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Plasmin Activity in Buffalo Milk

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ABSTRACT

The proteolytic activity of the indigenous proteinase, plasmin, in buffalo milk and casein was examined under different conditions, e.g. pH, NaCl, lysine or 6-aminohexanoic acid and heat treatments. The plasmin activity, which was primarily associated with the casein fraction, showed large variations between different types of casein preparation; rennet casein had greater plasmin activity than acid or centrifugal casein. The breakdown of buffalo casein by the indigenous proteinase was generally similar to that of bovine casein but minor differences were observed, and buffalo casein hydrolyzate produced by indigenous plasmin corresponded to that of a bovine plasmin-treated sample. Holding the milk at pH in the range 4·8–8·0 for 4 h had no effect on the distribution of plasmin activity between casein and whey, while at pH 4·6 or lower the plasmin activity was completely dissociated from the micelles but remained stable in the supernatant over pH range 4·0–8·0. There was little activity in the casein from milk samples treated with 3–15% NaCl but incubation of casein solutions in the presence of 1–15% NaCl caused proteolysis in all samples but the extent decreased with increasing NaCl concentration. 6-Aminohexanoic acid was more effective in dissociating plasmin from casein micelles than lysine and the micelles were almost devoid of plasmin after treatment with 0·2 M concentrations of either reagent. Heat treatment at 70°C for 10 min increased the plasmin activity in milk while heating at 80°C for 10 min caused losses of only 15 and 40% of the original activity in milk and casein solution, respectively. While heating at higher temperatures caused greater losses of plasmin activity in buffalo milk, some activity still survived in casein dispersions following heating at 90°C for 10 min.

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INTRODUCTION

The presence of indigenous proteolytic activity in milk was first demonstrated by Babcock and Russell (1897) and in recent years numerous publications on various aspects of indigenous milk proteinase have appeared and have been summarized in a series of reviews (Humbert & Alais, 1979; Fox, 1981; Visser, 1981; Grufferty & Fox, 1988*a*). It is now established that the major proteinase in normal bovine milk, referred to as alkaline milk proteinase, is actually plasmin (Kaminogawa *et al.*, 1972; Richardson & Pearce, 1981). Electrophoretic studies have also shown that plasmin is the major proteinase in human, ovine and caprine milks, so that this enzyme is probably the major protease in the milks of most or all mammals (Alchanidis *et al.*, 1986). Plasmin is predominantly bound to the casein micelles in milk, as is its zymogen, plasminogen (De Rham & Andrews, 1982; Richardson, 1983) and according to Korycka-Dahl *et al.* (1983), there is nearly eight times more plasminogen than plasmin in bovine milk. The close association of the enzyme with the casein substrate could make casein degradation more efficient. In bovine milk, plasma preferentially hydrolyses β-casein and, to a lesser degree, α_{s1} and α_{s2}-caseins (Eigel *et al.*, 1979) while κ-casein, β-lactoglobulin and α-lactalbumin do not seem to be attacked (Chen & Ledford, 1971; Eigel, 1977). According to Snoeren and Van Riel (1979) and Andrews (1983), β- and α_{s2}-caseins are the caseins most susceptible to degradation by plasmin. Le Bars and Gripon (1989) showed that plasmin releases several pH 4·6-soluble peptides from α_{S2}-casein and that hydrolysis of the protein occurred at basic regions containing lysine residues. The presence of several proteins, e.g. γ_1-, γ_2- and γ_3-caseins and some proteose peptones in milk is due to the selective proteolysis of bovine β-casein by plasmin (Reimerdes, 1983; Andrews & Alichanidis, 1983). Such hydrolysis can affect the manufacturing properties of milk, e.g. reduce the yield and quality of cheese (Fox, 1981) and of caseinate products (Richardson, 1982).

The plasmin activity in acid whey was reported to be dependent on both the pH and the duration of exposure to low pH values before curd separation (Richardson & Elston, 1984). Grufferty and Fox (1988*b*) found that plasmin activity was completely removed from the casein micelles in bovine milk which was held for 1 h at pH $\leq$ 4·6, while holding the milk at pH 4·8 or above had virtually no effect on the distribution of plasmin activity. The addition of lysine or 6-aminohexanoic acid at a concentration 0·1 M causes dissociation of plasmin from casein, suggesting that 'lysine binding sites' in plasmin and plasminogen are involved in the binding of these proteins to casein (Richardson, 1983). Addition of NaCl to milk to a concentration of 1 mole/litre completely dissociated plasmin activity from

the micelles (Grufferty & Fox, 1988*b*). The heat stability of isolated milk proteinase is dependent on the pH at heating (Dulley, 1972) and the heat stability of plasmin in micellar casein was reduced as the pH was increased in the range 6–9 (Grufferty & Fox, 1988*c*). Several authors have reported that the plasmin activity in milk almost fully survives typical pasteurization conditions and that a substantial proportion of the activity survives even UHT processing (Snoeren *et al.*, 1979; Richardson, 1983; Alichanidis *et al.*, 1986).

Most of the reports mentioned above refer only to milk proteinase or plasmin in bovine milk and as far as can be ascertained there is virtually no published information on the activity of plasmin in buffalo milk. In this paper, a preliminary investigation on the activity of plasmin in buffalo milk is reported with a view to determining its characteristics under different conditions.

MATERIALS AND METHODS

Milk samples

Raw bulk buffalo milk from an Egyptian herd, which was centrifugally defatted at 2000*g* for 30 min at 20°C and lyophilized, was used throughout. Raw bovine skim milk was obtained from the pilot plant of University College, Cork.

