Further Studies with Human Plasminogen

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Plasminogen is the inactive precursor of plasmin, a proteolytic enzyme of plasma. The most potent preparations of plasminogen described so far have been obtained by Kline's (1953) procedure, which involves extraction of Cohn's plasma fraction III with 0.05N-sulphuric acid and subsequently adjustment of the extract to pH 11 with sodium hydroxide. This method yields material which is insoluble at neutral pH although the reported isoelectric point is about 5.6 (Shulman, Alkjaersig & Sherry, 1958).

Derechin (1962) purified plasminogen by an alternative procedure not involving the treatment of the proteins with sulphuric acid or sodium hydroxide and obtained a preparation which is soluble at neutral pH. A further fractionation of this plasminogen preparation and a study of the different fractions obtained is described here.

MATERIALS AND METHODS

Reagents. L-Lysine monohydrochloride was supplied by Hopkin and Williams Ltd., diethylaminoethylcellulose was Whatman DE 50, referred to below as DEAE-cellulose, streptokinase (Varidase) was from Lederle Laboratories, and casein (Hammarsten) was from British Drug Houses Ltd.

Buffers. Ammonium acetate buffer was prepared as follows: 57.6 ml. of acetic acid was added to 1800 ml. of water followed by 35% (w/v) ammonia solution (Hopkin and Williams Ltd., analytical reagent) until the pH of the mixture was 9.0; this solution was designated '0.5M-ammonium acetate buffer' and was used as a stock solution from which diluted buffers were made. Phosphate buffer (pH 7.8, *I* 0.1) contained Na_2HPO_4 (16 mM), KH_2PO_4 (14 mM), and NaCl (50 mM). Veronal buffer (pH 8.6, *I* 0.05) contained sodium barbiturate (50 mM) and barbituric acid (20 mM).

Estimation of nitrogen. Samples of protein solutions were digested in 2 ml. of sulphuric acid (microanalytical reagent) with the catalyst mixture recommended by Chibnall, Rees & Williams (1943), and nitrogen estimations were made according to Ma & Zuazaga (1942).

Plasminogen assay. Plasminogen was estimated after converting it into plasmin with streptokinase, as described by Derechin (1961, 1962).

Plasminogen. Fraction R (Derechin, 1962), prepared by the batch adsorption of fraction BL on DEAE-cellulose and elution, was used as starting material for the purification described here. Fraction BL (Derechin, 1962) was used as one of the controls in electrophoresis.

Column chromatography. The protein solution (fraction R), at a concentration of about 2 g./100 ml. in 0.02M-

ammonium acetate buffer, was applied to a column of DEAE-cellulose equilibrated with the same buffer. The amount of protein applied was calculated to be between one-third and one-fifth of that (130–150 mg. of protein/g. of adsorbent) required to saturate the cellulose. The same buffer was then passed through the column to remove the unadsorbed material.

Elution was then performed first with 0.02M-ammonium acetate buffer containing lysine (0.025M) and then with the same buffer containing lysine (0.25M), as shown in Fig. 1.

Fractions of 15 ml. were collected, the flow rate being 105 ml./hr. The extinction of these solutions was read at 280 m μ to establish the position of the peaks, and fractions were pooled as shown in Fig. 1. Solutions were dialysed overnight against 10 vol. of water and then freeze-dried.

Paper electrophoresis. The protein fractions in veronal buffer were applied by a capillary pipette along a straight line on Whatman 3MM paper. A potential difference of 110V (d.c.) was applied across a 22 cm. length of paper for 13 hr. Fig. 2 shows photographs of such paper strips for fractions and controls after drying and staining with Naphthalene Black. Some fractions were run in duplicate on a single sheet, one half of each sheet being dried and stained and the other cut transversely into strips 0.5 cm. wide. The activity of the plasminogen contained in each was studied as follows: each 0.5 cm. strip was immersed in 4 ml. of casein solution (2 g./100 ml.) in a test tube; 0.1 ml. of streptokinase (50 units/ml.) was then added and the tubes were incubated at 40° for 4 hr. (fractions R2 and R3) or 12 hr. (fraction BL). At the end of this period 2 ml. of 1.7N-perchloric acid was added, the mixture being shaken at intervals for 20 min. and then filtered through washed Whatman filter paper no. 1. The extinction of the filtrates was read at 276 m μ against a blank prepared with a sample of the casein solution incubated alone at 40° for 12 hr. and precipitated and filtered as above. The extinction of the filtrates was plotted against the distance from the point of application of the sample in the paper.

