Purification and Properties of a Fibrin Cross-Linking Transamidase from Rabbit Liver*

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ABSTRACT: Rabbit liver has proved to be a rich source of fibrin cross-linking enzyme. Pooled rabbit livers, previously perfused until free of blood, were homogenized in 0.25 M sucrose and subjected to high-speed centrifugation. The fibrin cross-linking enzyme was purified from the high-speed supernatant by isoelectric precipitation and ion-exchange chromatography. Overall purification was 100-fold. The enzyme is Ca^{2+} dependent, heat labile, and is inhibited by *p*-mercuribenzoate, iodoacetamide, and some primary amines. The rabbit liver enzyme readily incorporates [^{14}C]-glycine ethyl ester into casein, thus resembling in this and other properties both the guinea pig liver transglutaminase and the plasma clot-stabilizing enzyme.

In recent years there has been put forward increasing evidence for the occurrence in mammalian tissues of enzymes capable of cross-linking (stabilizing) fibrin clot (Tyler and Lack, 1964; Tyler and Laki, 1966; Laki *et al.*, 1966). The similarities between these tissue enzymes and the well-known plasma enzyme (LL factor, FSF, factor XIII) which, in the presence of Ca^{2+} and thrombin brings about the formation of a urea-insoluble fibrin clot, have already been pointed out (Tyler and Laki, 1966).

The fibrin cross-links brought about by the plasma enzyme appear to be the result of a transamidase reaction (Loewy *et al.*, 1964, 1966) though the precise details of this reaction remain uncertain (Pisano *et al.*, 1966; Lorand *et al.*, 1966a; Doolittle and Fuller, 1967). On the other hand, the well-characterized transglutaminase from guinea pig liver (Waelsch and Mycek, 1962; Folk and Cole, 1966a,b) has been shown to cross-link fibrin (Tyler and Laki, 1966; Lorand *et al.*, 1966b), presumably at certain glutamine residues. A survey of a number of mammalian tissues carried out by the assay method described earlier (Tyler and Laki, 1966) indicated that this type of transglutaminase was not the only enzyme responsible for fibrin cross-linking activity (H. M. Tyler *et al.*, unpublished data). Rabbit liver has very little detectable transglutaminase, yet is a good source of cross-linking activity (Tyler, 1967). This tissue has therefore been chosen as the starting material for a more detailed study of this activity. The purification and some properties of a cross-linking enzyme from rabbit liver are here described.

Materials and Methods

Enzyme Preparation. An adult rabbit (New Zealand white) was given an intravenous injection of heparin (2000 units, 0.1 ml of solution) 5 min prior to being killed by decapitation and bled out. A midline incision was made to expose the liver which was perfused with ice-cold 0.25 M sucrose–5 mM EDTA solution (250 ml). This removed all traces of blood and the liver became pale and chilled. The liver was then removed, weighed, and placed in ice-cold sucrose–EDTA solution. The livers from three rabbits were treated in this way and were finally pooled, chopped, and homogenized batchwise using a Potter–Elvehjem homogenizer (Potter and Elvehjem, 1936) at 1500 rpm for 2 min. About 180 g wet wt of liver was homogenized in the sucrose–EDTA solution to give a final volume of 700 ml. The homogenate was subjected to high-speed centrifugation (30,000 rpm for 90 min in a Spinco preparative ultracentrifuge) and the resulting supernatant was brought to pH 5.0 with acetic acid. The pH 5.0 precipitate was collected by centrifugation and extracted with phosphate buffer (0.05 M, pH 6.5) as has been described for the experiments with guinea pig liver transglutaminase (Folk and Cole, 1966a,b; Tyler and Laki, 1966).

