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RAPID METHOD FOR PREPARATION OF HUMAN FIBRINOGEN LABELED WITH ^{125}I

Richard A. Peabody, Tyler Halse, and Makis J. Tsapogas

Veterans Administration Hospital, Albany, New York

A method for the purification of human fibrinogen from fresh frozen plasma which can be completed in less than 5 hr is described. An initial cryoprecipitation step is followed by two selective precipitations of fibrinogen with polyethylene glycol 4,000. The average purity of 62 preparations was 93%. Yields of over 50% of the fibrinogen present in the starting plasma were routinely obtained. The fibrinogen obtained is suitable for labeling with ^{125}I. A procedure for labeling fibrinogen, modified from work of others, which makes the isolation and radioactive labeling of fibrinogen complete within one working day is described in this study.

Among other applications ^{125}I-labeled fibrinogen has been found useful in the detection of venous thrombosis (*1,2*) and early rejection of renal transplants (*3*). There is a need for a quick, reliable procedure by which fibrinogen to be used for labeling with ^{125}I can be purified from human plasma. This has been accomplished by a number of workers using selective precipitation either by freezing (*4*), ammonium sulfate (*5*), glycine (*6,7*), or other salts. These procedures are technically tedious as the fibrinogen precipitates are difficult to solubilize and multiple reprecipitations requiring 8–12 hr are needed to achieve high purity. Polyethylene glycol has been used to precipitate fibrinogen from human antihemophilic factor concentrates (*8*). A study of the conditions necessary to precipitate fibrinogen selectively with polyethylene glycol after an initial cryoprecipitation step is the basis of the present work. Easily soluble fibrinogen of greater than 93% purity is produced in high yield in less than 5 hr from fresh frozen plasma. An outline of the technique has been reported (*1*).

MATERIALS

Fresh frozen plasma was obtained from the Northeastern New York Red Cross Blood Center. Each unit was taken from a donor who had given blood with no incidence of disease to the recipients. Each unit was negative for Australia antigen. Polyethylene glycol 4,000 (a linear polymer of 3,000–3,700 molecular weight) was obtained from J. T. Baker Chemical Company and Tris (hydroxymethyl) aminomethane from Sigma Chemical Company. Epsilon aminocaproic acid (EACA) and glycine were provided by Eastman Organic Chemical Corp. The sodium citrate was a product of Mallinckrodt Chemical Company. Iodine-125 was obtained carrier-free in sterile water from Cambridge Nuclear Corp., Cambridge, Mass.

ISOLATION PROCEDURE

All solutions used were sterile. Pipets, tubes, and glassware used in all stages of isolation were also sterile to reduce the risk of introducing bacteria and pyrogens into the preparation.

One unit of fresh frozen plasma was crushed and transferred to a 400-ml beaker. Caprylic alcohol was added (0.1 ml/100 ml plasma) to reduce foaming. A sufficient volume of 53% alcohol (chilled to −10°C) was slowly added in the slush to bring the concentration of alcohol to 3%. The slush was warmed at room temperature until only a thin layer of lumps of ice was present and the temperature was about −1°C. The solution was centrifuged at 825 g for 30 min in a refrigerated centrifuge at 4°C. The cryoprecipitate was dissolved in an amount of 0.02 *M* Tris pH 7.0 equal to ⅛ the volume of the original plasma. One-half molar sodium citrate was then added to make the solution 0.02 *M* in sodium citrate

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For reprints contact: Richard A. Peabody, Research Service, VA Hospital, Albany, N.Y. 12208.

which was spun at 5,000 g for 10 min at room temperature to remove insoluble protein. All steps thereafter were carried out at room temperature. Sufficient solid polyethylene glycol (PEG) was added to reach a concentration of 8% (W/V) over a 1–2 min period to minimize denaturation. Stirring was continued for 15 min to ensure complete solution of PEG and maximum precipitation of fibrinogen. The solution was then centrifuged for 15 min at 5,000 g. The precipitate was dissolved in 0.10 *M* EACA-0.2 *M* glycine pH 6.5 in an amount equal to ⅛ the volume of the original plasma. A small amount of insoluble protein was removed by centrifugation at 5,000 g for 10 min. PEG 4,000 was again added slowly to the clarified solution to a final concentration of 8%, stirring continuously for 15 min after the PEG was added. The fibrinogen precipitate was collected by centrifuging for 15 min at 5,000 g and this was easily dissolved in 0.13 *M* NaCl-0.02 *M* citrate pH 7.4. The concentration of fibrinogen was measured by a modification of the procedure of Ratnoff and Menzie (*9*) in which the fibrin is collected on a glass stirring rod to facilitate its isolation from solution. The purity of the preparation was tested by the method of Atencio, Burdick, and Reeve (*10*). Solutions containing 15–18 mg/ml of fibrinogen were stored at −20°C unless labeling with ^{125}I was to proceed immediately.

EVALUATION OF FIBRINOGEN PREPARATION

For 62 consecutive preparations over a 4-year period, the mean clottable protein was 93% (s.d. ± 4%). The purity of 57 out of 62 preparations was 90% or greater. In the other five this ranged from 71 to 86%.

