Vh. The initial applied voltage was 100 V, and it was gradually increased to a final voltage of 8,000 V. After the first-dimensional IEF, an IPG gel strip was placed in an equilibration solution (6 M urea, 2% SDS, 50% v/v glycerol, 2.5% acrylamide, 1.5 M Tris-HCl; pH 8.8) containing 5 mM TBP, for 20 min with gentle shaking. The second-dimensional separation was performed on 8~16% linear gradient SDS polyacrylamide gels. The gels were placed in an ISO-DALT system (Hoefer Scientific Instruments, San Francisco, CA, USA). The gels (200×250×1.0 mm) were run overnight at 10~15 mA per gel, until the bromophenol blue marker dye had disappeared at the bottom of the gel.

Staining and Image Analysis of 2-D Gels

After 2-DE, the gels were stained with colloidal Coomassie brilliant blue (CBB) G-250. The gels were fixed for 1 h in a fixation solution (30% v/v methanol, 10% v/v acetic acid), staining the gel with colloidal CBB G250 for 24 h; this was followed by destaining with 1% acetic acid. The gels were analyzed using Melanie III software (Swiss Institute for Bioinformatics, Geneva, Switzerland). Calculations were applied to the percent-volume parameter representative of the protein expression. Variations in abundance were calculated as the ratio of average values (% vol), between the two classes. The process for validating variant proteins was carried out by human operators.

Sample Preparation for MALDI-TOF Mass Spectrometry Analysis

In-gel digestion was performed mainly as previously described (Shevchenko *et al.*, 1996), with some modifications. For Coomassie-stained proteins, the gel slab was destained by using 120 μl of wash solution (50% v/v acetonitrile, 25 mM NH_4HCO_3; pH 7.8). The gel pieces were then dehydrated with 50 μl of acetonitrile and dried for 30 min with a vacuum centrifuge. The dried gel pieces were rehydrated with 5 μl of trypsin solution (trypsin at a concentration of 0.0012 μg/μl, in 25 mM NH_4HCO_3; pH 7.8). Digestion was performed at 37℃ overnight.

After completion of the digestion, the supernatant was transferred to another Eppendorf tube. To extract residual peptides, the gel pieces were sonicated for 20 min at 30℃ in a solution of 50% acetonitrile/0.5% TFA. Extracted peptides were used for MALDI-TOF analysis. Mass spectrometric analysis of peptide mass fingerprinting (PMF) was performed using a Voyager-DE STR MALDI-TOF-MS (PerSeptive Biosystems, Framingham, MA, USA). Approximately 1 μl of extracted peptide solution from each gel spot piece, together with the same volume of matrix solution (10 mg/ml α-ciano-4-hydroxycinnamic acid, 0.1% v/v TFA, and 50% v/v ace-

tonitrile), were loaded onto a 96-well MALDI sample plate and crystallized. For each sample, an average of 500 spectra was obtained; scans were performed twice. Spectra were automatically calibrated upon acquisition, using an external three-point calibration. Peak assignment was performed manually using DataExplorer™ software, which is part of the Voyager-DE STR MALDI-TOF-MS software package (PerSeptive Biosystems). Spectra were saved as peak table files, to facilitate a search against an online non-redundant protein sequence database (SWISS-PROT and/or NCBI [2008/ 05/01, Data Bank]).

RESULTS

Analysis of Placenta Proteomes by 2-DE

Differential protein expressions in the milk of pregnant and non-pregnant dairy cattle were evaluated using 2-DE analysis of total whey protein extracts. The milk samples were obtained from dairy cattle 35 days after AI, and they were sorted as being pregnant or non-pregnant, after pregnancy tests were carried out. Also, major milk proteins (e.g. caseins) were removed by lowering the pH just after milk collection; in this experiment, only whey proteins were used for 2-DE analysis. The 2DE gel images obtained from each 2-mg protein sample from pregnant whey and non-pregnant whey are shown in Fig. 1. In 2-DE, a large number of proteins were separated across the entire pI and MW ranges of the gel. By using ImageMaster 5.0 software after staining with Coomassie blue, approximately 500 ~600 protein spots were detected per gel. The expression levels of variant protein spots were statistically analyzed and are represented by the mean of percent-

