AN INACTIVE PRECURSOR OF STREPTOCOCCAL PROTEINASE*

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In a previous report (1) it was shown that, under suitable conditions, some strains of group A streptococci produce in broth cultures an extracellular proteolytic enzyme. In certain respects the enzyme resembles papain and the cathepsins in that it achieves maximal activity under reducing conditions and is inactivated by iodoacetic acid. In broth cultures the reducing conditions necessary for the enzymatic activity are brought about by the presence of living microorganisms and under such conditions the proteinase attacks some of the products of bacterial metabolism, notably the type-specific streptococcal M antigen.

Production of the proteinase by streptococci depends to some extent upon the conditions under which they are cultivated. The type of peptone incorporated in the medium has a marked effect in this regard: Thus, while Pfanstiehl and proteose peptone favor production of the enzyme, in neopeptone broth most strains produce only minimal concentrations of the proteinase. More recent experience with the peptone dialysate broth described by Dole (2) has shown that, unlike the undialyzed peptone, dialysate of Pfanstiehl peptone does not favor production of the enzyme. In examining these facts in further detail, it was found that the addition of an experimental commercially produced protein hydrolysate[1] to the neopeptone or to the peptone dialysate broths resulted in increased production of proteinase by the streptococci. Furthermore, addition of the protein hydrolysate to inactive filtrates prepared from cultures grown in either neopeptone broth or peptone dialysate broth resulted in the appearance of the streptococcal proteinase which, presumably, had previously been present in an inactive form. When the protein hydrolysate was found to contain a small amount of trypsin, these findings immediately suggested an analogy with the activation of trypsinogen and chymotrypsinogen by trypsin. Further experiments were therefore carried out to determine whether streptococcal proteinase is first formed in cultures as an inactive precursor and to study the behavior and mode of activation of this form of the enzyme.

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[1] This was first tried at the suggestion of Dr. R. Archibald.

Throughout the present paper the term "precursor" refers to that form of the enzyme in culture filtrates which displays no activity even when tested under optimal reducing conditions. The terms "active enzyme" and "proteinase," on the other hand, are used for that form which exhibits full proteolytic activity under these optimal reducing conditions.

Methods

Cultures.—Throughout the work to be described in this report a single strain of streptococcus has been used. This strain, "5797," is a group A hemolytic streptococcus with the T antigen of type 8; no M antigen has been identified. The results obtained with this microorganism have been confirmed with several other strains of group A streptococci belonging to different types.

The cultures were grown either in modified Todd-Hewitt broth containing 2 per cent of peptone or in the peptone dialysate medium described by Dole (2).

Estimation of Streptococcal Proteinase in Broth Cultures.—In previous studies (1) the presence of streptococcal proteinase in culture filtrates has been determined by the capacity of the enzyme to destroy the serological reactivity of streptococcal M extracts. This method does not readily lend itself to quantitative estimations. With partially purified preparations, estimation of the tyrosine liberated by the proteinase from a casein substrate has proven satisfactory; in crude culture filtrates, however, substances occur which interfere with the colorimetric method for determining the tyrosine concentrations. In the past, the coagulation of milk has been used as a means of estimating quantitatively the activity of chymotrypsin (3) and papain (4); in the present work, a similar method has been used in estimating streptococcal proteinase in broth cultures. Examination of a large number of different culture filtrates showed that their capacity to destroy the serological reactivity of M extracts was invariably paralleled by their clotting action on milk. The clotting time is inversely proportional to the concentration of the enzyme and this provides a simple method for making a rough estimate of the proteinase content of culture filtrates. A typical experiment of this kind is described below.

Strain 5797 was grown in Pfanstiehl peptone broth for 24 hours at 37°C. This culture was then filtered through a Coor's filter candle[2] (No. 3) and suitable dilutions of the filtrate were made in phosphate buffer, pH 7.0. As a suitable substrate for this enzyme preparation, a 10 per cent suspension of dried skimmed milk powder[3] was made in distilled water with the aid of a Waring blendor. To 4 volumes of this suspension was added 1 volume of 0.5 molar neutral sodium thioglycollate. Throughout this report this is referred to as milk-thioglycollate mixture. 0.3 cc. aliquots of the diluted culture filtrate and milk-thioglycollate mixture were mixed in round-ended agglutination tubes (9.5 mm. in diameter), and incubated in a water bath at 37°C. The tubes were inspected at frequent intervals during incubation and the time noted at which the first sign of coagulation appeared in the reaction mixtures (Table I).

