ENZYMATIC ACTIVITY OF STAPHYLOCOAGULASE

II. DISSOCIATION OF PLASMA CLOTTING FROM TRIBUTYRINASE ACTIVITY

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Drummond and Tager (1959) have shown that highly purified preparations of staphylocoagulase exhibit esterase activity as measured by the breakdown of tributyrin. Coagulase, like thrombin, ultimately leads to the conversion of fibrinogen to fibrin. Since Sherry and Troll (1954) have discovered esterase activity in purified thrombin, the finding of enzymatic activity in purified coagulase immediately raised the question of whether the thrombin activity of coagulase is a necessary, intrinsic activity of the coagulase molecule, or whether it is in fact a separable entity. The present studies were designed to resolve this question.

MATERIAL AND METHODS

Assays of plasma clotting activity. Tube dilution method. The method of assay (Tager and Hales, 1947) is based upon the clotting of noninhibitory citrated human plasma by dilutions of coagulase. Coagulase is diluted in 2 per cent peptone-saline, 1:10 in the first tube and serially 2-fold in the succeeding tubes. An equal volume of citrated human plasma (0.5 ml) is added to each tube, and the series incubated at 37 C. The course of the reaction is followed by grading the firmness of the clot, from 1 + to 4 +, in each tube at intervals up to 18 hr. The activity of coagulase is expressed as the reciprocal of the clotting titer as read at 18 hr, with a correction being made for the firmness of the clot.

Timed reaction method. This method also relies upon the clotting of citrated human plasma by coagulase, but bases its end point on the carefully timed first appearance of clotting (Tager and Lodge, 1951). To an appropriate sample of coagulase, an equal volume of citrated plasma is added at 37 C, and the reaction timed with a stop watch for the visible indication of clot formation, either an increase in viscosity or formation of fibrin.

Immunization of rabbits with purified staphylococagulase. Healthy young adult rabbits of approximately the same age and weight were immunized by weekly, subcutaneous injections of purified coagulase (2.5 mg) adsorbed on aluminum phosphate (1.5 mg) as an adjuvant (Boake, 1956). Immune sera were drawn by cardiac puncture at intervals after 4 weeks, and assayed for anticoagulase. Normal sera were drawn prior to immunization.

The antibody neutralization method employed was that described by Lominski and Roberts (1946). It involves reacting a known quantity of purified coagulase, diluted in 2 per cent peptone-saline, with a series of doubling dilutions of the serum under study. Following incubation at 37 C for 90 min, citrated human plasma was added, and the tubes observed for clotting. In this manner, the effect of the antisera on the speed and degree of clotting may be observed.

Fractionation of normal and immune rabbit sera. In order to eliminate the interfering action of serum lipase, antibody fractions were purified from both normal and immune rabbit sera (Deutsch, 1950). These purified fractions were checked for their ability to neutralize coagulase clotting. The neutralizing activity of the derived fractions essentially equalled that of the parent serum.

Zone electrophoresis of coagulase. A 2.0-ml portion of purified coagulase containing 40 mg of protein in the appropriate buffer was applied to a 25 by 375 cm column containing cellulose as the supporting medium (Floidin and Kupke, 1956). The zone electrophoresis apparatus employed was essentially identical to the LKB model 3340, described by Svensson (1954). After 18 to 20 hr
at 25 C, at a current of 30 ma, the column was removed, and fractions of 1.2 to 1.5 ml were collected automatically. These fractions were subsequently assayed for plasma clotting by the tube dilution technique, for tributyrinase by the manometric method, and for protein by the Lowry method (Lowry et al., 1951).

**Manometric assay of tributyrinase.** All studies were executed under the conditions already described (Drummond and Tager, 1959).

**Preparations of staphylocoagulase.** The same two preparations of staphylocoagulase described in the preceding paper (Drummond and Tager, 1959) were employed in these studies. Care was taken to use the same identical preparation for all antibody studies.

**RESULTS**

**Differential heat inactivation of coagulase tributyrinase and plasma clotting activities.** Samples of coagulase (0.072 mg per ml, pH 7.1) were held at 37 C and 56 C, respectively, in thermoregulated water baths. Samples of 5 ml were removed after 30 min, 2 hr, and 5 hr to an ice bath at 0 C. Upon removal of the final samples, all were assayed for tributyrinase and plasma clotting by the methods described, the latter by the tube dilution method. The control was a similar coagulase held at 0 C until assay.

