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THE PRINCIPAL PROTEASE SYSTEM IN BOVINE MILK

by

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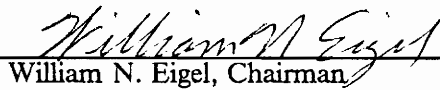

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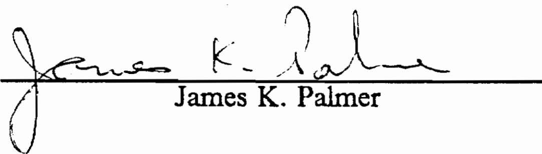

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THE PRIMARY PROTEASE SYSTEM IN BOVINE MILK

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(ABSTRACT)

Plasmin is the principal endogenous protease in bovine milk. Distribution of overall proteolytic potential among milk fractions was determined using Coumarin Substrate as a synthetic substrate. Casein containing fractions had a higher amidolytic potential. However, preparation of casein by acid treatment produced increased dissociation of plasminogen and plasmin from casein. The variable results obtained from milks of different cows could be due in part to the influence of inhibitors and activators of the fibrinolytic system present in milk.

We have shown, for the first time, the occurrence of α_2-M in bovine skimmilk (using SRID) at a level (using ELISA) of 1.54 +/- 0.91 mg/ml. This inhibitor appeared to primarily associate with the acid whey fraction. A high level (< 1 mg/ml) of α_2-M was also detected in human skimmilk. The other major fibrinolytic inhibitor, α_2-AP, as well as the complex formed between this inhibitor and plasmin were also shown to occur in human milk.

We used a coupled colorimetric assay to demonstrate the occurrence of a fibrin-independent plasminogen activator

(similar to u-PA) in bovine skimmilk. The occurrence of a u-PA like activator in bovine milk was further confirmed in co-polymerized gel electrophoresis. Moreover, u-PA could also be detected in a sample of human skimmilk. However, the electrophoretic gel patterns also contained additional zones of clearing which may be due to the occurrence of other activators in bovine milk. These plasminogen activators may be fragments of u-PA, or t-PA (shown to occur in sow milk) which retain catalytic activity.

The occurrence of such high levels of α_2-M (~ 4% of the total protein) and plasminogen activators may be of tremendous significance to the dairy industry, as they may not only influence plasmin-mediated proteolysis of milk proteins, but may also interfere with the action of milk clotting enzymes.

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"I can write another dissertation on the basis of what I owe."

Anonymous.

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LIST OF ABBREVIATIONS

α_2-AP	α_2-Antiplasmin
AU	Arbitrary units
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
DID	Double immunodiffusion assays
DMSO	Dimethyl sulfoxide
EACA	Epsilon amino caproic acid
ELISA	Enzyme-linked immunosorbent assay
IU	International units
kD	Kilo daltons
α_2-M	α_2-Macroglobulin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline, with Tween
Pg	Plasminogen
Pn	Plasmin
PU	Plough units
SBTI	Soybean trypsin inhibitor
SDS	Sodium dodecyl sulphate
SFP	Soluble fibrin products
SRID	Single radial immunodifussion assays
TIU	Trypsin inhibitor units
Tris	Tris(hydroxymethyl)aminomethane
t-PA	Tissue-plasminogen activator

u-PA	Urokinase-like plasminogen activator
WHO	World health organization
<	Less than
>	Greater than

INTRODUCTION

Plasmin, the principal fibrinolytic enzyme, has also been identified to be the principal endogenous enzyme in bovine milk. Plasmin occurs in bovine milk along with its zymogen, plasminogen, and a number of enhancing and inhibiting factors. Very little unambiguous information is currently available on these activating and inhibiting factors occurring in milk.

Plasmin is a thermo-resistant enzyme, has a broad pH optimum, and is active at refrigerator temperatures. Thus, the occurrence of plasmin in milk is of considerable concern to the dairy industry. The cheese-industry is particularly concerned as plasmin-mediated proteolysis of caseins may result in reduced cheese yields and may also adversely affect a variety of cheese-making parameters. However, a more comprehensive picture of the significance of plasmin in milk will emerge only when some of the major components comprising the fibrinolytic system in milk have been identified and quantified.

The primary objective of our study was to identify and quantify the principal components regulating the action of plasmin in bovine milk, by using highly sensitive and specific assay procedures.

2.0 LITERATURE REVIEW

The phenomenon called fibrinolysis was first discovered by Christensen and Kaplan (1,2,3) who noted the presence of an activated factor in plasma which had the ability to resolve thrombi. The activated factor was called plasmin and the proenzyme was named plasminogen. Plasmin, a serine protease [E.C. 3.4.21.7.], is normally found in mammalian serum and plays a principal role in the fibrinolytic system. However, since plasmin has a relatively broad specificity and has been found in a variety of tissues and body fluids, plasminogen may play a role in a variety of other physiological processes like tissue remodelling, as well.

2.1 PLASMINOGEN ORIGIN AND STRUCTURE

The liver has been reported (4,5) to be the primary site of plasminogen biosynthesis but the origin has yet to be firmly established. Native human plasminogen, Glu-plasminogen (Glu-pg), with glutamic acid as the N-terminal (6,7,8) and asparginine as the C-terminal amino acids (9) has a molecular weight of 83 kilodaltons (kD) (15). Plasmin cleaves Glu-pg at Arg(68)-Met(69), Lys(77)-Lys(78), and Lys(78)-Val(79) resulting in the release of the so-called "preactivation peptides" and the generation of Lys-plasminogen (Lys-pg) (10,11,12) with a

molecular weight of 82.4 kD (15). The native plasminogen binds to fibrin far less effectively than does Lys-pg (13).

Magnusson et al (14) have reported the occurrence of five kringles in native plasminogen. The kringles have a characteristic triple disulfide structure (Fig 1). The release of the preactivation peptide induces a marked conformational change in the five kringles (20) and results in an increased affinity of plasminogen for fibrin (19).

2.1.1 Plasminogen Activation

Proteolytic cleavage of single-chained plasminogen is necessary for conversion to active plasmin (Pn), which consists of a heavy(A) (MW ~ 60 kD) and light(B)-chain (MW ~ 20 kD) connected by a disulfide bond between Cys(557)-Cys(565) (17). Plasminogen activators specifically cleave the Arg(560)-Val(561) bond (9,15-18) which is located in a region of the polypeptide chain which is amino terminal relative to the active center. The active center of plasmin has been localized in the B-chain (22). The five kringles present in plasminogen are located in the A-chain of plasmin (23). Plasminogen activation may occur through either of two alternative pathways. In one, Glu-pg is converted to Glu-pn by plasminogen activators followed by an autocatalytic conversion to Lys-pn. In the second, plasmin catalyzes the conversion of Glu-pg to Lys-pg followed by conversion to Lys-pn by plasminogen activators (24).

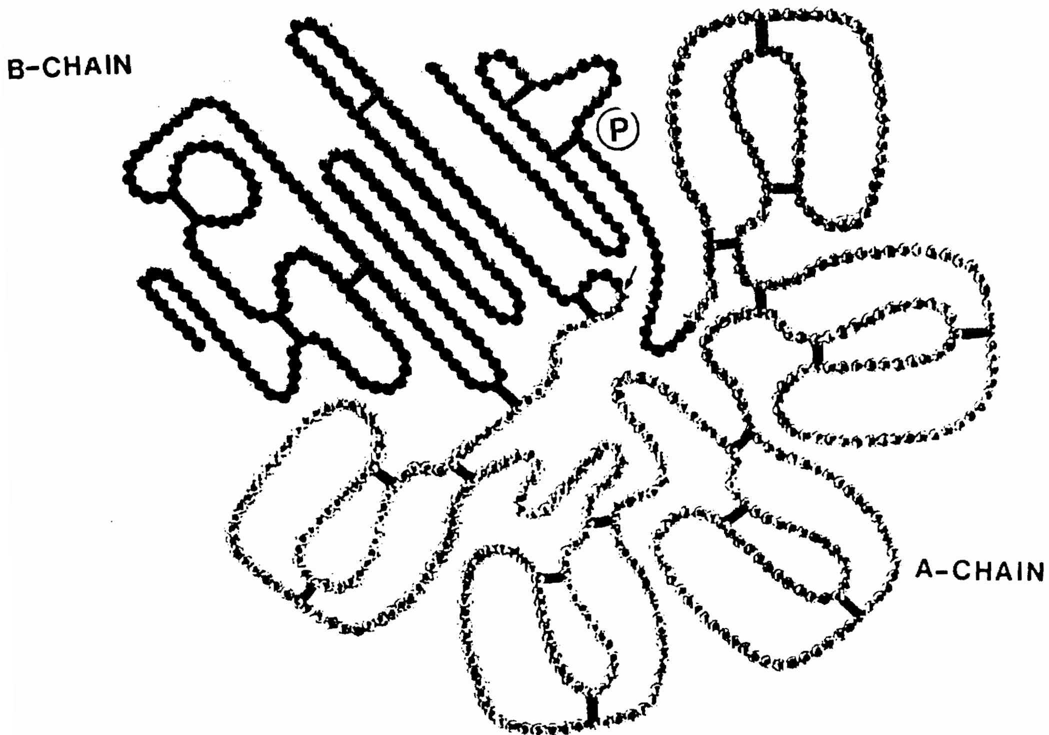


Figure 1. The primary structure of human Glu-Plasminogen. (P) represents the "pre-activaion peptides." Arrow indicates the peptide bond cleaved by plasminogen activators. A-chain contains the "Kringles" and B-chain the active site. (17).

Residues present at the N-terminus of the plasmin heavy chain, which react with α_2-antiplasmin (a plasmin inhibitor) and fibrin (a plasmin substrate), are closely related and may be present as a single regulatory site which also interacts with lysine and its analogs (25). The plasmin A-chain is necessary to stabilize the 3-dimensional structure of the active center located in the light(B) chain (21) (Fig 1). A high degree of sequence homology has been observed between the N-termini of both heavy and light chains of plasmin isolated from various animals (26). Homology also exists among residues at the N-terminus of plasminogen isolated from different sources, suggesting that similar proteolytic cleavages are involved in the activation of all plasminogens (27).

2.1.2 Plasmin Specificity

Plasmin is a serine protease and has the ability to hydrolyze a variety of proteins. Plasmin has trypsin-like specificity in that hydrolysis of lysyl and arginyl bonds present in proteins are catalyzed (28,29). Further, plasmin has a preferential specificity for bonds containing lysyl residues (30).

2.2 ANTIPLASMINS AND ANTIACTIVATORS

Though plasmin activity is essential for a number of biological functions, unregulated action would result in the

destruction of a variety of essential protein components in cells and tissues. Since 10% of all plasma proteins are proteinase inhibitors (64), the existence of plasmin inhibitors should be no surprise. Two types of fibrinolytic inhibitors have been identified: antiplasmins, which inhibit plasmin, and antiactivators, which inhibit plasminogen activators. The two major antiplasmins known to occur in human plasma have been identified as α_2-antiplasmin and α_2-macroglobulin (65,66). However, detection and identification of antiactivators has been difficult due to interference by antiplasmins as well as other plasma protease inhibitors such as antithrombin and α_1-antitrypsin. However, with the development of new and extremely sensitive assay procedures, recent reports on the occurrence of antiactivators in cultured bovine (126) and rabbit (67) endothelial cells, cultures of human plasma (68), and washed human platelets (69) have appeared. These antiactivators have yet to be fully characterized.

