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Identification of diagnostic protein markers of subclinical mastitis in bovine whey using comparative proteomics

Yanjie Bian, Ying Lv, Qingzhang Li

College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, China
qzli@neau.edu.cn

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Abstract

The proteomics of inflammatory response in whey from cows with subclinical mastitis were analysed. Whey protein lysates were separated on 24 cm dry IPG strips (pH 3-10 linear) and 24 cm dry IPG strips (pH 4-7) using two-dimensional electrophoresis. The results indicated that the whey proteins in milk from cows with subclinical mastitis are different from those in milk from healthy cows. All protein spots were found to have biologically relevant changes in relative abundance during subclinical mastitis using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry analysis, including β-1,4 galactosyltransferase, β-2 microglobulin, complement 3, α-1-acid glycoprotein, β-lactoglobulin A, α-S1 casein precursor , β-casein B, and serotransferrin precursor. The mRNA expression of these genes was verified by quantitative real-time PCR. These proteins are involved in signal transduction, binding, transport, and immune defence activity. The results suggest that the markers may be used for the diagnosis of subclinical mastitis.

Keywords: proteomic, subclinical mastitis, bovine whey, diagnostic markers.

Introduction

Mastitis is well recognised as one of the major devastating diseases of adult dairy cows, and 90% of mastitis cases are subclinical (30). Subclinical mastitis causes tremendous losses in the international dairy industries associated with reduced production and quality of milk (28). One hallmark feature of subclinical mastitis, irrespective of the bacterial agent, is a substantial increase in somatic cell count, mainly neutrophils, in milk (8). Although subclinical mastitis is more prevalent than clinical mastitis in developing countries, it is not manifested by visible changes in the mammary glands or in milk, so it is not easily recognised by farmers (22).

Up to now, many management practices have been used to reduce the occurrence of the disease (11). However, subclinical mastitis is still a significant problem in dairy production worldwide. The incidence rate of subclinical mastitis still has not been controlled efficiently, so its diagnosis appears especially important. A biomarker is generally considered as an indicator of normal biological processes, pathological processes, or pharmacological responses to therapeutic interventions (18). The interest in establishing biomarkers for bovine subclinical mastitis stems mainly from the need to better characterise the mechanisms of the disease, to identify reliable biomarkers to facilitate early detection, and to reveal potential novel targets for the development of alternative therapeutics.

Comparative proteomic analysis is an effective means for identifying disease-associated proteins and discovering disease marker molecules. The use of comparative proteomics to analyse differential milk protein expression in whey from milk collected from healthy cows and cows with subclinical mastitis, is highly significant for its effectiveness in finding biological marker molecules and elucidating the pathological mechanisms of the disease. Research has taken place into protein expression patterns in whey from milk from healthy cows and cows with clinical mastitis, and it was found that some identified proteins were associated with the mastitis (14, 18). These results indicated that protein expression patterns changed in whey from subclinical mastitis cases, and that proteomic methods could be used to find new diagnostic markers of the disease.

Because of the dynamic range of proteins present in bovine milk, the identification of low-abundance proteins is difficult. For their identification, whey protein lysates were separated on both 24 cm dry immobilised pH gradient (IPG) strips (pH 3-10 linear) and 24 cm dry IPG strips (pH 4-7) using 2-DE. Protein expression profiles indicating the host response during subclinical mastitis infections were distinguished from different local and systemic responses to infections caused by different bacterial pathogens (6). Up till now, in the subclinical mastitis field there have been few studies on the changes in bovine whey observed by application of proteomics. The objectives of the present study were to identify more differentially expressed proteins in whey from cows with local subclinical mastitis, and to search for new biomarkers which could be useful for the early detection of the disease.