The yield of purified fibrinogen averaged over 50%. In general, the recovery was better when the fibrinogen concentration in the original plasma was greater than 200 mg% (Table 1).

The major contaminating protein is gamma globulin as determined by immunoelectrophoretic analysis. Residual PEG which may be present in the fibrinogen solutions has been shown to be nontoxic (*8*).

It has been determined that the plasminogen content of the purified fibrinogen is 0.21 CTA units/mg fibrinogen; approximately one-third the plasminogen present per milligram fibrinogen in normal plasma. Plasminogen was determined by a caseinolytic method (*11*).

RADIOIODINATION OF FIBRINOGEN

Fibrinogen was labeled with ^{125}I by a procedure modified from McFarlane (*5*) by using the glycine-sodium chloride buffer described by Atencio and coworkers (*10*). In a typical run 100 mg of fibrinogen in 15 ml of citrate-saline buffer pH 7.4 was mixed with 1.8 ml of 0.9 *M* NaCl–0.9 *M* glycine buffered at pH 8.5 and chilled to 0°C. In a separate tube 1,000 μCi of carrier-free ^{125}I in 1–1.5-ml sterile water was mixed with 0.3 ml of dilute carrier iodine chloride (*5*) and chilled to 0°C. After a 30-min interval to allow equilibration of isotope with carrier, 1.3 ml of 0.8 *M* NaCl–0.8 *M* glycine buffered at pH 9.5 at 0°C was added to the ^{125}I-labeled iodine chloride and the combined solution was added rapidly to the fibrinogen solution by jetting it in with a capillary pipet. The reaction mixture was held for 30 min at 0° to assure complete reaction of the components. Free ^{125}I was removed by dialysis against 0.13 *M* sodium chloride–0.02 *M* sodium citrate pH 7.5 at 4°. Four to 5-hr dialysis was sufficient when 500 ml of buffer was replaced every 90 min. Free ^{125}I was 1% or less at this point, determined by McFarlane's method (*5*). The specific radioactivity of the labeled fibrinogen obtained ranged from 7–14 μCi/mg fibrinogen. There was often a slight decrease, up to 2%, in the apparent purity of the fibrinogen obtained. This was ascribed to interference of iodination in the clotting reaction.

TABLE 1. TYPICAL DATA ON FIBRINOGEN ISOLATION

Prep.	Plasma vol. (ml)	Fibrinogen* (mg %)	Yield† %	Purity† %
1	260	257	50	91
2	225	118	40	97
3	200	270	70	86
4	200	187	62	89
5	200	216	57	94
6	200	270	82	92
7	200	234	55	90
8	200	163	52	92
9	200	266	58	96

* Determined on frozen plasma in plastic tubes attached to the unit of fresh frozen plasma.

† Determined on final solution of fibrinogen in citrate-saline as described in text.

DISCUSSION

Fibrinogen can be purified by repeated ammonium sulfate precipitation (*5*) and by other selective precipitation methods (*6,7*). When using ammonium sulfate in particular, the procedure is tedious in that the fibrinogen precipitates dissolve with difficulty and takes a day or more to be completed. The procedure described in this study is based on selective precipitation with polyethylene glycol, yields readily soluble precipitates of fibrinogen, and is completed within 5 hr. Further purification by column chromatography may be required if the fibrinogen is to be

used for purposes other than the detection of thrombosis.

The purification of fibrinogen by a procedure involving ammonium sulfate, polyethylene glycol 6,000, and cold ethanol precipitation has been described by Polson, et al. Although macroglobulin contamination was shown to be 5% or less, the preparations obtained were only 85% clottable with thrombin (*12*). A procedure involving repeated ammonium sulfate precipitation has recently been reported as suitable to purify autologous human fibrinogen for labeling with ^{125}I and to be used in the donor for special studies (*13*). This procedure, in our hands, has failed to yield fibrinogen of sufficient purity for radioisotope labeling and use in detection of thrombosis.

The isolation and labeling of 200 mg of fibrinogen can be completed in one day if overnight dialysis is used to remove free ^{125}I. This may be done more conveniently on successive days to minimize the chance of denaturing ^{125}I-fibrinogen by prolonged dialysis at 4°.

It has been reported that polyethylene glycol may have a nonspecific stabilizing effect on proteins (*12*). The labeled fibrinogen preparations were stored at −20°C for up to 4 months with no loss in fibrinogen content. After this time the ^{125}I content of the preparations was too low to be useful because of the 60-day half-life of ^{125}I. Recent work has shown that iodine chloride preparations of ^{125}I-labeled fibrinogen show the least changes in their chemical and biological properties and may therefore be the best for use in in vivo tracer studies (*14*). The metabolism and distribution of such preparations of labeled fibrinogen in man have been described by Takeda (*15*).

Labeled fibrinogen prepared as described in detail in this paper has been used in over 150 patients with symptoms and signs of deep venous thrombosis or to screen postoperative patients for this condition (*1*, and unpublished work). In only two patients (1%) have side reactions appeared and these suggested a sensitivity to the autologous fibrinogen. There has been no incidence of hepatitis in any of the patients who received ^{125}I-labeled fibrinogen prepared as described here.

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