AN ELECTROPHORETIC STUDY OF A STREPTOCOCCAL PROTEINASE AND ITS PRECURSOR

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In a previous report (1) methods were described for isolating from culture filtrates of group A streptococci a proteolytic enzyme and its precursor. Each, isolated in crystalline form, was characterized in terms of enzymatic activity and immunological specificity and the conditions defined under which the precursor is converted to the active enzyme.

It is the purpose of the present communication to describe an electrophoretic examination of the enzyme and its precursor, and to show that the two substances may also be distinguished, one from the other, by their behavior in an electric field.

Methods

Sources of Material.—Both the proteinase and its precursor were obtained from culture filtrates of a group A streptococcus, strain "5797," the same strain as has been used in previous published work on this subject. The streptococci were cultivated and the enzyme or its precursor isolated in crystalline form by methods which have already been described (1).

Serological Tests.—Serum was taken from rabbits immunized with either precursor or proteinase preparations. Previously it has been shown that precursor antiserum reacts with the active enzyme as well as with the precursor (1). This antiserum was therefore absorbed with the active enzyme so as to react specifically with the precursor protein. Details of these procedures were given in a previous report (1). Precipitin tests were carried out by using the capillary pipette technique (2).

Electrophoresis.—The electrophoretic studies were carried out in a Longsworth scanning apparatus. Acetic acid, cacodylic acid, diethyl barbituric acid (veronal), and the corresponding sodium salts, made by appropriate additions of sodium hydroxide, were used to form the buffer solutions against which the protein preparations were dialyzed for electrophoresis at values of pH between 5 and 8.6. All these buffer solutions were prepared at an ionic strength of 0.100; this required the addition of neutral sodium chloride in the region between pH 7 and 8, owing to the relatively low solubility of diethyl barbituric acid near 0°C., the temperature at which electrophoresis is carried out. The values of pH reported in this paper are those measured at room temperatures (23–25°) in accordance with the usual procedure.

In a number of cases, portions of material corresponding to the several electrophoretic components present were withdrawn from the cell at the end of the experiments for serological examination, in order to determine their nature.

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EXPERIMENTAL

Electrophoretic Examination of the Active Proteinase

The proteinase used in these experiments was crystallized from concentrated preparations obtained by precipitation of the active material from culture filtrates with ammonium sulfate (1). As has already been reported (1), extraneous protein may be removed from the crude, concentrated, starting material by the addition of sodium thioglycollate and subsequent incubation at 37°C. for 12 to 18 hours. This treatment results in the digestion of the extraneous protein by the streptococcal proteinase, and the digestion products may subsequently be removed by dialysis.

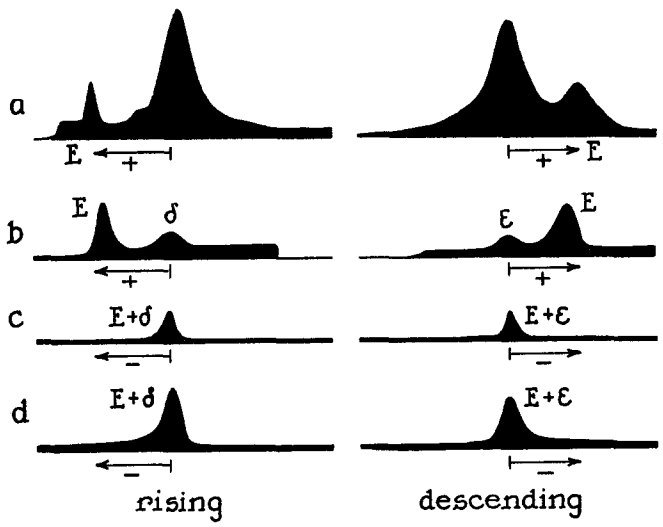

FIG. 1. Crude starting material pH 5.0, (*a*) Before autodigestion; (*b*) After autodigestion and dialysis, $U_E = 4.3 \times 10^{-5}$. Recrystallized active enzyme, (*c*) pH 8.45, $U_E = -0.032 \times 10^{-5}$; (*d*) pH 8.50, $U_E = -0.050 \times 10^{-5}$.

