Lait (1990) 70, 191-203

Original article

Purification of bovine milk alkaline proteinase and comparison with purified bovine blood plasminogen or plasmin

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(Received 29 January 1990; accepted 16 February 1990)

Summary — The bovine blood plasminogen (Plg) has been purified by affinity chromatography on Lysine-Sepharose. An electrophoresis pattern revealed some minor contaminant bands. The possibilities of contamination and activation of Plg have been examined; the likely causes of the heterogeneousness are:
1) an *in vivo* activation just before or during slaughter of the animals;
2) a partial activation during purification;
3) a self-activation or a self-hydrolyse which is a characteristic of proteinases or
4) a contamination of the chromatography column.

The milk alkaline proteinase was purified by affinity chromatographies on Lysine-Sepharose and an immunoadsorbant (rabbit anti-bovine plasminogen serum) from whole casein and on enzyme-enriched fraction. The purified fractions were partially heterogeneous and presented some minor components which are plasmin-chains. This sustains the assumption that self-activation or activation caused by traces of a whey activator in the whole casein occur. Blood and milk purified fractions were compared (electrophoresis, double-immunodiffusion, glycoprotein staining). This paper presents a novel method to purify the milk alkaline proteinase and to confirm the similarities between blood- and milk proteinase.

milk alkaline proteinase / blood plasminogen or plasmin / purification / affinity chromatography / immunological reaction

Résumé — Purification de la protéase alcaline du lait et comparaison avec la plasmine ou le plasminogène sanguins bovins purifiés. *Le plasminogène du sang bovin a été purifié par chromatographie d'affinité sur Lysine-Sepharose. Il présente une bande majeure et des bandes mineures en électrophorèse et un arc contaminant en double immunodiffusion. Les possibilités de contamination ou d'activation du plasminogène sont examinées; ces causes d'hétérogénéité seraient :*
1) une activation in vivo *au moment du prélèvement du sang,*
2) une activation partielle pendant la purification,
3) une autoactivation ou autolyse caractéristique des protéases, ou
4) une contamination du support chromatographique.
La chromatographie d'affinité sur Lysine-Sepharose et la chromatographie d'affinité sur immunoadsorbant (antisérum antiplasminogène bovin obtenu chez le lapin) ont été utilisées pour purifier la protéase alcaline du lait à partir de la caséine entière et d'une fraction protéique enrichie en enzyme. Les préparations sont hétérogènes en électrophorèse. En milieu dissociant, l'électrophorèse révèle des bandes mineures correspondant aux chaînes lourde et légère de la plasmine. Comme dans le cas du plasminogène sanguin il est permis de penser à une autoactivation, ou à une activation provoquée par des traces d'un activateur lactosérique dans la caséine. Les préparations contiennent

donc du plasminogène et de la plasmine du lait ou apparue pendant le cycle de purification. Les enzymes du lait et du sang sont comparées par électrophorèses en gel de polyacrylamide et par immunodiffusion radiale. La protéase alcaline est, comme le plasminogène, une glycoprotéine. Ce travail présente donc une nouvelle voie de purification de la protéase alcaline du lait et montre de nouveau qu'elle est l'enzyme du sang.

protéase alcaline du lait bovin / plasminogène ou plasmine du sang / chromatographie d'affinité / électrophorèse / immunochimie

INTRODUCTION

Alkaline proteinase (MAP) is the major component of the milk endogenous proteinase system. The properties of this enzyme have been reviewed by Humbert and Alais (1979) and more recently Miranda and Gripon (1986); Grufferty and Fox (1988a). This proteinasic activity may affect various dairy products by modifying rheology properties (gelation of UHT milk, viscosity of custards), flavour properties (apparition of bitterness), or diminishing cheese yield.

The study of molecular and kinetic properties has shown that milk alkaline proteinase displays certain similarities to blood plasmin. Kaminogawa *et al* (1972) were the first workers to report an analogy between these 2 enzymes.

Many enzyme purification experiments have been conducted (Humbert and Alais, 1979) and different protocols or methods of purification have been compared (Von Halpaap *et al,* 1977; Reimerdes *et al,* 1981a; Manji and Kakuda, 1986). Some authors have examined the sensibility of these endopeptidases to different effectors (Kaminogawa *et al,* 1972; Hofmann *et al,* 1979; Reimerdes *et al,* 1981a,b; Rollema *et al,* 1981, 1983). Others have studied its specific effects on caseins (Snoeren and Van Riel, 1979; Aimutis and Eigel, 1982; de Rham and Andrews, 1982; Andrews and Alichanidis, 1983, etc).

In this paper, the purification of blood and milk enzymes by affinity chromatography on Lysine-Sepharose will be described. Milk alkaline proteinase was also purified by affinity chromatography on immunoadsorbant using an antiplasminogen serum. Finally the products obtained by these purifications will be compared. Figure 1 depicts the experimental proceedings.

