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PURIFICATION OF UROKINASE BY AFFINITY CHROMATOGRAPHY

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Summary

Commercially available urokinase (EC 3.4.99.26), though highly active, is still contaminated with unrelated proteins and degradation fragments of urokinase. Further purification of a urokinase preparation by chromatography on benzamidine-Sepharose is described. The final preparation consisted of two molecular forms of urokinase with molecular weights of respectively 31 000 and 54 000. The 54 000-dalton urokinase appears to be composed of two protein chains, one of which is the 31 000-dalton urokinase. A monospecific antiserum against urokinase was raised.

Introduction

Urokinase (EC 3.4.99.26) is a plasminogen-activating enzyme present in urine [1—3] and produced in the kidney [4—6]. Urokinase has been widely used in the investigation of the kinetics of plasminogen activation and is also used in assays of fibrinolytic inhibitors. The extensive purification of urokinase has been described [7—9], but the methods are less suitable for the ordinary laboratory because they require large amounts of urine. Commercial preparations have therefore been used. Though highly active such preparations are not homogeneous [10]. This paper describes a quick purification of urokinase from a commercial preparation by affinity chromatography.

Material and Methods

Starting material. The contents of 10 ampoules of Urokinase Reagent, 10 000 Ploug units each (Leo Pharmaceutical Products) were dissolved in 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.0)/0.4 M NaCl.

Preparation of affinity column. 7.5 g of CH-Sepharose 4 B (Pharmacia Fine

Chemicals) was washed in 1.5 l of 0.5 M NaCl in accordance with the manufacturer's instructions. 1 g of dry gel gives 4 ml of swollen gel with 10—14 μmol/ml of coupled spacer groups. Distilled water was added to the gel to a final volume of 90 ml. 150 mg of *p*-aminobenzamidine · HCl (Sigma No A-1384) in 5 ml of distilled water was then added and the pH was adjusted to 4.5 with 1 M HCl. 1.7 g of *N*-Ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimidehydrochloride (Merck-Schuchardt) dissolved in 2—3 ml of distilled water was added and the mixture was gently stirred for 24 h. pH was checked during the first hour and maintained at about 5. The gel was then thoroughly washed with 0.1 M acetate, (pH 4)/1 M NaCl alternating with 0.1 M Tris · HCl (pH 8)/1 M NaCl. It was checked that all of the carboxyl groups were substituted with *p*-aminobenzamidine by reacting a sample of the washed gel with glycine methyl ester [11,12]. The gel was equilibrated with 0.1 M sodium phosphate buffer (pH 7.0)/0.4 M NaCl and packed in a 1.6 × 40-cm column (Pharmacia Fine Chemicals) to a height of about 12 cm. After the urokinase had been eluted with 0.1 M acetate (pH 4.0)/0.4 M NaCl (see Results), the column was washed with 0.1 M Tris · HCl (pH 8.0)/1 M NaCl alternating with 0.1 M acetate (pH 4.0)/1 M NaCl, several changes, to remove tightly bound protein, if any. The column was then again equilibrated with 0.1 M sodium phosphate buffer (pH 7.0)/0.4 M NaCl. It could be used many times and has been preserved for more than six months.

Polyacrylamide gel electrophoresis. This was performed in sodium dodecyl sulfate according to the method of Weber and Osborn [13] with and without β-mercaptoethanol. The acrylamide concentration in the gels was 10%. The gels were either stained for protein or cut into slices 1 mm thick. The slices were analyzed for urokinase activity by placing them directly onto fibrin plates. Molecular weights were determined on reduced samples with the use of phosphorylase A, transferrin, IgG, heavy and light chain and hemoglobin as markers.

Fibrin plates. These were prepared from human plasminogen-containing fibrinogen (KABI, Stockholm) according to the method of Nilsson and Olow [14] and from bovine plasminogen-free fibrinogen (Poviet Producten, Oss, The Netherlands) in the same way, but with plasminogen-free thrombin. It was checked that fractions active on plasminogen-containing fibrin plates were not active on the plasminogen-free plates. Activator activity was expressed in Ploug units with Urokinase Reagent (10 000 Ploug units per ampoule) as standard.