Comparable electrophoretic patterns of such a preparation, before and after "autodigestion," are shown in Figs. 1 *a* and 1 *b* respectively, where δ and ϵ are the familiar relatively immobile boundaries which do not correspond to any component, and E is the boundary due to active proteinase. From the partially purified material thus obtained, the proteinase was separated in crystalline form by treatment with ammonium sulfate as previously described (1).

A five times recrystallized preparation of the proteinase was examined electrophoretically at pH levels of 5.0, 7.8, 8.45, and 8.5; typical patterns are shown in Figs. 1 *c* and 1 *d*. The material appeared to be electrophoretically homogeneous within this pH range; the electrophoretic mobility of the active material under these conditions is shown in the lower plot of Fig. 3, in which

the motility, U, has the dimensions of $\frac{\text{cm.}}{\text{sec.}} / \frac{\text{volt}}{\text{cm.}}$ From these data the isoelectric point of the active material was found to be at pH 8.42 at an ionic strength of 0.1 which, as mentioned above, was kept constant in all the electrophoretic work reported in this paper.

Electrophoretic Examination of the Proteinase Precursor

Preparation of Material for Electrophoresis.—In concentrated solutions the proteinase precursor becomes converted autocatalytically to the active enzyme. This reaction is inhibited in the presence of ethanol or sodium iodoacetate (1).

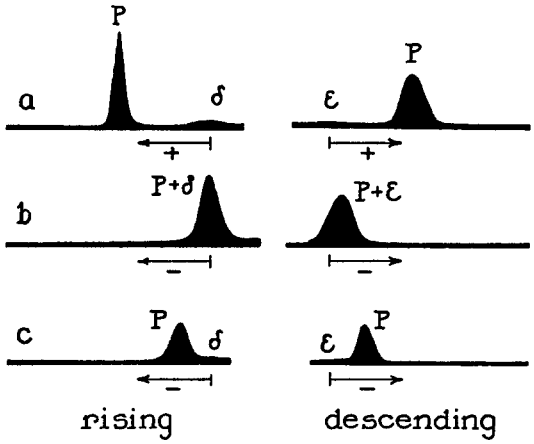

FIG. 2. Precursor, (*a*) pH 5.0, $U_P = 2.72 \times 10^{-5}$; (*b*) pH 7.3, $U_P = -0.074 \times 10^{-5}$; (*c*) pH 8.6, $U_P = -0.94 \times 10^{-5}$.

In the initial stages of the present investigation, precursor preparations were freed from the ammonium sulfate in which they had been crystallized by dialysis against 20 per cent ethanol in the cold. The active material was then dried from the frozen state. For electrophoresis, 150 mg. of the dried material was dissolved in 15 cc. of an appropriate buffer solution containing sodium iodoacetate in a concentration of 10^{-3} M.

Examination of such preparations at pH 8.6 revealed two components, although at lower pH values the material appeared homogeneous. It seemed possible that the inhomogeneity of these preparations resulted from some change in the precursor protein produced by either the ethanol treatment or the drying procedure.

Fresh material was therefore prepared for electrophoresis in the following manner. Crystals of the precursor protein were dissolved in an aqueous 10^{-3} M solution of sodium iodoacetate, pH 7.0, and dialyzed against an iodoacetate solution of the same concentration until free from ammonium sulfate; this solution was then dialyzed for electrophoresis against the appropriate buffer solution, which also contained sodium iodoacetate.

Although it avoided the use of ethanol and drying from the frozen state, this method of preparation had an obvious disadvantage in that the exact concentration of the precursor solution was not known. Owing to the presence of iodoacetate, assay of enzymatic activity was not possible, but an approximate estimate of the concentration of this preparation was made by serological methods: the serological reactivity of the preparation was tested by precipitation with a precursor-specific immune rabbit antiserum and compared with that of a precursor solution of known concentration.