With the lower dilutions of the culture filtrate, *i.e.* those with a clotting time of less than 30 minutes, coagulation of the substrate occurred quite sharply and the clotting time could be determined with a fair degree of precision by inspecting the reaction mixtures with the aid of a hand lens. From Table I it can be seen that in these tubes the ratio, clotting time: reciprocal

[2] Coor's filter candles are similar in type and porosity to Chamberland candles of the same numerical designation.

[3] Merrell-Soule powdered skimmed milk, made by The Borden Company, New York.

of filtrate dilution, tended to be fairly constant. With higher dilutions of the filtrate, coagulation of the substrate became more gradual and the clotting time correspondingly less precise. This was reflected in the clotting time: reciprocal of filtrate dilution ratio which departed more and more from the constant. It follows from this that the clotting time is a reliable measure of proteinase activity in undiluted filtrates of cultures producing a good yield of the enzyme; it is in such instances that this method has been used in the experiments reported here. Where small yields of the enzyme have been obtained in broth cultures, serial twofold dilutions of the filtrate have been mixed with equal volumes of the milk-thioglycollate mixture and incubated at 37°C. for 18 hours. The highest dilution causing coagulation of the milk in this time has been taken as an index of the proteinase content of the filtrate.

It should be mentioned here that the milk-clotting test is not applicable to highly concentrated preparations of the streptococcal proteinase. With these preparations, digestion of the casein proceeds very rapidly and clotting does not occur. Proteinase in these concentrations has not been found in the crude culture filtrates so far examined.

TABLE I

Coagulation Time of Milk-Thioglycollate Mixture in Presence of Streptococcal Culture Filtrate

	Milk-thioglycollate mixture (0.3 cc.) + culture filtrate (0.3 cc.)										
Final dilution of filtrate....	1:2	1:4	1:6	1:8	1:10	1:12	1:16	1:20	1:24	1:32	1:40
Coagulation time, *min.*.	4	8	12	19	20	32	47	53	85	125	145
Ratio $\frac{\text{Coagulation time}}{\text{Reciprocal of filtrate dilution}}$	2	2	2	2.38	2	2.67	2.94	2.65	3.54	3.91	3.63

In all experiments the final reaction mixtures, pH 7.0, were incubated at 37°C. after the addition of the milk-thioglycollate mixture (skimmed milk + neutral sodium thioglycollate in final concentration of 0.1 M).

The use of the milk-clotting test as a measure of proteinase concentration is admittedly open to criticism. The coagulation of milk by proteolytic enzymes is not fully understood, and in the past there have been differences of opinion as to whether the same mechanism is responsible for both digestion and coagulation. It is, however, generally accepted in the case of crystalline chymotrypsin that the milk-clotting and proteolytic activity are due to one and the same enzyme. This probably also holds good for the streptococcal proteinase: With this enzyme, both milk coagulation and proteolysis occur only under reducing conditions or in the presence of cyanide; both activities are inhibited by iodoacetic acid; ammonium sulfate treatment of proteinase preparations removes from solution both activities to an equal extent in the same fractions and both are found in preparations of the proteinase which have been separated from crude material by electrophoresis; finally, as will be shown below, the treatment with trypsin of inactive preparations containing enzyme precursor results in the appearance of both proteolytic and milk-clotting activity. For these reasons it appears likely that proteolysis and the coagulation of milk are expressions of activity of the same enzyme. That assumption has been made in this work, and the milk-clotting ability has been taken as a measure of the proteolytic activity of the enzyme preparations under investigation.

EXPERIMENTAL

Effect of Different Peptones on Yield of Proteinase in Broth Cultures.—Streptococci were cultured in broth containing either Pfanstiehl peptone or neopeptone; in the case of Pfanstiehl peptone either undialyzed material or peptone dialysate (2) was used.

The cultures were incubated at 37°C. for 24 hours after which they were filtered and the filtrates titrated for streptococcal proteinase as described under Methods. The streptococci appeared to grow equally well in all the media. The hydrogen ion concentration of the Pfanstiehl peptone and neopeptone culture filtrates corresponded to pH 6.5 (approx.); that of the Pfanstiehl dialysate filtrate to pH 7.3 (approx.).

TABLE II

Effect of Different Peptones on Yield of Proteinase in Broth Cultures

Culture medium	Final dilution of filtrate							
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
	Coagulation time after adding milk-thioglycollate mixture							
Pfanstiehl peptone broth	+++	+++	++	++	+	±	±	−
" " dialysate broth	+	±	±	−	−	−	−	−
Neopeptone broth	+	±	−	−	−	−	−	−

In all tables the following symbols have been used to indicate rate of coagulation:

+++ = coagulation within 15 minutes
++ = " " 60 "
+ = " " 4 hours
± = " " 18 "
− = no coagulation within 18 hours

Control filtrates heated to 100°C. for 5 minutes caused no coagulation in 18 hours.

