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Plasmin-mediated proteolysis of casein in bovine milk

(plasminogen/β-casein/urokinase)

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ABSTRACT **Plasminogen was found to be present in bovine milk by crossreactivity between rabbit antiserum to plasminogen and casein prepared from milk by acid precipitation. This result was further supported by recovery of intact ^{125}I-labeled plasminogen from rabbit milk after its intravenous injection. Freshly isolated whole bovine casein was observed to undergo slow autoproteolysis at 37°C. Polyacrylamide gel electrophoresis revealed gradual disappearance of major caseins accompanied by appearance and increase in intensity of numerous electrophoretic bands. This autoproteolysis was inhibited by low concentrations of ε-aminocaproic acid (0.1 mM) and diisopropyl fluorophosphate (1 mM); catalytic amounts of urokinase accelerated the process. Autoproteolysis of isolated bovine β-casein was shown by both urea and sodium dodecyl sulfate gel electrophoresis to result in formation of γ_1- and γ_2-caseins. Similar electrophoretic bands were formed when β-casein was degraded by plasmin prepared from bovine blood serum. These results support the hypothesis that bovine plasmin occurs in milk and is identical to alkaline milk protease.**

Plasminogen is the zymogen of the proteolytic enzyme plasmin which is responsible for the dissolution of fibrin clots in blood. In humans and other species several organs have been shown to contain considerable amounts of plasminogen activator activity (1–3). However, plasminogen itself is normally considered to be a constituent only of mammalian blood plasma (4). The presence of a naturally occurring protease in milk was first reported in 1897 (5). Casein, prepared by acid precipitation of skim milk, contains most of the proteolytic activity found in milk (6). Kaminogawa *et al.* (7) have suggested that this alkaline milk protease may actually be plasmin transported from bovine plasma across mammary epithelial cells.

β-Casein, a major casein fraction present in bovine milk, consists of a single polypeptide chain of 209 amino acids (8). Results of amino acid analyses, molecular weight determinations, peptide mappings, and end-group analyses first indicated a close relationship between several minor casein fractions (γ_1-, γ_2-, and γ_3-casein) and β-casein (9). Comparison of the amino acid sequence of the first 10 to 15 residues at the NH_2 terminus of each of these minor caseins with the primary structure reported for β-casein revealed that γ_1-, γ_2-, and γ_3-caseins are actually identical to residues 28–209, 106–209, and 108–209 of β-casein, respectively (8, 9). Eigel (10) demonstrated formation of γ_1-, γ_2-, and γ_3-caseins by incubation of bovine β-casein with plasmin. In this investigation we have found that plasminogen can be transferred from blood into milk and that low levels of plasmin do occur in milk.

MATERIALS AND METHODS

Preparation of Plasminogen and Plasmin. Bovine plasminogen was prepared by affinity chromatography from fresh citrated whole blood (11). Rabbit plasminogen was prepared by affinity chromatography (12) from lyophilized rabbit serum (10 g) suspended in 500 ml of phosphate-buffered saline (0.1 M sodium phosphate, pH 7.2/0.15 M NaCl). Plasmin was prepared by incubation of plasminogen with urokinase (UKase) at 37°C for 15 min. The incubation mixture contained plasminogen (2.4 mg/ml) in 0.05 M sodium tetraborate, pH 8.4, and UKase (100 Plough units/ml).

Preparation of Casein Fractions. Whole milk, obtained from the complete milking of a single cow, was used to prepare skim milk by centrifugation (7000 × *g*, 23°C, 15 min). Whole casein was prepared by adjusting the pH of the skim milk to 4.6 with 3 M HCl and was washed three times with distilled water prior to lyophilization. β-Casein was prepared from whole casein by the urea method of Hipp *et al.* (13). The genetic variant of β-casein was determined by vertical polyacrylamide gel electrophoresis at both pH 9.2 (14) and pH 3.0 (15) in 4.5 M urea.

