THE ACTION OF THROMBIN ON SERUM ALBUMIN

G. Murray Thelin* and Robert H. Wagner+

Departments of Pathology and Biochemistry, University of North Carolina, Chapel Hill, N. C.

Received September 21, 1959

In 1951, Bailey et al published the first direct evidence that thrombin acts as a protease in the conversion of fibrinogen to fibrin. Bettleheim and Bailey (1952) showed subsequently that thrombin catalyzes the hydrolysis of two peptides from the fibrinogen molecule. In addition, thrombin has been reported to attack casein (Schultz and Schwick, 1951) and probably gelatin (Kay, 1951). Guest and Ware (1950) found that thrombin, at a concentration 10,000 times that needed for coagulation, would dissolve fibrin. The lysis of fibrin by thrombin has been confirmed by Seegers and coworkers (1958) who used thrombin with the highest activity yet obtained, 4100 units per mg. Bailey and Bettleheim (1955) reported no effect on rabbit myosin or ovalbumin. Sherry and Troll (1954) have shown that thrombin catalyzes the hydrolysis of tosylarginine methyl ester. It has also been reported to hydrolyze lysine ethyl ester slowly (Ehrenpreis et al, 1957). It appears that thrombin has a limited proteolytic activity with substrate requirements similar to trypsin but more specific. The present report showing a limited digestion of albumin by thrombin is part of a study to determine the possible usefulness of thrombin in studies of protein structure.

MATERIALS AND METHODS

MATERIALS - 1. Albumin was a crystallized bovine serum preparation from Pentex Inc, Kankakee, Ill. Only one component was detected with paper electro-

* Public Health Service Research Fellow of the National Heart Institute.

+ This investigation was supported in part by a Senior Research Fellowship (SF-302) from the Public Health Service and by a Grant, H-1648, from the National Heart Institute of the National Institutes of Health, Public Health Service (K. M. Brinkhous).

phoresis of a 2% or a 10% solution in pH 7.3 phosphate buffer with ionic strength of 0.05. No antithrombin activity could be demonstrated in this product. 2. Heated albumin was prepared by heating at a specified pH in a 65°C water bath for 12, 60, or 180 minutes. Investigation showed no increase of TCA-soluble N in heated albumin compared with non-heated albumin. Gorini and Audrain (1956) reported that essentially all S-S bonds are disrupted in 5 minutes at 60°C, pH 7.9. 3. Resin thrombin-Bovine Topical Thrombin (Parke-Davis) was purified on Amberlite IRC-50 columns using a modification of Rasmussen's (1955) technique. The thrombin was dissolved in 0.05 M phosphate buffer pH 6.0 and applied to a column equilibrated with the same buffer. The bulk of the protein was then eluted with this buffer. Phosphate buffers 0.3 M, pH 7.0 and 0.4 M, pH 8.0 were then used as eluting agents. The thrombin was eluted with the last buffer and dialyzed vs. 0.075 M NaCl to remove other salts. Activity was measured by the method of Seegers and Smith (1942). After dialysis the activity was 1500 units/ml and 500 units/mgm protein. 4. Citrate thrombin-a preparation of canine prothrombin was activated to thrombin with sodium citrate according to Seegers (1949). After activation the thrombin was dialyzed vs. 0.001 M NaCl in order to remove the citrate. The resulting preparation assayed 500 units thrombin/mgm protein.

METHODS - Action of thrombin on albumin. 1. The liberation of amino groups was followed electrotitrimetrically by maintaining the pH of the reaction mixture at 8.8 with additions of standard NaOH. The reaction mixture consisted of 200 mgm albumin and 10 mg (5000 units) of resin thrombin in 10 ml 0.075 M NaCl. Ten ml of reaction mixture were placed in a 20 ml beaker containing the electrodes of a Beckman Zeromatic pH meter. The delivery tip of a Gilmont microburet was introduced to deliver the increments of base. A few ml of mineral oil were layered on top of the solution in order to exclude atmospheric CO_2. The solution was stirred magnetically and the reaction allowed to proceed at 25°C. 2. As an alternative procedure the TCA-soluble N of a thrombin-albumin incubation mixture was analyzed. After the proper incubation time at 38°C, one half volume of 20% trichloroacetic acid (TCA) was added and after 30 minutes at room temperature the precipitate was removed by filtration. After thorough wet ashing, the total

nitrogen of the supernatant was estimated colorimetrically by nesslerization.

3. The digestion products were also studied with electrophoresis. Albumin, heated 12 minutes at 65°C, was incubated for 24 hours at 38°C with Topical Thrombin at a final concentration of 500 units per ml. The reaction mixture was then subjected to electrophoresis on Whatman #1 paper between silicone-treated glass plates (Kunkel, 1951). Glucose was used as a marker for electroosmosis. Phosphate buffer at pH 7.05 and 0.05 ionic strength was used.

RESULTS

The hydrolysis of heated and non-heated albumin by resin thrombin followed electrotitrimetrically is shown in Fig. 1. Control titration values for thrombin alone and heated albumin alone were very small and identical. These values probably represent slow absorption of atmospheric CO_2. During the 24 hour course of

