J. Dairy Sci. 84:1786–1790

Plasminogen Activation System in Goat Milk and its Relation with Composition and Coagulation Properties

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ABSTRACT

The activity of plasmin (PL), plasminogen (PG), and plasminogen activator (PA) and their correlation with goat milk components and milk clotting parameters were investigated. Seven late-lactating Saanen goats were used to provide milk samples that were analyzed for PL, PG, and PA activity (colorimetric assay) fat, protein, noncasein nitrogen, nonprotein nitrogen, casein content, and somatic cell count (SCC). Milk clotting parameters (rennet coagulating time = coagulation time; K20 = firming rate of curd; A30 = curd firmness) were measured with a formagraph. Average milk yield and composition were similar to those previously observed in other studies. Plasmin, PG, and PA activity, expressed as units/ml, were, respectively, 20.04 ± 0.94, 3.21 ± 0.04, and 1154 ± 57.61. Plasminogen activity was surprisingly low compared with other species (bovine, ovine), but it was consistent with the high activity of PA. A negative significant correlation was observed between PL and milk casein content. The correlation coefficients between PL and casein/protein ratio and PA and casein/protein ratio were negative and significant. A positive significant correlation was observed between PL and rennet clotting time and PA and rennet clotting time. Also positive was the correlation between PL and K20 and PA and K20. The plasmin activity was negatively correlated with A30. High plasmin and plasminogen activator activity in goat milk appeared to be negatively related with coagulating properties in late lactation, most probably via degradation of casein due to plasmin activity.
(**Key words:** plasminogen activation system, coagulation properties, goat milk)

Abbreviation key: NCN = noncasein nitrogen, **PA** = plasminogen activator, **PG** = plasminogen, **PL** = plasmin.

INTRODUCTION

The number of goats in Italy is approximately 1.2 million (ASSO.NA.PA, 2000), and goat milk is mostly processed for cheese manufacturing. The cheese-making qualities of milk depend on many factors, the most important of which are the concentrations of intact casein and fat. Milk in which casein has been broken down by proteolytic enzymes is of less value to cheese manufacturers (Lucey and Kelly, 1994). Furthermore, in bovine milk, prehydrolysis of milk protein adversely affects the flavor and texture of cheese (bitterness and astringent off-flavor, longer clotting time, reduced curd firmness, high moisture content) (Fox, 1992; Harwalkar et al., 1993). Plasmin (**PL**) (EC 3.4.21.7), a serine-proteinase, appears to be the predominant native proteinase in milk, and it is mainly associated to casein micelles, which represent its substrate (Bastian and Brown, 1996). Plasmin occurs in milk together with its inactive zymogen, plasminogen (**PG**) (Bastian et al., 1991). The chain of reactions leading to plasminogen activation is regulated by a complex network of molecular interactions between plasminogen activators (**PA**) (EC 3.4.21.31) (tissue-type and urokinase-type) and at least three types of specific PA inhibitors (Politis, 1996). Inhibitors of plasmin were also reported (Precetti et al., 1997). The plasminogen activation system seems to be involved in tissue remodeling events that occur during the gradual involution of mammary gland. In fact, stage of lactation affects PL and PA activities: late lactation is associated with higher activity of PL and PA (Baldi et al., 1996). Plasmin in milk is responsible for the hydrolysis of α- and β-caseins (Aslam and Hurley, 1997; Trujillo et al., 1997). This detrimental effect could be more pronounced in dairy goats, since, having a seasonal breeding they progress through lactation in a synchronous manner, therefore all animals are at the same stage of lactation at a given time. The health status of the mammary gland also affects PL and PA activity, which increase during mastitis (Bastian and Brown, 1996; Gilmore et al., 1995). Most of the existing studies on the plasminogen activation system were carried out on bovine milk, and available information is scarce for other species; in particular, there is almost no information regarding goat milk.

Received August 7, 2000.
Accepted April 2, 2001.
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The aim of this study was to evaluate, in goat milk, the activity of plasmin, plasminogen, and PA and their correlation with milk production and composition and with coagulation properties of milk.