Material and Methods

Animals and sample preparation. The study was conducted on 42 Holstein dairy cows located on farms in the region of Harbin, China. Quarter milk samples from ll lactating cows were procured and subjected to somatic cell count (SCC), California mastitis test (CMT), and microbiological examinations to identify subclinical mastitis. Small translucent colonies with α- or β-haemolysis, suspected of being *Streptococcus agalactiae*, were subcultured for microscopic analysis by Gram-stained smears, and the CAMP test was used for taxonomical classification. *Escherichia coli* was short-rod and preliminarily identified as Gram-negative bacteria by Gram-stained smears, and *Escherichia coli* colonies in eosin-methylene blue (EMB) medium were small and had a metallic green sheen. *Staphylococcus* colonies were identified by haemolysis test, Gram-stained smears, and catalase and coagulase tests. The detailed procedure of bacteriological culture and identification of bacterial species were performed according to standard bacteriological procedures (17, 25). Healthy cows and cows with subclinical mastitis were identified as previously described (27). Then the pH of each milk sample, serum amyloid A (SAA), and haptoglobin (Hp) were determined. SAA concentration was assayed using the multispecies Tridelta Phase ELISA SAA kit (Tridelta Development Ltd, Ireland), and Hp concentration was assayed by the Phase ELISA Hp kit (Tridelta Development Ltd, Ireland).

Three samples were randomly chosen from control (n = 17) and subclinically mastitic (n = 25) cows, then the samples were immediately centrifuged (8 000 g, 20 min) at 4°C and the fat layer was carefully removed. Then the defatted milk was treated with acetic acid (4 M) to adjust the pH to 4.6 to precipitate the casein. The samples were stored at -80°C. Proteins were extracted according to a previously described procedure (4).

Isoelectric focusing. Protein samples were centrifuged (12,000 g, 10 min) to remove insoluble materials and then rehydrated at 450 μg in 450 μL rehydrate buffer containing: 9 M urea; 2% 3- ((3-cholamidopropyl) dimethylammonio)-1-propanesulfonic acid (CHAPS); 18 mM dithiothreitol (DTT), and 0.5% Ampholytes (GE Healthcare, USA). Three experimental replicates were performed for each whey sample from a single animal. Euroclone IPG strips (24 cm) with a linear pH range of 3.0-10.0 and 4.0-7.0 were rehydrated overnight in a buffer containing: 9 M urea; 4% CHAPS; 18 mM DTT; 10 mM TRIS, and 0.5% Ampholytes. Isoelectric focusing was performed using the Ettan IPGphor III IEF system (GE Healthcare, USA) at 20°C. Proteins were then focused under the following conditions: 250 V for 50 min, 500 V for 30 min, 1,000 V for 1.5 h, and 10,000 V for 10 h.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). When completing the first-dimension electrophoresis, the IPG strips were equilibrated first with 15 mL of equilibration buffer, containing: 0.375 M Tris-HCl, pH 8.8; 6 M urea; 20% glycerol; 2% SDS; 0.15 g of DTT, and a trace of bromophenol blue, for 15 min, followed by a second equilibration with 0.45 g of iodoacetamide replacing DTT, for 15 min. The strips were washed with deionised water and blotted to remove excessive equilibration buffer, embedded in low melting temperature agarose, and then transferred onto 12.5% acrylamide gels. The second-dimension electrophoresis was conducted with the Ettan Daltsix Electrophoresis System (GE Healthcare, USA). Gels were run at constant power first with 1W/strip for 70 min and then with 15 W/strip until the bromophenol blue reached the bottoms of the gels. After runs, gels were stained with Fast Silver Stain Kit (Beyotime, China).

Image analysis and identification of proteins. Gel images were acquired with a calibrated scanner (ImageScanner III, GE Healthcare, USA) at 300 dpi, and then the images were analysed using ImageMaster 2D Platinum 6.0 and 7.0 Software (GE Healthcare, USA) according to the manufacturer's instructions. Statistical analysis software within this software package was used to identify spots, which were up- or down-regulated under specified conditions (32). The procedure was repeated three times for each of the three experimental replicates of the whey sample. The backgrounds of gel images were removed, and then protein spots were detected automatically. After image analysis, whey protein spots expressed differentially between subclinically mastitic and control whey were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-TOF) peptide mass fingerprinting as previously described (15). The resultant peptide mass fingerprint was compared against the National Center for Biotechnology Information non-redundant (NCBInr) and SWISS-PROT databases using the Mascot search engine. The search criteria for acceptable matches were better than

95% confidence, and peptide sequence identity score greater than 30 ($P < 0.05$). The peptide mass tolerance was set at 0.1Da. The fixed and variable modifications were carbamidomethylation - cysteine, phosphorylation - serine, and oxidation - methionine. A maximum of one missed tryptic cleavage was allowed. Protein matches were considered to be valid if five valid matched peptides emerged with Mascot scores summing to more than 61. Valid peptides were required to have all of the major peaks in the spectra assigned (3).