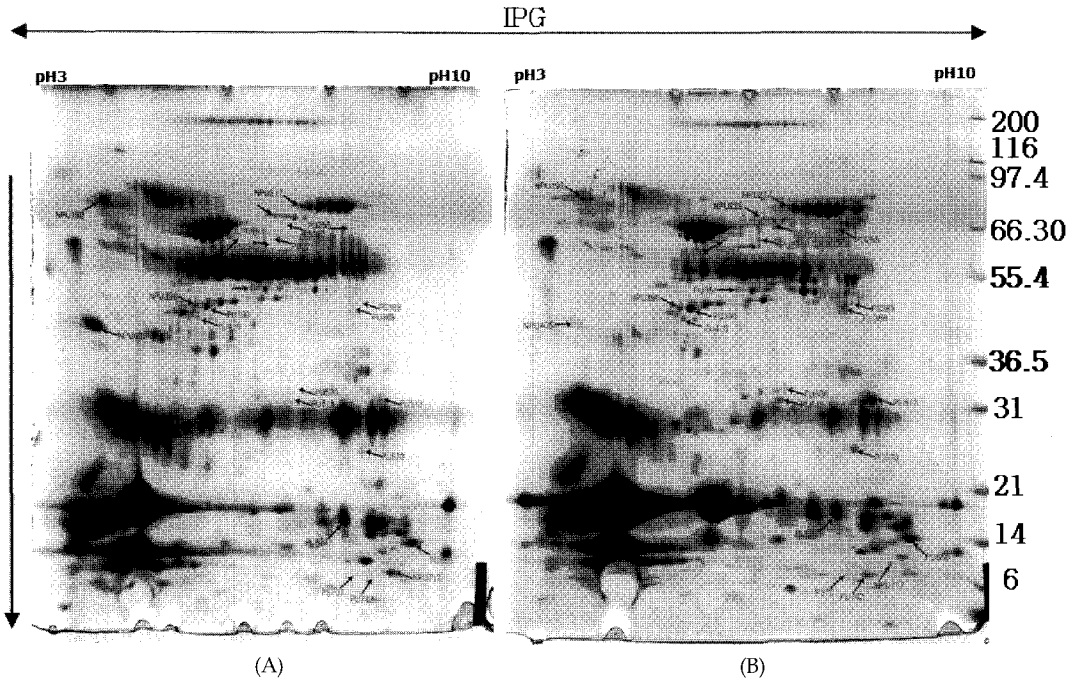

Fig. 1. 2-D PAGE protein separation of pregnant and non-pregnant whey, as visualized with CBB staining. The first dimension was in 18 cm 3~10 IPG, and the second dimension was in an 8~16% gradient gel. A mean of spots was enumerated with ImageMaster 5.0 software. (A) non-pregnant whey proteome, (B) pregnant whey proteome.

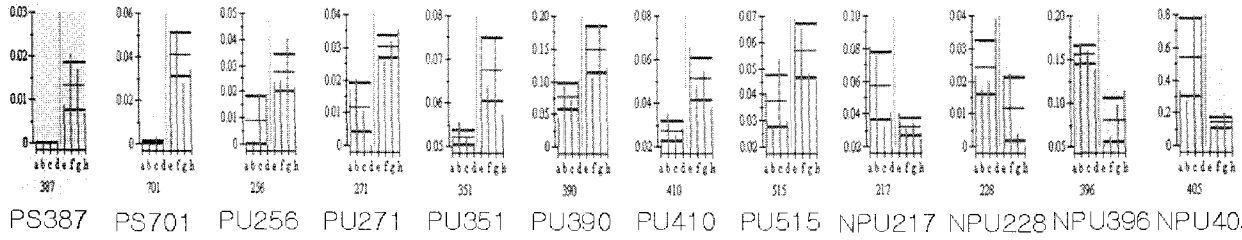

Fig. 2. Differential expression of proteins in percent-volume histograms. PS: pregnancy-specific spots, PU: up-regulated spots in pregnancy, NPU: down-regulated spots in pregnancy.

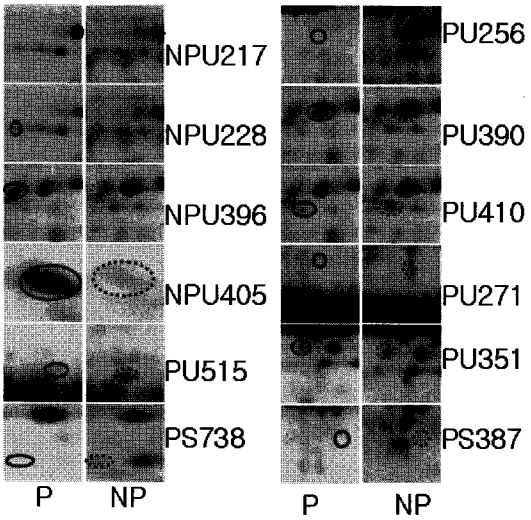

Fig. 3. Protein spots differentially expressed in milk, between pregnant and non-pregnant cattle. A black dotted line represents down-regulation and a solid line represents up-regulation. NPU: down-regulated spots in pregnancy, PU: up-regulated spots in pregnancy, PS: pregnancy-specific spots.