MATERIALS AND METHODS

Materials

Sephadex G-25 Superfine, Agarose C and Lysine-Sepharose were from Pharmacia Fine Chemicals (Uppsala, Sweden). Activated Ultrogel ACA 22 was from IBF Pharmindustrie (Clichy, France). Acrylamide and Bis-acrylamide

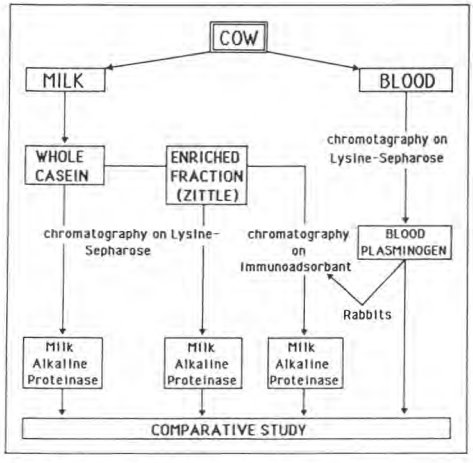

Fig 1. Experimental proceedings.
Plan d'expérimentation.

were from BDH (Poole, England). Urokinase (15 000 UI/ml) was from Choay (Paris, France). Freund complete adjuvant was from Gibco (New York, USA).

Methods

Preparation of blood plasma

The bovine blood is collected immediately after cutting the animal's throat. The blood is sampled in a receptable containing 0.13 mol•l^{-1} trisodium citrate and slightly stirred. The citrated blood (1 part of citrate solution + 9 parts of blood) is centrifuged (4 000 *g*, 30 min) at 5 °C. The citrated plasma is again centrifuged at 3 000 *g*, 10 min before being frozen.

Purification of blood plasminogen (Plg)

Plasminogen was purified from 500 ml of citrated plasma by affinity chromatography on Lysine-Sepharose according to Deutsch and Mertz (1970). The chromatography was carried out at 5 °C on a 2.5 x 15 cm column at a flow-rate of 75 ml•h^{-1} in 0.05 mol•l^{-1} Tris/0.01 mol•l^{-1} $ZnSO_4$/0.02% NaN_3 pH 8 buffer. The non-adsorbed proteins were eluted with 0.5 mol•l^{-1} NaCl – Tris buffer, and then plasminogen was desorbed by 0.2 mol•l^{-1} ε aminocaproic acid – Tris buffer. Finally the gel is washed with 6 mol•l^{-1} urea – Tris buffer before equilibrated in original Tris buffer. All the buffers of the affinity step contained 0.001 mol•l^{-1} Zinc sulphate to prevent the plasminogen activation (Dano and Reich, 1979).

The ε-aminocaproic acid was then eliminated at 5 °C by chromatography of obtained proteinous fraction on 5 x 100 cm Sephadex G-25 column in 0.01 mol•l^{-1} Tris/0.02% NaN_3 pH 8 buffer at a flow rate of 70 mol•l^{-1}.

Antibody production

The antibody to plasminogen was obtained intravenously and subcutaneously injecting rabbits with 5.5 mg of plasminogen dissolved in 1.5 ml of a 1/1 mixture of physiological saline and Freund's adjuvant. Four intravenous shots (2.5 mg.l^{-1}/rabbit) were given after 24, 31, 48 and 59 d. Blood was retrieved 6 d after the last injection.

Isolation of IgG was accomplished by a classical treatment of the blood serum by 3 $(NH_4)_2SO_4$ precipitations. The IgG were then dissolved in 0.1 mol.l^{-1} Na_2HPO_4/0.15 mol.l^{-1} NaCl buffer at pH 7.5. The antibody solution thus obtained was then used for affinity chromatography and for immunological analyse. Immuno-double diffusion according to Ouchterlony (1949) was used to test antigenic properties.

Obtention of the milk alkaline proteinase-enriched fraction

The freshly drawn milk was skimmed by centrifugation, and the whole casein was prepared using acid precipitation according to Nitschman and Lehman (1947). The different steps are specified in figure 2. The enriched-fraction (FZ) was prepared according to Zittle (1965) by acidificaton of casein to pH 3.5 with 7N H_2SO_4, $(NH_4)_2SO_4$ precipitation and freeze-drying.

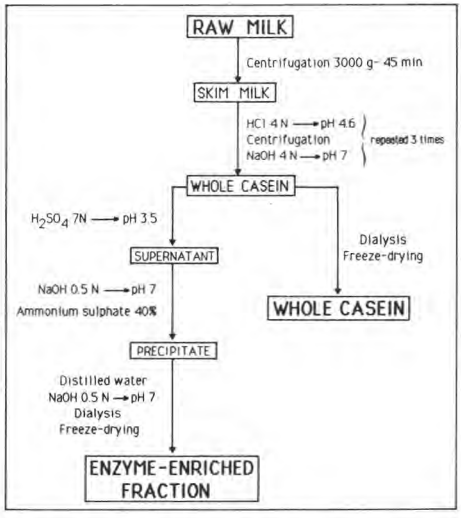

Fig 2. Procedure of preparation of whole casein and MAP-Enriched Fraction (FZ).
Préparation de la caséine entière et de la fraction (FZ) enrichie en protéase alcaline du lait.