A comparison of the effects of temperature on the two activities is given in figure 1, in which percentage residual activity is plotted as a function of time of heating at the two temperatures studied. It is apparent that the tributyrinase is significantly more susceptible to the inactivating influence of elevated temperatures than is the clotting activity. After being heated at 37 C for 5 hr, only 51 per cent of the tributyrinase activity remains as compared to 91 per cent of the plasma clotting activity. The same period of heating at 56 C inactivates 93.5 per cent of the tributyrinase but only 25 per cent of the plasma clotting activity.

**Differential trypsin inactivation of tributyrinase and plasma clotting activity.** Earlier studies (Tager, 1948) have revealed that the plasma clotting activity of coagulase is extremely susceptible to destruction by purified trypsin. Trypsin, at concentrations of 4 μg per ml, under certain specified experimental conditions, was shown to reduce the clotting activity 99 per cent after only 10 min incubation with coagulase at 37 C.

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**Figure 1.** The differential heat inactivation of coagulase tributyrinase and plasma clotting activities. Ordinate, percentage of residual activity. Abscissa, minutes of heating of purified coagulase in the absence of substrate at 37 and 56 C. Conditions in text.

A 0.2-ml sample of purified coagulase (0.072 mg) was added to 0.1 ml (0.002 mg) of crystalline trypsin and 0.1 ml of distilled water. After incubation at 37 C for 40 min, 0.1 ml (0.02 mg) of soybean trypsin inhibitor was introduced to terminate the proteolytic action of trypsin. Controls consisted of: (a) coagulase, without either trypsin or soybean trypsin inhibitor, held at 37 C for 40 min; (b) coagulase, without either trypsin or soybean trypsin inhibitor, held at 22 C for 40 min; and (c) coagulase, held at 37 C for 40 min, to which soybean trypsin inhibitor was subsequently added to determine the effect, if any, of soybean trypsin inhibitor on coagulase activity. Tributyrinase and plasma clotting of the trypsin-inactivated coagulase, as well as of all controls, were subsequently assayed. Plasma clotting was measured by the tube dilution method, with results expressed as reciprocals of the clotting titers.

A comparison of the titers given in table 1 reveals that the plasma clotting ability of coagulase...
Effect of trypsin inactivation on the plasma clotting activity of coagulase

<table>
<thead>
<tr>
<th>Coagulase Titer*</th>
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<tbody>
<tr>
<td>Coagulase at 22 C (control).............. 200</td>
</tr>
<tr>
<td>Coagulase at 37 C (control).............. 200</td>
</tr>
<tr>
<td>Coagulase + soybean trypsin inhibitor at 37 C.............. 200</td>
</tr>
<tr>
<td>Trypsin-inactivated coagulase at 37 C. .............. 0</td>
</tr>
</tbody>
</table>

* Reciprocal of clotting titer.

is totally destroyed by tryp tic action under the experimental conditions specified. The concentration of coagulase was 14.4 to $0.5 \times 10^{-4}$ mg per ml, in the first and sixth tubes, respectively, of the titration series. Both soybean trypsin inhibitor and heat are without effect on plasma clotting in this experiment.

Figure 2 shows the time course of the trypsin-inactivated coagulase tributyrinase on a substrate of $1 \times 10^{-6}$ tributyrin, with the controls described. Coagulase, with neither trypsin nor soybean trypsin inhibitor added, is rather susceptible to a 40-min exposure to 37 C, losing 22 per cent of its activity when compared to a similar control held at 22 C for 40 min. Calculated on the basis of the heat-inactivated control, it appears that, under these experimental conditions, trypsin is capable of a 44 per cent inactivation of tributyrinase. On the basis of the more appropriate soybean trypsin inhibitor control, held at 37 C for 40 min, the inhibition due to trypsic action is 41 per cent. Because of the lag period, all calculations were made on the basis of the final slopes of the curves.

It can be seen, therefore, that trypsin inactivation also serves to differentiate the two demonstrable activities of coagulase. Conditions of concentration of both trypsin and coagulase, time of preincubation and temperature, resulting in the total elimination of the plasma clotting activity inactivates the tributyrinase only 41 per cent.

Differential effects of certain enzyme inhibitors on tributyrinase and plasma clotting activity. To determine the effects of known enzyme inhibitors on the tributyrinase of coagulase, the enzyme, at a concentration of 0.072 mg per ml, was incubated for 90 min at 37 C with an equal volume of the inhibitor at a concentration twice that in the final diluted reaction mixture. Following incubation, a 2.0-ml sample was assayed for tributyrinase activity by the manometric method. Controls, in addition to the usual enzyme and substrate controls, consisted of coagulase incubated at 37 C for 90 min in the absence of the inhibitor.