Immunological Study. Antiserum to bovine plasminogen was raised by weekly subcutaneous injection of 1 ml of plasminogen (10 mg) in phosphate-buffered saline containing 50% Freund's complete adjuvant into a New Zealand White rabbit. Five weeks after the first injection, blood was collected by cardiac puncture, allowed to coagulate for 30 min at 23°C, and centrifuged at 1500 × *g* for 10 min (16). The globulin fraction was prepared by two successive precipitations at 50% ammonium sulfate saturation (17) followed by washing with phosphate-buffered saline. After extensive dialysis against distilled water at 4°C, the globulin fraction was recovered by lyophilization.

Double diffusion (Ouchterlony) plates (18) were prepared with 1% agarose, 0.05% sodium azide, 0.5% Triton X-100, and 0.2 M glycine in phosphate-buffered saline (17). Protein and buffer samples (20 μl) were placed in the wells and allowed to diffuse for 2–4 days at 23°C.

Plasminogen Transport. Rabbit plasminogen (8 mg) was labeled with ^{125}I by the method of Helmkamp *et al.* (19) and was dialyzed exhaustively against distilled water at 4°C until the radioactivity of the dialysate was at background level. After dialysis against phosphate-buffered saline at 4°C, an aliquot (3 ml) was injected into the ear vein of a New Zealand White rabbit on the ninth day of lactation. The four nursing young were removed overnight and the milk was collected by manual expression the following morning. The milk was diluted 1:3 with distilled water and casein was prepared by adjusting the pH to 4.6 with 1 M HCl. Casein was dispersed in 15 ml of 0.3 M Tris·HCl at pH 8.0 and mixed with 500 ml of rabbit serum. Rabbit plasminogen was prepared as described above. Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis of rabbit plasminogen was conducted in 7.5% acrylamide slabs (20) and gels were stained with 0.05% Coomassie blue. Gels were destained electrophoretically, dehydrated under reduced pressure, and exposed to x-ray film for 7 days.

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Abbreviations: EACA, ε-aminocaproic acid; iPr_2P-F, diisopropyl fluorophosphate; UKase, urokinase; $NaDodSO_4$, sodium dodecyl sulfate.

Autoproteolysis of Whole Casein. Casein was prepared in both the presence (added to milk at the time of collection) and absence of ϵ-aminocaproic acid (EACA) (0.1 mM). Casein or β-casein was dissolved in 0.05 M sodium tetraborate (pH 8.4) at a final concentration of 10 mg/ml. Casein solutions were incubated alone or with added EACA (0.1 mM), EACA (0.1 mM) and diisopropyl fluorophosphate (iPr_2P-F) (1 mM), or UKase (100 Plough units/ml) at 37°C for 48 hr. Merthiolate (0.02%) was added to each sample to inhibit microbial growth. A heated casein solution (80°C, 10 min) served as a control. Aliquots (200 μl) were removed after 12, 24, and 48 hr of incubation and were mixed with equal volumes of 8 M urea containing 0.2% 2-mercaptoethanol prior to electrophoresis.

Proteolysis of β-Casein by Plasmin. β-Casein was dissolved in 0.05 M sodium tetraborate (pH 8.4) to a final concentration of 10 mg/ml. The solution was heated at 80°C for 10 min, cooled, and then incubated at 37°C with plasmin (0.28 mg/ml) prepared from plasminogen isolated from bovine blood. Aliquots (200 μl) were taken at various intervals (0, 2, 4, 15, and 30 min) and mixed with equal volumes of 8 M urea containing 0.2% 2-mercaptoethanol.

Electrophoresis. Proteolysis of whole casein and β-casein was monitored by disc gel electrophoresis in 4 M urea at pH 9.6 (21). Molecular weights of various proteins were estimated by $NaDodSO_4$ gel electrophoresis in 7.5 and 10% polyacrylamide gels (17). Myosin (prepared from rabbit skeletal muscle), alkaline phosphatase, bovine serum albumin, ovalbumin, chymotrypsinogen, ribonuclease, and cytochrome *c* were used as standards for estimation of molecular weights.