2.2.1 Alpha$_2$-Macroglobulin (α_2-M)

α_2-Macroglobulin, which was first isolated by Schultze and colleagues (97), is stable within the pH range 5 - 8.4 and has the unique ability to bind and inhibit a variety of serine -, thiol -, carboxyl -, and metallo - proteinases (94,95,96). Proteins presumed to be homologous with human α_2-M have been isolated from a number of

mammalian species, including the cow (92), horse (98,99), pig (100), and mouse (106). α_2-Macroglobulin consists of identical subunits linked in pairs by disulfide bonds. Two such pairs associate noncovalently to form the native tetramer (95) which has a molecular weight of approximately 725 kD (93). α_2-Macroglobulin differs from other protease inhibitors not only by possessing an unusual ability to bind a variety of proteases, but also by leaving the active site of the bound enzyme intact. This latter property produces a variable pattern of inhibition; hydrolytic activity of bound enzymes is usually inhibited in the presence of high molecular weight substrates but only moderate to low inhibition, depending upon the bound enzyme, occurs when assayed with low molecular weight synthetic substrates (102).

Two models have been proposed to explain the mechanism by which α_2-M inhibits proteases. In the model proposed by Barrett and Starkey, binding of the protease to α_2-M produces a conformational change which serves to "trap" and sterically block the enzyme (94). Evidence supporting this "trap" hypothesis has also been reported by others (101,103,108). However, this observed structural alteration and change in conformation of α_2-M does not exclude crosslinking of protease to α_2-M (85), the second method proposed for inhibition. Feinman and co-workers found that α_2-M lost inhibitor ability after methylamine treatment,

suggesting that the lysyl amino groups present in proteolytic enzymes might be attacking nucleophiles at the activated glutamate site resulting in formation of an ε-lysyl- -glutamyl bond (86). However, rapid incorporation of methylamine did not result in loss of trypsin binding to α_2-M (85). Probably, both entrapment as well as formation of a covalent link with the thiolester act in concert to form an irreversible α_2-M-enzyme complex (85).

To account for the wide specificity of α_2-M, Salvensen and Barrett proposed the presence of a "bait region" on both subunits (109). The versatility of α_2-M is due to the occurrence of a wide range of amino acids in this bait region. These amino acids are highly accessible and their composition satisfies the specific requirements for all proteases known to be inhibited by α_2-M (87). Peptide bond cleavage in one subunit induces conformational change in the entire molecule making the complex more compact.

Though α_2-M contains two binding sites for each protease, not all enzymes have been found to bind in a 2:1 stoichiometry. Howell et al suggested that binding of protease molecules is dependent on the rate at which the cognate protease cleaves the scissile bond in the bait region (110). Trypsin cleaves α_2-M 100-times faster than plasmin and binds in 2:1 stoichiometry, while plasmin binds in a 1:1 ratio. Thus after the trap has been sprung by interaction of the first protease molecule with the bait

region, binding of the second protease molecule depends on whether it encounters a closing trap or not. This, in turn, is dependent on how fast the protease can cleave the appropriate bond in the bait region. If this cleavage is slow, the trap will close and leave out the second protease molecule (88).

Plasmin is one of the largest proteases known to be entrapped by α_2-M. However, the maximum size of protease which can be effectively bound is not known (86). Even plasmin's lysine binding sites appear to lie partially or completely outside the trap (102,111,112), since the affinity of α_2-M-plasmin complex for sepharose-lysine can be demonstrated. The exposed portion of plasmin has been identified to be the Kringle 1 -- Kringle 4 region of plasmin (89). Hence, some antigenic sites on plasmin remain exposed even on complex formation with α_2-M (102,112). Some endopeptidases, such as, pancreatic kallikrein, complement C1 s, hog renin and Factor XIIa are not inhibited by α_2-M (114-117). Whether the bait region does not have the required specificity or these proteases are too large to be entrapped is not currently known (113).

Another interesting characteristic of α_2-M is that the cognate protease is protected from inhibition by large molecular weight protease inhibitors such as soybean trypsin inhibitor (SBTI) (MW ~20 kD) and plasma α_1-protease inhibitor (MW ~ 50 kD) (119). However, low molecular weight

inhibitors, such as aprotinin (MW ~ 6,500) can inhibit the enzyme trapped in the α_2-M complex (118). Harpel et al (102) suggested that α_2-M functions not only as a protease inhibitor but also serves to preserve a portion of the biological activity of the complexed enzyme in presence of circulating inhibitors. He also found that α_2-M-plasmin complexes do have some activity against fibrinogen (MW ~ 340 kD). The mechanism by which plasmin bound in α_2-M reacts with fibrinogen, while being protected from interacting with smaller molecular weight proteins like SBTI is not currently known.

Urinary-PA forms both a reversible, nonspecific (104), as well as an irreversible (125), complex with α_2-M resulting in loss of catalytic activity. α_2-Macroglobulin also forms covalent complexes with the one- and two- chain forms of t-PA (107). However, immunoreactivity of the t-PA fraction is not retained.

Although active against both plasmin and plasminogen activator, α_2-M does not appear to be the primary inhibitor in either case. The primary physiological role of α_2-M is yet to be elucidated. Some indications exist to suggest that α_2-M acts as a modulator during many proteolytic reactions that accompany inflammation (90) and/or as a modulator in immune responses (105). Additionally, one of the more important roles may involve the receptor-mediated endocytosis of α_2-M-protease complexes (91).

2.2.2. Alpha$_2$-Antiplasmin (α_2-AP)

Although α_2-M has antiplasmin activity, α_2-antiplasmin has been identified as the major physiological plasmin inhibitor in human plasma (66,70,71). It is present at a concentration of 1μM and exists as a single-chain glycoprotein with an MW of approximately 70 kD (72) which is rapidly inactivated below pH 5.7 but is stable between pH 6.0 and 9.0 (72). Human plasma contains two types of α_2-AP which are immunologically indistinguishable. Only one form binds plasminogen. The other form does not bind plasminogen (makes up ~ 40% of the total) but nevertheless inactivates plasmin, though less effectively (73,74,75). The plasminogen binding form is produced in the liver (78) and is converted to the nonplasminogen binding form by cleavage of an Arg-Gly bond situated towards the C-terminus (79).

Plasmin forms a equimolar complex with α_2-AP which is devoid of either proteinase or esterase activity (66,71,72). For complex formation to occur, plasmin should have available both a free lysine-binding site as well as a free active site (76,77). Thus, plasmin which is actively involved in breaking down fibrin clots is inhibited to a much lesser extent than is free plasmin (82). Inhibition by α_2-AP involves two successive reactions: a very fast reversible second-order reaction followed by a slower irreversible first-order reaction (77). The reaction

between α_2-AP and plasmin is one of the fastest protein-protein reactions known, with the second-order rate constant ($4x10^{-7}$ M^{-1} s^{-1}) approaching the theoretical limit for diffusion controlled reactions (77). This reversible first step involves both an interaction between the lysine binding sites in the plasmin A-chain and corresponding sites in the α_2-AP molecule as well as a reaction between the active site of plasmin and the reactive site in α_2-AP. The reactive site in the inhibitor has been identified to be the Arg(364)-Met(365) bond (80).

The initial complex formation induces a conformational change which perturbs selected epitopes on the inhibitor and, subsequently, limited proteolysis of the plasmin-α_2-AP complex by excess plasmin further modulates the inhibitor moitey by destroying specific antigenic sites. The plasmin-α_2-AP complex now contains new antigenic structures called neoantigens (81).

α_2-Antiplasmin also slowly interacts with both u-PA (83) and t-PA (84) to form inactive complexes in purified systems. Whether this antiactivator activity of α_2-AP has any physiological importance is still unclear. The principal physiological function appears to be that of an antiplasmin.

2.2.3 Antiactivators

The existence of specific antiactivators has been

recently demonstrated in a number of tissues and cultured cells. Plasminogen activator-inhibitor 2, an inhibitor of u-PA, first identified in 1970 (120), has now been purified (121). This antiactivator, reacts with both u-PA and t-PA but not with plasmin or thrombin (122). Other specific antiactivators isolated include protease nexin from human fibroblasts (123,124) and PAI-1 from bovine endothelial cells (126,127) and human platelets (69). Both PAI-1 and PAI-2 have been shown to occur in human plasma (128,129). One characteristic feature of these inhibitors, apart from their specificity, is their stability to acid and denaturing conditions (130).

2.3 PLASMINOGEN ACTIVATORS

Plasminogen activators are serine proteases which specifically recognize the zymogen, plasminogen, and catalyze conversion to plasmin, the active enzyme. Plasminogen activators are known to be widely distributed in nature and can be found in microorganisms as well as most tissues and body fluids in higher animals. Plasminogen activators are classified as either fibrin independent, urokinase-like plasminogen activator (u-PA), or as fibrin dependent, tissue-plasminogen activator (t-PA). Vascular endothelial cells appear to be an important source of activator present in blood and in several tissues while urinary tract epithelial cells appear to be an important

source of u-PA (31).

2.3.1 Urokinase-like Plasminogen Activator (u-PA)

Macfarlane and Pilling first reported the presence of fibrinolytic activity in normal urine (32). Sobel and co-workers identified the activity as a plasminogen activator and provided the name Urokinase (33). Urokinase-like PA (u-PA) catalyzes activation of acid-treated Glu-pg better than native, untreated plasminogen (34). However, Lys-Pg is converted at the same rate as Glu-Pg indicating that release of the preactivation peptide does not alter the activatibility of plasminogen by u-PA (21). Urokinase-like plasminogen activator has been isolated in high (MW 54.7 kD) and low (MW 31.5 kD) molecular mass forms (35,36). The heavier form consists of two polypeptides, one with an MW of 33 kD and the other, 19 kD (37). The lighter form has only one chain (37) and is identical with a portion of the high molecular weight form (38). The heavier species has a specific activity of 93,500 CTA units/mg and the lighter species of 218,000 CTA units/mg (28). Whether u-PA originates from kidney cells or other sources has not yet been definitively determined (39). Three forms of u-PA are known to occur: a zymogen (pro-uPA), an active enzyme, and an enzyme-inhibitor complex (39). Human pro-uPA has no catalytic activity. However, low catalytic activity has been detected in pro-uPA from several

other mammalian sources (40). Reasons for differences in activity of the pro-enzyme forms have not yet been elucidated. Plasmin mediated cleavage of pro-uPA at the Lys(158)-Ile(158) bond, produces a conformational change resulting in the exposure of the active site and conversion to the active form (41). Although the pro-enzyme has a high affinity for fibrin (42) and enhanced catalytic activity in the presence of fibrin (43), the active enzyme does not depend on fibrin for activity (44). The mechanism of high affinity for fibrin by the Pro-uPA is not yet clearly understood.

2.3.2 Tissue-Plasminogen Activator (t-PA)

Tissue-plasminogen activator is a serine protease with a molecular weight of 57 kD to 69 kD (45,31) which is stable to both acid treatment and heating at 60 C for 30 min (56). Like u-PA, t-PA is also synthesized and secreted as a single-chain polypeptide which is converted to the two-chain form by limited plasmic cleavages at Arg(3)-Ser(4) and Arg(278)-Ile(279) (46,48). The active center in the human t-PA is located in the B-chain (47). Although the single-chain form appears likely to possess some catalytic activity (47,52) resulting from a partially exposed active site (49,50,51), some reports indicate that monochain t-PA is inactive (49).

The heavy chain of 2-chain t-PA is proposed to contain two

kringle structures which appear to confer high affinity for fibrin (47). The catalytic efficiency increases substantially in the presence of fibrin (47). Further, t-PA mediated plasminogen activation is greatly enhanced by addition of cyanogen bromide digests of both fibrin and fibrinogen (53), poly-Lys (54), and some denatured proteins (55).

