Role of endogenous enzymes in proteolysis of sheep milk

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ABSTRACT

The aim of the present study was to determine the role of milk endogenous proteolytic enzymes in sheep milk cheesemaking ability during lactation. Plasmin, plasminogen, and plasminogen activator in ewe bulk milk were not significantly affected by stage of lactation, probably because of the good health of the ewe udders throughout lactation as indicated by somatic cell count, which never exceeded 600,000 cells/mL. Elastase content increased significantly during lactation, whereas cathepsin showed the greatest content in mid lactation. Early and mid lactation milk showed impaired renneting parameter compared with late lactation milk, probably because of greater α -casein degradation, brought about by cathepsin, and lesser fat and casein (CN) milk contents. Changes in macrophage and neutrophil levels in ewe bulk milk during lactation were also investigated. Macrophages minimally contributed to leukocyte cell count in milk and had the greatest levels at the beginning of lactation. An opposite trend was recorded for polymorphonuclear neutrophilic leucocytes (PMNL) that increased throughout lactation, showing the greatest value in late lactation. Urea-PAGE of sodium caseinate (NaCN) incubated with isolated and concentrated PMNL at 37°C after 48 h at pH 8 showed massive casein degradation that could be ascribed to proteases yielded by PMNL. The increase of PMNL percentage and elastase content in milk, despite the relatively low SCC, suggests that PMNL and elastase underwent a physiological increase associated to the remodeling of mammary gland in late lactation.

Key words: endogenous enzyme, proteolysis, milk coagulation properties, leukocyte differential count

INTRODUCTION

Several studies have focused on endogenous milk enzymes; in particular, some proteolytic enzyme systems such as plasmin and cathepsin D have been well characterized in bovine milk for their origin and role in

milk and cheese. Plasmin is the main native proteolytic enzyme; milk contains the complete plasmin system as plasmin, plasminogen, and a complex system of activators and inhibitors. This system plays a major role in milk casein breakdown, reducing the yield of cheese and casein owing to the loss of proteose-peptones in whey (Fox and Kelly, 2006). Other endogenous proteolytic enzymes associated with somatic cells in milk are elastase, cathepsins, and collagenase (Kelly and McSweeney, 2002). Cathepsin D is an acid aspartic proteinase and, as with plasmin, it is part of a complex system, including inactive precursors (Fox and Kelly, 2006). The level of cathepsin D in milk is correlated with SCC (O'Driscoll et al., 1999) and is associated with milk PMNL and macrophages (Owen and Campbell, 1999). This enzyme contributes to casein breakdown and displays a proteolytic activity similar to that of chymosin. Elastase is a neutral serine-type proteinase mainly associated with PMNL; the potential significance of elastase for proteolysis and quality of milk has been investigated, and cleavage specificity toward bovine α_{S1} - and β -CN has been determined (Considine et al., 1999, 2000). Endogenous milk enzymes are thus technologically significant for the deterioration of milk quality through proteolytic disruption of intact casein.

As previously mentioned, many of the enzymes in milk originate from somatic cells; their presence in milk as active enzymes suggests that their leakage or secretion occurs depending on several physiological or external influences (i.e., stage of lactation or onset of intramammary infection). There has been interest in recent years in evaluating differential as opposed to total SCC as an index of milk quality, on the basis that the latter has the disadvantage of being the sum of several parameters that can vary independently (Kelly and Fox, 2006). It is worth investigating whether specific enzymatic activities could be associated with different somatic cell types in ewe bulk milk, because variations in the activity of milk enzymes are important not only for their physiological role but also as indicators of milk quality in terms of coagulation properties. To the best of our knowledge few studies have been conducted on this topic in small ruminant species.

The aim of this study was to determine the role of milk endogenous proteolytic enzymes in the cheese-

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making ability of sheep milk during lactation. Given that leukocyte populations in milk affect the amount and type of endogenous proteolytic enzymes, changes in macrophage and neutrophil levels in milk were also investigated.

