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STUDIES ON THE PURIFICATION AND ACTIVATION OF PLASMINOGEN (PROFIBRINOLYSIN)*

With the availability of a method of purifying plasminogen from human Fraction III,[8] the therapeutic use of this enzyme in thrombotic conditions became a distinct possibility. Before attempting the clinical use of purified plasminogen, however, it appeared advisable to investigate the report[3] that the enzyme responsible for the fibrinolytic activity can be separated from the agent producing proteolysis since this would make available a more desirable preparation for animal and clinical use.

In addition, an investigation of the activation of plasminogen by streptokinase was considered desirable in view of the observation of Christensen[2] that when plasminogen is activated by streptokinase, the solution obtained contains only a small percentage of the lytic activity which appears if the activation is carried out in the presence of substrate. The convincing demonstration by Cliffton, *et al.*[4,5,6] that purified plasminogen, after activation by streptokinase in the absence of protective substrate, will dissolve clots in animals would make even more impressive the potent fibrinolytic power of this enzyme if Christensen's report is correct.

MATERIALS AND METHODS

Plasminogen (profibrinolysin) was prepared from plasma Fraction III kindly released for our use by the American National Red Cross and supplied through the generosity of Dr. Tillman Gerlough of E. R. Squibb and Sons. Purified profibrinolysin is stable in refrigerated acid solution (pH 2-4), as an air-dried powder, or it may be dried with alcohol and ether as suggested by Cliffton and Cannamela.[8]

Streptokinase (Lederle) was kindly supplied by Dr. Frank B. Ablondi of Lederle Laboratories. The preparation used contained 3,400 streptokinase units per mg. of powder.

Commercial soluble casein was reprecipitated once, washed with water, alcohol, and ether and stored *in vacuo*. For use, it was dissolved in phosphate-saline buffer and standardized to 5 per cent by Kjeldahl analysis.

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Phosphate-saline buffer. 0.1 M sodium phosphate, pH 7.4, in 0.9 per cent sodium chloride. One ml. of 1:1000 merthiolate was added to each liter of solution.

Proteolytic activity was determined under the conditions described by Remmert and Cohen,[9] and their definition of a unit is used, e.g. that amount of enzyme producing an increase of 450γ of trichloroacetic acid (TCA) soluble tyrosine in a medium of 4 per cent casein between the 2d and 62d minutes. Tyrosine-like substances were estimated by determining the absorption of the TCA filtrates at 280 mμ. Remmert and Cohen have shown that the increase in this chromogenic material parallels the increase in TCA soluble nitrogen in this system.

Quantitative fibrinolytic assays were performed essentially according to the method of Christensen.[1]

TABLE 1. COMPARISON OF METHODS OF PREPARATION OF PLASMINOGEN (PROFIBRINOLYSIN) FROM 10 GMS. OF FRACTION III

Method	*Total N mg.*	*Proteolytic activity units*	*Specific activity P.U./mg. N*	*Fibrinolytic activity units*
A. Original[8]	15.0	553	37	0.8×10^6
B. Modified[8]	0.15	...	...	1.6×10^4
C. Direct addition of phosphate, pH 6.0	6.5	390	60	.....
D. Direct addition of phosphate, pH 8.5	6.0	385	64.2	.....

RESULTS

COMPARISON OF METHODS OF PREPARATION

A solution from 40 gms. of Fraction III was prepared as described in the original method up to the point of precipitation in the presence of phosphate. The solution was then divided into four equal portions which were treated as follows:

A—Dialyzed against 0.25 M phosphate, pH 6.0 for 10 minutes and transferred to the refrigerator. The precipitated enzyme was collected by centrifugation the following day and dissolved in 25 ml. of distilled water plus 2 drops of N HCl. This is the originally described procedure.[8]

B—Dialyzed against 0.15 M phosphate, pH 6.0 for 10 minutes and transferred to the refrigerator. The precipitated enzyme was collected by centrifugation the following day, carefully dispersed in 25 ml. of distilled water, and filtered through medium-fast filter paper. The use of 0.15 M

phosphate and the avoidance of the use of HCl to dissolve the final product are the modifications described by Cliffton and Cannamela.[8]

C—When analyses of the solutions obtained from the dialysis bags showed the phosphate to be .0002 in both A and B, 1 ml. of 0.02 M phosphate, pH 6.0, per 100 ml. of solution, was added directly to the third portion. The final pH was about 7.5. The precipitate obtained as in A and B was dissolved in 25 ml. of water plus 2 drops of N HCl.

D—The same as C except that the pH of the phosphate added was 8.5

The results obtained are shown in Table 1. It will be noted that only 1-2 per cent of the nitrogen and of the fibrinolytic activity was obtained

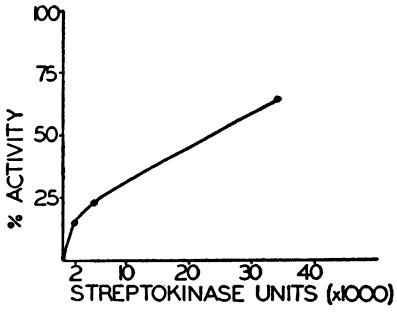

FIG. 1. The proteolytic activity of plasminogen after activation by varying amounts of streptokinase. The activity produced by 850 streptokinase units per mg. of enzyme in the presence of casein is designated as 100 per cent.

