A FACILE SEPARATION OF FRAGMENTS D AND E FROM THE FIBRINOGEN/FIBRIN DEGRADATION PRODUCTS OF THREE MAMMALIAN SPECIES

James P. Chen, Helen M. Shurley and Martha F. Vickroy
Department of Human Biological Chemistry and Genetics
The University of Texas Medical Branch
Galveston, Texas 77550

Received September 9,1974

Summary: A simple and efficient method of obtaining D and E fragments from the plasmin degradation products of human, canine and bovine fibrinogens and human fibrin is described. The method utilizes QAE-Sephadex for ion-exchange chromatography.

INTRODUCTION: The D and E fragments with molecular weights of 83,000 and 50,000 represent the major products after prolonged digestion of fibrinogen by plasmin. A method of obtaining D and E in high yields would be of importance in studies of the structure of fibrinogen and the role D and E play in the biologic function of the whole molecule which is the focal point of the coagulation system. In recent years, it has become increasingly apparent that fragments D and E constitute regional biological units in the structure and conformation of fibrinogen (1,2).

Marder et al. (3)have reported the use of Pevikon block electrophoresis (4) to purify D and E fragments based on the marked difference in their electrophoretic mobilities. We have found Pevikon block electrophoresis to be useful in fractionating relatively large amounts (ca. 50 mg) of fibrinogen/fibrin degradation products (FDP)*. However, using this method large portions of FDP overlap in the protein elution profile necessitating repeated electrophoretic runs for complete separation of D and E. Marder et al. (3) have also found DEAE-cellulose chromatography, the method originally used by Nussenzweig et al. (5),to be time-consuming and inefficient for this purpose. We here report a simple and efficient method of clearly separating D and E in a single step from comparatively small amounts (3-15 mg) of FDP. This method utilizes QAE-Sephadex (6) for ion-

* FDP: The plasmin degradation products of fibrinogen and fibrin.

exchange chromatography of FDP and has been applied successfully to the fractionation of FDP from three mammalian species, viz., human, canine and bovine.

MATERIALS AND METHODS: Human fibrinogen which was prepared from normal plasma by ethanol precipitation (7) and fractional precipitation with 25% saturated ammonium sulfate (8) was kindly supplied by Dr. D. J. Baughman of Ortho Research Foundation. Canine fibrinogen, isolated from plasma by the freeze-thaw method of Ware et al. (9), was purified further by fractional precipitation with 25% saturated ammonium sulfate (8). Bovine fibrinogen was prepared from bovine plasma by successive precipitation with 23% and 20% saturated ammonium sulfate. Prior to isolation, each plasma was treated with 30% barium sulfate suspension to remove prothrombin (10). The techniques used to proteolyze fibrinogen and fibrin, to prepare rabbit anti-fibrinogen antisera, and the immunochemical methods have been previously described in detail (2, 11, 12). The proteolysis was terminated after 24 hr at 37°C with soybean trypsin inhibitor at a final concentration of 1 mg/ml.

The FDP were fractionated by selective elution from QAE-Sephadex A-50, using a chromatographic method adapted from the procedure described by Joustra and Lundgren (6) for the preparation of immunochemically pure human IgG.* The 30-ml capacity column was fashioned from the barrel of a disposable syringe fitted at the bottom with a disc of stainless steel wire mesh (United Surgical, Largo, FL). Over this a piece of nylon filter cloth was held securely in place by a gasket cut from the plunger-tip of the same syringe. The swollen gel of QAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ) was packed into this column and equilibrated with ethylene diamine-acetic acid buffer (ionic strength 0.1, pH 8.0). This starting buffer was prepared by mixing 4.4 ml of ethylene diamine, 146 ml of 1 M acetic acid, 2.6 g of sodium azide and adjusting to

* A technical data sheet from Pharmacia Fine Chemicals, Inc. entitled "New Rapid Procedure for Preparation of Human IgG" gives a summary of their method with additional comments on practical aspects of the procedure.

pH 8.0 with dropwise addition of ethylene diamine before diluting to 2 liters with water.