MATERIALS AND METHODS

Experimental Design and Milk Sample Collection

The experiment was conducted from April to July 2007 in an intensively managed flock of Comisana ewes located in southern Italy. Ewes involved in the trial lambd in the winter of 2007. Ewes were housed on straw litter; they grazed and were supplemented with hay and concentrate. Ewes were healthy at the beginning of the trial and were checked for health by veterinarians throughout the experiment. Ewes showing any sign of clinical mastitis were excluded from milking. Ewes were milked using pipeline milking machines. Three sampling cycles were performed during early, mid, and late lactation (<70 d, from 110 to 130 d, and >160 d in lactation, respectively). For each sampling cycle, 3 samples were collected in triplicate on 3 consecutive days. A total of 27 bulk milk samples were collected and analyzed for fat, total protein, and lactose (MilkoScan 133B, Foss Electric, Hillerød, Denmark), and for pH value (GLP 21 Crison, Madrid, Spain). Total N, noncasein nitrogen (NCN), and NPN were determined by standard procedures using the Kjeldahl method (IDF, 1993). Casein nitrogen was calculated as the difference between total protein and NCN; whey protein was calculated as the difference between NCN and NPN. Somatic cell count was determined using a Fossomatic Minor (Foss Electric) according to the International Dairy Federation standard (IDF, 1995). Milk renneting characteristics (clotting time, rate of clot formation, and clot firmness after 30 min) were measured using a Foss Electric Formagraph.

Determination of Enzymatic Content in Milk

Plasmin (PL) and plasminogen (PG) activities were determined according to the method of Baldi et al. (1996); the dissociation of PL and PG from casein micelles was obtained by incubation of skim milk with 50 mM of ϵ -aminocaproic acid for 2 h at room temperature (Korycka-Dahl et al., 1983). A standard curve was prepared to convert PL (Sigma Chemical Co., St. Louis, MO) activity to PL concentration by plotting changes in absorbance against concentrations of PL over a range from 0 to 16 mg/L. Plasmin, PG, and plasminogen activator (PA) concentrations were reported as milligrams per liter of milk.

Cathepsin D activity was determined according to the Sigma enzymatic assay of cathepsin D (EC 3.4.23.5) based on the methods of Perlmann and Lorand (1970) and Smith and Turk (1974). The dissociation of acid serum from milk was obtained by incubation of skim milk with 30 μ L of acetic acid 33% (vol/vol) for 10 min at room temperature. After incubation, 30 μ L of 3.3 M sodium acetate was added and the mixture was centrifuged at $14,000 \times g$ for 30 min. The supernatant was recovered and used for the enzymatic assay of cathepsin D. The reaction cocktail consisted of 20 mL of deionized water, 5 mL of citrate buffer 400 mM (pH 2.8), and 20 mL of hemoglobin solution 2.5% (wt/vol; H2625, Sigma Chemical Co.). The mixture was equilibrated at 37°C, and the pH was adjusted to 3.0 with 100 mM HCl. The reaction mixture consisted of 900 μ L of reaction cocktail, 900 μ L of 5% TCA (vol/vol), and 200 μ L of milk acid serum. The solution was filtered through a 0.45 μ M syringe filter in a quartz cuvette. A similar mixture without sample served as negative control. The reaction mixture was incubated at 37°C for 5 h, and absorbance at 280 nm was measured at 30-min intervals using a UV-Vis spectrophotometer (Lambda 25, PerkinElmer Inc., Waltham, MA). A standard curve was prepared to convert cathepsin activity to cathepsin concentration by plotting changes in absorbance against concentrations of cathepsin over a range from 0 to 16 mg/L. Cathepsin concentration was reported as milligrams per liter of milk.

Elastase activity was determined according to the Sigma enzymatic assay of elastase (EC 3.4.21.36) based on the method of Bieth et al. (1974); the dissociation of acid serum from milk was performed as described previously. The reaction mixture consisted of 100 μ L of Tris-HCl 0.1 M (pH 8.0), 20 μ L of N-succinyl-alanyl-alanyl-p-nitroanilide (S4760, Sigma Chemical Co.), and 24 μ L of milk serum. A similar mixture without sample served as a negative control. A standard curve was prepared to convert elastase (Sigma Chemical Co.) activity to elastase concentration by plotting changes in absorbance against concentrations of elastase over a range from 0 to 16 mg/L. Elastase concentration was reported as milligrams per liter of milk.