Enzymes and inhibitors

Bovine plasmin, urokinase and soya-bean trypsin inhibitor (SBTI) were obtained from Sigma Chemical Co. Ltd (Poole, Dorset, UK). Stock solutions (1 mg/ml) of bovine plasmin or urokinase in H_2O were prepared immediately before use. SBTI was added to milk or casein solutions at a level of 1 mg/ml when it was desired to inhibit plasmin activity, i.e. controls.

Casein preparations

Casein was prepared from skim milk by precipitation with HCl at pH 4·6, by coagulation with rennet or by ultracentrifugation at 30 000*g* for 2 h at 20°C in a Sorvall Model RD-5B centrifuge. The micellar casein pellet was dispersed in 0·2M-Na_3 citrate buffer, pH 7·0, to a volume equal to that of the original milk samples.

Measurement of plasmin activity

The release of plasmin activity from the casein micelles in milk after various treatments was assessed by measuring the residual plasmin activity in casein or supernatant prepared from that milk. Plasmin activity was measured qualitatively by the extent of proteolysis in incubated samples using polyacrylamide gel electrophoresis (PAGE) in a vertical slab unit (Shandon Products Ltd, Runcorn, Cheshire, UK) by the method of Andrews (1983). Plasmin activity in milk, casein solutions or supernatants was quantified by measuring the release of the fluorescent compound, 7-amino-4-methyl-coumarin (AMC), from the synthetic substrate *N*-succinyl-L-alanyl-L-phenylalanyl-L-lysine-7-amido-4-methyl coumarin (Sigma Chemical Co.) by the method of Richardson and Pearce (1981). Plasmin activity was expressed in AMC units (nanomoles of AMC released per min/ml). Thimerosal (10 mg/ml) was added to all milk and casein samples to prevent bacterial growth.

RESULTS AND DISCUSSION

The level of plasmin activity in buffalo milk and its casein preparations

The plasmin activities in buffalo milk and in whey or casein fractions indicated (Table 1) that plasmin was associated primarily with the casein fraction. Large differences in plasmin activity were observed between the different types of casein. The plasmin activity in rennet casein was much

TABLE 1
Distribution of Plasmin Activity in Raw Buffalo Milk and its Casein Preparations

	Plasmin activity (AMC unit/ml)[a]
Milk	0·160
Ultracentrifugation:	
Casein dispersion	0·120
Ultracentrifugal serum	0·022
Acid precipitation:	
Casein dispersion	0·083
Acid whey	0·045
Rennet coagulation:	
Curd dispersion	0·200
Whey	0·030

[a] Means of triplicate determinations.

higher than in acid or centrifugal casein. Dispersed bovine micellar and acid casein contained 0·11 and 0·06 AMC unit/ml, respectively, which were slightly lower than for buffalo casein (Table 1). Similar variations in plasmin activity between different preparations of bovine casein were also reported by Richardson and Elston (1984). The conditions used for the preparation of acid casein caused a loss of some plasmin activity from the casein and consequently the plasmin activity in acid whey was higher than that of rennet or centrifugal whey. It has been reported (Grufferty & Fox, 1988*b*) that methods used to recover casein from milk that employ a neutral pH or minimize the exposure of casein to low pH result in the retention of plasmin in the casein fraction.

Although the plasmin activity (AMC unit/ml) in buffalo milk was higher than in centrifugally prepared casein, much greater proteolysis occurred in the micellar casein dispersion than in the milk from which this casein was prepared, as indicated by gel electrophoresis (Fig. 1). Plasmin, plasminogen and plasminogen activators are associated with the casein micelles while plasmin inhibitors are in the serum phase (see Grufferty & Fox, 1988*a*). Thus, removal of the casein micelles by centrifugation followed by redispersion in citrate buffer would increase plasmin activity during storage compared to milk, due to the absence of inhibitors. Addition of urokinase, which converts plasminogen to plasmin, to buffalo milk or its micellar casein dispersion, increased the proteolytic activity about 3·5 and 5 fold, respectively (Table 2). The results in Table 2 also show that the native as well as the urokinase-activated activity was largely suppressed by addition of SBTI. These results are consistent with those obtained for bovine milk (De Rham & Andrews, 1982; Richardson, 1983), i.e. that the major indigenous proteolytic activity in buffalo milk is due to plasmin and especially its zymogen, plasminogen.

TABLE 2
Plasmin Activity in Raw Buffalo Skim Milk and Micellar Casein after Addition of Urokinase

Sample	*Plasmin activity (AMC unit/ml)*	
	Control	*After activation with urokinase*
Skim milk	0·160	0·570
Skim milk + SBTI	0·030	0·035
Micellar casein	0·120	0·490
Micellar casein + SBTI	0·020	0·020
Serum fraction	0·024	0·028
Serum fraction + SBTI	0·019	0·020

SBTI, Soya bean trypsin inhibitor.

