THE CRYSTALLIZATION AND SEROLOGICAL DIFFERENTIATION OF A STREPTOCOCCAL PROTEINASE AND ITS PRECURSOR*

By S. D. ELLIOTT,‡ M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

PLATE 10

(Received for publication, May 31, 1950)

Grown under suitable conditions, group A streptococci may produce an extracellular proteolytic enzyme similar in some respects to papain and the cathepsins (1). In a previous communication (2), evidence was presented suggesting that the proteinase was derived from an inactive precursor but it was emphasized that further evidence for this relationship awaited the isolation of both in a more highly purified state. It is the purpose of this report to describe methods by which both the proteinase and its precursor have been isolated in crystalline form and to characterize each in terms of biological activity and immunological specificity.

Methods

Cultures.—A group A streptococcus, strain "5797," has been used throughout this work. This strain of streptococcus was selected because it produced a good yield of the proteinase and had been used in previous published work on this subject (2). The cultures were grown in dialysate broth (3) in the preparation of which Pfanstiehl peptone was used. The medium was enriched by the addition of a mixture of growth factors and salts referred to as "addition mixture" and identical with that used by Adams and Roe for the growth of pneumococci (4).

Estimations of Total Protein in Enzyme Preparations.—These were made by the use of the phenol reagent. To 0.4 cc. of enzyme preparation appropriately diluted in water were added 0.6 cc. phenol reagent (Folin and Ciocalteu) diluted 1:3 in water, and 1.6 cc. of a 14 per cent solution of Na_2CO_3. In a control tube, 0.4 cc. water was substituted for the enzyme preparation. The intensity of the blue color developed after 10 minutes was measured in a Coleman spectrophotometer and translated into tyrosine equivalents by comparison with a standard curve.

Proteinase Determinations.—The proteolytic activity of culture fluids and of the fractions obtained in the course of their purification was measured by their ability either to digest casein (1) or to clot milk (2).

Digestion of Casein.—To 2.7 cc. of a 2 per cent solution of casein pH 8, was added 0.15 cc. enzyme preparation. Of the mixture 0.95 cc. was taken into each of two tubes; to one was added 0.05 cc. KCN (0.4 M, pH 7.0), to the other 0.05 cc. H_2O. A control tube contained

* This work was supported by a grant from the Helen Hay Whitney Foundation.

‡ Visiting Investigator at The Rockefeller Institute for Medical Research. Formerly Reader in Bacteriology at London Hospital Medical College.

0.9 cc. casein solution, 0.05 cc. KCN, and 0.05 cc. H_2O. All three tubes were incubated at 37°C. for 60 minutes. To each tube was then added 1.0 cc. of 5 per cent trichloracetic acid, and the resulting precipitate removed by centrifugation. The supernate from each tube was boiled for 30 minutes to remove HCN and then made up to its original volume with H_2O. The concentration of soluble tyrosine was then determined by the same method as was used in estimating the total protein.

Hydrolysis of Benzoyl Arginineamide.—The activity of more highly purified enzyme preparations was measured by the hydrolysis of α-benzoyl-*l*-arginineamide hydrochloride following the method of Bergmann, Fruton, and Pollock for the estimation of papain (5). The substrate was prepared from benzoyl-*l*-arginine (5), for a liberal supply of which I am indebted to Dr. Stanford Moore.[1]

Proteinase precursor was estimated from its milk-clotting activity following treatment for 60 minutes with crystalline trypsin in a final concentration of 0.001 mg. per cc. at 37°C. and pH 8.0 (2).

Precipitin tests were carried out using the capillary pipette technique (6).

EXPERIMENTAL

Production of Proteinase Precursor in Broth Cultures of Group A Streptococci.—The optimal conditions for production of precursor are found in broth cultures which achieve maximal growth in a slightly acid environment: at a neutral or alkaline reaction little or no precursor is liberated into the culture fluid. This is in striking contrast to the conditions under which streptokinase and desoxyribonuclease are elaborated by streptococci, for both these metabolites are produced during growth in a neutral or slightly alkaline milieu. The following procedure has therefore been adopted.

One liter of dialysate broth, pH 7.8, is inoculated with 10 cc. of a young broth culture of strain 5797. After about 10 hours' incubation at 37°C. the following solutions, sterilized by filtration, are added: 1 liter of dialysate broth previously warmed to 37°C.; 200 cc. of "addition mixture" (see Methods) (4); 200 cc. of a 50 per cent solution of glucose; sufficient 10 per cent sodium bicarbonate solution to bring the reaction to neutrality. Incubation of the culture is then continued at 37°C. and the reaction allowed to fall to a level between pH 5.5 and 6.5, within which range it is thereafter maintained by frequent additions of sodium bicarbonate solution. Periodically, samples are taken from the culture and tested for proteinase precursor as described under Methods. Incubation of the main streptococcal culture is continued until a trypsin-treated sample, in a dilution of 1 in 5, clots an equal volume of milk-thioglycollate mixture in 10 minutes. This stage is reached about 8 to 10 hours after the addition of glucose and "growth factors." The culture is then chilled, filtered, and the precursor precipitated by the addition of ammonium sulfate to 0.8 saturation. Delay at this stage may result in precursor being converted to active proteinase autocatalytically under the reducing conditions produced by the heavy bacterial growth (2).

The streptococci harvested from the culture may be used for further precursor production by suspending them in 2 liters of a dialysate of Pfanstiehl peptone, pH 7.0, containing 5 per cent of glucose but without any additional nutrients. The suspension is incubated at 37°C. and, by the addition of sodium bicarbonate, the reaction maintained between pH 5.5 and 6.5

[1] I am indebted to Dr. J. S. Fruton and to Dr. J. Weisiger for advice and help in preparing this compound.

until a suitable yield has been obtained. This usually takes from 2 to 4 hours. When not in use, the streptococci are preserved as a thick suspension in broth or peptone dialysate at −40°C. By serial passage in glucose peptone dialysate the cells from an original culture of 2 liters may yield an additonal 10 liters of potent precursor-containing filtrate.