The inhibitor effects on plasma clotting were studied by incubating 0.5-ml samples of the enzyme, at a concentration three times that of the final assay dilution, and the inhibitor, at a concentration twice that of the incubation mixture. After incubation, 0.5 ml of noninhibitory citrated human plasma was added, and the clotting activity assayed by the timed reaction method described. The control was coagulase at the appropriate concentration incubated at 37 C in the absence of the inhibitor.

The enzyme inhibitors studied were not employed in solutions of the same concentration. Factors influencing the choice of concentration in each case were the solubility of the compound.
and its range of effectiveness as shown by preliminary investigation. Tetraethylpyrophosphate alone of the five inhibitors reported had to be studied in a buffered system because of its extreme acidity. In general, the buffers tested, Veronal and phosphate at pH 7.0, delayed clotting as compared to a distilled water medium, and consequently were not employed except in the case mentioned where the pH effects were found to be even more deleterious.

The findings of these studies suggest that neither the tributyrinase nor the plasma clotting of coagulase is dependent upon essential sulfhydryl groups. Table 2 reveals that p-chloromercuribenzoate, at the maximal concentration employed, $2.5 \times 10^{-2}$ M, is without significant effect on either activity of coagulase. Sodium fluoride, a known metal inactivator, at $5.0 \times 10^{-1}$ M, appears to enhance plasma clotting while significantly inhibiting the tributyrinase. The actions of three alleged lipase or esterase inhibitors differ considerably in their effects on the two activities. Tri-o-cresyl phosphate, a cholinesterase and tissue lipase inhibitor (Augustinsson, 1950; Rona and Ammon, 1926) inhibits plasma clotting 30 per cent and tributyrinase 25 per cent at a concentration of $2.5 \times 10^{-1}$ M, thus having a similar effect on both activities. Atoxyl or sodium arseniate (Myers and Mendel, 1953), at $2.5 \times 10^{-2}$ M, and tetraethylpyrophosphate (Jansen et al., 1949), at $5.0 \times 10^{-1}$ M, inhibit the plasma clotting but not the tributyrinase.

**Differential neutralization of tributyrinase and plasma clotting activity by specific antibody fractions.** Characterization of the antibody fractions. Fractionation of immune rabbit serum by the Deutsch ethanol procedure yielded two preparations which are designated $\gamma$-globulin-containing and $\beta$-globulin-containing fractions. The electrophoretic patterns of these two serum fractions are shown in figure 3, superimposed upon the immune serum from which they were derived. The $\gamma$-globulin-containing fraction, corresponding to the precipitate C described by Deutsch, can be seen to contain predominantly $\gamma$-globulin, but in addition a faster-moving component, presumably $\beta$-globulin. The $\beta$-globulin-containing fraction derived from immune rabbit serum under the experimental conditions described by Deutsch contains predominantly $\beta$-globulin.

Neutralization by $\gamma$-globulin. Samples of 1.0 ml of a 1:160 dilution of a 20 mg per ml stock coagulase solution were incubated for 90 min at 37 C with 2.5 ml of a $\gamma$-globulin fraction dilution, found by previous experiment to neutralize plasma clotting completely over a period of at least several hours. Of this 3.5 ml incubation mixture, 2.0 ml were assayed manometrically for tributyrinase under the conditions of assay already described. The usual enzyme and substrate controls were run as well as a control of antisem plus substrate, without coagulase. The degree of neutralization of tributyrinase was determined by a comparison of the reaction velocities of coagulase incubated with and without antiserum, the percentage neutralization being

![Figure 3. Paper electrophoresis of immune rabbit serum before and after fractionation by the Deutsch procedure.](http://jb.asm.org/)