Ultracentrifuging. A Spinco model E (electrically driven) ultracentrifuge was used with diagonal schlieren optics and phase plate. The rotor temperature was recorded continuously and unless otherwise stated was approx. 18°. Sedimentation coefficients reported here in Svedberg units (s) were calculated from the movement of the maximum ordinate of the peak corrected for the viscosity and density of water at 20°. The partial specific volume, $\bar{v}$, was assumed to be 0.725.

RESULTS

The material passing unadsorbed through the column (fraction R1) amounted to 1–2% of the total applied (Fig. 1), its main component as indi-

cated by paper electrophoresis (Fig. 2) being of the γ -globulin type, but sedimenting with $S_{20,w} = 2.8$ s. Some plasminogen was present in this fraction. The specific activity showed considerable variation between different columns but was lower than the starting material in all instances.

The most active plasminogen fractions were found in two neighbouring peaks which appeared after adding lysine (0.025M) to the eluent buffer (fractions R2 and R3, Fig. 1). The specific activity (Derechin, 1961) varied between 6000 and 7800 for fraction R2 and between 7800 and 9000 for fraction R3. The last-named specific activity corresponds approximately to a 500-fold increase in potency relative to plasma, or about twice the potency of that described by Derechin (1962).

By paper electrophoresis fractions R2 and R3 were shown to contain mainly material migrating alongside the β -globulin serum fraction, the main component of fraction R3 migrating on paper slightly faster than the main component of fraction R2. About 20% of the protein applied and not less than 50% of the total activity were recovered in fractions R2 and R3 taken together. The α -type globulins which are very abundant in fraction BL and still present in fraction R were not found in fractions R2 or R3. The material eluted with 0.25M-lysine (fraction R4) did, however, contain α -globulin predominantly, the main electrophoretic component being an α_2 -globulin, though α_1 -globulin and a continuum of material between α_2 - and the γ -globulins were also observed. This fraction possessed an activity similar to that of the applied fraction R.

Fractions R2 and R3 showed several components in the ultracentrifuge patterns (Fig. 3). In addition to small amounts of a rapidly sedimenting

component of about 9s and material which did not completely separate from the meniscus, the bulk of the protein gave an asymmetric peak, comprising a main (slower) component and a smaller proportion of a slightly faster component.

Fig. 2. Paper electrophoresis of fractions R, R1, R2, R3 and R4. Undiluted human serum and fraction BL (see Materials and Methods section) were used as controls. Except for fraction R4 (2.7 g./100 ml.) and serum, protein concentrations were about 1.1 g./100 ml. in veronal buffer, pH 8.6. Approx. 0.05 ml. of each solution was used.

Fig. 3. Ultracentrifuge patterns of fractions R2 (1.3 g./100 ml.) and R3 (1.07 g./100 ml.) in phosphate buffer, pH 7.8 (I 0.1 and 0.3 respectively).

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Fig. 1. Extinction, E , plotted against tube no. for elution of plasminogen (fraction R) from DEAE-cellulose.

Fig. 4. Plots of sedimentation coefficient (S_{20}^0) against protein concentration of fractions R3 (open symbols) and R2 (filled symbols). Each shape of symbol corresponds to a different preparation.

The main component of fraction R2 did not show any apparent dependence of the sedimentation coefficient on protein concentration (Fig. 4), whereas that of fraction R3 gave a linear plot of S against c which could be represented by $S_{20,w} = 4.84 - 0.43 c$. The value of $S_{20,w}^0$ obtained for fraction R2 was 4.13s.