Crystallization of Proteinase Precursor.—The precursor is completely precipitated from culture filtrates by the addition of ammonium sulfate to 0.8 saturation. It is readily soluble in 0.5 saturated ammonium sulfate at 2°C. and pH 5.0; it is relatively insoluble in this concentration of salt at 22-37°C. and pH 8.0. Under the latter conditions the precursor crystallizes from sufficiently concentrated solutions in the form of long needles (Fig. 1). In the cold it fails to crystallize but precipitates in amorphous form at ammonium sulfate concentrations exceeding 0.55 saturation.

The solubility of the precursor in cold, half-saturated ammonium sulfate is an important feature of the purification procedure: it makes possible the early separation of highly colored, insoluble, extraneous material; furthermore, ammonium sulfate in this concentration prevents the autocatalytic conversion of precursor to proteinase. After initial precipitation from culture filtrates, therefore, the precursor is kept in half-saturated ammonium sulfate, and its crystallization or solution achieved by alterations of temperature and hydrogen ion concentration.

In order to study the precursor in greater detail it was obviously desirable to separate the crystalline material from ammonium sulfate. Dialysis against water was impracticable owing to the rapid conversion of precursor to proteinase which takes place in highly concentrated aqueous solutions. This reaction is inhibited in the presence of ethanol. It was therefore possible to remove ammonium sulfate from the recrystallized precursor preparations by dialysis against 20 per cent ethanol in the cold. When salt-free, the precursor was precipitated from solution by increasing the ethanol concentration to 70 per cent. The precipitate was removed by cold centrifugation and rapidly dissolved in water at 2°C., immediately frozen, and then dried under vacuum from the frozen state.

To 10 liters of a suitable precursor-containing culture filtrate was added 5610 gm. $(NH_4)_2SO_4$ to produce 0.8 saturation. After 2 days at 2°C. the precipitate was removed by filtration with filtercel through paper and dissolved in 50 cc. of cold 0.45 saturated $(NH_4)_2SO_4$, pH 5.0. Insoluble material was discarded and the clear filtrate dialyzed at room temperature against 0.5 saturated $(NH_4)_2SO_4$, pH 8.0. Crystallization commenced within an hour of the temperature reaching approximately 22°C. and was even more rapid at a temperature of 37°C. By increasing the salt concentration to 0.55 saturation, over 70 per cent of the precursor was recovered in crystalline form within 3 days.

After washing the crystals at room temperature in 5 to 10 volumes 0.5 saturated $(NH_4)_2SO_4$, pH 8.0, recrystallization was achieved by dissolving them in 25 cc. cold 0.5 saturated $(NH_4)_2SO_4$, pH 5.0, discarding insoluble material, and then placing the clear solution at room temperature and pH 8.0, under which conditions crystallization commenced almost immediately.

After six recrystallizations the precursor solution was dialyzed against 20 per cent ethanol at 2°C. until salt-free. The ethanol concentration was then increased to 70 per cent, the resulting precipitate removed by cold centrifugation and dissolved rapidly in 20 cc. ice cold water. The solution was then immediately frozen and dried under vacuum from the frozen state. It yielded 169 mg. of a white material with a nitrogen content of 15.19 per cent.

Desoxyribonuclease Activity of Recrystallized Precursor Preparation.—Group A streptococci produce desoxyribonuclease (7, 8), and a high degree of activity attributable to this enzyme was found in the culture filtrates used as a source of proteinase precursor. As pointed out by McCarty (8), measurement of nuclease activity provides a delicate and simple test for the efficacy of fractionation procedures employed in the isolation of other extracellular streptococcal products. This is especially so in the case of the proteinase precursor, since both are precipitated from solution in approximately the same range of ammonium sulfate concentration.

The nuclease activity of a precursor preparation after six recrystallizations was approximately 0.01 per cent of that of the original crude ammonium sulfate concentrate.[2] On the basis of the known activity of purified preparations of streptococcal desoxyribonuclease it was calculated that this enzyme was responsible for less than 0.03 per cent of the dry weight of the recrystallized precursor preparation.

The precursor content of the recrystallized precursor preparation was approximately 3 per cent that of the original. In considering the relatively poor yield of precursor after repeated recrystallization, it should be stated that only about 50 per cent is recovered by crystallization from half-saturated ammonium sulfate. The advantage of a greater yield from 0.55 saturation is offset by the proportionate decrease in purity. In particular, contamination with desoxyribonuclease was significantly greater in precursor preparations crystallized at the higher salt concentration.

Proteinase Production in Broth Cultures of Group A Streptococci.—In the production of active proteinase the procedure is the same as that outlined for the preparation of proteinase precursor, but incubation of the culture is continued until the enzyme precursor has been converted autocatalytically into active proteinase under the reducing conditions prevalent in the culture during the later stages of growth.

Crystallization of Proteinase.—Like its precursor, the proteinase is completely precipitated from broth culture filtrates by 0.8 saturation with ammonium sulfate. In contrast to the precursor, at lower salt concentrations the proteinase is relatively less soluble in the cold than at room temperature. From suitably concentrated solutions it crystallizes in the form of short needles at 0.15 saturation with ammonium sulfate, pH 8.0, and 2°C. (Fig. 2). More than 70

[2] These and subsequent nuclease determinations were kindly made by Dr. Maclyn McCarty.

per cent of the active material is recovered in crystalline form when the salt concentration is raised to 0.40 saturation. At room temperature or at pH 5.0 approximately 0.25 saturation with ammonium sulfate is necessary to initiate crystallization.

To 10 liters of dialysate broth culture filtrate was added 5610 gm. $(NH_4)_2SO_4$ to produce 0.80 saturation. After 2 days at 2° C. the precipitate was recovered by filtration with filtercel through paper and taken up in 100 cc. of 0.1 saturated $(NH_4)_2SO_4$ adjusted to pH 5 by the addition of HCl. Insoluble material was discarded and the clear filtrate adjusted to pH 8.0. Crystallization commenced within 30 minutes at 2°C. Small increments of $(NH_4)_2SO_4$ were made until, after 3 days, 0.4 saturation had been reached.

