HETEROGENEITY OF HUMAN FIBRINOGEN: POSSIBLE RELATION TO PROTEOLYSIS BY THROMBIN AND PLASMIN AS STUDIED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

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Received June 2, 1970

Summary: SDS-Polyacrylamide gel electrophoresis of human fibrinogen or fibrin revealed two α-chain species which differed in molecular weight by 3000. With prolonged incubation, thrombin solutions proteolyzed fibrin by attack on α-chains to cause a molecular weight loss of 5000. The initial α-chain products were then further degraded. Purified plasmin digestion of fibrinogen differed in that the initial α-chain molecular weight loss was 3000. This may be responsible for the minor α (A)-chain component found in "native" fibrinogen. With plasmin, further α-chain degradation was followed by β-chain digestion with a molecular weight decrease of 4000. Neither intact α-chains nor intact β-chains were required for clotting of fibrinogen. Fibrinogen of high ethanol solubility lacked intact α-chains.

Although fibrinogen is too large to penetrate the usual gels, polyacrylamide gel electrophoresis of sulfitolyzed fibrinogen and fibrin has been used to detect heterogeneity in each of the three constituent chain types (Brummel and Montgomery, 1970; Lorand, et al., 1969; Takagi and Iwanaga, 1969). Modified methods were used since S-sulfo derivatives are poorly soluble in non-denaturing solvents.

In the present report, an alternate method has been used to investigate human fibrinogen and its derivatives. Disulfide bonds were reduced with 2-mercaptoethanol and polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS). This served to separate the polypeptide chains according to their molecular weights (Shapiro, et al., 1967).

Materials and Methods

Fibrinogen was precipitated at 2°C from citrated plasma (New York

Blood Center) by addition of glycine to 2.1 M (Kazal, et al., 1963; Mosesson and Sherry, 1966). Solutions contained 0.2 M 6-aminocaproic acid to inhibit plasmin. Fraction I-4 was obtained by the Blombäcks' procedure (Blombäck and Blombäck, 1956), while fibrinogen of greater solubility was precipitated from I-2 supernatant by addition of ethanol to 20% (cf. Mosesson and Sherry, 1966). The precipitate was dissolved to 3 mg/ml in 0.055 M sodium citrate - 0.2 M 6-aminocaproic acid, pH 7.4, and subfractions were precipitated at 0-8% (Fig. 1, E) and 8-20% ethanol, -4°C. The 8-20% subfraction was again fractionated at 0-8% and 8-20% ethanol (Fig. 1, F and G, respectively).

Fibrinogen samples dissolved in 0.055 M sodium citrate, pH 7.4, were clotted for one hour at 20°C by dilution with 3 to 6 volumes of bovine thrombin (Parke Davis Topical, 10 u/ml) in 0.015 M sodium phosphate - 0.075 M sodium chloride - 0.1 M 6-aminocaproic acid, pH 6.4. Percent clottability was determined as previously described (Mills, et al., 1964). In some experiments, thrombin concentration was varied and purified human thrombin (courtesy of Dr. Kent Miller, National Children's Cardiac Hospital, Miami, Florida) was used.

Plasmin digestion was performed at 20°C with fraction I-4 (9 mg/ml) in 0.055 M sodium citrate, pH 7.4. Purified human plasmin (a 50% glycerol solution kindly supplied by Dr. Alan Johnson, American National Red Cross Laboratory, New York, New York) was added in quantities below 2% of the total protein. At various time intervals, aliquots were removed and clotted in the presence of 0.1 M 6-aminocaproic acid as previously indicated.

The SDS-gel technique of Shapiro, Viñuela, and Maizel (1967) was carried out essentially as described by Weber and Osborn (1969). Gel concentration was reduced from 10% to 9% for ease of handling. From 7 to 15 μg of protein were applied and electrophoresed at 7 ma per tube for 16 hours. Gels were stained for 5 minutes and destained by washing in 7% acetic acid. A linear relationship was found between the logarithm of molecular weight (MW) and relative migration distance of the marker proteins used. Relative to aldolase (MW 40,000) = 1.00, the mobility values were 0.68 for catalase (MW 60,000), 0.59 for plasma albumin (MW 66,000) and 0.34 for phosphorylase a (MW 94,000).