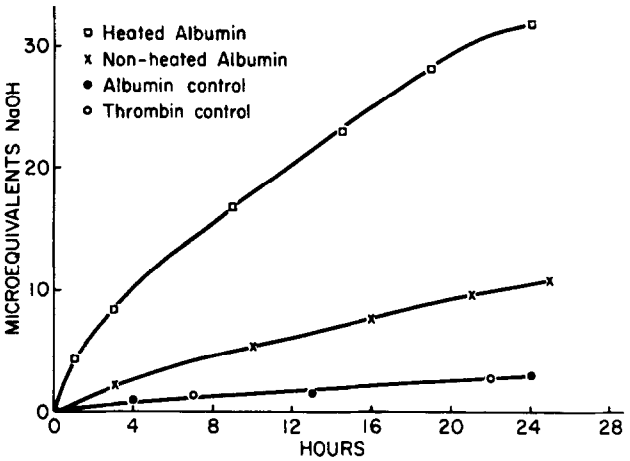

Figure 1. Proteolysis of albumin by a purified thrombin preparation. Reaction mixture consisted of 200 mgm albumin and 5,000 units of thrombin in 10 ml of 0.075 M NaCl. Heated albumin was prepared by heating for 180 min. at 65°C a 3% solution in 0.075 M NaCl after adjusting to pH 9.0 with NaOH. The controls contained the proper amount of either thrombin or heated albumin in 10 ml of 0.075 M NaCl. All experiments were done in duplicate.

the reaction the titration of the thrombin and heated albumin mixture required 28.9 μeq NaOH. Titration of the mixture of thrombin and non-heated albumin required only 7.8 μeq. For comparison heated and non-heated samples of albumin were digested for 24 hours with trypsin at a final concentration of 0.05 mg trypsin/ml. Other conditions were similar to those used in the thrombin experiments. Titra-

tion of the digestion mixture containing the heated albumin required 167 μeq of NaOH, while the trypsin digest of the non-heated albumin required 133 μeq. All the above figures were corrected for the controls.

It was assumed that the pK' of the liberated amino groups was 7.6. This is approximately the value used by Richards (1955) for ribonuclease. At pH 8.8, approximately 94% of the liberated amino groups would be titrated. A molecular weight of 65,000 was assumed for bovine albumin (Creeth, 1952, and Tanford et al, 1955). On the basis of these assumptions, it was calculated that the thrombin caused an average of 10.0 splits per molecule of heated albumin and 2.7 per molecule of non-heated albumin. The trypsin caused an average of 57.6 splits per molecule of heated albumin and 46.0 per molecule of non-heated albumin.

The appearance of TCA-soluble N in albumin-thrombin incubation mixtures is recorded in Table I. Twenty-four hour incubation of the non-heated and the two heated samples of albumin, in the absence of thrombin, produced a negligible increase in the TCA-soluble N.

TABLE I

The appearance of TCA-soluble albumin nitrogen in albumin-thrombin incubation mixture. The four ml reaction volume contained 32 to 39 mgm albumin, 2,000 units citrate thrombin, and pH 7.5 phosphate buffer. Final ionic strength 0.03, temperature 38°C.

Type of Albumin Used	TCA-Soluble N*			Albumin N in Reaction Mixture	Albumin N Appearing as TCA-Soluble N in 24 Hours
	Micrograms			Milligrams	Per cent
	0 hrs	24 hrs	Increase		
Non-heated	305	356	51	5.12	1.0
Heated+(12 min)	249	412	163	5.91	2.8
Heated+(60 min)	284	513	229	6.17	3.8

*Corrected for enzyme control.
+The albumin was heated (65°C) at a concentration of 2.7% in pH 7.23 phosphate buffer with ionic strength of 0.08.

Paper electrophoresis of an albumin-thrombin reaction mixture showed a new large diffuse spot migrating towards the cathode. Also, a poorly resolved family of peptides appeared between albumin and the origin.

DISCUSSION

Pre-treatment of albumin, by heating, greatly increases its susceptibility

to hydrolysis both by thrombin or trypsin. This is probably due to greater accessibility of labile bonds within the molecule which are exposed by molecular unfolding following rupture of S-S bonds. On any basis of comparison used, trypsin digests albumin or heated albumin more quickly and more thoroughly than thrombin. The limited but definite proteolytic activity of thrombin suggests the usefulness of thrombin as a reagent in protein structure studies. A thrombin digest of a large protein would contain a few large fragments that could be separated before further digestion. An investigation of the albumin bonds split by thrombin is in progress.

REFERENCES

Bailey, K., and Bettleheim, F. R., Biochim. et Biophys. Acta, 18, 495 (1955).
Bailey, K, Bettleheim, F. R., Lorand, L, and Middlebrook, W. R., Nature, 167, 233 (1951).
Bettleheim, F. R., and Bailey, K., Biochim. et Biophys. Acta, 9, 578 (1952).
Creeth, J. M., Biochem. J., 51, 10 (1952).
Ehrenpreis, S., Leach, S. J., and Scheraga, H. A., J. Am. Chem. Soc., 79, 6086 (1957).
Gorini, L., and Audrain, L., Biochim. et Biophys. Acta, 19, 289 (1956).
Guest, M. M., and Ware, A. G., Science, 112, 21 (1950).
Kay, J. H., Trans. Conf. Blood Clotting and Allied Problems, ed. J. E. Flynn, New York. Josiah Macy, Jr., Foundation, 4, 72 (1951).
Kunkel, H. G., and Tiselius, A., J. Gen. Physiol., 35, 89 (1951).
Rasmussen, P. S., Biochim. et Biophys. Acta, 16, 157 (1955).
Richards, F. M., Compt. rend. trav. lab. Carls. Sér. chim., 29, 322 (1955).
Schultze, H. E., and Schwick, G., Z. physiol. Chem., 289, 26 (1951).
Seegers, W. H., Proc. Soc. Exptl. Biol. Med., 72, 677 (1949).
Seegers, W. H., Levine, W. G., and Shepard, R. S., Can. J. Biochem. and Physiol., 36, 603 (1958).
Seegers, W. H., and Smith, H. P., Am. J. Physiol, 137, 348 (1942).
Sherry, S., and Troll, W., J. Biol. Chem., 208, 95 (1954).
Tanford, C., Swanson, S. A., and Shore, W. S., J. Am. Chem. Soc., 77, 6414 (1955).