The extract (90 ml) was then chromatographed on a DEAE-cellulose (Carl Schleicher & Schuel Co., 0.87 mequiv/g) column (42 × 2.2 cm) using 5 mM Tris-HCl–2 mM EDTA solution (pH 7.5), as eluent, with a NaCl gradient (0–1.5 M). Active fractions were pooled and brought to 70% saturation with ammonium sulfate. The protein precipitate was collected by centrifugation, taken up in 20 ml of Tris-EDTA (as above), and dialyzed against this solution overnight. Rechromatography of this preparation on DEAE-cellulose was carried out under similar conditions to the above experiment, except for a less steep NaCl gradient. Active fractions were pooled and concentrated as before. The final product was dialyzed against

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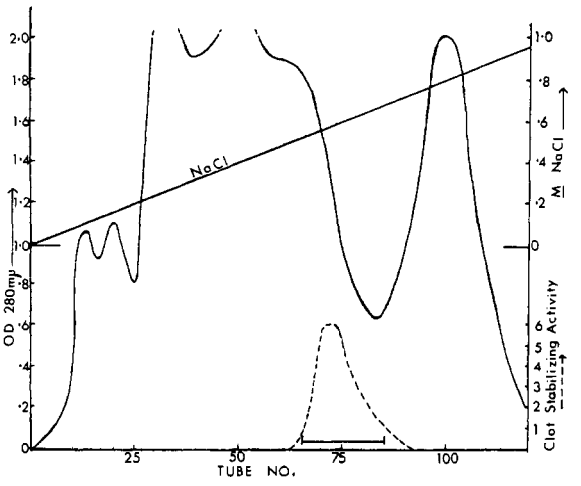

FIGURE 1: Chromatography of rabbit liver enzyme on a DEAE-cellulose column. The rabbit liver enzyme (90 ml) was pumped into a DEAE-cellulose column (43 × 2.2 cm). Elution was with 5 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and with a linear NaCl gradient (0–1.5 M). Fractions (3 ml) were collected and assayed for clot-stabilizing activity (- - - -). Absorbance of the fractions at 280 mμ was measured in 1-cm cuvets (———). The fractions were also assayed for transglutaminase and fractions 70–76 inclusive were found to contain a trace of this activity.

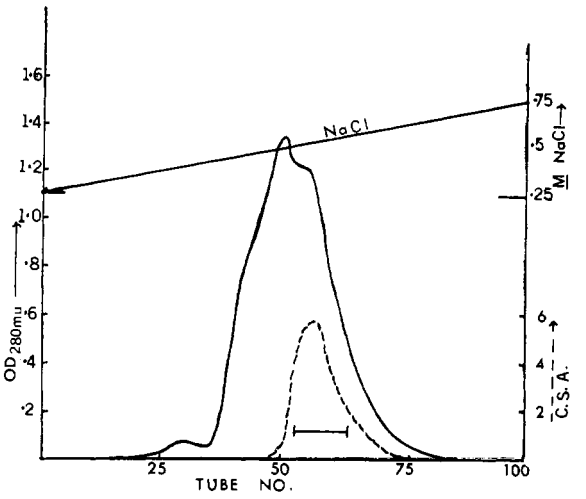

FIGURE 2: Rechromatography of partially purified rabbit liver enzyme on a DEAE-cellulose column. Partially purified rabbit liver enzyme (36 ml) was pumped into a DEAE-cellulose column (43 × 2.2 cm). The experimental procedure was the same as that described in Figure 1, except that the NaCl gradient was from 0.25 to 0.75 M. $OD_{280\,m\mu}$ (———); clot-stabilizing activity (- - - -). Transglutaminase was detected in trace amounts in fractions 56–62 inclusive.

5 mM Tris–2 mM EDTA and stored frozen in 1-ml aliquots. Activity decreased to almost zero after storage at −10° for 10 weeks. Further purification details are in Figures 1 and 2. Over-all purification was approximately 100-fold; recoveries tended to be low (10–15%).