2.3.3 Streptokinase

Streptokinase is one of the principal plasminogen activators of bacterial origin and is obtained from culture filtrates prepared from ß-hemolytic streptococci (57). Early studies led to identification of plasmin as the protease with fibrinolytic activity, which resulted from activation catalyzed by streptokinase (58). The name, however, is a misnomer since streptokinase is not an enzyme. Although streptokinase is a single chain polypeptide of molecular weight 47 kD (59), the mechanism proposed for activity does not involve proteolytic cleavage (59). Streptokinase forms a stoichiometric complex with plasminogen. Complex formation results in exposure of the active site in plasminogen which then catalyzes conversion of non-complexed plasminogen to plasmin. Streptokinase can activate the plasminogens isolated from humans, cats, and monkeys, but has no activity towards plasminogens isolated from cows, sheep, pigs, mice, and rats. However, the human

plasminogen-streptokinase complex can activate the plasminogens isolated from different species.

2.3.4 Staphylokinase

Staphylokinase is an extracellular protein produced by many strains of staphylococci which can convert human plasminogen to plasmin (60,62) by a mechanism similar to streptokinase (61). Staphylokinase is not only unable to activate bovine plasminogen, but, unlike streptokinase, the human plasminogen-staphylokinase complex also is not able to activate bovine plasminogen (63).

2.4 FIBRINOLYTIC SYSTEM IN MILK

At least twenty enzymes have been reported to be native constituents of healthy bovine milk (131). These enzymes are non-microbial in origin and may be present in milk either as a result of *de novo* synthesis in the mammary gland (132) or by passage from blood into milk (133).

Protease activity was first shown to be present in milk in 1897 (134). However,this proteolytic activity was thought to be of microbial origin until the late 1950´s (135,136). Biochemical characterization led Kaminogawa et al to suggest that the milk protease then called, Alkaline Milk Protease, is identical to plasmin (137). More conclusive evidence that alkaline milk protease is indeed plasmin was obtained by demonstrating that immunological

cross-reactivity occurred between whole bovine milk and antiserum to bovine plasmin (139). Although protease activity has been shown to be associated with the casein fraction (138), some plasmin activity is also associated with the milk fat globule membrane (140). Blood could well be the major source of plasminogen in milk since, ^{125}I-labelled plasminogen has been shown to be transferred from blood across mammary epithilia into milk (139). Similarities in proteolytic profiles generated by blood and milk adds further support to this possibility (141). Most likely plasminogen is similar to the immunoglobulins and passes from blood into milk by the transcellular route (139,142). However, de novo synthesis in the mammary gland has not yet been ruled out (154). Both plasminogen and plasmin have been shown to occur in milk (139,143).

Bovine milk may also contain other proteases including a thrombin-like enzyme (144), an aminopeptidase (145), and a Cathepsin-like acid protease (146). However, the identity of non-plasmin-like protease activity in milk, independent of microorganisms, awaits further characterization.

Plasminogen activators are known to occur in a variety of body tissues and fluids in a number of different animals. The presence of plasminogen activator activity was observed in human milk by Astrup and Sterndroff (147) and in bovine milk by Korycka-Dahl (149). Activator activity in bovine milk has been shown to be associated with the casein

fraction (149,150). The major plasminogen activator in human milk has been identified as t-PA (148). The identity of plasminogen activator in bovine milk has not yet been established.

Protease inhibitors, like trypsin inhibitors, are present in both bovine and human milks (151,152,153). The plasmin inhibitory activity of ß-lactoglobulin has been documented (137,171). Most inhibitory activity appears to be associated with the serum fraction of milk (149). However, very little information is available on the other inhibitors present in milk.

2.4.1 Plasmin Mediated Proteolysis in Normal Fresh Milk

The principal protease in bovine milk has a wide pH optimum, with maximum activity occurring over the pH range 6.5 - 9.0 (154). Proteolytic activity is maximal at calving and decreases in about five days to 10 - 20% of the peak activity (166). Plasminogen, the zymogen of the active enzyme, accounts for the majority of the proteolytic potential in milk (149). Richardson and Pearce (143) have reported that plasmin and plasminogen occur in bovine milk at levels of 0.14 - 0.73 μg/ml and 0.55 - 2.75 μg/ml, respectively. The levels and ratios at which these occur in milk depend upon a variety of factors such as the stage of lactation and health of the cow (156,157). Plasminogen in milk is converted to plasmin by the action of activators.

Activation may occur while the milk is temporarily stored in the lumen (158) or at any time after milking (159). Proteolytic fragments may also be secreted directly into the lumen (160).

In milk, casein is the primary substrate for plasmin. Many minor proteins and peptides identified in milk occur due to the action of plasmin on the major caseins and proteins in the fat globule membrane. ß-Casein contains 11 or 12 lysyl and 4 or 5 arginyl residues/mole providing 15 - 17 susceptible sites for plasmin. It is now established that γ-Caseins are the most prevalent minor caseins in bovine milk and are produced by the proteolytic breakdown of ß-casein (174,178). The γ1-, γ2-, and γ3- caseins have been identified as specific C-terminal fragments of ß-casein resulting from plasmin-mediated cleavages at Lys 28, Lys 105, and Lys 107, respectively (176). In fact, the Milk Protein Nomenclature Committee has recommended that the term γ-casein no longer be used and that the proteolytic fragments be identified as breakdown products of ß-casein. Thus γ1-, γ2-, and γ3- caseins have been renamed ß-casein (fragment 29 - 209), ß-casein (fragment 106 - 209), and ß-casein (fragment 108 - 209), respectively (175). The corresponding N-terminal fragments released during proteolysis of ß-casein contribute to the proteose-peptone fraction in milk. Proteose-peptone component 8F represents the fragment containing residues 1 - 28, which is produced during

formation of ß-casein (f 29 - 209) (161). The fragment containing residues 1 - 105 and 1 - 107 have been identified as proteose-peptone component 5 (177). At one time residues 29-105 and 29-107 were proposed to be proteose-peptone 8S (162), however, subsequent work has shown that PP 8S is not a breakdown product of ß-casein and may actually be a primary gene product (163). Other susceptible bonds in ß-casein may be hydrolyzed more slowly because they occur in environments unsuitable for plasmin action (164).

α_{s1}-Casein, which contains 14 lysyl and 6 arginyl residues, is also hydrolyzed by plasmin, although to a lesser extent than ß-casein. The Lambda-caseins have been identified as containing some breakdown products resulting from plasmin action on α_{s1}-casein (165). However, the lambda- casein fraction is rather ill defined and awaits further characterization. Other breakdown fragments of α_{s1}-casein have not yet been identified. Based on the amino acid sequence, α_{s2}-casein could appear to contain several regions which may be susceptible to attack by plasmin (179). These sites are the Lys(76)-His(77), Lys(113)-Arg(114), Lys(136)-Lys(137), Lys(149)-Lys(150), and Lys(165)-Lys(166) bonds. Thusfar, no proteolytic fragments of α_2-casein have been identified in milk. Kappa-casein is essential to the stability of casein micelles in milk and appears to be very resistant to proteolysis by plasmin (166,167).

In isolated casein systems, plasmin attacks ß-casein

more rapidly than either α_{s1}- or α_{s2}- caseins, while kappa-casein remains largely intact. However, α_{s1}-casein is more rapidly and extensively degraded in milk than are the other caseins (168). One explanation proposed is that the micellar α_{s1}-casein may protect the susceptible sites of ß-casein from exogenous plasmin (168). Thus, the rate at which caseins are degraded in milk may be a reflection of their distribution in the micelle as well as the permeability of the micelle to plasmin (169).

Nevertheless, milk does contain large quantities of breakdown products produced by the action of plasmin on ß-casein. A relatively large portion of ß-casein exists in a nonmicellar form and may be more susceptible to plasmin action (161).

Compared to the caseins, whey proteins are resistant to plasmin mediated proteolysis probably as a result of their rigid globular conformation (170). In fact, ß-lactoglobulin (ß-Lg) actually appears to be an inhibitor of plasmin. Native ß-Lg has been shown to inhibit plasmin degradation of whole casein (137) heat-denatured, but not native, ß-Lg inhibits plasmin degradation of isolated α_{s2}- and ß- caseins (171).

Plasmin has also been shown to hydrolyze some of the major milk-fat-globule-membrane proteins. Membrane polypeptides of MW 155-, 70-, and 34- kD are degraded by plasmin to polypeptides of MW 92-, 20-, 18-, 12-, and 8.5 kD

(140). Proteose-peptone 3 has been proposed to be produced by plasmin degradation of membrane polypeptides (172). Nejjar et al have recently shown that the hydrophobic fraction of proteose peptones are immunologically identical with the fat-globule-membrane proteins (173). Thus some polypeptides comprising the proteose peptone fraction appear to be fragments of proteins in the fat globule membrane as well as fragments of ß-casein.

2.4.2 Plasmin Activity in Heat-treated Milk

Plasmin is a thermo-resistant enzyme (154) which has been reported to express enhanced activity in pasteurized milk (155). Increased activity could occur as a result of inactivation of a heat-labile inhibitor (155,180), from inactivation of an antiactivator producing enhanced plasminogen activator activity (181), or due to increased accessibility of the susceptible bonds resulting from partial denaturation of the substrate (182). Plasmin in milk may even be partially resistant to conditions existing in some ultra-high-temperature treatments (183,184).

Plasmin forms a more stable complex with casein than does plasminogen (185) which might explain why heat inactivation of plasmin lags behind that of plasminogen (186). The thermo-resistance of plasmin has been attributed to the occurrence of a number of stabilizing intermolecular -S-S- bonds. Inactivation of plasmin by ß-Lg during heating

has been proposed to occur through interaction of activated free -SH groups on ß-Lg with the -S-S- bridges of plasmin (186). Plasmin is more thermostable in the absence of ß-Lg (186).

2.4.3 <u>Plasmin Activity in Cold-stored Milk</u>

Besides being very thermostable, plasmin is also active at refrigerator temperatures (154). Casein hydrolysis has been reported to be even more extensive at 4° C than at 37° C and is probably due to the immobilization of both enzyme and substrate at the micelle surface at higher temperatures (187). Storage of milk at 4° C for 48 h produces extensive transfer of ß-casein from the micellar to the non-micellar phase so that almost 30-60% of the total ß-casein occurs in the soluble phase (188,189). During this time the gamma-casein content of milk increases about 25% (187). Cold-storage also increases levels of α_{s1}- and -caseins in the non-micellar phase (188). Whether this affects the extent to which the α_{s1}- and κ- caseins are hydrolyzed by plasmin is not known.

2.4.4 <u>Plasmin Activity in Abnormal Milk</u>

During mastitic infection and advanced stages of lactation the level of blood constituents which are transferred into milk increase dramatically (168). These milks have a much higher proteolytic potential than does

milk from normal, healthy cows. Milk from cows with subclinical mastitis contain plasmin as the principal protease, whereas, milk from mastitic animals also have increased levels of non-plasmin-like enzymes (157,190). Milk from mastitic animals contains constituents derived not only from blood but also from the large numbers of cells which pass into milk from infected mammary tissue. The major proteolytic enzymes of these cells, primarily composed of polymorphonuclear leucocytes (191), are elastase, cathepsin, and collagenase (192). However, the extent to which these cells are capable of enhancing proteolysis depends not only on their numbers, but also on their activity (194). These cells may also contain intra-cellular protease inhibitors (195,196) whose release may affect extent of proteolysis in milk. Increased proteolytic activity observed in late lactation milk has been attributed to increased levels of active plasmin rather than enhanced conversion of plasminogen by activators in milk (149,193).

These studies indicate that milk contains at least a few of the components that comprise the complex fibrinolytic system in blood. However, only some of these components have been identified or quantified. Furthermore, little information is available on interactions which occur among the various components which make up the principal proteolytic system in bovine milk.

This information would be of considerable interest to the dairy industry, as extensive proteolysis may result in greatly reduced yields and adversely affect a variety of cheese-making parameters. Furthermore, high levels of general antiproteases like α_2-M may inhibit action of milk clotting enzymes. Additionally, the peculiar mode of α_2-M-mediated inhibition may result in an inaccurate estimation, especially when measured using synthetic substrates.

