PROTEOLYTIC ACTIVITY DETERMINED WITH A SUBSTRATE TAGGED WITH RADIOACTIVE IODINE*

BY N. RAFAEL SHULMAN† AND HENRY J. TAGNON

(From the Sloan-Kettering Institute for Cancer Research and the Department of Medicine, Memorial Hospital, New York)

(Received for publication, March 31, 1950)

The proteolytic enzyme, plasmin, is occasionally found in the plasma of patients with a variety of pathologic conditions. Its presence is detected by observing the complete dissolution of the clot of fibrin (fibrinolysis) formed by the coagulation of blood (1). Methods based on time of fibrinolysis are unsuitable for detecting quantities of plasmin that are insufficient to produce complete lysis of the clot. The method described in this communication was developed to increase the sensitivity of the fibrinolytic test and to establish it on a quantitative basis. The method consists, essentially, of using fibrin tagged with radioactive iodine as a substrate and of relating increases of radioactivity in the non-clottable protein fraction to proteolytic activity.

Materials and Methods

The following reagents were used.

*Thrombin*—A commercial preparation called Topical Thrombin, produced by Parke, Davis and Company (2).

*Trypsin*—A crystalline trypsin preparation, obtained from the Plaut Research Laboratory. It contained approximately 50 per cent magnesium sulfate.

*Plasmin*1—A preparation from bovine plasma prepared by the method of Loomis *et al.* (3).

*Fibrinogen*—Fraction I from bovine plasma2 (4) was further purified by the method of Ware, Guest, and Seegers (5). The clottable nitrogen of the final preparation constituted at least 88 per cent of the total nitrogen. The fibrinogen was kept frozen at −10° as a 1 to 2 per cent solution in

---

* This work was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, and by the American Cancer Society.

† Damon Runyon Clinical Research Fellow and Resident in Medicine at Memorial Hospital, New York. We are indebted to Dr. Wendell C. Peacock for valuable suggestions in radioactive techniques.

1 Kindly supplied by Dr. Eugene Loomis.

2 Supplied by Armour and Company.
0.01 M phosphate buffer in physiological saline containing 0.1 per cent sodium citrate at pH 7.5.

Iodination of Fibrinogen—The radioactive iodine used was I\(^{131}\) with a half life of 8 days obtained as a carrier-free sodium iodide solution. 1 mc. was equivalent to approximately 10\(^{-8}\) gm. of iodine. The iodinating solution was prepared as follows: To 1 mc. of radioactive iodine in 0.25 to 0.5 ml. of water were added 0.6 ml. of 0.002 M sodium iodide and 0.6 ml. of 0.02 M sodium nitrite. The solution was acidified to below pH 4 by the addition of 0.5 N hydrochloric acid. After a few minutes the solution was neutralized by the addition of 0.25 N sodium hydroxide and diluted to a volume of 25 ml. with \(\frac{1}{15}\) phosphate buffer, pH 7.5.

The iodinating solution was added drop by drop with continuous stirring over a 10 minute period at room temperature to 25 ml. of approximately 1.4 per cent fibrinogen solution. The mixture was kept at room temperature for 10 minutes and then dialyzed against 0.01 M phosphate buffer in physiological saline containing 0.1 per cent sodium citrate at pH 7.5. Dialysis was carried out at 4\(^\circ\) against a total of 10 liters of buffer for 2 days, with 5 liters of buffer each day. The dialyzing bag was rotated continuously by means of an electric motor.

Following dialysis, the solution was heated to 37\(^\circ\) to dissolve the fibrinogen that partly precipitated in the cold and was centrifuged at 2000 r.p.m. for several minutes to eliminate a small amount of undissolved protein. The iodinated fibrinogen stock solution was stored in small lots at -10\(^\circ\).

Properties of Fibrinogen Tagged with Radioactive Iodine—The clottable fraction of the fibrinogen solution took up approximately 5 per cent of the total radioactive iodine used in the iodination. This corresponds roughly to 1 atom of I\(^{131}\) for 180,000 molecules of fibrinogen and 1 atom of carrier iodine for 12 molecules of fibrinogen. After dialysis, the clottable fraction contained approximately 75 per cent of the total radioactivity of the iodinated fibrinogen solution. This proportion fluctuated between 70 and 80 per cent for different batches but remained constant for each batch during the period of storage (6 weeks). The proportion remained unchanged after incubation of the solution for 1 to 2 hours at 37\(^\circ\) over the range, pH 6.3 to 8.7, as well as after a 10-fold dilution. The proportion of radioactivity contained in the clottable fraction of the iodinated fibrinogen solution could not be increased by dialysis prolonged beyond the 2 day period.

