THE TRANSPEPTIDASE SYSTEM WHICH CROSSLINKS FIBRIN BY γ-GLUTAMYL-ε-LYSINE BONDS.

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Fibrin stabilizing factor[1] (FSF) is the precursor of a transpeptidase (FSF*) which catalyzes the reaction of amines with certain γ-glutamyl bonds in fibrin (Lorand and Ong, 1966 a and b; Lorand et al., 1966; Lorand et al., 1968). If the attacking nucleophile is that of another fibrin molecule, crosslinking of the fibrin gel ensues so that it becomes insoluble in 1% monochloroacetic acid, resembling the natural clot (Lorand, 1950).

For purposes of purifying FSF, crude concentrates may be obtained by a variety of methods, utilizing precipitation by ether, alcohol or ammonium sulfate; an isoelectric step of about pH 5.4 is a common feature of all procedures (Lorand, 1954; Lorand and Jacobsen, 1958). Heating at 56° for 3 min differentially removes fibrinogen (Loewy et al., 1957), though the possible effect of heating on the FSF molecule itself is an open question. Further purification is achieved by chromatography on diethylaminoethyl (DEAE) cellulose either by gradient (Lorand, 1961) or by step-wise elution (Loewy et al., 1961). We now find a linear NaCl gradient to 0.2M (in 0.05M Tris-HCl buffer, pH 7.5) most satisfactory. Though the pattern and size of inactive peaks varies somewhat from preparation to preparation, elution of the FSF activity itself at around 0.05M NaCl seems to be very reproducible.

Occasionally the chromatographic fractionation by itself yields a product of high purity as indicated, for example, by zonal centrifugation (Fig. 1).

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[1] In relation to the scheme of blood clotting, a committee recommended the name "factor XIII" for this precursor "component present in circulating blood or plasma" (Thromb. Diath. Haemorrh., Suppl. 13, p. 428, 1963).

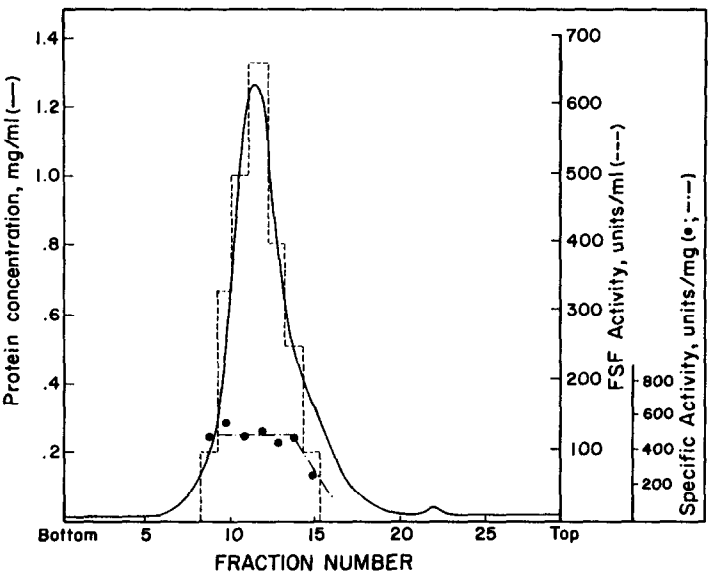
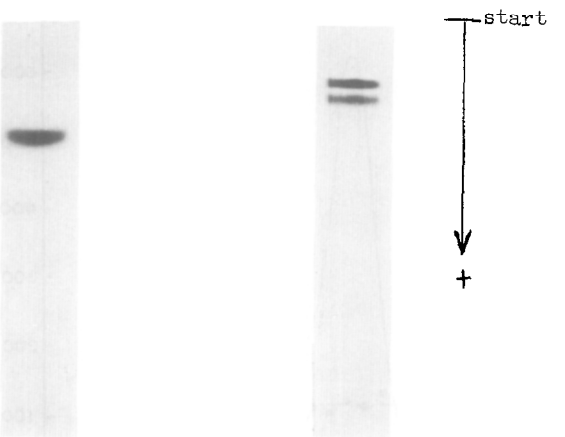


Fig. 1. Centrifugation of chromatographed FSF in a linear sucrose-density gradient (to 40% $^{w}/_{v}$). Spinco SW-65 rotor; 44,000 rpm x 18.5 hr. FSF was measured, after activation with thrombin, on fibrin substrate (see Lorand, 1964).