Purificaton of milk alkaline proteinase (MAP)

Affinity chromatography on Lysine-Sepharose

It was purified at 5 °C from whole casein or enzyme-enriched fraction according to Deutsch and Mertz (1970) on a 1.5 x 5 cm column. After loading of 50 mg of protein in 2 ml of 0.05 mol.l^{-1} Tris/0.001 mol.l^{-1} $ZnSO_4$/0.14 mol.l^{-1} NaCl/0.02% NaN_3/pH 8 buffer the elution was stopped to facilitate the affinity linkings in the gel. Then the elution was continued at 50 ml.h^{-1} with the same Tris buffer. The non-absorbed protein were eliminated by washing at 15 ml.h^{-1} with 0.4 mol.l^{-1} NaCl-Tris buffer. Milk alkaline proteinase was desorbed with 0.1 mol.$^{-1}$ lysine pH 8 solution at a flow-rate of 50 ml.h^{-1}. Finally, the gel was washed with 6 mol.l^{-1} urea which desorbes proteinous substances.

Immunoaffinity chromatography

The slurry of activated Ultrogel ACA 22 was washed successively with 0.5 mol•l^{-1}, then 0.1 mol•l^{-1} sodium phosphate buffer pH 7.5 containing 0.15 mol•l^{-1} sodium chloride. The ligand, antibody to plasminogen was fixed onto the matrix during 18 h at 4 °C while being stirred. Then the slurry was washed with the last buffer, the proportion of proteins not bound was evaluated in the eluate, measuring absorbance at 280 nm. The coupling yield was about 60%. The mixture gel-ligand was poured into the (1.5 x 5 cm) column and washed with the same buffer.

Three ml of FZ proteins solution (10–30 mg•ml^{-1}), previously equilibrated in the 0.1 mol•l^{-1} KH_2PO_4/0.15 mol•l^{-1} NaCl/0.02% NaN_3 pH 7 buffer, were loaded onto the column at room temperature. After a first washing step at the flow-rate of 10 ml•h^{-1} with 0.2 mol•l^{-1} glycine/0.15 mol•l^{-1} NaCl pH 2.4 buffer at 55 ml•h^{-1}, dropped into tubes previously filled with 1 mol•l^{-1} potassium phosphate buffer pH 8 and stirred. The proteins were rapidly dialyzed and frozen. After chromatography the gel was washed with 10% dioxan at a flow-rate of 55 ml•h^{-1} before equilibrating in the phosphate buffer.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on vertical gel slabs were carried out according to Hillier (1976) and Laemmli and Favre (1973) respectively. Gels were also specifically stained with Fuchsine according to Kapitany and Zebrowski (1973) to detect glycoproteins.

Detection of endopeptidasic activity

Detection occurred at 37 °C in Petri dishes using a method similar to that reported by Laurence and Sanderson (1969). The mixture consisted of gelose (1.5%) and reconstituted-milk (30%), in a Tris/HCl buffer (0.05 mol.l^{-1}; pH 7.5) containing 0.01% merthiolate. A 10 ml aliquot of agar mixture was poured into the dish on a level table. After cooling at room temperature 3 mm-diameter wells were punched in the agar gel and filled with 10 µl of protein samples.

Activation of plasminogen by urokinase (UK)

Activation was sustained for 20 min at 37 °C. The reaction mixture contained 100 µl of urokinase (750 UI = 525 Plough) and 50 µl of proenzymatic fraction solution (10 µg of protein). UK-activated Plg is plasmin (Plm).

Abreviations for plasminogen and plasmin molecules

We shall use the abreviations according to the common nomenclature: for instance Glu_1-Plg for Glutamic$_1$-Plasminogen, Lys_{77}-Plg for Lysine$_{77}$-Plasminogen. The indicated amino acid corresponds to the NH_2-terminal residue, and the number to its position in the primary sequence of plasminogen.

RESULTS

Characteristics of purified blood plasminogen

The plasminogen obtained by chromatography on Lysine-Sepharose presents on

PAGE pattern major bands with feeble mobility (fig 3A, slot a). A sample of plasminogen previously activated by UK results in a much more complex pattern (fig 3A, slots b and c).

In SDS-PAGE, the plasminogen (fig 3B, slot a) shows several bands: a major band of protein with weak mobility and a molecular weight greater than 90 kDa and, minor bands with higher mobility, 1 of which corresponds to 60 kDa. By activating the plasminogen with UK, the 90 kDa band disappears as 2 major bands (60 kDa and 25 kDa) appear progressively (fig 3B, slot b).

