Chapter 8

Proteomic Analysis of Goat Milk

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70082

Abstract

The advancement of electrophoresis and chromatography, along with technological developments in mass spectrometry, has widened the potential application of proteomics to study milk from smaller ruminants. The aim of this chapter is to provide an in-depth overview of the development and progress of proteomics applications in goat milk. After examining various proteomic approaches that are currently applied to this field, we narrow our focus on proteomic investigations of mastitis in goat milk. A summary of protein modulation in goat milk during experimentally-induced endotoxin mastitis is discussed. Because the molecular function of proteins is disrupted during disease due to changes in post-translational modifications, we also review the phosphorylation of caseins, which are the predominant phosphoproteins in milk, and discuss the implications of casein modifications during mastitis. These results offer new insights into the changes of protein expression in goat milk during infection.

Keywords: goat milk, mastitis, proteomics, casein phosphorylation

1. Introduction

Milk is an important biological fluid and an essential nutrient for young mammals and humans during their lifetime. It provides macro- and micro-nutrients and is an important source of antimicrobial and immunoregulatory agents [1]. Typically, the U.S. dairy industry relies on cows as the main source of milk and other dairy products. However, cow milk has been implicated in the increasing rates of protein allergies in infants [2, 3], causing gastrointestinal disorders in adults [4], and contains insufficient concentrations of iron [5], thus an interest in finding alternatives to cow milk has emerged.

Goat milk is an excellent source of macro- and micro-nutrients, and proteins that are more easily digested, presumably due to the higher essential fatty acid contents [6]. It is also less allergenic than cow milk, as it was shown to help children at risk of food allergies [7]. While not very popular in the United States, in developing countries where cow milk is not readily available or affordable, goat milk accounts for more than 50% of milk production [8]. In Europe, goat milk is processed mostly for cheese manufacturing [9]. Considering the economic importance in developing countries and the inherent health benefits, goat milk might be a reliable alternative, if not a replacement, for cow milk [10].

Proteins are the key components of milk with many diverse cellular functions. For example, casein micelles provide essential amino acids that are vital for energy, tissue growth, and cellular function. In addition, some proteins can act as hormones, whereas others display antimicrobial properties. Proteomics is the large-scale study of the protein contents of cells and tissues [11]. One of the most promising outcomes of proteome analysis is the discovery of protein biomarkers, which are specific proteins or protein isoforms, whose expression levels change significantly during disease conditions [12]. The identification of these biomarkers in accessible body fluids such as milk could eventually enable farmers and veterinarians to monitor diseases and expand treatment options.

Mastitis, the focus of several prior veterinary proteomics studies, is defined as inflammation of the breast or udder tissues that is typically caused by invading bacteria. The first proteomic investigation of bovine milk mastitis was conducted by Baeker et al., where they used a comparative proteomics to identify expressed proteins in normal and mastitic bovine milk [13]. This was followed by several other research investigations to identify differentially expressed proteins in cow milk, either following experimentally induced infection or during naturally occurring mastitis [14–16]. Quantification of expressed proteins in clinically healthy cows and cows with experimentally-induced coliform mastitis was also reported using both liquid chromatography tandem-mass spectrometry (LC-MS/MS)-based label-free approach [17] and isobaric peptide tags for relative and absolute quantification (iTRAQ) [18]. As a result, biochemical mechanisms and inflammatory-related biomarkers, especially acute phase proteins (APPs), were identified [19–21].

Like other ruminant species that are managed for milk production, goats are also affected by mastitis; however, much less is known about the goat innate immune response to mastitis pathogens or about subsequent changes in goat milk protein expression over the course of a clinical infection. Although some of the apparent clinical signs of mastitis in goats, including udder swelling and redness, increased rectal temperature, and changes in the appearance of the milk are similar to those observed in dairy cattle, limited knowledge of the host response during mastitis in goats exists. Nonetheless, other hallmarks of clinical mastitis in dairy cattle include elevated milk somatic cell counts (SCC), reduced appetite, reduced milk production, increased heart rate, and changes in blood chemistry [22, 23].

Initial reports of proteomic evaluations of goat milk were limited to the analyses of casein fractions and the determination of the molecular weights of major whey proteins [8, 24]. Later attempts to identify differentially expressed proteins in goat milk employed gel-based assays followed by enzymatic digestion of isolated proteins and matrix-assisted laser desorption/ionization

mass spectrometry MALDI-MS [25, 26] or further refinements of gel-based assays followed by LC-MS/MS [27]. Until recently, no studies have focused on the proteomic analysis of goat milk protein modulation during clinical mastitis. The first study of goat milk protein modulation over the course of experimentally-induced mastitis using proteomics was recently been reported by our group [28]. Since the molecular function of proteins can be disrupted during the disease due to the changes in post-translational modifications (PTMs), we also evaluated the phosphorylation status of caseins [29].

This chapter will provide an overview of common proteomic approaches and the application of proteomics to the detection of caprine milk proteins. Likewise, a brief description of the advances in our current understanding of goat mastitis and of associated inflammatory biomarkers detected in goat milk will follow a summary of our proteomic investigations of goat milk during the course of experimentally-induced mastitis. Moreover, the phosphorylation of caprine casein proteins and their potential implications as markers of disease will be discussed.

2. Proteomics approaches

The proteomic field is divided into two main analytical methodologies: the top-down and the bottom-up. The top-down approach relies on the analysis of intact proteins and corresponding fragmentation within the MS. In contrast, the bottom-up approach, which has been increasingly adopted by the proteomics community, proceeds through analysis of peptides that are generated outside the MS. The identified peptide sequences are then reassigned to the proteins they originate from, through database searching. To reduce sample complexities, a protein mixture can also be fractionated by gel-based approaches prior to MS analysis. The spots from the gel are excised and are subjected to an in-gel digestion. In the following sections, gel electrophoresis, MS, and database searching will be briefly discussed.