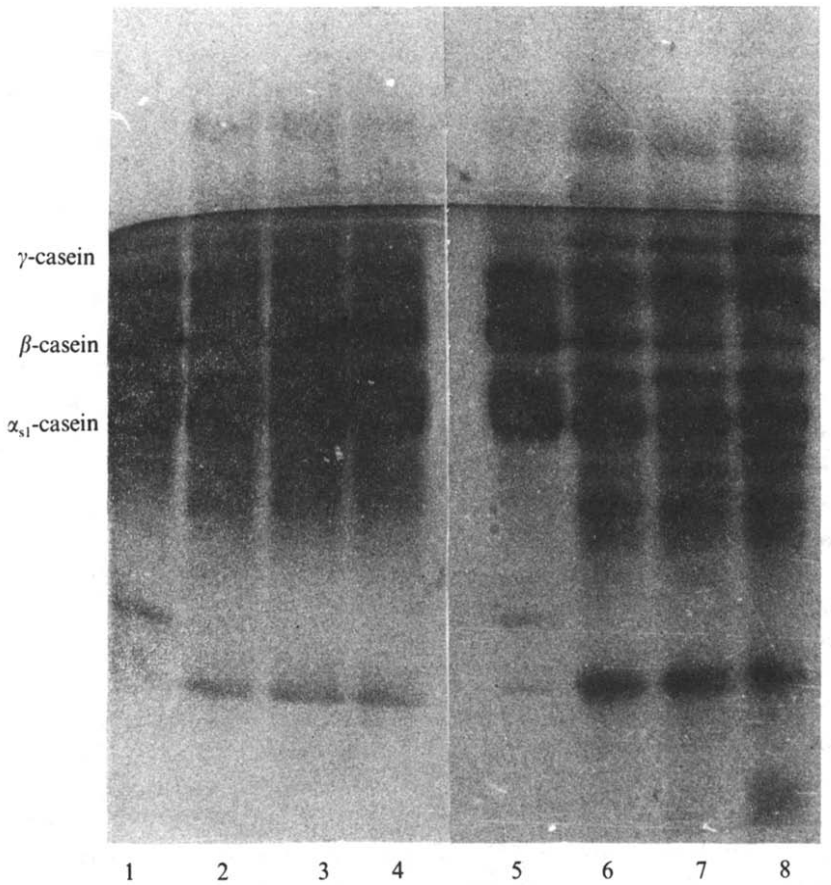


Fig. 1. Gel electrophoretograms of buffalo milk and micellar casein dispersion after incubation at 6·8 and 37°C. The controls contained soya bean trypsin inhibitor (SBTI) to inhibit plasmin activity. 1, Control milk + SBTI; 2, 3, 4, milk after incubation for 1, 2 and 3 days, respectively; 5, control casein + SBTI; 6, 7, 8, casein after incubation for 1, 2 and 3 days, respectively.

Specific action of indigenous proteinase on buffalo casein

The effect of indigenous milk proteinase on the electrophoretic pattern of milk proteins as a function of time is shown in Figs 1, 2(a) and Fig. 3. It is clear that, under the experimental conditions, β-casein was hydrolyzed faster than α_{s1}-casein with the concomitant appearance of bands corresponding to γ-caseins; after incubation for 60 h, the β-casein was hydrolyzed almost completely. The present results are similar to the published data for the action of indigenous proteinase on bovine caseins (Noomen, 1975; Andrews, 1983; Richardson, 1983; Grufferty & Fox, 1988*b*). It has been demonstrated by Weinstein and Doolittle (1972) that plasmin cleaves lysine-

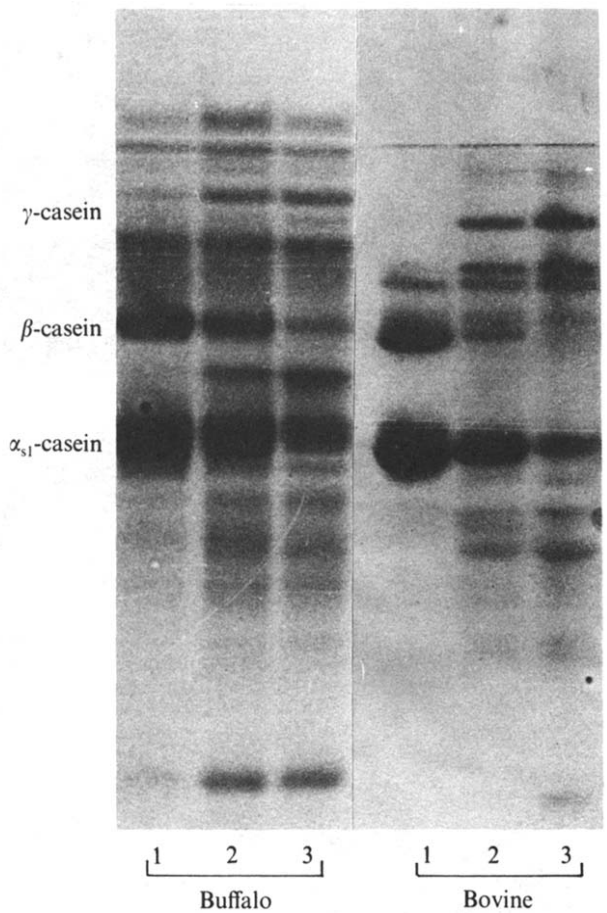


Fig. 2. (a) Comparison of the proteolytic activity of indigenous proteinase in buffalo and bovine casein dispersions (pH 7·0) as revealed by polyacrylamide gel electrophoresis. 1, Control casein + SBTI; 2 and 3, casein after 30 and 60 h incubation at 37°C, respectively.

containing peptides just after lysine residues, and Andrews (1983) noted that bovine β-casein is hydrolyzed by plasmin primarily at three sites where at least one of the residues present is Lys. Similar basic sequences also occur in buffalo β-casein (Addeo *et al.*, 1977), i.e. there are 11 lysine residues, which would be expected to be the preferred cleavage sites for indigenous proteinase on buffalo β-casein. However, the validity of this assumption is uncertain because β-casein contains 11 or 12 Lys residues (depending on the genetic variant) and α_{s1}-casein, which is only slightly susceptible to plasmin action, contains 14 Lys residues, indicating that other structural factors play important roles.