TABLE I

Molecular Weights of the Polypeptide Chains of Fibrinogen and Clottable Derivatives

Chain Designation	Molecular Weight
α (A) major	73,000
α (A) minor	70,000
β (B)	58,000
γ	47,000
α-1	71,000
α-2	68,000
α-3	66,000
α-4	64,500
β	56,500
β'	52,000

Results and Discussion

In Table I are listed the molecular weight (MW) values of the polypeptide chains determined after reduction of disulfide bonds and electrophoresis in the presence of SDS. With human fibrinogen (fraction I-4), two α (A)-chain bands (one major and one minor in amount) were seen, while β (B)- and γ-chains each appeared to be homogeneous (Fig. 1, A). The α (A)-chain MW values were slightly higher than previously determined by ultracentrifugation (McKee, et al., 1966). After clotting for 15 minutes, the same pattern as Fig. 1 A was obtained except that both the fibrin α-chain bands (designated as α-1 and α-2) and the β-chain band were reduced in MW by about 2000, as expected for release of N-terminal fibrinopeptides of 1500 MW (Blomback et al., 1966). Thus, the α (A)-

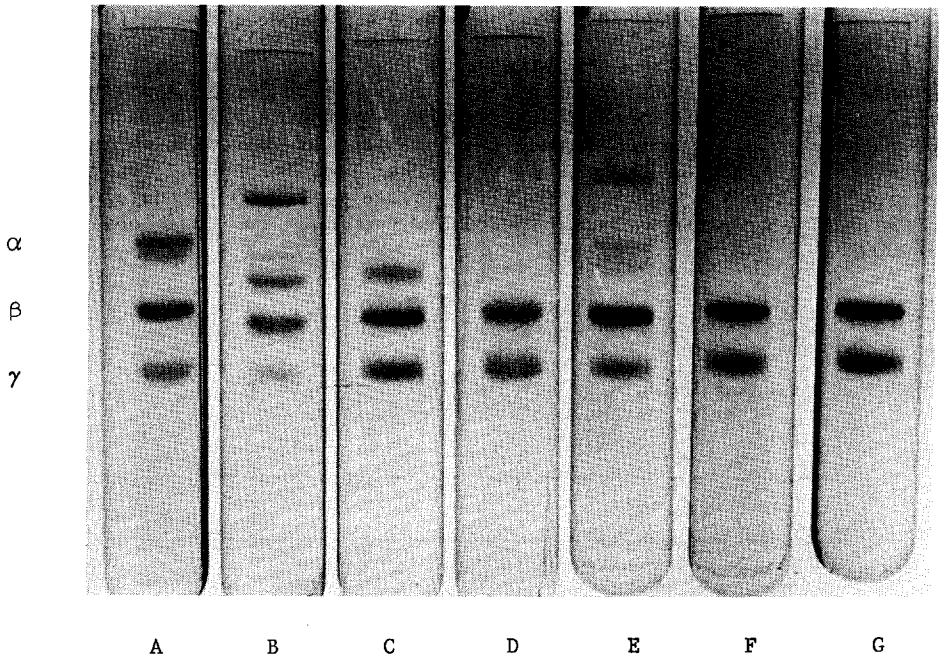

Fig. 1. SDS-Polyacrylamide gel electrophoretic patterns of reduced fibrinogen and clottable products of its digestion by thrombin and plasmin. The lower the molecular weight, the further the migration toward the anode (bottom). A. Fibrinogen (fraction I-4). B. Intermediate bovine thrombin digest. C and D. Intermediate and prolonged human thrombin digests. E, F and G. Clots from fractions of increased ethanol solubility, 93%, 88% and 65% clottable.

chain minor component had not been derived from the major component by removal of N-terminal peptides since thrombin acted on both in the same manner.

After clotting for one hour, a new band, α-3, appeared, which after six hours was the major α band (Fig. 1, B). (A small amount of α-4, MW 64,500, was barely detectable at this time). Furthermore, a band of about 88,000 MW (present earlier in trace amounts, cf. also Fig. 2, A-G) became easily visible. This was associated with the reduced staining intensity of the γ-chain region. The high MW species seems to be a product of two α-chains cross-linked by Factor XIII (fibrin stabilizing factor), a presumed contaminant in the commercial bovine thrombin.[1] This