Immunization of rabbits. Each rabbit was injected on 2 occasions, the second 3 weeks after the first, with 1 ml of the peak fractions of purified urokinase, emulsified with 1 ml of Freund's complete adjuvant. IgG from the rabbit antisera and that from the control rabbit sera were prepared by the method of Steinbuch and Audran [15].

Neutralization experiments. Increasing dilutions of urokinase or tissue culture medium were incubated with IgG from, respectively, antiserum and normal rabbit serum as a control. After having been incubated for 1 h at room temperature and overnight at +4°C the samples were examined for urokinase activity on fibrin plates.

Double diffusion in agarose. This was performed according to the method of Ouchterlony [16].

Immunoelectrophoresis. This was run in 1% agarose (Litex) in 0.075 M barbital buffer, pH 8.6.

Protein determination. This was done according to the method of Lowry et al. [17].

Fetal human kidney. This was cultured in a purely synthetic medium (Parker 199) as described previously [18].

Results

100 000 Ploug units of urokinase activity (specific activity 12 500 Ploug units/mg) contained in 1.5 ml of 0.1 M phosphate buffer (pH 7.0)/0.4 M NaCl were applied to the affinity column (Fig. 1). Most of the protein passed the column unabsorbed together with only 1—2% of the original activity. The column was washed until no absorbance at 280 nm was detected. Urokinase was then eluted by changing the buffer to 0.1 M acetate (pH 4.0)/0.4 M NaCl. In the peak fraction of the chromatogram shown, the protein concentration was 0.148 mg/ml and the urokinase activity 14 000 Ploug units/ml, corresponding to 94 100 Ploug units per mg protein. The recovery of activity from the column was 85—90% of that applied.

The chromatographic fractions were analyzed on sodium dodecyl sulfate polyacrylamide gels for protein and activity. The activity of urokinase was shown to be well preserved in 0.1% SDS without mercaptoethanol. The fraction passing the column unabsorbed was similar in appearance to the redissolved urokinase from the ampoule. There were 10—12 bands in the gels both with

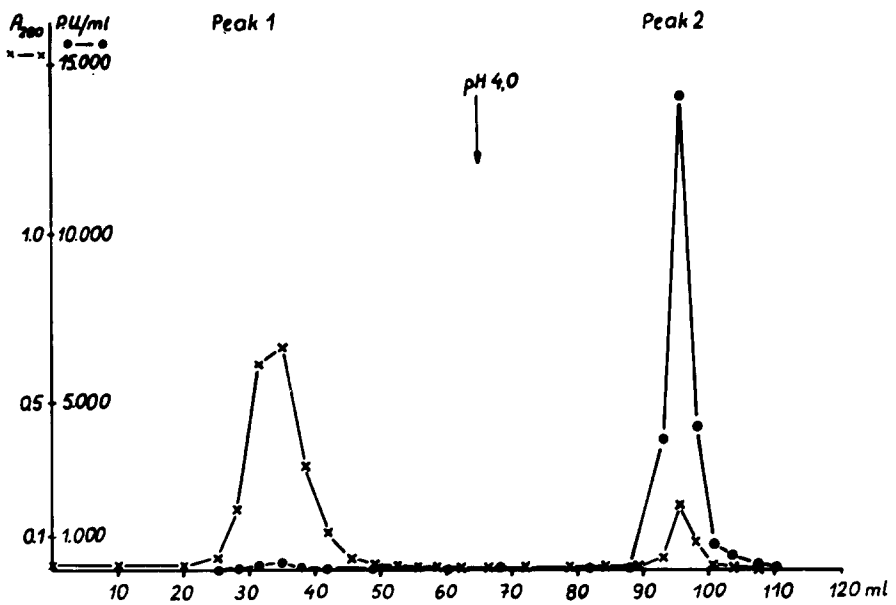

Fig. 1. Chromatography of a commercial urokinase preparation on a 1.6 × 12-cm column of benzamidine-Sepharose. Flow rate 42 ml/h. For explanation see text. X———X, A_{280} nm; ●———●, urokinase activity, expressed in Ploug units/ml.

and without mercaptoethanol (fig. 2). Molecular weights ranged from above 60 000 to small fragments, the smallest with mobilities as fast as that of the dye marker (bromophenol blue). Urokinase activity, determined on unreduced samples, was spread through the whole gel, and although maximal in the middle part, it was present also among the smallest fractions.