RNA extraction and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Total RNA from bovine whey was extracted using Trizol reagent (Invitrogen, USA) and purified by phenol/chloroform extraction. Then the purified RNA was treated with DNase I (QIAGEN, USA). The total RNA sample (0.5 μg) was transcribed into cDNA by Thermoscript reverse transcriptase (TaKaRa, China). RT-PCR analysis was performed with an ABI PRISM 7300 RT-PCR System (Applied Biosystems, USA) using 96-well microwell plates in a total volume of 20 μL. The β-actin was used as the reference gene. The primers for validating genes were as follows: B4GALT1:sense 5'-CAGTGCCC TCCACCACAACA-3', antisense 5'-CCATAGTC TAACTGCTGACGCTGT-3'; B2M:sense 5'-GGGCT GCTGTCGCTGTCTG-3', antisense 5'-CCTCTTCT AATTTAGCCTCGTCAGT-3'; C3:sense 5'-TGTC CCTTACAGCCTTTGTTCTCAT-3', antisense 5'-GT TCCTCCCAGCGGTTCTTTT-3'; α1-AGP:sense 5'-GAGGTGACCCAGGAGCAGAAGA-', antisense 5'-AACCGAGGGAACTGAGTTACAAACA-3'; β-lactoglobulin A:sense 5'-CCACCCTCCAGGA CACACC-3', antisense 5'-GATGGTCCTTGG CAGGTCCG-3'; CSN1:sense 5'-CTGTCTTGTG GCTGTTGCTCTTG-3', antisense 5'-TTGGGCA TGGATTCCCTCTT -3'; β-CNB:sense 5'-GGC TATGGCTCCTAAGCACAAAG-3', antisense 5'-AGGAAACATGACAGTTGGAGGAAGA-3'; TF: sense 5'-TGGTTTCGCTGGTCTGAGTGAT-3', antisense 5'-GCTG TGGGAAAGAGCAAATGAAT-3'; β-actin: sense 5'-CCGCAAGGACCTCTACGC-3', antisense 5'-CATGCCAATCTCATCTCGTTTT -3'. RT-PCR reactions were operated at 95°C for 10 s, followed by 40 cycles for 5 s at 95°C, and 60°C for 31 s using the two-step RT-PCR method. RT-PCR analysis was performed according to the procedure in an earlier report (20).

Statistical analysis. Statistical data and individual differences among groups were analysed using ANOVA by Statistical Package for the Social Sciences (SPSS) 17.0 software (IBM, USA). Results were reported as mean ± SD. For all statistical analyses, a value of $P < 0.05$ was considered significantly different, and $P < 0.01$ was considered extremely significantly different.

Results

As shown in Table 1, cows were identified by clinical symptoms, as well as the results of SCC, CMT, and bacteriological culture of milk samples from particular quarters of the mammary gland. Clinical mastitis was diagnosed in eight cows, 25 cows displayed subclinical mastitis, and 17 cows were healthy controls. The pH of milk was higher in cows with subclinical mastitis than in control cows (Fig. 1A). SAA and Hp concentrations were statistically higher in subclinically mastitic cows compared to healthy cows (Fig. 1B).

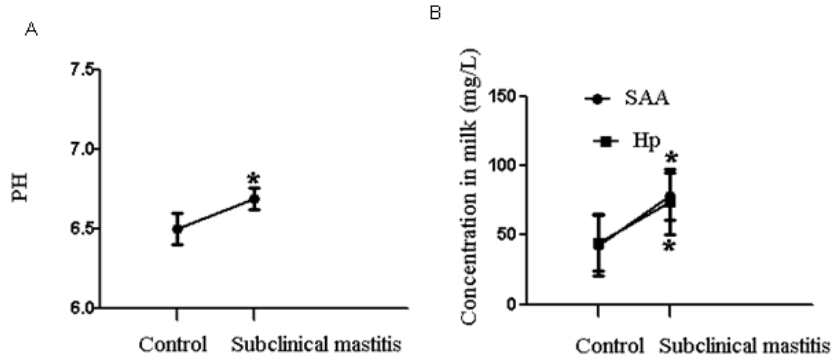
The proteomics analysis of whey from cows with subclinical mastitis and control cows was performed using 2-DE with three separate biological replicates. Five differentially expressed protein spots of interest were found on the gels (pH 3 to 10 linear), and the results are shown in Fig. 2A. The picture of amplification and the 3D view of the five differentially expressed proteins are shown in Figs 2B and C respectively. Because most of the protein spots on the gels displayed pH from 4 to 7, for improving the resolution of 2-DE maps at the pH area, narrow pH gradient dry strips (pH 4 to 7, 24 cm) were also used to screen the expressed proteins. The gels are shown in 3A, and three new expressed proteins were found (Figs 3B and C).

