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Proteolysis and partial dephosphorylation of casein are affected by high somatic cell counts in sheep milk

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ABSTRACT

Mastitis, an inflammatory affection of the mammary gland, causes an increase of milk somatic cells. The individual milk composition changes while that of bulk milk is remarkably constant, particularly in the lactose content. Milk samples were taken from 240 udder halves, with low ($<500 \times 10^3$/ml), medium ($500–1000 \times 10^3$/ml) and high ($1000–2000 \times 10^3$/ml) somatic cell count (SCC) of a flock of 120 primiparous Sarda ewes. A proteomic study has been conducted on milk from healthy udder halves ($<500 \times 10^3$/ml) as control group and infected udder halves (SCC $> 1000 \times 10^3$/ml). In milk with high SCC, the majority of the peptides measured by ESI-Q-TOF MS arose from the four casein fractions by the decreasing order of affinity for plasmin (PL): i.e. β-casein (CN) > α_{s2}-CN > α_{s1}-CN ≫ κ-CN. The focus of the present work was on phosphopeptides (CPP) released by milk enzymes. In infected milk, α_{s2}-CN and β-CN derived CPP were enriched on hydroxyapatite and seventeen CPP and twenty partially dephosphorylated CPP were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. Although MALDI-TOF analysis faces quantitative limitations in the detection of highly phosphorylated peptides, it was, however, useful for identifying partially or fully dephosphorylated peptides. These results are well correlated with the alkaline phosphatase (ALP) activity in milk. Therefore, in addition to ALP, PL activity is indicated as valid predictor of endogenous proteolysis in infected sheep's milk.

1. Introduction

Mastitis defined as "an inflammatory reaction of the mammary gland" (IDF, 1987) represents a marker of the sanitary state of the udder. Invasion of pathogens activates the immune defense in the udder, manifesting an increase of the secretion of cytokines and chemokines for a successful defense against invading pathogens (Goldammer et al., 2004). In parallel, somatic cell count (SCC) increases in milk (Poutrel, 1981). The lack of data on small ruminants could lead to errors in the diagnosis of subclinical intramammary infection and in the application of discriminatory standards for sheep and goat milk. For this reason, the EU Directives did not establish threshold values for SCC in ewe and goat milk. Nevertheless, a limit of 1500×10^3 cells/ml has been recommended for bulk tank milk of small ruminant (Barbosa et al., 1994). On the other hand, the milk of healthy ewes often contains more than 1000×10^3 SCC/ml of milk (Bufano, Dario, & Laudadio, 1996).

The normal composition of milk somatic cells may vary with several factors: animal age, flock size, parity, flock management, milk yield, season, physiological stress and, especially, stage of lactation (Menzies & Ramanoon, 2001; Orman, Günay, Balci, & Koyuncu, 2011; Yagi et al., 2004). It can reach very high levels during the colostral period and at the end of lactation (Dulin, Paape, Schultze, & Weinland, 1983; Fruganti, Ranucci, Tesei, & Valente, 1985), although the low mean SCC of the uninfected glands of Israeli-Assaf sheep did not change in the course of the lactation (Leitner et al., 2001).

The variations of SCC induced by mentioned factors are generally of less importance than the degree of infection. The most important cause of SCC variation in milk is the infection status of the mammary gland, although it is very hard to establish the level of SCC indicative of an inflammation of the mammary gland. SCC has been used in the determination of subclinical mastitis (SCM) in sheep (Gonzalez-Rodriguez, Gonzalo, San Primitivo, & Carmenes, 1995). Values exceeding 1000×10^3 cells/ml

Abbreviations: ACN, acetonitrile; ALP, alkaline phosphatase; AMBIC, ammonium bicarbonate; CN, casein; CPP, phosphopeptides; DHB, 2,5-dihydroxybenzoic acid; DTT, dithiothreitol; ESI-Q-TOF MS, electrospray ionization-quadrupole time of flight-tandem mass spectrometry; HA, hydroxyapatite; HPLC, high performance liquid chromatography; LC–MS, liquid chromatography mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PA, phosphoric acid; PL, plasmin; PMNs, polymorphonuclear neutrophils; PP, proteose–peptones; SCC, somatic cell count; SCM, subclinical mastitis; TFA, trifluoroacetic acid; UTLIEF, ultra-thin layer isoelectric focusing.

