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Urokinase-plasminogen activator from bovine plasma obtained by cellulose sulfate precipitation

Ativadores de plasminogênio do tipo uroquinase, extraídos de plasma bovino por precipitação com sulfato de celulose

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SUMMARY

Bovine plasma treated with cellulose sulfate produces multiple forms of plasminogen activators. The most fibrinolytic active fraction was isolated by preparative electrophoresis. Amidolytic and radial immunodiffusion tests with specific antibodies revealed that the most active plasminogen activator possesses properties similar to those of urokinase.

UNITERMS: Bovine: Plasma; Sulfates; Plasminogen activator; Monoclonal antibodies.

INTRODUCTION

The plasminogen activators are proteases which catalyze the conversion of plasminogen to plasmin. Attempts to isolate and characterize plasminogen activators have long been reported (Astrup[2], 1996; Matsuo *et al.*[13], 1981; Wijingaards *et al.*[25], 1982). There are three families of plasminogen activators, the single-chain urokinase type plasminogen activators (Scu-PA) (Wilson *et al.*[26], 1980; Wunt *et al.*[27], 1982; Stump *et al.*[22], 1986), the double-chain urokinase type plasminogen activators (TC-UK and U-PA) (Lijnen *et al.*[12], 1987) and the tissue plasminogen activators (t-PA) (Bernick; Kwaan[3], 1969; Rijken *et al.*[19], 1979; Bernick *et al.*[4], 1981; Rijken; Collen[20], 1981; McMullen; Fujikawa[15], 1985). Scientific literature points out that bovine plasma treated with sulfate polysaccharide produces a fibrinolytically active precipitate (Rothschild *et al.*[21], 1975; Miles *et al.*[16], 1981; Takada *et al.*[23], 1994) containing a mixture of proteins with different molecular weights. In the present study, bovine plasma was treated with cellulose sulfate; the resulting precipitate had various active fibrinolytic forms and the fraction with the highest molecular weight was also the most active one in the zymographic tests. The objectives of the study were to isolate the most fibrinolytic active fraction and to determine its immunological nature, using specific antibodies and amidolytic tests with chromogenic substrates.

MATERIAL AND METHODS

Activation of the bovine plasma fibrinolytic system

Bovine plasma was diluted 1:10 with distilled water at 2ºC and treated with cellulose sulfate (Astrup *et al.*[1], 1944) to a 25 µg/ml final concentration. After 24 hours the resulting precipitate was separated by centrifugation at 13.200 x g and washed with small quantities of 0.1 M glycine buffer, pH 9.6, containing 5% NaCl until dissolution. This solution was exhaustively dialyzed with distilled water; the entire content of the dialysis bag was submitted to lyophilization and then kept frozen at -8ºC. Samples of this material were extracted twice with 0.1 M phosphate buffer, pH 7.3. The final precipitate was mixtured in the glycine-NaCl-buffer, pH 9.6, until dissolution. The resulting solution contained the crude plasminogen activators (CPA).

ABBREVIATIONS: CPA: crude plasminogen activator
PA: plasminogen activator
TPA: tissue plasminogen activator
UK: urokinase

Zymographic analysis

Preparative SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed according to Laemmli[11] (1970). The acrylamide gradient gel concentration was 6-13%. After the procedure (20mA, 4-5hrs), the gel was sliced lengthwise and one segment was stained with Coomassie Brilliant Blue G. The remaining gel was washed with 50 mM TRIS-HCl buffer, pH 7.7, and 0.1 M NaCl containing 2.5% Triton X-100 and gently rotated for two and a half hours at 45ºC to remove the SDS. To determine the fibrinolytic activity of the protein fractions, separated from the gel by preparative electrophoresis, a fibrin-agarose Petri dish was used. The position of the plasminogen activators in the gel had been established by the zymographic technique of Granelli-Pipperno; Reich[9] (1978).

