Studies on the Proteolytic and Antiproteolytic Activity of Blood Serum

I. Activation of Plasminogen with Trypsin

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Denys and Marbaix¹ showed that normal blood serum can be rendered proteolytic by treatment with chloroform. Somewhat later Delezenne and Pozerski² made the same observation. They also showed that the trypsin inhibiting effect of serum is diminished by shaking with chloroform. It has also long been known that the addition of certain tissue cells can render serum proteolytic. (For references see Permin³.)

Tillett and Garner⁴ showed that certain β -hemolytic streptococci produce a substance that can break down fibrin clots. Milstone⁵ found that this substance does not break down fibrin clots prepared from highly purified fibrinogen and that a serum factor and a streptococcus factor are needed for the break-down of fibrin clots. He also showed that this serum factor is to be found in the euglobulin fraction of serum.

According to Christensen⁶ the serum factor is an enzymogen, which can be enzymatically activated to a proteolytic enzyme. Christensen and MacLeod⁷ named the precursor plasminogen, the active enzyme plasmin, and the streptococcus factor streptokinase. Ratnoff⁸, however, claims that plasminogen reacts stoichiometrically with streptokinase to give plasmin.

Christensen⁹ confirmed Delezenne and Pozerski's experiment and also showed that after the trypsin inhibitor has been destroyed by chloroform the activation curve for plasmin is of an autocatalytic type.

Recently Lewis and Ferguson¹⁰ claimed to have prepared a plasminogen activator from blood serum.

The experiments reported below show that on the addition of trypsin to a crude plasminogen fraction part of the trypsin added is immediately inhibited, that the remaining free trypsin has the power to activate plasminogen, and that the rate of activation is dependent on the remaining trypsin activity.

EXPERIMENTAL

Temperature. All experiments were performed at $25^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$.

Buffer solutions. Buffer A was prepared from sodium acetate (final concentration 0.029 *M*) and sodium diethyl-barbiturate (final concentration 0.032 *M*) and HCl to give a pH of 7.5. Buffer B = buffer A but contains also CaCl_2 in a final concentration of 0.27 *M*.

Trypsin. Crystalline trypsin (Worthington lab.) was dissolved in 0.0025 *N* HCl and diluted in buffer B ten minutes before use. Trypsin is stabilized by the presence of Ca-ions as shown by Bier and Nord¹¹.

Plasminogen. 40 ml human blood serum was diluted with distilled water to a specific resistance of 700 ohms and adjusted to pH 5.2 with *N* acetic acid. It was allowed to stand for 1 hour at $+4^{\circ}\text{C}$ and was then centrifuged after which the precipitate was dissolved in 20 ml buffer B. To this solution 6 ml ether was added and the test tube was placed in an ethanol-carbon dioxide bath at -30 – -35°C from which it was removed at short intervals and turned upside down to secure a satisfactory mixture during freezing. The test tube was kept at this temperature for about 15 minutes and then placed in a refrigerator where it was allowed to thaw slowly at $+4^{\circ}\text{C}$. The ether layer was then sucked off and discarded. The aqueous layer was again frozen and the ether was evaporated with the aid of a water-suction pump.

Streptokinase. The hemolytic streptococcus H 46 A * was grown on a synthetic substrate. The streptokinase was then highly purified but was still not quite free from plasmin inhibitor.

Fibrinogen was prepared from oxalated pig's plasma according to the freezing method of Ware, Guest and Seegers¹² and dissolved in buffer A to a concentration of 0.17%.

Thrombin Topical (Parke & Davis) was dissolved in buffer A. The sample used contained a trypsin inhibitor that inhibited about 5 γ trypsin/ml.

Determination of plasmin activity. To 0.5 ml fibrinogen was added 0.1 ml buffer A and 0.1 ml buffer B and 0.2 ml of the solution to be tested and within 0.07 minutes 0.1 ml thrombin, which clotted the fibrinogen within 0.10 minutes. Immediately after the addition of thrombin the tube was shaken. In this way air bubbles were included in the clot and the lysis time was thus easy to record as the time when the air bubbles had risen to the surface.

For the concentrations of plasmin used in these experiments there is a fairly good proportionality between the inverted lysis time and concentration. Concentrations were, however, calculated from a standard curve relating concentration to lysis time. The fibrinolytic unit for plasmin was taken so that one unit will lyse the clot in 10 minutes under the conditions used. Because of spontaneous lysis less than 0.02 fibrinolytic units cannot be measured. Details of the standardization of plasmin and the influence of ions and of coagulation time will be published later in this series.

Determination of trypsin activity was made essentially according to Anson's¹³ hemoglobin method but with hemoglobin in buffer A instead of in phosphate buffer. The extinction values of the trichloroacetic acid filtrate were measured spectrophotometrically at 2800 Å. The trypsin concentration was checked according to Kunitz¹⁴ with 5 times crystallized soy bean trypsin inhibitor (Worthington lab.).

* Obtained by courtesy from Dr Christensen, New York.

Interaction of trypsin and plasmin. In order to measure the trypsin and the plasmin activity separately in a mixture of the two enzymes, it is desirable to have either different substrates, which are attacked only by one of the enzymes or to have potent inhibitors that act selectively on the two enzymes. Grob¹⁵ showed that plasmin does not attack hemoglobin in strong urea solution. This was confirmed in the present experiments using Anson's hemoglobin method for determining trypsin. Streptokinase-activated plasmin containing 20 fibrinolytic units/ml had no demonstrable activity against hemoglobin. Thus trypsin can be determined separately when mixed with plasmin.