Comparison of the breakdown of buffalo casein with that of bovine casein under the experimental conditions showed (Fig. 2(a)) that the extent of

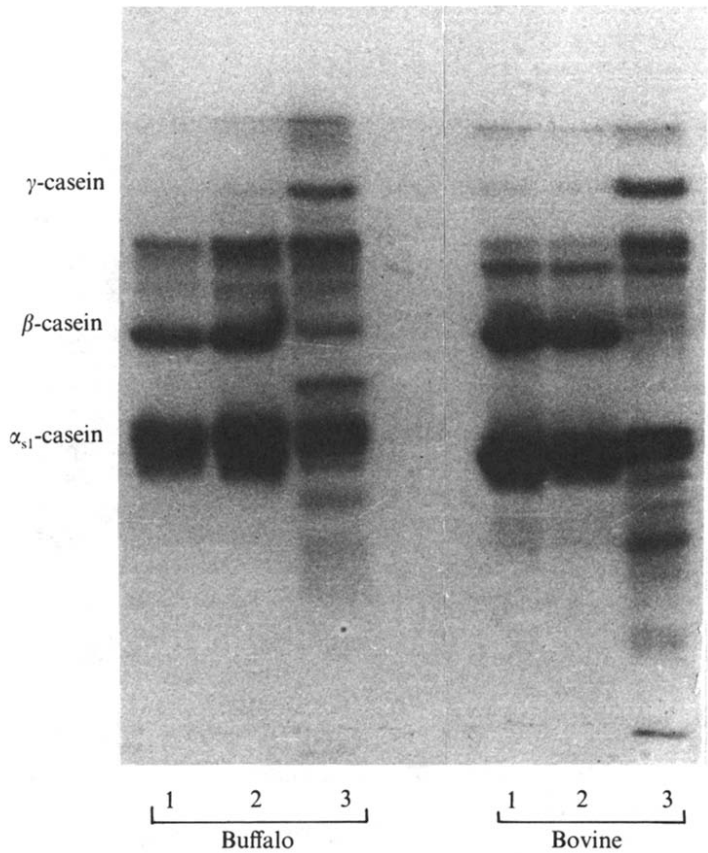


Fig. 2—*contd.* (b) Gel electrophoretograms of freeze dried pH 4·6-insoluble fractions prepared from buffalo and bovine control or plasmin-hydrolysed casein. 1, Control casein + SBTI; 2, control + SBTI pH 4·6-insoluble fraction; 3, plasmin-hydrolysed sample, pH 4·6-insoluble fraction.

hydrolysis of both types of casein was generally similar but with some minor differences. A band with a very low electrophoretic mobility was apparent in the stacking gel and a heavily stained band with a slightly lower mobility than α_{s1}-casein was detected in the gel electrophoretic profile of buffalo casein hydrolyzate but these were absent from the profile of hydrolyzed bovine casein. Examination of pH 4·6-insoluble fractions of both buffalo and bovine casein hydrolyzates by gel electrophoresis showed (Fig. 2(b)) that α_{s1}- and γ-casein were the major components insoluble at pH 4·6. The heavily stained band, with a slightly lower mobility than α_{s1}-casein, remained a clear feature in the pH 4·6 precipitate from buffalo casein hydrolyzate. To establish whether indigenous buffalo plasmin may have a slightly different specificity from bovine plasmin or whether the two types of casein had different susceptibilities to plasmin action, the hydrolysis of redispersed

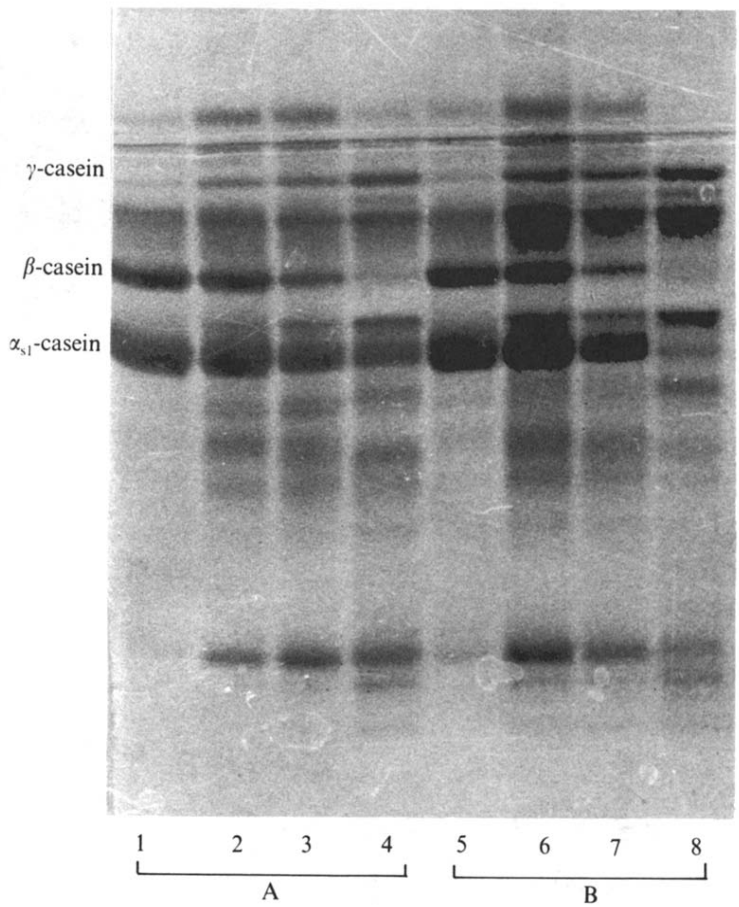
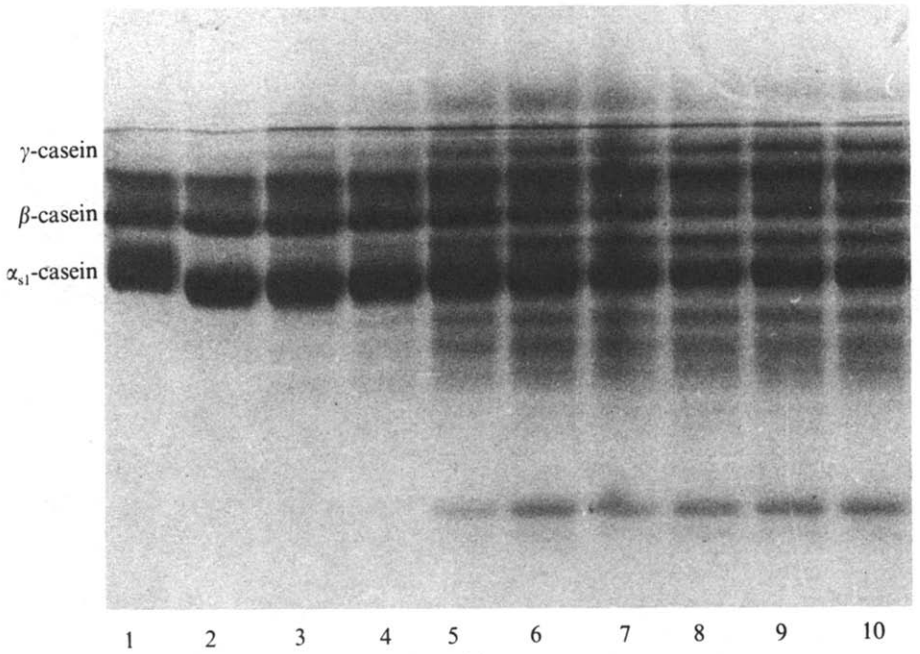
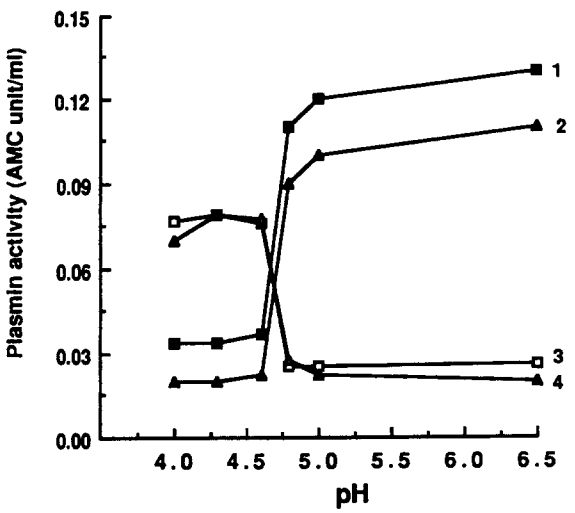