Generally, eight different protein spots were successfully identified by MALDI-TOF-MS. The eight protein spots were matched to eight different proteins by searching against the NCBInr and SWISS-PROT databases (Table 2). In the Table 2 the spot number corresponds to the numbers in Figs 2A and 3A. The β-1,4 galactosyltransferase (B4GALT1), β-2 microglobulin (B2M), complement 3, and α-1-acid glycoprotein
(α1-AGP) were over-expressed in subclinical mastitis; β-lactoglobulin A, casein precursor (CSN1), β-casein B (β-CNB), and serotransferrin precursor (TF) were down-regulated in subclinical mastitis. Most of these over-expressed proteins in subclinically mastitic cows participate in host immune defence and antibacterial activities. Down-regulated proteins in subclinically mastitic cows are mainly proteins related to milk protein synthesis.

These eight proteins' relative mRNA expression level changes in subclinically mastitic bovine whey were assessed by qRT-PCR. Results showed that mRNA levels of B4GALT1, B2M, complement 3, and α1-AGP were significantly up-regulated in the subclinically mastitic group compared with the control group, while the mRNA expression of β-lactoglobulin A, CSN1, β-CNB, and TF in the subclinically mastitic group was lower than in the control group (Fig. 4). The mRNA expression level of all tested genes was consistent with the tendency of their protein expression changes.

Table 1. SCC (mean ± SD), CMT, and results of bacteriological examination

Parameter	Control (n = 17)	Subclinical mastitis (n = 25)
SCC ($\times 10^4$ cells/mL)	5.7 ± 4.1	34.1 ± 7.5**
CMT	negative	doubtful (n = 18), positive (n = 7)
Microbiological examinations	negative	*Staphylococcus aureus* (n = 13), *Staphylococcus epidermidis* (n = 2), *Streptococcus agalactiae* (n = 9), *Escherichia coli* (n = 1)



* and ** indicate values with significant differences from the control group at P < 0.05 and P < 0.01 respectively

Fig. 1. Milk pH (A) and SAA and Hp concentration (B) in cows with subclinical mastitis and controls

