POLYPEPTIDE CHAIN INVOLVED IN THE CROSS-LINKING OF STABILIZED BOVINE FIBRIN

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Summary: The γ-chain peptide among the three chains of the fibrin molecule participates to the formation of cross-linkages which stabilize the fibrin clot through the action of activated FSF. The cross-linkage in stabilized fibrin may be formed with one or two pairs of γ-chain peptides, because the molecular weight of "X" isolated from S-sulfo-stabilized fibrin seems to be approximately 150,000 to 200,000, judging from the elution volume on a Sepharose 6B column.

Fibrinogen consists of three pairs of polypeptide chains (Blombäck and Yamashina, 1958), α(A), β(B) and γ. In the conversion of fibrinogen to fibrin by thrombin, fibrinopeptides A and B are released from the N-terminal parts of the parent molecule. The resulting fibrin is subsequently transformed to stabilized fibrin in the presence of activated FSF[1] and calcium ions. The enzymatic nature of the stabilization of the fibrin clot has recently been established (cf. Loewy et al., 1961; Lorand and Konishi, 1964). One difference between fibrin and stabilized fibrin is found in their solubilities in 5 M urea or 1 % monochloroacetic acid. The latter is insoluble whereas the former is soluble in these solvents. Recent studies on these protein molecules have revealed that insoluble stabilized

[1] FSF is the abbreviation of Fibrin Stabilizing Factor. The enzyme is also called as fibrinase, plasma transglutaminase and Factor XIII.

fibrin but not soluble fibrin contains a cross-linkage with an isopeptide bond, which is formed from the γ-amido group of glutamine and the ε-amino group of lysine residues (Lorand et al., 1968; Matatić and Loewy, 1968; Pisano et al., 1968, 1969). The number of ε-(γ-glutamyl) lysine isopeptide bond in the stabilized fibrin has been determined to be 1.2-2.8 moles per molecule (mol. wt., 330,000).

There is, however, no information regarding which chains of the three polypeptide sequences, α, β and γ, in the fibrin molecule participate in formation of the cross-linking. This problem is crucial to an understanding of fibrin stabilization on a molecular level. In present paper, we will show some evidences that the γ-polypeptide chain of fibrin participates in the cross-linkages of stabilized fibrin.

MATERIALS AND METHODS

Bovine fibrinogen (Armour Pharmaceutical Co., Lot, M9M8363) was purified by Laki's method (Laki, 1951) and it was subsequently converted to fibrin by the method of Donnely et al. (1953). Bovine FSF was prepared according to the method of Loewy et al. (1961) and purified further by column chromatography on DEAE-cellulose (Lorand et al., 1968). Sepharose 6B was purchased from Pharmacia, Co., Uppsala, Sweden. Urea was recrystallized with aqueous 70 % ethanol.

The preparation of stabilized fibrin was as follows: 10 ml of 1.5 % fibrin solution, 20 units of bovine thrombin (Park Davis, Co.), 1.5 ml of purified fibrinase (0.9 mg/ml), 10 ml of 0.05 M Tris-HCl buffer containing 0.1 M NaCl, pH 7.5, 22 mmoles of L-cysteine and 0.22 mmoles of $CaCl_2$, in a total volume of 42.5 ml, were incubated at room temperature. After incubation

for 2-3 hours, the mixture was added to an equal volume of 2 % monochloroacetic acid and suspension was stirred vigorously for 12 hours in a cold room. The stabilized fibrin thus produced was collected by centrifugation and washed two times with water. To cleave the disulfide bridges of the stabilized fibrin, it was first suspended in 5.0 M guanidine-HCl and then dissolved by increasing the pH of the suspension to 8.5 with 1.0 N NH_4OH and sulfitolized essentially according to the method of Henschen (1963). The S-sulfo-stabilized fibrin preparation was dialyzed against the buffers recommended by Pechère et al. (1958) and used in the present experiments. The S-sulfo-derivatives of bovine fibrinogen and fibrin were also prepared by the almost same procedures (cf. Iwanaga et al., 1969).