Leukocyte Differential Count

Milk samples were centrifuged at $2,000 \times g$ for 30 min at 4°C in 50-mL centrifuge tubes. The fatty fraction and supernatant were removed, and each pellet was dissolved in 500 μ L of PBS, pH 7.4, + 0.02% (wt/vol) NaN_3 . Recovered pellets were centrifuged twice at $400 \times g$ for 15 min at 4°C. A concentration of at least 10^6 cells/mL was obtained.

Cell pellets were shared into 2 aliquots to quantify the populations of lymphocytes, macrophages and PMNL in milk. A first aliquot of 150 μL of cells was labeled with 5 μL of mouse anti-human CD14:R-phycoerythrin (**RPE**; MCA1568PE, Serotec, Oxford, UK) + 10 μL of mouse anti-bovine CD11b:fluorescein isothiocyanate (**FITC**; MCA1425F, Serotec) at room temperature for 30 min, to quantify milk PMNL. A second aliquot of 150 μL of cells was labeled with 5 μL of mouse anti-human CD14:RPE + 10 μL of mouse anti-bovine CD5:FITC at room temperature for 30 min to quantify milk lymphocytes and macrophages. Cells were then resuspended in PBS (pH 7.4) + NaN_3 (0.02%, wt/vol) to a volume of 1 mL. The cell suspension was then centrifuged at $400 \times g$ for 5 min, the supernatant was poured off, and the pellet of cells was resuspended in 800 μL of PBS. All these samples were acquired by flow cytometry (Cell Lab Quanta SC, Beckman Coulter Inc., Fullerton, CA). Linear amplification of the forward scatter and side scatter light signals was set with logarithmic amplification of the fluorescence signals. An excitation wavelength of 488 nm was used.

Milk lymphocytes, macrophages, and PMNL were selected for analysis by gating on the forward scatter and side scatter dot plot. Fluorescence of FITC and RPE was measured through 530/30 (fluorescence 1, FL1) and 585/42 (fluorescence 2, FL2) band pass filters, respectively. Then, FL1 versus FL2 was used to determine the proportions of CD14/CD11b and CD14/CD5. Approximately 10,000 events were collected for each sample.

Macrophage and PMNL Isolation

Separation of macrophages from milk somatic cells was performed according to Caroprese et al. (2008) by a magnetic positive separation (EasySep, StemCell Technologies, Vancouver, Canada) using monoclonal mouse IgG anti-ovine macrophages (MCA919, Serotec).

Isolation of PMNL from milk cells was performed by negative selection, using monoclonal mouse IgG anti-ovine macrophages and monoclonal mouse IgG anti-bovine lymphocytes. Cells were targeted for selection by using a mix consisting of the mouse IgG anti-ovine macrophages (750 μL at a concentration of 25 $\mu\text{g}/\text{mL}$) and of the IgG anti-bovine lymphocytes (750 μL at a concentration of 25 $\mu\text{g}/\text{mL}$), mouse IgG₁ directed against dextran, rat monoclonal antibody against the Fc region of mouse IgG₁ supplied by the kit (EasySep), and PBS (pH 7.4) + NaN_3 (0.02% wt/vol). Concentrated cells were resuspended in at least 100 μL of PBS (pH 7.4) + NaN_3 (0.02% wt/vol) in a 12- $\times$ 75-mm polystyrene tube, according to their concentration. The tetrameric antibody complex was added to the cell suspension at

100 $\mu\text{L}/\text{mL}$ of cells and incubated at room temperature for 15 min. Magnetic dextran iron nanoparticles were then added to the cell suspension at a concentration of 100 $\mu\text{L}/\text{mL}$ and incubated at room temperature for 10 min. Cells were resuspended in PBS (pH 7.4) + NaN_3 (0.02%) up to a total volume of 2.5 mL and then mixed in the tube according to supplier's instructions. The tube was then placed into the magnet for 10 min and the supernatant fraction, containing PMNL, was recovered in a second tube by firmly inverting the magnet and tube. The first tube was again placed into the magnet twice for 5 min, the supernatant was recovered again and centrifuged at $400 \times g$ for 10 min. The supernatant was poured off and the PMNL were suspended in 2 mL of PBS (pH 7.4) + NaN_3 (0.02% wt/vol).

