The Assay of Human Plasminogen with Casein as Substrate

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Casein has been widely used for the assay of proteolytic enzymes since Kunitz (1947) described his procedure for the measurement of the activity of trypsin and chymotrypsin. Several procedures have since been designed for the assay of plasmin, the most widely used being that of Remmert & Cohen (1949). These workers activated plasminogen with excess of streptokinase in the presence of casein and then casein was added to the reaction mixture. After incubation the protein was precipitated and the tyrosine content of the filtrates estimated. A proteolytic unit was defined as the amount of enzyme producing an increase of 450 μ g. of acid-soluble tyrosine in an hour at 35° in a medium containing 4 g. of casein/100 ml.

For work on the purification of human plasminogen it was intended to use an assay based on that described by Müllertz (1955), who purified casein and measured hydrolysis by the increase in extinction at 275 m μ after precipitating protein with perchloric acid.

With our procedure, when a balance sheet was made of the activities of fractions obtained during the course of purification experiments, the total activity exceeded that of the starting material by 12–20%. Moreover, for any particular preparation, the estimated activity varied with the time allowed for the interaction of the enzyme with the substrate: the longer the incubation the higher the resulting activity. By examining samples taken at short intervals from the incubation mixture, the course of the reaction was found to be triphasic: an initial non-linear region of increasing slope, then a linear region followed by a region of decreasing slope. From this it was clear that an assay based on a single sample taken at a fixed time from the initiation of the reaction would be unreliable because of the initial non-linear phase. Many experiments were made under different conditions, with several plasmin preparations. It was found that a closer agreement between the total activity of the fractions and that of the starting material was obtained when the slope of the linear part of the curve was used for calculating the activity.

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The present work was undertaken to establish the validity of an assay based on this calculation.

MATERIALS AND METHODS

Buffers. Phosphate buffers (*I* 0.2) containing Na⁺ and K⁺ ions and covering the range pH 7.0–8.0 were prepared according to Sørensen (1909). Phosphate–NaCl: 0.19 M–NaCl (900 ml.), phosphate buffer, pH 7.6, 100 ml. Acetate–phosphate buffers, pH 4–6 (Kekwick & Mackay, 1954).

Plasminogen. Plasminogen was prepared from out-dated citric acid–sodium citrate–dextrose plasma as described by Derechin (1960) for the fraction BL. After precipitating fibrinogen from 2 l. of plasma the supernatant solution was adjusted to pH 5.3 with 0.5 M-acetic acid, the volume made up to 4 l. with water and ether was added to a concentration of 10% (v/v) at 0°. The resulting precipitate, which contained prothrombin and plasminogen, was redissolved in 150 ml. of 0.049 M-trisodium citrate; the volume was made up to 750 ml. with water and ether was added to a concentration of 10% (v/v) at –3°. The precipitate was redissolved in 1 l. of acetate–phosphate buffer, pH 4.0; the pH of the solution was adjusted to 5.15 with acetate–phosphate buffer, pH 6.0, and the volume made up to 6 l. with water at 0°. The precipitate was dissolved in 1 l. of phosphate–NaCl and portions (100 ml.) of this solution were shaken with 200 ml. of ether at –3° in transfusion bottles (540 ml.). The precipitate was removed by centrifuging and plasminogen was precipitated from the bottom solution by dialysis for 36 hr. against three changes of 4 l. of acetate–phosphate buffer, pH 5.15.

Streptokinase. A commercial preparation (Varidase, Lederle) was used. The activity (Christensen, 1949) was taken to be that indicated on the vials. Fresh solutions were prepared daily in phosphate buffer, pH 7.6.

Plasmin. Plasminogen solutions were mixed with optimum amounts of streptokinase, in the proportion 4:1 (v/v) respectively, and were allowed to stand for 10 min. at room temperature (18–20°) to complete the activation.