Results of Electrophoresis.—Typical electrophoretic patterns of precursor material prepared in this way are shown in Fig. 2 *a*, *b*, and *c*, at pH values of 5.0, 7.3, and 8.6 respectively. Under these conditions of pH and concentration the material presented a single peak, P, which appeared to be homogeneous.

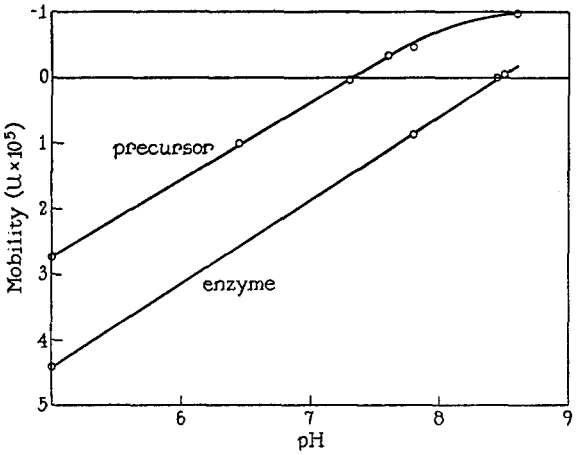

FIG. 3. Mobility *vs.* pH curves for precursor and active enzyme.

The electrophoretic mobilities of a precursor preparation at various pH levels are shown in the upper curve of Fig. 3. From these data the isoelectric point was found to be at pH 7.35.

Conversion of Precursor to Proteinase

By Autocatalysis.—It has previously been shown that the proteinase precursor is converted to the active enzyme autocatalytically in the presence of sulfhydryl compounds. This was now confirmed by examining electrophoretically a precursor preparation before and after incubation with sodium thioglycollate.

The enzyme precursor used in this experiment had been treated with alcohol preparatory to drying from the frozen state. A 1.0 per cent solution of the dried material was examined by electrophoresis at pH values of 6.45, 7.6, and 8.6. Sodium iodoacetate in a final concentration of 10^{-3} M was added to the solution in order to prevent autocatalytic conversion of the precursor at this stage of the experiment.

The material recovered after electrophoresis was dialyzed free from buffer salt and sodium iodoacetate, and redried. Of this dried material, 150 mg. was then dissolved in 10 cc. veronal buffer at pH 8.45. To this solution was added 1.0 cc. neutral sodium thioglycollate solution, 1.0 M, and the mixture brought to 37°C. Incubation was continued until the solution no longer reacted serologically with a precursor-specific antiserum; this required about 6 hours' incubation. The solution was dialyzed at 5°C. against a veronal buffer at pH 8.45, and 10^{-3} M in iodoacetate and then reexamined by electrophoresis. Under these conditions the precursor should have a mobility of about -1×10^{-5}, while the enzyme should be practically immobile, assuming that its electrophoretic behavior is uninfluenced by the presence of sodium iodoacetate.

TABLE I

Conversion of Precursor to Proteinase by Autocatalysis in Presence of Sodium Thioglycollate

Antigen used in precipitin tests	Precipitin reactions Antiserum prepared with														
	Precursor (R_2)							Proteinase (R_1)							
	Antigen diluted							Antigen diluted							
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:1	1:2	1:4	1:8	1:16	1:32	1:64	3:128
Precursor	++	++	++	++	++	+	±	±	±	±	−	−	−	−	−
Precursor treated with thioglycollate	−	−	−	−	−	−	−	++	++	++	++	++	+	+	±

± to ++ indicates various strengths of precipitin reactions.

The precursor preparation used in electrophoresis was diluted tenfold before making serial twofold dilutions for precipitin tests.

In all precipitin tests antigen dilutions were made in saline containing sodium iodoacetate (10^{-3} M).

In this experiment it was found that both the serological and electrophoretic evidence indicated that the precursor had disappeared. In its place was found an immobile, homogeneous electrophoretic component having the serological specificity of the active enzyme (Table I).