Separation of the plasminogen activator with the highest Fibrinolytic activity (PA)

The most fibrinolytic active fraction according to the zymographic tests was removed from the washed gel using as a guide the stained segment containing standards of known molecular weights. Six or seven pieces of this gel with the most fibrinolytic active material were submitted to electrophoretic elution by the method of Laemmli[11] (1970). The resulting solution contained the most active plasminogen activator (PA).

Fibrinolytic activity

Six ml of a 0.05 M barbital buffer, pH 7.75 containing 1.66 mM $CaCl^2$, 0.686 mM $MgCl^2$ and 93.36 mM CaCl plus fibrinogen to a final concentration of 1 mg/ml were placed on 8 x 8 cm Petri

dishes. The fibrinogen was prepared according to Doolittle *et al.*[7] (1967) or Blomback; Blomback[5] (1957). Fibrin was formed by adding 100 µl of 20 UI/ml thrombin (Topstasine Roche). The fibrinolytic active material (30 µl) was placed in the quadrants of the Petri dishes. The results were compared to an International Reference Preparation (IRP) of UK from NIBSC.

Preparation of antibodies

White, New Zealand, non-pregnant rabbits weighing 3-4 kg were subcutaneously injected with 400-500 µg of the solution containing the plasminogen activators, or 250-450 µg of the most fibrinolytic active plasminogen activator according to the zymographic tests, or 48 IU of UK-standard, per inoculation. All antigens were prepared with Freund's adjuvant (1:1). After 6 or 7 inoculations, the rabbits were bled and the antiserum titers were assayed by radial immunodiffusion analysis (Ouchterlony; Nilsoon[18], 1973).

Purification of the gamma fraction

Sera from immunized and non-immunized rabbits were submitted to chromatography by the method of Miles[17] (1986). DEAE-Sephadex A-50 balanced with K_2HPO_4 buffer, pH 8, was used. The resulting eluates were pooled, concentrated and then submitted to a second chromatography on Sepharose 4B bound to concanavalin A and balanced with Na_2HPO_4 buffer, pH 7.4, containing 0.4 M $CaCl_2$.

Amidolytic assays with the chromogenic substrate

Amidolytic assays were carried out with the solution containing the plasminogen activator, as well as with the most fibrinolytic active plasminogen activator, according to Frieberger[8] (1982). S-2444 (L-pyroglutamyl-glycyl-L-arginine-p-nitroanilide) (ORTHO-DIAGNOSTICS), which is UK-specific according to Matsuo *et al.*[14] (1983), was used as chromogenic substrate.

Protein determination

Protein content was assayed by the microbiuret of Itzhaki; Gill[10] (1964), Fraction V-Sigma bovine albumin (1 mg/ml) was used as standard.

RESULTS

Diluted bovine plasma treated with cellulose sulfate produces a fibrinolytic active precipitate. After dialysis, lyophilization and extraction, a solution containing plasminogen activators (CPA) is obtained. The electrophoresis of CPA can be visualized in Fig. 1 (A-a_2). After electrophoresis, the polyacrylamide gel was purified from SDS and then used in the zymographic tests in order to identify the protein fractions with fibrinolytic activity (Fig. 1 C-c_1). CPA was found to contain a number of proteins with different fibrinolytic properties showing molecular weights ranging from 30.000 to 85.000. The activator with the highest molecular weight in the gel (A-a_2, B-b_2, see arrow) was also the most fibrinolytic active component, according to the zymographic tests (C-c_1). After elution from the polyacrylamide gel, this fraction was submitted to electrophoresis. Fig.1 B-b_1 shows the electrophoretic profile of this plasminogen activator and Fig.1 C-c_2 shows its fibrinolytic activity after electrophoretic elution. The gamma fraction of the anti-CPA, anti-PA, and anti-UK antisera was obtained by chromatography as shown in Materials and Methods. Radial immunodiffusion tests were carried out to determine the immunological nature of the plasminogen activator studied. The precipitation lines that can be seen in the agarose gel show that both CPA and PA were immunologically recognized using the gamma fraction of the UK-standard antiserum (Figs. 2 A and B).

