FIBRIN CLOT STABILIZING ENZYMES FROM GUINEA PIG LIVER

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The plasma enzyme (Laki-Lorand Factor, FSF, Factor XIII) which, in the presence of thrombin and calcium brings about the formation of an insoluble fibrin clot (Laki & Lorand, 1948; Loewy, et al., 1961), appears to be a transamidase. The enzyme is thought to catalyze a cross-linking reaction between the N-terminal glycine residues and the amide group of certain glutamine or asparagine residues, in adjacent chains of fibrin monomers; there is a concomitant release of ammonia and possibly also a decrease of carbohydrate (Loewy, et al., 1964; Chandrasekhar, et al., 1964; Lorand & Jacobsen, 1964; Loewy, et al., 1966).

Certain similarities between this plasma enzyme and the tissue transamidase described in a different context by Waelsch and co-workers (Sarkar, et al., 1957; Mycek, et al., 1959; and Clarke, et al., 1959) have been noted by Loewy, et al., (1964). The actual presence of fibrin clot stabilizing activity in some mammalian tissues, notably liver, has been demonstrated by Tyler & Lack, (1964), and Sayers, et al., (1965). Recently, a guinea pig liver transglutaminase has been purified and characterized (Folk & Cole, 1965, 1966[a & b]), and shown to connect together the proteins actin and tropomyosin (Derrick & Laki, 1966). It was therefore of interest to re-examine the clot stabilizing activity of tissue

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extracts to see if this correlated with transamidase activity.

This communication reports preliminary experiments which strongly suggest that highly purified transglutaminase from guinea pig liver is capable of stabilizing fibrin clot and also that the earlier reports of a "tissue FSF" are, in part, consistent with the presence of tissue transamidase activity.

Materials and Methods - Bovine fibrinogen (Armour) was first purified by the method of Laki (1951) and the product was further purified by a series of ammonium sulfate precipitations as described by Loewy, et al., (1961). The final product was free of detectable plasma FSF, as judged by solubility in 8M urea after clotting in the presence of 10mM $CaCl_2$ and 10mM cysteine. Stock fibrinogen solution in 0.3MKCl was stored frozen; before use, it was thawed and diluted to 5 mg/ml with borate-saline buffer, 0.2M, pH 7.8 (Palitzsch, 1915).

Clot stabilizing activity was assayed by serial 0.2 ml twofold dilutions of the test solution in borate-saline buffer. To the test solutions were then added fibrinogen solution (0.2 ml, 5 mg/ml) and $CaCl_2$ (0.1 ml, 0.05M); clotting was carried out by the addition of 0.02 ml of thrombin (2 N.I.H. units). After incubation for 15 min. at 37°, urea solution (1 ml, 8M) was added and the clot stabilizing activity assessed visually one hour later, in terms of the number of twofold dilutions of the test solution required to still achieve an insoluble clot. Control fibrin clots, which did not contain enzyme, dissolved in 8M urea within 20 min. Inhibition of clot stabilizing activity was studied briefly, using serial dilutions of putrescine or carbobenzoxy-L-glutaminylglycine solutions.

Highly purified guinea pig transglutaminase was kindly given by Dr. J. E. Folk (National Institute of Dental Research). This enzyme was assayed by a modified method (Laki, unpublished) based on that of Folk & Cole (1965), using CBZ-L-glutaminylglycine as substrate.

Subcellular fractions of guinea pig liver were prepared after homogenization of the liver in 10 vols. of 0.25 M sucrose. The fractions were prepared by the method of de Duve & Bethet (1954) and the nomenclature is that adopted by de Duve, et al., (1955) and Dingle (1961) for rat liver. Further experiments, involving chromatography of partially purified transglutaminase on DEAE-cellulose columns, were carried out according to the method of Folk & Cole (1966[a]).

Results and Discussion - By serial dilutions of a solution of highly purified transglutaminase, it was found that approximately 6μg of enzyme protein would stabilize a 1 mg fibrin clot. It was also found that a synthetic substrate for transglutaminase, CBZ-L-glutaminylglycine, would inhibit the stabilizing action of 6-8 μg of enzyme, at a concentration of 5mM. Putrescine (2.5mM), known to be incorporated into proteins by transglutaminase (Derrick & Laki, 1966), was also found to inhibit the clot stabilizing activity of transglutaminase. This is presumably due to competition with the N-terminal glycine groups in fibrin monomer (Lorand & Jacobsen, 1964). These results strongly suggest that highly purified transglutaminase is capable of introducing cross-linkages into fibrin, presumably at glutamine residues, so bringing about a fibrin insoluble in 8M urea. In this respect, it appears similar to the plasma enzyme (Loewy, et al., 1966).

Subcellular fractionation of guinea pig liver homogenate in 0.25M sucrose indicated that the clot stabilizing activity remains predominantly in the high speed supernatant, as has been reported for transamidase activity (Clarke, et al., 1959). Furthermore, on bringing the high speed supernatant to pH 5 and centrifuging the resultant precipitate, stabilizing activity was found to be associated with the precipitate and could be extracted in 0.05M phosphate buffer, pH 6.5, as is known for the transglutaminase (Clarke, et al., 1959; Folk & Cole, 1966). These findings are summarized in Fig. 1 and suggest an association