The three most active fractions of peak 2 were pooled. The electrophoretic pattern is shown in Fig. 2 to the right. Two bands of protein were seen in the absence of mercaptoethanol. Both had urokinase activity (Fig. 3). The mobility of the wider band corresponded to a protein chain with a molecular weight of 32 000; that of the other to a molecular weight of 54 000. In the presence of mercaptoethanol one main component was seen (Fig. 2). The molecular weight was determined as 31 000. But there was also a fainter component with a molecular weight of 20 000.

Antiserum raised in rabbits against the purified urokinase fraction gave one precipitation line with the starting material on double diffusion (Fig. 4) and on immunoelectrophoresis (Fig. 5). When immunization was continued beyond two injections, the antisera from some rabbits gave a second band on immuno-

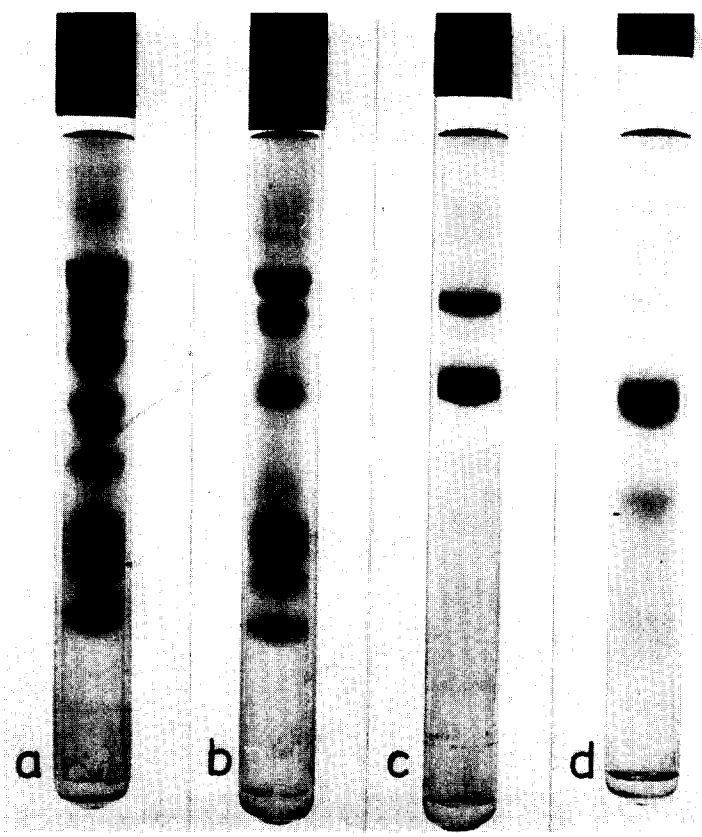

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of: (a) peak 1 run without mercaptoethanol; (b) peak 1 treated with mercaptoethanol; (c) peak 2 run without mercaptoethanol; (d) peak 2 treated with mercaptoethanol.

