ENZYMATIC ACTIVITIES ASSOCIATED WITH CLOTTING OF FIBRINOGEN BY STAPHYLOCOAGULASE AND COAGULASE-REACTING FACTOR AND THEIR INHIBITION BY DIISOPROPYLFLUOROPHOSPHATE

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ABSTRACT

Drummond, Margaret C. (Emory University, Atlanta, Ga.) and Morris Tager. Enzymatic activities associated with clotting of fibrinogen by staphylocoagulase and coagulase-reacting factor and their inhibition by diisopropylfluorophosphate. J. Bacteriol. 83:975-980. 1962.—The chemical mechanism of fibrinogen clotting by staphylocoagulase and its plasma factor (CRF) involves a preliminary stage of proteinolysis, analogous to that found in thrombin-catalyzed fibrinogen clotting. Coagulase-CRF also exhibits N-α-toluene-p-sulfonyl-L-arginine methyl esterase activity in addition to its fibrinogen-clotting and proteolytic activities. These enzymatic activities have been further studied by their responses to certain enzyme inhibitors, and from the standpoint of their possible interrelationships.

Studies in this laboratory (unpublished data) have demonstrated that the clotting of purified bovine fibrinogen by staphylocoagulase with its plasma factor (CRF) is accompanied by the liberation of nonprotein nitrogen. As Lorand (1952) found in thrombin-catalyzed systems, proteinolysis is initiated before visible indications of clot formation, and does not stop with gel formation. The reaction reaches completion when the levels of nonprotein nitrogen released approach about 3% of the total nitrogen. Similar findings, obtained with the coagulase-CRF system, would indicate, therefore, that clotting induced by coagulase-CRF involves the limited type of proteinolysis now recognized to be characteristic of thrombin.

Although coagulase alone has no esterase activity on the synthetic substrate, N-α-toluene-p-sulfonyl-L-arginine methyl ester (TAME) (Tager, 1956), Haughton and Duthie (1959) demonstrated that when coagulase is previously incubated with the plasma factor, or CRF, esterase activity is revealed. It thus appears that coagulase-CRF, in addition to clotting, exhibits two enzymatic activities, proteolytic and esterolytic. It is significant that the same three activities are associated with other proteins endowed with coagulating properties, namely thrombin (Eagle and Harris, 1937; Bailey et al., 1951; Sherry and Troll, 1954), trypsin (Schwert et al., 1948), and certain snake venoms (Blomback and Yamashina, 1958). In view of the parallelism of associated enzymatic reactions of diverse clotting agents, it was deemed of interest to study in greater detail the action of coagulase-CRF on TAME from such standpoints as the kinetics of the reaction, the effect of prior incubation of the reactants, and the action of certain inhibitors, such as soy bean trypsin inhibitor (SBTI) and diisopropylfluorophosphate (DFP). Studies of the action of DFP were deemed of special relevance. This agent has been shown to inhibit a variety of esterases and, in particular, proteases with esterase activity, such as trypsin and thrombin (Miller and Van Vunakis, 1956). In the present studies, therefore, we have sought to establish the possible effects of this inhibitor on the protease-esterase, as well as on the coagulating, activities of coagulase-CRF, in the hope of further elucidating the relationships of these three activities of the coagulase-CRF moiety.

MATERIALS AND METHODS

Purified coagulase. The two preparations of coagulase employed were purified from culture filtrates of Staphylococcus aureus 104 by the method described earlier (Tager, 1948). The final purified preparations, after dialysis and lyophilization, contained 0.06 mg of protein (Lowry et al., 1951) per mg (dry wt). The clotting activity of these preparations was measured by the method of Tager (1948), in which 0.5-ml samples of noninhibitory human plasma were added to 0.5 ml of serial twofold dilutions of...
coagulase. The titer was read as the reciprocal of the dilution of the last tube which showed a solid (invertable) clot after incubation at 38 C for 24 hr. The two coagulase preparations employed had titers of 1024 and 2048 per mg of dry material, and both appeared homogeneous upon ultracentrifugation at 50,700 rev/min (Spinco analytical ultracentrifuge).

Purified plasma factor. CRF preparations were purified by column chromatography (Tager, 1956a) of Seitz-filtered human plasma. Of the two preparations used in these studies, one contained 0.067 mg of protein per mg (dry wt) and the other 0.045 mg. The level of thrombin contamination of these products was about \( \frac{1}{16} \) to \( \frac{1}{32} \) of a unit per ml as measured by the clotting of fibrinogen and comparison with clotting by dilutions of purified bovine thrombin.