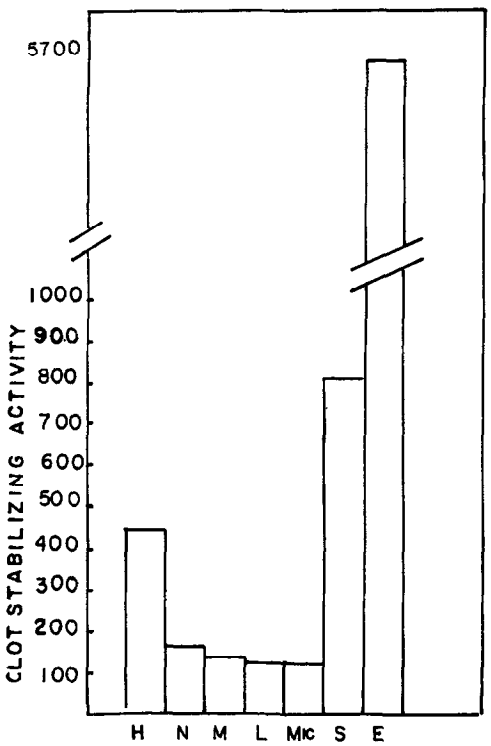

Fig. 1 The relative clot stabilizing activities of guinea pig liver fractions: Homogenate (H), nuclear (N), mitochondrial (M), lysosomal (L), microsomal (Mic.), supernatant (S), and a pH 6.5 extract (E) of the pH 5 precipitate. Activity is expressed in arbitrary units, based on the protein content and the minimal dilution of test solution required to stabilize a 1 mg fibrin clot in 15 min. at 37°.

between the clot stabilizing activity and transglutaminase. They also confirm earlier work with mouse liver (Tyler & Lack, 1964; Tyler, unpublished data).

Partially purified transglutaminase was prepared essentially according to Clarke, et al., (1959) and was further purified by chromatography on DEAE-cellulose as described by Folk & Cole (1966[a]). A typical chromatogram is shown in Fig. 2 and the results again indicate the identity between clot

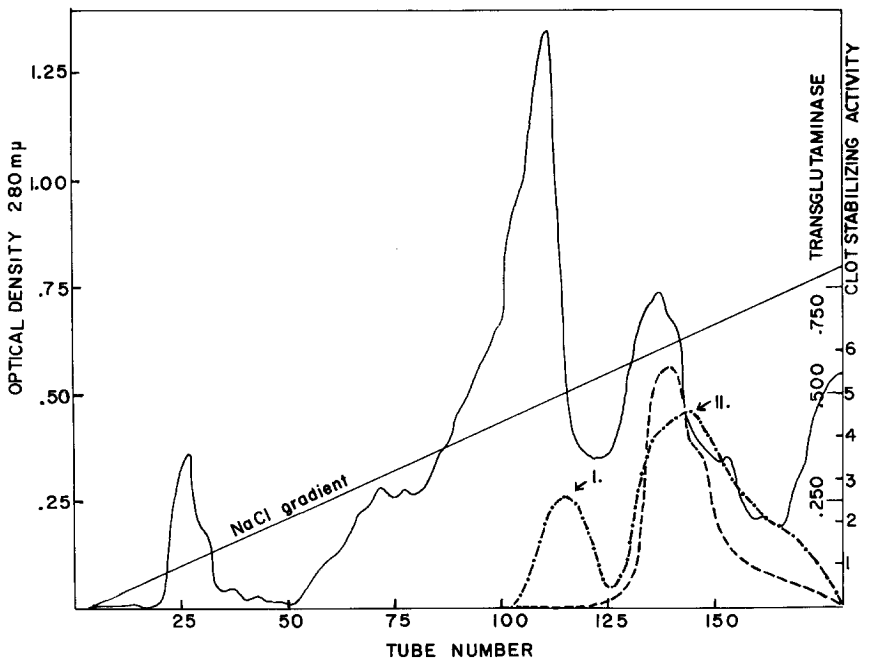

Fig. 2 Chromatography of partially purified transglutaminase on a DEAE-cellulose column (43 X 2.2 cm.): Elution was with 5mM tris-HCl buffer pH 7.5 containing 1mM EDTA, and with a linear NaCl gradient (0 to 0.8M). Absorbance of the fractions at 280mμ was measured in 1 cm. cuvettes. Clot stabilizing activity is shown as the number of twofold dilutions required to still achieve an insoluble 1 mg fibrin clot. Transglutaminase was determined by incubating 0.05 ml of test solution with tris-acetate buffer pH 6.0 (5μmoles), $CaCl_2$ (1μmole), glutathione (2.5μmoles), NH_2OH (25 μmoles) and CBZ-L-glutaminylglycine (20μmoles), in a final volume of 0.25 ml. After 15 min. at 25°C, the reaction was stopped by addition of 1.0 ml of the $FeCl_3$-trichloracetic acid -HCl reagent (Folk & Cole, 1965). Absorbancies were measured at 525mμ in 1 cm. cuvettes (Right ordinate).

_____ O.D. 280mμ;

____.____.____. Fibrin clot stabilizing activity;

____ ____ ____ ____ Transglutaminase (O.D. 525mμ).

stabilizing activity (Peak 2) and transglutaminase. In addition to the main peak of stabilizing activity, which closely corresponds to that of transglutaminase, an earlier and smaller peak (Peak 1) of stabilizing activity was consistently found which was not associated with transglutaminase. This secondary clot stabilizing activity is being further investigated. It is possible that a number of tissue transamidases exist, of which more than one can bring about cross-linking of fibrin monomers, but of which only one is specific for the synthetic CBZ-L-glutaminylglycine used in these experiments.* It is hoped that further study of different tissues will resolve this question and also show whether or not such enzymes play a wider role in the final stages of protein synthesis.

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* While this manuscript was in preparation, Lorand, et al., (1966) reported that a transglutaminase preparation made according to Waelsch & Mycek (1962) stabilized bovine fibrin clot and clotted lobster plasma. Not knowing that their crude preparation contained two kinds of stabilizing enzyme, only one of which can be identified as transglutaminase, these authors attributed clot stabilizing activity only to transglutaminase.

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