The clottable nitrogen of the iodinated fibrinogen solution did not vary significantly during the period of storage, indicating that the amount of iodine used in these experiments did not alter the clottability of fibrinogen by thrombin.
Little or no uptake of radioactive iodine occurred when no carrier iodine was used. However, when the carrier iodine was increased to 20 times the amount used in these experiments, the fibrinogen solution became unstable and showed increasing precipitation on storage. When the amount was increased to 220 times, the fibrinogen precipitated completely in 20 minutes. The amount of radioactive iodine used was very small compared to the amount of non-radioactive iodine, the ratio of the 2 being approximately 1:15,000. It was, therefore, possible to increase the radioactivity in the fibrinogen by increasing the proportion of $I^{131}$ to carrier iodine without significantly altering the total iodine content of the preparation.

**Fig. 1.** Proteolysis of iodinated fibrinogen by plasmin. Reaction carried out at pH 7.4. Substrate, 0.08 per cent iodinated fibrinogen solution. Thrombin, 1 ml. = 100 units. Total volume in each tube, 1.1 ml.; temperature 37°; incubation time, 50 minutes.

**Fig. 2.** Proteolysis of iodinated fibrinogen by plasmin. Conditions as in Fig. 1, except that 0.12 per cent iodinated fibrinogen solution is the substrate. Thrombin, 1 ml. = 50 units.

**Determination of Proteolytic Activity**—The reaction was carried out in test-tubes (10 X 70 mm.) immersed in a constant temperature water bath. The stock iodinated fibrinogen solution was diluted with buffer to a concentration of 0.08 per cent. This constituted the substrate. For measurement of proteolytic activity, varying amounts of a plasmin solution were added to 0.5 ml. of substrate. The total volume of the reaction mixture was kept constant by the addition of suitable amounts of buffer solution. Coagulation was produced in each tube by adding 0.1 ml. of thrombin and stirring with a glass rod. A solid clot formed within 15 seconds after the addition of thrombin. After incubation, the supernatant solution of the partly digested clot was separated by breaking the clot with a glass rod and filtering through Whatman No. 1 paper. Filtration was completed.
in 1 minute. Measurement of β particles was made on a 0.5 ml. aliquot of each filtrate, which was placed in a suitable container for the Geiger counter, made alkaline with 0.1 ml. of 0.1 N sodium hydroxide, and dried before counts per minute were determined.

Results

Plasmin Activity As Measured By Radioactivity of Non-Clottable Fraction of Substrate—Varying amounts of plasmin were incubated with a constant amount of substrate and the radioactivity in the supernatant solution was measured. The same experiment was repeated twelve times in three groups of four determinations. In every experiment the number of counts per minute obtained with 0.015 mg. of plasmin was arbitrarily selected as a reference value. For every experiment, a ratio was made of the counts obtained with each amount of plasmin to the reference value. The mean ratio for each amount of plasmin and the 2-σ variation of the ratio were determined. The results are shown in Fig. 1. By using a ratio of counts per minute, the results obtained for substrates containing different amounts of radioactivity are comparable and the graph may be considered a standard curve with standard variations representing the action of plasmin as measured by radioactivity. Larger amounts of plasmin could be measured in a similar fashion by increasing the amount of substrate.

Effect of Time of Incubation As Measured by Radioactivity of Non-Clottable Fraction of Substrate—When a constant amount of plasmin was incubated with a constant amount of substrate for varying periods of time, radioactivity in the non-clottable fraction increased with time. The re-
suits, representing the average values of three experiments, are shown in Fig. 2. The enzyme activity was found to increase at a constant rate for at least 50 minutes.

Comparison of Plasmin and Trypsin Activity—For purposes of comparison, varying amounts of plasmin and crystalline trypsin were incubated with a constant amount of substrate. The results, representing the average values of five experiments, are shown in Fig. 3. The curves were parallel. 25 mg. of the trypsin preparation used produced 0.1137 mg. of tyrosine in 10 minutes at 25° when assayed by the method of Anson and Mirsky (6). By comparing these results with those of Fig. 1 it was determined that increments of plasmin activity equivalent to $2.5 \times 10^{-9}$ to $4.7 \times 10^{-9}$ hemoglobin trypsin units could be measured.