Casein. Purified casein was prepared by a method based on the procedure of Müllertz (1955). Commercial casein (20 g.; Hammersten, British Drug Houses Ltd.) was dissolved in water (600 ml.) by adding 0.2 N-NaOH slowly so that at no stage did the pH exceed 8. The solution was boiled and after cooling (2–4°) was brought to about pH 2.0 with N-HCl. At this temperature the precipitate that formed near the isoelectric point was much finer and redissolved more easily on decreasing the pH, than that at room temperature. The solution was then treated with 900 ml. of 0.17 M-perchloric acid and the resulting precipitate was washed four times with water (1 l.), suspended in

phosphate buffer, pH 7.6 (200 ml.), and dialysed at 2–4° for 3 days against six changes of the same buffer. The precipitate dissolved completely overnight. This method provided a medium of constant ionic composition and removed undesirable perchlorate ions from the washed precipitate. After the dialysis the protein concentration of the solution was measured with a dipping refractometer and adjusted to the required concentration. It was then filtered through Seitz EK filters to give a clear solution. The yield of casein was 50–60%.

Solutions of commercial casein were prepared by suspending it in phosphate buffer, pH 7.6, and dialysing against the same buffer at 2–4° as already described. Some of these solutions were boiled. Occasionally commercial casein solution was prepared by dissolving in phosphate buffer, pH 7.6, and readjusting the pH to 7.6 with 0.2N-NaOH.

Assay procedure. Plasmin was added to a casein solution at 40° in the proportion 1:3 (v/v). At convenient intervals samples (4 ml.) of this reaction mixture were withdrawn and pipetted into 2 ml. of 1.7M-perchloric acid at room temperature and shaken at intervals for 20–30 min. until most of the precipitated protein fell spontaneously to the bottom of the tube. The suspension was filtered and the extinction of the filtrate was read at 276 m μ in 1 cm. cells with a Unicam model SP. 600 spectrophotometer. Readings were made for each mixture against a blank prepared by adding 1 ml. of plasmin and 3 ml. of casein solution to 2 ml. of 1.7M-perchloric acid. For establishing the reaction curves, times were measured from the moment of adding the plasmin to casein. The protein precipitated from the samples by perchloric acid could be removed by centrifuging or filtering. Centrifuging did not give particle-free material and solutions were therefore filtered through washed Whatman no. 1 paper. Unwashed paper introduced material with apparent absorption at 276 m μ . This effect was considerable when Whatman no. 3 or no. 42 was used.

Unless otherwise stated the conditions of the tests were: temperature 40°; pH 7.6; *I* 0.2; casein concentration 2.25 g./100 ml. in the reaction mixture.

RESULTS

Conditions for the activation of plasminogen to plasmin

Protection of plasmin by the casein in the activation mixture. Remmert & Cohen (1949) activated plasminogen with streptokinase for 10 min. in a medium containing 1.35 g. of casein/100 ml., at 35°. No reason was given for the presence of the substrate in the activation mixture. Kline (1954) observed that casein and other substrates prevent the inactivation of the plasmin formed by streptokinase activation of plasminogen. This effect of casein was studied at several temperatures. The results agreed with Kline's (1954) conclusions when activation was carried out above 30°. In these circumstances a higher plasmin activity was obtained in the presence of casein. However, when the activation took place at or below 30° the plasmin activity obtained was uninfluenced by the presence of casein, and reached the maximum value.

Incubation time for full activation. Plasminogen samples were activated with an optimum concentration of streptokinase at room temperature (18–20°) for 10, 30 and 60 min. respectively before being added to casein (Fig. 1). To obtain results for zero activation time plasminogen and streptokinase were added separately to casein. The linear part of the reaction curves obtained had the same slope in all instances, but with zero activation time the linear phase was 2 min. delayed with respect to the curve given by the other three samples. This suggests that under the experimental conditions used, 2 min. is required for maximum activation to occur.

Fig. 1. Time required for maximum activation of streptokinase-plasminogen mixtures. Streptokinase in the activation mixture: 200 units/ml. Plasminogen in the reaction mixture: 0.15 mg./ml. $\circ$, 10, 30 and 60 min.; $\bullet$, 0 min. The interval between the intercepts of the extrapolations to the abscissa indicates the minimum time for maximum activation.

Fig. 2. Determination of optimum streptokinase concentration. Activation was for 10 min. at room temperature. Streptokinase in the activation mixture: 2000 ($\bullet$), 660 (Δ), 200 ($\times$), 66 ($\blacksquare$), 25 ($\circ$) and 5 ($\blacktriangle$) units/ml. Plasminogen in the reaction mixture: 0.15 mg./ml.