In immunodouble diffusion, Plg shows 2 arcs against anti-serum (fig 4A). The internal arc, corresponds to the precipitin reaction between Plg and its antibody, the external to the precipitin reaction between bovine immunoglobulins and antiserum. This contaminant arc is also present when the reaction of diluted milk to antiserum is tested (fig 4B, 4C). Whole casein, ß-lactoglobulin and serumalbumine give no precipitin reaction. When Plg and Plm obtained with UK are tested, the contaminant arcs are in continuity with those formed between antiserum and bovine immunoglobulin. The continuity of the principal arcs of Plg and Plm, points to a partial identity (fig 4D). Antigenic determinants are localized on the NH_2-terminal peptide of Plg (1–76) which is eliminated by activation with UK. No precipitin reaction occurs between antiserum and UK.

Comparative study of purified fractions (Plg and MAP)

Electrophoretic behaviour

The 2 milk enzymatic fractions obtained by the 2 ways of chromatography on Lysine-Sepharose and on immunoadsorbant do not present PAGE patterns similar to that of blood Plg (fig 5A). The former show, as Plm, quicker bands (relative mobility =

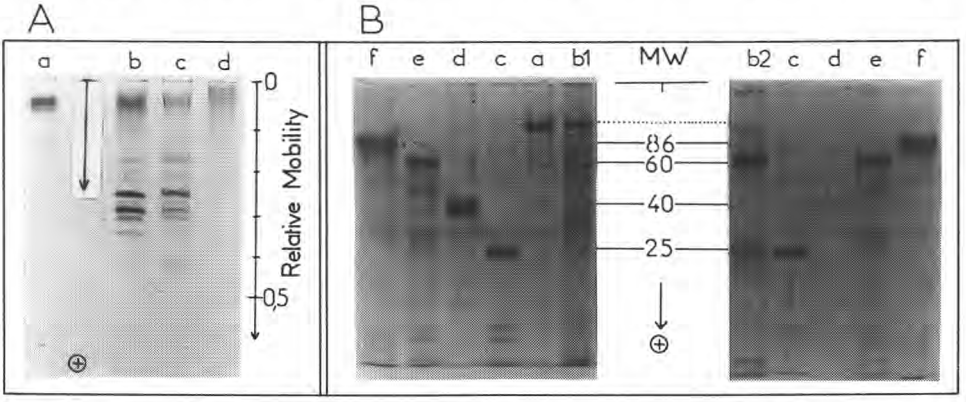

Fig 3. Electrophoretic study of blood enzyme. **A** PAGE pattern of blood Plg (a), of activated Plg (b, c) and of UK (d). **B** SDS-PAGE pattern of blood Plg (a), of little activated Plg (b_1) and of total activated Plg (b_2). Standard Proteins: chymotrypsinogen (c), aldolase (d), catalase (e) and bovine transferrin (f). Molecular weights in kDa.

*Etude électrophorétique de l'enzyme du sang. **A** En gel natif : plasminogène sanguin purifié (a), activé en plasmine (b, c) et urokinase (d). **B** En milieu dissociant (SDS + mercaptoéthanol) : plasminogène sanguin purifié (a), partiellement (b_1) et totalement activé (b_2) en plasmine. Protéines étalons : chymotrypsinogène (c), aldolase (d), catalase (e) et transferrine bovine (f). Les poids moléculaires sont exprimés en kDa.*

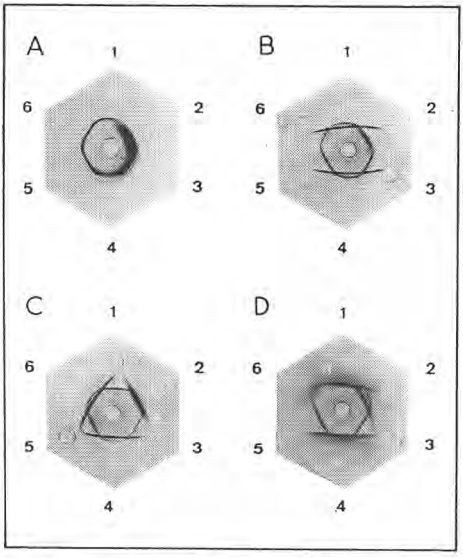

Fig 4. Immunodouble diffusion patterns demonstrating the presence of a contaminant protein in antigenic Plg preparation **(A, B),** in dialysed milk **(C)** and in Plm **(D).** Center wells contain anti-Plg serum; **A** 1-6 : dilution 1/1 to 1/32 of blood Plg 8.5 mg.ml^{-1}; **B** 1,4 : Plg (1 mg.ml^{-1}); 2,5 : immunoglobulin (1 and 0.5 mg.ml^{-1}); 3.6 : dilution 1/2, 1/4 of dialysed milk; **C** 1,3,5 : dilutions 1/1, 1/2 and 1/4 of dialysed milk; 2,4,6: blood Plg (1, 0.5 and 0.25 mg.ml^{-1}); **D** 1,4: blood Plg (0.5 mg.ml^{-1}); 2: Plm; 3,5: immunoglobulins (0.5 mg.ml^{-1}).
Mise en évidence par double immunodiffusion de la présence d'un contaminant protéique dans la préparation antigénique de plasminogène ***(A, B),*** *dans le lait dialysé* ***(C)*** *et dans la plasmine* ***(D).*** *Les puits centraux contiennent le sérum anti-plasminogène.* ***A*** *1-6 : dilutions 1/1 à 1/32 du plasminogène sanguin (8,5 mg.ml^{-1});* ***B*** *1,4 : plasminogène sanguin (1 mg.ml^{-1}); 2,5 : immunoglobulines (1 et 0,5 mg.ml^{-1}); 3,6 : dilutions 1/2 et 1/4 du lait dialysé;* ***C*** *1,3,5 : dilutions 1/1, 1/2 et 1/4 du lait dialysé; 2,4,6 : plasminogène sanguin (1, 0,5 et 0,25 mg.ml^{-1});* ***D*** *1,4 : plasminogène sanguin (0,5 mg.ml^{-1}); 2 : plasmine; 3,5 : immunoglobulines (0,5 mg.ml^{-1}).*