Typical titration results with the three different media are shown in Table II. It will be seen that, while a good yield of proteinase was obtained in the undialyzed Pfanstiehl peptone broth culture, only low concentrations were found in cultures made in broth containing either Pfanstiehl peptone dialysate or neopeptone.

Effect of Trypsin on Culture Filtrates.—Streptococci were grown in broth containing either neopeptone, Pfanstiehl peptone, or Pfanstiehl peptone dialysate and the culture filtrates tested for streptococcal proteinase before and after the addition of trypsin.

To 0.9 cc. samples of 24 hour culture filtrates were added 0.1 cc. amounts of a solution of crystalline trypsin in water; the final concentration of trypsin was 0.01 mg. per cc. The hydrogen ion concentration of the filtrates was the same as those of the corresponding preparations in the preceding experiment. Two series of control tubes received either 0.9 cc. filtrate and 0.1 cc. water or 0.9 cc. heated filtrate and 0.1 cc. trypsin solution; the heated

filtrate had been held at a temperature of 100°C. for 5 minutes. The mixtures were incubated at 37°C. for 15 minutes and then titrated for proteinase.

It will be seen from Table III that those filtrates which by themselves showed little enzymatic activity were strongly proteolytic after incubating with trypsin. Trypsin, itself, under ordinary conditions does not coagulate milk and, in the concentrations used in these experiments, had no visible effect on the milk substrate. It is, however, reasonable to suppose that the trypsin converted a precursor of streptococcal proteinase into the enzyme in a manner analogous to that in which chymotrypsinogen is converted by trypsin into chymotrypsin. Further experiments were therefore performed to determine the effect of varying conditions upon the reaction.

TABLE III

Conversion of Precursor to Proteinase by Trypsin

Treatment of culture filtrate*	Final dilution of treated filtrate							
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
	Coagulation time after adding milk-thioglycollate mixture							
Neopeptone culture filtrate + water	+	±	±	−	−	−	−	−
" " " + trypsin	+++	+++	++	++	+	+	±	−
" " " heated + "	−	−	−	−	−	−	−	−
Pfanstiehl culture filtrate + water	+++	+++	++	+	+	±	±	−
" " " + trypsin	+++	+++	++	++	+	±	±	−
" " " heated + "	−	−	−	−	−	−	−	−
Pfanstiehl dialysate culture filtrate + water	±	−	−	−	−	−	−	−
" " " " + trypsin	+++	++	++	+	±	±	−	−
" " " " heated + "	−	−	−	−	−	−	−	−

For interpretation of symbols − to +++ see footnote to Table II.

* Treated filtrates were diluted and added to milk-thioglycollate mixture after treatment at 37°C. for 15 minutes.

Effect of Varying Conditions on Conversion of Precursor to Proteinase by Trypsin.—An experiment was designed to show the influence of trypsin concentration, hydrogen ion concentration, and temperature on the conversion of precursor to proteinase by trypsin.

To each of nine 2.7 cc. samples of a neopeptone broth culture filtrate was added 0.3 cc. of a solution of crystalline trypsin suitably diluted in distilled water. In one series of three tubes, the concentration of trypsin in milligrams per cubic centimeter was 0.01, 0.001, and 0.0001 respectively, and the pH of each mixture adjusted to 6.5 (Table IVA); in a second series of three tubes, in which the concentration of trypsin was 0.002 mg. per cc., the pH of the mixtures was adjusted to 3.5, 4.3, and 6.5 respectively (Table IVB). Both series of tubes were then incubated at 37°C. The third series of three tubes contained trypsin in a final concentration of 0.002 mg. per cc. and the pH of each mixture was adjusted to 6.5; one tube was then incubated at 37°C., another at 24°C., and the third at −5°C. (Table IVC). At intervals, 0.3 cc. samples were taken from each of the nine tubes, neutralized, and added to an equal volume

of the milk-thioglycollate mixture. These mixtures were then incubated at 37°C. and the coagulation time noted.

It can be seen from Table IVA that, in the filtrate used in these experiments, the enzyme precursor was fully converted into the proteinase after incubation for 1/4 hour at 37°C. and pH 6.5 with trypsin in a concentration of 0.01 mg. per cc. The reaction proceeded more slowly in the presence of smaller concentrations of trypsin (Table IVA) or when carried out at a lower temperature (Table IVC). The effect of decreasing pH (Table IVB) is less readily interpreted for, at pH 3.5, the small amount of proteinase produced as the result of 15 minutes' incubation with trypsin was almost completely destroyed during the succeeding 18 hours. A similar, if slower, destruction of the enzyme may have occurred at pH 4.3 and this accounted in part for the apparently slow rate of production.