Determination of optimum concentration of streptokinase. With a constant activation time of 10 min. at room temperature, the streptokinase concentration of the activation mixture was varied from 5 to 2000 units (Fig. 2).

The slope of the curves increased with time, and with streptokinase concentration up to 200 units/ml. At 200 units/ml. the slope was clearly linear and remained constant for mixtures containing 200–660 units/ml., falling from this constant value by 15% at 2000 units/ml. There was thus a broad optimum range of streptokinase concentration between 200 and 660 units/ml. in the activation mixture, in which maximum activation occurred with an activation time of 10 min.

In a further experiment a suboptimum concentration of streptokinase (25 units/ml.) was tested for periods of 10–200 min. A control containing the optimum concentration of streptokinase (200 units/ml.) was activated for 10 min. Provided that sufficient time was allowed, plasminogen was maximally activated at a streptokinase concentration of 25 units/ml.

Fig. 3. Influence of temperature on enzyme activity. Streptokinase in the activation mixture: 200 units/ml. Plasminogen: 0.17 mg./ml. in the reaction mixture. (A) Reaction curves at 20° (■), 28° (×), 30° (□), 35° (▲), 40° (●), 45° (○) and 50° (△). (B) Rate of enzyme action plotted against temperature. Calculations were made from the slope of the linear part of the reaction curves of Fig. 3A.

Optimum temperature and pH for the proteolysis of casein

A study of the effect of temperature on the reaction curves showed that the steepest linear slope occurred at 45° (Fig. 3A). However, at 40° the concentration of the products of reaction rose to a higher value than at 45° before the linear phase of the reaction ended. This suggested that at 45° there was a significant loss in enzyme activity during the course of the measurements. There was more obvious inactivation of the enzyme at 50°.

In Fig. 3B it can be seen also that the activity at 40° was about 2.5 times that at 30° and that in this region the activity increased linearly with temperature.

An examination of the effect of pH on the activity showed that the optimum occurred between 7.4 and 7.6, which is in agreement with the results of other workers (Christensen & MacLeod, 1945; Remmert & Cohen, 1949).

The standard conditions selected for the assay of plasmin with casein accordingly were pH 7.6, temperature 40°.

Fig. 4. Influence of casein concentration on the proteolysis by plasmin. Streptokinase: 200 units/ml. in the activation mixture; plasminogen: 0.15 mg./ml. in the reaction mixture. (A) Reaction curves with 0.5 (×), 1.0 (▲), 2.0 (□), 3.0 (○), 4.0 (△) and 5.0 (●) g. of casein/100 ml. of the reaction mixture. (B) Rates of enzyme action calculated from the slope of the linear part of the curves (Δ) and from single determinations after proteolysis for 10 (○), 20 (●) and 30 (□) min. The apparent lag period (▲) was obtained by extrapolation of the linear part of each curve to the abscissa.

Effect of substrate concentration

Six casein concentrations varying from 0.5 to 5 g./100 ml. were tested (Fig. 4A). The higher the substrate concentration the longer the lag period before the linear part of the curve was reached. On the other hand, the lower the substrate concentration the shorter the linear phase of the reaction, though the slope of the linear part was very little affected. A tenfold increase in substrate concentration resulted only in a 7% decrease in the slope of the linear section of the curve. However, when the rate of the reaction was calculated on the basis of a single reading, a fivefold increase in casein concentration produced apparent differences of the order of 25–80% in the activity of the same enzyme preparation.

It was clear that the substrate concentration was not critical for the calculation of activity from the slope of the linear part of the reaction curve. A final concentration of about 2 g. of casein/100 ml. was chosen. With it the lag period was not too long and allowed sufficient time for the assay, provided that the plasminogen concentration was adjusted accordingly. Lower casein concentrations were occasionally found useful for assaying weak plasminogen preparations. In no instance was a casein concentration less than 1 g./100 ml. used.

Effect of plasminogen concentration

When the plasminogen concentration was increased at constant casein concentration, the slope of the linear portion of the reaction curve increased proportionally and the lag period was progressively shortened. When the rate calculations were made from a single reading at 20 min., the rate-enzyme concentration curve was convex to the abscissa (Fig. 5).