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have been suggested as a base level for the estimation of SCM in ewes (Fthenakis, El-Masannat, Booth, & Jones, 1991). Moreover, three sanitary categories have been proposed relating to the SCC of ovine bulk tank milk: good (SCC < 500×10^3), average (SCC between 500×10^3 and 1000×10^3) and bad (SCC > 1000×10^3) (Gonzalo et al., 2000).

The SC are predominantly white blood cells or leukocytes, including lymphocytes, polymorphonuclear neutrophils (PMNs) and macrophages, which serve as important components in the mammary defense against primarily bacteria (Paape, Bannerman, Zhao, & Lee, 2003). In nearly bacteria-free milk, macrophages (Concha, Holmberg, & Astrom, 1986) and epithelial cells (Leitner, Shoshani, et al., 2000) are the predominant cell type (35–79%) followed by PMNs (3–26%), lymphocytes (10–24%) and epithelial cells (2–15%) (Sohn et al., 2007). After pathogens invade the mammary gland, macrophages release chemoattractants, which trigger the migration of PMNs from the blood toward the infection in the gland. The PMNs are increased from a basal level of 5–25% of the total cell population to approximately 90% of the total cell population. Upon host infection, PMNs ingest and destroy invading pathogens via reactive oxygen species and a wide range of proteolytic enzymes (Paape, Wergin, Guidry, & Pearson, 1979). There is evidence that the oxidative stress associated with clinical mastitis may liberate massive quantities of lysosomal proteolytic enzymes from the PMNs (Le Roux, Laurent, & Moussaoui, 2003). These proteolytic enzymes include neutral and acidic proteases, such as elastase and cathepsins B and D (Considine, Healy, Kelly, & McSweeney, 2004), which, in turn, might increase proteolysis and cause damage to the mammary tissue (Le Roux et al., 2003). During inflammation, an increased secretion of lysosomal enzymes, such as N-acetyl-β-D-glucosaminidase (NAGase), was observed as the major source of mammary epithelial cells (Leitner, Chaffer, et al., 2004; Leitner, Merin, et al., 2004). Therefore, it is difficult to distinguish between leukocyte-derived and epithelial cell-derived enzymes during SCM. Certain enzymes, such as the predominant indigenous milk proteinase plasmin (PL), originate from the blood and enter the milk, indicating the leakage of blood proteins into the milk. PL preferentially cleaves polypeptide chains following lysine (Lys) residues and, to a lesser extent, arginine (Arg) residues (Ueshima, Okada, & Matsuo, 1996). β-Casein (CN) is the preferred substrate for PL and milk proteose–peptones (PP) and γ-caseins result from the enzymatic hydrolysis of native β-CN (Andrews, 1983). β-CN (f1–28) CPP, produced by PL during milk storage, range from 8% to 12% PP and match the formation of γ_1-CN and β-CN (f29–209) (Andrews, 1978). β-CN (f1–28) CPP are resistant to further degradation by PL (Andrews, 1983). α_{s1}-CN (McSweeney, Olson, Fox, Healy, & Højrup, 1993) and α_{s2}-CN (Le Bars & Gripon, 1989) are also susceptible to PL hydrolysis and the former gives rise to λ-CN (Aimutis & Eigel, 1983). κ-CN appears to be quite resistant to plasmin action (Diaz, Gouldsworthy, & Leaver, 1996), whereas cathepsin B and the acid proteinase, cathepsin D, produce the glycomacropeptide, κ-CN (f106–169).