Concomitant, radial immunodiffusion tests were performed with the anti-CPA and anti-PA gamma fractions versus t-PA extracted from a culture of myeloma cells. In these tests, the antisera had no immunological identity with the plasminogen activator used (t-PA).

Immunological nature of the plasminogen activator was confirmed by other tests: 1- total inhibition of the fibrinolytic activity of the UK-standard when incubated with the anti-CPA gamma fraction, with the gamma fraction from the sera of non-immunized rabbits used as control; 2- amidolytic activity of the CPA and PA solutions against the S-2444 urokinase-specific chromogenic substrate, which reacted both with CPA and PA, but not with the t-PA standard. These results are presented in Table1.

The method yielded 200-300 mg CPA/ml bovine plasma, corresponding to 1-1.5 mg/ml of fibrinolytic material; the fibrinolytic activity of CPA was 0.4 IU/ml and the fibrinolytic activity of PA was about 0.6 IU/ml.

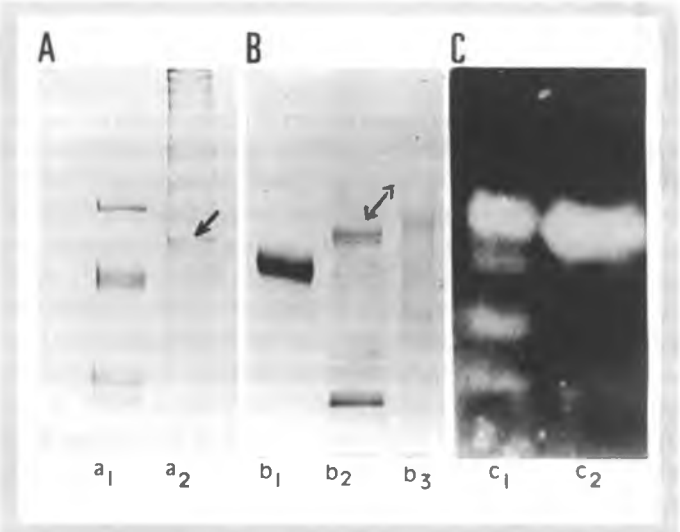

Figure 1
SDS-PAGE (Sodium sulfate-polyacrylamide gel electrophoresis) combined with fibrinolytic analysis in fibrin-agarose dishes.

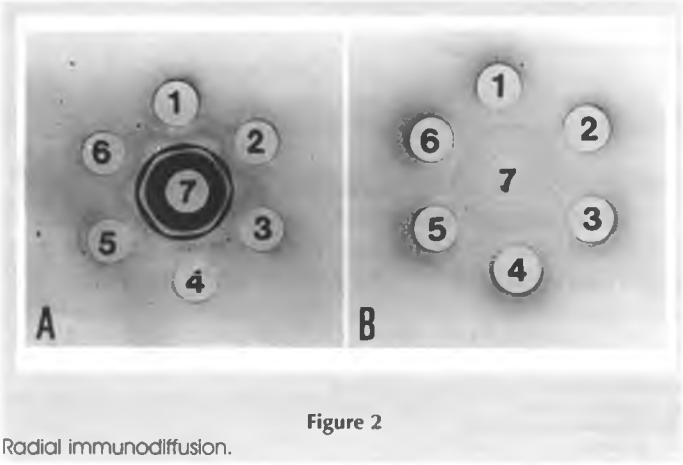

Figure 2
Radial immunodiffusion.

Table 1
Amidolytic activities of bovine plasminogen activators. São Paulo, 1993.

Enzyme species	Amidolytic activity (S-2444)	Specific activity O.D. 405/min/mg
UK	120	0.011
CPA	185.5	0.017
PA	480	0.044
t-PA	-	0.0003

The data are expressed as UI per mg protein obtained by comparison with the International Reference Preparation of Urokinase (see Methods).