The crystals were washed in cold 0.4 saturated $(NH_4)_2SO_4$ at pH 8, redissolved in 70 cc. of water, and insoluble material discarded. The clear solution was then brought to pH 8 by the addition of NH_4OH and to 0.4 saturation with $(NH_4)_2SO_4$. With successive recrystallizations the active material became progressively less soluble in water but dissolved readily in saline, which was therefore used as a solvent in the later stages of the purification.

After six recrystallizations the active material was dissolved in 25 cc. saline and dialyzed against saline until free from $(NH_4)_2SO_4$. The solution was then frozen and dried under vacuum, yielding 525 mg. of a white powder of which 212.5 mg. was NaCl by calculation. The nitrogen content of the dried product after correction for NaCl was 15.35 per cent.

Isolation of Crystalline Proteinase from Undialyzed Broth Culture Filtrates.—Although the use of dialysate broth is advantageous in that it excludes much extraneous protein from culture filtrates, it is, nevertheless, possible to crystallize the proteinase from concentrated solutions prepared from the filtrates of cultures in undialyzed media. A concentrated enzyme solution is prepared by ammonium sulfate precipitation of the active material. After removal of residual ammonium sulfate by dialysis the enzyme preparation is incubated at 37°C. for several hours in the presence of a suitable activator such as sodium thioglycollate. By this procedure the extraneous protein is digested by the active proteinase and the digestion products may subsequently be removed by dialysis. Thereafter, the proteinase readily crystallizes from solution under the same conditions as have already been described for preparations made from dialysate broth cultures.

Evidence of Effective Separation Following Repeated Recrystallization of Proteinase. 1. Ratio of Total Protein to Proteolytic Activity.—Evidence of separation of the proteinase from extraneous protein may be found by comparing the total protein with the proteolytic activity in successive enzyme preparations following recrystallization. Total protein was estimated by the use of the phenol reagent, and proteolytic activity by the digestion of casein as described under Methods.

From Table I it will be seen that a considerable elimination of extraneous protein resulted from the first crystallization of the proteinase. Thereafter, successive recrystallizations did not result in any significant alteration in the tyrosine ratios.

2. Desoxyribonuclease Activity of Recrystallized Proteinase Preparations.—As with the precursor, so with the proteinase, desoxyribonuclease activity was used as a measure of the separation achieved by crystallization. The first crystallization resulted in a 100-fold decrease in nuclease activity; after five recrystallizations none was demonstrable in a highly concentrated proteinase preparation which retained more than 50 per cent of the original proteolytic activity.

In comparing this result with the less satisfactory separation of precursor

TABLE I

Comparison of Proteolytic Activity and Total Protein Content of Proteinase Preparations before and after Crystallization

Enzyme preparation	Proteolytic activity Milliequivalents of tyrosine	Total protein Milliequivalents of tyrosine	Ratio $\frac{\text{Proteolytic activity}}{\text{Total protein}}$
1st concentrate	0.138×10^{-3}	0.118×10^{-3}	1.17
Mother liquor after 1st crystallization	0.145×10^{-3}	0.195×10^{-3}	0.74
Solution of crystals 1st crystallization	0.148×10^{-3}	0.053×10^{-3}	2.79
Solution of crystals 2nd crystallization	0.190×10^{-3}	0.087×10^{-3}	2.18
Solution of crystals 3rd crystallization	0.130×10^{-3}	0.050×10^{-3}	2.60
Solution of crystals 4th crystallization	0.153×10^{-3}	0.062×10^{-3}	2.47
Solution of crystals 5th crystallization	0.170×10^{-3}	0.085×10^{-3}	2.0

from nuclease it must be remembered that the precursor crystallizes from solution at the same concentration of ammonium sulfate as precipitates the nuclease (0.5 saturation). The proteinase, on the other hand, crystallizes at lower salt concentrations, between 0.15 and 0.4 saturation, under which conditions an effective separation would be expected.

Milk-Clotting Activity of Precursor and Proteinase.—As already reported, the streptococcal proteinase coagulates milk and does so most readily in the presence of sulfhydryl compounds or cyanide (2). The proteinase precursor is inactive but may be converted into the active enzyme either by trypsin or, autocatalytically, by incubation under reducing conditions.

The clotting activity of precursor and proteinase preparations, each six times recrystallized, is shown in Table II.

In these experiments the enzyme preparations were dissolved and serial dilutions made in saline; in the absence of saline, milk coagulation was greatly retarded. The precursor preparations in a concentration of 1.0 mg. per cc. were first incubated at 37°C. and pH 8.0 with either trypsin or sodium thioglycollate for 60 minutes, and serial dilutions then made in saline. Equal volumes of the diluted enzyme preparation and of milk substrate, with or without

TABLE II

Coagulation of Milk by Crystalline Proteinase and Precursor

Enzyme preparation	Proteinase activator* added to milk	Proteinase or precursor concentration, *mg. per cc.*							
		0.5	0.25	0.125	0.063	0.032	0.012	0.006	0.003
		Coagulation time, *min.*							
Proteinase	+						4	8.5	20
"	−						>60	>60	>60
Precursor	+	50	50	>60	>60				
"	−	No clot							
Precursor after preliminary incubation with trypsin	+	4	5	8	13	19	32		
Precursor after preliminary incubation with thioglycollate	+		2.5	4	7	14	36		

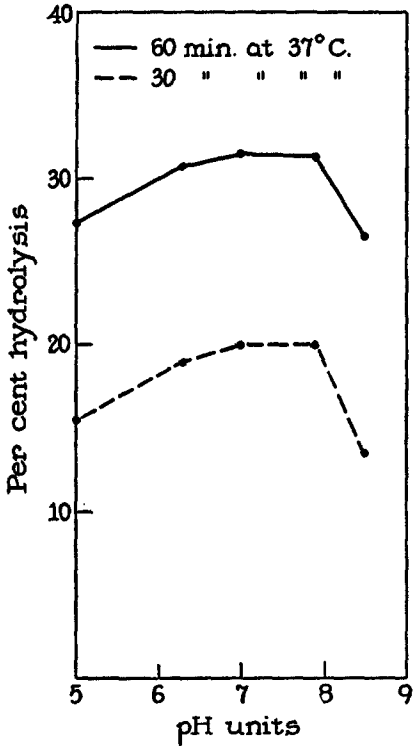
* Sodium thioglycollate in a final concentration of 0.1 M.

sodium thioglycollate, were then incubated together at 37°C., pH 7.0, and the coagulation time noted as previously described (2). Sodium thioglycollate was used in a final concentration of 0.1 M and crystalline trypsin in a concentration of 0.001 mg. per cc.