RM = 0.30, 0.34 and 0.08–0.12, the last one being large). The 2 fractions have the same patterns but those obtained by immunochromatography display a more pronounced and quicker band (*RM* = 0.60) as well as minor bands (*RM* = 0.74, 0.85) with equally higher velocities.

In SDS-PAGE (fig 5B-CS) the enzymatic fraction obtained from casein by affinity on Lysine-Sepharose (slot b) shows a band which is slightly ahead of Plg (slot a) and minor rapid bands which are also present in Plg. The enzymatic fraction obtained from FZ by immunoadsorbant consists of a principal band (*RM* = 0.16) and a faint doublet corresponding to the 2 glycosyled Plg variants. Fuchsine staining shows Plg and the milk enzymatic preparation to be glycoproteins (fig 5B-FS). Most plasmin bands (*RM* = 0.1–0.3, on slot d on fig 5B-FS) also contain carbohydrates.

Endopeptidasic activity

The 2 enzymatic fractions obtained from FZ by chromatography on Lysine-Sepharose and on immunoadsorbant produce a proteolytic area in agar-milk plate. Plg and milk enzymes obtained on Lysine-Sepharose show no detectable activity after a 24 h incubation period. All the tested preparations but not UK itself reveal caseolytic activity after UK-activation.

Immunological relationships

In immunodouble diffusion, 3 preparations obtained from FZ by affinity on immunoadsorbant show precipitin reactions (fig 6A,B,C) with 2 arcs. The first arc is in continuity with the principal one of Plm but not with those of Plg. The second is in continuity with that formed by the contaminant of Plg or Plm.

Preparation number 2 shows a third internal arc, which is in partial continuity with principal arc of Plg and Plm (fig 6B).

Milk enzyme obtained from whole casein by chromatography on Lysine-Sepharose gives a preciptin arc in conti-