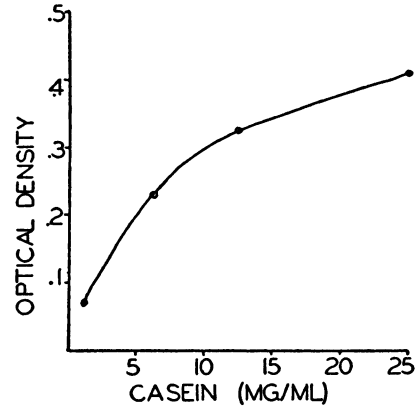

FIG. 2. The proteolytic activity of plasminogen after activation by 850 units of streptokinase per mg. of enzyme in the presence of varying concentrations of casein.

in experiment B (the modified method). The proteolytic activity of this weak solution was not measurable. When, instead of filtering the final precipitate prepared by the modified technique, it was dried with alcohol and ether, no difference in fibrinolytic or proteolytic activity was found as compared to material prepared by the original method. Upon dilution to the concentration of enzyme used by Cliffton and Cannamela (.0056 mg. N/ml.), proteolytic activity was not measurable, irrespective of the method of purification.

It will also be observed (Table 1, C and D) that the direct addition of phosphate resulted in a smaller yield of more highly purified material. At present, the pH of the final solution from which precipitation of the enzyme takes place is varied from 6.0 to 8.5 depending on whether maximum yield

or purity is desired. Above pH 8.0, the enzyme adheres tenaciously to glass surfaces.

THE ACTIVATION OF PLASMINOGEN BY STREPTOKINASE

When purified plasminogen was incubated at pH 7.4 with streptokinase, the activity obtained was far below that which appeared if casein were present during the activation (Fig. 1). Even with enormous amounts of streptokinase, full activity was not obtained. From Figure 2, it may be seen that the activity which appeared was directly related to the concentration of casein present during the activation. It may be noted in passing that the assay method of Remmert and Cohen calls for the presence of a casein concentration of 11.5 per cent during the activation. This concentration is not optimal and may explain the fact that when the enzyme is diluted, the proteolytic activity does not decrease proportionately.

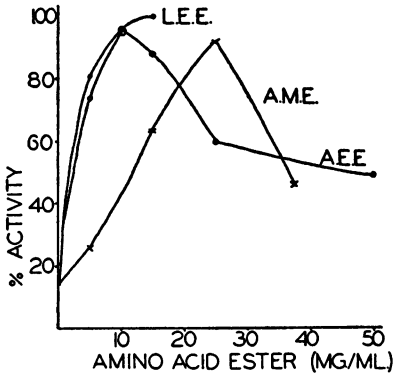

FIG. 3. The protective action of arginine methyl ester (x), arginine ethyl ester (o), and lysine ethyl ester (•) hydrochlorides. The activity produced by 850 units of streptokinase per mg. of enzyme is designated as 100 per cent.

Since the presence of casein renders the preparation unsuitable for repeated intravenous administration, other substrates of plasmin were tested for protective action. Troll, Sherry, and Wachman[10] have reported that certain esterified basic amino acids are split by plasmin. Therefore, arginine methyl ester (AME),* arginine ethyl ester (AEE), and lysine ethyl ester (LEE) were investigated. In carrying out the tests, the hydrochlorides of the ester compounds were dissolved in buffer, adjusted to pH 7-8 and brought to final volume before the addition of streptokinase powder. The plasminogen was dissolved at pH 3.5, and the solution was carefully adjusted to clarity at about pH 8.6. Distilled water was then added to bring the solution to the final concentration desired, usually 4 mg. per ml. Equal volumes of the two solutions were then mixed and incubated at 37.5° C. for 10 minutes. Aliquots, usually 0.4 ml. of the activated mixture, were added to 4 ml. of 5 per cent casein solution and sufficient buffer to make a final

* I am indebted to Dr. Joseph S. Fruton for a supply of AME; the other esters were synthesized by Mr. Jack Graff, Department of Biochemistry, Yale Medical School.

volume of 5 ml. Two ml. aliquots were withdrawn at 2 and 62 minutes and added to 2 ml. of 10 per cent TCA solution. The TCA precipitate was incubated at 37.5° C. for 10 minutes, 5 ml. of water were added, and the suspension was filtered through Whatman No. 3 filter paper.