It will be seen that the clotting activity of the proteinase is greatly enhanced by the addition of sodium thioglycollate. With the precursor preparations, clotting activity became manifest after treatment with trypsin or sodium thioglycollate. The late coagulation which occurred when untreated precursor was incubated with milk in the presence of thioglycollate is attributable to the autocatalytic conversion of precursor to proteinase during the initial stages of incubation.

It will be noted that the clotting activity of the precursor sample treated with trypsin was less than that of the sample treated with thioglycollate. Whether this is due to incomplete conversion of precursor to proteinase by trypsin or to other unknown factors has not been established.

Hydrolysis of α-benzoyl-l-Argininineamide by Crystalline Proteinase.—It has been shown by Bergmann and Fruton (9) that proteinases exhibit differing chemical specificities in their capacity to hydrolyze certain synthetic polypep-



TEXT-FIG. 1. Hydrolysis of benzoyl arginineamide by streptococcal proteinase in presence of cysteine at various pH levels. Enzyme concentration 0.1 mg. proteinase N per cc.

tides. In the presence of a suitable activator the streptococcal proteinase hydrolyzes α-benzoyl-*l*-arginineamide, but has no activity against *l*-leucylglycylglycine (1). These observations resulted from experiments with crude enzyme preparations prepared by ammonium sulfate precipitation of culture filtrates. The activity of the proteinase against benzoyl arginineamide has now been confirmed with a six times recrystallized preparation of the enzyme.

α-benzoyl-*l*-arginineamide was used throughout in a concentration of 0.05 mM per cc. The following buffer solutions were employed in a final concentration of 0.01 M: sodium citrate,

pH 5.0 and 6.3; sodium phosphate, pH 6.95 and 7.86; sodium veronal, pH 8.6. Cysteine was employed as an activator in a final concentration of 0.02 M. The proteinase was used in a final concentration of 0.1 mg. enzyme nitrogen per cc. Hydrolysis of the substrate was measured by the microtitration of liberated carboxyl groups according to the method of Grassmann and Heyde (10).

The results of a typical experiment are illustrated in Text-fig. 1. This shows the percentage hydrolysis of benzoyl arginineamide after incubation for 30 minutes and 60 minutes with the proteinase at 37°C. in the presence of cysteine at various hydrogen ion concentrations. In the absence of cysteine no hydrolysis occurred. In the presence of cysteine it will be seen that the enzyme was active over a pH range of 5.0 to 8.6, but that hydrolysis of benzoyl arginineamide occurred optimally between pH 7 and 8. It has previously been shown that the proteinase is unstable at 37°C. outside the range of hydrogen ion concentration used in this experiment (2).

Activity of Proteinase Precursor against Benzoyl Arginineamide.—When incubated with milk under reducing conditions the precursor is autocatalytically converted to active proteinase which subsequently clots the milk. This system is complex and does not lend itself readily to precise analysis. The use of benzoyl arginineamide as a substrate afforded a means of following more closely the change in activity which occurs when the precursor is incubated either under reducing conditions or with trypsin. A thrice recrystallized precursor preparation was used in these experiments.

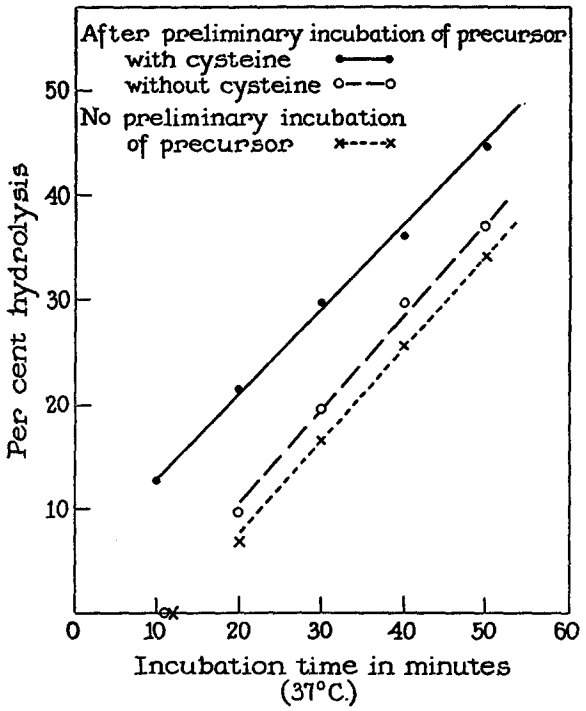
1. Conversion of Precursor to Proteinase under Reducing Conditions.—The activity of the precursor against benzoyl arginineamide was tested without prior treatment and after previous incubation with and without cysteine.

In 5.1 cc. cold citrate buffer (0.02 M, pH 6.0), 24 mg. precursor was dissolved, and divided equally into three parts—A, B, and C. To A was added 0.4 cc. cysteine solution (0.1 M, pH 7.0); to B and C, 0.4 cc. water. A and B were incubated at 37°C. for 60 minutes and then chilled; C was kept chilled throughout. Now 0.4 cc. cysteine solution was added to B and C, and 0.4 cc. water to A. Finally, 41.5 mg. benzoyl arginineamide was dissolved in the contents of each tube, and hydrolysis followed during incubation of all three at 37°C. The final concentration of precursor nitrogen was 0.5 mg. per cc.

The results of this experiment are illustrated in Text-fig. 2. In preparation A, containing precursor subjected to preliminary incubation with cysteine, hydrolysis of benzoyl arginineamide proceeded from the outset of the final period of incubation; here, precursor had been converted to proteinase during its preliminary incubation with cysteine. In C, containing precursor not subjected to any preliminary treatment, there elapsed a period of from 10 to 20 minutes at 37°C. under reducing conditions before hydrolysis became apparent. Clearly, during this time, precursor was being converted to proteinase, for thereafter hydrolysis proceeded at the same rate as in A. Preliminary incubation for 60 minutes in the absence of cysteine (C) did not result in the conversion

of precursor to proteinase, although some slight change may have occurred as shown by the somewhat shorter period necessary for the initiation of hydrolysis as compared with preparation C when both were subsequently incubated with benzoyl arginineamide under reducing conditions.