2.1. Electrophoresis

Classical proteomic approaches take advantage of protein fractionation by using gel-based assays. 1D-SDS/PAGE and two-dimensional electrophoresis (2-DE) have provided direct separation technologies and contributed to the better understanding of the global milk proteome. 2-DE involves separation by isoelectric focusing in the first dimension, followed by SDS-PAGE in the second dimension. 1D-SDS/PAGE offers a number of important advantages as it produce sharp, molecular weight-separated bands, which increase the dynamic range of the mixture analysis. Fractionation of the complex mixture by spreading it out over 10–20 gel slices dramatically increases the depth of analysis, and hence the number of identified proteins. 2-DE is useful to optimize the separation of proteins of similar molecular weight but with different isoelectric points, which are not resolved using SDS/PAGE. 2-DE provides higher resolution compared to SDS/PAGE and is the best choice for the analysis of phosphorylated or glycosylated proteins as their isoelectric point can shift. In fact, 2-DE is the only technique that provides a visualization of PTM. On the other hand, low abundance, highly charged and hydrophobic proteins such as membrane proteins cannot be well resolved in

2-DE. 2-DE is often time-consuming and requires sample cleanup prior to analysis. In both 1D- and 2-DE analyses, the bands or spot map are visualized by protein staining with Coomassie. The targeted bands from 1D spots or spots from 2D gels are sequenced and identified by mass spectrometry. In a bovine mastitis study, Boehmer et al. performed an optimized protein separation with 2D electrophoresis to analyze whey from control and mastitic bovines. The experiments were conducted with a conventional proteomic approach using 2-DE as a choice of separation method, coupled by MALDI-TOF analysis for the identification [30].

2.2. Mass spectrometry

Although gel-based assays are useful for fractionating complex mixtures and for removing contaminants from a sample that might interfere with the MS analysis, gel-free approaches are simple and straightforward. Using a gel-free approach, a complex protein mixture is directly digested in solution prior to MS analysis. Reduction and alkylation steps, though not required prior to enzymatic cleavage, provide greater peptide yields and can enhance peptide sequence coverage. Prior to MS analysis, peptides can be fractionated using different forms of chromatography including reverse-phase, strong and weak ion exchange, size exclusion, and affinity capture chromatography. Although reverse-phase chromatography is the most widely used method of LC separation in proteomics, affinity capture chromatography is commonly used to enrich or isolate intact proteins, or to investigate PTMs prior to MS analysis.

There are different types of MS instruments for the analysis of a particular type of sample, each with different scanning speed and resolution capabilities. Nonetheless, all MS instrument systems have three common and distinct components: an ionization source for the generation of ions, a mass analyzer for the separation of ions, and a mass detector for the detection of ions.

In regards to ionization, it is important to note that soft ionization techniques such as MALDI and electrospray ionization (ESI) have revolutionized the analysis of large biomolecules. In MALDI, a sample is mixed with a UV-absorbing crystalline matrix, most commonly derivatives of cinnamic acid, and spotted onto a metal target plate. The plate is then inserted into the MS instrument, where it is placed in a vacuum and irradiated with a UV laser. The matrix absorbs the irradiation, which simultaneously heats, volatilizes, and ionizes the sample [16]. Once ionized, the proteins or peptides are analyzed in a mass analyzer, which is typically time-of-flight (TOF) in a MALDI instrument. The TOF mass analyzer is ideal for MALDI because it has a virtually unlimited mass range, which is advantageous because MALDI yields singly charged molecular ions that can have a high mass-to-charge (m/z) ratio [30]. TOF instruments can also operate in tandem mode, using a second mass analyzer to monitor fragment ions (i.e., TOF/TOF).

In contrast to MALDI, ESI produces multiply charged species for each molecule. This is due to the mechanism of ion formation in ESI in which an electrical field leads to the transfer of ions from a solution into the gaseous phase. The transfer of an ionic species from solution into the gas phase by ESI involves three steps that include the dispersion of charged droplets, followed by solvent evaporation, and finally the ejection of the ion from the highly charged droplets [31]. Although the generation of multiply charged ions complicates the mass analysis, it greatly enhances fragmentation potential, which is necessary for structure elucidation. Ideal

mass analyzers for ESI are single or triple quadrupoles and quadrupole ion traps. Because it is a liquid-based method, ESI is more compatible with the LC separations and has consequently became the standard ionization method in LC-MS/MS experiments. With the advent of ultra-performance liquid chromatography (UPLC), chromatographic separations have improved in terms of both the resolving power and the speed of the separation. Nanoflow LC (nLC) is now also widely used in proteomics because it is amenable to LC columns with a smaller internal diameter, which increases the column backpressure and results in greater sensitivity [32].

2.3. Bioinformatics

The accurate identification of proteins and peptides in a complex mixture using tandem mass spectrometry, data can be achieved by database searching strategies. The databases are normally protein sequences translated from genomic data. Considering a database containing potential proteins that could be present in the sample, these proteins are digested in silico by a search engine. For example, for tryptic peptides, the search engine would calculate the masses of all peptides that could be produced by cleavages after lysine and arginine residues and would create a virtual peptide database [33]. Similarly, for the identification of peptides in the sample, the search engine first filters all potential peptides with the same mass, then performs an in silico fragmentation of each of these peptides. After matching peptide sequences with the list of fragment masses observed in the MS/MS spectrum, the search engine assigns a score [33].

The success of MS-based proteomics analyses depends on the availability of complete and accurately annotated databases containing the gene and protein sequence information for the animal species of interest. Unfortunately, as of yet, only a limited number of annotated genome sequences are available for the goat. In recent years, despite an increase in the number of proteomic investigations of various body fluids from the goat, identifying these proteins is recognized as a greater technical challenge [34]. In the absence of annotated protein databases, researchers are forced to extrapolate their data from the bovine genome or other mammalian protein databases [28]. If a given database does not contain the amino acid sequences of all of the proteins in the sample, suitable matches cannot be made; thus the proteins will remain unidentified.

