Distribution of Plasminogen Activator Forms in Fractions of Goat Milk

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ABSTRACT

Distribution of plasminogen activator forms in fractions of goat milk was examined. Raw milk was centrifuged to separate skim milk, cream, and a somatic cell pellet. Somatic cell extracts were obtained by sonication. Skim milk was centrifuged to separate milk serum and casein micelles. Activity of plasminogen activator was detected in casein, serum fractions, and in association with somatic cells. Plasminogen activator forms in milk casein had approximate molecular weights of 75,000, 50,000, and 30,000. The predominant forms of plasminogen activator in milk serum and in association with milk somatic cells had molecular weights of 30,000 and 50,000. Based on fibrin dependency and inhibition of activity in the presence of amiloride, the forms at 30,000 and 50,000 represent urokinase-plasminogen activator, and the form at 75,000 represents tissue-plasminogen activator.

(**Key words**: plasminogen activator, goat milk)

Abbreviation key: **EACA** = ϵ-aminocaproic acid, **MW** = molecular weight, **PA** = plasminogen activator, **t-PA** = tissue PA, **u-PA** = urokinase PA, **V 7127** = Val-Leu-Lys-*p*-nitroanilide.

INTRODUCTION

Proteolysis in milk negatively affects virtually all dairy foods. Milk in which casein has been broken down by proteolytic enzymes is of less value to cheese manufacturers (10). However, plasmin is essential in ripening of some cheese varieties, such as Swiss, Havarti, and Saint Paulin (3). Surface-active properties of β-casein and its fragments improved following proteolysis by plasmin (17). Plasmin, the most important milk protease, occurs in milk with its inactive proenzyme, plasminogen (2, 5, 7, 12, 13).

Plasminogen activators (**PA**) (EC 3.4.21.31) are serine proteases that are responsible for the conversion of plasminogen to plasmin (6, 9, 11, 14, 18); their role and significance in milk are under intense scrutiny. Multiple molecular forms of the two types of PA, tissue PA (**t-PA**) and urokinase PA (**u-PA**), exist (15, 16, 19). These PA types have different structural properties, immunochemical specificities, and sensitivity to fibrin and amiloride (15, 19). Unlike u-PA, t-PA is preferentially activated in the presence of fibrin fragments (15, 19). The activation occurs through binding of activator and plasminogen to fibrin to form a cyclic complex (15, 19). Amiloride inhibits the activity of u-PA, but not t-PA (16). Amiloride blocks the activity of bovine u-PA, but not bovine t-PA (6).

Lu and Nielsen (9) reported the presence of five proteins, presumably u-PA, in bovine milk obtained from healthy quarters that were capable of activating plasminogen with molecular weights (**MW**) of 93,000, 57,000, 42,000, 35,000, and 27,000. Others (6) reported that the major type of PA associated with the casein micelles in bovine milk obtained from healthy or mastitic quarters was t-PA. Heegard et al. (6) reported that t-PA was 100-fold more abundant than u-PA in bovine milk from healthy or mastitic quarters. Zachos et al. (18) reported the presence of t-PA in association with bovine milk somatic cells. No information is available on the presence and the type of PA in goat milk.

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The objective of the present study was to determine the quantity and type of PA in different fractions of goat milk (casein, serum, and somatic cells).

MATERIALS AND METHODS

Origin of Milk Samples

Thirteen Saanen goats housed at the Miner Institute (Chazy, NY) were used to provide milk samples. Milk was collected separately from all goats four times during mid to late lactation. Samples were immediately transported to our laboratory. The SCC for each individual milk sample was determined with a Foss-o-Matic somatic cell counter (Foss Food Technology, Eden Prairie, MN).

Treatment of Milk Samples

Milk samples (5 ml) were centrifuged at 2000 × *g* for 15 min to produce skim milk and a somatic cell pellet; cream was discarded. Skim milk was centrifuged at 100,000 × *g* for 1 h at 4°C to obtain the supernatant milk serum fraction and the casein pellet. The latter was reconstituted to the original volume (5 ml) in a 50-m*M* Tris buffer (pH 8.0). Reconstituted casein micelles were divided into aliquots. The first aliquot was immediately processed for PA determinations. The second aliquot was utilized for experiments related to dissociation of PA from the casein micelles.

