

PLASMINOGEN ACTIVATOR ACTIVITY IN SECRETIONS FROM CLINICALLY NORMAL, MASTITIC, LACTATING AND DRIED-OFF UDDERS OF DAIRY COWS

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ABSTRACT

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Using the composite fibrin plates method, plasminogen activator (PA) activity was found in secretions from clinically normal, mastitic, lactating and dried-off udders of dairy cows. The possible involvement of the fibrinolytic system in the physiology and pathology of the bovine mammary gland is discussed.

INTRODUCTION

Normal bovine milk from healthy cows shows proteolytic activity (Humbert & Alais, 1979; Visser, 1981), hydrolyzing beta-casein to gamma-casein (Tomich, Eigel & Chibber, 1976; Eigel, 1977), proteose peptone fragments (Andrews, 1978; 1979) and other, non-identified products.

After initial suggestions that the milk protease may exhibit trypsin- (Kiermeier & Semper, 1959/1960) and chymotrypsin-like activity (Kaminogawa, Yamauchi & Tsugo, 1969), the enzyme apparently was finally purified (Chen & Ledford, 1971; Kaminogawa, Sato & Yamauchi, 1971) and identified as plasmin (Kaminogawa, Mizobuchi & Yamauchi, 1972; Eigel, Hofmann, Chibber, Tomich, Keenan & Merz, 1979; De Rahm & Andrews, 1982).

The presence in milk of plasmin depends on the availability in milk of PA, which releases plasmin from plasminogen.

Plasminogen was found in milk by Tomich *et al.*, (1976), Halpaap, Reimerdes & Klostermeyer (1977) and Eigel *et al.* (1979).

It has been shown that the type and activity of PAs may differ, depending on its tissue source (Astrup, 1956a, b) and the condition of the tissue (Förster, 1969; Ryan, Nashioka & Dawber, 1971; Todd, 1959, 1979; Quigley, Ossowski & Reich, 1974; Marcus, Takita, Camiolo Corasanti, Evers & Hobika, 1980; Evers, Patel, Madeja, Schneider, Hobika & Camiolo, 1982).

Tissue PA activity has been observed in extracts and tissue from involuting mammary glands of rats and mice (Ossowski, Biegel & Reich, 1979) and in abnormal breast milk (Astrup, 1953; Astrup & Sterndroff, 1956).

By mixing urokinase with bovine milk, Tomich *et al.* (1976) showed that available plasminogen could be activated to plasmin by means of artificially added PA (urokinase). However, the presence and activity in bovine milk of natural PAs from a mammary or other tissue source remained undetermined until recently, when Korycka-Dahl, Ribadeau Dumas, Chene & Martel (1983) claimed to have found PA activity associated with casein micelles of milk from mastitis-free cows.

The above-mentioned investigations on plasminogen, proteolytic, plasmin, and PA activities in milk were mainly aimed at assessing the significance of such activities from a technological dairy point of view. However, a detailed knowledge of fibrinolytic activities in mammary secretions seems necessary of further research on bovine mastitis, especially work on the pathogenesis and the therapy of the disease.

Different types of bovine udder secretions were therefore investigated for the purpose of assessing the general feasibility of tentative determinations on fibrinolysis and related activities in such secretions.

MATERIALS AND METHODS

Experimental animals

The 47 individual quarter milk samples investigated were collected from 45 Friesland-type dairy cows which were generally healthy, in good condition and free from tuberculosis and brucellosis. Samples were collected from 27 clinically normal udder quarters of 20 lactating and 7 dried-off cows kept at the Veterinary Research Institute, and from 20 clinically mastitic quarters of 18 lactating cows of a large commercial dairy farm. The animals differed in age, number of lactations, milk production and stage of lactation.

Clinical diagnosis of mastitis

Clinically normal and mastitic quarters were selected by means of conventional veterinary techniques applicable to the clinical examinations of the bovine udder (Heidrich & Renk, 1967).

Particular attention was given to the case history and symptoms, such as, among others, changes in the consistency of udder tissue, pain reaction during palpation, possible increase in the temperature of the udder skin, rapid decrease in milk production and changes in milk. The clinical cases of mastitis diagnosed varied from acute to subacute to chronic types.

Sixteen of the 18 clinically mastitic cows had been treated by means of antibiotic remedies administered intracisternally for one or more days prior to sampling.

Collection and handling of samples

Regardless of treatments previously administered to mastitic quarters, aseptic foremilk samples were collected from the individual clinically selected quarters by means of a sampling technique similar to that described by Giesecke & Viljoen (1974), except that 3 ml samples were used. No samples were collected by means of cisternal puncture, and special sample containers (i.e. sterile tubes with screw caps suitable for freezing⁽¹⁾) were used.