3. Proteomic research in caprine milk

Milk is composed of three main components: casein, whey proteins, and milk fat globule membrane (MFGM). Using differential centrifugation and ultracentrifugation, it is possible to isolate these three fractions. While the application of proteomics in the milk of larger animals has attracted many research groups, the role of proteomics in the milk of goat remained limited. In recent years, with the advancements of available technologies in proteomics, there has been a growing interest to unravel the dynamic structure of goat milk protein contents. Proteomic research in caprine milk has been applied in the areas of identifying major proteins, comprehensive analysis, MFGM, and identification of PTM (**Table 1**).

	Techniques	Matrix	Proteomic study	References[1]
Major proteins	2-DE & MALDI-TOF	Milk	Casein profile	[24]
	nLC-MS/MS	Cheese	Comparative proteomics/ adulteration	[35]
	1-DE & LC-MS/MS	Skim milk	Comparative proteomics	[27]
	MALDI-TOF	Raw milk	Comparative proteomics	[25]
	2-DE & MALDI-TOF	Skim milk	Farm animals principle proteins	[36]
	2-DE & nLC-MS/MS	Skim milk	Comparison of healthy and LPS induced	[28]
	1DE/2-DE/HPLC	Skim milk	Comparative proteomics Brazilian breeds	[37]
	MALDI-TOF	Skim milk	Anti-inflammatory/anti-allergic properties	[3]
	MALDI-TOF-MS	Skim milk	Fingerprinting of principle proteins	[26]
	MALDI-TOF-MS	Skim milk	Fingerprinting of major proteins/adulteration	[38]
	2-DE/ELISA	Colostrum/skim milk	IgG/IgM bindings profile & quantification	[39]
	UPLC-XevoTQS	Milk powder	Absolute quantification (MRM) Whey powder	[40]
Comprehensive	SCX & nLC-MS/MS	Whey	Farm animals comparative & quantification	[41]
	CPLL, 1DE & nLC-MS/MS	Milk	Comprehensive of low abundance proteins	[6]
	2-DE & MALDI-TOF & LC-MS/MS	Milk	Comparison study sheep and goat	[42]
MGFM	1D SDS/PAGE	MFGM	Protein composition of MFGM	[43]
	1DE &MALD-TOF	MFGM	Assessment of protein composition	[8]
	LC-MS/MS	MFGM	Proteome profile and biological activity	[44]
	nLC-MS/MS	MFGM	Colostrum & mature milk	[45]
	2-DE/nLC-MS/MS	MFGM	Quantification of mammalian	[46]

	Techniques	Matrix	Proteomic study	References[1]
PTM	1DE & nLC-MS/MS	Skim milk	Comprehensive caseins phosphoproteome	[29]
	Affinity chromatography & nLC-Chip-QTOF-MS	Skim milk	Lactoferrin N-glycans in human and bovine	[47]
	nLC-MS/MS	MFGM	N-glycosylation comparison in mammals	[46]
	LC-MS/MS	MFGM	Phosphoproteome analysis	[48]

[1]Literature references of each study: 2-DE, two-dimensional; 1-DE, one-dimensional; nLC, nano liquid chromatography; UPLC, ultra-performance liquid chromatography; SCX, strong cation exchange; CPLL, combinatorial peptide ligand libraries; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; MRM, multiple-reaction-monitoring.

Table 1. Proteomic investigations of caprine milk.

3.1. Identifying major proteins

One of the first proteomic studies in goat milk was reported by Roncada et al., where they used two dimensional electrophoresis (2-DE) combined with MALDI-TOF and ESI-ion trap mass spectrometers to analyze α_{S1}-casein alleles. They determined casein polymorphisms as the key characteristics in the cheese manufacturing industry [24]. Major goat milk proteins including caseins (α_{S1}, α_{S2}, β-, and κ-casein) and some of the whey proteins (albumin, lactoferrin, β-lactoglobulin, and α-lactalbumin) are highly abundant and have been widely studied [8, 25, 37]. Several groups compared the data from proteomics studies of goat milk with those of cow and other species, but their reports involved only the characterization of major proteins [27, 36, 41]. In one of 2-DE analyses [36], highly abundant proteins in each animal species display their own unique pattern [36]. This group further highlighted significant interspecies differences in milk from different ruminants and identified β-lactoglobulin as the major whey protein in many ruminants including goat.

The use of goat milk as a substitute for cow milk for allergic people has been also recently reported [3, 10]. In some parts of the world, milk of donkey or goat is used in newborn and infant feeding because they are less allergenic than cow milk. Accordingly, Di Girolamo et al. used fingerprinting of major milk proteins by MALDI-TOF MS coupled to a robust statistical analysis to determine adulteration and unintended contamination of donkey milk and goat milk [26]. Peptide mass fingerprinting is a simple methodology in proteomics where proteins are cleaved with a protease, such as trypsin. The identification is accomplished by matching the observed peptide masses from MS data to the theoretical masses derived from a sequence database [49]. Comparative proteomic approaches were used to study colostrum and milk of goats, cows, and sheep to determine chemical composition and immunoglobulin concentration [39]. The study revealed that despite the similar immunoglobulin concentrations in colostrum and milk from the three studied species, differences in several immune components can be detected.