Fig. 3. Comparison by gel electrophoretograms of buffalo casein following hydrolysis by native milk proteinase (A), or added bovine plasmin (B). All samples were adjusted to pH 7·0 and incubated at 37°C. A, Control casein + SBTI (slot 1), samples incubated for 30 h (slot 2), 60 h (slot 3) or 90 h (slot 4); B, control casein + SBTI (slot 5), samples incubated for 2 h (slot 6), 4 h (slot 7) or 8 h (slot 8).

micellar buffalo casein by indigenous proteinase during storage was compared with that occurring after addition of bovine plasmin to redispersed buffalo casein micelles (Fig. 3). Nearly all bands arising from the action of indigenous proteinase were also formed in a shorter time in the bovine plasmin-treated samples. Both enzymes hydrolyzed casein to give similar bands with low electrophoretic mobilities, i.e. γ-casein, a peptide with a slightly lower mobility than α_{s1}-casein and several peptides with higher mobilities than α_{s1}-casein. Therefore, it is probable that the differences between the electrophoretograms of hydrolyzates of buffalo and bovine casein are due to the different susceptibilities of the caseins to the action of milk proteinase.



(a)



(b)

Fig. 4. (a) Gel electrophoretograms of casein prepared from buffalo milks that were held at pH 4·0 (slot 2), 4·5 (slot 3), 5·0 (slot 4), 5·5 (slot 5), 6·0 (slot 6), 6·5 (slot 7), 7·0 (slot 8), 7·5 (slot 9) or 8·0 (slot 10) for 4 h at 20°C prior to centrifugation at 30 000*g* for 2 h. The casein pellets were dispersed in 0·2M-citrate buffer pH 7·0 and incubated at 37°C for 3 days. A control casein sample + SBTI (slot 1) prepared from milk at pH 6·5 was incubated under the same conditions. (b) Effect of pH on the release of plasmin from the casein micelles in buffalo and bovine milks which were held at different pH values (4·0–6·5) for 4 h at 4°C. The milks were centrifuged at 30 000*g* for 2 h and the plasmin activity measured in the dispersed casein pellets and supernatants: 1, dispersed buffalo casein; 2, dispersed bovine casein; 3, buffalo supernatant; 4, bovine supernatant.

Effect of pH on the release of plasmin activity from casein micelles

To examine the effect of pH on the release of plasmin from buffalo casein micelles, milk was adjusted to pH values in the range from 4·0 to 8·0 and held for 4 h at 4°C. Caseinate or supernatant obtained after ultracentrifugation was readjusted to pH 7·0 and the plasmin activity then determined. Extensive proteolysis occurred in the casein dispersions prepared from milk samples throughout the pH range 5·5–8·0 (Fig. 4(a) slots 5–10), indicating that there was little or no loss of plasmin activity from the casein micelles in this pH range. Grufferty and Fox (1988*b*) have also noted a similar effect of pH on the release of plasmin activity from the casein micelles in bovine milk. The caseinate prepared from buffalo milk at pH 4·0 or 4·5 underwent very little proteolysis (slots 2 and 3), indicating that most of the plasmin activity was removed from the micelles under these conditions. Comparison of the effect of varying the pH in the range 4·0–5·0 on the release of plasmin activity in both buffalo and bovine milk using the coumarin peptide substrate showed (Fig. 4(b)) that plasmin activity was retained completely in the casein micelles at pH 5·0. At pH 4·8, there was little loss of plasmin activity but at pH 4·6, 4·4 or 4·0 the plasmin activity was completely released from the micelles. Thus, no significant difference was observed between the effect of pH on the release of plasmin activity from buffalo or bovine casein micelles. Under the conditions used in these experiments, the plasmin system seems to be stable over the pH range 4·0–8·0, since the plasmin activity released from the micelles at low pH remained relatively stable in the supernatant. Kaminogawa *et al.* (1972) showed that isolated plasmin is stable over the pH range 4·0–9·0. It has also been demonstrated (Richardson & Elston, 1984; Grufferty & Fox, 1988*b*) that most of the plasmin activity which was removed from the bovine casein micelles at pH values below 4·6 remained stable in the supernatant.