Buffers. The TAME (obtained from H and M Chemical Co., Ltd., Santa Monica, Calif.) hydrolysis experiments were carried out in 0.25 m 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 8.5. All clotting studies were done in 0.067 m \( \text{Na}_2\text{HPO}_4\)-\( \text{NaH}_2\text{PO}_4 \) buffer (pH 6.8).

Assay of clotting activity of coagulase-CRF. Dilutions of coagulase-CRF were made in phosphate buffer (pH 6.8), 0.4 ml of which was added to 0.2 ml of 0.25% bovine fibrinogen (Armour fraction I) in 0.9% NaCl at 23 to 25 C. The reaction was timed from the mixing of the reagents until the appearance of visible signs of clotting (flocculation or fibrin formation). Each system of coagulase-CRF or coagulase-CRF-inhibitor was studied at several doubling dilutions to avoid the zones of inhibition which may occur when either protein is present in excess.

Assay of TAME esterase activity. The TAME esterase of coagulase-CRF was determined by the colorimetric measurement of the residual ester after hydrolysis. The method used was that of Hestrin (1949) and of Sherry and Troll (1954), with a single modification. For reproducibility, it was found necessary to carry out the reaction between ester and hydroxylamine for a minimum of 30 min instead of the 2 min prescribed by Hestrin for the reaction with acetylcholine. Readings were made on a Coleman Junior spectrophotometer at 540 m\( \mu \), and compared with those of a standard TAME solution. Results are expressed as mmoles of TAME hydrolyzed in a given time period under the conditions described.

RESULTS

Although the work of Haughton and Duthie (1959) has established the TAME esterase activity of coagulase-CRF, few details were given in regard to the kinetics of this reaction. It was of interest, therefore, to investigate certain of these kinetics, preliminary to the inhibition studies, in view of the variability of the protein preparations employed and the difference in the chemical methods for the assay of TAME hydrolysis.

Rate of hydrolysis of TAME by coagulase-CRF

![Graph](http://jb.asm.org/)  
**FIG. 1.** TAME hydrolysis by coagulase-CRF, by CRF alone, and by coagulase alone, as a function of time. Coagulase, 0.87 mg protein per ml; CRF, 0.064 mg protein per ml. Temperature, 38 C; pH, 8.5.

**FIG. 2.** Rate of hydrolysis of TAME by coagulase-CRF as a function of substrate concentration. Concentration of coagulase and CRF as mg protein per ml, 0.87 and 0.064, respectively. Michaelis constant (K_m) of the reaction, \( 2.8 \times 10^{-3} \text{ m} \).
as a function of time. Coagulase and CRF were combined in 0.1-ml amounts (containing 0.27 and 0.054 mg, respectively, of protein per 0.1 ml of pH 6.8 phosphate buffer), and incubated for 20 min at 38 C. To this incubation mixture were added TAMe in tris buffer, and tris buffer (both warmed to 38 C) sufficient to give a final volume of 1 ml and a final TAMe concentration of 0.015 M. The pH for hydrolysis was 8.5, and the temperature 38 C. Controls run simultaneously consisted of: (i) coagulase and phosphate buffer (0.1 ml each), previously incubated at 38 C for 20 min, and (ii) CRF and phosphate buffer (0.1 ml each) also previously incubated. To each of these controls were added the TAMe substrate and tris buffer as above. A control for spontaneous substrate hydrolysis was 0.08 mg, and the level of fibrinogen clotted, is sufficient to effect less than 7% hydrolysis of TAMe under the experimental conditions described.

Optimal substrate concentration for hydrolysis of TAMe by coagulase-CRF. Reaction velocities were studied over a range of substrate concentrations from 0.5-3.5 x 10^{-2} M, the coagulase and CRF concentrations and other experimental conditions being as described above. Higher concentrations of the substrate were contraindicated in view of the insolubility of TAMe under these conditions. The results of this study are presented in Fig. 2. The K_m of the reaction was found to be 2.8 x 10^{-3} M (Lineweaver and Burk, 1934).