Proteomics is instrumental in detecting milk adulteration as adulteration has been a significant problem in the dairy industry. In a comparative proteomic study, different cheese samples obtained from milk of cow, sheep, and goat were analyzed using HPLC-chip-MS/MS [35]. The authors found κ-casein to have a unique primary structure and suggested that it could be used to determine the origin of milk in different cheese samples. In another study, a MALDI-TOF-MS platform was used to profile milk samples for the rapid detection of illegal adulterations caused by the addition of either nondeclared cow milk, milk of other species, or the addition of powdered milk to the fresh counterpart [38]. For this purpose, peptide and protein markers of cow, water buffalo, goat, and sheep milk were identified and the effects of thermal treatment-associated adulterated milk samples were evaluated. This study introduced an independent, complementary peptide profiling measurement and extended proteomic approaches to the analysis of thermal treatment. Yang et al. analyzed milk whey samples obtained from a number of species including goat, cow, buffalo, yak, and camel. They detected certain proteins as the characterizing traits for a given species that could be used to evaluate adulteration [41].

3.2. Comprehensive analysis

Proteomic investigation is challenging due to the wide dynamic range of protein expression where the presence of high abundance protein masks or prevents the detection of low abundance proteins. Recently, the most comprehensive proteomic dataset of goat milk has been reported by Cunsolo et al. [6]. This group fractionated the total milk samples using combinatorial hexapeptide ligand libraries (CPLL; such as ProteoMiner) at different pH levels to reduce the dynamic range of protein concentrations. They identified 452 unique gene products including many low abundance proteins in goat milk. Their success was also related to the use of further fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), high resolution, nLC-MS/MS, and an advanced bioinformatics platform [6]. New strategies employing multi-enzyme digestion coupled with CID and ETD for protein sequencing and characterization was also used to increase sequence coverage and localization of PTMs [50]. In a separate study, using two complementary proteomic approaches, Anagnostopoulos et al. investigated the milk whey of three Greek sheep and goat breeds. They identified about 600 protein groups, most of which were involved in nutrient transport and immune system responses [42]. These findings provide the most comprehensive description of the goat milk proteome that can be utilized to build a goat protein sequence database. As it was pointed out by Soares et al. the success of proteomic-based investigations largely depend on the availability of complete and annotated databases containing the gene and protein sequence information for different animal species [51]. Hence, the data generated from these recent, more comprehensive proteomics analysis of the goat milk proteome could facilitate the detection and characterization of additional milk proteins in future proteomics analyses of goat milk.

While proteomic analyses have been used to qualitatively identify adulterants in milk, no reliable, selective, and sensitive method existed to obtain absolute quantification. In a clever analysis, Chen et al. used multiple reaction monitoring (MRM) to quantify milk adulteration

when cow milk was added to sheep or goat milk [40]. In this study, two peptides derived from β-lactoglobulin were chosen as the protein markers. Similar isotopically labeled peptides as internal standards were designed and synthesized to minimize the matrix effect, which led to more accurate quantification results. MRM has been used for many years to measure and quantify small molecules, drugs, and metabolites. However, the application of MRM to obtain absolute quantitation of proteins is relatively new and offers great potential to the field.

3.3. Analysis of MFGM

Goat milk is a great source of essential fatty acids, which are concentrated in MFGM along with a complex mixture of proteins, glycoproteins, and enzymes [52]. Although MFGM proteins account for a small fraction of the total milk protein, they have been extensively studied in human and bovine milk. In previous bovine MFGM studies, many health benefits including anticancer, antimicrobial, and antiviral effects have been attributed to the glycoproteins [53]. Therefore, there is a need to characterize this important component in goat milk.

The protein composition of goat MFGM has been described by several groups [8, 43, 44, 53]. Cebo et al. used 1DE and MALDI-TOF-MS and reported butyrophilin, lactadherin, mucin, and lectin as major MFGM proteins in goat milk. Interestingly, lactadherin from goat milk consisted of a single polypeptide chain whereas 2 polypeptide chains were detected in bovine milk [8]. In addition, the MFGM of colostrum and mature milk was also investigated [45]. As expected, the acute phase proteins were higher in colostrum MFGM, signifying the importance of colostrum intake to the immune system of newborns.

In a more recent 2016 study by Yang et al., the N-glycoproteome of MFGMs, obtained from a group of mammals' milk including human, Holstein, Jersey, buffalo, yak, camel, horse, and goat was investigated. They found that protein components of MFGM fractions from ruminants were more similar to each other when compared to nonruminants [46]. In a comprehensive analysis of goat milk MFGM, Henry et al. reported the use of high-resolution LC-MS/MS to expand the MFGM proteome in goat milk to 442 functional groups [48]. The main focus of their study was to probe the phosphoproteome of goat MFGM that will be covered in the next section.

3.4. Post-translational modifications (PTM)

PTMs are chemical modifications that play a key role in functional proteomics. The characterization of PTMs, although challenging, is very important as they regulate protein function and control numerous important biological processes. A large number of different PTMs have been reported, but by far, phosphorylation and glycosylation are the most important and well-studied [54]. Since phosphorylation has a low stoichiometry, there is a need for enrichment of phosphopeptides in an analysis of a complex mixture. The current method to enrich phosphopeptides is based on affinity purification using phosphospecific antibodies immobilized-metal affinity chromatography (IMAC). Zhong et al. optimized the selective isolation of mono- and multi-phosphorylated peptides by using different forms of iron ions [55].

In this study, they selected α-casein and two synthesized mono- and di-phosphopeptides as a model system to demonstrate that $NiZnFe_2O_4$ was highly selective for multi-phosphopeptides whereas Fe_3O_4, $NiFe_2O_4$, and $ZnFe_2O_4$ had a higher affinity for mono-phosphopeptides. Along with the improvements of IMAC for phosphoproteomic experiments, instrument enhancements including improved acquisition speed allowed the identification of many more phosphopeptides per analysis. As mentioned before, in a comprehensive analysis of goat milk MFGM phosphoproteome, Henry et al. used TiO_2 for enrichment of the MFGM samples. Using nLC-MS/MS and high resolution mass spectrometer, they characterized the phosphorylation of several key mammary gland proteins in goat MFGM. This group, leveraging the strengths of high resolution and faster acquisition time, reported the detection of 271 sites of phosphorylation on 124 unique goat MFGM proteins [48].