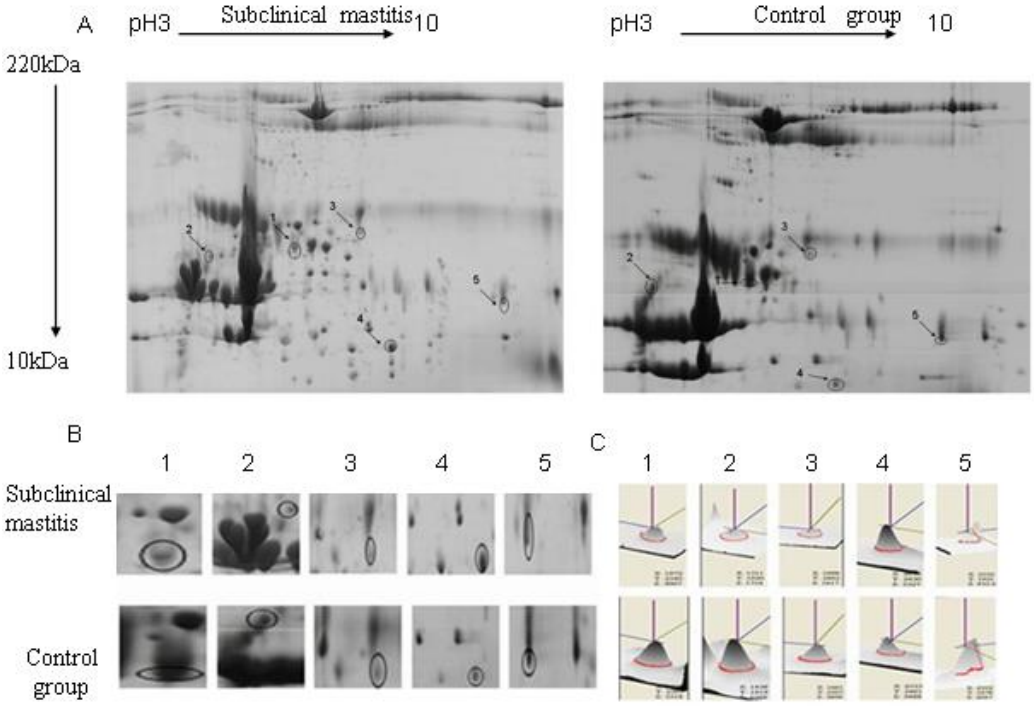


Fig. 2. 2-DE maps (pH 3-10) of whey from subclinically mastitic and control cows. (A) Comparison of the gels (pH 3-10) of the subclinical mastitis group with the control group. The expressed proteins are labelled with numbers and arrows; (B) Amplification of the five expressed proteins in Fig. 2A; (C) The 3D view analysis of proteins in Fig. 2B by ImageMaster 2D Platinum 6.0. Peak volume represents the expression amount of protein

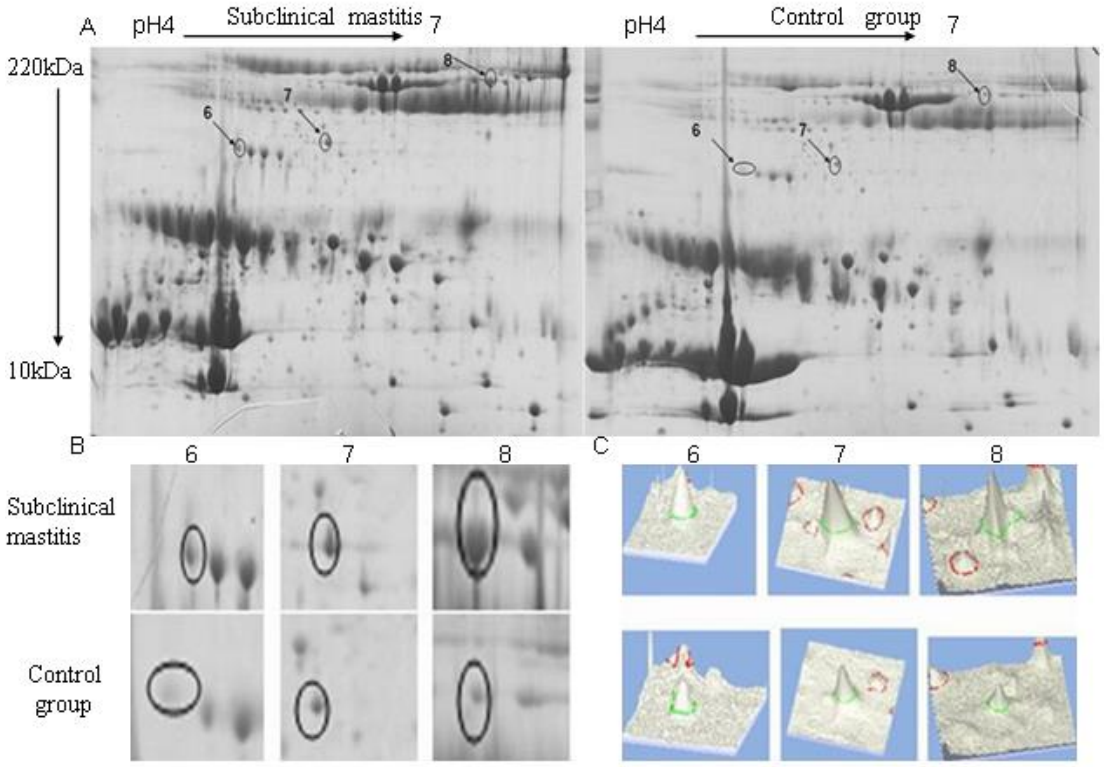


Fig. 3. 2-DE maps (pH 4-7) of whey from subclinical mastitis and control cows. (A) Comparison of the gels (pH 4-7) of the subclinical mastitis group with the control group. Differentially expressed proteins are labelled with numbers and arrows; (B) Close-up of the three differentially expressed proteins in Fig. 3A; (C) Results of 3D view analysis of proteins in Fig 3B by ImageMaster 2D Platinum 7.0