To evaluate the purity of isolated PMNL by cytometry, 2 aliquots of cells (100 μL) were labeled with 10 μL of mouse anti-bovine CD 11b:FITC at room temperature for 30 min; cells were then resuspended in PBS (pH 7.4) + NaN_3 (0.02%) to a volume of 1 mL. The cell suspension was then centrifuged at $400 \times g$ for 5 min, the supernatant was poured off, and the pellet of cells was resuspended in 800 μL of PBS. Approximately 5,000 events were collected for each sample on flow cytometry (Cell Lab Quanta SC, Beckman Coulter Inc.).

Urea-PAGE of Milk and Na Caseinate

Skim bulk milk samples were incubated at 37°C up to 48 h and analyzed by alkaline urea-PAGE using stacking and separating gels prepared according to Andrews (1983). The samples diluted with sample buffer (1:10) were heated to 50°C for 5 min and then loaded on the gels. The run was performed in a continuous buffer system using a Protean II xi vertical slab gel unit (BioRad, Watford, UK).

A 4% ovine Na caseinate (**NaCN**) in Tris buffer solutions (pH 8, 6.5, and 5) were used to investigate the proteolytic activity of lysed macrophages and PMNL during early, mid, and late lactation. Whole CN and NaCN were prepared from a sample of ovine skim milk according to Mulvihill and Fox (1977). Sodium caseinate was dissolved in 0.1 M Tris buffer (Sigma Chemical Co.) and 0.05% (wt/vol) NaN_3 . Lysed macrophages and PMNL were incubated in NaCN buffer up to 48 h at 37°C. The effects of macrophage and PMNL proteolytic activity on NaCN were evaluated by urea-PAGE. The gels were stained according to the method of Blakesley and Boezi (1977) with Coomassie Brilliant Blue G250. The destained gels were acquired by the Gel Doc EQ system (BioRad) using a white light conversion screen and analyzed with the Quantity One software (BioRad) to determine the signal intensity (optical density) of the defined bands. Identification of bands was done by

Table 1. Least squares means of chemical composition and renneting parameters of sheep milk

Item	Stage of lactation			SEM	Effect
	Early	Mid	Late		Stage of lactation
pH	6.71 ^b	6.56 ^{ab}	6.51 ^a	0.06	*
SCC, log ₁₀ cells/mL	5.85	5.75	5.85	0.06	NS
Fat, %	5.62 ^a	5.83 ^{ab}	6.11 ^b	0.1	**
Protein, %	5.08 ^a	5.42 ^b	5.59 ^c	0.05	***
Lactose, %	4.67	4.7	4.75	0.04	NS
Casein, %	3.89 ^a	4.22 ^b	4.34 ^b	0.05	***
Whey protein, %	1.19 ^a	1.20 ^a	1.25 ^b	0.01	***
Clotting time, min	19.34 ^b	17.90 ^{ab}	15.95 ^a	0.94	***
Rate of clot formation, min	1.43 ^b	1.36 ^a	1.32 ^a	0.02	**
Clot firmness at 30 min, mm	52.59 ^b	59.32 ^a	60.98 ^a	1.40	***

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

comparison with the NaCN standard. With the sum of the intensity of the defined bands in a lane set at 100%, the relative quantity of each band was determined as the percentage of the signal intensity of the defined band in a lane.

Statistical Analyses

Data were processed by ANOVA, using the GLM procedure (SAS Institute, 1999). The following model was used:

$$y_{ij} = \mu + \alpha_i + \beta_{ij} + \varepsilon_{ij},$$

where μ was the overall mean, α was the effect of stage of lactation ($i = 1$ to 3), β was the sample variation within the stage of lactation, and ε was the error.