Effect of NaCl on the release of plasmin activity from casein

Samples of buffalo milk were held at 4°C for 8 h in the presence of 0, 1, 3, 5, 10 or 15% NaCl prior to centrifugation at 30 000*g* for 2 h and dispersion of the pellets in 0·2M-citrate buffer, pH 7·0. β-Casein was extensively hydrolyzed in the control sample but little activity was retained in the caseinates prepared from the milk samples treated with 1–15% NaCl (Fig. 5(A)). In fact, more proteolysis appeared to occur in the casein dispersions from milk samples treated with 10 or 15% NaCl than with lower concentrations of NaCl, but this does not appear to be due to plasmin since the electrophoretic profile of NaCl-treated milk with added SBTI showed similar results. The effect of NaCl on the activity of indigenous plasmin was also examined to establish

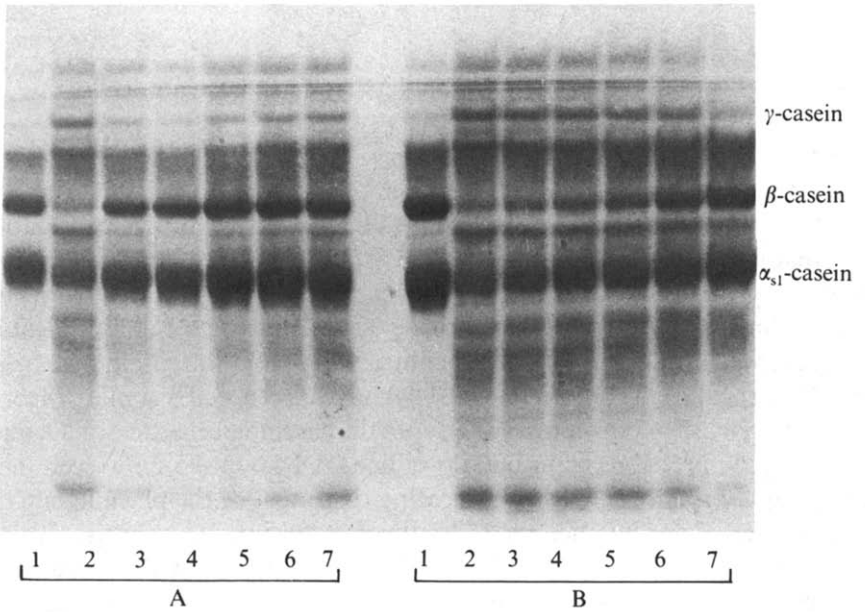


Fig. 5. (A) Gel electrophoretograms of casein prepared from buffalo milk that had been treated with different NaCl concentrations for 8 h prior to centrifugation at 30 000*g* for 2 h. The pellets were dispersed in 0·2M-citrate buffer, pH 7·0 and incubated at 37°C for 3 days. (B) Gel electrophoretograms of casein solutions, pH 7·0, incubated for 3 days at 37°C in the presence of different NaCl concentrations. 1, Control casein + SBTI; 2, 0%; 3, 1%; 4, 3%; 5, 5%; 6, 10%; 7, 15% NaCl.

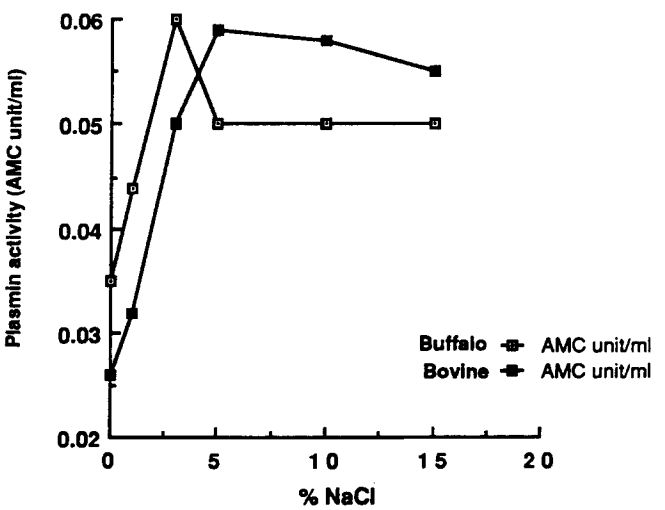


Fig. 6. Effect of adding NaCl to buffalo or bovine milk at 20°C for 8 h on the release of plasmin from the casein micelles. The plasmin activity was determined in the supernatant after separation of casein by centrifugation at 30 000*g* for 2 h using the coumarin peptide substrate.