The conversion of precursor to proteinase which takes place under reducing conditions is probably autocatalytic in nature, but the initial stages of the

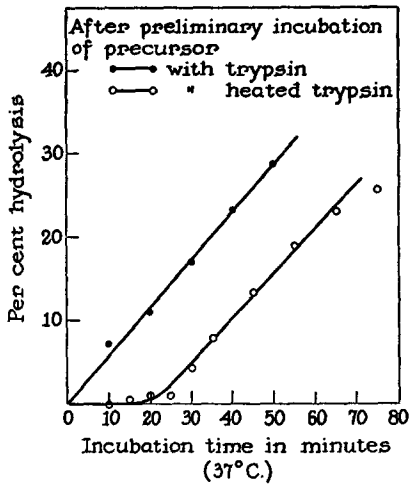


TEXT-FIG. 2. Conversion of precursor to proteinase under reducing conditions. Hydrolysis of benzoyl arginineamide in presence of cysteine at pH 6. Enzyme concentration 0.5 mg. precursor N per cc.

reaction are difficult to detect by the hydrolysis of benzoyl arginineamide. In experiments similar to the above, but using the precursor in lower concentrations, a corresponding increase was noted in the time required for conversion to the active proteinase. For example, at pH 6.0, from 20 to 40 and from 80 to 100 minutes were necessary for precursor nitrogen concentrations of 0.2 and 0.1 mg. per cc. respectively.

2. Conversion of Precursor to Proteinase by Trypsin.—The conversion of precursor to proteinase by trypsin has already been described. This reaction has now been confirmed using benzoyl arginineamide as a substrate for the streptococcal enzyme.

Eight mg. precursor was dissolved in 3.8 cc. phosphate buffer 0.025 M, pH 8, and divided equally between two tubes. To one was added 0.25 cc. of a solution of crystalline trypsin to give a final concentration of 0.001 mg. per cc.; to the other was added the same amount of trypsin previously inactivated by heating to 100°C. for 5 minutes at pH 8. Both tubes were now incubated at 37°C. for 25 minutes and then chilled. In each, 41.5 mg. benzoyl arginineamide was then dissolved before adding 0.35 cc. cysteine solution, pH 7.0, such that its final concentration was 0.04 M, Both tubes were now reincubated at 37°C. and the hydrolysis of benzoyl arginineamide followed. A control tube without precursor but containing benzoyl arginineamide, trypsin, cysteine, and buffer was included in the experiment. The final concentration of precursor nitrogen was 0.25 mg. per cc.



TEXT-FIG. 3. Conversion of precursor to proteinase by trypsin. Hydrolysis of benzoyl arginineamide in presence of cysteine at pH 8.0. Enzyme concentration 0.25 mg. precursor N per cc. 0.001 mg. trypsin per cc.

The results of this experiment are presented in Text-fig. 3. In the tube containing precursor which had been subjected to preliminary incubation with trypsin, hydrolysis of the benzoyl arginineamide started immediately; in the tube containing precursor treated with heated trypsin, hydrolysis did not commence until after 15 to 30 minutes' incubation under reducing conditions; in the control tube containing trypsin but no precursor, no hydrolysis of the substrate was apparent after 24 hours' incubation. It may be inferred that the precursor was converted to proteinase during its preliminary incubation with trypsin. In the tube containing heated trypsin this did not occur but, as in the previous experiment, the conversion of precursor to active enzyme took place during the initial 15 to 30 minutes of its incubation with benzoyl arginineamide under reducing conditions.

Serological Reactions of Precursor and Proteinase.—It seemed possible that further information concerning the relationship of the precursor to the active

enzyme might be obtained from a study of their serological reactions with antisera prepared against both substances. In the experiments to be described a thrice recrystallized precursor preparation and a six times recrystallized proteinase preparation were used throughout.

Two rabbits, R_1 and R_2, were immunized with the precursor preparation. During a period of 2 weeks each rabbit received four 1.0 mg. doses, followed by three 5.0 mg. doses. On each occasion the dried precursor material was dissolved in 1.0 cc. saline and injected intravenously. Two rabbits, R_3 and R_4, were immunized with the active enzyme. R_3 received during a period of 3 weeks five 1.0 mg. doses; R_4 in a period of 2 weeks received two doses each of 1.0 mg., two of 2.0 mg., and three of 5.0 mg. In both rabbits the proteinase was given in the form of a saline solution of which 1.0 cc. was injected intravenously on each occasion. Each of the four rabbits was bled 5 days after receiving its final injection.

TABLE III

Precipitin Reactions with Precursor and Proteinase Antisera

Antiserum			Precipitin reactions Antigen diluted							
Prepared with	Absorbed with	Tested with	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Precursor (R_2)		Precursor	+++	+++	++++	++++	+++	++	++	±
"		Proteinase	±	+	+	++	++	+	+	±
"	Proteinase	Precursor	++	++	+++	+++	+++	++	++	+
"	"	Proteinase	−	−	−	−	−	−	−	−
Proteinase (R_4)		Precursor	+	+	++	++	+	±	±	−
"		Proteinase	+	++	+++	++	++	+	+	±
"	Precursor (once)	Precursor	−	−	±	−	−	−	−	−
"	" "	Proteinase	−	−	−	+	+	+	±	−
"	Precursor (twice)	Precursor	−	−	−	−	−	−	−	−
"	" "	Proteinase	−	−	−	−	±	±	−	−

In all precipitin tests, precursor and proteinase were used in an initial concentration of 1 mg. per cc. saline.

± to ++++ indicates various strengths of precipitin reactions.