MATERIALS AND METHODS

Origin of Milk Samples and Compositional Analysis

Seven Saanen goats in late lactation (approximately 170 DIM), matched for parity and milk production in the previous lactation, were used to provide milk samples in a 2-mo study. Goats were milked twice daily using a rotating milking machine. Individual milk yield was recorded weekly, and at the same time milk samples were collected during the morning and evening milking. One aliquot of the samples was immediately analyzed for major milk components and coagulation properties and another aliquot was frozen and stored at –80°C until analysis. Milk samples were analyzed for fat, protein (Milkoscan-Foss Electric, Hillerod, Denmark) and SCC (Fossomatic-Foss Electric). Total nitrogen, noncasein nitrogen (**NCN**) and NPN were determined by standard procedures (IDF, 1964). Casein nitrogen was calculated as the difference between total protein and NCN. Whey protein was calculated as the difference between NCN and NPN. All nitrogen results were expressed as protein equivalent using a conversion factor of 6.38. The pH of each milk sample was also measured with a potentiometer.

Determination of Plasmin, Plasminogen, and Plasminogen Activator Activities

Activities of PL, PG, and PA were determined with a method described by Baldi et al. (1996). Briefly, PG and PA activities were defined as the plasmin activity that was generated after the addition of 30 plough units (2.5 μl) of urokinase (U8627; Sigma Chemical Co., St. Louis, MO) or plasminogen (50 μg/ml; P5661; Sigma Chemical Co.), respectively. Plasmin activity was measured without added urokinase or plasminogen. Plasminogen and PA activity was calculated by subtracting endogenous PL activity. Assays were performed in duplicate in 250 μl of 0.1 *M* Tris-HCl buffer (pH 7.4), 0.6 m*M* Val-Leu-Lys-*p*-nitroanilide (V7127; Sigma Chemical Co.), and 5 μl of sample for PA determination or 30 μl of sample for PL and PG determination. An analysis in which sample was replaced by buffer was used as control to detect spontaneous breakdown of the substrates; in all cases, spontaneous hydrolysis was negligible. The reaction mixture was incubated at 37°C for 3 h and A_{405} was measured at 30-min intervals with a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA). The rate of *p*-nitroanilide formation was calculated from the linear portion of the absorbance versus time curve. Plasmin, PG, and PA activities were expressed as units, one unit being the amount of enzyme that produces a change in absorbance at 405 nm of 0.1 in 60 min.

Table 1. Least square means and standard error of milk composition, coagulation parameters and plasmin, plasminogen, and plasminogen activator activities.

n = 112	Means	SEM
Fat, %	2.92	0.08
Protein, %	3.18	0.04
Casein, %	2.42	0.05
Casein number[1]	75.82	0.71
Whey protein, %	0.59	0.02
Nonprotein nitrogen, %	0.24	0.02
Log SCC·10^{-3}	2.8	0.07
pH	6.78	0.01
RCT[2], min	14.60	0.49
K20[3], min	5.53	0.42
A30[4], mm	21.36	0.70
Plasmin, Units/ml	20.04	0.94
Plasminogen, Units/ml	3.21	0.04
Plasminogen activator, Units/ml	1154.29	57.61

[1]Casein nitrogen as % of total nitrogen.
[2]RCT = Rennet clotting time.
[3]K20 = Rate of firming.
[4]A30 = Curd firmness.

Coagulation Properties of Milk

Milk coagulation properties for each milk samples were measured at 35°C with a Formagraph (Foss Electric) as previously described (Bastian et al., 1991). From the formagraph tracing, which illustrates development of viscosity in the milk after rennet addition, rennet clotting time was determined as the time from rennet addition to the beginning of coagulation. Rate of firming (**K20**) was defined as the time for clotting until the amplitude had reached 20 mm on the recording chart. Curd firmness (**A30**) was defined as the amplitude of the trace following 30 min following rennet addition.

Statistical Analysis

Least square means and standard error for the considered variables were calculated using the SAS system package (1996). Correlation coefficients were also calculated to investigate relationships between variables. Log transformation was applied to SCC before analysis.