volume histograms (Fig. 2). The differentially expressed protein spots between pregnant and non-pregnant whey proteins are shown in Fig. 3. In comparing pregnant and non-pregnant whey protein spots, a total of 12 spots were identified as being differentially expressed proteins; six of these were up-regulated in pregnant whey, four spots were up-regulated in non-pregnant whey, and two spots were pregnancy-specific.

Identification of Differentially Expressed Spots

To identify the proteins of differentially expressed spots, the unprocessed spectra of samples after tryptic digestion, followed by MALDI-TOF MS, were evaluated using DataExplorer™ software; this resulted in the capture of monoisotopic peaks (Fig. 4). The tryptic peptide masses were used to identify protein candidates in the web-based searching software ProFound (http://129.85.19.192/ profoundbin/WebProFound.exe). A total of 12 spots were identified as known proteins, via SWISS-PROT and NCBI database-searching. The search results were evaluated on the basis of accepted standards that take into account the number of peptides matched to a candidate protein, the coverage of a candidate protein's sequence by matching peptides, and the agreement of experimental and theoretical pI and M_r with derived values.

The identified proteins are presented in Table 1. In comparing pregnant and non-pregnant whey samples, out of the 12 spots identified as being differentially expressed proteins, six spots were up-regulated proteins in pregnant milk samples (e.g., bovine lactoferrin, NADH dehydrogenase subunit 2, and serum albumin precursor); four spots were up-regulated proteins in non-pregnant samples (e.g., bovine potassium voltage-gated channel subfamily A member 4, AMP-activated protein kinase gamma subunit, and serum albumin precursor); and two spots were pregnancy-specific proteins (i.e., cytosolic NADP-dependent isocitrate dehydrogenase and TBK1 similar to TANK-binding kinase TBK1). These proteins function are known to be iron-binding glycoproteins, immune proteins, metabolic stress-sensing protein kinases, carrier proteins, or molecule transport or membrane proteins.

DISCUSSION

The protein expression patterns of pregnant milk (at Day 35) and non-pregnant milk proteins were compared using 2-DE analysis. Through extensive analysis with MALDI-TOF, several proteins differentially expressed in the milk of dairy cattle were identified. The

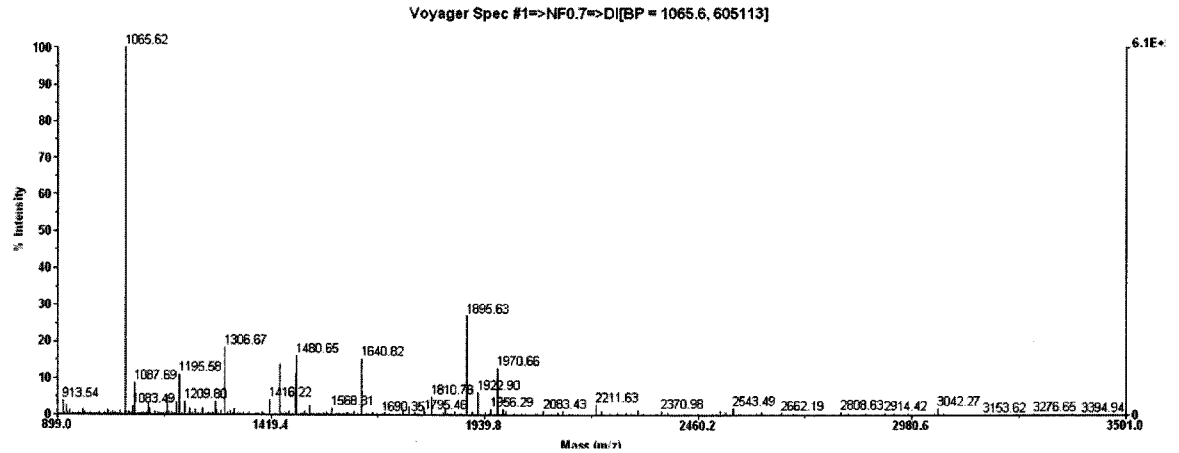

Fig. 4. MALDI-TOF-MS spectra obtained for PU515 spots. Database searching allowed the identification of albumin, as seen in Table 1.