Materials. UKase was obtained from Calbiochem. Bovine serum albumin, ovalbumin, and cytochrome *c* were from Sigma. Carrier-free ^{125}I (100 mCi/ml; 1 Ci = 3.7×10^{10} becquerels) was from Amersham/Searle; chymotrypsinogen was from Pharmacia; lyophilized rabbit serum was from Miles.

RESULTS

Patterns obtained on Ouchterlony double-diffusion plates indicated that plasminogen occurs in bovine milk (Fig. 1). Antiserum raised to bovine plasminogen was monospecific and produced a single precipitin line with isolated bovine plasminogen. In contrast, with bovine casein it produced double precipitin lines. These double precipitin lines may have been the result of different concentrations of plasminogen and plasmin in bovine milk. Activation of plasminogen is a distinct possibility because plasminogen activator is known to occur in milk (22). No immune response was detected with 0.05 M tetraborate (pH 8.4) which was used to disperse the samples.

A low level of radioactivity (450 cpm/200 μl) was detected in rabbit milk after intravenous injection of ^{125}I-labeled plasminogen (^{125}I-plasminogen). Casein was recovered by acid precipitation, dissolved in 0.3 M Tris·HCl (pH 8.0), and combined with rabbit serum. Rabbit plasminogen, prepared by affinity chromatography, was reasonably homogeneous by $NaDodSO_4$ gel electrophoresis (Fig. 2*A*) and had a molecular weight of 91,000. Autoradiograms made from $NaDodSO_4$ gel electrophorograms contained a single zone of radioactivity that comigrated with rabbit plasminogen (Fig. 2*B*). These results indicated that at least some of the radioactivity recovered in rabbit milk could be attributed to the presence of intact ^{125}I-plasminogen.

When whole casein was incubated at 37°C for 48 hr, several changes were observed in the electrophoretic patterns (Figs. 3 and 4). The intensity of bands corresponding to α_{s1}- and β-caseins decreased and, concurrently, the intensity of bands

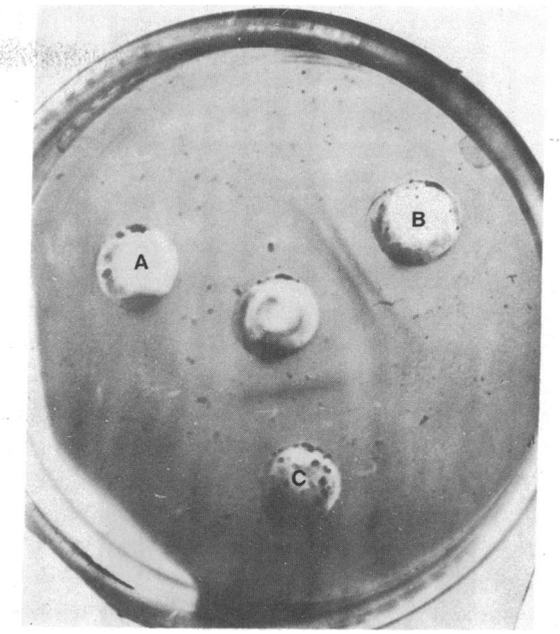

FIG. 1. Ouchterlony double-immunodiffusion analysis of bovine plasminogen and whole casein. The center well contained antiserum raised to bovine plasminogen in a New Zealand White rabbit. Peripheral wells: A, 0.05 M sodium tetraborate at pH 8.4; B, whole casein; C, plasminogen.

similar in mobility to γ_1- and γ_2-caseins increased. To determine if any of these changes could be attributed to the presence of plasmin and its zymogen in milk, we studied the effects of inhibitors (EACA and iPr_2P-F) and an activator (UKase) of the fibrinolytic system on the autoproteolysis of casein. Almost complete inhibition of proteolysis was obtained with casein prepared from one cow and incubated in the presence of 0.1