No substrate is known that is attacked by plasmin but not by trypsin, neither is an inhibitor available that inhibits trypsin but not plasmin. The results of earlier experiments, however, showed that fibrinogen prepared according to the method used here seems to be contaminated with an inhibitor which reacts rapidly with trypsin but only slightly with plasmin (Jacobsson, unpublished experiments).

This might explain why under the experimental conditions used the highest amount of free trypsin present possessed only a relatively slight fibrinolytic activity as compared with the fibrinolytic activity of the activated plasmin. Thus a solution containing 20 γ trypsin/ml had a fibrinolytic effect equal to 0.6 fibrinolytic units/ml.

Activation experiments. To each of a set of test tubes containing 2 ml plasminogen was added 2 ml of trypsin of varying concentration. 0.1 ml aliquots were drawn from each tube immediately after mixing and 2 hours later and tested for trypsin activity.

Table 1. shows that the same amount of trypsin was immediately inhibited in each tube, and that afterwards the trypsin activity remained unchanged for at least 2 hours.

Table 1.

γ Trypsin / ml			
Total trypsin	Free trypsin		Trypsin inhibited
	immediately	after 2 hours	
37.0	19.5	19.4	17.5
33.3	16.2	16.2	17.1
29.6	11.8	11.6	17.8
25.9	7.9	7.7	18.0
22.2	4.4	4.4	17.8
18.5	1.1	1.2	17.4

From each tube also 0.2 ml aliquots were withdrawn immediately and after varying intervals and tested for plasmin activity. The plasmin activity rose with time in all tubes (Fig. 1). The rate of plasminogen activation was very slow in the tube containing only 1.1 γ free trypsin/ml and the plasmin activity was not measurable until after 3 hours. The maximum activities of the tubes with 4.4 and 7.9 γ free trypsin/ml were 6 and 7 fibrinolytic units obtained after 6 and 2 hours respectively. Plasminogen in two control tubes without trypsin did not become spontaneously active even after 24 hours.

Fig. 1. The plasmin activity in fibrinolytic units/ml trypsin-plasminogen mixture as a function of time.

DISCUSSION

The interceptions of the time-activity curves with zero time lie at somewhat higher values than those expected from the values of free trypsin. The reason for this is not clear.

The rate of activation as measured by the slope of the initial part of the activation curves rises with increasing concentration of free trypsin in the trypsin-plasminogen mixture. The rate of activation is, however, not quite proportional to the concentration of free trypsin. High concentrations of free trypsin seem to produce a proportionally greater plasminogen activating effect than low concentrations. This is at least partly due to an unavoidable experimental error: the activation proceeds during the time of lysis. Thus the moment corresponding to the measured fibrinolytic activity is not the moment when the trypsin-plasminogen mixture is added to fibrinogen but at a time somewhat later, which cannot be exactly determined. The trypsin inhibitors present in fibrinogen and thrombin counteract this effect relatively more with those trypsin-plasminogen mixtures containing lower concentrations of free trypsin than with those containing higher concentrations of free trypsin. Thus this experimental error has its greatest effect on the curves representing the highest concentrations of trypsin.

As seen from the upper curves on Fig. 1 plasmin is not stable in the presence of trypsin. This may possibly explain that the final plasmin activity is lower, when the activation rate is slow, and higher when the activation is more rapid. The highest plasmin activity obtainable with trypsin as an activator was 13 fibrinolytic units/ml as compared to 17 units when streptokinase was used as an activator.

The enzyme precursor, that can be activated to a fibrinolytic enzyme by streptokinase has been named plasminogen by Christensen and MacLeod. The enzyme precursor activated by trypsin in the experiments above is obtained from the same serum fraction as plasminogen. As the trypsin-activated enzyme is similar to plasmin in respect to its action on fibrin and on hemoglobin in urea it may be assumed to be plasmin. Of course these experiments do not prove conclusively that it is the same precursor which is activated by streptokinase and by trypsin.

SUMMARY

When varying amounts of trypsin are added to a constant amount of a crude plasminogen fraction, a constant amount of trypsin is immediately inhibited. Then the remaining free trypsin activates plasminogen to plasmin. The rate of activation increases with increasing concentration of free trypsin.

Addendum: After this article had gone to press Kocholaty, W., Ellis, W.W., and Jensen, H. *Blood* 7 (1952) 882 have also shown that trypsin can activate plasminogen to plasmin.

REFERENCES

1. Denys, J., and de Marbaix, H. *Cellule* 5 (1889) 197.
2. Delezenne, C., and Pozerski, E. *Compt. rend. soc. biol.* 55 (1903) 327.
3. Permin, P. M. *Undersøgelser over fibrinolytiske enzymer* (Diss.) Copenhagen 1949.
4. Tillett, W. S., and Garner, R. L. *J. Exp. Med.* 58 (1933) 485.
5. Milstone, J. J. *Immunol.* 42 (1941) 109.
6. Christensen, L. R. *J. Gen. Physiol.* 28 (1945) 363.
7. Christensen, L. R., and MacLeod, C. M. *J. Gen. Physiol.* 28 (1945) 559.
8. Ratnoff, O. D. *J. Exp. Med.* 87 (1948) 211.
9. Christensen, L. R. *J. Gen. Physiol.* 30 (1947) 149.
10. Lewis, J. H., and Ferguson, J. F. *Proc. Soc. Exptl. Biol. Med.* 78 (1951) 184.
11. Bier, M., and Nord, F. F. *Arch. Biochem.* 33 (1951) 320.
12. Ware, A. G., Guest, M. M., and Seegers, W. H. *Arch. Biochem.* 13 (1947) 241.
13. Anson, M. L. *J. Gen. Physiol.* 22 (1939) 79.
14. Kunitz, M. *J. Gen. Physiol.* 30 (1947) 291.
15. Grob, D. *J. Gen. Physiol.* 33 (1949) 103.

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