RESULTS AND DISCUSSION

Milk Production, Major Milk Components, and Coagulation Properties of Milk

The overall means and standard error for major milk components are summarized in Table 1. These values

are within the ranges reported by others (Lucaroni et al., 1993; Morand-Fehr and Hervieu, 1999). The average milk SCC of approximately 6.3×10^5/ml was in a common range for goat milk (Galina et al., 1996; Schuppel and Schwope, 1999); a high SCC is considered normal in goat milk, particularly in late lactation (Wilson et al., 1995; Zeng and Escobar, 1995). On the other hand, a relationship between goat milk SCC, udder health, and milk quality has been reported (Contreras et al., 1996; Marti et al., 1998; Sung et al., 1999; White and Hinkley, 1999). Average milk production during the experimental period was 1.72 ± 0.09 kg/d.

Milk rennet clotting time, rate of firming, and curd firmness are summarized in Table 1. These results are similar to those reported by others (Ambrosoli et al., 1988; Lucaroni et al., 1993; Marti et al., 1998) but different to those reported by Espinoza and Calvo (1998) and Lopez-Fandino and Olano (1998). Moreover, our results on goat milk differ from those previously reported for bovine milk (Bastian et al., 1991; Politis and Ng-Kwai-Hang, 1988).

Plasmin, Plasminogen, and Plasminogen Activator Activity

Overall means for PL, PG, and PA activities are reported in Table 1. Plasmin activity had an overall mean of 20.04 units/ml with a standard error of 0.94. To our knowledge, there are no previous data in literature for comparing our results for goat milk. The only paper on this subject (Marti et al., 1998), reported an average activity for PL of 20.1 (regardless to the units of measurements) in goat milk with less than 800,000 SCC/ml. It is very difficult to compare data between our experiment and those obtained previously for other species since, in the literature, results about PL were mostly expressed as change in absorbance/hour, or as an arbitrary unit. However, our results on PL activity were 17 to 50% higher than those observed during late lactation, using the same method, and the same expression of results, in bovine milk (Baldi et al., 1996) and 45% higher than in ovine milk (Chiofalo et al., 1999). The activity of PG (Table 1) was surprisingly low, with an overall mean of 3.21 units/ml and with a standard error of 0.04. In fact, PG activity is always reported to be in excess with respect to PL activity (Baldi et al., 1998; Dupont et al., 1998; Fantuz et al., 2001; Politis et al., 1992). Inactive PG, which occurs in most body fluids, serves as an unlimited supply of proteolytic activity, "a proteolytic reservoir" as reported by Politis (1996). Such a low PG activity could be due to a low influx from blood onto the mammary gland or to a rapid local conversion of PG in active PL by the action of PA. Overall mean PA activity was 1154.29 units/ml with a standard error of 57.61. The presence of both tissue PA and urokinase PA in goat milk has already been reported (Politis et al., 1994), but a quantitative comparison with that study is impossible because PA activity was expressed in different units. In our research, PA activity was much higher than that reported in ovine and bovine milk (Baldi et al., 1996, 1998; Fantuz et al., 2001), partly confirming the hypothesis that, in goat milk, most of the inactive PG was apparently converted in active PL. However, caution should still be exercised, and further work is needed to characterize goat PG and the activity of PL and PA inhibitors.

Correlation Between Plasminogen Activation System and Milk Composition and Coagulation Properties

Correlation coefficients between PL, PG, PA, and milk composition and coagulation properties of milk are presented in Table 2.