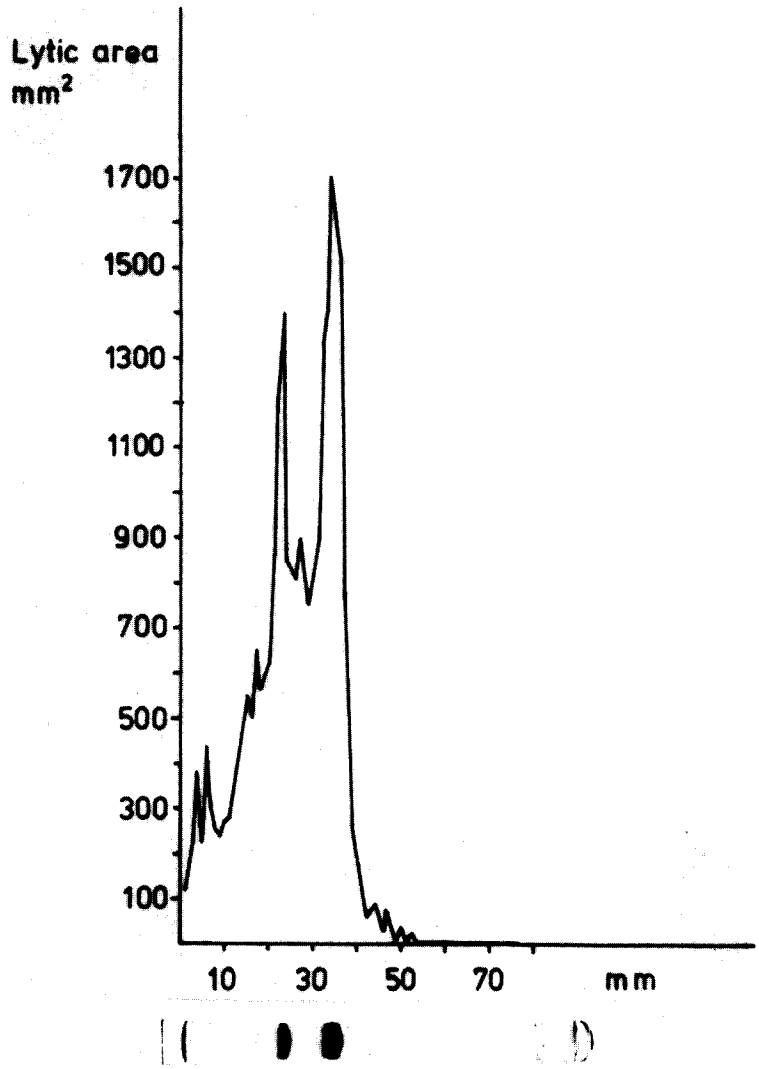

Fig. 3. SDS-polyacrylamide gel electrophoreses of purified urokinase in the absence of mercaptoethanol. One gel was stained for protein and a second gel with the same migration of the dye marker was cut into slices 1 mm thick, which were analyzed for urokinase activity on fibrin plates.

diffusion. 0.2 ml of an IgG preparation from the anti-urokinase antiserum was incubated with 0.5 ml of urokinase solution. The urokinase activity in the mixtures was 1250 or 625 Ploug units/ml. There was an almost complete neutralization of urokinase activity, as the residual activity after incubation was 2 or 1 Ploug unit/ml, respectively. IgG from normal rabbit serum used in the same way did not affect urokinase activity. 0.2 ml of the anti-urokinase IgG was also incubated with 0.5 ml of culture medium from a tissue culture of fetal human kidney. The urokinase activity in the mixtures was 40 or 20 Ploug units/ml. After incubation the activity was 1 or <1 Ploug unit/ml, respectively. IgG from normal rabbit serum did not affect the urokinase activity of the medium.

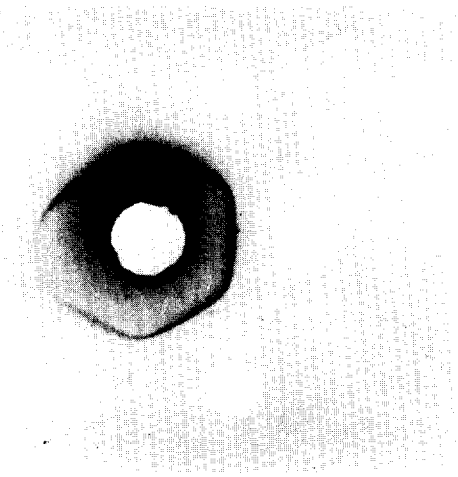

Fig. 4. Double diffusion in agarose. The antiserum was deposited in the central well and urokinase in the outer wells. 1 ampoule of Urokinase Reagent was reconstituted with 1 ml of NaCl and dilutions from 1/1 to 1/32 put in the wells.

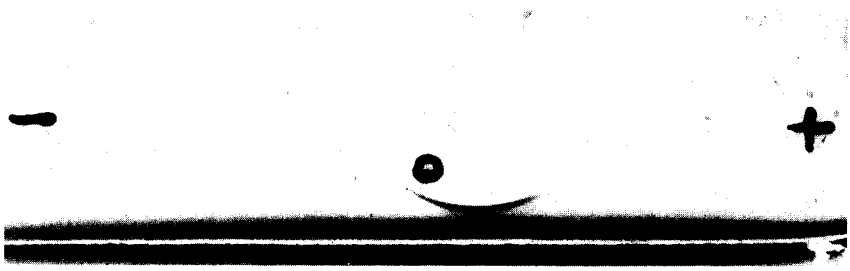

Fig. 5. Immunoelectrophoresis of Urokinase Reagent. One band is seen with slight anodal mobility in the system used.