The somatic cell pellet from the centrifugation (2000 × *g*, 15 min) was washed twice with PBS (.15 *M* NaCl, .01 *M* NaH_2PO_4, pH 7.2). Total cell numbers and cell viability were measured in a sample (20 μl) by addition of 5 μl of .25% (wt/vol) Trypan blue and by counting the proportion of cells that were capable of excluding the dye. Typically, 64 to 72% of recovered cells were viable. Three milliliters of PBS containing 10^6 viable cells/ml were then sonicated (Heat Systems-Ultrasonics Inc., Farmingdale, NY) for 45 s. The sonicate was stored at −20°C until use.

Determination of PA Activity in Milk Fractions

A colorimetric assay, previously used to determine PA activity in milk somatic cells (18), was validated to measure PA activity associated with the casein and serum fractions of milk. The assay system utilizes the PA that is present in the respective fraction to convert exogenously supplied inactive plasminogen to active plasmin. Plasmin, so produced, is subsequently allowed to attack the chromogenic substrate Val-Leu-Lys-*p*-nitroaniline (**V 7127**) adjacent to Lys and to liberate the free chromophore, *p*-nitroaniline. In this system, changes in color are directly related to amounts of plasmin and, therefore, indirectly to PA activity.

Assays were performed in 250 μl of 100-m*M* Tris buffer (pH 8.0) containing plasminogen (50 μg/ml; Sigma Chemical Co., St. Louis, MO), .6 m*M* V 7127 (Sigma Chemical Co.), and 1 to 2 μl of milk serum, milk casein, or milk somatic cell extract. Preliminary experiments indicated that PA activity was maximal at .6 m*M* of V 7127, 50 μg/ml of exogenous plasminogen, and pH 8.0; these conditions were maintained throughout subsequent assays. Because of the very small amounts of casein added in the reaction mixture, correction for turbidity was not made. Under these conditions, casein enhanced PA activity (11). The reaction mixture was incubated for 3 h, and absorbance at 405 nm was measured at 30-min intervals using a microtiter plate (Bio-Tek Instruments, Burlington, VT). The rate of *p*-nitroaniline formation was calculated from the linear part of the curve for absorbance versus time. A sample without plasminogen served as a control. Preliminary experiments indicated that PA activity was linear for up to 3 h of incubation and between 0 and 5 μl of sample volume.

Dissociation of PA from Casein Micelles

The second aliquot of reconstituted casein micelles in 50 m*M* Tris buffer (pH 8.0) was incubated for 3 h in the presence of 50 m*M* ε-aminocaproic acid (**EACA**) or 1 *M* NaCl. Earlier studies (1, 6) suggested that these treatments could result in dissociation of PA from the casein micelles and transferral into the buffer medium. Treated casein micelles were centrifuged at 100,000 × *g* for 1 h at 4°C to obtain the supernatant buffer containing the transferred PA and the casein pellet. The latter was reconstituted to the original volume in a 50 m*M* Tris buffer (pH 8.0). Then, PA activity

was determined in buffer and casein fractions by the method described. Prior to PA determination, the Tris buffer that contained 1 *M* NaCl was dialyzed overnight using cellulose dialysis tubing (Sigma Chemical Co.) against 1000 volumes of the same buffer without NaCl; the external medium was changed once.

Effect of Fibrin and Amiloride on PA Activity

The PA activity of each milk fraction was determined in the presence of fibrin (20 μg/ml) or amiloride (1 m*M*). Preliminary experiments indicated that, at these concentrations, fibrin and amiloride optimally affected PA activity. All other details are as described.

Characterization of PA Activity

To characterize the PA protein that is present in each milk fraction, the methodology utilized included localization of PA within the electrophoretic lane following SDS-PAGE, extraction of the protein, and determination of its activity, fibrin dependency, and MW. Furthermore, the effect was determined of amiloride, a specific u-PA inhibitor (6, 16), on PA activity.

Proteins that were present in milk serum, extracted from casein micelles, or present in milk cell extracts were resolved through a 10% acrylamide resolving gel and 4% acrylamide stacking gel (14). Samples were not reduced or boiled. This procedure was necessary to preserve the integrity of the PA molecule, which contains disulfide bridges (14, 15, 19). Samples were then applied to the gels in the mini-gel apparatus (Bio-Rad, Richmond, CA), using MW markers in adjacent lanes. The MW standards included β-galactosidase, 116,000; fructose-6-phosphate kinase, 84,000; pyruvate kinase, 58,000; ovalbumin, 45,000; lactic dehydrogenase, 36,500; and triose-phosphate isomerase, 26,500 (Sigma Chemical Co.). Electrophoresis was conducted in a buffer of .02 *M* Tris and .15 *M* glycine (pH 8.3) at 120 V until the tracking dye reached the bottom of the gel. When electrophoresis was complete, the gel portions containing MW standards were cut off the gel, stained for 2 h in 1% (wt/vol) Coomassie blue and 50% (wt/vol) TCA, and then destained in 20% (vol/vol) acetic acid. The distance of migration of each protein in these standards from the bottom of the well was recorded. The remaining portion of the gel containing the fractionated proteins of the cell extracts was processed for localization of PA activity.