By Trypsin.—Conversion of the precursor to the active enzyme may be brought about by trypsin. Under these circumstances the conversion is incomplete and the yield of active enzyme approximately half that produced by autocatalysis. Complete conversion to the active enzyme may be achieved by incubation of trypsin-treated precursor preparations in the presence of sodium thioglycollate (1).

An experiment was designed to show by electrophoresis the change produced in the enzyme precursor as the result of its treatment with trypsin.

The precursor preparation used in this experiment had not previously been treated with ethanol or subjected to drying from the frozen state. It contained sodium iodoacetate (10^{-3}

M) and the same concentration of this salt was present in the buffer solution against which it was dialyzed prior to electrophoresis. Preliminary serological tests showed that the solution contained approximately 2.0 per cent of precursor protein.

15 cc. of the precursor solution was first submitted to preliminary electrophoretic examination at pH 7.8. To another 15 cc. sample of the same material was added crystalline trypsin in a final concentration of 0.01 mg. per cc., ten times the concentration previously found necessary under these conditions to convert this amount of precursor to active proteinase. The mixture was incubated at 37°C. for 1 hour and then dialyzed against veronal buffer pH 7.8 for 48 hours at 5°C. before electrophoresis.

Figs. 4 *a* and 4 *b* show the electrophoretic patterns of this material before and after treatment with trypsin. It will be seen that before treatment the preparation contained two components, A and B; samples of each were re-

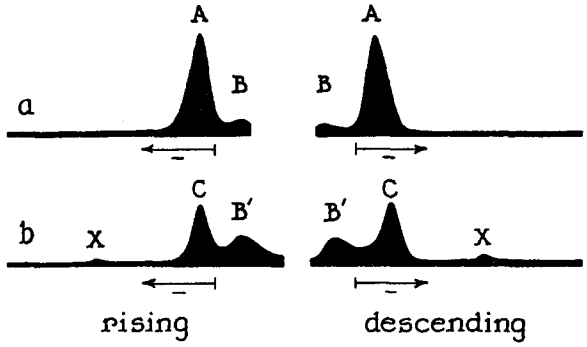

FIG. 4. (*a*) Precursor before treatment with trypsin, pH 7.8, $U_A = -0.47 \times 10^{-5}$; $U_B = 0.98 \times 10^{-5}$; (b) after treatment with trypsin, pH 7.8, $U_C = -0.76 \times 10^{-5}$; $U_{B'} = 1.16 \times 10^{-5}$.

moved from the cell after electrophoresis and identified by serological tests as precursor and active enzyme respectively. These serological reactions together with those of the parent-precursor preparation before and after trypsin treatment are shown in Table II. It has already been pointed out that precursor preparations have been found always to contain a small amount of active proteinase. The failure of this to appear as a separate peak in the earlier electrophoretic patterns was probably due to the lower concentration of the precursor solutions used in those experiments. In the present experiment an approximately 2 per cent precursor solution was used and it is apparent from the size of the peak that only a small amount of active proteinase was present.

Electrophoresis of the precursor material after treatment with trypsin showed three components, B′, C, and X in Fig. 4 *b*. A sample of component B′ separated after electrophoresis was identified serologically as active proteinase. This component had the same electrophoretic mobility ($U = 1 \times 10^{-5}$) as the minor component, B, present in the preparation examined before treatment with

trypsin (Fig. 4 *a*); its increased concentration following treatment with trypsin indicates that some conversion of precursor to proteinase had indeed occurred. Component C was of interest in that, although it had the serological specificity of precursor, its electrophoretic mobility ($U = -0.76 \times 10^{-5}$) differed significantly from that of the precursor component separated and identified before trypsin treatment ($U = -0.47 \times 10^{-5}$). Further, it may be assumed that this component could not be converted to active proteinase by trypsin, an excess