A 15 mg sample of FDP was applied to the QAE-Sephadex column and eluted with starting buffer. The ethylene diamine buffer at pH 5.0 yielded a second peak. The third peak was eluted with sodium acetate buffer (ionic strength 0.2, pH 4.4) prepared by mixing 300 ml of 1M acetic acid and 200 ml of 1M sodium acetate and made up to 1 liter. After each elution, the column was washed thoroughly until eluate OD_{280} reading was less than 0.03. Regeneration of the QAE-Sephadex required washing with 2 to 3 bed volumes of the pH 8.0 starting buffer. All tubes containing D or E fragments were pooled, concentrated by negative pressure dialysis, and dialyzed against normal saline.

RESULTS AND DISCUSSION: QAE-Sephadex chromatography was performed using a stepwise gradient of three buffers, (a) ionic strength 0.1, pH 8.0, (b) ionic strength 0.1, pH 5.0, and (c) ionic strength 0.2, pH 4.4. In each of the fibrinogen or fibrin digestion mixtures studied, D fragment was eluted into two fractions with the pH 8.0 and pH 5.0 buffers at ionic strength 0.1. E fragment was eluted solely with the ionic strength 0.2, pH 4.4 buffer (Figure 1). The purity of isolated D and E fragments was examined by immunoelectrophoresis against monospecific rabbit anti-fibrinogen antisera (Figure 2). The FDP of human and canine fibrinogens clearly separated into fragments D and E according to the criteria of immunoelectrophoresis. However, the electrophoretic mobility of bovine FDP appeared somewhat different and D fragments in the second fraction of QAE-Sephadex chromatography were occasionally slightly contaminated with E fragments.

It is interesting to note that the D fragments of all three species were eluted in the stepwise gradient by two different pH levels (8.0 and 5.0). This is probably due to the heterogeneity of D resulting from slightly different points of proteolysis. These varying points of plasmin digestion for D fragment are antigenically indistinguishable

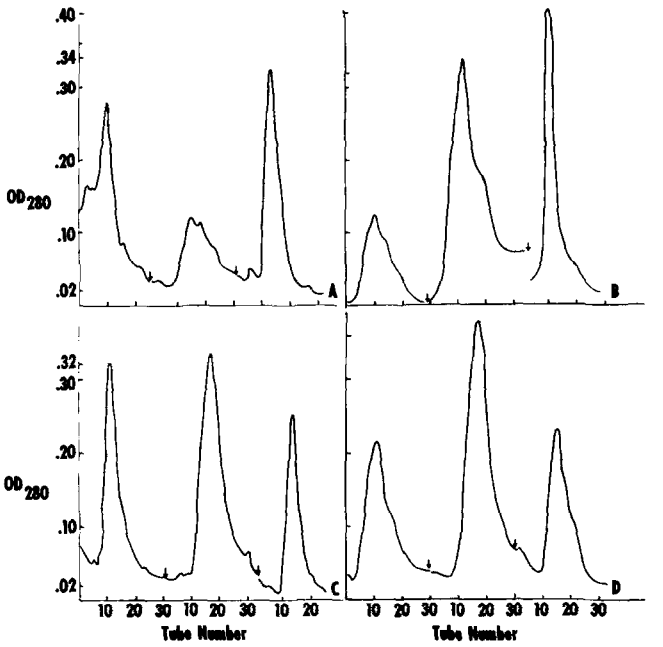

Figure 1. Elution profile of the QAE-Sephadex A-50 chromatography of the plasmin degradation products of human fibrinogen (A), human fibrin (B), canine fibrinogen (C) and bovine fibrinogen (D). Fractions of 3 ml per tube were collected. Arrows indicate elution buffer changes in the stepwise gradient.

---

when tested with anti-fibrinogen antiserum in Ouchterlony analysis. Therefore, neither the first nor the second peak appears to represent higher molecular weight Y fragments.

Arnesen (13) has, by isoelectric focusing, estimated the isoelectric point of fragment E to be about pH 4.9. He reported also that fragments D derived from the 18-hr digest of human fibrinogen appeared in 6 distinct bands in the pH range of about 6.6 to 7.2. Thus, the starting pH was chosen to approximate one pH unit above the isoelectric points of D fragments. The second elution pH was about 1.5 pH units below the isoelectric points of D. Since proteins generally dissociate from ion exchangers at about 0.4-0.6 pH units from their isoelectric points at ionic strength 0.1 (14), the third elution pH for E fragments was set at pH 4.4. However, some E fragments remained bound to the anion exchanger (at ionic strength 0.1) necessitating an increase to 0.2 ionic strength of the last eluting buffer.