All four rabbits developed precipitins against the material with which they had been inoculated. The reactions of the serum from one rabbit of each pair are shown in Table III. It will be seen that in each case the serum reacted strongly with the homologous antigen. The serum prepared against the precursor (R_2) reacted less strongly with the proteinase preparation, and the same applied to the reaction of the proteinase antiserum (R_4) with the precursor preparation. The significance of these observations was clarified by the results of a cross-absorption experiment in which the precursor antiserum was absorbed with the proteinase and the proteinase antiserum with the precursor.

The concentrations of precursor and proteinase used in absorbing the sera were in each case approximately twice the concentration necessary for optimal precipitation. To 0.9 cc. precursor antiserum (R_2) was added 0.1 mg. proteinase in 0.1 cc. saline; to 0.9 cc. proteinase antiserum (R_4) was added 0.2 mg. precursor in 0.1 cc. saline. Both sera were left overnight at refrigerator temperature, after which the precipitate was removed by centrifugation. Precipitin reactions showed that the absorption of serum R_4 by precursor was incomplete. It was therefore reabsorbed with the same amount of precursor as before.

The results of precipitin tests with the absorbed sera are shown in Table III. It will be seen that a single absorption of the precursor antiserum (R_2) with proteinase removed all precipitins for the proteinase without altering significantly the serum's capacity to react with the precursor. This was in marked contrast to the result of the parallel experiment in which proteinase antiserum (R_4) was absorbed with precursor; after a single absorption the proteinase antiserum reacted only slightly with precursor but its capacity to react with the proteinase was also greatly reduced; after reabsorption it failed to react with precursor and gave only a very weak reaction with proteinase.

From the results of these experiments the following conclusions were drawn: the active proteinase behaved immunologically as a single antigen; the precursor preparation had two antigenic components, one of which was specific and the other serologically identical with the proteinase antigen.

Changes in Serological Activity of Precursor after Treatment with Trypsin or Thioglycollate.—It has already been shown that treatment with trypsin or sodium thioglycollate induces a marked alteration in the enzymatic activity of the proteinase precursor. This change is attributed to the conversion of the precursor to the active enzyme. Since these two substances have been shown to differ immunologically, it was of interest to follow in a precursor preparation the changes in serological reactivity that might occur as a result of its treatment by these methods. Two experiments were performed with this objective; a six times recrystallized precursor preparation was used in each.

To 1.8 cc. of a saline solution of precursor, 1.0 mg. per cc., was added 0.2 cc. of a solution of crystalline trypsin such that its final concentration was 0.001 mg. per cc. To another 1.8 cc. of the precursor solution was added 0.2 cc. 1.0 M sodium thioglycollate solution. The reaction of both mixtures was adjusted to pH 8.0 before incubating them for 4 hours at 37°C. Serial dilutions of the untreated and the trypsin-treated preparations were made in saline; similar dilutions of the thioglycollate-treated preparation were made in saline containing sodium iodoacetate, 10^{-4} M, in order to prevent proteolytic activity from interfering with the precipitin reactions.

The serological reactions of the precursor preparations before and after incubation with either trypsin or sodium thioglycollate are shown in Table IV. The preparations were tested against two immune sera described in the previous experiment: serum R_2, prepared against precursor, had been absorbed with proteinase and retained only precursor-specific antibody; serum R_4 had

been prepared against the proteinase. It will be seen that before treatment the precursor preparation reacted strongly with both sera and may therefore be regarded as containing both precursor and proteinase antigens. Treatment with trypsin led to a slightly weaker reaction with the precursor antiserum (R_2) and a stronger reaction with the proteinase antiserum (R_4). It has already been shown that complete conversion of precursor to proteinase is not achieved by treatment with trypsin. Incubation with sodium thioglycollate, by which full conversion to proteinase is achieved, resulted in complete loss of reactivity with the precursor antiserum and a very strong reaction with the proteinase

TABLE IV

Conversion of Precursor to Proteinase by Treatment with Trypsin or Thioglycollate

Antigen used in precipitin tests	Precipitin reactions Antiserum prepared with															
	Precursor* (R_2)								Proteinase (R_4)							
	Antigen diluted								Antigen diluted							
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Precursor	++	++	+++	+++	+++	++	++	+	++	++	+++	+++	++	++	+	±
Precursor treated with trypsin	+	+	++	++	++	++	+	+	+++	+++	+++	+++	++	++	+	±
Precursor treated with thioglycollate	−	−	−	−	−	−	−	−	++++	++++	++++	++++	+++	++	+	±
Proteinase	−	−	−	−	−	−	−	−	++++	++++	++++	++++	+++	+++	+	±

* This serum had been absorbed with proteinase and consequently reacted specifically with precursor.
± to ++++ indicates various strengths of precipitin reactions.

antiserum. Indeed, the reactions of this preparation were identical with those of the crystalline proteinase.

Under reducing conditions precursor is converted to proteinase. This reaction is autocatalytic and is initiated by small amounts of proteinase always present in precursor preparations (2). The enzymatic activity of the proteinase is inhibited by iodoacetic acid (1). In the next experiment, therefore, the serological reactivity of a precursor preparation incubated under reducing conditions was compared with that of an identical preparation to which, in addition, sodium iodoacetate had been added.

To 3.2 cc. of a solution of precursor in saline, 1.0 mg. per cc., was added 0.4 cc. neutral sodium thioglycollate, 1.0 M. The mixture was divided equally into two parts: to one was added 0.2 cc. neutral sodium iodoacetate, 10^{-3} M; to the other, 0.2 cc. saline. Both solutions were adjusted to pH 7.0 and incubated at 37°C. At intervals, samples were taken from each and serially diluted in saline containing neutral sodium iodoacetate in a concentration of 10^{-4} M.

The diluted samples were then tested for precipitation with two hyperimmune rabbit sera: serum R_2, previously absorbed with proteinase, contained only precursor-specific antibody; serum R_5 was from a rabbit immunized with a proteinase preparation. Although the latter serum reacted specifically with the proteinase, it was not sufficiently strong to form a visible precipitate with the small amount of active enzyme in the precursor preparation at the beginning of the experiment.