TABLE II

Conversion of Precursor to Proteinase by Trypsin

Antigen used in precipitin tests	Precipitin reactions Antiserum prepared with																	
	Precursor (R_2)										Proteinase (R_1)							
	Antigen diluted										Antigen diluted							
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Precursor	++	++	++	++	++	++	+	+	±	−	+	+	+	±	−	−	−	−
Component A	++	++	++	++	++	++	+	+	+	±	+	+	+	+	+	±	−	−
Component B	±	±	−	−	−	−	−	−	−	−	++	++	++	++	+	±	−	−
Precursor treated with trypsin	++	++	++	++	++	+	+	±	−	−	+	+	+	+	+	+	±	−
Component B′	−	−	−	−	−	−	−	−	−	−	+	++	++	++	++	++	+	±
Component C	+	++	++	++	++	++	++	+	+	±	+	+	+	+	+	±	−	−

± to ++ indicates various strengths of precipitin reactions.

The precursor preparation used in electrophoresis was diluted tenfold before making serial twofold dilutions for precipitin tests. Serial twofold dilutions of components A, B, B′, and C were made without preliminary dilution.

of which had been used in this experiment. Presumably, component C was a modified form of the precursor protein produced as a result of the treatment by trypsin. The very small, fast moving component X (Fig. 4 *b*) was not identified. It may correspond, either to a small quantity of impurity or to a breakdown product, other than C, resulting from the treatment with trypsin.

The results of the foregoing experiment suggest that treatment of the proteinase precursor with trypsin results in the production of two new electrophoretic components: one represents the active enzyme, the other, a modified form of the original precursor protein from which it differs in electrophoretic

mobility but which it resembles in serological specificity. Before ascribing this change in mobility to the action of trypsin it was essential to know whether the mobility of the precursor protein remained constant at pH 7.8 in the absence of trypsin. Two further electrophoretic examinations of this precursor preparation were therefore made 5 and 41 days respectively after the experiment in which part of it had been treated with trypsin; *i.e.*, 10 and 46 days after the stock solution had been prepared. This stock solution in veronal buffer pH 7.8 contained sodium iodoacetate in a concentration of 10^{-3} M and had been kept throughout at 5°C. The mobilities of the two components, precursor and active enzyme, determined at each examination of this material are shown in Table III.

TABLE III

Effect of Time at pH 7.8 (Veronal Buffer) on Mobility of Enzyme Protein

Preparation	Time at pH 7.8 (veronal buffer)	Electrophoretic mobility $U \times 10^5$	
		Precursor component	Active proteinase component
	days		
Precursor	2	−0.47	+0.98
"	10	−0.45	+1.12
"	46	−0.57	+1.37
"			
Treated with trypsin	5	−0.76	+1.16

It will be seen that during the first 10 days no significant change occurred in the mobility of either component. The observations on the effect of trypsin on this preparation were made on the 5th day of this period and the resulting changes in mobility may therefore be ascribed to the action of trypsin. After 46 days a small but significant increase had occurred in the mobility of each component. The electrophoretic pattern showed no change in the relative concentrations of precursor and active enzyme.

A similar but more pronounced change of mobility occurred in another precursor preparation maintained at 5°C. in veronal buffer pH 8.6 for 38 days. During this period the mobility of the precursor component increased from -0.96 to -1.84 $U \times 10^5$.

In neither case was there any change in the electrophoretic pattern of the untreated material that would indicate conversion of precursor to active enzyme. Indeed, both precursor preparations contained sodium iodoacetate in order to minimize this possibility. A single small colony of a mould was seen in the first preparation at the end of the 46 day period. The second preparation proved to be bacteriologically sterile on culture.

The reason for this change in mobility of untreated precursor preparations is not clear but it may be due to the effect of pH on the stability of the enzyme

protein. Evidence has already been presented to show increasing instability of both the proteinase and its precursor with increasing deviation of pH from neutrality (3).