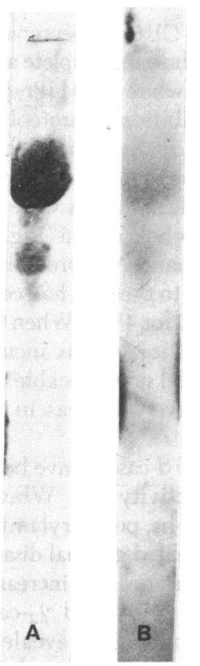

FIG. 2. $NaDodSO_4$ gel electrophoresis of rabbit plasminogen prepared from rabbit casein dissolved in blood serum. (*A*) Stained gel. (*B*) Autoradiogram of gel. The casein was prepared from rabbit skim milk obtained after injection of ^{125}I-plasminogen into an ear vein of a lactating rabbit.

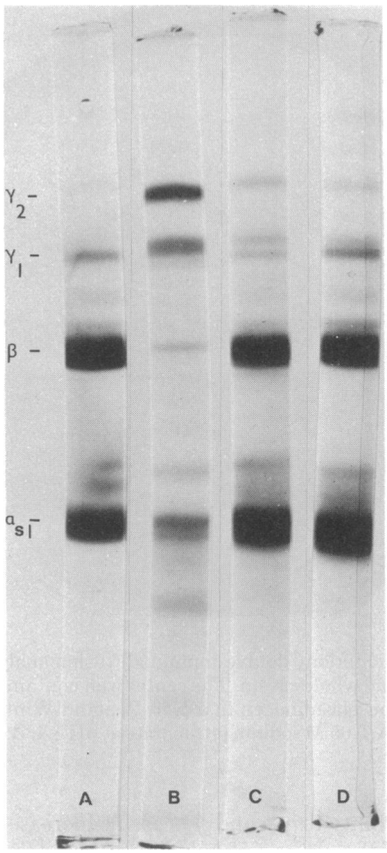

FIG. 3. Disc gel electrophoretic patterns. Lanes: A, native whole casein; B, casein after 48-hr incubation at 37°C; C, casein incubated with EACA (0.1 mM) for 48 hr at 37°C; D, casein incubated in the presence of EACA (0.1 mM) and iPr_2P-F (1 mM) for 48 hr at 37°C. Incubation mixtures contained casein (10 mg/ml) in 0.05 M sodium tetraborate (pH 8.4) with 0.02% Merthiolate.

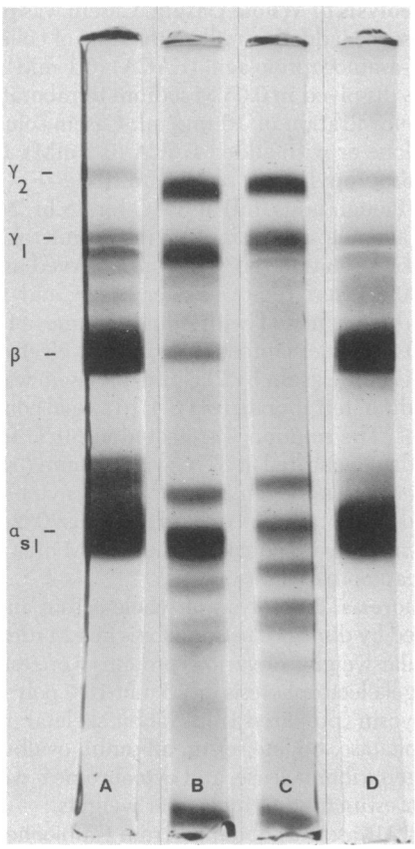

FIG. 4. Disc gel electrophoretic patterns. Lanes: A, native whole casein; B, casein after 48-hr incubation at 37°C; C, casein after 48-hr incubation at 37°C in the presence of UKase (100 Plough units/ml); D, heat-treated casein (80°C, 10 min) incubated with UKase (100 Plough units/ml) for 48 hr at 37°C. Incubation mixtures contained casein (10 mg/ml) in 0.05 M sodium tetraborate (pH 8.4) with 0.02% Merthiolate.