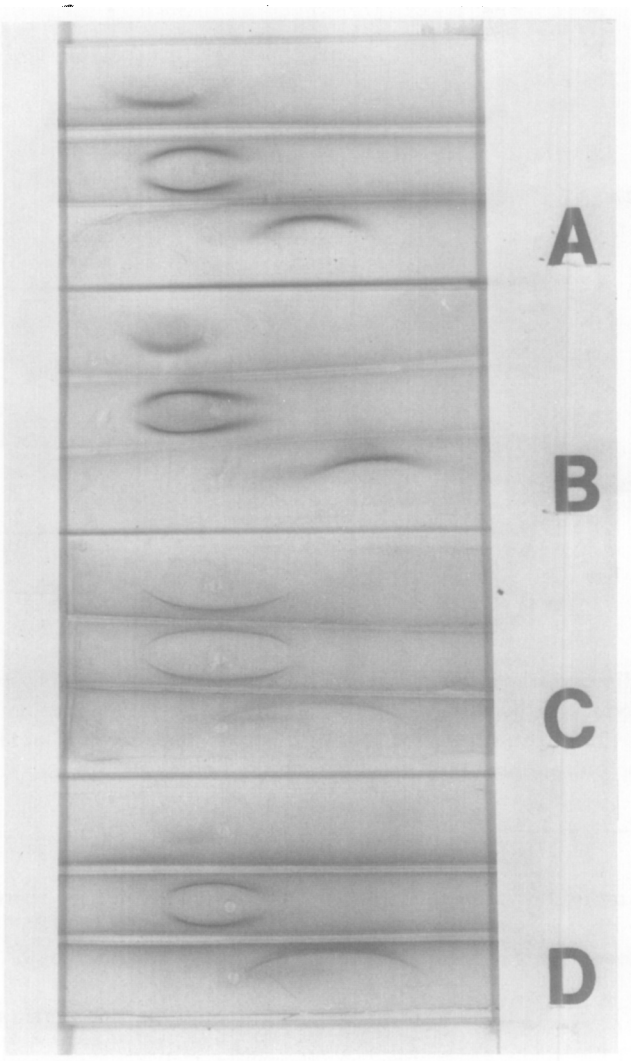

Figure 2. Immunoelectrophoretic patterns show the isolated fractions of D and E fragments from human fibrinogen (A), human fibrin (B), canine fibrinogen (C) and bovine fibrinogen (D). The antigens were tested with rabbit antisera directed against the fibrinogen of each corresponding species.

This work was supported by grants from American Heart Association (Texas Affiliate), Ortho Research Foundation and Robert A. Welch Foundation Grant H-380.

REFERENCES:

1. Marder, V.J., Budzynski, A.Z., and James, H.L. (1972) J. Biol. Chem. 247, 4775-4781.
2. Pizzo, S.V., Schwartz, M.L., Hill, R.L., and McKee, P.A. (1973) J. Biol. Chem. 248, 4574-4583.
3. Marder, V.J., James, H.L., and Sherry, S. (1969) Thromb. Diath. Haemorrh. 22, 234-239.
4. Muller-Eberhard, H.J. (1960) Scand. J. Clin. Lab. Invest. 12, 33-37.
5. Nussenzweig, V., Seligmann, M., Pelmont, J., and Grabar, P. (1961) Ann. Inst. Pasteur 100, 377-389.
6. Joustra, M., and Lundgren, H. (1969) Protides Biol. Fluids, Proc. Colloq. 17, 511-515.
7. Blomback, B., and Blomback, M. (1959) Arkiv Kemi 10, 415-443.
8. Laki, K. (1951) Arch. Biochem. Biophys. 32, 317-324.
9. Ware, A.G., Guest, M.M., and Seegers, W.H. (1947) Arch. Biochem. 13, 231-236.
10. Nanninga, L.B. (1946) Arch. Neerl. Physiol. 28, 241-276.
11. Pizzo, S.V., Schwartz, M.L., Hill, R.L., and McKee, P.A. (1972) J. Biol. Chem. 247, 636-645.
12. Chen, J.P., Hutchison, H.T., Nanninga, L. B., and Guest, M.M. (1974) Biochim. Biophys. Acta (Submitted).
13. Arnesen, H. (1971) Scand. J. Haematol. Suppl. 13, 43-47.
14. Lampson, G.P., and Tytell, A.A. (1965) Anal. Biochem. 11, 374-377.