2.5 OBJECTIVES

The primary objectives of this study were (a) to identify and quantitate some of these activating and inhibiting factors in bovine milk, and (b) to study the distribution of various components of the fibrinolytic system among milk fractions.

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Bovine plasminogen (free of EACA), bovine plasmin, N-succinyl-alanyl-phenylalanyl-lysyl-7-amido-4-methyl coumarin (Coumarin Peptide), 7-amido-4-methyl coumarin, gelatin, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), Tween-80, Tween-20, ammonium persulfate, tris (hydroxymethyl) aminomethane (Tris), TEMED, dimethyl sulfoxide (DMSO), acrylamide, N-N´-methylene-bis-acrylamide, Sephrose 4B, Sephacryl S200, agarose, L-lysine, soybean trypsin inhibitor (SBTI), alkaline phosphatase, substrate for alkaline phosphatase (Sigma 104-105), horseradish peroxidase, glutaraldehyde, and Freund´s Complete and Incomplete reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Peroxidase substrate (ABTS) was from Perry Lab. Inc., Gaithersburg, Md. Ultrapure SDS (Sequanal grade), and the BCA protein assay reagent were from Pierce Chem. Co., Rockford, Il. Antihuman urokinase (anti u-PA) was from Alpha-Therapeutic Corp., Los Angeles, Ca. Antihuman antiplasmin, antihuman α_2-antiplasmin-plasmin complex, antihuman α_2-macroglobulin (anti α_2-M), and human urokinase (low molecular weight) were obtained from Calbiochem, San Diego, Ca. Antihuman tissue-plasminogen activator (anti t-PA) (from the ovary), antiporcine t-PA (from the heart), Chromozyme-Pl, Chromozyme-UK (substrates

for plasmin and u-PA, respectively), and soluble fibrin products (SFP) were from American Diagnostica Inc., Greenwich, Ct. Ninety-six well ELISA plates were from Dynatech, Alexandria, Va. Plates for Single immunodiffusion were from Miles Lab. Inc. and for Double Immunodiffusion were from Falcon, Oxnard, Ca. Amicon Concentrators were from Grace and Co., Danvers, Ma. Low and High molecular weight markers were from Pharmacia Inc., Piscataway, NJ. Dialysis tubing was from Spectrum Medical Industries, Inc., Los Angeles, Ca. Sodium sulfate, sodium phosphate (mono-, and di- basic), trisodium citrate, cyanogen bromide, sodium bicarbonate, sodium bicarbonate, and Triton X-100 (electrophoretic grade) were from Fisher Scientific Co., Raleigh, N.C. All other chemicals were reagent grade.

3.2 COLLECTION AND TREATMENT OF MILK SAMPLES

Milk was obtained by complete milking of healthy or mastitic (cell counts > 10^6) cows from the university herd. Additionally, porcine milk was obtained from the university swine center and human milk from a local volunteer. Milk was skimmed by centrifuging at 10,000xg for 15 min and sodium azide (0.02% w/v) was added to all samples. A portion of the bovine skim milk was treated with 0.1M trisodium citrate so as to disperse the casein micelles (CSM fraction).

3.3 PREPARATION OF WHOLE CASEIN AND WHEY

Whole casein was obtained from bovine skimmilk by isoelectric precipitation at pH 4.6 using 1N HCl. Casein was separated from whey by centrifugation at 5,000xg for 15 min and then filtration through cheese cloth. The volume of whey obtained was measured. Casein was dispersed in an equivalent amount of water or 0.1M trisodium citrate while the pH was maintained between 7.0 and 7.4 to dissolve the casein (acid casein fraction). The pH of the acid whey was adjusted to 7.0 by addition of 1N NaOH (acid whey fraction).

Casein micelles were prepared from bovine skim milk by ultracentrifugation at 100,000xg for 60 min at 7-10° C. The volume of the supernatant was measured and micelles were dispersed in an equivalent volume of either water or 0.1M sodium citrate while maintaining the pH at ~ 7.0 (casein micelles). The supernatant was divided into 2 portions, one of which (ultracentrifuge supernatant) was used without further treatment. The other portion was adjusted to pH 4.6 with 1 N HCl to precipitate non-micellar casein. Casein was removed by centrifugation at 5,000xg for 15 min and the pH of the casein-free supernatant was adjusted to 7.0 (acid supernatant). Sodium azide (0.02% w/v) was added to all samples. Aliquots of all samples were stored at -20° C until further use.

3.4 BCA PROTEIN ASSAY

The protein content of samples was determined using the BCA protein assay reagent and the colorimetric procedure recommended by the supplier (Pierce Chem. Co.). Bovine serum albumin was used as standard.

3.5 PREPARATION OF BOVINE PLASMA

Fresh bovine blood was collected at the university abattoir in the presence of 0.4% sodium citrate and placed on ice. The cooled blood was centrifuged at 5,000xg for 30 min at 0 - 4°C and the supernatant recovered. Plasma was recovered by centrifuging the supernatant at 27,000xg for 60 min at 0 - 4°C and stored at -20°C until further use.

3.6 ISOLATION OF BOVINE PLASMINOGEN

Bovine plasminogen was prepared by affinity chromatography (200). Sepharose 4B was activated with cyanogen bromide solution (10% w/v) while maintaining the pH at 11.0. A 10% (w/v) lysine solution in 0.1M phosphate buffer, pH 9.2, was added and mixed overnight at 4° C. After extensive washing with 0.1M phosphate buffer, pH 8.0, the activated Sepharose was poured into a column and 500 ml of bovine plasma was added. The column was subsequently washed with 0.3M phosphate buffer, pH 8.0, until A_{280} was approximately less than 0.05. Plasminogen was eluted using an EACA gradient with 0.1M sodium phosphate, pH 8.0, in the

mixing chamber and 0.001M sodium phosphate, pH 8.0, containing 0.005M EACA, in the gradient chamber. The A_{280} of collected fractions was obtained; peaks were pooled and dialyzed extensively (6,000-8,000 MW cut-off) and then lyophilized.

3.7 <u>ISOLATION OF BOVINE α_2-MACROGLOBULIN</u>

Alpha$_2$-macroglobulin was prepared from bovine plasma by PEG precipitation procedure (201). All purification steps were carried out on plastic surfaces and in the presence of SBTI, to avoid glass-induced activation of plasma proteases of the Hageman-dependent pathways.

One liter fresh bovine plasma was mixed with 30 ml crude SBTI solution (50 mg crude SBTI dissolved in 30 ml 0.02 M phosphate buffer, pH 7.4, containing 0.1 M NaCl) and stored at -20°C until further use. Complex formation of prothrombin with α_2-M was prevented by barium salt adsorption of blood coagulation factors II, VII, IX, and X. Barium chloride (0.1 M) was added to 500 ml of thawed plasma and mixed at room temperature for 10 min. The precipitate was removed by centrifugation at 2000 rpm for 15 min at room temperature. Barium sulfate (50 mg/ml plasma) was added to the supernatant and mixed at room temperature for 10 min. The precipitate was removed by centrifugation at 2,000 rpm for 15 min at room temperature. The barium sulfate adsorption was repeated a second time. The majority of

plasma fibrinogen was removed by addition of 2 volumes of phosphate buffer (0.02 M, pH 7.4, 0.1 M NaCl) to the supernatant followed by the addition of PEG to a final concentration of 4% and mixing for 30 min at room temperature. The precipitate was removed by centrifugation at 9,000 rpm for 30 min at $4^{o}C$. The supernatant was recovered and treated with PEG (50%) to a final concentration of 12% and incubated for 18 h at $4^{o}C$. Crude α_2-M was recovered by centrifugation at 9,000 rpm for 30 min $4^{o}C$.

Crude α_2-M was further purified by dissolving the 4-12% PEG precipitate in 90 ml KBr (10 g KBr in 100 ml 25 mM Tris-HCl, pH 8.0, containing 25 mg crude SBTI)solution. Insoluble material was removed by centrifuging at 9,000 rpm for 30 min at $4^{o}C$. The low density lipoprotein fraction in the supernatant was removed by centrifugation at 40,000 rpm for 20 h at $5^{o}C$ (Beckman, 50 i rotor). The pellet was resuspended in 2 volumes 50 mM Tris-HCl, pH 8.0, and applied to DEAE-Cellulose (DE 52) columns. Pure α_2-M was eluted using an NaCl gradient with 50 mM Tris-HCl, pH 8.0,20 mM NaCl in the mixing chamber and 50 mM Tris-HCl, pH 8.0, 0.4 M NaCl in the gradient chamber. Purity and identity of α_2-M was established by gel electrophoresis and DID assays.

Stock α_2-M solution for ELISA was prepared by dispersing 50 mg α_2-M in 10 ml PBS/T (0.2 g KH_2PO_4, 0.2 g NaCl, and 0.5 ml Tween-20, in a total volume of 1 L, pH 7.4)

containing 10% (w/v) NaN_3 at 0-4 C. The α_2-M solution was then filtered and dialyzed against PBS/T overnight under refrigerator conditions.

3.8 PREPARATION OF ANTIBOVINE α_2-MACROGLOBULIN

Rabbits were immunized subcutaneously with 200 µg of purified bovine α_2-M dissolved in 0.25 ml sterile saline and 0.25 ml complete Freund´s adjuvant. The rabbits were given a booster with 200 µg of purified antigen in incomplete Freund´s adjuvant three weeks later. Blood was collected from the ear 14 days after the booster shot and was checked for sufficient titer by doubleimmunodiffusion assay. Rabbits were inoculated every 4 weeks with 100 µg antigen in incomplete adjuvant until sufficient titer was obtained. Rabbits were exsanguinated and blood was allowed to clot at room temperature for 1 h and was centrifuged at 1,000xg for 10 min. Supernatant (antisera) was aliquoted and stored at -20° C until further use.

3.9 PREPARATION OF IMMUNOGLOBULINS

The IgG component of the antiserum was precipitated by adding 10 ml of 36% sodium sulfate to a mixture of 5 ml antiserum and 5 ml PBS (0.005 M phosphate buffer, pH 7.4, 0.15 M NaCl). The solution was mixed for 30 min at room temperature and was centrifuged at 3,000xg for 10 min. The precipitate was washed twice with 18% sodium sulfate,

dissolved in 5 ml PBS, and dialyzed extensively (6,000-8,000 MW cut-off) against PBS at 0-4° C . The protein content of the IgG fraction was determined by BCA assay.

3.10 PREPARATION OF CONJUGATES

3.10.1 Alkaline Phosphatase Conjugates

Rabbit IgG-alkaline phosphatase conjugates were prepared by the method described by Voller et al (205). An aliquot (~ 2.5 mg) of alkaline phosphatase was centrifuged at 3,000xg for 10 min at 0-4°C. The pellet was mixed with 3 mg of rabbit anti bovine α_2-M IgG in 1 ml PBS and dialyzed extensively (6,000-8,000 MW cut-off) against PBS at 0-4°C. Glutaraldehyde (0.2% v/v) was added to the IgG-enzyme solution and was mixed gently for 2 h at room temp. The conjugate was then extensively dialyzed at 0-4 C, initiallly against several changes of PBS and subsequently against several changes of 0.05 M Tis-HCl, pH 8.0. After dialysis, the conjugate was placed on a column of Sephacryl S200 and eluted with 0.05 M Tris-HCl, pH 8.0. The peaks were pooled and concentrated to 4 ml using Amicon Concentrators. To this concentrated solution 1% (w/v) BSA was added and then subjected to dialysis against several changes of 0.05M Tris-HCl, pH 8.0. The conjugate was stored at 0-4° C until further use (197).