The results of this experiment are shown in Table V. It will be seen that in the sample which originally contained precursor and sodium thioglycollate no precursor was detectable serologically after 4 hours' incubation at 37°C. In-

TABLE V

Inhibitory Effect of Iodoacetate upon Conversion of Precursor to Proteinase

Antigen used in precipitin tests	Preliminary incubation of antigen at 37°C. for	Precipitin reactions Antiserum prepared with											
		Precursor* (R_2)						Proteinase (R_5)					
		Antigen diluted						Antigen diluted					
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:2	1:4	1:8	1:16	1:32	1:64
Precursor and thioglycollate	20 mins.	++	++	++	++	+	±	−	−	−	−	−	−	−
	60 "	++	++	++	++	+	±	−	−	−	−	−	−	−
	140 "	+	+	+	+	±	−	−	++	++	++	+	±	−
	4 hrs.	−	−	−	−	−	−	−	++	++	++	+	±	−
Precursor and thioglycollate and iodoacetate	20 mins.	++	++	++	++	+	±	−	−	−	−	−	−	−
	60 "	++	++	++	++	+	±	−	−	−	−	−	−	−
	140 "	++	++	++	++	+	±	−	−	−	−	−	−	−
	4 hrs.	++	++	++	++	+	±	−	−	−	−	−	−	−

± to ++ indicates various strengths of precipitin reactions.

* This serum had been absorbed with proteinase and consequently reacted specifically with precursor.

stead, this preparation now reacted strongly with the proteinase antiserum with which it had failed to react at the beginning of the experiment. It may be inferred that this change in reactivity resulted from the autocatalytic conversion of precursor to proteinase during incubation under reducing conditions. It will be noted that no such change occurred in the precursor preparation which contained sodium iodoacetate in addition to sodium thioglycollate. Here, the enzymatic activity of the proteinase was inhibited and hence the autocatalytic reaction could not take place.

The results of these experiments provide serological confirmation of the hypothesis that the proteinase precursor is converted to the active enzyme by incubation in the presence of sodium thioglycollate. Treatment of the precursor with trypsin results in a less complete conversion to the proteinase.

DISCUSSION

The conditions under which the proteinase precursor is produced merit comment. Clearly, it is elaborated within relatively narrow limits of hydrogen ion concentration suitable for multiplication of the streptococci but on the acid side of neutrality. In this regard the proteinase precursor differs strikingly from streptokinase and streptococcal desoxyribonuclease, both of which are produced during growth of streptococci in a neutral or slightly alkaline milieu although the nuclease is also formed under the acid conditions essential for the elaboration of the proteinase precursor. Recent reports have described the formation of streptolysin S by "resting" streptococcal cells (11–13). There is, at present, no evidence that the proteinase precursor is formed by "resting" cells. Admittedly, under suitable conditions, a good yield can be obtained when a massive inoculum of streptococci is added to a relatively simple medium consisting of peptone dialysate and glucose, but multiplication of the bacteria undoubtedly occurs. Although serial subculture in this medium results in continued production of the enzyme, this procedure cannot be continued indefinitely and is probably limited by the eventual exhaustion of nutrients carried over from the original culture medium.

Although recrystallized preparations of the precursor and proteinase have not so far been subjected to rigorous tests for purity, it would appear that a fair degree of purification has, indeed, been achieved. This is particularly so in the case of the active proteinase whose relative insolubility greatly facilitates its separation from the other constituents of culture filtrates. Greater difficulty has been experienced in the purification of the enzyme precursor which, although crystallizing readily, does so under conditions in which extraneous material precipitates from solution. Streptococcal desoxyribonuclease was found as a contaminant present in an amount corresponding to less than 0.03 per cent of the dry weight of a recrystallized precursor preparation: this represented an approximately 10,000-fold decrease in the amount originally present in the crude precursor concentrate. It may be mentioned here that precipitating antisera prepared against the precursor and proteinase failed to react with any other streptococcal products. On the other hand, an antiserum prepared against a partially purified preparation of streptococcal desoxyribonuclease gave a slight but definite reaction with the recrystallized proteinase precursor.

The results of the serological experiments with the precursor and proteinase confirm the relationship which had been postulated on the basis of their enzymological behavior. Furthermore, their serological reactivity has provided a means of following directly the transformation of one to the other. The effect of specific antiserum on the enzymatic activity of the proteinase has yet to be determined but from preliminary observations, it appears to be complicated by the capacity of the active enzyme to digest antibody protein.

It is of some interest that, on a weight basis, the proteinase precursor has

considerably less potential activity than has the proteinase. For example, the examination of recrystallized preparations showed that the precursor in a concentration of 0.1 mg. per cc., when converted to proteinase by autocatalysis, had milk-clotting activity corresponding to a proteinase concentration of approximately 0.01 mg. per cc. A similar difference was noted when enzymatic activity was measured by the hydrolysis of benzoyl arginineamide. The reason for this discrepancy is undetermined but it appears unlikely that it is due to impurity of the precursor preparations although these certainly contain traces of desoxyribonuclease. Several other possible explanations suggest themselves: the proteinase may be derived from only a small fraction of the precursor molecule or complex; there may be loss of activity in the process of converting one to the other; during the conversion process an inhibitor of the active enzyme may be liberated. These possibilities are undergoing investigation.

The similarity between the enzymes of the pancreas and the group A streptococci has already been the subject of comment (8). In the case of trypsin and the streptococcal proteinase the analogy may be carried a stage further. Both enzymes are derived from inactive precursors from which they are distinguishable on the basis of physical properties, biological activity, and immunological specificity. In both instances conversion of the precursor to the active enzyme is induced by an autocatalytic reaction initiated by minute amounts of the active proteinase from which, in each case, the precursor appears to be inseparable. Trypsin also converts the precursor of the streptococcal enzyme to the active proteinase. In this connection it will be recalled that trypsin has a similar action on chymotrypsinogen, which it converts to chymotrypsin. Chymotrypsin itself, on the other hand, does not induce this transformation, nor does it convert the streptococcal proteinase precursor to the active enzyme (2). Chymotrypsin, moreover, does not hydrolyze benzoyl arginineamide, a substrate readily split by both trypsin and the streptococcal proteinase. It seems possible that the capacity of these enzymes to convert their precursors to the active proteinases may be a reflection of their substrate specificities.