Bulk milk SCC and isolated macrophage and PMNL counts were transformed into logarithms to normalize their frequency distributions before performing statistical analysis. Values recorded in milk before incubation were used as covariates for percentage composition of the CN and CN degradation products measured after 24 and 48 h of incubation. When significant effects were found (at $P < 0.05$), the Student t -test was used to locate significant differences between means. Linear simple correlations between renneting parameters and milk constituents, and endogenous enzymes were also investigated.

RESULTS AND DISCUSSION

Milk Composition

Sheep milk is destined totally for dairy product manufacture; raw milk is often used to preserve nutritional features and indigenous microflora during cheese ripening. The role of endogenous enzymes is critical

during milk storage and before cheesemaking, because enzymes can cleave caseins and impair milk coagulation properties.

In this study, stage of lactation significantly affected fat, protein, casein, and whey protein percentage in milk (Table 1); in general an increase was observed for these components with the advancement of lactation. This effect could be ascribed to a progressive reduction in the volume of milk yielded, which resulted in a concentration of milk constituents. In mid and late lactation, lower pH values could be related to the greater casein content that contributes to milk acidity. Another factor responsible for pH reduction in late lactation milk, although not investigated in the present survey, could be the decrease of milk calcium content, which has been observed in sheep milk during the summer season (Sevi et al., 2004). Somatic cell counts did not display significant differences throughout lactation, always being $<600,000$ cells/mL. Sevi et al. (1999) stated that a SCC of 700,000/mL is the threshold of acceptability of sheep milk destined for cheesemaking. Thus, based on milk SCC, the flock used in the present study produced good-quality milk in terms of hygienic and processing quality, so changes in enzyme proteolytic activity in milk depended on stage of lactation and were not affected by ewe udder health.

Endogenous Enzymes in Milk and Milk Electrophoresis

The major enzyme present in milk is the PL-PG system, which is under the control of a complex system of inhibitors and activators. Other proteolytic enzymes in milk originate from somatic cells whose lysosomes contain elastase, collagenase, and cathepsins (Verdi and Barbano, 1991).

Plasmin, PG, and PA in milk were not significantly affected by stage of lactation (Table 2). It has been

Table 2. Least squares means of plasmin, plasminogen, plasminogen/plasmin, total plasminogen activator, elastase, and cathepsin D in sheep milk during lactation

Item	Stage of lactation			SEM	Effect
	Early	Mid	Late		Stage of lactation
Plasmin, mg/L	0.84	1.32	1.48	0.18	NS
Plasminogen, mg/L	1.34	1.24	1.87	0.18	NS
Plasminogen/plasmin	0.76	0.80	1.31	0.17	NS
Plasminogen activator, mg/L	2.27	2.59	2.80	0.14	NS
Elastase, mg/L	0.10 ^a	0.19 ^b	0.95 ^c	0.09	**
Cathepsin D, mg/L	2.41 ^b	3.24 ^c	2.04 ^a	0.06	***

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

** $P < 0.01$; *** $P < 0.001$.

reported that milk somatic cells can convert PG to PL through urokinase-PA; stable levels of PL-PG system throughout lactation could be explained, in part, by the lack of differences in SCC. However, an increase in the content of the aforementioned enzymes occurred with the advancement of lactation probably because of the gradual involution of the mammary gland (Caroprese et al., 2007). Levels of elastase and cathepsin displayed a different trend throughout lactation; the content of elastase increased significantly, whereas cathepsin showed the greatest content in mid-lactation milk. Cathepsin D is the second proteinase identified in milk that is ostensibly a lysosomal enzyme but is present in acid whey (Larsen et al., 1996). The correlation between SCC and cathepsin D activity is controversial. O'Driscoll et al. (1999) suggested that cathepsin activity in milk is correlated with SCC but whether this reflects an increased amount of enzyme or increased activation of pro-cathepsin remains to be determined. In contrast, Somers et al. (2003) reported that cysteine protease activity was increased only in individual cow milk samples with SCC >800,000 cells/mL, not in milk with lower SCC. Somatic cells destroy bacteria by lysosomal enzymes (Paape et al., 2003) that degrade bacterial constituents such as proteins and cell-wall polysaccharides (Kelly and Fox, 2006).