3.10.2 Horseradish Peroxidase Conjugates

Rabit IgG-horseradish peroxidase conjugates were prepared by mixing 5 mg of antibovine α_2-M IgG in 1 ml PBS with 12 mg of the enzyme. A 1% aqueous solution (0.05 ml) of gluteraldehyde was then added and mixed with gentle stirring for 2 h at room temperature. The conjugate was then dialyzed, purified, concentrated, and stored as described in the procedure for preparing the phosphatase conjugate.

3.11 FLUOROMETRIC ASSAY FOR PLASMINOGEN AND PLASMIN

Plasmin activity in various milk fractions was determined by fluorometric assay using Coumarin Peptide as substrate and 7-amino methyl coumarin as standard (143,199). The combined amidolytic activity of plasminogen and plasmin was determined after treatment with urokinase (24 IU/0.1 ml) at 37° C for 15 min. Amidolysis, mediated by non-plasmin like enzymes, was determined by treatment with Aprotinin (2.8 TIU/0.1ml). Samples were incubated with substrate for 30 min at room temperature and the reaction stopped by treatment with aprotinin (2.8 TIU/ml incubation mixture). Difference in fluorescent intensities between the samples and blanks (not treated with substrate) were obtained at excitation and emission wavelengths of 380 and 460 nm, respectively, using an Aminco-Bowman Spectrophotofluorometer. Whey samples were dialyzed

overnight (6,000-8,000 cut-off) at 0-4°C against 0.05M sodium citrate to remove low molecular weight components which might contribute to fluorescence.

3.12 IMMUNOASSAYS FOR INHIBITORS

3.12.1 Immunodiffusionassays

Double immunodiffusion was performed (205) using antihuman α_2-M, antibovine α_2-M, antihuman alpha$_2$-antiplasmin, or antihuman alpha$_2$-antiplasmin-plasmin complex at various dilutions ranging from undiluted to 1:5120. Various skimmilk (bovine or human) dilutions (undiluted to 1:1024) were also used. The center well contained either the antiserum or the sample. If no precipitin lines were detected after incubation at 37°C for 2 days, one set of plates was stained with Coomasie Blue (0.5 % w/v solution in 25 % isopropanol, 10 % acetic acid). The other set was incubated for 5 more days and observed for precipitin line formation. Availability of purified bovine α_2-M, and human α_2-M allowed controls to be run with appropriate antisera.

Single radial immunodiffusion was performed (202) incorporating the following antisera antibovine α_2-M, antihuman α_2-M antiα_2-antiplasmin, or antiα_2-antiplasmin-plasmin complex. Various serial dilutions of the appropriate skimmilk (bovine, or human) were used.

Plates were incubated at 37°C for 18-24 h and the diameter of the rings were noted. Duplicate plates were stained with Coomasie Blue after 24 h of incubation.

3.12.2 Enzyme-Linked Immunosorbent Assay (ELISA)

A sequential, heterogeneous immunoassay (ELISA) was used to quantitate levels of α_2-M in bovine milk. The sandwich was formed by coating the wells of a 96-well microtiter plate with excess antibovine α_2-M IgG, followed by sequential treatments with sample (or standard), conjugate, and substrate. The concentrations of these components comprising the sandwich, as well as the incubation conditions, were determined to give optimum results with respect to slope, linearity, and correlation coefficient.

Anti α_2-M IgG was prepared from the antisera of two rabbits and contained 14.65 mg/ml and 17.00 mg/ml protein, respectively. Wells were coated using 100 µl of IgG from either antisera at a dilution of 1:400 (in coating buffer; a carbonate buffer prepared by mixing 5.1 ml 1 M $NaHCO_3$, 1.6 ml 1 M Na_2CO_3, and 0.2 ml 10 % NaN_3, in a total volume of 100 ml, pH 9.6, and stored at 0-4 C). Plates were incubated at 0-4°C for 16-18 h or at room temperature for 3-4 h. Wells were washed with coating buffer (3x2 min) and serial dilutions (of a 1:1000 dilution) of skimmilk (100 µl) were added to the wells. Plates were incubated at room temp

for 30 min. Wells were again washed with PBS/T (3x2 min). A 1:50 dilution of α_2-M-alkaline phosphatase conjugate (100 µl) in PBS/T was added to the wells. Plates were incubated at room temperature for 30 min. Wells were again washed with PBS/T (3x2 min). Disodium nitrophenyl phosphate (5 mg/ml of diehtanolamine buffer; the buffer contained 97 ml diethananolamine, 0.2 g NaN_3, 100 mg $MgCl_2.6H_2O$, pH 9.8, stored in dark at 0-4 C) was added to the wells (100 µl) and the plates were incubated for 60 min at room temperature. Reactions were stopped by the addition of 3N NaOH (100 µl) and the optical density was measured at 405 nm using a Titertek multiscanner.

Standard curves were obtained by adding serial dilutions (of a 1 µg/ml solution in PBS/T) of α_2-M (100 µl) to the wells instead of skimmilk samples.

Similar experiments were also performed to determine the distribution of α_2-M between acid casein and acid whey fractions obtained from the milks of 8 cows. Wells were coated with antiα_2-M IgG (1:400) followed by sequential treatments of sample (1:2000 and 1:4000), peroxidase conjugate (1:200), and Kirkegaard ABTS substrate. The reaction was stopped by adding 2% oxalic acid (100 µl) and the optical density was measured at 414 nm. Incubation and washing were performed as described for the experiments using alkaline phosphatase.

3.13 ASSAY FOR PLASMINOGEN ACTIVATORS

3.13.1 Colorimetric Assay

The coupled colorimetric assay (204) was used to detect the activity of plasminogen activators in milk and in various milk fractions. The assay was performed in 96-well ELISA plates, with the wells containing 25 µl of sample, 25 µl of soluble fibrin products (100 µg/ml), 25 µl of 1.6 mM SPEC-PL solution (a 5 mM solution in water diluted before use with Buffer Z, a 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM NaCl, 1 mM EDTA, and 0.01% Tween-80) and 25 µl plasminogen (1.5 WHO Units/ml). The turbidity effect was eliminated by measuring differences in optical density at 2 wavelengths (405 and 492 nm) using a Titertek Multiscanner. Readings were obtained periodically for 24 hours. All the samples were also assayed in the absence of exogenous plasminogen. The difference in optical densities obtained in the presence and in absence of exogenous plasminogen served as a measure of net plasminogen activator activity. The activity of fibrin-independent activators was detected by excluding the SFP from the incubation mixture.

The plasminogen activation potential in milk fractions was also assayed utilizing SPEC-UK (Cbo-L-( )Glu(α-t-BuO)-Gly-Arg-pNA.2AcOH), a chromogenic tripeptide, more specific for activators than plasmin (Km 2-4 orders of magnitude lower for activators). Inclusion of

aprotinin in the incubation mixture allowed the effect of plasmin to be eliminated without affecting the action of the activators. The assay was performed in 96-well ELISA plates containing 25 µl sample, 25 µl 1.6 mM SPEC-UK (a 5 mM solution. in water diluted before use with Buffer Z), 25 µl SFP (100 µg/ml), 15 µl aprotinin (15-30 TIU/ml), and 10 µl Buffer Z. Fibrin independent activators were detected by excluding the SFP from the incubation mixture. The optical density was measured as described above for the coupled assay. The ability of u-PA to release the chromophore from the substrate in the presence of aprotinin (.225-.450 TIU/well) was ensured by including controls containing different levels of u-PA (7.5-45 IU/well) in a total incubation volume of 100 µl.

3.13.2 Electrophoretic Assay

Copolymerized substrate electrophoresis (203) was used to determine the presence of plasminogen activators in milk. The substrate (gelatin concentration of 1 mg/ml) was copolymerized with a 10% acrylamide solution prepared as suggested by Laemmli (206). Gels also contained 15 WHO Units of plasminogen and 0.1% ultrapure SDS. Prior to application to the wells, skimmilk samples (obtained as described earlier) and human low molecular weight u-PA (1000 PU/ml) were treated with twice the volume of sample buffer (2.5 ml 0.5 M Tris-HCl, 5 ml 10% ultrapure SDS, and 10 ml

H_2O, pH 8.6) and allowed to stand for 15 min at room temp. Electrophoresis was performed under refrigerator temperature at 14 mA. A solution of 0.025 M Tris, 0.192 M Glycine, 0.1% ultrapure SDS, pH 8.6, served as electrode buffer.

After electrophoresis, gels was immersed in 2.5% Triton-X and incubated for 1 h at room temperature with gentle shaking. Gel slabs were immersed in 0.1M glycine, pH 8.3, and stored overnight at 0-4 C. Gels were stained with 0.1% amido black. Proteolysis independent of plasminogen activation was monitored by running control gels which did not contain plasminogen.

3.13.3 Immunodiffusionassays

The immunologic identity of plasminogen activators in human and porcine skimmilk was established by performing DID and SRID assays using antihuman u-PA, antihuman t-PA, or antiporcine t-PA. The procedure described in section 3.12.1 was followed.

4.0 RESULTS AND DISCUSSION

4.1 ENDOGENOUS PROTEASES IN BOVINE MILK

The average distribution of proteolytic activity (Table 1) was determined in fractions prepared from the individual milks of 4 cows by the fluorometric assay (individual cow values shown in appendix). Although general trends were similar in all cows, specific values obtained were variable (appendix). Greater amidolytic activity was detected (due to endogenous plasmin) in casein micelles (12.71 AU) than in isoelectric casein (9.94 AU). Correspondingly, the acid whey fraction obtained during preparation of isoelectric casein contained higher activity (8.41 AU) than did supernatant fractions (5.92 AU) obtained during preparation of casein micelles. Thus, the method used to isolate fractions does affect the distribution of amidolytic potential. Previously, Richardson (181) had found that preparation of casein by acid treatment resulted in dissociation of plasmin and plasminogen from casein. However, the percentage of plasmin dissociating from casein in this study is higher (~22%) than previously reported (~14%). This discrepancy could be due to differences in the time that samples were exposed to acid. Distribution of plasminogen (measured as the difference in the fluorescence intensities before and after treatment with u-PA) among different milk fractions paralleled the distribution of

Table 1. Distribution of Amidolytic Activity Among Milk Fractions.

Sample	Activity (AU)[a]			
	Plasmin	Plasminogen and Plasmin	Plasminogen[b]	Non-plasmin
CSM[c]	11.38	18.48	7.10	8.17
Casein micelles[c]	12.71	26.73	14.02	7.19
Acid casein[c]	9.94	19.40	9.46	7.66
Supernatant	5.92	7.30	1.38	6.11
Acid supernatant	5.67	6.71	1.04	2.82
Acid whey	8.41	13.81	5.40	3.15
Dialyzed acid whey	3.76	7.10	3.34	3.21

[a]Activity expressed in Arbitrary Units (AU); 1 AU = 10^{-7} M amino methyl coumarin released/ml of sample.
[b]Difference in fluorescent intensity before and after treating with u-PA.
[c]Fluorescent intensity of casein containing fractions have been multiplied by 2.5 to account for competition between casein and coumarin peptide (143).

plasmin (Table 1). Moreover, approximately 20% of the total amidolytic potential was contributed by aprotinin-resistant proteases present in milk fractions. This is in agreement with Rollema (206) who reported that about 20% of proteolytic activity in milk directed towards S-2251 (H-D-Val-Lys-Lue-pNA) is due to enzymes other than plasmin. This aprotinin-resistant amidolysis could be mediated by plasminogen activators, since aprotinin inhibits plasmin but not plasminogen activators. Although synthetic substrates are more susceptible to action by plasmin, hydrolysis by activators can occur, though to a lesser extent. The aprotinin-resistant amidolysis could also be due to proteases other than plasmin and plasminogen activators known to occur in milk.