Hitherto, enzyme precursors have been described only for the proteinases of animal origin—pepsin, trypsin, chymotrypsin, and plasmin. The streptococcal proteinase differs from these in that it achieves maximal activity only in the presence of sulfhydryl compounds or cyanide. In this respect it more closely resembles the intracellular cathepsins of animals and the vegetable proteinases, papain, ficin, and bromelin, although for these enzymes precursors have not been described.

SUMMARY

Grown in dialysate broth at a pH between 5.5 and 6.5, some strains of group A streptococci elaborate the precursor of a proteolytic enzyme. Within this range of hydrogen concentration the precursor is also produced when the

streptococci are suspended in a peptone dialysate containing glucose and incubated at 37°C. The precursor does not appear to be produced at a neutral or alkaline reaction.

Methods are described whereby the precursor and proteinase have been isolated in crystalline form. The precursor crystallizes from half-saturated ammonium sulfate at pH 8.0 and a temperature of 22°C. or higher; the proteinase crystallizes from 0.15 saturated ammonium sulfate at pH 8.0 but does so most readily at refrigerator temperature. The degree of purification achieved by these procedures is discussed.

The activity of purified preparations of the precursor and of proteinase has been tested against α-benzoyl-*l*-arginineamide and, with this as a substrate, the conversion of precursor to proteinase by autocatalysis or by trypsin has been confirmed.

Immunological experiments are described, the results of which provide evidence of the distinct antigenic specificity of the precursor and proteinase; the conversion of precursor to proteinase has been followed by means of serological tests.

BIBLIOGRAPHY

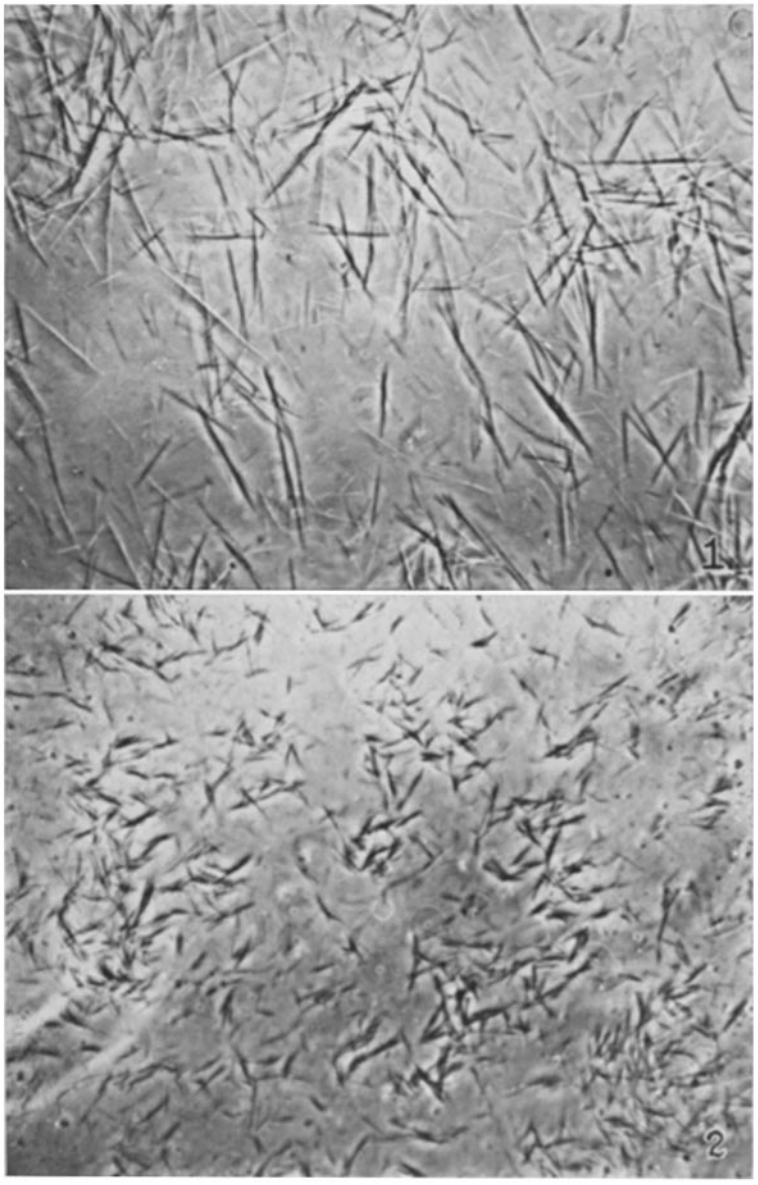
1. Elliott, S. D., *J. Exp. Med.*, 1945, **81,** 573.
2. Elliott, S. D., and Dole, V. P., *J. Exp. Med.*, 1947, **85,** 305.
3. Dole, V. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63,** 122.
4. Adams, M. H., and Roe, A. S., *J. Bact.*, 1945, **49,** 401.
5. Bergmann, M., Fruton, J. S., and Pollock, H., *J. Biol. Chem.*, 1939, **127,** 643.
6. Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78,** 127.
7. Tillett, W. S., Sherry, S., and Christensen, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68,** 184.
8. McCarty, M., *J. Exp. Med.*, 1948, **88,** 181.
9. Bergmann, M., and Fruton, J. S., *Advances Enzymol.*, 1941, **1,** 63.
10. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, 1929, **183,** 32.
11. Ito, R., Okami, T., and Yoshimura, M., *Japan. Med. J.*, 1948, **1,** 253.
12. Bernheimer, A. W., *J. Exp. Med.*, 1949, **90,** 373.
13. Hosoya, S., Hayashi, T., Mori, Y., Homma, Y., Egami, F., Shimamura, M., Yagi, Y., and Suzuki, Y., *Japan. J. Exp. Med.*, 1949, **20,** 35.

EXPLANATION OF PLATE 10

The photographs were made by Mr. L. V. Foster of Messrs. Bausch and Lomb to whom my thanks are due.

FIG. 1. Crystals of streptococcal proteinase precursor. Phase contrast. × 560.

FIG. 2. Crystals of streptococcal proteinase. Phase contrast. × 560.



(Elliott: Streptococcal proteinase and precursor)