On the Primary Structure of Human Fibrinogen

I. Two-dimensional "Finger Prints" of Tryptic Digests of Sulfitolyzed Fibrinogen and Fibrin

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Work in this laboratory has for some time concentrated on the elucidation of the chemical structure of fibrinogen. N-Terminal analyses have indicated that the molecule of 340 000 unit weight is built up by three pairs of peptide chains.[1] After sulfitolysis of the disulfide bonds [2] in bovine fibrinogen three chains (A, B, and C *) of fibrinogen have been isolated.[3,4] The molecular weight of these subunits as determined by means of ultracentrifugation is between 50 000 [2,5] and 65 000.[5,6] The data thus indicate that the formula of the molecule is $A_2B_2C_2$. This makes it likely that the molecular weight of 340 000 determined for fibrinogen is for a dimeric form of the molecule. The structure of human fibrinogen seems to be analogous to that of bovine fibrinogen. Thus the molecule is built up by three peptide chains [1,2,7] and the formula for the 340 000 unit weight seems also to be $A_2B_2C_2$. However, in this formula A is the sum of three A-chain analogues (A, AP and Y).[7] The reason for this microheterogeneity of the A-chains in human fibrinogen has not yet been elucidated.

In order to study the primary structure of fibrinogen a preliminary fragmentation of the molecule is necessary. The present report will deal with tryptic (EC 3.4.4.4) digestion of sulfitolyzed fibrinogen and fibrin and separation of the tryptic peptides by two-dimensional electrophoresis-chromatography. Similar studies on isolated chains are in progress and will be reported shortly. Sequence studies of the isolated tryptic peptides will also be dealt with in forthcoming publications.

* The nomenclature of the chains is based upon the following definition: A-chain of fibrinogen: Fibrinopeptide A split off from the N-terminal end. B-chain of fibrinogen: Fibrinopeptide B split off from the N-terminal end. C-chain of fibrinogen: Chain not attacked by thrombin. The N-terminal amino acid of this chain is usually tyrosine.

Human fibrinogen was prepared from pooled citrated plasma according to the glycine method.[8] The fibrinogen (fraction I−4) used in this study had a purity of 97− 100 %, as judged from the coagulability. Fibrin was obtained from the fibrinogen by clotting with bovine thrombin (EC 3.4.4.13) (200 NIH-units/mg) under the following conditions: 0.5 % protein in 0.15 M sodium chloride or 0.15 M ammonium acetate, pH adjusted to 7, 2 NIH-units of thrombin/ml, room-temperature, 6−9 h. The clot was collected on a Büchner funnel, triturated and washed several times with water.

Sulfitolysis (*cf.* Ref. 2) of the protein was done in the following way: To a solution of 1 g of fibrinogen or fibrin in 75 ml of 9 M urea was added under vigorous stirring first 14 ml of freshly prepared, saturated sodium sulfite and then in portions 5 ml of 1 M cupric sulfate. The pH was never allowed to rise above 9.0 or fall below 8.0; it was adjusted with 2 N hydrochloric acid or concentrated ammonia solution. The final pH was kept at 9.0. The reaction mixture was left at room temperature for 2 h. The sulfitolyzed protein was separated from the reagents by means of dialysis in the cold for 12 h against each of the following solutions: (a) 0.05 N hydrochloric acid brought to pH 9.0 with ammonia, (b) and (c) 0.01 N hydrochloric acid, 0.01 M versene brought to pH 8.5 with ammonia, (d) and (e) 0.005 N hydrochloric acid, 0.005 M versene brought to pH 8.5 with ammonia, (f) and (g) water brought to pH 8.5 with ammonia. After dialysis the clear solution was freeze-dried or pervaporated to a protein concentration of between 1.0 and 1.7 %.

For the digestions a 0.5 % solution of trypsin (3 × recrystallized preparation, Lot 6110 of Worthington Biochemical Corporation) in 0.005 N hydrochloric acid was used. The substrate solution was made by dissolving the freeze-dried, sulfitolyzed fibrinogen and fibrin in 8 M urea and thereafter diluting to 2 M urea; the pH was adjusted to 8.5 with ammonia solution. Alternatively, the pervaporated protein solution was used as substrate and the digestions were performed in the absence of urea. The conditions in some digestions are summarized in Table 1. The incubation mixture (volume 5−10 ml) was titrated automatically in the pH-stat (Radiometer, type TTT 1 c) with 0.2 N sodium hydroxide at pH 8.5, 37°, in an atmosphere of air.

Table 1. Tryptic digestions of sulfitolyzed human fibrinogen and fibrin at different conditions.

Material	Ratio substrate to enzyme (w/w)	Substrate concentration (%)*	Incubation time (min)	Alkali consumption**
Sulfitolyzed fibrinogen	60:1	0.71 (2 M urea)	128	98
	100:1	0.87 (2 M urea)	216	85
	100:1	1.16 (water)	184	103
	140:1	1.40 (water)	216	104
	150:1	1.21 (water)	296	128
Sulfitolyzed fibrin	86:1	1.15 (2 M urea)	328	86
	100:1	0.99 (water)	178	104
	170:1	1.67 (water)	104	110
	170:1	1.67 (water)	256	126

* Substrate concentration determination was based on the extinction coefficient ($E_{1\ \mathrm{cm}}^{1\ \%}$) at 282 m$\mu$: 16.51 and 16.84 for bovine fibrinogen and fibrin, respectively (13).

** Equivalents NaOH/unit weight fibrinogen. The unit weight was assumed to be 170 000. Figures uncorrected for ionization of amino groups.