Results obtained may have been affected by one or more components of the assay system. For example, aprotinin may not be able to react with plasmin due to interference by milk proteins. Thus, plasmin in the ultracentrifuge supernatant could be protected from aprotinin by non-micellar casein, resulting in a high value (6.11 AU) compared to values obtained in casein-free supernatant (2.82 AU) and acid whey (3.15 AU). Amidolysis produced by endogenous plasmin did not seem to be affected by removal of non-micellar casein from the supernatant (5.92 and 5.67 AU, before and after casein removal, respectively). However, casein in the supernatant might have competed with Coumarin

Peptide producing an underestimation of plasmin activity. Dialysis of acid whey resulted in a decrease of both plasmin and plasminogen activities.

However, these results do show that plasmin-like activity is present in all milk fractions and that the distribution of activity depends upon the method used to prepare the fractions. However, little information has been gained about the levels and activities of fibrinolytic activators and inhibitors which may also occur in milk. These activators and inhibitors could contribute to the high variance in plasmin activity detected among milk fractions from different cows. Thus a clearer picture of the fibrinolytic system in milk could be obtained by identifying the principal plasminogen activators and antiplasmins.

4.2 ANTIPLASMINS AND ANTIACTIVATORS IN MILK

Blood has been shown to be a source of plasminogen and plasmin in milk. Since enzyme mediated reactions are highly regulated, other components of the fibrinolytic system may also be present in milk. Efforts to identify and detect inhibitors in milk were directed towards α_2-macroglobulin and α_2-antiplasmin, the principal inhibitors known to occur in blood.

4.2.1. α_2-Macroglobulin

Samples of skimmilk prepared from healthy cows

produced single precipitin lines in DID assay against antibovine α_2-M (not shown). These results were the first indication that α_2-M actually occurs in bovine milk. An ELISA was developed to further verify these results and to quantify the levels at which α_2-M occurs in bovine milk. Very good correlation coefficients, linearities, and slopes were obtained for both the standard curves and various skimmilk sample dilutions (Table 2). The 10 skimmilk samples examined contained 1.54 +/- 0.91 mg α_2-M/ml (Table 3) (appendix shows calculations for one sample), which comprises over 4% of the total protein in milk. This level of α_2-M occurrence is much higher than expected, based on the relatively low levels of plasminogen and plasmin (< 3.5 μg/ml) reported to occur in milk (143). A SRID assay was developed and was used to determine the level of α_2-M in a sample of human milk and a correspondingly high level (< 1 mg/ml) was detected (Fig 2).

Elevated levels of α_2-M detected in both human and bovine milks might suggest a major role in preventing damage to mammary tissue mediated by plasmin and by proteases (cathepsin, elastase, collegenase) released by leucocytes which are present in mammary alveoli in response to infection. High levels of α_2-M could inhibit the activities of both plasminogen activators and plasmin and protect milk proteins from extensive degradation. Further, the ability of α_2-M bound enzymes to hydrolyze small

Table 2. Mean Absorbance and Correlation Coefficients for α_2-M ELISA.

	α_2-M (μg/ml)	A_{405}	
		a	b
Standard curve	10.00	1.194	0.780
	5.00	0.897	0.429
	2.50	0.524	0.261
	1.25	0.273	0.162
		r_a=0.963[a]	r_b=1.00[b]

Skimmilk Cow number	Mean Vol.($x10^{-3}$)[c]	Mean A_{405}[d]	Correlation coefficient
1	5.83	0.965	0.990
2	4.69	0.891	0.959
3	5.83	0.526	0.904
4	2.92	0.975	0.947
5	5.83	1.006	0.965
6	5.83	0.813	0.953
7	4.69	0.670	0.977
8	4.69	0.845	0.981
9	2.92	0.822	0.990
10	4.69	0.685	0.978

[a]For cows 1-8.
[b]For cows 9 and 10.
[c]Total volume of each sample divided by total number of observations.
[d]Total absorbance of each sample divided by total number of observations.

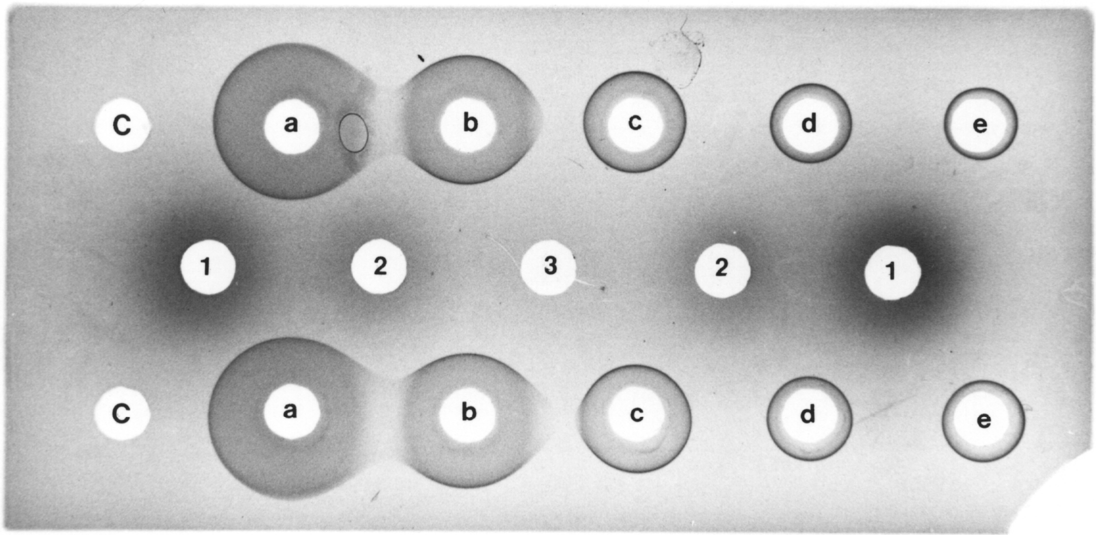
Table 3. α_2-Macroglobulin in Bovine Skimmilk as Determined by ELISA.[a]

Cow number	α_2-M (mg/ml)[b]
1	1.22
2	1.36
3	0.47
4	2.46
5	1.29
6	0.96
7	0.89
8	1.26
9	3.63
10	1.84
Average	1.54 +/- 0.91
Range	0.47 - 3.63

[a]1:400 coating antibody; 1:50 alkaline phosphatase conjugate.

[b]Value of α_2-M in µg/ml obtained from mean absorbance of the sample, and the standard curve, was multiplied by the dilution factor.

Figure 2. Detection of α_2-M in Human Skimmilk. 0.25% (V/V) antibovine α_2-M co-polymerized in 1% agarose, containing 2.5% polyethylene glycol 3,350. C, PBS (10 µl); a, b, c, and d, undiluted, 1:1, 1:2, and 1:4 dilutions of stock (1 mg/ml) α_2-M (10 µl); e, 1:10 dilution of stock α_2-M (10 µl); 1, 2, and 3, 1:1, 1:2, and 1:4 dilutions of human skimmilk. Gel stained with 0.5% amido black 10B in 45:45:10, methanol:acetic acid:water, and destained with 45:45:10, methanol:acetic acid:water.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molecular weight, but not large molecular weight, substrates may also affect assays which use synthetic substrates to measure proteolytic potential in milk. Thus, α_2-M bound plasmin, while showing little or no activity towards casein, may still rapidly hydrolyze synthetic substrates like Coumarin Peptide, Chromozyme-PL, and S-2251. These assays may produce an overestimation of plasmin mediated proteolytic potential in milk. On the other hand, free α_2-M in milk may bind exogenous plasminogen activators and prevent activation of all the endogenous plasminogen in milk. Inaccurate values for plasminogen would then be obtained when measured as a difference in amidolytic potential before and after treatment with u-PA. For the same reason, assay of activators by addition of exogenous plasminogen to milk may not give accurate results. Thus, the antiproteolytic potential of milk should be considered when interpreting data (as in Table 1) obtained using synthetic substrates to assay for proteolytic potential.

The α_2-M ELISA was also used to determine the distribution of α_2-M activity between casein and whey fractions. α_2-Macroglobulin appears to associate to a much greater extent with whey than with the casein fraction (Table 4). Although α_2-M effectively inhibits a large number of proteases, not all are affected (114-117). Whether bovine α_2-M is effective against milk clotting enzymes would be of considerable interest to the cheese

Table 4. α_2-Macroglobulin ELISA of Bovine Milk Fractions.[a]

	A_{414}	
Cow number	Whey	Casein
1	0.984	0.378
2	1.281	0.455
3	1.019	0.383
4	1.140	0.291
5	0.990	0.285
6	0.904	0.240
7	0.623	0.202
8	0.756	0.303
Average	0.962	0.317
SD	0.207	0.083

[a]1:400 coating antibody; 1:4000 sample; 1:200 peroxidase conjugate.

industry.

4.2.2 α_2-Antiplasmin

The occurrence of α_2-M in bovine milk suggests that the other principal inhibitor of the fibrinolytic system, α_2-AP, might also be present. However, the only commercially available antisera had been prepared against human plasma α_2-AP. Moreover, antisera prepared against the human plasmin-α_2-AP complex was also available. We did not observe any cross-reactivity between these human antisera and the corresponding bovine antigens in DID assay using aliquots of cow skimmilk (not shown). Moreover, negative results were again obtained when a sample of human milk was used in the DID assay (not shown). However, when the more sensitive SRID assay was used, human milk produced a light ring in plates containing human anti α_2-AP, as well as human antiplasmin-α_2-AP complex (not shown). These precipitin rings were difficult to observe because background staining made the precipitin rings difficult to photograph. However, these results do provide strong evidence that α_2-AP occurs in human milk both in the free form as well as complexed with plasmin. Plasmin should have available both a free lysine binding site and a free active site, to be inactivated by α_2-AP (76,77). Thus, the occurrence of both free α_2-AP and free plasmin in human milk suggests that some of the plasmin is protected through interactions with other

milk proteins, such as casein, mediated by the plasmin A-chain. Plasmin bound to casein may still retain the ability to act on other casein molecules, even in the presence of α_2-AP.

Similar experiments could not be conducted with bovine milk because purified bovine α_2-AP and the corresponding antisera, is not currently available. However, since both the bovine and human fibrinolytic systems and milk systems are qualitatively similar, α_2-AP is very likely to also occur in bovine milk. If true, the primary role for α_2-M in the mammary gland may not be to function as an antiplasmin (that role being played by α_2-AP) but as a general antiprotease. This system would allow plasmin to maintain patency of the mammary gland while ensuring that extensive tissue and protein breakdown does not occur when a battery of proteolytic enzymes are released by leucocytes in response to mammary infection.

Both human α_2-M, and α_2-AP are known to inhibit t-PA and u-PA (83,84,107,125). Thus, high levels of α_2-M detected in bovine milk (Table 3) might represent a significant antiactivator potential.

The effect of α_2-AP in bovine milk on proteolysis mediated by endogenous proteases is currently not known. However, development of an ELISA for detection and quantification of α_2-AP in various bovine milk fractions, as well as during various stages of lactation and during

mastitic infection, may further the understanding of the role of α_2-AP in milk. Moreover, since α_2-AP is rapidly inactivated below pH 5.7 (72), the proteolytic potential of milk whose pH is < 5.7 during the production of various cheeses and cultured products may be altered.