Discussion

In addition to its plasminogen-activating activity urokinase has an effect on synthetic substrates [19—21]. The esterolytic activity and the plasminogen activation are inhibited by some synthetic compounds [22,23], e.g. benzamidine derivatives. Synthetic inhibitors coupled to Sepharose have been used in affinity chromatography of urokinase [24]. However, endeavours to purify urokinase directly from urine or culture media by this method have so far failed.

We used affinity chromatography as the final step in the purification starting with a commercial urokinase preparation. *p*-Aminobenzamidine was coupled to Sepharose through a 6-carbon spacer. The matrix thus produced efficiently bound the urokinase activity applied in a small volume. Increasing of the salt concentration above 0.5 M caused some leakage of urokinase activity. This shows that the binding of urokinase to the column is rather weak. Although the method served its purpose as a final purification step, it is probably not suitable for isolating urokinase from very dilute sources.

It is known that commercially available highly active urokinase preparations are not pure, but contaminated with other proteins [10]. The present study showed that it also contains smaller protein fragments, certainly degradation products, with urokinase activity. These degradation products may be the result of the so-called uropepsin activity of urine. White et al. [8] also noted forms of urokinase with low molecular weights during their purification procedure.

By far the largest amount of activity was, however, linked to two molecular forms present in the purified fraction. This could conveniently be analyzed in sodium dodecyl sulfate polyacrylamide gels, as urokinase activity was found to be well preserved in 0.1% sodium dodecyl sulfate. The two forms apparently had molecular weights of 31 000—32 000 and 54 000. The first form was most probably the urokinase described by Burges et al. [25], White et al. [8] and Ogawa et al. [9], while the second may correspond to the urokinase of Lesuk et al. [7] and to the S 2 type of White et al. [8].

White et al. [8] felt that their S 2 type might be an association of the lower-molecular-weight urokinase with an inert protein, but they were unable to dissociate it into smaller molecules by varying the pH, by 6 M urea or by succinic anhydride. Our study shows that the higher-molecular-weight urokinase is composed of smaller components. Demonstration of the components required breaking of disulfide bonds. The higher-molecular-weight urokinase appeared to be composed of the 31 000-dalton urokinase and of a smaller chain with a molecular weight of about 20 000. It is not yet known whether this "light chain" is a degradation product of native urokinase or an unrelated protein. The higher molecular weight urokinase may also very well be a naturally occurring form of urokinase.

The specific activity of our preparation was 94 100 Ploug units/mg protein. This corresponds to 134 600 CTA (Committee on Thrombolytic agents) units/mg or 140 200 International units/mg. The specific activity is difficult to compare with that obtained by other workers, as we had two components, one of which had a higher molecular weight. The molar amount of protein in our study is thus smaller, for which reason the specific activity may be underestimated when compared with the purest preparations described [8,9].

Immunization with our purified fraction gave a monospecific antiserum, provided that the immunization was confined to two injections. It thus appears that the "light chain" of the 54 000-dalton urokinase contains only antigenic determinants that are present in the "heavy chain". White et al. [8] also found immunological identity between their S 1 and S 2 urokinases. The electrophoretic mobility in agarose of the higher and lower molecular weight urokinase seems to be similar. Minor impurities in the preparation may be present although not detected, since extended immunization sometimes resulted in the appearance of an unrelated antibody. The present results are also in accord with the findings of Ogawa et al. [9], who reported one band on double diffusion with their specific antiserum and a urokinase preparation. The various forms of urokinase demonstrated immunologically by Day and Ball [26] may represent degradation forms of urokinase with different immunological properties in urokinase preparations.

A urokinase purer than is commercially available is desirable for many rea-

sons, e.g. in scientific work where interference of unrelated proteins or degradation products must be excluded, and as antigenic material for obtaining specific anti-urokinase. A monospecific antiserum may prove very useful in the investigation of the presence and release of fibrinolytic activators in tissues and tissue cultures [27—29]. The present work describes a simple method for obtaining a molecularly well-defined urokinase. It should be easy, if desired, to separate the higher-molecular-weight urokinase from the lower-molecular-weight urokinase [8] provided sufficient starting material is available.

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