Localization of PA Within Electrophoretic Gels

The position of PA within the electrophoretic lanes was determined as described elsewhere (14). Briefly, this procedure involved three sequential steps: 1) cutting the gel into pieces, 2) extracting the protein from each gel piece into Tris buffer after removal of the SDS, and 3) assaying all of the extracts for PA activity. This methodology takes advantage of the fact that PA are not irreversibly inactivated by SDS (14, 15).

Electrophoretic lanes were cut into approximately 5-mm slices. Each gel slice was incubated in .1 *M* Tris buffer (pH 8.0) containing .5% Triton X-100 to remove SDS. After 1 h, the Tris buffer was discarded, and the slices were allowed to continue soaking in .5 ml of the same Tris buffer for an additional 16 h to elute the proteins. Aliquots of 100 μl of the gel extracts were tested for PA activity as described.

Statistical Analysis

Differences between PA activity in the presence or absence of fibrin or amiloride were evaluated using Student's *t* test ($P < .05$).

RESULTS AND DISCUSSION

Distribution of PA

Distribution of PA activity in the casein and serum fractions of goat milk was examined. The PA activities, expressed as absorbance per hour, were 1.1 ± .10 and .7 ± .05, respectively. In bovine milk from healthy or mastitic quarters, the majority of PA was in the casein fraction (6). Activity of PA of goat milk was associated with the somatic cell fraction. Mean PA activity associated with the somatic cell fraction of goat and cow milks expressed per 100,000 somatic cells was .12 ± .006. Goat milk contains higher SCC than cow milk, and the majority of these cells are neutrophils (4). Conceivably, the high SCC may contribute to the high PA activity in the serum fraction of

goat milk because the viability of milk somatic cells in goat milk is approximately 64 to 72%. Somatic cells in the process of dying probably release their intracellular contents and contribute to PA in the serum fraction of goat milk.

Effect of Fibrin and Amiloride on PA Activity

The effect of fibrin and amiloride on PA activity in each fraction of goat milk was examined. Figure 1 shows that PA activity in the casein fraction did not increase in the presence of fibrin, but, in fact, decreased by 10% ($P < .05$). As expected, control experiments showed that addition of fibrin led to a 7-fold increase in t-PA activity, but had no effect on u-PA activity (data now shown). These results apparently indicate that t-PA is not present in the casein fraction of goat milk, but caution should be exercised because others (3) demonstrated that PA in casein micelles was bound to κ-casein and α_{s2}-casein. Conceivably, the activity of PA that is bound to casein may be unaffected by fibrin. Activity of PA associated with the casein fraction declined ($P < .01$) in the presence of 1 m*M* amiloride, indicating that u-PA was present in the casein fraction of goat milk.

Effects of fibrin and amiloride on PA activity associated with the serum fraction of goat milk were examined. Figure 1 shows that addition of fibrin had no effect ($P > .05$) on PA activity. This lack of effect indicates that t-PA is not present in the serum fraction. Amiloride inhibited ($P < .05$) PA activity (Figure 1), indicating the presence of u-PA in milk serum.

Effects of fibrin and amiloride on PA activity within the somatic cell fraction of bovine milk are presented in Figure 1. In the presence of fibrin, PA activity did not increase ($P > .05$), indicating that t-PA is not present in somatic cell extracts. Amiloride inhibited ($P < .05$) PA activity (Figure 1), indicating the presence of u-PA in goat somatic cell extracts. Heegard et al. (6) reported that the main type of PA in bovine milk somatic cell extracts was u-PA.

Dissociation of PA from Casein Micelles

The effect of fibrin on PA activity associated with the casein micelles cannot be interpreted unequivocally. Therefore, if possible, the PA should be dissociated from the micelles and transferred to some type of solution. This transfer has been achieved following treatment of micelles with EACA or NaCl (1, 6).