Table 2. MS identification of differentially-expressed proteins in whey of subclinically mastitic and control cows

Spot no.	Protein	Fold change	Accession no.	Percentage of coverage	Mascot score	Confidence
1	TF	-2.09	gi:114326282	34%	624	100%
2	CSN1	-1.59	gi:31341348	16%	61	96.006%
3	β-lactoglobulin A	-2.46	gi:23200515	66%	163	100%
4	B2M	+2.29	gi:110347591	70%	148	100%
5	β-CNB	-2.18	gi:13661026	19%	84	98.967%
6	α1-AGP	+2.01	gi:159895415	39%	238	100%
7	Complement 3	+1.56	gi:99028969	52%	101	100%
8	B4GALT1	+2.37	gi:21450879	45%	90	99.779%

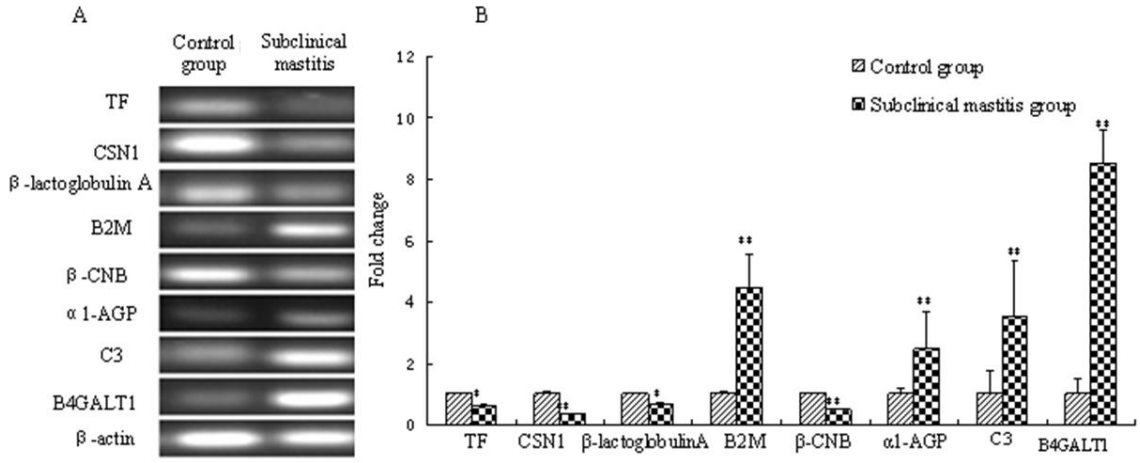


Fig. 4. The mRNA expression change of proteins different in the subclinically mastitic group compared with the control group. (A) Agarose gel electrophoresis of the mRNA expression change of the different proteins; (B) Relative expression level of genes tested by RT-PCR. β-actin was used for normalisation control under the same conditions. The ratio of the control groups was regarded as one. Values are means ± SD (n = 3 per group). * and ** indicate values with significant differences from the control group at $P < 0.05$ and $P < 0.01$ respectively

Discussion

Proteomics analysis has been gradually developed in the study of complex pathological processes at the protein level, especially to detect low-abundance proteins and those for which no ELISA or antibody currently exists. In this study, eight differentially expressed proteins were found in the proteomics analysis, including B4GALT1, B2M, complement 3, α1-AGP, β-lactoglobulin A, CSN1, β-CNB, and TF. These identified proteins are mainly involved in signal transduction, binding and transport, and immune defence activity. The changes in the mRNA level of all identified proteins indicated that the differences in protein level may be due to transcriptional level regulation when subclinical mastitis occurs.

B4GALT1 is a Golgi resident type II membrane protein existing in two sub-cellular compartments where it performs two distinct functions. The protein's principal task as a biosynthetic enzyme is to catalyse the transfer of galactose from UDPGAL to GLCNAC in the presence of Mn2+, forming the product N-acetyllactosamine (23); its other role as a recognition molecule on the cell surface is to participate in cellular interactions by binding to appropriate glycoside substrates on the adjacent cell surface and in the extracellular matrix (29). B4GALT1 is highly expressed in the mammary gland of mammals. Over-expression of B4GALT1 leads to impaired lactation as a result of reduced branching and differentiation, and to elevated apoptosis, while deleting surface B4GALT1 enhances branching and differentiation, and reduces apoptosis (12). B4GALT1 is also involved in a variety of pathological phenomena. There was previous evidence suggesting that the expression of B4GALT1 was enhanced by its inflammatory status, which is induced by nerve injury (21), tumour necrosis factor-α (TNF-α) treatment (10), and lipopolysaccharide stimulation (24). In agreement with these results, here we detected B4GALT1 in 2-DE gels from both subclinically mastitic and normal whey, and B4GALT1 was increased in whey when inflammation occurred.