mM EACA (Fig. 3, lane C). Caseins prepared individually from two other cows did not show as complete an inhibition by EACA (not shown). However, when 1 mM iPr_2P-F was also included during incubation, inhibition of proteolysis was complete for caseins prepared individually from all three cows (Fig. 3, lane D). When casein was incubated in the presence of UKase (100 Plough units/ml), similar changes were observed in the electrophoretic pattern of whole casein (Fig. 4, lane C). However, UKase appeared to enhance the proteolysis of casein because the band corresponding to β-casein had completely disappeared after incubation at 37°C for 48 hr. When the casein solution was initially heated to 80°C for 10 min, incubation with UKase at 37°C for 48 hr produced no noticeable change in the electrophoretic pattern of native whole casein (compare Fig. 4, lanes A and D).

Some preparations of β-casein have been reported to contain traces of proteolytic activity (23). When β-casein A^2 was incubated at 37°C for 48 hr, polyacrylamide gel electrophoresis (pH 9.6, 4 M urea) revealed gradual disappearance of β-casein A^2 with time and appearance and increase in intensity of bands with mobilities similar to γ_1- and γ_2-caseins A^2 (not shown). $NaDodSO_4$ gel electrophoresis revealed formation of three polypeptide bands during autoproteolysis of β-casein A^2. Molecular weights assigned to two of these degradation products (21,600 and 14,900) compare favorably with values for γ_1- and γ_2-caseins A^2 (24). The molecular weight of the third degradation product (about 10,500) fell below the lowest molecular weight marker (cytochrome *c*). Addition of UKase to the incubation mixture did not alter disc gel (pH 9.6, 4 M urea) and $NaDodSO_4$ gel electrophoretic patterns (Table 1). However, addition of UKase did appear to enhance the rate of β-casein A^2 degradation. Incubation of β-casein A^2 with bovine plasmin resulted in complete disappearance of β-casein A^2 from disc gel patterns after just 4 min at 37°C. Degradation of β-casein A^2 by plasmin resulted in formation and increase in intensity of electrophoretic bands (pH 9.6, 4 M urea) with mobilities similar to γ_1- and γ_2-caseins A^2. $NaDodSO_4$ gel electrophoresis indicated the formation of three polypeptide bands possessing molecular weights similar to those obtained during autoproteolysis of β-casein A^2 (Table 1).

Table 1. Molecular weights of products formed by proteolysis of β-casein A^2

Band identity	Molecular weight		
	a	*b*	*c*
β-Casein A^2	27,000	26,000	26,000
γ_1-Casein A^2	21,600	20,500	21,000
γ_2-A^2 and γ_3-A Caseins	14,900	13,800	14,900

Molecular weights were determined by $NaDodSO_4$/polyacrylamide gel electrophoresis. The appearance of these electrophoretic bands followed (*a*) incubation of β-casein A^2 at 37°C for 48 hr, (*b*) incubation of β-casein A^2 with UKase (100 Plough units/ml) for 24 hr at 37°C, and (*c*) *in vitro* proteolysis of β-casein A^2 by exogenous plasmin for 4 min at 37°C.

DISCUSSION

Previous studies (7) have indicated that similarities exist between bovine milk protease and plasmin in optimum pH, pH stability, heat stability, and response to various inhibitors. Immunodiffusion studies, reported herein, provide more definitive evidence for the presence of plasminogen in milk. Moreover, recovery of intact plasminogen from milk after intravenous injection not only provides additional evidence for the presence of plasminogen in milk but also demonstrates one possible mechanism for the occurrence of plasminogen in milk. Transfer of plasminogen across the mammary epithelium must occur through either a transcellular or a paracellular route. In the rabbit a paracellular route exists throughout lactation (25); however, only certain ions (Na^+, Cl^-, and K^+) and sugars (such as lactose and sucrose) have been reported to move between epithelial cells in this fashion (26). More likely, plasminogen passes from blood into milk by the transcellular route in a manner similar to that hypothesized for the immunoglobulins (27). We have found plasminogen to be associated with preparations of secretory vesicles isolated from lactating bovine mammary gland (unpublished data).