**TABLE 2**

Comparative action of enzyme inhibitors on tributyrinase and plasma clotting activities of coagulase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc.</th>
<th>Percentage Change* from Control</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Clotting</td>
</tr>
<tr>
<td>p-Chloromercuri-</td>
<td>$2.5 \times 10^{-3}$</td>
<td>$-8.9$</td>
</tr>
<tr>
<td>benzoate...........</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium fluoride...</td>
<td>$5.0 \times 10^{-1}$</td>
<td>$+26.0$</td>
</tr>
<tr>
<td>Atoxyl.............</td>
<td>$2.5 \times 10^{-2}$</td>
<td>$-53.2$</td>
</tr>
<tr>
<td>Cresyl phosphate...</td>
<td>$2.5 \times 10^{-1}$</td>
<td>$-30.1$</td>
</tr>
<tr>
<td>Tetraethylpyro-</td>
<td>$5.0 \times 10^{-1}$</td>
<td>$-21.2$</td>
</tr>
<tr>
<td>phosphosphate......</td>
<td></td>
<td></td>
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* Negative values denote inhibition, positive ones stimulation.
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Figure 4. Neutralization of tributyrinase activity by a γ-globulin-containing fraction (Deutsch ppt C) of immune rabbit serum, time course of the reaction. Open circles denote the tributyrinase control reaction (no added antiserum fraction), and the closed circles, the serum-inactivated tributyrinase reaction. Neutralization studies were carried out under the conditions already described, employing two different β-globulin-containing fractions (precipitate B of immune rabbit serum). It was of interest to know whether or not it would be possible to achieve greater than 66 per cent neutralization of tributyrinase activity by increasing the antibody concentration. This question was resolved experimentally by carrying out the tributyrinase neutralization with varying concentrations of the antibody fraction (figure 5). Increasing the antibody concentration increases the percentage neutralization of tributyrinase to the point of about 66 per cent neutralization. This inhibition appears maximal, since a further two-fold increase in antibody concentration fails to effect a corresponding increase in percentage neutralization.

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The highest concentration of $\beta$-globulin available failed to affect clotting neutralization (table 3) but inhibited the tributyrinase approximately 60 per cent (figure 6).

**Separation of the plasma clotting and tributyrinase activities by zone electrophoresis.** The evidence suggests that the clotting and the esterase activities of purified coagulase are separable. However, these observations do not preclude the possibility that a single coagulase molecule may possess several reacting groups, and that the dissociation of activities by heat, enzymatic digestion, enzyme inhibitors, and purified antibodies represents a differential action on two measurable activities of the same molecular entity. This, then, might be analogous to, for example, the loss of toxic properties of toxin in the conversion to toxoid, but with the retention of antigenic activity by both preparations. Definitive clarification of this point required attempts at further purification to determine whether the two activities, clotting and the splitting of tributyrin, could be physically recovered separately from each other. Attempts at separation, therefore, were undertaken by the use of zone electrophoresis and fractional elution from cellulose columns.

In view of the successful purification of thrombin on a cationic exchange resin (Rasmussen, 1955), efforts were made to achieve separation of the two activities of coagulase on Amberlite IRC-50 (XE-64), kindly supplied by Rohm and Haas, under several different experimental conditions, none of which proved successful. These conditions included the use of phosphate buffers at pH 6.5 and 7.6, and of 0.05 M phosphate buffer at pH 7.0, with elution by 0.3 M phosphate, pH 8.0. In each of these studies, both activities, as well as protein, emerged simultaneously.

**Figure 6.** Neutralization of tributyrinase by a $\beta$-globulin-containing fraction (Deutsch ppt B) of immune rabbit serum, time course of the reaction. Open circles denote the tributyrinase control reaction (no added antiserum), and the crosses, the inactivated reaction.

| Antiserum Dilutions | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | none |
|---------------------|-----|-----|------|------|------|-------|-------|
| Clotting time in min* |
| Immune serum         | NC† | NC  | NC   | 180  | 50   | 40    | 25    | 25   |
| Ppt C‡               | NC  | NC  | NC   | 180  | 60   | 30    | 25    | 25   |

* Time required for formation of firm 4+ clot.
† NC denotes no clot at 180 min.
‡ Reconstituted to original serum volume.
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Figure 7a. Separation of the plasma clotting and tributyrinase activities of staphylocoagulase by cellulose column zone electrophoresis. Open circles denote tributyrinase activity (X20); closed circles, the plasma clotting activity. Experimental details in text. The arrow designates the clotting titer of coagulase which, in a standard serial dilution, fails to demonstrate tributyrinase activity. Plasma clotting factor migrate together (figure 7a) in response to the conditions of electrophoresis described, it is apparent that the peaks of maximal purification do not coincide (figure 7b). This fact would, therefore, suggest a true dissociation of the two activities found associated in purified coagulase. With the experimental conditions described, it has not been possible to recover appreciable quantities of either activity in the complete absence of the other, because of the considerable overlapping of the two activities.

