Clotting of Bovine Fibrinogen by Liver Transamidase

By Judith Farrell and K. Laki

Casein, in conjunction with labeled glycine ethyl ester, proved to be a suitable material in the study of liver transglutaminase. The liver enzyme catalyzes the incorporation of the labeled amine into the glutamine residues of the casein and thus can easily be followed in a scintillation counter. Since transglutaminase catalyzes the cross-bonding of the fibrin clot, we explored the possibility of using fibrinogen instead of casein for the study of transglutaminase. In the course of these studies, we observed that after an initial incorporation of glycine ethyl ester into fibrinogen, it began to lose the labeled amine, and in the same time the reaction mixture developed turbidity, indicating that the enzyme catalyzed the cross-bonding of fibrinogen molecules.

The experiments in this paper show that indeed the liver transglutaminase connects fibrinogen molecules into an urea insoluble clot.

Materials and Methods

Bovine fibrinogen was prepared according to Laki from Cohn fraction I purchased from Armour Pharmaceutical Co., Chicago. A 2.85 per cent stock solution in 0.3 M KCl was kept in a freezer. No clot stabilizing activity was detected in this preparation without thrombin.

The highly purified guinea pig liver transglutaminase, kindly supplied by Dr. J. E. Folk (National Dental Institute) contained 2 mg. enzyme/ml. solution and was also kept frozen.

Labeled glycine ethyl ester (GEE) was purchased from New England Nuclear Corp., Boston. Stock solution was made up with unlabeled GEE to give a solution containing 7 μCi. and 1μM. per 10 μl. This stock solution was kept frozen. With each experiment, 5 μl. of this solution was also counted.

Parke-Davis thrombin topical containing 1000 units per ml. was used without further purification.

Results

Incorporation of GEE into Bovine Fibrinogen

The reaction mixture in three separate sets consisted of 0.05 ml. of 0.2 M CaCl₂, 0.05 ml. 0.04 M EDTA, 0.4 ml. 2.85 per cent fibrinogen, 0.05 ml. glycine ethyl ester, and 0.4 ml. 0.2 M Tris buffer. After the reaction mixtures were adjusted to pH 6.04, 7.06, and 8.00, 0.1 ml. transglutaminase was added. The reaction was incubated at 37°C. Aliquots of 0.1 ml. of each reaction mixture were removed at various times into centrifuge tubes containing 6.0 ml. 18 per cent trichloroacetic acid. The precipitates were placed in an ice bath to induce flocculation. These precipitates were then centrifuged for five minutes and


First submitted December 22, 1969; accepted for publication February 5, 1970.


804 Blood, Vol. 35, No. 6 (June) 1970
Fig. 1.—Time course of incorporation of GEE into fibrinogen at pH 6○, 7□ and 8△. 15,700 counts per minute represent four GEE residues incorporated into one mole of fibrinogen (MW:3.3×10⁶).

washed with 5 ml. of 7.5 per cent TCA. This washing procedure was performed three times; the precipitate was finally dissolved in 0.6 ml. 0.2 N NaOH. Mixed with 0.5 ml. of this solution was 10.0 ml. Bray’s scintillation fluid. Radioactivity was measured in a Nuclear Chicago liquid scintillation counter. The results are shown in Fig. 1.
The Clotting of Fibrinogen by Transglutaminase

A reaction mixture containing 10 ml. 2.85 per cent fibrinogen, 2.0 ml. 0.2 M Tris buffer (pH 7.0), 1.0 ml. 0.2 M CaCl₂, 1.0 ml. 0.04 M EDTA and 0.7 ml. transglutaminase was put on a 37°C water bath for 3½ hours, after which the thick clot that had formed was removed and put into 10 ml. 0.2 M NH₄HCO₃. The supernatant still contained 120 mg. of protein. Freezing and thawing yielded more clot. This process was repeated until no more clots were formed. Then the supernatant was tested for peptides by paper electrophoresis.