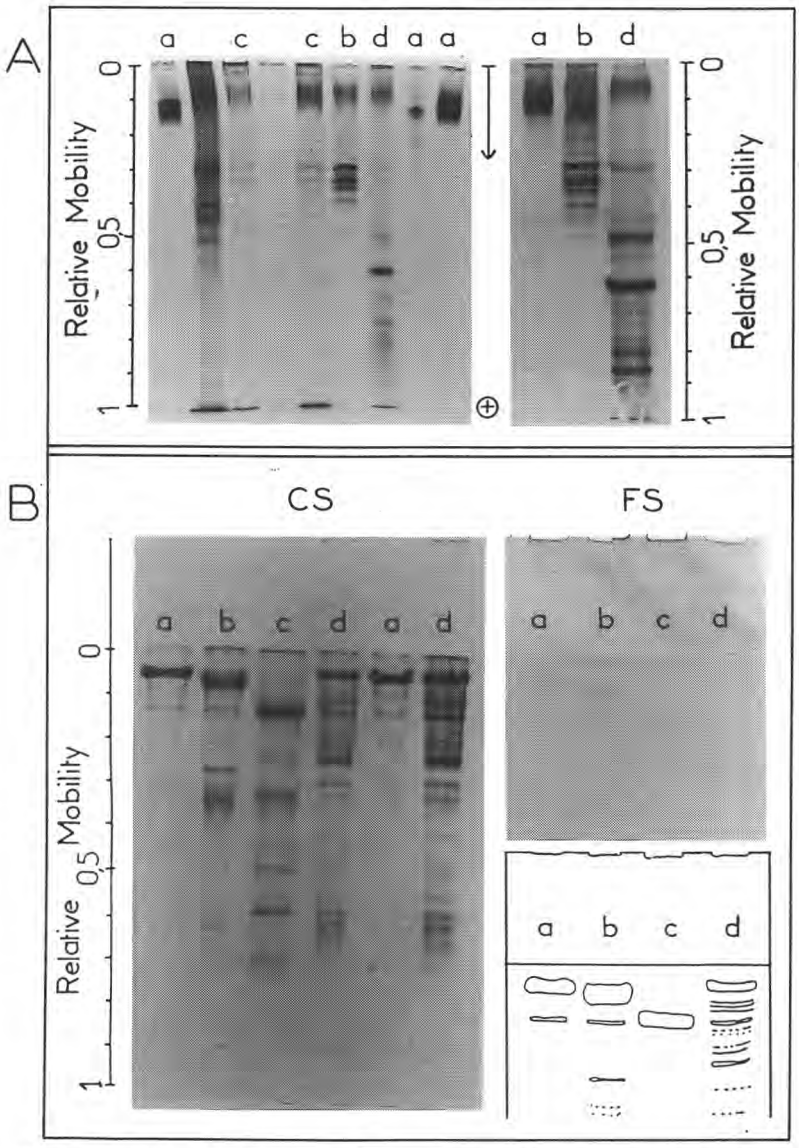

Fig 5. Electrophoretic study of milk enzyme. **A** : Comparative PAGE patterns of blood Plg (a), of UK-activated Plg (b), of MAP obtained from FZ by affinity chromatographies on Lysine-Sepharose (c) and on immunoadsorbant (d). **B** : Comparative SDS-PAGE patterns with Coomassie (CS) or Fuchsine (FS) staining of blood Plg (a), of MAP obtained from casein by chromatography on Lysine-Sepharose (b), of MAP obtained from FZ by chromatography on immunoadsorbant (c) and of UK-activated Plg (d).

*Etude électrophorétique de l'enzyme du lait. **A** : Comparaison en gel natif du plasminogène sanguin (a), du plasminogène activé par l'urokinase (b), de la protéase alcaline du lait obtenue à partir de la fraction enrichie FZ par chromatographie d'affinité sur Lysine-Sépharose (c) et sur immunoadsorbant (d). **B** : Comparaison en milieu dissociant après coloration au bleu de Coomassie (CS) ou à la Fuchsine (FS) du plasminogène sanguin (a), de la protéase alcaline du lait obtenue par chromatographie sur Lysine-Sepharose à partir de la caséine entière (b), ou par chromatographie sur immunoadsorbant à partir de la fraction FZ (c) et du plasminogène activé par l'urokinase (d).*

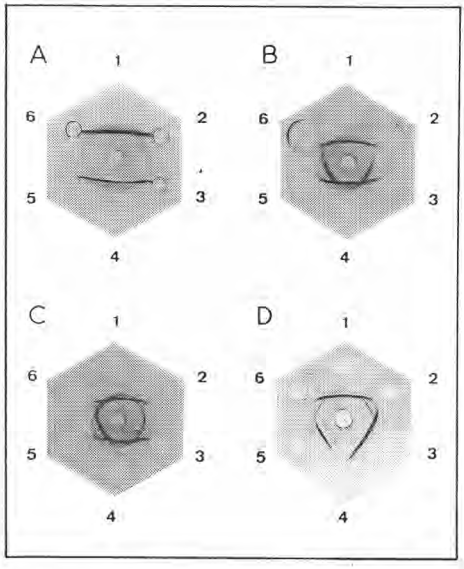

Fig 6. Immunodouble diffusion patterns resulting from the interaction of anti-Plg serum (center wells) and the different MAP preparations obtained from FZ by chromatography on immunoadsorbant **(A, B, C)** and from whole casein by chromatography on Lysine-Sepharose **(D).** A : 1,4 : Plg (0.5 mg.ml^{-1}); 2,5: MAP preparation no 1 diluted 1/1 and 1/2; 3,6: MAP preparation no 2 diluted 1/2 and 1/; B : 1,4 Plg (0.5 mg.ml^{-1}); 2,6: MAP preparation no 2, dilution 1/1; 3 : immunoglobulin (0.25 mg.ml^{-1}); 5 : UK-activated Plg; C : B with MAP preparation no 3, dilution 1/1; D : 1,3 : Plg (0.5 mg.ml^{-1}); 2,6 MAP; 5 : immunoglobulins (0.5 mg.ml^{-1}).
*Mise en évidence par double immunodiffusion de la réaction entre le sérum anti-plasminogène (puits centraux) et les différents lots de protéase alcaline du lait (MAP) obtenus à partir de la fraction FZ par chromatographie sur immunoadsorbant **(A, B, C)** et à partir de la caséine par chromatographie sur Lysine-Sepharose **(D).** A : 1,4 : plasminogène sanguin (0,5 mg•ml^{-1}); 2,5 : lot de MAP n° 1, dilution 1/1 and 1/2; 3,6 : lot de MAP n° 2, dilution 1/2 and 1/1; B : 1,4 : plasminogène sanguin (0,5 mg•ml^{-1}); 2,6 : lot de MAP n° 2, dilution 1/1; 3 : immunoglobulines (0,25 mg•ml^{-1}); 5 : plasminogène activé par l'urokinase; C : B avec le lot de MAP n° 3, dilution 1/1; D : 1,3 : plasminogène sanguin (0,5 mg•ml^{-1}); 2,6 : MAP; 5 : immunglobulines (0,5 mg•ml^{-1}).*

nuity with Plg (fig 6D). Contaminant is also present.