Plasmin, PG, and PA activities were not significantly correlated with milk production. A negative correlation between PL with milk casein and casein number indicated that a higher concentration of PL was associated with a lower casein content and casein number in milk. The correlation between PA and casein number was negative and significant, but between PA and casein content did not reach the significant threshold. Plasmin activity, as well as PA activity, significantly correlated with rennet clotting time and K20, but the correlation coefficients with A30 was significant only for PL activity. These data indicated that high levels of PL and PA in goat milk are associated with a deterioration of coagulating properties of milk, most probably due to casein proteolysis related to PL activity. In fact, PL activity in milk results in a production of heterogeneous peptides (γ-caseins and proteose-peptones) generated from α_s- and β-casein degradation (Aslam and Hurley, 1997; Bastian and Brown, 1996; Trujillo et al., 1997). This phenomenon is more evident in goat milk from late-lactating animals when the proportions of individual caseins altered as lactation progress: α- and β-caseins decreased and γ- and κ-caseins increased (Brown et al., 1995). Previous works found that milk with poor rennet properties contained a large amount of γ-casein (Okigbo et al., 1985). The positive correlation observed between PG activity and casein content and casein number (Table 2) would suggest that a lower conversion of inactive PG in PL was associated with a lower casein degradation, but this hypothesis was not confirmed because correlation coefficient between PG and PL activities did not reach the significant threshold. Correlation coefficients between NPN and PL, PG, and PA activities were not significant. Correlation between PA and PL

Table 2. Correlation[1] coefficients between plasminogen activation system and some milk major components and coagulation properties.

n = 112	Casein, %	Casein number[2]	Whey protein	pH	SCC	RCT[3]	K20[4]	A30[5]
Plasmin	–0.24*	–0.50**	0.65**	0.33**	0.38**	0.22*	0.45**	–0.22*
Plasminogen	0.89**	0.23*	NS	–0.23*	NS	NS	NS	0.34**
Plasminogen activator	NS	–0.269*	0.38**	0.34**	0.49**	0.52**	0.38**	NS

[1]Only significant correlations are shown.
[2]Casein nitrogen as % of total nitrogen.
[3]RCT = Rennet clotting time.
[4]K20 = Rate of firming.
[5]A30 = Curd firmness.
*$P < 0.05$.
**$P < 0.01$.
NS: Nonsignificant.

was positive and significant (r = 0.43; $P < 0.01$). The positive correlations observed between milk pH and PL, PA (Table 2), and SCC (r = 0.23; $P < 0.01$) were in agreement with previous findings in bovine milk (Aaltonen et al., 1988; Le Roux et al., 1995) These results can be explained by a direct casein degradation due to PL activity, considering correlation between PL activity and casein content, together with a leakage of serum components from blood to milk. In fact, the positive correlation observed between PL and total milk protein content (r = 0.273; $P < 0.01$) could be attributed to the leakage of serum protein from blood onto milk due to an increase in permeability of the mammary epithelium during the declining phase of lactation (also called gradual involution). This hypothesis is strengthened by the positive correlation between PL and PA activities with whey protein percentage (Table 2). High PL and PA activities in late lactation were reported to be related to the increased permeability of mammary epithelium via disruption of tight junction between mammary epithelial cells (Stelwagen et al., 1994). In fact, the primary effect of plasmin in tissue remodeling events is the breakdown of matrix and basement membrane proteins (Politis, 1996).

The association between SCC and PL and PA activities (Table 2) confirms the fact, already observed in bovine and ovine milk, that proteolytic activity in milk also relates to the health status of the mammary gland (Baldi et al., 1996; Gilmore et al., 1995; Le Roux et al., 1995). However, it must be noted that healthy dairy goats with healthy udders produced milk with more than 1×10^6 SCC/ml particularly in late-lactation stages (Zeng and Escobar, 1995).

CONCLUSIONS

In summary, the present study evaluated the plasmin, plasminogen, and plasminogen activator activities in milk from late-lactating Saanen goats and the correlation with milk composition and coagulation properties of milk. Only residual plasminogen activity appeared to be detectable; most of the plasminogen appeared to be converted in active plasmin by the action of the high level of plasminogen activator activity. High plasmin and plasminogen activator activities were associated with a deterioration of coagulation properties of milk, most probably because of protelysis of casein by plasmin activity together with a leakage of serum protein from blood to milk.

ACKNOWLEDGMENTS

Research supported by a grant from University of Camerino, M.U.R.S.T. 60% (A. Baldi) and C.N.R. 1999 (F. Polidori).

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