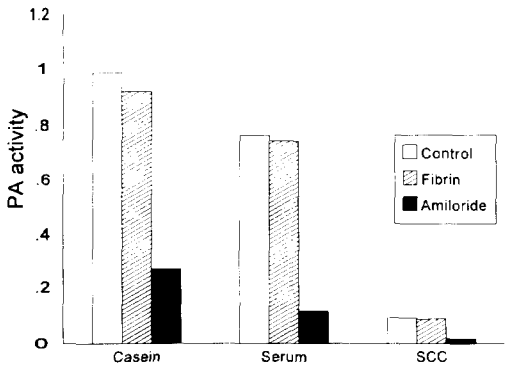

Figure 1. Effect of fibrin (20 μg/ml) and amiloride (1 m*M*) on plasminogen activator (PA) activity in the casein, serum, and SCC fraction of goat milk. Activity of PA was expressed as change in absorbance per hour. Results are means of five independent experiments. Standard errors were <5% of the mean.

The effect of EACA and NaCl on PA activity associated with casein micelles was examined. Table 1 shows that addition of EACA (50 m*M*) and NaCl (1 *M*) decreased PA activity in association with casein micelles by 59 and 85%, respectively. This decrease was due to dissociation of PA from micelles and transfer to the buffer. The NaCl solution that contained the dissociated PA from the casein micelles was dialyzed and retained for further charac-

TABLE 1. Effect of various treatments on dissociation of plasminogen activator activity from casein micelles.

Treatment	Plasminogen activator activity[1]			
	Casein		Buffer	
	— Change in absorbance (/h) —			
	$\bar{X}$	SE	$\bar{X}$	SE
Casein control	1.08	.06	0	0
EACA,[2] 50 m*M*	.44	.06	.55	.08
NaCl, 1 *M*	.16	.06	.82	.08

[1]Results of 10 independent determinations.

[2]ϵ-Aminocaproic acid.

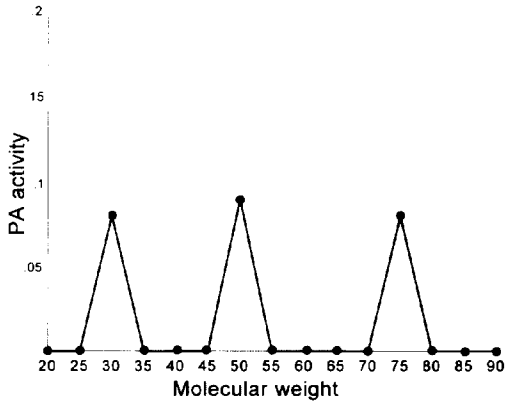

Figure 2. The SDS-PAGE distribution of plasminogen activator (PA) activity extracted from casein micelles following treatment with 1 *M* NaCl for 3 h. Activity of PA was assayed in 5-mm gel pieces and expressed as change in absorbance per hour. Results are from a single representative experiment.

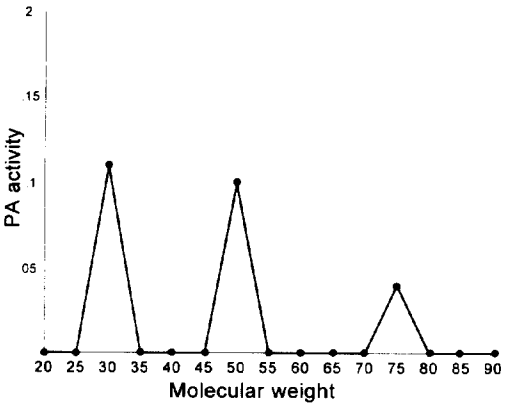

Figure 3. The SDS-PAGE distribution of plasminogen activator (PA) activity in the milk serum fraction of goat milk. Activity of PA was assayed in 5-mm gel pieces and expressed as change in absorbance per hour. Results are from a single representative experiment.

terization of PA. Heegard et al. (6) reported that treatment with NaCl effectively removed PA from bovine casein micelles. Baldi et al. (1) reported that EACA removed some (20%) of the PA from the casein micelles. However, treatment of micelles with EACA did not remove PA from casein micelles, suggesting that the binding of PA to bovine casein micelles was resistant to EACA (6). The differences between these studies cannot be explained.

Characterization of PA

The methodology to characterize the PA protein in each milk fraction included localization of the PA protein within electrophoretic lanes following SDS-PAGE of proteins in each fraction. Following SDS-PAGE, proteins were extracted from gel pieces, and their MW were determined. The effect was tested of fibrin and on the three peaks of PA activity (Figures 2, 3, and 4) extracted from the gel pieces. Results reported in Table 2 are from the peaks obtained following NaCl extraction of PA from casein micelles.