DISCUSSION

Problems linked to blood plasminogen purification

The concentration of εACA used (0.2 mol.l^{-1}) is higher than those used by many authors for the purification of different mammalian blood plasminogens (dog, rabbit, goat, sheep...); *eg* Brockway and Castellino (1972) have separated the 2 glycosyled variants of human Plg using an εACA-gradient 0.007 to 0.02 mol.l^{-1}. The high concentration of εACA solution does not prevent the proteinous contamination of the column by fibrinogen when chromatographies are repeated. To dissolve this fibrinous deposit, Hatton and Regoeczi (1974) used 0.2 mol.l^{-1} Lysine. In the present work a 6 mol.l^{-1} urea solution was used.

It is necessary to eliminate εACA of Plg-preparation by gel filtration as this acid is a plasmin inhibitor as well as a competitive inhibitor of urokinase and streptokinase for the activation of the proenzyme (Ablondi *et al,* 1959; Castellino, 1981). Gel filtration chromatography is more rapid than dialysis.

PAGE reveals minor bands which are certainly Plg-degradation products. In SDS-PAGE there are not 2 bands corresponding to the 2 glycosyled forms of bovine Plg (Schaller *et al,* 1985) but several small bands with higher mobilities. Several explanations may be proposed:

— A partial activation of Plg occurs during gel filtration steps; yielding a mixture of Plg + Lys – Plg (86 kDa) + H-chain (60 kDa) and L-chain (25 kDa) of Plm. By

chromatography on Lysine-Sepharose and Lysine or Arginine gradients, Radcliffe and Heinze (1978) have purified a human Plg activator. εACA may also elute an activator which hydrolyses Plg during experimental steps, in which case the preparation is heterogenous.

— In neutral solution serine-proteinases often hydrolyse themselves (Walsh and Wilcox, 1970).

— The contamination occurs during the Plg-preparation. According to Thorsen (1975), the degraded forms of Plg, bearing a strong affinity to fibrin, can carry fibrin or fragments of fibrin. Thus, Maillard and Favreau (1977) have observed with immunoelectrophoresis that mouse Plg purified by chromatography on Lysine-Sepharose contains traces of α- and γ-globulins. Hatton and Regoeczi (1974) have observed that Lysine-Sepharose retains IgG molecules desorbed with 0.2 mol.l^{-1} Lysine. The present work reports on the contamination by γ-globulin through immuno double diffusion. Connell and Porter (1971) have demonstrated that rabbit plasmin hydrolyses immunoglobulins, hence traces of plasmin in the preparation may retain very small quantities of substrate.

— Activation of Plg occurs just before or during slaughter of the animals. Yamamoto *et al* (1982, 1983) have shown that human plasma contains 78.5% native Plg, 15.5% Plm and 6.1% modified forms *(ie* Lys_{77}-Plg). The proportion of modified forms increases (50%) after strenuous exercise. Yamamoto *et al* (1984) reported that Lys_{77}-Plg constituted the major part of modified forms and that their half-life fell within the range of 5-30 min. Wiman *et al* (1983) reported a half-life of 5 min. Mc Nicol and Douglas (1972) announced the activator role of "stress" in human blood. The "stress" and strenuous exercise of cows before slaughter, have been evidenced. There is reason to assume that their physiological state is akin to that reported by Yamamoto *et al* (1982, 1983, 1984).

Purification of MAP and comparisons with Plg

MAP being associated with casein (Eigel *et al,* 1979), may also be found in the fat globule membrane (Hofmann *et al,* 1979). According to Slattery (1976) or Schmidt (1980) casein Kappa is abundantly present in the superficial layer of the micelle. Although being subject to controversy (Humbert and Alais, 1979) MAP could be associated to the casein Kappa. Nevertheless, most authors use the method of Zittle (1965) in the initial step of MAP purification, with acidification to pH 3.5 (Humbert and Alais, 1979). Von Halpaap *et al* (1977) acidified the micellar casein to pH 2.0. Grufferty and Fox (1988b) have shown the necessity of pH being inferior to 4.6 in order to desorb Plg from the micellar complex.

The elution profiles of affinity chromatography on Lysine-Sepharose were identical when loads were whole casein or FZ. It was necessary to stagnate elution during 1 h when loads descended into the column, to permit affinity fixation on the ligand. The elution of adsorbed proteins was brought about using 0.1 mol•l^{-1} Lysine because 0.2 mol•l^{-1} εACA was not efficient. During the chromatography on immunoadsorbant, Glycine does not totally elute proteinous substances; the residual proteinous substance adsorbed on the gel, not eluted by 0.2 mol•l^{-1} Glycine is desorbed by 10% dioxanne or equilibrating in phosphate buffer.