4.3 ACTIVATORS IN MILK

4.3.1 Colorimetric Assay

The direct assay utilizing SPEC-UK, which is more specific for u-PA than t-PA, was not sensitive enough (A_{405}-A_{492} was < .05 in samples treated with aprotinin) to detect activators present in milk. Thus, the coupled assay, which involves detection of plasmin activity released by action of endogenous activators on exogenous plasminogen, was used to detect the occurrence of plasminogen activators in milk (Table 5). The mean activating potential (A_{405}-A_{492}) detected in the samples of skimmilk was 0.122 in the presence of both exogenous plasminogen and SFP. Compared to the value of 0.054 obtained in the absence of both (Table 5), this value indicates that endogenous activators present in bovine milk do convert exogenous plasminogen to plasmin. Sufficient controls were included to ensure that exogenous plasminogen was not responsible for the release of the chromophore detected in the assay. The reason that endogenous activators convert exogenous, but not

Table 5. Colorimetric Assay for Plasminogen Activators in Bovine Skimmilk.[a]

Treatment	Absorbance[b] (A_{405} - A_{492})	
	Buffer	u-PA
Pg; SFP	0.122 +/- 0.028	1.587 +/- 0.950
Pg	0.110 +/- 0.019	1.540 +/- 0.041
SFP	0.054 +/- 0.023	0.249[c]
None	0.054 +/- 0.021	0.236 +/- 0.105

[a]n = 4.
[b]To account for absorbance due to turbidity, A_{492} was subtracted from A_{405}.
[c]n = 2.

endogenous, plasminogen is not immediately apparent. Endogenous plasminogen may not be easily accessible to endogenous activators and may interact with other milk proteins or with α_2-M or α_2-AP in milk (Table 5). However, exogenous activators, such as u-PA, can activate endogenous plasminogen (A_{405}-A_{492} .054, and 0.236, respectively, before and after treatment with u-PA). Controls containing only u-PA and the substrate were used to show that the substrate was not hydrolyzed by exogenous u-PA (A_{405}-A_{492} < .029). Inclusion of SFP in the incubation mixture had no effect on either the activation of exogenous plasminogen (0.122 and 0.110, respectively, in the presence and absence of SFP) or on activation of endogenous plasminogen (0.05 in both cases) (Table 5).

Soluble fibrin products were included to enhance the activity of fibrin-dependent activators (t-PA). Since, SFP had little effect on levels of plasminogen activation (endogenous, and exogenous) (Table 5), very little t-PA activity may actually be present in milk. Alternatively, milk protein may satisfy the fibrin dependence of t-PA by interacting with the two kringles (assuming bovine t-PA also has the same structure as human t-PA) of the t-PA heavy chain.

Exogenous u-PA also produced a marked increase in optical density when exogenous plasminogen was present (Table 5). Thus, exogenous activators can activate exogenous

plasminogen both in the presence and absence of SFP. However, levels of α_2-M occurring in milk will affect the extent of plasminogen conversion by u-PA as well as the ability of plasmin to act on other milk proteins (as opposed to small substrates).

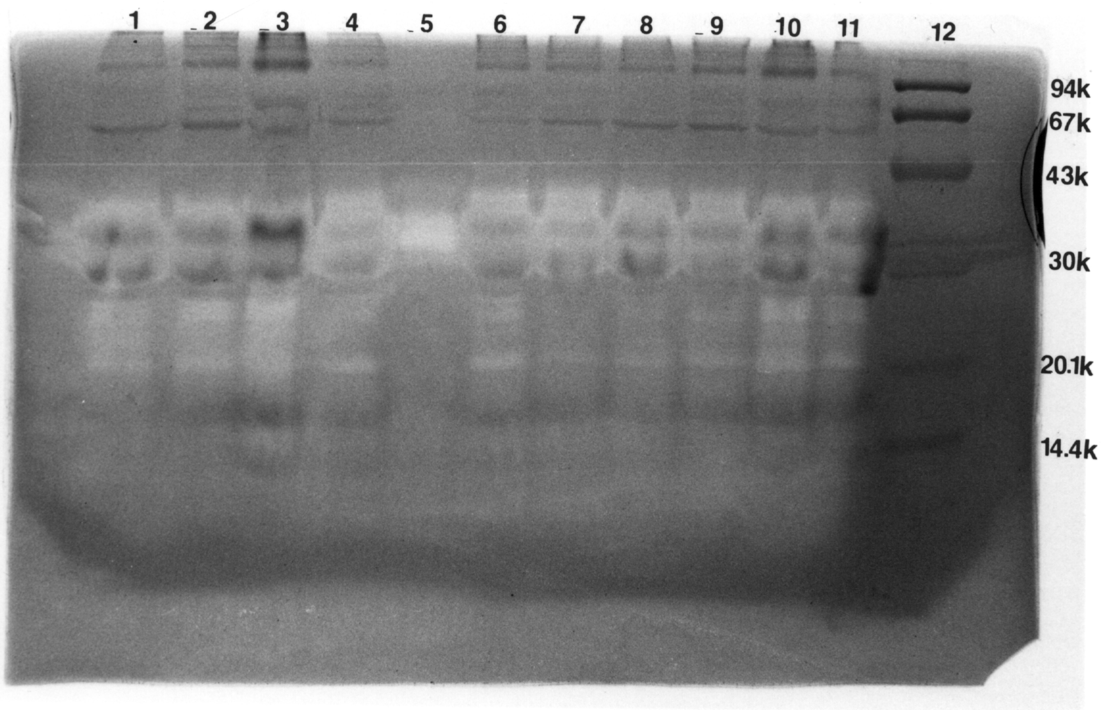
4.3.2 Co-polymerized Gel Electrophoresis

Co-polymerization of plasminogen and gelatin in an electrophoretic gel matrix was used to detect and further identify plasminogen activators which occur in milk. Ultrapure SDS was incorporated into all gels to reversibly inhibit all proteases and to dissociate enzyme-inhibitor complexes (eliminating interference by inhibitors). Thus, low levels of activator can be detected even in the presence of inhibitors. Activators present in samples will concentrate in narrow bands during electrophoresis. After SDS removal from gels (by treatment with Triton X-100, which also stabilizes the plasminogen activators) any activator present would renature and may be expected to activate plasminogen incorporated into the gel matrix. Plasmin would then hydrolyze the *in situ* substrate, gelatin. Thus, when gels are stained and subsequently destained, clear zones of lysis would appear where active proteases are present. This electrophoretic procedure also allows differentiation between two or more activators present in milk since each will appear as separate, distinct bands based on separation

due to differences in molecular weight.

All milk samples produced clear zones of lysis in a band co-migrating in the 30 kD range (Fig 3). Low molecular weight human u-PA also produced similar lysis zones (Fig 3). When copolymerized plasminogen was eliminated from the gel matrix, no zones of lysis could be detected in either milk samples or controls (Fig 4). These results indicate that bovine milk contains one class of activators which are probably analogous to human low molecular weight u-PA (MW 31.5 kD). No lysis zones were detected in the molecular weight range expected for t-PA (human t-PA has a molecular weight of 57-69 kD). This observation would appear to support the failure of SFP to enhance plasminogen activators activity in the colorimetric assay (Table 5). However, although plasminogen activators similar to u-PA would appear to be the primary source of activity, gels with copolymerized plasminogen also contained other clear zones of lysis which were totally absent in gels not containing plasminogen (Figs 3,and 4). These lysis zones should not be due to the presence of streptokinase and staphylokinase which are known to be inactive towards bovine plasminogen (59,63). If the zones of lysis observed in plasminogen containing gels was due to other endogenous proteases (like trypsin) or bacterial proteases, similar zones should also be observed in gels which do not contain plasminogen. These additional zones of lysis would appear

Figure 3. Detection and Identification of Plasminogen Activators and Proteases in Bovine Skimmilk. Gel contained 10% acrylamide, 0.1% ultrapure SDS, 15 WHO Units co-polymerized bovine plasminogen, and 0.1% co-polymerized gelatin. Lanes 1, 2, 3, 4, and 8, 9, 10, 11 contained 20 µl, and 10 µl, respectively of skimmilk from four healthy cows. Lanes 6 and 7, 10 µl skimmilk from two other healthy cows. Lane 5, 2 Plough Units low molecular weight human u-PA. Lane 12, molecular weight standards (Phosphorylase b, BSA, Ovalbumin, Carbonic anhydrase, SBTI, α-Lactalbumin). Gel was washed, incubated, stained, and destained as described in Materials and methods.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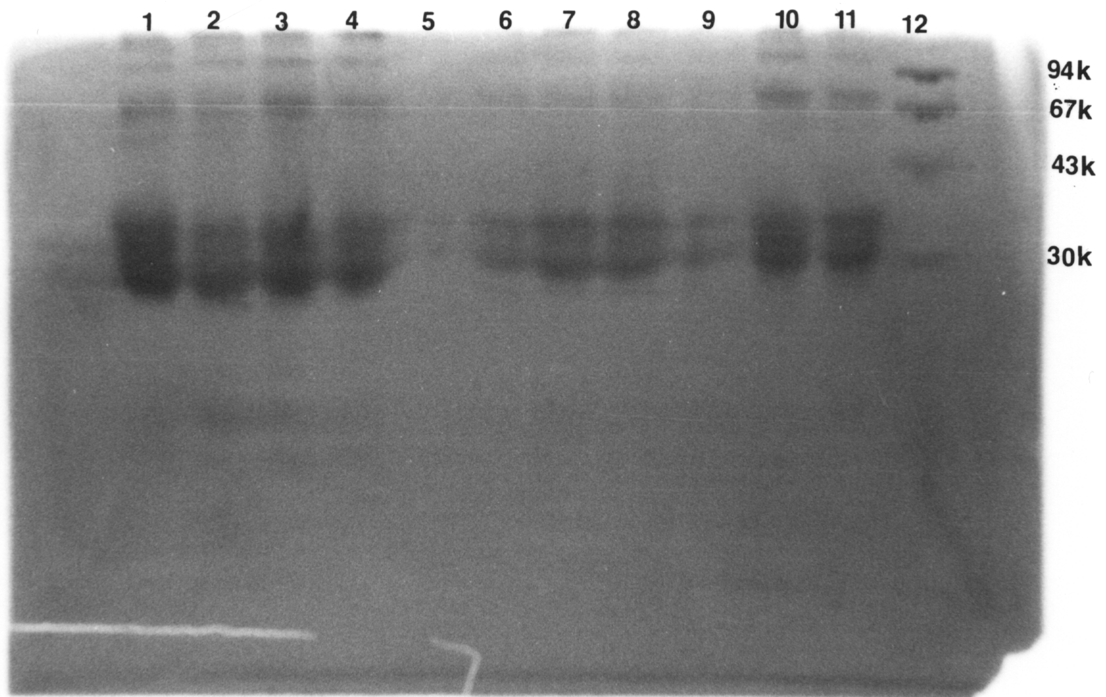
12
94k
67k
43k
30k
20.1k
14.4k

Figure 4. Detection of Plasminogen-independent Proteases in Bovine Skimmilk. Bovine plasminogen was not co-polymerized with the gel. Electrophoresis was performed as indicated in Figure 3. Note complete absence of gelatinolysis in all lanes.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to represent additional plasminogen activators present in bovine milk.

4.3.3 Single Radial Immunodiffusion

In order to further identify plasminogen activators present in milk, antisera developed against porcine t-PA and human t-PA and u-PA were incorporated into SRID agar. Both samples of porcine skimmilk produced immunodiffusion rings with several serial dilutions (Fig 5). The sample of human skimmilk produced rings with both t-PA (Fig 6) as well as u-PA (Fig 7). Thus, if bovine milk is analagous, the zone of lysis produced in the co-polymerized gel (Fig 3), which co-migrates with u-PA, would appear to actually be u-PA.

Similar experiments could not be performed with bovine milk samples, as the corresponding bovine antisera are not currently available. Our attempts to isolate u-PA from bovine urine were unsuccessful due to loss of u-PA activity during dialysis.

Figure 5. Detection of t-PA in Sow Skimmilk. C, PBS (10 µl); 1-8, serial dilutions of sow skimmilk (10 µl). Gel composition, and staining and destaining procedures were as described in Figure 2.