The distribution of the plasminogen activity for fractions BL and R3 on paper after electrophoresis (Fig. 5) showed a maximum slightly on the anode side of the point of application but still within the region of the γ -globulin. This maximum corresponded in the stained strips of paper to a faint and narrow protein band which could be distinguished in most runs (Fig. 2). Less activity was associated with the bulk of the protein.

DISCUSSION

Certain enzymes consist of a group of components, all having the same substrate specificity, which may be resolved into closely related but distinguishable molecular types (Market & Møller, 1959). Several types of plasmin, each with a different specificity, have been suggested (Markus & Ambrus, 1960). However, it has not been shown that several proteolytic enzymes, all active against casein, are formed by streptokinase activation of

Fig. 5. (a) Distribution of plasminogen activity (for units, see text) after electrophoresis on paper plotted against distance from the point of application of the fractions BL (O) and R3 (Δ). (b) Diagrammatic version of the paper stained with Naphthalene Black. This should be compared with Fig. 2.

plasminogen. In this discussion it has been assumed that we are dealing with a single caseinolytic proenzyme capable of being activated by streptokinase.

The purification of plasminogen with DEAE-cellulose and lysine-containing buffers was first employed by Wallen & Bergström (1959, 1960), who used Cohn's fraction II + III as their starting material. Unfortunately no detailed elution pattern was given and this prevents a comparison with our results.

In the present work plasminogen preparations were obtained with the highest activities ever reported. However, paper electrophoresis suggests that, even in these cases, the major protein components are inactive and possibly unrelated to plasminogen. It may be objected that the distribution of activity on paper refers to a proactivator rather than to plasminogen (Müllertz & Lassen, 1953; Troll & Sherry, 1955). However, it is still uncertain whether this proactivator is indeed different from human plasminogen. The results presented here have therefore been interpreted in terms of the simpler hypothesis that streptokinase activates human plasminogen directly.

Since the plasminogen activity is associated with only a small proportion of the total protein the

determination of the physical properties of plasminogen must await the preparation of much purer specimens. Similarly other plasminogen preparations for which somewhat lower potencies have been reported (Shulman *et al.* 1958; Hagan, Ablondi & Renzo, 1960; Davies & Englert, 1960; Shulman, 1961) must contain much inactive or inactivated material, and the physicochemical behaviour of such material may not be representative of that of plasminogen.

The results presented in Fig. 4 indicate the presence of two main differently-sedimenting components; differences in paper-electrophoretic behaviour are also associated. The scatter of sedimentation results shown by Shulman *et al.* (1958) might well result from the occurrence of unresolved mixtures of the same components. However, as pointed out above, such results may have no relevance for plasminogen.

Michl & Slotta (1961) studied the distribution of the plasminogen activity after electrophoresis of Kline's preparation in starch gel with urea, the solvent being tris buffer, pH 8.5. They found that the bulk of the protein contained almost all the activity and migrated towards the cathode.

This result differs from ours in two respects: (a) after the electro-osmotic effects have been taken into account the mobilities of the active materials are significantly different; (b) in our work the maximum of plasminogen activity does not coincide with the position of maximum protein content. Electrophoretic differences could arise from the presence of urea in one of the buffer systems, but it seems possible that complex formation of the active material with an inactive protein in one or both cases is also involved.

Although plasminogen prepared from plasma fractions treated with sulphuric acid or sodium hydroxide at extreme pH values (Christensen & Smith, 1950; Kline, 1953) can still be activated into plasmin, it is not unlikely, in view of the solubility behaviour of these preparations (Alkjaersig, Fletcher & Sherry, 1958; Slotta, Michl & Santos, 1961/1962) that the original molecule has been considerably modified. Our preparations also may be altered to some extent, and the different electrophoretic and ultracentrifugal components could derive from a single native protein by a degradation process.

SUMMARY

1. Plasminogen has been purified by column chromatography with diethylaminoethylcellulose.
2. The most active fractions were eluted with buffers containing 0.025 M-lysine resulting in preparations up to 500 times more potent than plasminogen in plasma.
3. By paper electrophoresis it was shown that the active material is a minor component of the most active protein fractions.

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