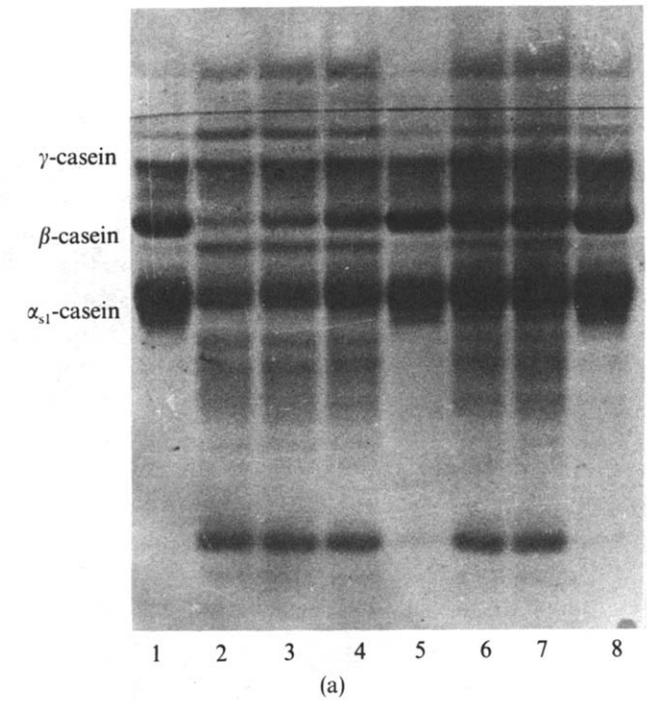
whether increasing the ionic strength may inhibit or denature plasmin. Incubation of dispersed buffalo casein micelles at 37°C for 3 days in the presence of 0–15% NaCl showed (Fig. 5(B)) that NaCl had an inhibitory effect on plasmin ≥ 3% but some plasmin activity was observed in the presence of 15% NaCl. Grufferty and Fox (1988*b*) reported that although addition of NaCl (~ 5·8%) released plasmin activity from the casein micelles in bovine milk, it had no effect on plasmin activity in casein solutions. When the plasmin activities in the supernatant prepared from NaCl-treated samples of buffalo and bovine milks were compared using the coumarin peptide substrate (Fig. 6), it was found that the plasmin activity in the supernatants from both milks increased to a similar extent with increasing salt concentration up to 5%, above which plasmin activity remained relatively constant in the bovine supernatant, but decreased slightly in that from buffalo milk.

Effect of lysine and 6-aminohexanoic acid on the release of plasmin from casein

Richardson (1983) and Grufferty and Fox (1988*c*) showed that adding lysine or 6-aminohexanoic acid to bovine milk up to 0·1 M dissociated plasmin from the casein micelles. In the present study, lysine or 6-aminohexanoic acid were added to buffalo milk at 0·05, 0·1 and 0·2 M and held for 8 h at 20°C prior to centrifugation at 30 000*g* for 2 h. The results in Fig. 7(a) show that 6-aminohexanoic acid was more effective than lysine in dissociating plasmin from the casein micelles. At 0·05 and 0·1 M, lysine removed very little plasmin and casein was extensively hydrolyzed in comparison to the slight proteolysis which occurred in dispersed casein micelles prepared from milk treated with 6-aminohexanoic acid at the same concentration. Micelles prepared from milks treated with 0·2 mole/litre of either reagent were almost devoid of plasmin activity. These results were further confirmed by measuring the plasmin activity in the supernatants from buffalo milk treated with these reagents (Fig. 7(b)). Richardson (1983) demonstrated that 'lysine binding sites' in plasmin and plasminogen appear to be involved in the binding of these proteins to casein, since they were dissociated from the casein fraction of milk by 6-aminohexanoic acid or lysine.

Effect of heat treatment on the indigenous proteinase system in buffalo milk

The data in Fig. 8(a) (slots 3 and 10) and 8(b) show that heat treatment at 70°C for 10 min increased the plasmin activity in either skim milk or casein micelle dispersion during incubation at 37°C for 3 days compared to unheated samples. It has been suggested (Noomen, 1975; De Rham &



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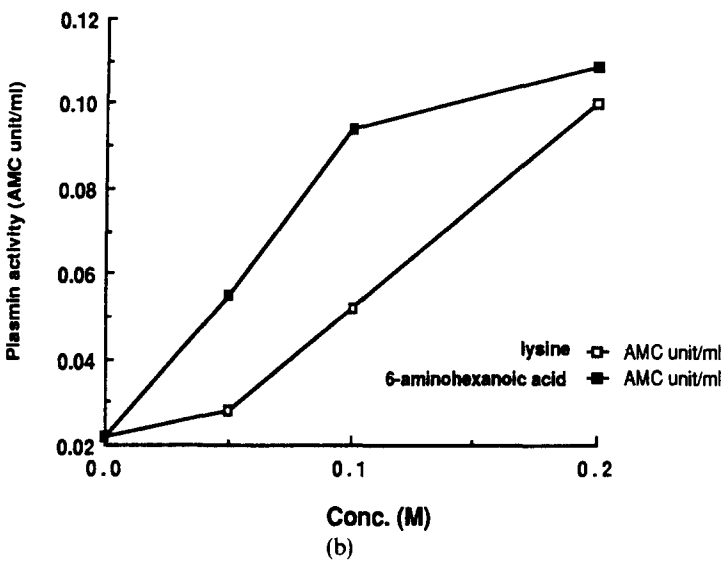
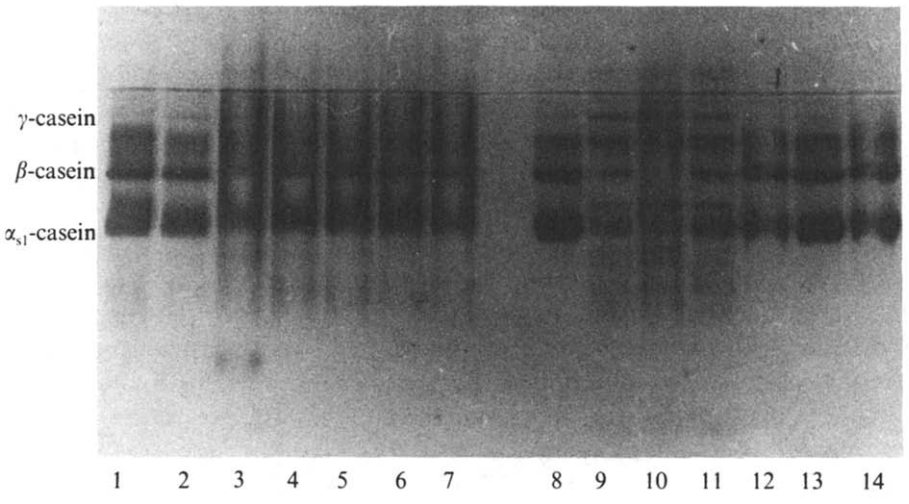