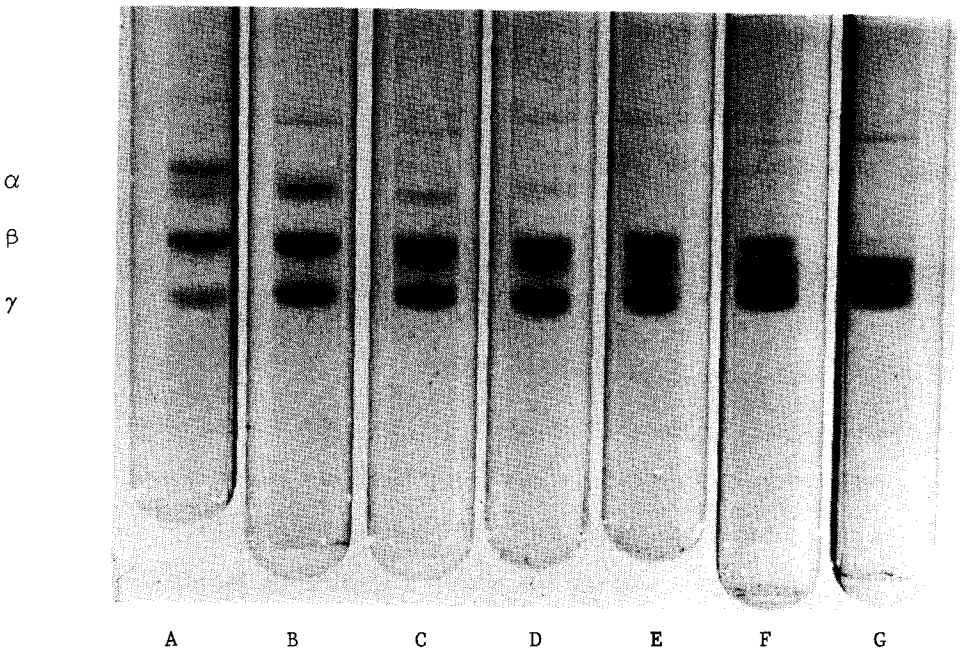

Fig. 2. Electrophoretic patterns (as Fig. 1) of clottable material from fibrinogen digested with plasmin for increasing periods of time, 0 to 2 hours, A to G. Digest clottability: A. 96% (control), B. 86%, C. 82%, D. 78%, E. 70%, F. 64%, G. 40%.

[1] The same product has been found in plasma clots and fibrin stabilized in the presence of calcium and cysteine (unpublished observations). A γ-chain product has also been identified on gel ecectrophoresis by Takagi and Iwanaga, (1970) in stabilized (bovine) fibrin. Lorand et al. (1969) have given a different interpretation of their gel patterns.

product was not found when purified human thrombin was used, even after prolonged incubation times (Fig. 1, C and D). With human thrombin, the α-chain pattern underwent the same changes to yield primarily α-3 chain with small amounts of α-1 and α-4 visible at 18 hours (Fig. 1, C). Large quantities of either human or bovine thrombin (100 u/ml) caused almost total disappearance of the α-chain bands in 24 hours (shown for human thrombin, Fig. 1, D).

An experiment was designed to determine whether proteolysis of α-chains by thrombin solutions was related to contamination by plasmin. Aliquots of fibrinogen were incubated with human plasmin for various time intervals, and were removed and clotted for one hour in the presence of 0.1 M 6-aminocaproic acid, pH 6.4, to retard further lysis. Gel patterns of the clottable protein of each aliquot are shown in Fig. 2, arranged in order of increasing degree of lysis (A to G). The first change observed was the conversion of α-1 chain into α-2 rather than α-3, as found with fibrin digested by thrombin solutions. Although this could indicate that lysis was caused by different enzymes, it could also be the result of a different susceptibility of fibrinogen in solution as compared to polymerized fibrin.

As with fibrin lysed by thrombin solutions, the α-chain bands subsequently disappeared. At least two large fragments may have been produced from α-chains: one indicated by an apparent broadening and increased staining of the γ-chain region, the other, a product of 25,000 MW observed in gels electrophoresed for shorter periods.

The second major change noted with plasmin digestion was conversion of the β-chain to a derivative (here designated as β'-chain). Thus, it is apparent that a clot may form from fibrinogen products in which neither α (A)- nor β (B)-chains are intact.

It has been suggested by Mosesson and Sherry, 1966, that plasma fibrinogens of high ethanol solubility may be products of plasmin digestion.

The gel patterns of clottable material from three fibrinogen fractions of increased ethanol solubility are shown in Fig. 1, E to G (in the order of increasing solubility and clotting time, cf. Mosesson and Sherry, 1966). These fractions are characterized by a lack of intact α-chains although small amounts of α-1 and α-3 chains remain in the sample of Fig. 1, E.

Further experiments will be required to determine what relationship the present data have to the previous reports of heterogeneity in S-sulfo derivatives. There are some difficulties in using these derivatives. In addition to their poor solubility properties, extensive dialysis is required to remove copper ions used in sulfitolysis and under certain conditions artifacts are formed (Brummel and Montgomery, 1970). The SDS-gel electrophoresis technique is simple to perform. This method should prove useful in the study of both normal and abnormal fibrinogens and the products of their interactions with various enzymes.

Acknowledgements: Supported by a grant-in-aide from the New York Heart Association. Dr. Mills is a New York Heart Association Fellow.

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