With muscle lactic dehydrogenase (Sigma) and catalase (Mann) as markers, FSF sediments between these two proteins. Activity of lactic dehydrogenase was maximal in fraction 15, that of FSF in 12 and catalase in 9. If the relationship given by Martin and Ames (1961) may be applied, a molecular weight of about 156,000 - 195,000 is obtained for FSF. This, of course, would imply the bold assumption that the frictional coefficient and partial specific volume of FSF are similar to those of the markers.

The purity of the active FSF fraction obtained by zonal centrifugation may be further investigated by disc-gel electrophoresis. In Fig. 2a, the FSF peak shown in Fig. 1 was analyzed. This protein appeared to be quite homogeneous.

In many instances, however, the product of DEAE-cellulose chromatography gives a more complex pattern in zonal centrifugation than that shown in Fig. 1. Several inactive peaks are present, varying in size and position



2a
5 mA
6.7 μg FSF

2b
3.5 mA
7.6 μg (FSF-FSF*)

Fig. 2. Disc-gel electrophoresis (Canalco) of FSF samples purified by DEAE-cellulose chromatography and zonal centrifugation. For explanation see text. Canalco Tris-glycine buffer of pH 8.6; 2 hr; 1% amidoblack stain.

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from one preparation to another. Contaminating protein peaks are most often seen to center around fractions 7, 15, and 22. More significantly, however, under such circumstances the FSF fraction (fraction 12) itself contains appreciable proportions (up to 60%) of the activated FSF* species.

Since the FSF and FSF* activities did not separate in zonal centrifugation, the sedimentation coefficients of the two proteins must be quite similar, suggesting only a relatively minor alteration of the zymogen during conversion to the active transpeptidase. When such a spontaneously occurring mixture (about 1:1) of FSF and FSF*, isolated by zonal centrifugation, is examined by disc-gel electrophoresis (Fig. 2b) two major components appear, FSF* being the slower component. The electrophoretic separation is the first physico-chemical evidence to show that the precursor (FSF) is different from the thrombin-activated species (FSF*). In fact, electrophoresis provides the simplest tool for studying the FSF→FSF* transformation and also for separating these two

molecules with different isoelectric points on a preparative scale. Details on these aspects will be reported in due course.

Apart from thrombin, which is seemingly the natural activator of FSF in plasma (Lorand and Konishi, 1964; Konishi and Lorand, 1966), trypsin can also be used (Konishi and Takagi, 1967). It will be recalled that the kinetic specificities of thrombin and trypsin are very similar, indeed (Kézdy et al., 1965). As in the case with other zymogens (see e.g. Rimon et al., 1966), water insoluble enzymes (e.g. Bar-Eli and Katchalski, 1963) as activators are of particular advantage. Activation may be rapidly terminated by removal of the activator without the necessity of adding inhibitors. Fig. 3 shows that FSF can be activated by insoluble polytyrosyltrypsin (IPTT); copolymaleic acid ethylenetrypsin acted similarly. When trypsin derivatives are added in much