DISCUSSION

The present study has provided additional evidence that the streptococcal proteinase and its precursor are two substances, distinct and separable. Subjected to electrophoresis, mixtures of the two proteins present patterns in which each appears as a separate component with characteristic mobility and isoelectric point. Withdrawn from the mixture after electrophoresis, each component can be identified by its distinctive immunological specificity. Under appropriate conditions, the precursor is converted to the active enzyme with a corresponding change in electrophoretic mobility. Furthermore, it has previously been shown that the two substances can be distinguished one from the other on the basis of biological activity, solubility, and crystal form (1).

In view of these distinguishing characteristics of the enzyme and its precursor, it is, perhaps, of interest to consider the evidence suggestive of an immunological relationship between the two substances. It has previously been shown that the active enzyme behaves immunologically as a single antigen, whereas precursor preparations have two antigenic components, one specific, the other serologically identical with the proteinase antigen (1). In the experiments described here, the precursor antiserum (R_2) had been absorbed with proteinase and hence reacted specifically with precursor; the proteinase antiserum (R_5) reacted both with proteinase and with precursor preparations. Hitherto, it had seemed probable that the reaction between proteinase antiserum and precursor preparations was due to the small amount of active enzyme with which the latter had been found always to be contaminated; this contaminating proteinase appeared as a separate component in electrophoretic patterns of a precursor preparation described in this report (Fig. 4). It was therefore surprising to find that a sample of the precursor component withdrawn from the cell after electrophoresis of this preparation also reacted with the proteinase antiserum (R_5), as well as with the precursor specific antiserum (R_2). Two possible explanations suggest themselves: first, that the precursor sample was contaminated with active enzyme either accidentally, during its withdrawal from the cell, or unavoidably, due to incomplete separation of the two components by electrophoresis; second, that the apparently homogeneous precursor component had two antigenic groups, one reacting with the precursor, the other with the proteinase antiserum. As regards the first interpretation, it may be mentioned that samples of the proteinase component which were also withdrawn from this preparation (Fig. 4, B and B′) contained little or no precursor as shown by the fact that they reacted weakly, if at all, with the specific precursor antiserum (R_2). Furthermore, samples of the precursor component withdrawn from the cell after electrophoresis of other preparations

always reacted with both proteinase and precursor antisera. The second interpretation, although to some extent speculative, is supported by the knowledge that complex substances may induce the production of more than one kind of antibody. It, therefore, seems possible that, despite the many distinguishing characteristics between the enzyme and the precursor from which it is derived, an immunological relationship exists between the two substances.

The nature of the change in chemical structure which accompanies conversion of the precursor to the active enzyme remains unknown, but it appears that by inducing this change with trypsin a modified form of the precursor is also produced. This differs from the original precursor protein in electrophoretic mobility but resembles it in serological specificity; it is resistant to trypsin but can be converted to the active enzyme by the streptococcal proteinase itself (1). For this reason the modified form of the precursor was not seen in preparations converted autocatalytically to the active enzyme for, under such conditions, conversion to the active enzyme is complete.

SUMMARY

An electrophoretic study of crystalline preparations of a streptococcal proteinase and its precursor established their isoelectric points at pH values of 8.42 and 7.35 respectively (ionic strength 0.10).

Preparations of the proteinase appeared to be electrophoretically homogeneous over a pH range of 5 to 8.5. Precursor preparations contained a relatively low concentration of the active enzyme visible as a separate peak in electrophoretic patterns of sufficiently concentrated solutions.

Autocatalytic conversion of precursor to active enzyme was complete and resulted in a corresponding change in the electrophoretic pattern.

Treatment of precursor preparations with trypsin produced incomplete conversion to the active enzyme and resulted in the formation of a modified precursor protein. This differed from the parent substance in electrophoretic mobility and in susceptibility to trypsin, but resembled it in immunological specificity and, as previously shown, in susceptibility to conversion to active enzyme by autocatalysis.

Serological reactions of precursor and active enzyme components withdrawn from the cell after electrophoresis are described. It appears that the precursor protein may have two antigenic groups, one specific, the other shared by the active enzyme which behaves as a single antigen.

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