B2M is a chief component of the immune system. All normal karyocytes, especially lymphocytes, can synthesise B2M. It is essential for assembly of the peptide–major histocompatibility complex (MHC) and can influence the ability of TCRs to engage that complex (2). B2M was reported to be involved in the activation of T-cells by antigen, presenting cells *via* direct contact with the CD8 molecule, and playing a role in viral resistance and anti-tumour immune response (19). Our result showed that B2M increases in subclinical mastitis. B4GALT1 and B2M might be associated with the host response presented in whey from bovine milk during subclinical mastitis.

The α1-AGP and complement 3 have been previously identified in bovine milk by 2-DE coupled with MALDI-TOF/MS, most frequently in clinical mastitis samples (5). Bovine α1-AGP is an acute phase protein (APP) with anti-inflammatory properties (9). It belongs to the lipocalin protein family as do α1-microglobulin, complement component 8, prosta-glandin D synthase. It was also revealed that α1-AGP increased in serum during bovine mastitis, but was not identified in mastitic milk (13). Ceciliani *et al.* (7) reported that α1-AGP was identified in colostrum and normal milk of cattle. An increase in α1-AGP in subclinically mastitic whey was congruent with reports of increased production of APP. This concerned APP production during coliform mastitis and the subsequent leakage of APP into the milk as a result of some action of blood proteins exosmosed to the mammary gland

when mammary gland inflammation occurred (1). The detection of content changes of α1-AGP has important significance for diagnosis of disease. C3 increased in subclinically mastitic whey samples compared with control group samples. The result was compatible with the previously characterised role of the complement system in innate immunity (26). Complement 3 is an acute phase factor produced by the liver, secreted by activated macrophages at inflammation sites and by adipocytes and has a significant impact on the immune system (33). The complement system is involved in pathological processes of some transformation reactions, autoimmune diseases, and autoimmune disorders. Complement 3 has a role in bactericidal action and promoting inflammatory response. So it was not novel to find that complement 3 was up-regulated in the whey of cows suffering from subclinical mastitis. Although it needs to be further validated, we can presume that the pathogenic microorganism of subclinical mastitis provides surface in contact and an active complement pathway from complement 3 to carry out effective immune defence in the organism.

Conversely, the concentrations of the abundant milk proteins, which were β-CNB, β-lactoglobulin A, and CSN1 decreased in whey from subclinically mastitic cows. The results were supported by findings described in bovine clinical mastitis (31). Bovine subclinical mastitis induces reduction in the concentrations of TF, which are negative acute phase reactants. The condition was different from that previously described in bovine clinical mastitis (27). It might be correlated with bacterial antagonism while inflammation happened in the early stage. When the mammary gland was in an inflammatory and injured condition, TF decreased expeditiously with the help of a pro-inflammatory cytokine (16). The finding of the existing differences in milk protein patterns between subclinically mastitic and healthy cows is very useful in the search for specific biomarkers. The result showed that 2-DE followed by MALDI-TOF MS is a powerful strategy to identify the proteins. Though further evaluations would require additional refinement of sample preparation and analysis strategies, the findings provide information that could verify the usefulness of inflammatory biomarkers in diagnosis of subclinical mastitis.

In conclusion, in the current study, eight proteins were identified successfully in milk whey from subclinically mastitic and healthy cows. These proteins were B4GALT1, B2M, complement 3, α1-AGP, β-lactoglobulin A, CSN1, β-CNB, and TF. The proteins are mainly involved in signal transduction, binding and transport, and immune defence activity. Though the validation of a single biomarker specific to bovine subclinical mastitis may not be feasible, the establishment of biomarker profiles could be valuable for early disease detection and veterinary therapeutics, and expand existing knowledge of the biological mechanisms involved in bovine subclinical mastitis.

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