(b)

Fig. 7. (a) Gel electrophoretograms of casein prepared from buffalo milk which was treated with lysine or 6-aminohexanoic acid for 8 h, prior to centrifugation at 30 000*g* for 2 h. The casein pellets were dispersed in citrate buffer, pH 7·0 and incubated at 37°C for 3 days. Control casein + SBTI (1); casein prepared from untreated milk (2); casein prepared from milk containing 0·05M (3); 0·1M (4); 0·2M (5) lysine or 0·05M (6); 0·1M (7); 0·2M (8) 6-aminohexanoic acid. (b) Dissociation of plasmin from the casein micelles in buffalo milk after incubation at 20°C for 8 h in the presence of lysine or 6-aminohexanoic acid. The plasmin activity was determined in the supernatant after separation of casein by centrifugation at 30 000*g* for 2 h using the coumarin peptide substrate.



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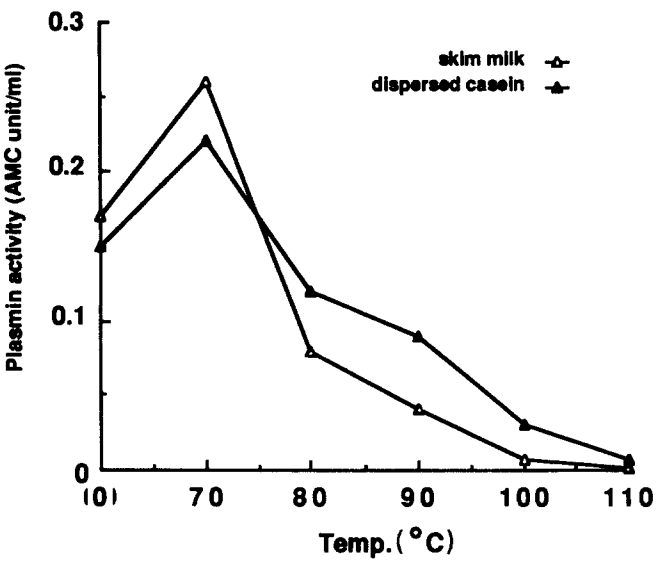

(b)

Fig. 8. (a) Gel electrophoretograms of buffalo milk and dispersed casein micelles following heating at different temperatures and incubation at 37°C for 3 days. A control milk and casein sample (unheated) were incubated under the same conditions but with SBTI added to inhibit plasmin activity. 1, Control milk. 2, Unheated milk. 3, 4, 5, 6, 7, Milk heated for 10 min at 70, 80, 90, 100 or 110°C, respectively. 8, Control casein. 9, Unheated casein. 10, 11, 12, 13, 14, Casein heated for 10 min at 70, 80, 90, 100 or 110°C, respectively. (b) Activity of plasmin in buffalo milk and dispersed casein following heating at different temperatures for 10 min. Plasmin activity was determined in milk and dispersed casein using the coumarin peptide substrate after storing the heated samples at 37°C for 3 days.

Andrews, 1982; Andrews & Alichanidis, 1983) that heat treatment of bovine milk at temperatures in the range used in the present study increased the plasmin activity in bovine milk during subsequent storage, probably owing to the destruction of inhibitors of plasminogen activator (Richardson, 1983).

Humbert and Alais (1979) reported that purified plasmin is completely inactivated on heating at 80°C for 10 min. In the present study, heating buffalo milk or casein dispersion at 80°C for 10 min caused losses of only about 55 and 22% of the original plasmin activity, respectively (Fig. 8(b)). Similar results were reported by Grufferty and Fox (1988*c*) who suggested that the plasmin system appears to be more heat-stable in bovine milk or micellar casein dispersions than the purified enzyme in aqueous solution. Alichanidis *et al.* (1986) maintained that the presence of casein in solutions of porcine plasmin provided very substantial substrate protection for the enzyme during heat treatment. The results in Fig. 8(b) show that residual plasmin activity was greater in the casein dispersion than in milk after heating at ≥ 80°C for 10 min. It is also notable that while heating at relatively high temperatures (≥ 100°C for 10 min) caused a complete loss of plasmin activity in buffalo milk, some activity still survived in the dispersion of buffalo casein micelles after heating at 100°C for 10 min (Fig. 8(b)). The present data are consistent with those reported by Grufferty and Fox (1988*c*) and Rollema and Poll (1986) who found that the heat-stability of indigenous proteinase was lower in bovine milk than in a micellar casein dispersion prepared from that milk. It has been established (Snoeren *et al.*, 1980; Grufferty & Fox, 1986; Rollema & Poll, 1986) that the free sulphydryl groups of β-lactoglobulin, which become available following heat denaturation, may interact via thiol-disulphide interchange with plasmin, resulting in a decrease in enzyme activity.

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