The data shown in Figure 3, in which the activity obtained in the presence of casein is set at 100 per cent, demonstrate the protective action of these substances. The decline in protective action at high concentrations of the esters probably represents the effect of competition for the substrate. An additional experiment was performed to demonstrate the protective action of LEE in fibrinolytic tests. Two portions of a plasminogen solution were activated with streptokinase, one with and the other without LEE. The unprotected solution yielded 28.5 per cent of the proteolytic and 26.2 per cent of the fibrinolytic activity as compared to the protected solution.

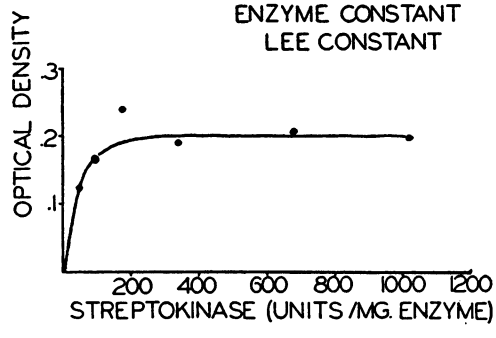

FIG. 4. The proteolytic activity of plasminogen after activation by varying amounts of streptokinase per mg. of plasminogen in the presence of 15 mg./ml. of lysine ethyl ester hydrochloride (.072M).

The arginine esters formed precipitates during the activation, and efforts to elute the enzyme from the precipitates proved fruitless. However, the precipitate formed with LEE was smaller in amount and could be removed with no loss of enzyme activity by centrifugation at pH 8.5. The final enzyme solution was water clear. The plasmin obtained was filterable through a Berkefeld filter but there was some loss in the process.

Using the optimal concentration of LEE hydrochloride, .072 M (15 mg./ml.), it was found, as is shown in Figure 4, that full activation of plasminogen in proteolytic tests was obtained with 2-300 units of streptokinase per mg. of enzyme (0.13 mg. of enzyme N). This amount of streptokinase produces little activated material in the absence of the protective compound (Fig. 1).

STABILITY OF PLASMIN

Plasmin solutions in the presence of LEE were stable for 24 hours at 4° C. and lost activity slowly thereafter until about 20 per cent remained after one week. Frozen solutions showed no loss of activity in two weeks.

TOXICITY STUDIES

Eleven mice were injected intravenously with 150 mg. of neutralized LEE hydrochloride per kg. of body weight.* This amount was calculated to be five times the quantity which will be used. No evidence of toxicity was obtained during a two-week observation period. At autopsy, all the organs appeared to be normal.

When dogs were injected intravenously with plasmin activated in the presence of LEE, the electrocardiogram was normal and blood pressure was unaffected.† When these studies are considered in conjunction with those of Cliffton, *et al.*,[4,5,6] it would appear that plasmin activated with streptokinase in the presence of LEE can safely be administered by the intravenous route. The observation of Cliffton, *et al.*[5] that small quantities of plasmin produce a temporary shortening of the clotting time, was confirmed.

DISCUSSION

We have been unable to confirm the claim that slight modifications of the original method of preparation of plasminogen produce a separation of a proteolytic from the fibrinolytic enzyme.[3] In addition to the evidence obtained earlier by Remmert and Cohen[9] and by Christensen and MacLeod[2] that the pH and temperature optimum for proteolytic and fibrinolytic activities and stability are identical, and that during purification the two activities increase proportionately, we have found that the degree of protection afforded by LEE is the same and that the lysis time is increased when a competing substrate, casein, is added to the enzyme system. On the basis of our studies, the failure of Cliffton and Cannamela to detect proteolytic activity in their preparations appears to have been the result of their using solutions which were too dilute to produce measurable activity in this type of assay. In adequate concentration, plasmin prepared by the modified method possessed the same specific proteolytic activity as the material obtained by the original method.

The observation that plasmin is rapidly destroyed in the absence of substrate has been described by Christensen.[1] The fact that the addition of substrate after the activation does not produce additional activity provides evidence that this is not a phenomenon involving the activation of plasminogen but involves protection of the active enzyme. Furthermore, when plasmin in the presence of substrate is precipitated and redissolved in water, there is an immediate destruction of the active enzyme.

* I should like to thank Dr. D. D. Bonnycastle for his assistance in the mice studies.
† Part of a study being conducted in conjunction with Dr. L. Nahum.

The demonstration of the dependence of protective action on the concentration of substrate may help to eliminate one of the variables which has made the assays of streptokinase and fibrinolysin somewhat erratic and has given rise to controversy concerning the kinetics of the activation process. In addition, it has been possible to show that the quantity of streptokinase needed for activation is considerably less than has hitherto been used. Since streptokinase is a somewhat toxic material,[8] this fact is of importance in the preparation of plasmin for biological use.

SUMMARY

1. It has been found that casein and basic amino acid esters will protect plasmin from destruction.

2. A method is described for the preparation of a plasmin solution which is stable and clear at a physiological pH. Toxicity studies with this preparation have been carried out. No harmful effects were obtained.

3. Evidence is presented which supports the conclusion that the proteolytic and fibrinolytic activities are manifestations of the actions of the same enzyme.

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