To prepare the supernatant for electrophoresis, an equal amount of 15 per cent TCA was added in the cold and allowed to stand in ice for 10 minutes before being centrifuged for five minutes at 1500 rpm. The precipitate was then discarded and the supernatants washed free of TCA with ice cold ether. These washings were repeated five more times, and the volume brought down to 2.0 ml. in a rotary evaporator. Nine ml. of acetone was added, and the solution allowed to flocculate. It was centrifuged for five minutes at 1500 rpm and the precipitate stored overnight in the freezer. The following morning the precipitate was dissolved in 1 ml. distilled H₂O and washed through a Sephadex G-25 column (150 ml. volume) with 150 ml. distilled H₂O. The fraction from 45-75 ml. was collected and evaporated to dryness in a rotary evaporator. The residue was transferred quantitatively in 3.0 ml. H₂O to a centrifuge tube and evaporated again to dryness. The residue was dissolved in 200 ml. distilled H₂O and applied to a paper for electrophoresis. A control of 10 μl. cofibrin was also applied as another spot. This paper was prepared for electrophoresis by wetting it with Tris buffer (pH 6.4) and put in the apparatus for two hours at 2500-V. The paper was dried and then put into an α-naphthol solution (50 mg./400 ml. absolute alcohol). The paper was again allowed to dry before spraying with the Sakaguchi reagent (0.10 ml. 1 per cent NaOH 0.75 ml. hypochlorous acid). No peptides were visible when compared with those of cofibrin released in normal clotting.

The clots meanwhile were suspended in 0.2 M NH₄HCO₃ and allowed to stand for three hours before 0.01 ml. thrombin was added. The mixture was then allowed to stand for four more hours at room temperature while a slight transparent clot formed. After standing overnight in a freezer and thawing in the morning, the clot was removed and the supernatant was examined for the presence of peptides as described above. By carrying out paper electrophoresis as developed in this laboratory for the identification of cofibrin, we could demonstrate the two components of cofibrin (fibrinopeptide A and B) in the supernatant.

The Inhibition of the Transamidase-induced Clotting by GEE

Two reaction mixtures having a pH of 7.06 were made up similar to that described above. A mixture without glycine ethyl ester served as control. Samples were taken from the reaction mixtures and the previous procedure followed. The remainders of the incubation mixtures were left standing at 37°C. Within two hours a firm clot was noted in the control mixture.
DISCUSSION

The experiments presented in this paper show that the highly purified transglutaminase from guinea pig liver clots bovine fibrinogen. It is very unlikely that the clotting is due to a thrombinlike contamination in the enzyme preparation, because no peptides are liberated in the clotting process. On the other hand, the fact that the transglutaminase-catalyzed incorporation of glycine ethyl ester into fibrinogen inhibits the clotting reaction strongly suggests that the clotting is brought about by the transglutaminase. We can surmise the nature of the bond formed between fibrinogen molecules from the known specificity of the enzyme. Transglutaminase catalyzes a reaction between glutamine residues and amino groups. Recent experiments of Bray and Laki suggest that in addition to glutamine residues the enzyme may utilize a carbohydrate chain bound asparagine as well.

Lorand et al. noticed that a liver extract clotted citrated lobster plasma, and attributed clotting to transglutaminase present in their crude liver preparation. The finding reported here makes it likely that in Lorand and coworkers experiments, it was the transglutaminase in the liver extract which clotted lobster plasma. The analogous behavior of lobster plasma and bovine fibrinogen has interesting implications for the evolution of the clotting systems. However, clear-cut evidence with purified preparations is needed before their analogy of the lobster plasma to bovine fibrinogen is established.

To close this discussion, we would like to point out that Alving and Laki observed that during pregnancy in rabbit uterus the activity of the clot stabilizing enzyme increased severalfold. In view of the possibility of clot formation by this enzyme, an investigation of the effect of birth control pills seems advisable.

SUMMARY

It was found that pure liver transamidase (transglutaminase) clotted bovine fibrinogen without prior release of cofibrin (fibrinopeptide) by cross-bonding fibrinogen molecules.

ACKNOWLEDGMENTS

We are indebted to Dr. A. Benkő for his advice during the course of these experiments and to Miss Sarah Holstein for her skillful assistance.

REFERENCES


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