RESULTS AND DISCUSSIONS

Fig. 1 shows disc electrophoretic patterns of S-sulfo-derivatives of fibrinogen and fibrin performed under the optimal condition for resolution of α-, β- and γ-chain peptides (Takagi and Iwanaga, 1969). As seen in Fig. 1-1, each of three chains in S-sulfo-fibrin was resolved and no other strong protein band in the gel could be observed. However, the pattern of S-sulfo-stabilized fibrin (Fig. 1-2) was different to that of S-sulfo-fibrin. There was no protein band corresponding to the position of γ-chain peptide, whereas the bands of α- and β-chains were evident in the gel. Moreover, a new protein band designated as "X" was found in the gel. This band, "X", could not be detected in S-sulfo-fibrinogen as shown in Fig. 1-3. These results suggest that the γ-chain peptide of fibrin must be involved in the formation of cross-linkages which stabilize the fibrin clot. The electrophoretic behaviour of "X" located in the gel were examined

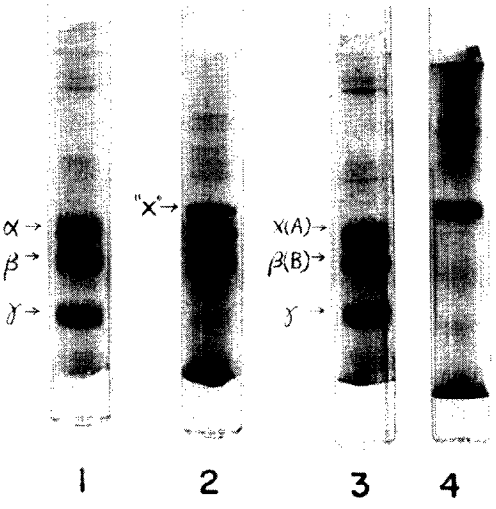

Fig. 1. Electrophoretic patterns of S-sulfo derivatives of bovine fibrinogen, fibrin and stabilized fibrin. Polyacrylamide gel electrophoresis was performed according to the method of Davis (1964), using 5.0 % gel and Tris-glycine buffer, pH 8.3, containing 8 M urea. The sample was dissolved in 0.1 M glycine-NaOH buffer, pH 9.5, containing 10 M urea and 10 mM EDTA (Takagi and Iwanaga, 1969). After the run, the gels were stained with Coomassie brilliant blue R 250. Migration is from top (cathode) to bottom (anode). 1 S-sulfo-fibrin. 2 S-sulfo-stabilized fibrin. 3 S-sulfo-fibrinogen. 4 "X" isolated from S-sulfo-stabilized fibrin.

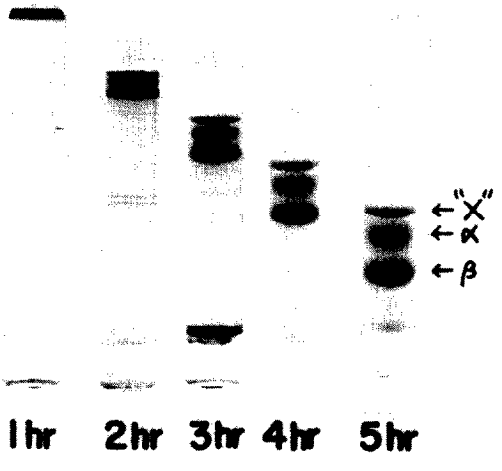

Fig. 2. Electrophoretic behaviours of S-sulfo-stabilized fibrin in polyacrylamide gel. The conditions used were identical to those of Fig. 1. The time period for each run is indicated.

(Fig. 2). Even in short period of electrophoresis, a narrow protein band ("X") was observed to separate from α- and β-chain peptides and the band behaved as a single homogeneous component during further electrophoresis in this system.