Other attempts to effect the physical separation of these two activities have utilized phosphate buffers of pH 6.6 and 7.6, of ionic strength of 0.1, as well as Veronal of pH 8.1 and ionic strength of 0.2. These efforts have proved completely unrewarding in that both activities emerge from the column simultaneously.

Figure 7b. The results of cellulose column electrophoresis of staphylocoagulase expressed as specific activity. The open circles represent tributyrinase activity per μg of protein (X20); closed circles, reciprocals of plasma clotting titers per μg of protein.

DISCUSSION

Since the mechanism of the clotting of citrated plasma by staphylocoagulase is poorly understood, the discovery of esterase activity in purified coagulase preparations was of considerable interest. The finding of a second activity in any purified protein preparation, however, poses the problems of whether the two activities represent the same functional groups on a single protein, different functional groups on the same protein, or different groups on different proteins. The evidence obtained from the studies described
would suggest that the esterase and plasma clotting activities of staphylocoagulase constitute separate molecular entities, and that the esterase here described is only fortuitously co-purified with the plasma clotting activity.

Studies of the differential effects of heat, trypsin, antiserum fractions, and certain enzyme inhibitors indicated this separation. Heat, trypsin, and antiserum, as well as three of the five inhibitors studied, suggested differential effects whereas only two of the enzyme inhibitors showed similar effects on the two activities of coagulase. The tributyrinase was found to be more heat labile than the clotting activity, being inactivated to a greater extent at each of the two temperatures studied. Concentrations of trypsin totally eliminating the clotting activity reduced the tributyrinase only 43 per cent. Concentrations of γ-globulin-containing antiserum fractions which completely neutralized the clotting activity inhibited the tributyrinase only 66 per cent. Of the enzyme inhibitors studied, both atoxyl and tetraethylpyrophosphate inhibited the clotting activity only, whereas sodium fluoride inhibited the tributyrinase while stimulating the plasma clotting.

Since the differential effects described suggested the probable dual nature of the two activities of coagulase, further attempts at fractionation and purification seemed indicated. In the absence of such investigations, the findings described could be interpreted only as signifying that the functional groups responsible for the two activities were different. The possibility would still exist that the two activities might simply reside on different portions of the same molecule. In the case of the two activities of coagulase, however, this problem has been in part successfully resolved by the application of zone electrophoresis. Cellulose column electrophoresis indicates a true dissociation of the tributyrinase and plasma clotting activities, despite the fact that thus far it has proved experimentally impossible to recover either activity in the complete absence of the other.

Paper electrophoresis of the two antibody fractions, precipitates B and C, derived from immune rabbit serum, has revealed that both contain a faster-moving component, presumably β-globulin, whereas only precipitate C possesses a considerable quantity of a slow-moving component, presumably γ-globulin. It should be pointed out that Deutsch likewise found traces of γ-globulin in his β-globulin precipitate B, but contrary to the present attempts, did succeed in achieving a γ-globulin preparation free of all traces of β-globulin. Although the present fractionation proved abortive in the original effort to obtain a preparation of γ-globulin only, several facts of interest emerge. In view of the findings that precipitate C contains both serum components and neutralizes both activities of coagulase, whereas precipitate B contains predominantly only one component and neutralizes only the tributyrinase, it is tempting to venture the following hypothesis: (a) that specific antitributyrinase is contained in the β-globulin fraction of immune serum, and (b) that specific antibodies to the coagulase component responsible for plasma clotting reside in the slow-moving γ-globulin fraction.

The studies with the enzyme inhibitors are of additional interest for the several problems they raise. For example, it appears from the results reported that plasma clotting is not sulfhydryl dependent, and that its activity is enhanced by a known metal inactivator, sodium fluoride. Furthermore, why the plasma clotting and not the esterase activity should be inhibited by atoxyl and tetraethylpyrophosphate, both alleged esterase inhibitors, is not immediately apparent. These findings suggest more subtle substrate specificities to esterase action than had been evident. In the absence of knowledge regarding the precise mechanisms of coagulase clotting of plasma, it may be that a more extensive study of the action of inhibitors on this system will be informative. Investigations relative to these several problems are currently in progress.

SUMMARY

Highly purified preparations of staphylocoagulase have been shown to possess esterase activity in addition to the plasma clotting activity. The differential effects of heat, trypsin, certain enzyme inhibitors, and antiserum fractions indicated the dual nature of these two activities. A physical separation of tributyrinase and the clotting principle has been achieved by cellulose column zone electrophoresis.

REFERENCES

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