Milk was incubated up to 48 h at 37°C to evaluate the effect of endogenous enzymes on casein hydrolysis; β -CN did not show evidence of appreciable hydrolysis, whereas α -CN underwent a slight degradation during incubation resulting in polypeptides with greater electrophoretic mobility than the intact α -CN (urea-PAGE is not shown). The quantities of β - and α -CN degradation products after 24 and 48 h of incubation at 37°C are reported in Table 3. The amount of β -CN degradation products was not significantly different among the stages of lactation, partly because of the lack of differences in the plasmin content of the milk, β -CN being the preferred substrate of plasmin activity. Considine et al. (1999) demonstrated that elastase

added exogenously to β -CN at pH 7.5 had broad cleavage specificity on this casein fraction, with some of the cleavage sites being identical to or near those cleaved by plasmin, chymosin, or cell envelope-associated proteinases of several strains of *Lactococcus*. Apart from its level, the contribution of elastase activity to the hydrolysis of β -CN fraction seemed to be negligible with respect of stage of lactation, because of the inhibitory effect of the milk pH being lower than the optimal pH value for elastase. Products of α -CN degradation displayed an increase from 0 to 24 h of incubation in all milk samples, whereas the same parameter underwent a decrease of 23% from 24 to 48 h only in mid-lactation milk, probably because of secondary hydrolysis of the fragment deriving from α -CN. Caseins have been reported to be degraded by leukocyte proteinases in the following order $\alpha > \beta > \kappa$ -casein (Considine et al., 2004). In particular, cathepsin D from milk degraded α_{S1} -casein to a peptide (α_{S1} -CN f24–199) with a proteolytic specificity similar to that of chymosin; the α_{S1} -CN f24–199 peptide is further degraded by cathepsin D in vitro (Hurley et al., 2000). Accordingly, the greatest cathepsin content detected in mid-lactation milk could be responsible for the disappearance of α -CN degradation products, thus supporting the hypothesis that cathepsin can play a role in proteolysis of milk during storage.

Milk Coagulation Properties

Milk renneting parameters (Table 1) improved with the advancement of lactation, probably due in part to greater α -CN degradation and in part to lower fat and protein contents found in early- and mid-lactation milk compared with late-lactation milk. Furthermore, the lower clot firmness detected in early-lactation milk could be an outcome of casein breakdown brought about by cathepsin, α_S -CN being a structural component of the casein micelle with a functional role in cheese curd formation (Walstra et al., 1984). This parameter is

Table 3. Changes in casein degradation products (β -CN and α -CN products¹) after 24 and 48 h of incubation at 37°C of sheep milk during lactation

Degradation products	Incubation, h	Stage of lactation			SEM	Effect
		Early	Mid	Late		Stage of lactation
β -CN, %	24	3.43	4.39	3.70	0.51	NS
	48	3.62	4.04	3.93	0.38	NS
α -CN, %	24	4.57	5.27	4.50	1.12	NS
	48	6.40 ^b	4.06 ^a	4.54 ^a	0.47	*

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).
¹ β -CN and α -CN degradation products were determined by urea PAGE and were measured as area of the individual electrophoretic bands as a percentage of total band area.
* $P < 0.05$.

the most critical coagulation property because it influences cheese quality, yield, and economic returns (Clark and Sherbon, 2000). As expected, casein content was negatively correlated with clotting time and rate of clot formation ($r = -0.54$, $P < 0.01$ and $r = -0.60$, $P < 0.001$, respectively) and positively correlated with curd firmness ($r = 0.71$, $P < 0.001$). It is worth noting that clotting time was positively ($P < 0.05$) correlated with cathepsin, confirming that casein hydrolysis carried out by cathepsin can impair the coagulating behavior of ewe milk.