Determination of optimal concentrations of coagulase and CRF for TAMe hydrolysis. Since little is known regarding the mechanisms of the interaction of coagulase with CRF, specifically in regard to which protein is enzyme and which substrate, studies were done to determine the optimal concentrations of both components for the TAMe hydrolysis reaction. With the TAMe concentration at 0.015 M and the experimental conditions as described, the following two experiments were carried out. In one, the CRF concentration was held constant at 0.08 mg protein per

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![Image](http://jb.asm.org/)

**Fig. 3.** Rate of hydrolysis of TAMe by coagulase-CRF as a function of the coagulase concentration. Concentration of CRF, 0.08 mg protein per ml.

**Fig. 4.** Rate of hydrolysis of TAMe by coagulase-CRF and by CRF in the absence of coagulase, as a function of the CRF concentration. Concentration of coagulase, 0.04 mg protein per ml.
ml of reaction mixture, while the coagulase concentration varied from 0.96 to 0.03 mg of protein per ml; in the second experiment, the coagulase concentration was maintained at 0.24 mg protein per ml, while the CRF concentrations were varied from 1.3 to 0.04 mg protein per ml. Since CRF alone, in the absence of coagulase, exhibits TAMe esterase activity, controls of CRF alone at the several concentrations were included.

The results obtained with constant CRF and varying coagulase concentrations are depicted in Fig. 3. The reaction velocity reaches a maximum at a coagulase concentration of 0.24 mg protein per ml, levelling off at higher concentrations. The TAMe hydrolysis by CRF alone is also shown.

Figure 4 shows the effects of varying CRF concentrations, both with coagulase and without. TAMe esterase activity increases sharply in both instances, being in all cases higher when coagulase is present. The maximal differences in nmoles of substrate hydrolyzed by coagulase-CRF and by CRF alone occur in the range of CRF protein concentration of 0.085 to 0.33 mg per ml.

**Effect of prior incubation of coagulase with CRF on the TAMe esterase reaction.** Haughton and Duthie (1959) reported a shortening of fibrinogen clotting times upon incubation of coagulase with plasma factor. They employed the same protocol in their TAMe experiments, although the effect of incubation was not described. The necessity of this preliminary period of incubation was studied.

Accordingly, 0.1 ml of coagulase (0.24 mg protein) in phosphate buffer was incubated with 0.1 ml of CRF (0.08 mg protein), also in buffer, for 20 min at 38 C. Other reactions consisted of: (i) each protein incubated separately before addition to the substrate, and (ii) CRF incubated with phosphate buffer. To these three tubes were added tris and TAMe as before.

The TAMe esterase activity of coagulase-CRF previously incubated together was consistently greater (Fig. 5) than that of the coagulase-CRF system in which the proteins were added simultaneously to the substrate. This difference in activity, however, was only of the order of 7 to 10%.

**Effect of SBTI on the TAMe esterase reactions.** To one of two tubes containing 0.1 ml each of coagulase (0.3 mg protein) and of CRF (1.3 mg protein) was added 0.1 ml (0.4 mg) of SBTI in phosphate buffer, and to the other 0.1 ml of buffer only. After incubation at 38 C for 30 min, tris and TAMe were added to give a final volume of 1 ml and a final TAMe molarity of 0.015. Simultaneously determined were CRF alone in the absence of coagulase, both with and without the inhibitor, as well as the usual controls of coagulase without CRF, and TAMe without either protein. Since the purpose of this experiment was to determine the effect of SBTI on esterase activity, the CRF concentration employed (1.3 mg protein per ml) was greater than optimal, to increase the amount of esterase activity.

The results (Table 1) confirm the observations of Haughton and Duthie (1959) that SBTI fails
to inhibit the TAMe esterase reaction of coagulase-CRF. Of special interest was the finding that this inhibitor also fails to inhibit the esterase of CRF. SBTI is known not to affect thrombin activity (Sarkar, 1960), but does inhibit proteolysis by plasmin and trypsin (Sherry and Troll, 1954). On this basis it would appear that the purified CRF preparations employed were not detectably contaminated by either plasmin or trypsin. On the other hand, the studies described earlier revealed that the level of thrombin contamination is less than 7%. The identity of the TAMe-hydrolyzing component of these purified preparations is as yet not known.