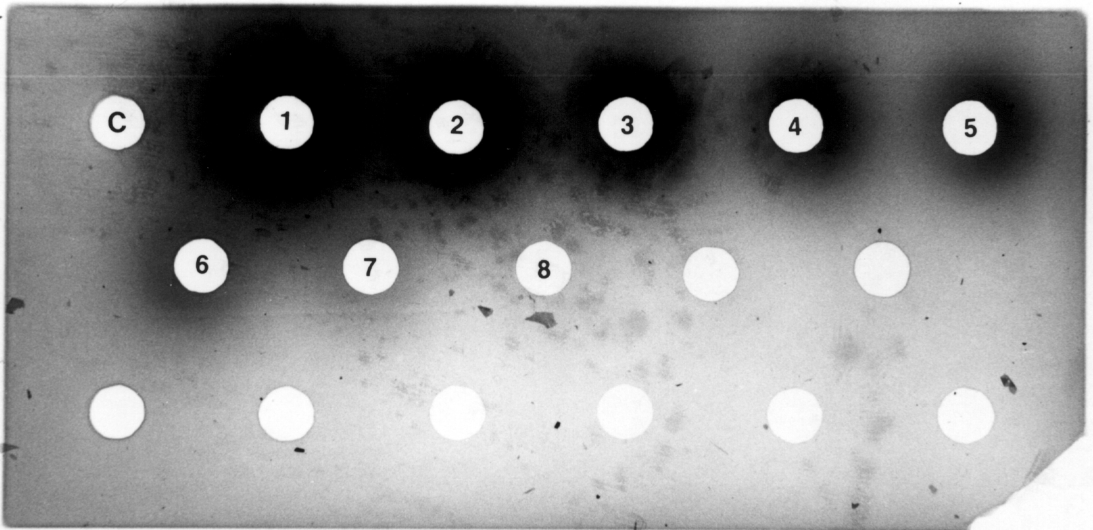
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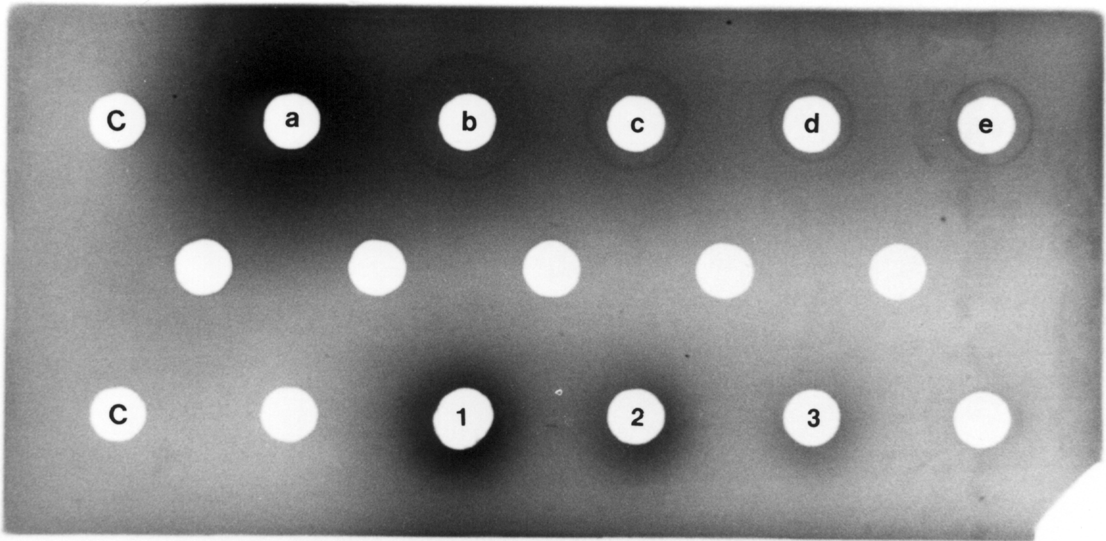
Figure 6. Detection of u-PA in Human Skimmilk. C, PBS (10 μl); a, b, c, and d, serial dilutions of stock (1000 PU/ml) human u-PA (10 μl); e, 1:10 dilution of stock u-PA (10 μl); 1, 2, and 3, 1:1, 1:2, and 1:4 dilutions of human skimmilk. Composition, and staining and destaining procedures were as described in Figure 2.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e
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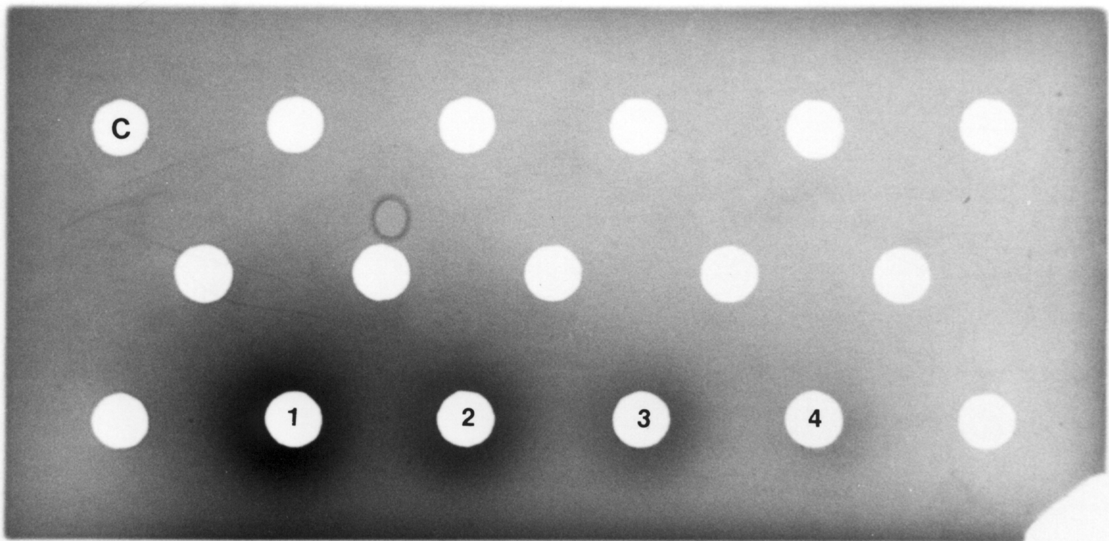
3

Figure 7. Detection of t-PA in Human Skimmilk. C, PBS (10 μl); 1, 2, 3, and 4, undiluted, 1:1, 1:2, and 1:4, dilutions of human skimmilk (10 μl). Composition, and staining destaining procedures were as described in Figure 2.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5.0 SUMMARY AND CONCLUSIONS

Fibrinolysis in mammals is mediated and regulated by a complex, multicomponent system in which plasmin is the principal proteolytic enzyme. Plasmin has also been identified as the primary endogenous protease in bovine milk (139,143) and been shown to occur in the milks of other species as well (148). A great deal of attention has been directed toward determining the significance of plasmin in milk. However, a clearer understanding of the role of plasmin in milk will only be possible when other components of the fibrinolytic system which occur in bovine milk have been identified and quantified. This information will be obtained only when highly specific and sensitive assays for the detection of activators and inhibitors in milk are developed.

The colorimetric assay was able to verify that plasminogen activators do occur in milk (Table 5). This assay has been successfully used to differentiate between fibrin-dependent (t-PA) and fibrin-independent (u-PA) activators in sera (204). Results obtained with this assay seem to indicate that most plasminogen activator activity in bovine milk is similar to u-PA and independent of fibrin. Little evidence was obtained for the presence of t-PA or fibrin-dependent activators as reported to occur in human milk (148). Further differentiation between u-PA and t-PA

was attempted with copolymerized substrate electrophoresis, which was found to be very convenient for detecting low levels of plasminogen activators, even in the presence of inhibitors. The sensitivity of the assay is greatly enhanced through concentration of activators into narrow bands which allows catalysis of plasminogen to plasmin with subsequent hydrolysis of gelatin. Exclusion of plasminogen from the gels allows differentiation of gelatinolysis mediated by proteases other than plasminogen activators. We have thus demonstrated, for the first time, that bovine milk samples possess an activator which co-migrates with human u-PA (Fig 3). Additional evidence for the presence of u-PA in bovine milk was obtained by demonstrating that human milk also contains a plasminogen activator which is immunologically identical to u-PA. Although u-PA appears to be a major source of plasminogen activator activity in bovine milk, a large number of additional zones of lysis also appeared in gel patterns. These zones of lysis may represent additional plasminogen activators present in bovine milk or fragments of t-PA which still retain the ability to activate plasminogen. Although no zones of lysis appeared in the molecular weight range reported for t-PA, a plasminogen activator immunologically identical to t-PA was shown to occur both in human and porcine milk. The primary role ascribed to u-PA is that of tissue remodelling, while t-PA is primarily for resolving thrombi (in the circulatory

system) and for maintaining duct patency (in the mammary gland).

A number of workers (143,149,159) have reported that milk contains low levels of plasmin and highly variable levels of plasminogen. However, this variability could well be due in part to the effect of inhibitors and activators which would affect assays based on enzyme activity. Again, the distribution of plasmin among various milk fractions can not be considered independently of the distribution of the other components of the fibrinolytic system. α_2-Macroglobulin has been identified, for the first time, as one of the antiproteases present in bovine milk. The surprisingly high level of α_2-M (1.54 mg/ml) detected in bovine milk indicates that the mammary gland may require a general protease inhibitor to prevent excessive tissue autodigestion. Furthermore, high levels of α_2-M may be necessary to contain the tissue damage caused by proteases released by leucocytes in response to infection. Thus, screening milk and serum samples to determine the effect of disease on levels of these components in milk may assist in gaining a greater understanding of the role of these components in healthy and mastitic udders. If α_2-M is an effective inhibitor of chymosin (rennin), it would be of tremendous significance to the cheese industry. If so, elevated levels could be expected to tremendously affect various cheese-making parameters such as clotting time,

texture, and yield. Additionally, human milk has been shown to also contain the specific plasmin inhibitor, α_2-AP. If present in bovine milk, α_2-AP as well as α_2-M, may be responsible for the variations in levels of plasminogen and plasmin reported in the literature (143,149,159).

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APPENDIX A

Distribution of Amidolytic Activity Among Milk Fractions from Individual Cows.

	Activity (Au)								
	Plasmin			Plasminogen + Plasmin			Non-Plasmin		
	a	b	c[1]	a	b	c[1]	a	b	c[1]
CSM	11.98	n.d.[2]	10.78	17.83	n.d.	19.13	9.73	n.d.	6.60
Casein Micelles	11.98	8.90	17.50	21.40	18.90	39.98	8.83	5.70	7.05
Acid Casein	13.33	6.63	9.85	18.70	15.28	24.23	7.50	8.43	7.05
Supernatant	6.23	n.d.	5.61	7.13	n.d.	7.46	5.69	n.d.	6.53
Acid Supernatant	7.48	3.92	5.61	7.48	4.83	7.83	2.64	3.01	2.82
Acid Whey	5.24	10.66	9.32	4.79	22.13	14.51	3.44	3.01	3.01
Dialyzed Acid Whey	3.44	4.10	3.75	4.79	7.02	9.50	3.44	3.37	2.82

[1]Milk from two healthy cows was combined and used in the assay.
[2]n.d.=not determined.

APPENDIX B

Sample ELISA Calculations for α_2-M in Bovine Skimmilk.

Standard α_2-M	µg/ml	A_{405}
	10.00	0.780
	5.00	0.429
	2.50	0.261
	1.25	0.162

r = 1.00

Skimmilk (from Cow 10)	µl/well	A_{405}
	10.00	1.068
	5.00	0.779
	2.50	0.550
	1.25	0.342
Mean	4.69	0.685

Thus, α_2-M in skimmilk (µg/well) = 8.64.
Dilution factor = 1/4.69 x 1000 = 213.33.
α_2-M in skimmilk (mg/ml) = 8.64 x 213.33 = 1.84.

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