DISCUSSION

A brief comparison of some methods of fibrinolytic system activation cited in the literature shows that the pioneering procedure of Rothschild *et al.*[21] (1975), using cellulose sulfate at the concentration of 4 µg/ml, generated a fibrinolytic material on the plate. This material was first submitted to fractionated precipitation with 30% ammonium sulfate and then chromatographed twice consecutively on Sephadex G-200 for further purification. The material was then submitted to SDS-polyacrylamide electrophoresis in which, because of its highly aggregating properties, it did not penetrate the space gel, so that plate fibrinolytic activity appeared only at the top of the gel, after the electrophoretic run.

In the present study, the method was submitted to some modifications in order to solve some of the problems cited earlier. We used cellulose sulfate at the concentration of 25 µg/ml, and the precipitate was solubilized in 0.1 M glycine buffer, pH 9.6, containing 5% NaCl. This salt concentration is of vital importance for a better yield, since it permits better solubilization of the material with fibrinolytic activity. During the subsequent purification step, the above material was dialyzed against distilled water, becoming insoluble again. Thus, to avoid loss of material, we lyophilized the entire content of the dialysis bag. The material thus prepared was again solubilized in the presence of salt whenever it was submitted to electrophoretic separation. With these modifications of the preparation of fibrinolytic material, we were able to obtain CPA fractionation into different plasminogen activators of different molecular weights, by preparative electrophoresis (Fig. 1-b_2).

Miles *et al.*[16] (1981), using human plasma and 50 mg/ml dextran sulfate, obtained a preparation with fibrinolytic activity equivalent to approximately 9 µg/ml purified plasmin. In the present study, CPA yield was 1-1.5 mg/ml using cellulose sulfate and bovine plasma.

In a recent study, Takada *et al.*[23] (1994) used a heparin like polysaccharide, i.e., heparan sulfate at the concentration of 10 µg/ml, and observed that this procedure was more effective for plasminogen activation by t-PA or u-PA than the use of other sulfated polysaccharides (dextran sulfate, chondroitin sulfate etc.). In the same study, the authors, using u-PA extracted from kidney cell cultures after plasmin activation, detected a fibrinolytic material with specific activity of about $15x10^4$ IU/mg. In the present study, our activator had a specific activity of about 470 IU/mg, i.e., much lower than the preparation activated with heparan sulfate. These data may suggest the presence of a plasminogen activator inhibitor (PAI) in our preparation, a hypothesis that was not tested.

On the basis of the results of the radial immunodiffusion tests with isolated monoclonal antibodies and of the amidolytic tests, we concluded that the nature of the isolated plasminogen activator is similar to that of urokinase. The process of purification and preparation of the active fibrinolytic material using cellulose sulfate-activated bovine plasma produced a low yield, as commented earlier. However, in view of the high costs involved in molecular engineering techniques for the production of plasminogen activators on an industrial scale, as stated by Dibner; Timmermans[6] (1986), we believe that the methodology presented here should be considered, since it does not involve large expenditures and allows working with a biological material that does not involve risks of contamination with AIDS, hepatitis or other viruses, besides representing an abundant raw material usually discarded.

Studies of PAI by the method of Van Hinsbergh[24] (1988) may lead to improved yields of the plasminogen activator preparation presented here.

RESUMO

Plasma bovino tratado com sulfato de celulose produz, em placas de fibrina, múltiplas formas de ativadores de plasminogênio. A fração fibrinoliticamente mais ativa foi isolada por eletroforese preparativa. Testes amidolíticos com substratos cromogênicos e testes de imunodifusão radial com anticorpos monoclonais revelaram que o ativador de plasminogênio isolado neste trabalho possui propriedades semelhantes às da uroquinase.

UNITERMOS: Bovinos; Plasma; Sulfatos; Ativador de plasminogênio; Anticorpos monoclonais.

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