**Inhibition of clotting and TAMe esterase activities of coagulase-CRF by DFP.** Samples of 0.1 ml of coagulase (1.2 mg protein) and 0.1 ml of CRF (0.41 mg protein) were incubated at pH 6.8 under the usual conditions of time and temperature. This incubation mixture was then buffered to pH 8.6 with tris buffer and incubated further for 15 min with dilutions of the inhibitor. After incubation with the inhibitor, tenfold dilutions of the inhibition mixture were made in pH 6.8 phosphate buffer for the assay of clotting activity. For the assay of TAMe esterase activity, prewarmed TAMe and tris buffer were added to a sample of the inhibition mixture, so that, in the reaction tubes, the final concentration of TAMe was 0.015 M, of coagulase was 0.12 mg protein per ml, and of CRF was 0.04 mg protein per ml. Samples were withdrawn after 30, 60, 90, and 120 min of incubation.

Since DFP is made in anhydrous isopropanol for stability, coagulase-CRF was incubated with isopropanol in the absence of DFP as a control. Simultaneously studied was the effect of DFP on CRF alone. Percentage inhibition was calculated on the basis of coagulase-CRF treated in the above manner except for the DFP.

Figure 6 shows the variation in the degree of inhibition of both the clotting and TAMe esterase activities of coagulase-CRF after 15 min of incubation with four different concentrations of DFP. Both activities appear to be affected to a similar extent by DFP under the conditions described. The TAMe esterase of coagulase-CRF is 50% inhibited by 2.1 × 10^{-4} M DFP, and clotting by 1.8 × 10^{-4} M DFP. Both of these two activities of thrombin are 50% inhibited by 2.0 × 10^{-4} M DFP (Miller and Van Vunakis, 1956).

**DISCUSSION**

The coagulase-CRF clotting system has been subjected to some of the biochemical and enzymatic analyses which have been so productive in the study of the action of thrombin on fibrinogen. Striking similarities have been demonstrated.

Fibrinogen clotting induced by both coagulase-CRF and by thrombin is accompanied by the release of nonprotein nitrogen to the extent of about 3% of the total nitrogen. Both clotting systems exhibit esterase activity on a synthetic substrate, TAMe. Both clotting systems, in regard to the two activities which they each exhibit, respond in a similar manner to two enzyme inhibitors, DFP and SBTI. Coagulase-CRF is unaffected by SBTI, both in its clotting (Tager, 1952) and in its TAMe esterase activity. On the contrary, the present studies reveal that both activities are inhibited by DFP. Thrombin is affected by these two inhibitors like coagulase-CRF, both activities being unaffected by SBTI but inhibited by DFP.

The action of DFP on the coagulase-CRF merits special emphasis. The inhibition of fibrinogen clotting and of TAMe esterase was found to occur to the same extent, 50% inhibition of both functions being obtained with 1.8-2.1 × 10^{-4} M concentrations of the inhibitor under the conditions described. This finding heightens the analogy of coagulase-CRF to the thrombin system. It also indicates that it becomes feasible, as proposed earlier by Glueck, Sherry, and Troll (1954) for prothrombin, to substitute a TAMe assay for the demonstration of coagulase-CRF activity in preference to the cumbersome, many-staged reaction of fibrinogen clotting, with its inherent dangers of unknown limiting factors.
It has been suggested (Sarkar, 1960) that the hydrolytic (i.e., TAME esterase) and clotting activities of a variety of coagulating materials seem always to be affected in the same way by a given inhibitor, that is, either both inhibited or both unaffected. This observation is further substantiated experimentally by the findings of this study with coagulase-CRF, still another clotting moiety. Such similarities, having occurred thus far without known exception (Sarkar, 1960), indicate a close relationship between clotting and esterase activities, and suggest the possibility of an active grouping in the molecule common to both activities.

The interrelationships of the clotting, esterase, and proteolytic activities have been under close scrutiny in studies of thrombin clotting. In terms of thrombin catalysis, the fibrinogen to fibrin conversion is believed to be a reaction consisting of several stages. The first stage is proteolytic, in which two peptides are cleaved from fibrinogen, leaving the residual protein with the proper charge for polymerization, or fibrin formation. This stage has been shown to occur even in the absence of subsequent polymerization. On the basis of studies with crystalline trypsin (Schwert et al., 1948), in which both the amidase and esterase activities were shown to be mediated by the same active surface configurations, it appears justifiable to equate proteolysis and esterase activities. In this work the amide and methyl ester of tosyl arginine were among the compounds studied. In view of the evidence presented in the current study for the coagulase-CRF system, and elsewhere for other clotting systems, there would appear to be merit in the hypothesis that proteolysis and esterase activity may be general attributes of coagulating materials, such as thrombin, snake venoms, trypsin, and now coagulase-CRF.

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LITERATURE CITED