The identification of PTMs is especially useful for the detection and characterization of acute phase proteins (APPs) during disease because APPs are subjected to modification. The N-glycan profiles of goat milk lactoferrin were compared with human and bovine milk using advanced mass spectrometry techniques [47]. The characterization of glycan composition established high mannose, hybrid, and complex N-glycans. Among the N-glycan compositions, 37% were sialylated and 34% were fucosylated. This group highlighted the existence of similar glycan composition between human and goat milk and discovered a novel glycan in goat milk that was not detected in human milk. A recent 2016 study investigated N-glycoproteome analysis of MFGMs from a number of mammals' milk [46]. They observed different glycosylation patterns in certain proteins that were previously reported with varying molecular weights based on the analysis by SDS-PAGE. They concluded that these discrepancies were the result of the differences in carbohydrate content of these proteins.

4. Mastitis and inflammatory-related biomarkers in goats

Mastitis is inflammation of the mammary gland that manifests in a wide range of physical indications, chemical changes in the milk, and pathological changes in the udder [56]. Clinical symptoms of mastitis include swelling and pain in the udder, increased rectal temperature, reduced feed intake and milk production, and the watery appearance or presence of clots in the milk. Mastitis research has drawn immense interest over the years because of its profound economic impact on the dairy industry. The incidence of clinical mastitis can be reduced by the application of management strategies, including a greater awareness of efficient milking and hygienic measures. However, despite the development of vaccines and other preventative methods, mastitis caused by Gram negative environmental pathogens and coliform bacteria remain problematic to treat and to manage [57]. Coliform bacteria are normal inhabitants of soil, manure, bedding, and water; thus, coliform mastitis can occur due to the contact of teats with the infected environment. Although innate immune responses in the mammary gland are effective to some extent, the mammary gland defense mechanisms can be compromised by environmental and physiological conditions [58]. Despite extensive knowledge of the bovine host response to mastitis pathogens and the effects of mastitis infection on the

bovine milk proteome, only a limited understanding of the goat innate immune response to mastitis pathogens or the subsequent changes in goat milk protein expression over the course of a clinical infection exists. Nonetheless, like other ruminants that are managed for milk production, goats are also susceptible to and affected by mastitis.

Soluble mediators of inflammation in bovine milk and plasma during clinical mastitis have been studied extensively using antibody-based strategies [59]. Although antibody-based methodologies are both quantitative and accurate, they have limited detection capabilities. Conversely, mass spectrometric-based proteomic technologies allow for the simultaneous analysis of a larger number of proteins without the reliance on antibodies. Using MS-based proteomics, a number of biomarkers including APPs were identified in bovine serum and milk, which were correlated with pain and disease status [19, 60]. The concentration of most APPs typically increased during infection or inflammation, and the increased levels were relatively stable and persisted for a number of days, or even weeks, after the original insult or stimulus [19]. Despite the fact that our knowledge of the modulation of the bovine milk proteome during mastitis continues to expand, very little comparative data exists on lactating dairy goats.

In regards to the study of the goat milk proteome, our group detected increases in haptoglobin (Hp), serum amyloid A (SAA), and lactoferrin in the milk of goats following an intramammary infusion of lipopolysaccharide (LPS) to induce coliform mastitis [28]. Other studies also documented significantly increased blood levels of Hp and SAA in an experimentally induced subacute ruminal acidosis in goats [61]. The majority of APPs are known to be glycosylated. Due to the high extent of its carbohydrate moiety, the APP alpha-1-acid glycoprotein (AGP) has been established as a biomarker of inflammation in goats [62]. AGP was also reported to potentially inhibit neutrophil migration to the site of infection, leading to inadequate bacterial clearance and resulting in increased risk of mortality [63]. Further, Heller et al. determined the species-specific reference intervals for four APPs including Hp, SAA, AGP, and lipopolysaccharide-binding protein (LBP), which is a soluble polypeptide that binds to bacterial LPS and increases its proinflammatory activity up to 1000 fold, in goat milk [64, 65].

4.1. Effects of experimentally-induced mastitis on the goat proteome

Modulations in the expression of goat milk proteins have been examined following an experimental induction of endotoxin mastitis by intra-mammary infusion with LPS. For details of challenge study and sample preparation, see materials and methods section in Ref. [28]. Crude milk samples were separated by 2DE prior to nLC-MS/MS analysis. The unique proteins identified following the 2DE analysis of skim milk from healthy goats and skim milk collected from the same goats 18 h post infusion with LPS are summarized in **Table 2**. In the absence of goat specific database, we used the Swiss-Prot other mammalia taxonomy, which includes only a limited number of goat sequences. Though some goat specific proteins were identified, the majority of the protein identifications were assigned to other species. As shown in this table, caseins constitute the most abundant proteins in milk; thus, a marked number of casein variants, specifically β- and α_{S2}-caseins, which were detected in 13 and 6 separate

Protein ID	Protein name	Species[1]	Peptides[2]	Sample[3]
Q28372	Gelsolin	Equine	2	Healthy
Q3SX14	Gelsolin	Bovine	6	LPS
P85295	Serum albumin	Caprine	6	Healthy
P14639	Serum albumin	Ovine	9	LPS
P18626	α_{S1}-Casein	Caprine	12	Healthy
P18626	α_{S1}-Casein	Caprine	15	LPS
P11839	β-Casein	Ovine	3	Healthy
P11839	β-Casein	Ovine	4	LPS
P04654	α_{S2}-Casein	Ovine	4	Healthy
P33049	α_{S2}-Casein	Caprine	5	LPS
P02670	κ-Casein	Caprine	6	Healthy
P02670	κ-Casein	Caprine	6	LPS
P02756	β-Lactoglobulin	Caprine	22	Healthy
P02756	β-Lactoglobulin	Caprine	10	LPS
P02694	Retinol-binding protein-1	Bovine	4	Healthy
P02694	Retinol-binding protein-1	Bovine	2	LPS
P00712	α-Lactalbumin	Caprine	3	Healthy
P00711	α-Lactalbumin	Bovine	2	LPS
Q4TZH2	Fatty acid-binding protein	Bovine	5	Healthy
Q6QAT4	β-2-microglobulin	Ovine	3	healthy
Q6QAT4	β-2-microglobulin	Bovine	4	LPS
Q29477	Lactotransferrin	Caprine	19	LPS
Q32PJ2	Apolipoprotein A-IV	Bovine	9	LPS
B6E141	Haptoglobin	Ibex	3	LPS