In both types of digestions the alkali consumption was almost complete after 2 h incubation, thereafter only a slow increase was observed over a period of 5 h. Further addition of enzyme did not increase alkali consumption at digestions on pervaporated solutions. However, when the digestions were performed in the presence of urea a slight increase occurred on a second addition of enzyme. The average numbers of bonds split as determined in the pH-stat, are seen in the last column of Table 1. The highest average number, 128, is about 80 % of the sum of arginine and lysine residues in human fibrinogen.[9] The split number of the sulfitolyzed protein is in reasonable agreement with that reported for native bovine fibrinogen by Mihalyi and Godfrey.[10]

When the pervaporated solution was digested (in the absence of urea), a fine gelatinous precipitate appeared. It was removed by centrifugation and washed with water and freeze-dried. This precipitate was obtained both from sulfitolyzed fibrinogen and fibrin. It constituted between 9 and 11 % (w/w) of the substrate. It was not further investigated at this time. The clear supernatant (P-1) was freeze-dried and constituted about 90 % (w/w) of the starting material.

A precipitate also appeared when digestions were performed in 2 M urea but in rather small amounts. The total digestion mixture was freeze-dried, dissolved in 5 % acetic acid and subjected to gel filtration on Sephadex G-10, equilibrated with 5 % acetic acid. Two peptide peaks were obtained, one coming through at about the void volume (U-1) and the second just in front of urea. The second peak was after freeze-drying found to contain the insoluble material (about 8 %) which has not yet been analyzed.

The freeze-dried material of the first peak (U-1) from the Sephadex G-10 column or the freeze-dried supernatant of the digest in the absence of urea (P-1) were subjected to two-dimensional paper electrophoresis-chromatography. 20 μl of freeze-dried digest dissolved in water (100 mg/ml) were applied to a sheet (35 × 48 cm) of filter paper (Munktell No. 302). High voltage electrophoresis was run in pyridine-glacial acetic acid-water, pH 5.5 (100:35:4865, v/v), at 2500 V, 50 mA, for 3 h with a tap-water cooled, horizontal apparatus (AB Analysteknik, Vallentuna, Sweden). Before electrophoresis excess buffer was removed by blotting with filter paper. After electrophoresis the paper was dried at room-temperature for 3 h and subsequently descending chromatography in butanol-pyridine-glacial acetic acid-water[11] (150:100:30:120, v/v) was carried out in a Shandon 500 chromatank at 22° for 21 h. The papers were then dried at room-temperature for 2 h, dipped into 0.3 % buffered ninhydrin-acetone[12] and left to develop for 24 h at room-temperature. Additional heating at 80° for 20 min did not further increase the number of spots.

Typical "finger prints" are shown in Fig. 1. The spots have been arbitrarily numbered from the cathodal part of the finger print. Fiftythree of the spots have

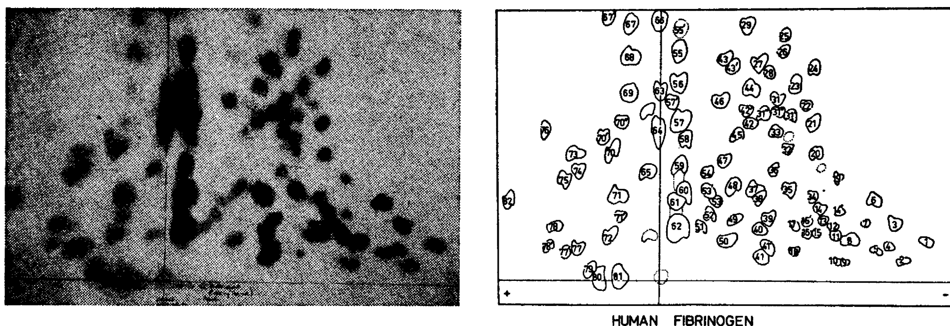

Fig. 1 a. Finger print of human fibrinogen. Left: Original. Right: Tracing with numbers. Spot No. 30 not recorded in this experiment.

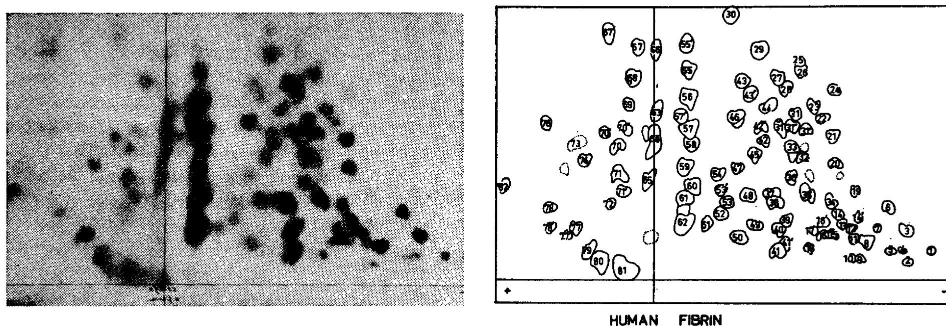

Fig. 1 b. Finger print of human fibrin. Left: Original. Right: Tracing with numbers.

excellent reproducibility. For 37 additional spots the chromatogram has to be run in triplicate in order to permit a reliable interpretation. Nine mainly faint spots, corresponding to acidic peptides, have not a satisfactory reproducibility. In all 90–100 spots can be detected. This agrees favourably with the average number of bonds split as determined in the pH-stat titration. The "finger print" of pooled fibrin was almost identical with that of fibrinogen. However, in fibrin one of the spots in the area Nos. 73–75 is missing, most probably due to the absence of fibrinopeptides A and/or Y, as these peptides are localized in this part of the chromatogram.

Further investigations on "finger prints" from normal individuals as well as different pathological states are in progress.

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