PAGE shows that the milk enzyme preparations are different from Plg but bear a resemblance to activated-plasminogen. On the SDS-PAGE pattern, the enzyme obtained from whole casein by

chromatography on Lysine-Sepharose has a slightly higher mobility than Plg (Asp_1-Plg) and is, in fact, Lys_{77}-Plg. The FZ-based enzyme, purified on immunoadsorbant has electrophoretic patterns different from those obtained with Plg or casein (fig 7). This preparation is slightly degraded, perhaps due to the acid elution (pH 2.3). It is better to use distilled water (Bureau and Daussan, 1981) or preferably neutral solutions with chaotropic ions.

Furthermore, a different pattern emerges with enzyme obtained from FZ on Lysine-Sepharose (fig 7, slot c): there is a doublet of H-chains which corresponds to the 2 glycosyled variants.

The 2 preparations purified from FZ contain proteins with molecular weights of 60 and 25 kDa. They are plasmin molecules because they also display endopeptidasic activity without UK-activation. Therefore plasmin was prepared instead of plasminogen. The acid elution proved, then, not to be solely responsible for this activation.

Zittle's method may be assumed to cause an activation of Plg or to allow this activation by an endogenous activator. The possible existence of small quantities of the whey Plg-activator (Korycka-Dahl *et al*, 1983) in our casein and our fraction (FZ) should not be excluded.

Manji and Kakuda (1986) have shown that the enzymatic preparation obtained by chromatography of casein on Lysine-Sepharose consists of 90% Plg and 10% Plm, indicating the possibility of self-activation.

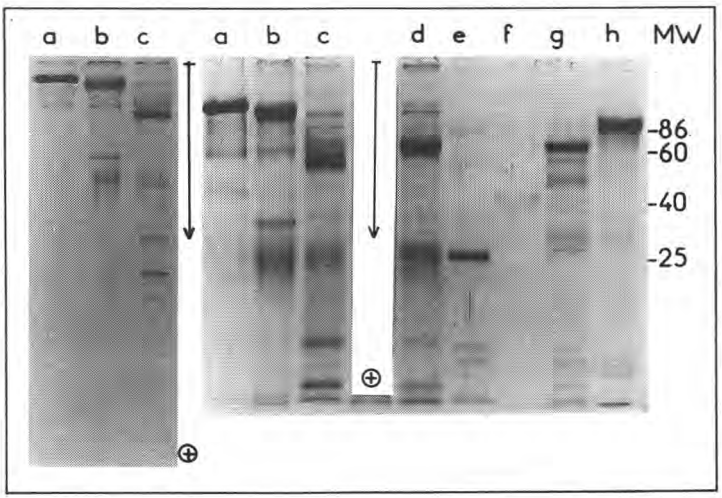

Fig 7. SDS-PAGE patterns of enzymatic preparations obtained by affinity chromatography on Lysine-Sepharose from blood (a = Plg), from whole casein (b) and from FZ (c). Slot (d) is UK-activated Plg. Standard proteins: chymotrypsinogen (e), aldolase (f), catalase (g) and bovine transferrin (h). Molecular weights in kDa.

Electrophorégramme en milieu dissociant (SDS + mercaptoéthanol) des préparations enzymatiques obtenues par chromatographie d'affinité sur Lysine-Sepharose à partir du sang (a), ou de la caséine entière (b) ou de la fraction enrichie FZ (c). Dépôt (d) : plasminogène sanguin activé par l'urokinase. Protéines étalons : chymotrypsinogène (e), aldolase (f), catalase (g) et transferrine bovine (h). Les poids moléculaires sont exprimés en kDa.

Immunodouble diffusion tests show that prepared milk alkaline proteinases are Plg, and sometimes Plm. In the latter case, there was an activation during experimental steps. Moreover, this technique detects immunoglobulins in antigenic solutions and consequently their antibodies in antiserum. In milk, immunoglobulins are localised in whey, but traces then remain in washed isoelectric casein.

There are different precipitin reactions with milk alkaline proteinase obtained from FZ. The third arc obtained in 1 preparation is reminiscent of the works of Eigel *et al* (1979) and Hofmann *et al* (1979) who reported 2 arcs between antiplasminogen serum and whole casein or fat globule membrane. They attributed these precipitin arcs to different concentrations of Plg, Plm and other modified forms with lower molecular weights. The antigenic solution may contain Asp_1, Lys_{77}, Val_{354}, Val_{442}, ... Plg or Plm, hence the antibodies obtained may cover a wide range. This problem may certainly be resolved using monoclonal antibodies.

CONCLUSION

In conclusion, milk alkaline proteinase was purified using a novel method (affinity chromatography on immunoadsorbant) and a more conventional one (affinity chromatography on Lysine-Sepharose). Today, only 1 and hardly satisfying disc-electrophoresis pattern has been reported (Kaminogawa *et al*, 1971) but the vertical gel slab electrophoresis is more reliable and our obtained patterns are also better. In the present study, the electrophoretic patterns and the immunological relationships confirm that milk alkaline proteinase is identical to blood plasminogen (or plasmin). This paper reports a novel method to demonstrate the similarities between milk and blood proteins.

ACKNOWLEDGMENTS

The authors gratefully acknowledge C Tankosic for her technical assistance.

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