Protein ID	Protein name	Species[1]	Peptides[2]	Sample[3]
P19661	Cathelicidin-3	Bovine	2	LPS
P22226	Cathelicidin-1	Bovine	3	LPS
P42819	Serum amyloid A	Ovine	4	LPS
P02584	Profilin-1	Bovine	2	LPS

[1]Species of highest scoring assignment from Swiss-Prot (http://www.uniprot.org/).
[2]Number of peptide assignments.
[3]Samples were either obtained from healthy goats or induced by LPS.

Table 2. Proteins detected in milk of healthy goats and experimentally induced with endotoxin mastitis (LPS).

spots at varying isoelectric points on the gel, respectively, were observed in the milk of the goats prior to LPS infusion. The presence of full lengths β- and α_{S2}-casein and in several spots of varying mass and isoelectric charges was most likely due to the presence of multiple fragments of the two dominant caseins in the skim milk as a result of proteolysis. Conversely, the α_{S1}- and κ-caseins were detected in only two spots on the gel. Similar to the protein expression profiles generated from milk samples collected prior to infection, the β- and α_{S2}-caseins dominated the profiles of the skim milk samples collected 18 h following LPS infusion. The number of β-casein fragments at lower-MW detected on the gel was reduced, but the number of α_{S2}-casein spots, both full length protein and corresponding fragments increased in the 18 h samples. In addition to the caseins and the whey proteins β-lactoglobulin and α-lactalbumin, the lower abundance proteins serum albumin, gelsolin, retinol binding protein, fatty acid binding protein, and β-2-microglobulin were likewise detected in goat skim milk samples collected just prior to challenge with LPS.

In sharp contrast to prior reports of bovine milk protein profiles during coliform mastitis, vascular-derived proteins such as complement factors and serotransferrin (known to leak into bovine milk following cytokine induction and the subsequent breakdown of the blood-milk barrier) were not detected in our analysis. Similarly, although serum albumin was detected, no significant increase in the abundance of the vascular-derived protein was apparent. However, along with the increases in SCC, several proteins with antimicrobial properties that are known to be found in the granules of neutrophils, the primary component of SCC during mammary infections, were detected in the goat skim milk samples at 18 h after induction of endotoxin mastitis including cathelicidin-1 and cathelicidin-3 and lactoferrin. The induction of the acute phase response during endotoxin mastitis in goats was also apparent as the APP haptoglobin (Hp) and SAA were likewise detected in the mastitic goat skim milk samples collected 18 h after intra-mammary infusion with LPS.

The intensity of the spot corresponding to serum albumin remained the same in the gels of the goat milk samples 18 h post challenge, which could indicate that the breakdown of the blood-milk barrier during endotoxin mastitis might not be as profound in goats as has been observed in dairy cattle. The inflammatory response was however, supported by elevated

SCC in the goat milk following inoculation with endotoxin, as well as by the presence of both antimicrobial and APPs. The results likewise provided preliminary information regarding protein modulations of goat milk during disease as well as added knowledge of the host response during endotoxin mastitis in goats [28].

4.2. Analysis of multiphosphorylation sites in caseins

Caseins exist in four different variants including α_{S1}-, α_{S2}, β-, and κ-casein. Despite little homology, there is a rare conserved sequence (SSSEE) present in α_{S1}-, α_{S2}, and β-casein that serves as a multiphosphorylation site [66]. This conserved sequence motif does not exist in κ-casein, although it does possess two phosphorylation sites embedded in the C-terminus of the protein. The existence of the conserved sequence domain in casein multi-phosphopeptides makes their detection more challenging as the two glutamic acid (E) residues further increase their hydrophilic nature. In the presence of other peptides, they often go undetected by mass spectrometry. Thus, a vast majority of researchers use IMAC to enrich multi-phosphopeptides prior to detection. Without using any enrichment strategies, we reported changes in the levels of caprine casein phosphorylation [29].

In our experiments, we used milk samples obtained from healthy goats before and after experimental induction of endotoxin mastitis with LPS. We isolated casein bands using 1D-SDS/PAGE prior to in-gel digestion and analysis by nLC-MS/MS. Despite their large size, the majority of these tryptic phosphopeptides eluted early during chromatographic separation. As well, many were not detected following database searching or were assigned a very low peptide score. Consequently, manual inspections of the MS/MS spectra were necessary to validate the identifications.

In addition to mono- and di-phosphorylation sites, a number of multiphosphorylation sites exist in α_{S1}-, α_{S2}-, and β-casein (**Figure 1**). As shown, the conserved SSSEE domain forms a hexa-phosphopeptide in α_{S1}-casein, a tetra-phosphopeptide in β-casein, and two different multi-phosphopeptides in α_{S2}-casein. We characterized 18 different phosphorylation sites from a series of mono- and multi-phosphopeptides.