There is a close relationship between the SCC and the level of CN hydrolysis. Mastitic milk has been efficiently detected by measuring the concentration of PL, which increases during infection (Kaartinen & Sandholm, 1986). PL activity in milk is positively correlated with SCC, although a small, but significant correlation between SCC and PL activities has been found even in healthy quarter milk samples with SCC as low as 250,000 cells/ml (Le Roux, Colin, & Laurent, 1995). An increase of the SCC from 100×10^3 cells/ml to 1300×10^3 cells/ml was associated with a 2.3-fold increase in PL (Politis, Ng Kwai Hang, & Giroux, 1989). In addition, alkaline phosphatase (ALP) activity is significantly higher during mastitis compared to normal milk (Mauriello et al., 2007; Yang et al., 2011). Therefore, ALP is suggested as a marker enzyme for mastitis (Akerstedt, Forsbäck, Larsen, & Svennersten-Sjaunja, 2011). The changes in phosphorylation depend on the increased amount of ALP and are associated with a high SCC (Piredda et al., 2000). By consequence, dephosphorylation could occur simultaneously at many different sites, as previously observed for ovine and caprine β-CN (Chianese, Mauriello, Moio, Intorcia, & Addeo, 1992; Neveu, Mollé, Moreno, Martin, & Léonil, 2002). Also, 2-DE has been used to study the ovine CN phosphoproteome (Mamone et al., 2003). However, it is much more difficult to screen for protein alterations than to comprehensively assess the disease-related proteome. Therefore, to determine CN degradation by PL and ALP activities and in turn its possible consequence on milk yield, as found in previous studies (Leitner, Chaffer, et al., 2004), high SCC milk was chosen from individuals with healthy and infected udders. Descriptive gel electrophoresis combined with immunoblotting was used to screen the CN bands. The direct analysis of proteins by electrospray ionization-quadrupole time of flight-tandem mass spectrometry (ESI-Q-TOF MS) allowed for the identification of partially dephosphorylated CN that are released in the milk of infected subjects. Furthermore, to improve our knowledge on the peptide bonds hydrolyzed by PL and the dephosphorylated SerP sites, HA-enriched tryptic CPP were studied. These CPP are useful for unambiguous identification of parent casein by MS. The presence of specific CN peptides and partially dephosphorylated CN/CPP may be possible indicators of mastitis in milk.

2. Materials and methods

2.1. Chemicals

Milk from ovine Sarda breed was obtained from local dairy farms. All animals were primiparous and homogeneous for lambing date, feeding scheme and stage of lactation. The collection of all the samples occurred at third month of lactation, ~12 weeks after lambing. Udder-halves of a flock of 120 sheep were hand milked (once daily) separately and a total of 240 individual milk samples were obtained. Samples were grouped by SCC, i.e. SCC < 500×10^3, 500×10^3 < SCC < 1500×10^3 and SCC > 1500×10^3. Milk samples did not show visible alteration such as flakes, clots, and a watery serum-like appearance. All samples were collected aseptically from each half-udder, immediately mixed with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and stored at 4 °C. Milk sample aliquots were taken for SCC testing with a Fossomatic counter (Fossomatic, Foss Electric, Hillerød, Denmark) and analyzed within 24 h.

On the basis of the SCC, each sample of milk was then blended to have three bulk milks corresponding to the pre-established SCC thresholds. We considered infected the milk containing an SCC exceeding 1000×10^3 cells/ml, while healthy the milk containing an SCC less than 500×10^3 cells/ml (Table 1). Moreover, 68% of sheep had both a healthy and an infected half-udder. The percentage of both infected udder halves was 12%, while that of healthy udder halves was 20%.

Isoelectric CN was obtained from skimmed milk after centrifugation at 3000 *g* for 10 min by the addition of acetate buffer according to the procedure of Aschaffenburg and Drewry (1959).

Tris(hydroxymethyl) aminomethane hydrochloride (Tris–HCl), potassium chloride (KCl), urea, trifluoroacetic acid (TFA), acetonitrile

Table 1
Measurement of phosphatase alkaline activity (milliunits per liter, mU/l) in milk samples with different SCCs.

Milk samples	SCC		ALP mU/l	
	Healthy half-udder	Infected contralateral half-udder	Healthy	Infected
IZ5073	79,000	1,041,000	767.0	1697.2
IZ5073	273,000	1,054,000	460.0	1763.4
IZ4346	46,000	4,897,000	308.5	2051.2
IZ4741	67,000	2,040,000	947.9	2652.9
OS6107	78,000	2,465,000	117.2	1011.8
0S7339	118,000	13,341,000	2548.1	11,492.0