Assays. Fibrin cross-linking activity and transglutaminase were assayed as described previously (Tyler and Laki, 1966). In this paper, transglutaminase refers only to that activity which couples NH_2OH to CBZ-glutaminyl-glycine (Folk and Cole, 1965). Transamidase activity was assayed by the incorporation of [^{14}C]GEE[1] into casein. Labeled GEE (4.12 mc/mM) was diluted with "cold" GEE to give a stock solution containing 100 μc/200 μmoles per 1 ml of 0.1 M Tris-HCl buffer (pH 7.95). Casein (Hammarsten) was dissolved in 0.2 M Tris-HCl buffer (pH 7.95) and dialyzed against this buffer. The casein solution was adjusted to 3% with buffer and stored frozen until use. Assays were carried out routinely in the presence of 10 mM $CaCl_2$ and 5 mM GSH. Aliquots of the incubation mixture were pipetted into 5 ml of 5% TCA and the precipitate was centrifuged. Washing with TCA followed by centrifugation was repeated three times and the final precipitate was taken up in 1 ml of 0.1 N NaOH. Aliquots of this solution were then pipetted into counting vials containing 10 ml of scintillation fluid (Bray, 1960) and radioactivity was counted on a Packard Tri-Carb liquid scintillation spectrometer (Model 500A). Results are expressed as counts per minute and are adjusted for zero-time controls, enzyme blanks, and background. Counting efficiency of ^{14}C was about 75%.

Experimental and Results

The final product, if assayed immediately after the final preparative step, sometimes contained slight transglutaminase activity. This rapidly deteriorated on storage, however, and the following studies were carried out on material devoid of detectable transglutaminase.

Heat Stability. Separate samples of stock enzyme solution (1.5 mg/ml) were heated at 45 and 65° for 0–30 min. Aliquots (50 μl) were removed at intervals and assayed for stabilizing activity in the usual standard clot system. No stabilizing activity remained after prior heating of the enzyme for 5 min at 65°, while at 45° the activity was fully destroyed after 30 min (Figure 3).

Sulfhydryl Inhibitors. Freshly prepared aqueous solutions of iodoacetate, iodoacetamide, and PMB were diluted out in 0.2-ml volumes with borate–saline buffer (pH 7.6). Aliquots (50 μl) of enzyme solution were then added to each tube, followed by the remaining constituents of the standard fibrin clot system. Solubility of the clots in 8 M urea was assessed visually 1 hr later. The results are shown in Table I and indicate that the enzyme preparation is inhibited by low con-

[1] Abbreviations used: GEE, glycine ethyl ester; TCA, trichloroacetic acid; PMB, *p*-mercuribenzoate, GSH, glutathione; CBZ, carbobenzoxy.

TABLE I: Inhibition of Rabbit Liver Transamidase by SH-Blocking Reagents.[a]

Inhibitor	Final Molarity in Clotting Mixture					
	1×10^{-3}	5×10^{-4}	1×10^{-4}	5×10^{-5}	1×10^{-5}	Nil
Iodoacetate	−	−	±	+	+	+
Iodoacetamide	−	−	−	±	+	+
PMB	−	−	−	−	±	+

[a] Mixture: 50 μl of rabbit liver enzyme, 2 mg of fibrinogen, 10 mM $CaCl_2$, inhibitor, 0.2 M borate–saline buffer (pH 7.6), and 20 μl (2 NIH units) of thrombin, in a final volume of 0.5 ml. (−) and (+) denote solubility and insolubility, respectively, of the clots in 8 M urea.

centrations of the SH-blocking reagents.

Transamidase Activity. A preliminary experiment showed that the enzyme preparation readily incorporated labeled GEE into casein. This reaction has been used in preference to the fibrin clot stabilization assay for the remaining characterization studies. Figure 4 shows the relationship between GEE incorporation and time, using different concentrations of enzyme. The reaction is also seen to be inhibited in the presence of 10^{-3} M iodoacetamide or in the absence of Ca^{2+} ions, and no GEE incorporation takes place if heated (65° for 5 min) enzyme is used.

pH Optimum. Transamidase activity was determined in the pH range 6–9, using 0.05 M Tris-HCl mixtures. With GEE as the amine donor and casein as the acceptor protein, the pH optimum was found to be at pH 8–8.2 (Figure 5).