Results with commercial preparations of casein

The behaviour of commercial casein, undialysed and dialysed for 3 days against phosphate buffer, was compared with that of purified casein. In several preparations of commercial casein, and of purified casein incubated at 40° for 2 hr., there was no increase in ultraviolet-absorbing material soluble in perchloric acid in the absence of plasmin. A certain amount of perchloric acid-soluble, ultraviolet-absorbing material present in the undialysed commercial preparations could be completely removed by dialysis. With different batches of casein there were slight variations in the rate of hydrolysis up to 15%, when the same enzyme preparation was assayed. The results suggested that the purification procedure of Müllertz (1955) had little effect on the accuracy of the assay: about 40–50% of the casein was lost during the purification.

Standard assay conditions

It was clear that the determination of the slope of the linear part of the reaction curve was the most reliable basis for the assay of plasmin with casein. The reagents are handled as described in the Materials and Methods section. In addition, when room temperature was higher than 30° a casein solution (3 g./100 ml.) was used as the solvent for streptokinase instead of phosphate buffer.

To determine the slope of the linear part of the reaction curve two procedures were adopted. When the activity of the enzyme preparation was unknown a complete curve was constructed, comprising not less than six points covering a period of 30 min. from the addition of plasmin to the substrate. A blank of water was then used in determining the extinction of the filtrates. When the order of activity of the enzyme preparation was known, the amount of enzyme in the reaction mixture was adjusted to ensure that three samples taken at intervals of 3–5 min. from one another would each give estimates falling on the linear portion of the reaction curve. In all, four samples were taken, the

Fig. 5. Relation between enzyme concentration and activity. Streptokinase: 200 units/ml. in the reaction mixture; plasminogen: 1.0 = 0.10 mg./ml. in the reaction mixture. (A) Reaction curves with four concentrations of plasmin: 1.0 (○), 0.75 (▲), 0.5 (■) and 0.25 (●). (B) Rates of enzyme activity calculated from the linear portion of the curves (●) and from a single determination after digestion for 20 min. (▲).

first, after proteolysis for 10 min., being used as a blank.

The plasmin unit defined here is that amount of enzyme which gives rise to an increase of 1×10^{-3} unit of extinction/min., measured at $276 \text{ m}\mu$ during the linear phase of the reaction with a casein concentration of about 2 g./100 ml. in phosphate buffer, pH 7.6 (I 0.2), at 40° .

The specific activity of a preparation is given by the equation: specific activity = $10^3(SF)/N$, where S = slope of the linear portion of the reaction curve in units of extinction/min., F = the overall dilution factor, N = mg. of N/ml. of the plasminogen solution assayed.

The overall dilution factor is the product of three terms: the dilution of the plasminogen solution with streptokinase at the activation stage, the dilution when the activated product is added to the casein substrate and the dilution when samples of the digestion mixture are added to perchloric acid. In the standard test these are 1.25, 4.0 and 1.5 respectively, leading to an overall factor of 7.5.

The use of suitable dilution factors also makes possible comparison between assays performed with different proportions of the reagents.

DISCUSSION

The reaction curve of the plasmin-casein interaction in these experiments is triphasic whatever the experimental conditions. This result is not due to incomplete activation of the enzyme. The presence of an inhibitor in the enzyme preparation would be expected to give a curve concave to the abscissa when rate of reaction is plotted against enzyme concentration. This did not occur (Fig. 5B) in curves based on estimates either by the linear-slope method or from a single reading; with the linear-slope method the enzyme activity is directly proportional to its concentration. The plots of the curves showing the effect of the substrate concentration based on a single reading after a set period (Fig. 4B) might suggest the presence of an inhibitor in the casein preparations. However, the slope of the linear part of the reaction curves is very little modified by a tenfold increase in substrate concentration and the reaction proceeds at its maximum rate for a longer period the higher the substrate concentration.

A similar type of reaction curve was obtained for the hydrolysis of redistilled ethyl mandelate by liver aliesterase by Willstätter, Kuhn, Lind & Memmen (1927). In this case, an increase in the substrate concentration by a factor of two approximately doubled the lag period. This was due to the presence in the substrate solution of small amounts of its keto ester, which had a strong affinity

for the enzyme but was hydrolysed at a much slower rate. The removal of the keto ester allowed the reaction to proceed linearly from the starting point.