Table 1. Milk proteins differentially expressed between pregnant and non-pregnant cattle, as identified by MALDI-TOF.

Spot ID	Est'd Z [a)]	Accession No.	Protein information	%	pI	kDa
Pregnancy-specific spot						
PS387	1.79	gi|4959708	Cytosolic NADP-dependent isocitrate dehydrogenase	40	6.1	47.2
PS701	1.44	IPI00707221.2	TBK1 similar to TANK-binding kinase TBK1	33	6.2	84.6
Up-regulation spot in non-pregnancy						
NPU217	2	gi|2501351	Transferrin	28	6.8	80.4
NPU228	1.37	gi|1168949	Bovine potassium voltage-gated channel subfamily A member 4	9	5.1	74.5
NPU396	1.67	IPI00708398.1	Serum albumin precursor	34	5.8	71.8
NPU405	1.49	IPI00710146.4	AMP-activated protein kinase gamma subunit	23	9.4	37.7
Up-regulation spot in pregnancy						
PU256	2.11	gi|504	Bovine lactoferrin	26	9.2	77.7
PU271	1.91	gi|5836032|	NADH dehydrogenase subunit 2	36	9.7	39
PU351	1.89	gi|30794280	Albumin	33	5.8	71.8
PU390	2.4	gi|30794280	Albumin	20	5.8	71.8
PU410	1.56	gi|1351907|	Serum albumin precursor	30	5.8	71.8
PU515	2.18	gi|30794280	Albumin	28	5.8	71.8

[a)] The Z score is the distance to the population mean, in units of standard deviation. It also corresponds to the percentile of the search in the random-match population. Conceptually, this "95th percentile" is different from "95% confidence," in that the search is a correct identification. (The following is a list of Z scores and their corresponding percentiles in an estimated random-match population: [(Z: percentile) 1.282:90.0, 1.645:95.0, 2.326:99.0, 3.090:99.9].)

expression levels of 12 spots on 2-DE gel differed significantly when those proteins were compared to the repeated 2-DE results of pregnant and non-pregnant milk samples. Bovine milk contains low levels of serum-genesis proteins such as albumin, immunoglobulins, and complement proteins; members of the IGF family; and lactoferrin, an iron-binding protein that also has antimicrobial properties (Lacy-Hulbert *et al.*, 1999; Korhonen *et al.*, 2000; Ward *et al.*, 2002). A few minor milk proteins are known to be present at increased levels during beestings secretion and drying-off (Hurley, 1989). However, differences in whey-protein ingredients in pregnant and non-pregnant milk whey have not been reported, to date; the current study is the first to do so.

In the current study, a pregnancy-specific up-regulated spot (PU256) was identified as lactotransferrin. Lactoferrin is an iron-binding glycoprotein of the transferrin family; it is expressed in most biological fluids and is a major component of innate mammalian immune systems (Legrand *et al.*, 2008). Lactoferrin, lactoperoxidase, and other, minor milk proteins have functions associated with host defenses (Smolenski *et al.*, 2007). Meanwhile, a spot up-regulated in non-pregnant whey (NPU217) was identified as transferrin. Transferrin is a glycoprotein with an approximate molecular mass of 76,500 kD; it carries iron from the intestine and reticuloendothelial system. It must be further investigated, why transferrin is more highly expressed in the milk of non-pregnant cattle than that of pregnant cattle. Serum albumin precursor and albumin were identified in both pregnant and non-pregnant whey. Generally, expressions of albumin subunits may be differentially regulated by the physiological conditions of the animals studied. Up-regulated albumin proteins were reportedly expressed in the blood and other tissues of pregnant cattle (Kim *et al.*, 2005; Woo *et al.*, 2005). TBK1 was identified in pregnancy-specific proteins that were known to have functions in controlling bacterial infection and providing viral immunity (Radtke & O' Riordan, 2008). Future researchers should examine more fully the relationship between this milk protein and pregnancy in dairy cattle.

Proteomic analysis has allowed us to visualize protein expression patterns and profiles in the blood or milk during bovine pregnancy. The results of the current study present composite profiles of key milk proteins whose expressions are affected by pregnancy; the results also reveal that several functional class proteins up- and down-regulate in the early stages of pregnan-

cy. These differentially expressed proteins may have a beneficial implication in the early diagnosis of bovine pregnancy.

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