Preincubation with Thrombin. Because the plasma transamidase requires activation by thrombin (Buluk *et al.*, 1961; Lorand and Konishi, 1964), it was of interest to determine whether or not the enzyme from rabbit liver could also be influenced by thrombin. A fresh enzyme preparation (20 μl) was incubated at 37° for 15 min with and without approximately 100 NIH units of highly purified bovine thrombin. Transamidase was then assayed in the usual way at 0, 20,

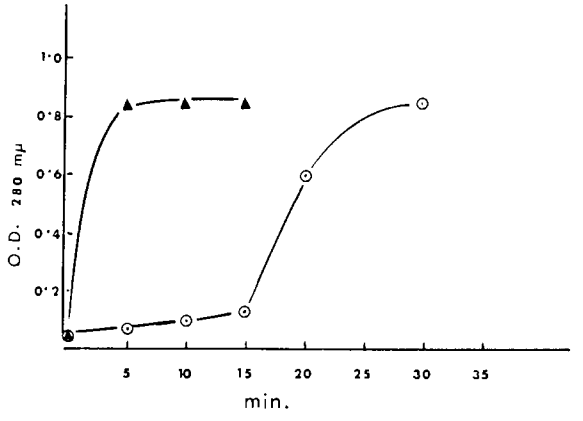

FIGURE 3: Heat stability of rabbit liver enzyme at 45 (⊙) and 65° (▲). Aliquots (50 μl) of heated enzyme were incubated in the standard 2-mg fibrin clot for 15 min at 37°. Urea (2 ml of 8 M) was then added and after 1 hr at room temperature the remaining fibrin clots were filtered through cotton plugs. The filtrates were read at 280 mμ against a reagent blank (no fibrin). The figure shows the increase of $OD_{280\ m\mu}$ (*i.e.*, decrease in fibrin stabilization) as a function of time of heating of the enzyme preparation.

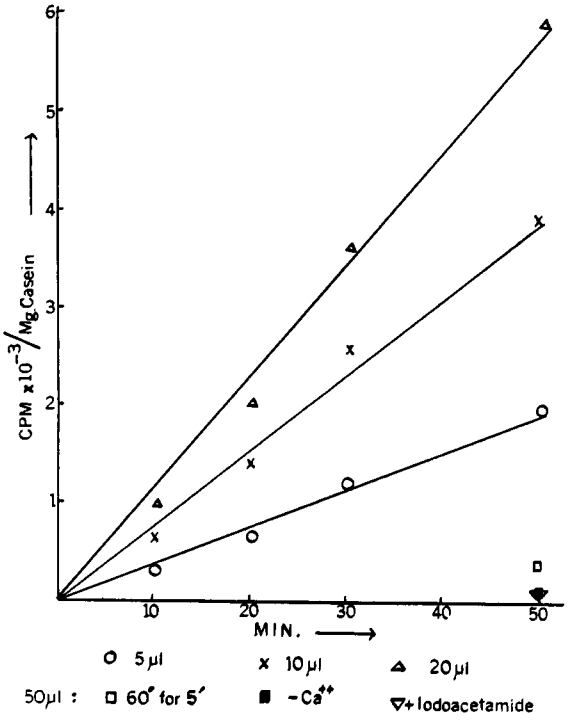

FIGURE 4: Incorporation of [^{14}C]GEE into casein by rabbit liver enzyme. The reaction mixtures contained 10 μmoles of $CaCl_2$ or 10 μmoles of EDTA, 5 μmoles of GSH or 1 μmole of iodoacetamide, 5 μc/10 μmoles of GEE, 3 mg of casein, and 5–20 μl of rabbit liver enzyme, made up to 1 ml with 0.2 M Tris-HCl (buffer pH 7.6). One reaction mixture had active enzyme substituted by 50 μl of rabbit liver enzyme preheated at 60° for 5 min. Aliquots were removed at various times and the amount of GEE incorporated was determined as described in the Methods section.