Examples of multi-phosphopeptides detected in our analysis are presented in **Figure 2**. The top MS/MS spectrum corresponds to a di-phosphopeptide (D58−K73) in α_{S1}-casein that was detected as a doubly charged ion (MH^{2+}= 951.32) corresponding to (MH^{+} = 1901.64, **Figure 2A**). In the α_{S2}-casein, a triply charged ion (MH^{3+} = 1039.89) with the monoisotopic mass (MH^{+} = 3117.08) was isolated and fragmented in a linear ion trap to produce the MS/MS spectrum as shown in **Figure 2B**. The bottom MS/MS spectrum corresponds to the tetra-phosphopeptide (E17−K43) that was detected in β-casein as a triply charged ion (MH^{3+} = 1103.10) corresponding to (MH^{+} = 3307.29, **Figure 2C**). The phosphorylation sites were accurately assigned in each multi-phosphopeptide despite the presence of multiple other serine and threonine resides. In α_{S1}-casein, we did not detect the hexa-phosphopeptide, which also contains the conserved domain. Likely, the proximity of numerous phosphorylated sites made it even more fragile. Nevertheless, we clearly characterized one mono- and one di-phosphopeptide in α_{S1}-casein [29].

http://dx.doi.org/10.5772/intechopen.70082

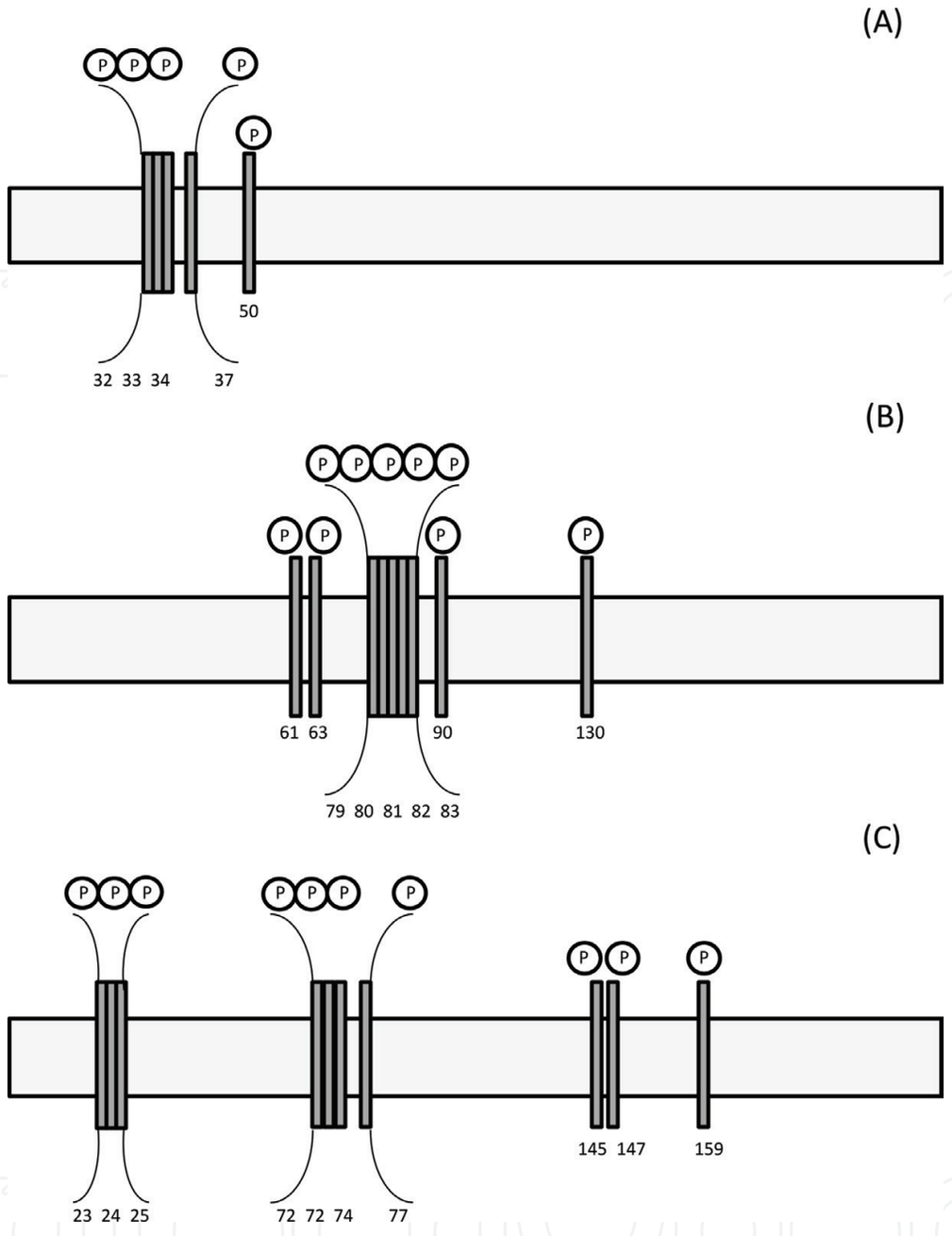

Figure 1. Identification of potential serine and threonine phosphorylation sites within β-casein (A), α_{S1}-casein (B), and α_{S2}-casein (C). The conserved sequence domains (SSSEE) that serve as the multiphosphorylation sites are present in all three caseins.