Immediately after its collection, each sample was placed in an insulated container with dry ice where it was frozen and stored for transportation. After sampling in a herd was completed, the samples were transported to the laboratory, where they were kept at -85 °C for further processing.

Laboratory investigation of samples

Materials

All reagents were of analytical grade unless otherwise specified. Plasminogen-enriched fibrinogen and plasminogen-free fibrinogen were from Poviet Chemicals (Leiden, Netherlands), urokinase from Leo Laboratories

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(Dublin, Ireland), plasminogen-free thrombin from Miles Laboratory, thrombin-containing plasminogen from Park Davis (USA), plasmin and trypsin from Sigma (USA). Other reagents were from Merck (West Germany).

Methods

Without exception, all the samples were investigated for general proteolytic activity as well as for the presence of PA and plasminogen by means of the composite fibrin plate assay (Franz, Coetzee, Anderson & Towers, 1979).

Plasminogen-rich as well as plasminogen-free fibrin plates were prepared according to Haverkate & Brakman (1975).

Urokinase-enriched, plasminogen-free (U⁺P⁻) plates were prepared according to Coetzee & Franz (1978).

Processing of the sample on plates

After defreezing and thorough mixing, 100 µl of each sample was placed in triplicate on each of the 3 types of plates, which were then incubated for 17 h at 37 °C. The diameter of lytic zones at the inoculation sites was then determined by means of a special measuring device (Haverkate & Brakman, 1975).

Evaluation of results of the composite fibrin plates assay

Results were interpreted as shown in Table 1.

TABLE 1 Diagnostic key for evaluating results obtained by means of the composite fibrin plate assay (Franz *et al.*, 1979)

Composite fibrin plates			
Diagnosis.....	P ⁺ plate	P ⁻ plate	P ⁻ U ⁺ plate
PA present	+*	-	-
Non-specific fibrinolysis	+	+	+
Plasminogen present.....	-	-	+**
Inconclusive results ***	±	±	±

Fibrinolysis present—(+)

Fibrinolysis absent—(-)

*—specific for PA activity

**—specific for plasminogen

***—inconclusive, owing to indistinct traces of fibrinolytic activity.

Standardization of composite fibrin plates

Positive and negative controls of the plates were performed as described by Coetzee & Franz (1978).

RESULTS

Clinically normal udders

Milk from clinically normal udders showed neither plasmin nor non-specific proteolytic activity (Table 2a). However, the secretions differed in their PA activity, which was negative, inconclusive and positive in 6, 8 and 6 of the milk samples respectively.

Clinically mastitic udders

An evaluation similar to the former evaluation of results from samples of clinically mastitic udders (Table 2b) suggested that 17 and 3 of the secretions showed either no or inconclusive plasminogen incidence, respectively, whereas all of them were negative for non-specific proteolytic activity.

Compared with the samples from clinically normal udders, none of those from clinically mastitic ones were negative, but were less frequently inconclusive (3×) and mostly positive (17×) for PA activity.

TABLE 2a Mean diameters (mm) of lytic zones measured in triplicate on each of 3 different types of composite fibrin plates used for assessing PA, fibrinolytic activity and plasminogen occurrence in milk samples from clinically healthy cows

Sample No.	Composite fibrin plate assay		
	P ⁺	P ⁻	P ⁻ U ⁺
1	19,0	-	-
2	-	-	-
3	20,5	-	-
4	31,0	-	-
5	-	-	-
6	13,5	-	-
7	±	-	-
8	29,0	-	-
9	±	-	-
10	±	-	-
11	±	-	-
12	±	-	-
13	±	-	-
14	±	-	-
15	±	-	-
16	±	-	-
17	21,0	-	-
18	-	-	-
19	-	-	-
20	-	-	-
Mean value.....	22,33	-	-
Std dev	6,54	-	-

Dried-off udders

Secretions from dried-off udders (Table 2c) indicated inconsistent patterns of negative and inconclusive plasminogen incidence and non-specific proteolytic activity, but, apart from the inconclusive result of Sample 1, all were positive for PA activity.

DISCUSSION

The composite fibrin plate assay (CFPA), normally used for medical work, facilitates tentative determinations on fibrinolysis and the occurrence of the related PAs, non-specific proteolytic activity and plasminogen (Haverkate & Brakman, 1975; Coetzee & Franz, 1978; Franz *et al.*, 1979).

The assay shows extremely high sensitivity levels to trypsin, urokinase, plasmin and others at concentrations even as low as 0,3 ng (i.e. 10⁻¹⁴ moles) per standard drop of 5 µl each used for inoculation. Because of the relative simplicity of the CFPA and the fact that in preliminary experiments it was not necessary to distinguish between urokinase type PA and tissue-bound PAs, it was the method of choice for work on fibrinolytic activities associated with mastitis.