Effect of pH—The optimal pH of the reaction was found to be 7.4 by this method. Below pH 6.3 the substrate precipitated, and above pH 8.7 the clotting time with large amounts of thrombin was longer than 1 hour. The pH curve obtained by this method (Fig. 4) was identical with the curve obtained by conventional methods.

Effect of Additional Amounts of Iodine and Iodide in Enzyme-Substrate Reaction Mixture—No effect on the reaction rate was observed when molecular iodine was added to the reaction mixture in a concentration 40 times that used in iodination or when iodide in the form of sodium iodide was added in a concentration 1000 times greater than the amount of iodine used in iodination.

DISCUSSION

Radioactive iodinated fibrinogen was easily prepared and could be used for a period of at least 6 weeks when stored in the cold. Plasmin and trypsin were tested on the clotted radioactive substrate and the data indicate that increases of radioactivity (as measured by $\beta$-ray counts) in the supernatant solution of the clot can be taken as a measure of enzyme activity. The amount of iodine present in the experimental preparation did not appear to modify the activity of the two proteolytic enzymes tested by this method. Partial precipitation of the substrate at pH 6.3 and failure of clot formation with large amounts of thrombin at pH 8.8 limited the measurement of enzyme activity to this range of pH. The amount of radioactivity in the tagged substrate was small and did not constitute a health hazard if a few simple precautions were taken.

Several methods have been proposed for measurement of plasmin and antiplasmin in plasma and plasma derivatives. The most common method used is based on a measurement of time for complete dissolution of a standard fibrin clot by the enzyme (7). This method has two limitations: first, the end-point is somewhat uncertain, especially when the time of
dissolution is prolonged; and second, amounts of enzyme which are too small to digest the clot completely cannot be measured because no end-point is reached.

The method described herein does not have these limitations. It permits measurement of amounts of enzyme that do not completely digest the clot and is based on the measurement of end-products of the reaction between plasmin and fibrin rather than on a measurement of time. The end-products are defined by the fact that they are not clottable by thrombin. Since these end-products are radioactive, they can be measured accurately and simply by a Geiger counter without the use of chemical or colorimetric techniques.

The sensitivity of the method depends in great part upon the use of thrombin as the agent for precipitating protein. In contrast to most protein precipitants used in proteolytic enzyme work, as for instance trichloroacetic acid, thrombin acts specifically in precipitating only fibrinogen by the formation of fibrin. The early split-products of fibrin which would be precipitated by reagents such as trichloroacetic acid are soluble in the presence of thrombin. The usefulness of such a highly specific protein precipitant as thrombin is especially apparent when the enzyme plasmin is used, since it is known that the products of plasmin digestion consist mostly of large molecular fragments that still have the characteristics of proteins (8). Another factor in the sensitivity of the method is the measurement of reaction products by the radioactivity they contain instead of by a colorimetric tyrosine measurement. The blank in the radioactive method depends solely upon the substrate and is not contributed to by the other reagents used. When the tyrosine method is used for the measurement of plasmin activity in plasma or plasma derivatives, the color-producing material in the reagents may make the value of the blank so high that experimental variations due to enzyme action become relatively insignificant.

**SUMMARY**

Fibrinogen was tagged with radioactive iodine by a simple method. A fibrin clot formed from the tagged fibrinogen was found to constitute an adequate substrate for certain proteolytic enzymes within the range, pH 6.3 to 8.8. The increase of radioactivity in the filtrate of this clot after incubation with enzyme was taken as a measure of proteolytic activity. In this way it was possible to measure accurately increments of proteolytic activity equivalent to $2.5 \times 10^{-3}$ to $4.7 \times 10^{-9}$ hemoglobin trypsin units. The method was more sensitive and more accurate than methods used previously for the detection and measurement of small amounts of plasmin and trypsin and may be applicable to other similarly reacting proteolytic enzymes.
BIBLIOGRAPHY

PROTEOLYTIC ACTIVITY DETERMINED WITH A SUBSTRATE TAGGED WITH RADIOACTIVE IODINE
N. Raphael Shulman and Henry J. Tagnon


Access the most updated version of this article at http://www.jbc.org/content/186/1/69.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/186/1/69.citation.full.html#ref-list-1