Differential Cell Count in Milk and NaCN Incubation with Isolated PMNL Cells and Macrophages

Percentages of the main leukocyte populations in milk (i.e., macrophages, PMNL, and lymphocytes) enumerated by flow cytometry are reported in Table 4. Lymphocytes were lowest at the end of lactation, with the percentage found in late lactation being almost half that observed in early and mid lactation; an opposite trend was recorded for PMNL, which increased throughout lactation. In agreement, PMNL are known to increase physiologically during late lactation (Pillai et al., 2001).

In this study macrophages contributed minimally to total SCC and this cell population was the highest in early-lactation milk. Changes in milk somatic cell populations, particularly the dynamics of PMNL and

macrophages, are still debated because no conclusive results have been obtained. Macrophages are reported to be the predominant cells in cow milk from a healthy udder (Kelly and Fox, 2006). The percentage of macrophages in individual ewe milk from noninfected udders was 57.33%; this cell type decreased during mid lactation and increased again near the dry period (Morgante et al., 1996). In individual ewe milk, Albenzio et al. (2004) found that PMNL and lymphocytes were predominant in milk with high SCC, whereas the macrophage percentage did not exceed the threshold of 4.30%. Caroprese et al. (2007) found that in ewe bulk milk, macrophages significantly decreased in late lactation. Cuccuru et al. (1997) found in individual milk that PMNL ranged between 30 and 40% when SCC was <100,000 cells/mL but increased to 70% when SCC exceeded 400,000 cells/mL; macrophage concentration had the opposite trend, displaying an increase when PMNL cells decreased. It is worth noting that, in this study, both PMNL and elastase displayed the same behavior during lactation supporting the hypothesis that both parameters could be related to the physiological state of the mammary gland. Thus, the elastase content in ewe bulk milk could be considered a reliable indicator of mammary gland involution.

The urea-PAGE electrophoretogram of NaCN incubated with isolated and concentrated macrophages and PMNL at 37°C after 48 h at pH 5, 6.5, and 8 is shown in Figure 1. Proteolysis of NaCN incubated

Table 4. Percentage of differential cell populations in sheep milk during lactation

Item	Stage of lactation			SEM	Effect
	Early	Mid	Late		Stage of lactation
Lymphocytes, %	43.02 ^a	40.57 ^a	26.15 ^b	3.52	***
PMNL, %	49.39 ^b	57.07 ^b	70.58 ^a	3.68	***
Macrophages, %	7.59 ^b	2.36 ^a	3.27 ^a	0.74	***

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).
*** $P < 0.001$.

Figure 1. Urea-PAGE electrophoretogram of sodium caseinate (NaCN) incubated with isolated and concentrated macrophages and PMNL at 37°C after 48 h at different pH. Lanes 1, 2, and 3: NaCN incubated with macrophages at pH 8, 6.5, and 5, respectively; lanes 4, 5, and 6: NaCN incubated with PMNL at pH 8, 6.5, and 5, respectively.

at different pH with isolated macrophages showed evidence of slight hydrolysis of α - and β -CN without relevant differences throughout lactation. In samples of PMNL isolated from mid- and late-lactation milk at pH 8, massive degradation of casein was reported, with caseins disappearing almost totally. This finding could be ascribed to proteases yielded by PMNL. When NaCN was incubated at pH 8, elastase displayed its proteolytic potential resulting in major degradation of casein. Considine et al. (1999; 2000) reported that β -CN was readily degraded by elastase and α_{S1} -CN showed at least 25 cleavage sites principally in the C- and N-termini of the molecule.

CONCLUSIONS

The study of changes in endogenous proteolytic enzymes in sheep milk during lactation suggests that the plasmin-plasminogen system does not vary significantly when SCC remains relatively low throughout lactation. The positive correlation between clotting time and cathepsin levels suggests that casein hydrolysis carried out by cathepsin can impair the coagulating behavior of sheep milk. Differential cell counts showed that PMNL increased physiologically with lactation, being largely

predominant in late-lactation milk. Changes in elastase levels in milk closely followed those found in PMNL suggesting that elastase concentration could be a reliable indicator of mammary gland involution in healthy udders. Proteolytic patterns of NaCN incubated with PMNL and macrophages isolated from sheep milk indicated that PMNL release lysosomal enzymes, which induce a more intense hydrolysis on casein in late than in early lactation.

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