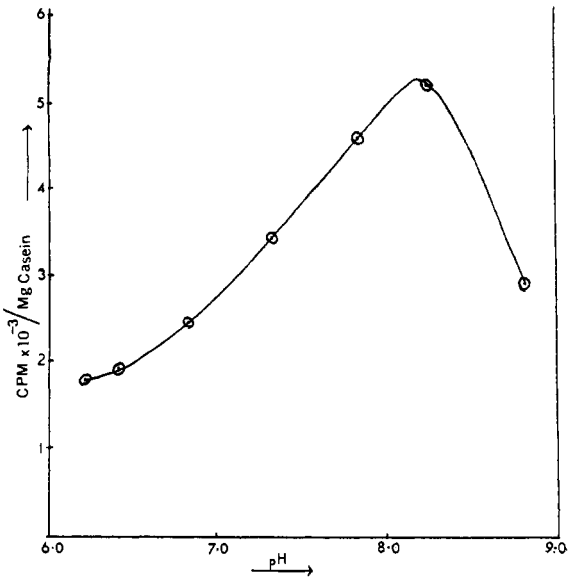

FIGURE 5: The pH optimum of rabbit liver enzyme. The reaction mixture contained 10 μmoles of $CaCl_2$, 5 μmoles of GSH, 5 μc/10 μmoles of GEE, 3 mg of casein, and 10 μl of rabbit liver enzyme, made up to 1 ml with 0.05M Tris-HCl mixtures in the pH range 5.6–9.2. The final pH of each mixture was checked prior to the addition of enzyme, and these final pH values are those shown in the figure. After 30-min incubation at 37° the incorporation of GEE was determined as before.

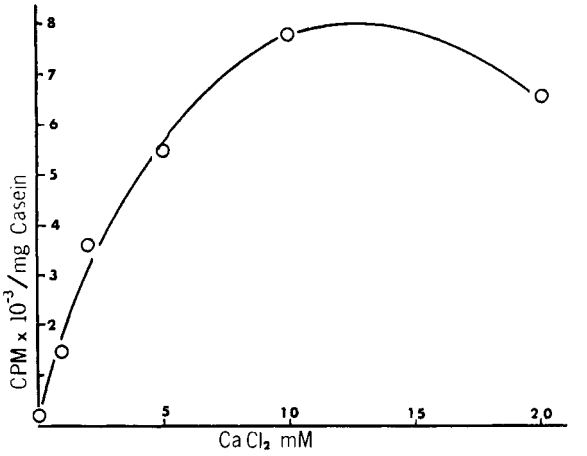

FIGURE 6: The effects of $CaCl_2$ concentration on rabbit liver enzyme activity. The reaction mixtures contained 0–20 μmoles of $CaCl_2$, 5 μmoles of GSH, 5 μc/10 μmoles of GEE, and 12 mg of casein, made up to 1 ml with 0.2 M Tris-HCl buffer (pH 7.95), the mixtures were incubated at 37°, and aliquots were removed at various times before determining the amounts of GEE incorporated. The curve shown is based on the figures obtained after 40-min incubation at 37°.

and 60 min. The counts obtained from the two series were within 2–6% of each other, indicating that preincubation with thrombin had no effect on the transamidase activity of the rabbit liver preparation.

$CaCl_2$ Concentration. Earlier experiments using both the fibrin-stabilizing test and the GEE incorporation system had shown that the enzyme required calcium for activity. An experiment was set up to determine the effect of varying the $CaCl_2$ concentration in the usual transamidase system. Final $CaCl_2$ concentrations in the assay system varied from 0 to 20 mM and the results show that with 20 μl of enzyme preparation and 12 mg of casein/1 ml of incubation mixture, maximum enzyme activity was reached at a $CaCl_2$ concentration of 10–15 mM (Figure 6).

Examination in the Analytical Ultracentrifuge. A 1% solution of purified enzyme preparation was made in 0.05 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, and this was dialyzed overnight at 4° against the same buffer solution. Some protein aggregation was visible after the dialysis and the enzyme solution was centrifuged to give an optically clear solution which was then examined in the analytical ultracentrifuge (Spinco, Model E) at 6.6°. The results (Figure 7) indicated the heterogeneity of the preparation. However, separate removal from the cell of the two main peaks at the end of the centrifugation, followed by subsequent transamidase assay, revealed that they possessed the same specific activity. Thus, it seems that the two main components (10.4 and 3.2 S) represent transamidase enzyme and one may be an aggregate. Visible aggregation had occurred after the dialysis at 4° and this might have been reduced had a higher salt concentration been used.

Discussion

Perfusion of the liver with a sucrose–EDTA solution was designed to minimize the possibility that the fibrin-stabilizing activity detected in liver extracts was derived from contaminating plasma or platelets (Kiesselbach and Wagner, 1966). In fact, we found that removal of blood did not significantly reduce the clot-stabilizing activity of crude liver preparations, so that the activity we measure can be considered due to a *bona fide* liver derivative.