To isolate "X" from S-sulfo-stabilized fibrin, gel-filtration on a Sepharose 6B column was performed. In this experiment, gel-filtrations of S-sulfo-fibrin and native fibrinogen in addition to S-sulfo-stabilized fibrin were also attempted in order to get information on the molecular size of "X". Using 8 M urea containing 0.005 M acetate buffer, pH 5.5, as the eluate, fibrinogen with molecular weight of about 330,000 was eluted in the earliest fractions corresponding to tube numbers 18-22 (Fig. 3). On the other hand, S-sulfo-fibrin with approximate molecular weight of 56,000 (Henschen, 1963; Johnson and Mihalyi, 1965; Mckee et al., 1966) appeared in the later fractions (tube numbers 23-29) as a single symmetrical peak like that given by fibrinogen. However, the elution pattern of S-sulfo-stabilized fibrin was evidently different from those of fibrinogen and S-sulfo-fibrin, showing two main peaks. The elution volumes of the second peak corresponding to tube numbers 23-29 were identical to that of S-sulfo-fibrin, suggesting similarity of the molecular weights of the two proteins. On the other hand, the elution volume of the first peak at tube number 22 was between the elution volumes of the fibrinogen and S-sulfo-fibrin. Using the fractions of the first and second peaks separated from S-sulfo-stabilized fibrin, their gel electrophoretic patterns were examined. As shown in Fig. 1-4, the first peak showed a single protein band and the mobility in the gel was identical to that of "X" resolved from whole S-sulfo-stabilized fibrin. The second peak showed two bands and each of them moved, respectively, to the positions

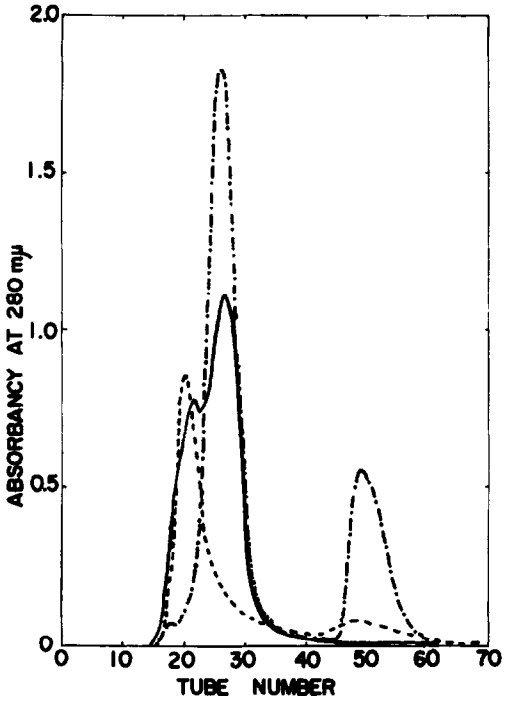

Fig. 3. Gel filtrations of fibrinogen, S-sulfo-fibrin and S-sulfo-stabilized fibrin on Sepharose 6B column. Each of three materials (50 to 70 mg protein) was applied to a column (3.5 x 45 cm) of Sepharose 6B, equilibrated with 0.005 M acetate buffer, pH 5.5, containing 8 M urea. Elution was performed with the equilibration buffer at a flow rate of about 10 ml per hour, and 5 ml fractions were collected at room temperature. Absorbancy at 280 mμ was measured with a Hitachi model 124 spectrophotometer. Fibrinogen, ——; S-sulfo-fibrin, -·-·-·-; S-sulfo-stabilized fibrin, ———.

corresponding to the α- and β-chain peptides, and no γ-chain peptide was detectable. These results indicate that "X" must contain γ-chain peptide as the cross-linked part of the stabilized fibrin. Although the characterization of "X" isolated from Sepharose 6B column is now under investigation, preliminary results on the N-terminal amino acid (PTC-methos) and the amino acid composition are identical to those obtained with γ-chain peptide isolated from S-sulfo-fibrin (Henschen,

1963). The two differ of course in molecular weight.

From the above results the following conclusion can be drawn: (1) The γ-chain peptide among the three chains of the fibrin molecule participates to the formation of cross-linkages which stabilize the fibrin clot through the action of activated FSF. (2) The cross-linkage in stabilized fibrin may be formed with one or two pairs of γ-chain peptides, because the molecular weight of "X" isolated from S-sulfo-stabilized fibrin seems to be approximately 150,000 to 200,000, judging from the elution volume on a Sepharose 6B column.

During the course of present work, Chen and Doolittle (1969) have reported that the stabilization of fibrin clot by activated FSF involves two different sets of cross-linked chains, one of which is γ-γ cross-linking and the other is α-γ cross-linking. The existence of the former type of cross-linking has been found independently by the present authors as mentioned above. So far, however, we have found no evidence to indicate the existence of the latter type of cross-linking in stabilized fibrin. Further studies on the chemical and physical characterization of the cross-linked chain involved in the stabilized fibrin will be required.

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