In the buffer obtained following extraction of PA from the casein micelles (Figure 2) and in the milk serum (Figure 3), activities were associated with three slices that corresponded to MW of 30,000, 50,000, and 75,000. Apparently, the same types of PA are present in casein micelles or milk serum. However, some important differences exist. In the casein fraction, all forms of PA are present in equal amounts (Figure 2), but, in the serum, the majority of activity is associated with the forms of MW of 30,000 and 50,000 (Figure 3). The presence of small amounts of t-PA in the

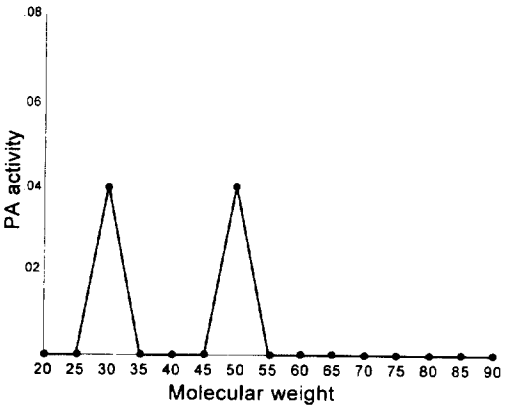

Figure 4. The SDS-PAGE distribution of plasminogen activator (PA) activity in association with milk somatic cell extracts. Activity of PA was assayed in 5-mm gel pieces and was expressed as change in absorbance per hour. Results are from a single representative experiment.

TABLE 2. Effect of fibrin and amiloride on the activity of various forms of plasminogen activator extracted from casein micelles following treatment with 1 *M* NaCl for 3 h.

Treatment	Plasminogen activator activity[1]					
	Peak 1[2]		Peak 2		Peak 3	
	Change in absorbance (/h)					
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Control	.11	.02	.09	.03	.09	.04
+ Fibrin, 20 μg/ml	.10	.02	.08	.03	.37	.09
+ Amiloride, 1 m*M*	.01	.01	.01	.01	.11	.07

[1]Results of four independent samples.

[2]Peaks 1, 2, and 3 correspond to molecular weights of 30,000, 50,000, and 75,000.

milk serum (Figure 3) is not consistent with the results in Figure 1 showing that addition of fibrin, which enhances t-PA activity, had no effect on PA activity in milk serum. The only possible explanation is that t-PA may be present in milk serum in association with soluble casein. Fibrin does not enhance the activity of PA bound to casein. In somatic cell extracts, activity occurred in two gel slices that corresponded to MW of 30,000 and 50,000 (Figure 4).

Activities associated with peaks of MW of 30,000 and 50,000 were not enhanced ($P > .05$) in the presence of fibrin but were inhibited in the presence of amiloride (Table 2). Considered collectively, these data suggest that the activity peaks of MW of 30,000 and 50,000 are attributed to different forms of u-PA. Forms of u-PA with similar MW in the bovine mammary gland have been identified previously (6, 9, 14). The activity associated with peak 3 (MW of 75,000) was enhanced 4.0-fold in the presence of fibrin but was not inhibited ($P > .05$) in the presence of amiloride (Table 2). These data suggest that the activity peak at MW of 75,000 is attributed to t-PA. High t-PA associated with the casein micelles from bovine milk were reported previously (6).

Sources of PA

The present study identified both types of PA in goat milk. Both types of PA, t-PA and u-PA, were associated with casein micelles and milk serum, but u-PA was the only form in milk somatic cells. Three potential sources of PA exist: 1) mammary epithelial cells, 2) endothelial cells, and 3) leakage of PA through the mammary epithelium from the blood. Mammary epithelial cells produce u-PA and t-PA in vitro (I. Politis, 1994, unpublished data). Whether those cells produce PA in vivo is not known. Endothelial cells produce t-PA (8) and should be considered to be a potential source of the enzyme. Leakage of t-PA through the mammary epithelium is also possible but seems to be a more realistic possibility during mastitis, when the permeability of the mammary epithelium is compromised and blood components cross this barrier.

Identification of u-PA within milk somatic cells is consistent with findings by Politis et al. (14), who reported that mammary macrophages produced u-PA. Macrophages are the predominant cell types in milk obtained from healthy quarters (14).

CONCLUSIONS

The present study revealed that t-PA was present in the casein and serum fractions of goat milk, but u-PA was present in all fractions (i.e., casein, serum, and somatic cells). The t-PA had MW of 75,000; two forms of u-PA had MW of 30,000 and 50,000. This information provides the basis for future studies on understanding the control of plasminogen activation in goat milk.

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