Results obtained for the presence of plasminogen in milk are consistent with observations on effect of inhibitors and an activator of the fibrinolytic system on the activity of the naturally occurring protease in bovine milk. EACA is a strong inhibitor of plasminogen activation (28) and, in low concentrations, is known to inhibit plasminogen activation (29). Its effect as an inhibitor of plasmin is not detectable at less than 10 mM (27, 28). In one cow, autoproteolysis was almost completely inhibited by addition of 0.1 mM EACA (Fig. 3, lane C) which indicates that very little plasmin was present in this casein preparation. Autoproteolysis observed in the absence of EACA can be attributed to the slow activation of plasminogen during incubation. This was further verified by the effect of UKase, a specific plasminogen activator (30), on autoproteolysis. UKase addition resulted in a more rapid disappearance of β-casein from the electrophoretic patterns (Fig. 4, lane C). UKase has no other known protein substrate and does not hydrolyze casein (31) (Fig. 4, lane D). The EACA only slowed autoproteolysis in casein prepared from the other two cows because more of the plasminogen had already been activated *in vivo*. When iPr_2P-F, an irreversible inhibitor of plasmin (32), was incubated with the casein preparations in addition to EACA, complete inhibition of autoproteolysis was achieved (Fig. 3, lane D). However, this does not completely eliminate the possible involvement of other serine proteases because iPr_2P-F would also inhibit their activity (33).

Our preparation of β-casein A^2 contained traces of milk protease activity. The products of autoproteolysis of β-casein A^2 were identified as γ_1- and γ_2-caseins A^2 by both polyacrylamide gel electrophoresis (pH 9.6, 4 M urea) and NaDodSO$_4$ gel electrophoresis (Table 1). Similar electrophoretic patterns were obtained in both systems when heat-treated (80°C, 10 min) β-casein A^2 was incubated with bovine plasmin. However, β-casein was hydrolyzed more rapidly by plasmin (4 min) than by the low levels of protease in milk (48 hr). Gordon and Groves (34) proposed that *in vivo* proteolysis of β-casein A^2 at Lys-28, -105, and -107 could account for the formation of γ_1-A^2, γ_2-A^2, and γ_3-A caseins in bovine milk. Although plasmin possesses trypsin-like specificity, it has been reported to have a preference for lysine residues (35). We were unable to detect formation of γ_3-casein A by disc gel and NaDodSO$_4$ gel electrophoresis after incubation of β-casein A^2 with plasmin. γ_3-Casein A comigrates with γ_1-casein A^2 in polyacrylamide gel electrophoresis (pH 9.6, 4 M urea) (21) and is not differentiated from γ_2-casein A^2 in NaDodSO$_4$ gel electrophoresis because they differ in size by only two amino acid residues (24). Polyacrylamide gel electrophoresis (pH 3.0, 4.5 M urea) has been used to identify γ_1-A^2, γ_2-A^2, and γ_3-A caseins as products of the degradation of β-casein A^2 by bovine plasmin (10).

Enzymatic properties of the bovine milk protease bear a close resemblance to those of the fibrinolytic enzyme plasmin, and results presented in this report strongly suggest plasmin generation in bovine milk from its precursor, plasminogen. The mechanism whereby milk plasminogen undergoes slow activation to plasmin under *in vitro* conditions is as yet obscure. Presumably, it would occur enzymatically via plasminogen activator(s) now known to be almost ubiquitous in mammalian cells (36). In this connection it is of interest to note that Astrup and Sterdorff (22) postulated the presence of plasminogen activators in human milk based upon an observable increase in the fibrinolytic activity of that fluid upon treatment with streptokinase.

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