Despite the lower apparent abundance, the multi-phosphopeptides shown in **Figure 2** were also detected in milk samples obtained from animals with experimentally-induced endotoxin mastitis. In α_{S2}-casein, we also detected a tetra-phosphopeptide (N62–K86) with the amino acid sequence NANEEEYSIRSSSEESAEVAPEEIK [29]. The phosphorylation sites were found at S72, S73, S74, and S77. However, this multi-phosphopeptide was never observed in the mastitic goat milk samples. Instead, this ion was isolated and fragmented in the linear ion trap in which we readily detected two unmodified peptides. The first tryptic

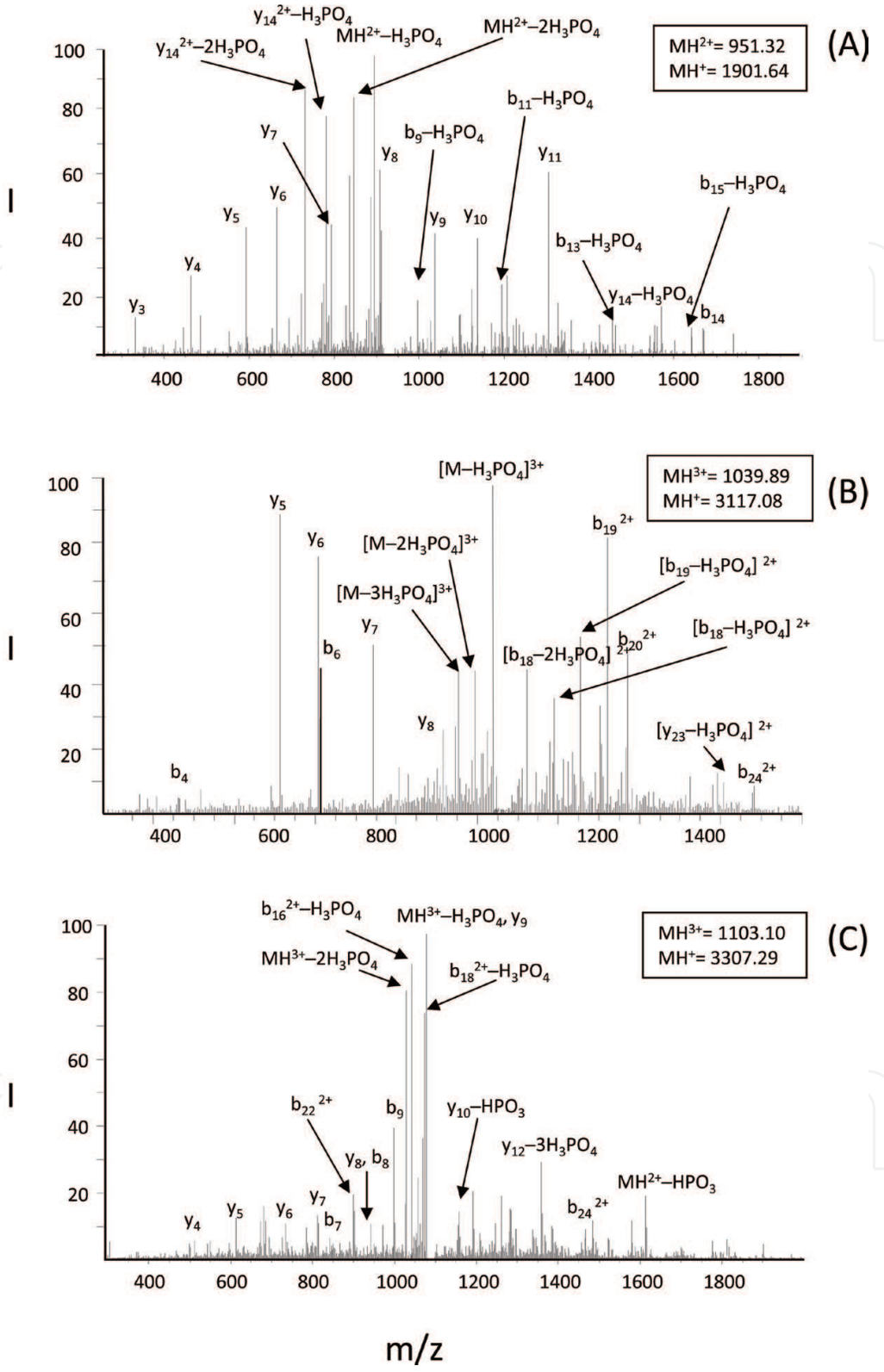

Figure 2. Tandem mass spectra of multi-phosphopeptides: (A) di-phosphopeptide in α_{S1}-casein, (B) tetra-phosphopeptide α_{S2}-casein, and (C) tetra-phosphopeptide β-casein. The *y*- and *b*-ions are marked in each spectrum with losses of phosphoric acid (neutral loss) probably owing to an in-source fragmentation.

peptide in the amino acid sequence (NANEEEYSIR) was detected as an intense doubly charged ion (MH^{2+} = 614.18 Da) corresponding to (MH^{+} = 1227.36 Da). The second dephosphorylated segment (SSSEESAEVAPEEIK) was detected as a doubly charged ion (MH^{2+} = 796.68 Da) corresponding to (MH^{+} = 1592.36 Da). These observations clearly indicated that the peptide remained dephosphorylated in mastitic goat milk. The attachment of multiple phosphate moieties might have sterically hindered the trypsin cleavage site and as a result, we observed the tetra-phosphopeptide as a missed cleavage. To this end, it has been reported that the proximity of the cleavage sites to the phosphorylated amino acids could impair tryptic digestion [67].

5. Conclusions

Advances in separation and mass spectrometry capabilities, enable our abilities to identify and characterize proteins and their PTMs. This chapter provides an overview of proteomics investigations in goat milk, from identifying major milk proteins to comprehensive analysis in different fractions and PTMs. Many challenges still exist, but technological advances have led to an increased in research contributing to a better understanding of the proteomic analysis of goat milk. Low-abundance proteins and disease-specific proteins have been identified as potential biomarkers. Using proteomics strategies, the efforts of our group and others have shed some light on the role of APPs during coliform mastitis and other diseases in goats. The host response during infection and related changes in the goat milk proteome remains comparatively limited. Nonetheless, our comparative proteomic analysis suggests that the caprine host response to endotoxin could differ from other ruminant species. Finally, our precise characterization of casein phosphorylation in goat milk before and after challenge with LPS offers new insights into protein modulations in goat milk during mastitis.

Disclaimer

The views expressed in this article are those of the author do not necessarily reflect the official policy of the Department of Health Human Services, the U.S. Food Drug Administration, or the U.S. Government.

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