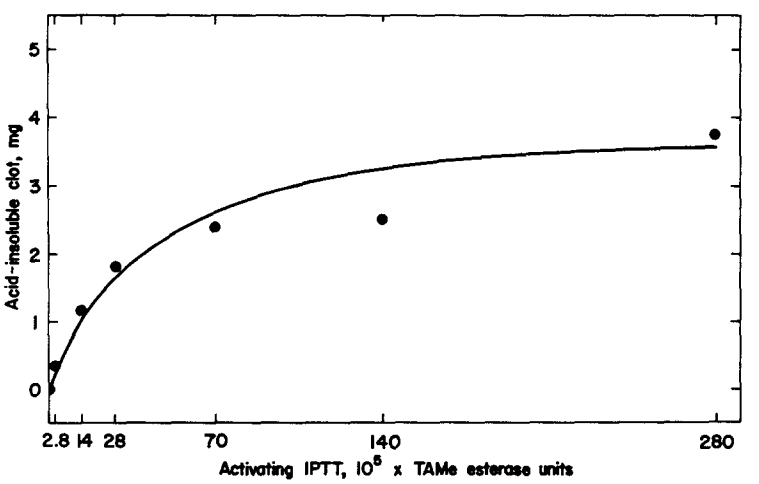


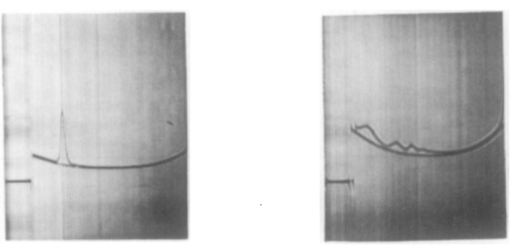
Fig. 3. Activation of FSF by a water-insoluble trypsin (IPTT). The trypsin activity is given in TAMe units, as μmoles of p-tosylarginine methyl-ester hydrolyzed per min. at 25° and pH 7.8; $(TAMe)_o$ = 0.04M. For methodology of FSF activation and measurement, see Lorand, (1964). Instead of thrombin, however, IPTT (as shown on the abscissa) was added to 8 units of FSF. The water-insoluble trypsin was removed by filtration (on Millipore, 0.22μ) after 10 min of activation. Then 4.5 mg of fibrin was admixed and allowed to crosslink for 30 min, at which time 1% monochloroacetic acid was added to test the solubility of the gel. Acid-insoluble clot residues are shown on the ordinate.

higher concentrations than shown on the abscissa, there is a lower yield of activity, possibly due to overdigestion.

It is now all the more clear that the FSF $\rightarrow$FSF* transformation belongs to the family of zymogen activations by limited proteolysis.

Considering the reaction of fibrin with extraneously added FSF*, the change in solubility characteristics in 1% monochloroacetic acid has always been interpreted to mean that covalent bonds between individual fibrin molecules formed. Nevertheless, definite physical proof for the existence of interfibrin linkages was still required. Therefore, ultracentrifuge experiments were carried out in a 2.5M guanidine-HCl solvent system in which fibrin gels, already insoluble in 1% monochloroacetic acid, could still be dissolved. Under such conditions, various clusters of crosslinked oligomers of fibrin can be clearly demonstrated (Fig. 4). This is the first physical evidence showing fibrin-to-fibrin linkages.

The nature and relative size of oligomeric crosslinked clusters appears to be a function of several factors. Those already investigated include the



54 min 58 min

Fig. 4. Schlieren-diagram of fibrin (left) and of crosslinked fibrin (right) in 2.5M guanidine-HCl, 25mM ethylenediaminetetraacetate-Na, 0.4M NaCl; pH 8; 20°; 59,780 rpm; 0.6% total protein in each.

concentration of FSF*, time of crosslinking, initial fibrin concentration and presence of competitive inhibitors of crosslinking. In addition to the crosslinked clusters, attention should also be drawn to the unexpected appearance of a slow peak of about 2S in the ultracentrifuge. This slow peak is appreciably larger in experiments where the initial reacting fibrin concentration is reduced ($<$1.5 mg per ml). Since this peak represents a boundary much less sharp than that of monomeric fibrin, there can be little doubt that the small sedimentation coefficient is due to a reduction in size rather than to an increase in asymmetry. The question thus arises whether the 2S species is a subunit of fibrin displaced in the course of transpeptidation or whether it reflects on the hydrolytic activity of FSF* itself. We are further investigating the properties of the 2S component and the significance of its production during the FSF*-fibrin reaction.

Turning to the chemical nature of the interfibrin crosslinks, it will be recalled that our earlier work already indicated the formation of γ-glutamyl-ε lysine bridges. The γ-glutamyl or acceptor group was delineated first (Lorand and Ong, 1966b) by carrying out a Lossen-rearrangement (according to Blumenfeld and Gallop, 1962) on the fibrin hydroxamate. The latter was produced by reacting fibrin, in the presence of FSF* and Ca^{2+}, with hydroxylamine which is a specific inhibitor of crosslinking (Lorand and Ong, 1966a). Participation of ε- amines of lysine as donor groups was deduced from results of two types of experiments. Lysine analogues with free ε-amino groups are the best inhibitors of crosslinking, competing against the natural donors on fibrin. Secondly, crosslinked fibrin, in contrast to fibrin which has not been reacted with FSF*, was shown to contain approximately 1 mole of lysine per 100,000 gm of protein which resists deamination (Lorand, 1965; Lorand et al., 1966; Lorand et al., 1968).