The observation of this 'anomalous' behaviour of casein when used as a substrate for proteolysis is not new. A lag period can be seen very clearly in the reaction curves published by Remmert & Cohen (1949); but in spite of this they based their assay procedure on a single reading after digestion for 60 min. Christensen (1954) also obtained a non-linear curve when he studied the action of trypsin, chymotrypsin and plasmin on casein. Müllertz (1955) on the contrary obtained no lag period; his curves are linear to the origin. The low casein concentration in the digestion mixture may explain this result. Although Müllertz designates a stock casein solution containing 3 g./100 ml. the actual concentration is likely to have been below 2 g./100 ml. because of losses occurring during purification. The casein concentration of the reaction mixture would consequently be below 1 g./100 ml. and at this concentration the lag period is rather short. Further, he measured the absorption for zero time from samples taken after adding the plasmin to casein. It is unlikely that such samples could be handled in much less than a minute and this procedure would also shorten the apparent lag period.

The type of reaction curve described here might be due to a complex sequence of interactions between plasmin and casein. Bergman (1938) has shown that the action of a proteolytic enzyme on a substrate is affected by a number of variables such as the length of the peptide chain, the ratio and sequence of the individual amino acid residues and the nature of the side chains. Changes in the peptide chains with the progress of the hydrolysis might determine a change in the rate of the reaction. A peptide bond hydrolysed at a low rate owing to the presence of certain groups in the neighbourhood might be hydrolysed at a higher rate after the removal of these groups. Also, the shortening of the peptide chain might have a similar effect.

The fact that casein is not a homogeneous protein makes the situation more complicated. Mellander (1939) showed that casein is composed of at least three electrophoretic components, which he designated as α , β and γ , in the order of decreasing mobilities at alkaline pH values. The triphasic-reaction curve obtained when casein is digested with plasmin might be explicable in part on the basis of a differential rate of hydrolysis of these components, an explanation similar to that given by Willstätter *et al.* (1927) for the hydrolysis of ethyl mandelate. There is no evidence at present available to support this suggestion.

Experimental work on the proteolysis with plasmin of separated casein components (Derechin, 1960) indicates that the protein composition of casein mixtures is a controlling factor determining the shape and slope of the curves.

SUMMARY

1. When the proteolysis of casein by plasmin is followed by measuring the increase in material with an absorption at $276\text{ m}\mu$ soluble in perchloric acid, a triphasic curve is obtained, the second part of which is linear.

2. The assay of plasminogen preparations from single determinations after a fixed time of proteolysis gives unreliable results because of the influence of the lag period before the linear portion of the curve is reached.

3. The length of the lag period before the linear portion of the reaction curve is reached is increased by increasing the substrate concentration or by decreasing the enzyme concentration.

4. The slope of the linear part of the curve is proportional to enzyme activity and is almost independent of substrate concentration over a tenfold range.

5. A standardized assay is described which gives reliable results, based on the slope of the linear part of the reaction curve.

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REFERENCES

- Bergman, M. (1938). *Chem. Rev.* **22**, 423.
Christensen, L. R. (1949). *J. clin. Invest.* **28**, 163.
Christensen, L. R. (1954). *Arch. Biochem. Biophys.* **53**, 128.
Christensen, L. R. & MacLeod, C. M. (1945). *J. gen. Physiol.* **28**, 559.
Derechin, M. (1960). Ph.D. Thesis: University of London.
Kekwick, R. A. & Mackay, M. E. (1954). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 286.
Kline, D. L. (1954). *Yale J. Biol. Med.* **26**, 365.
Kunitz, M. (1947). *J. gen. Physiol.* **30**, 291.
Mellander, O. (1939). *Biochem. Z.* **300**, 240.
Müllertz, S. (1955). *Biochem. J.* **61**, 424.
Remmert, L. F. & Cohen, P. P. (1949). *J. biol. Chem.* **181**, 431.
Sørensen, S. P. L. (1909). *Biochem. Z.* **21**, 131.
Willstätter, R., Kuhn, R., Lind, O. & Memmen, F. (1927). *Hoppe-Seyl. Z.* **167**, 303.