The data indicate that the fibrin clot stabilizing activity is due to a transamidase. Thus, it is possibly similar to the plasma clot stabilizing enzyme which is known to be a transamidase. The present rabbit liver preparations would not couple hydroxylamine to CBZ-glutaminyl-glycine, however, thus suggesting different specificity from the guinea pig liver transglutaminase. It is interesting to note that the plasma transamidase is also unable to catalyze formation of the hydroxamate derivative of CBZ-glutaminyl glycine.

Our data indicate an important difference between the rabbit liver enzyme and the plasma transamidase. The latter enzyme, if prepared under conditions which would preclude it coming into contact with thrombin, can be shown to be a thrombin-dependent enzyme (Buluk *et al.*, 1961; Lorand and Konishi, 1964). The

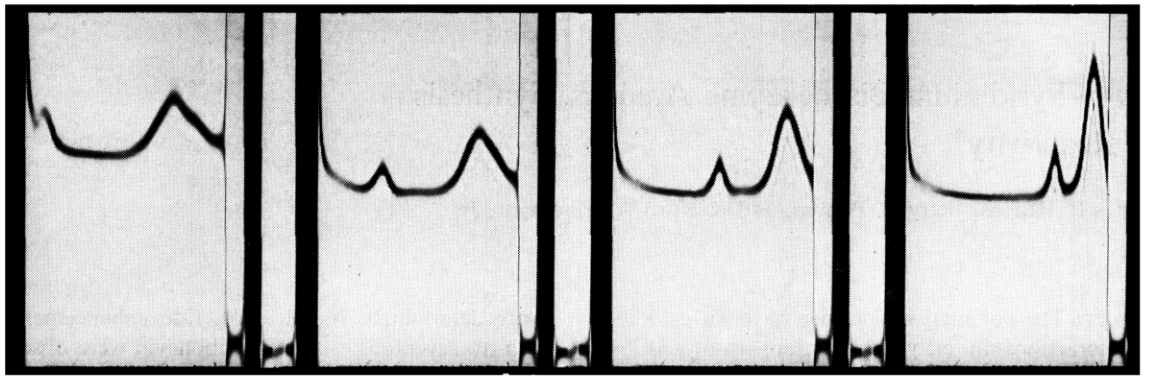

FIGURE 7: Ultracentrifugal analysis of purified rabbit liver enzyme. Schlieren diagrams of an ultracentrifuge run at 6.6° of purified rabbit liver enzyme. The protein content was 0.8% in 5 mM Tris-HCl buffer (pH 7.9) containing 2 mM EDTA. Photographs were taken at 19, 35, 51, and 67 min after reaching 60,000 rpm. Sedimentation proceeded from right to left.

rabbit liver enzyme, prepared free of blood and in the presence of EDTA, was shown to be independent of thrombin action and so must be judged different in this respect from plasma transamidase.

The liver enzyme does, however, share a number of properties with the plasma transamidase and the guinea pig liver transglutaminase. All three enzymes readily incorporate GEE into proteins such as casein, are dependent on Ca^{2+} ions for activity and are inhibited by SH-blocking agents. Our own studies on the varying $CaCl_2$ concentration served only to determine the maximum conditions for assay, the binding of calcium to casein making difficult any assessment of the role of this ion in the enzyme-activation process.

Though our purest preparation neither migrated as a single band on cellulose acetate electrophoresis at pH 8.6 (unpublished data obtained in conjunction with Dr. R. E. Alving) nor appeared homogeneous in the analytical ultracentrifuge, there was evidence to suggest the presence of enzymatically active aggregates. Nevertheless, at present, we cannot exclude the presence of inert proteins in our purest preparations. It is possible that the enzyme can be isolated in greater yield and purity by adopting some of the later techniques described by Folk and Cole (1966a,b) for the purification of guinea pig liver transglutaminase.

Our data show, however, that a transamidase from rabbit liver can be isolated which is similar to, but distinct from, plasma transamidase and guinea pig liver transglutaminase. Our preliminary experiments indicate that the same or similar enzymes exist in other tissues. Further work is required to establish its specificity with regard to the residues on the fibrin monomer which it cross-links, and also to determine its *in vivo* role.

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