Using the Beckman Amino Acid Analyzer, we have now directly demonstrated the presence of γ-glutamyl-ε-lysine in enzymatic digests of crosslinked fibrin, whereas no significant amounts of this dipeptide were found in similarly

treated fibrin which was not crosslinked. On Custom AA-15 resin column (0.9 x 54 cm) with 0.2N citrate buffer of pH 3.31 (66 ml per hour; 56° throughout), the position of the reference γ-glutamyl-ε-lysine dipeptide (Cyclo) is (approximately at 283 min) located in the wide trough between the leucine (~228 min) and tyrosine (~313 min) peaks. Because of the wide separation of the latter two, the column could be loaded with more than 15 mg of protein digest so that the presence of as little as 0.05 μmoles of the γ-glutamyl-ε-lysine dipeptide could be conveniently demonstrated. Both fibrin and crosslinked fibrin (~50 mg) were sequentially digested (37°) with pronase (3 x 1 mg; Calbiochem), Mn^{2+}-activated leucine aminopeptidase (2 x 0.25 mg; Worthington) and carboxypeptidase A (1 x 0.5 mg; Worthington), with enzyme additions made at 24 hr intervals. This digestion procedure reduced the proteins virtually to amino acids, but left the γ-glutamyl-ε-lysine dipeptide in crosslinked fibrin intact.

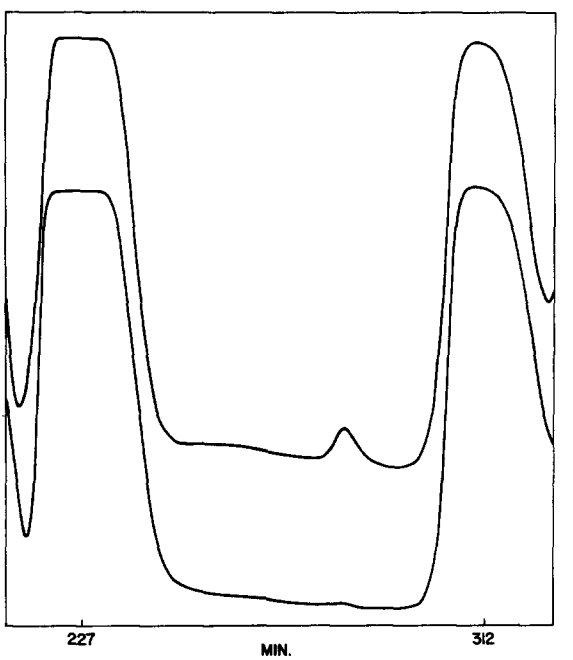


Fig. 5. Direct elution patterns of enzyme digests (15 mg each) of crosslinked fibrin (upper line) and fibrin (lower line). For details, see text. The γ-glutamyl-ε-lysine dipeptide derived from crosslinked fibrin elutes at about 283 min (between leucine and tyrosine). Peak height is at 0.1 OD with base line at 0.05 OD (570 mμ).

A comparison of the enzyme digests of fibrin and crosslinked fibrin is given in Fig. 5. The crosslinked material was obtained by reacting fibrin (5 mg/ml) with 12 units/ml of FSF* (pre-activated by thrombin), in the presence of 2mM $CaCl_2$ and 20mM cysteine at pH 7.5, 25° for 2 hours. A recovery of 1-1.4 mole of γ-glutamyl-ε-lysine per 330,000 gm of crosslinked fibrin could be calculated from the data in Fig. 5. Variations of crosslinking conditions in relation to the yield of γ-glutamyl-ε-lysine are being investigated.

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Note added

After submitting our manuscript (Feb. 14th) an article appeared in the latest issue of this journal (S. Matacic and A.G. Loewy, Biochem. Biophys. Res. Comm., 30, 356, 1968) which relates to the last paragraphs of our paper and describes another method for the identification of γ-glutamyl-ε-lysine. It should be emphasized, however, that unlike our work, which pertains to the direct crosslinking of isolated fibrin, the above report dealt with the formation of γ-glutamyl-ε-lysine in a system based on the simultaneous interaction of fibrinogen, Factor XIII and thrombin.