The results obtained using CFPA differ significantly from all former published data on the presence of plasminogen and free plasmin in the milk. The different sampling techniques used, for example, the immediate freez-

TABLE 2b Mean diameter (mm) of lytic zones, measured in triplicate on each of the 3 different types of composite fibrin plates used for a measurement of PA, fibrinolytic activity and plasminogen occurrence in milk from clinically mastitic cows

Sample No.	Composite fibrin plate assay		
	P ⁺	P ⁻	P ⁻ U ⁺
1	21,0	-	-
2	36,0	-	-
3	±	-	-
4	27,3	-	-
5	17,5	-	-
6	±	-	-
7	28,5	-	-
8	18,5	-	-
9	28,5	-	-
10	23,3	-	-
11	37,0	-	-
12	±	-	-
13	31,0	-	±
14	24,5	-	-
15	12,0	-	±
16	15,0	-	-
17	16,5	-	±
18	15,0	-	-
19	19,5	-	-
20	27,5	-	-
Mean value.....	23,46	-	-
Std dev.....	7,43	-	-

TABLE 2c Mean diameter (mm) of lytic zones, measured in triplicate on each of the 3 different types of composite fibrin plates used for a measurement of PA, fibrinolytic activity and plasminogen occurrence in dry udder secretion

Sample No.	Composite fibrin plate assay		
	P ⁺	P ⁻	P ⁻ U ⁺
1	19,5	±	±
2	±	-	-
3	25,5	-	-
4	27,0	±	±
5	29,0	-	-
6	20,5	-	±
7	14,5	-	±
Mean value.....	22,67	-	-
Std dev.....	5,45	-	-

ing of samples and storage in -85°C to prevent changes in the milk similar to those occurring during milk storage in the tanks, pasteurization and heating, most probably account for this. The object of our work was to investigate PA and non-specific proteolytic activity in physiological and pathological conditions of the dairy cow, not in milk after storage and processing.

Another problem is the interpretation of the results. The negative results on P⁻U⁺ plates do not necessarily mean that plasminogen and plasmin do not occur in the sample, but most probably that plasmin cleaved by urokinase from plasminogen has been inactivated by inhibitors. This explanation seems to be supported by the results published by Honkanen-Buzalski & Sandholm (1981) and Honkanen-Buzalski (1981) on the occurrence of antiproteolytic activity in normal and mastitic milk. Whether plasminogen can occur in the milk samples with increased PA activity, as was suggested by Korycka-Dahl *et al.* (1983), is a matter for discussion and further research. Most probably the presence of plasminogen in their samples was due to the thrombin and fibrinogen used in the assay being contaminated by plasminogen.

Our demonstration of PA activity in the dry udder secretion supports the results of Ossowski *et al.* (1979) published on the involution of the mammary gland in mice and rats. We likewise found PA activity in mastitic udder secretion, a result similar to that of Astrup & Sterndorff (1956), who were the first to discover PA activity in abnormal breast milk. The former data as well as those obtained in our work show that distinct levels of PA activity are common in secretions of involuted and abnormal mammary glands. However, exceptions from that finding are possible, as a few inconclusive results indicate. Factors affecting the latter finding may be differences in the duration and level of involution at the time of sampling of the dried-off cows and the determination of mastitis by clinical means of acknowledged limited diagnostic reliability (Giesecke & Van den Heever, 1974).

PA activity also occurred in 6 samples from clinically normal udder quarters. However, an equal number of samples showed no PA activity, whereas results from 8 samples were inconclusive. From work on subclinical mastitis (Giesecke & Van den Heever, 1974; Giesecke & Viljoen, 1974), it is reasonable to suspect that the 6 quarter samples positive for PA activity might have been affected by subclinical mastitis. But these results and similarly inconclusive ones on clinically mastitic and dried-off udders require further investigation.

The above-mentioned observations make it reasonable to conclude that PA activity in different types of bovine udder secretions has been conclusively demonstrated by means of the CFP. As a technique, it seems a practical one for further investigations on fibrinolysis associated with physiological and pathological states of udder health in dairy cows.

CONCLUSIONS

Plasminogen activator activity has been found in secretion from clinically normal and mastitic lactating and dried-off udders of dairy cows. The occurrence of the plasminogen activator in the udder secretion may suggest the involvement of the fibrinolytic system in the physiology and pathology of the bovine mammary gland. Further research on this subject will be of the greatest importance because of the possible diagnostic value of PA in milk for mastitis, as well as for the improvement of our fundamental knowledge of inflammation and regression of the bovine udder.

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