Effect of Hydrogen Ion Concentration on Stability of the Proteinase and Its Precursor.—In the preceding experiment, evidence was presented suggesting that both the proteinase and its precursor were unstable in acid solutions. An experiment was therefore designed to determine the influence of hydrogen ion concentration on the stability of the proteinase and its precursor.

To 18 cc. of a neopeptone culture filtrate containing enzyme precursor were added 2 cc. of a solution of crystalline trypsin in water (0.02 mg./cc.). The mixture was incubated at 37°C. for 1 hour, during which time all the precursor was converted to proteinase. It was then divided into five equal parts, the reactions of which were adjusted to pH levels of 3.52, 4.3, 6.5, 8.48, and 9.46 respectively. They were then incubated at 37°C. and duplicate samples from each taken at intervals, neutralized, and tested for their ability to coagulate an equal volume of milk-thioglycollate mixture.

In a parallel experiment, 4 cc. samples of the precursor-containing culture filtrate were adjusted to hydrogen ion concentrations corresponding to those already mentioned. They were then incubated at 37°C. and 0.9 cc. samples taken periodically and neutralized. To each was added 0.1 cc. of the trypsin solution, and these mixtures were incubated for a further period of 1 hour, thereby converting any remaining precursor to proteinase. Each sample was then tested, in duplicate, against the milk-thioglycollate mixture. The mean coagulation times are shown in Table V.

From the results shown in Table V, it can be seen that at pH 6.5 both the proteinase and its precursor were stable at 37°C. over a period of 18 hours. Increasing instability was observed at reactions which departed further from neutrality so that at pH 3.52 most of the activity of the proteinase was lost within 4 hours. It will be noted, however, that the precursor was still capable of yielding some active enzyme after 18 hours at this hydrogen ion concentration. Indeed, further experiments, not shown here, indicated that even after several days' exposure to this degree of acidity, precursor could still be detected in neopeptone culture filtrates.

Effect of Chymotrypsin on Proteinase Precursor.—In view of the striking effect of trypsin in converting the precursor of streptococcal proteinase into

TABLE IVA

Effect of Trypsin Concentration on Rate of Conversion of Precursor to Proteinase by Trypsin

Culture filtrate treated with trypsin (pH 6.5, 37°C.) at concentrations indicated	Concentration of trypsin, *mg./cc.*		
	0.01	0.001	0.0001
Length of treatment	Coagulation time (min.) at 37°C. after neutralizing and adding milk-thioglycollate mixture		
hrs.			
¼	15	120	300
1	15	60	240
4	15	30	90
18	15	15	15

TABLE IVB

Effect of pH on Rate of Conversion of Precursor to Proteinase by Trypsin

Culture filtrate treated with trypsin (0.002 mg./cc., 37°C.) at pH indicated	pH		
	3.5	4.3	6.5
Length of treatment	Coagulation time (min.) at 37°C. after neutralizing and adding milk-thioglycollate mixture		
hrs.			
¼	65	45	23
1	120	30	15
4	120–720	24	15
18	720	57	15

TABLE IVC

Effect of Temperature on Rate of Conversion of Precursor to Proteinase by Trypsin

Culture filtrate treated with trypsin (0.002 mg./cc., pH 6.5) at temperatures indicated	Temperature		
	−5°C.	24°C.	37°C.
Length of treatment	Coagulation time (min.) at 37°C. after neutralizing and adding milk-thioglycollate mixture		
hrs.			
¼	55	28	23
1	42	20	15
4	35	15	15
18	20	16	15

the active enzyme, an experiment was performed to determine whether chymotrypsin would have the same action.

To each of three 5 cc. samples of a neopeptone broth culture filtrate was added 0.05 cc. of a solution of crystalline chymotrypsin suitably diluted in distilled water so that the concentration of chymotrypsin in the three tubes was 0.01, 0.001, and 0.0001 mg. per cc. respectively.

Three control tubes were also included in this experiment: one contained culture filtrate without chymotrypsin; another, culture filtrate to which had been added trypsin in a final concentration of 0.01 mg. per cc; the third contained the filtrate of a neopeptone broth culture of a streptococcus which produced neither proteinase nor its precursor and to this filtrate was added chymotrypsin in a final concentration of 0.01 mg. per cc. All six tubes were incubated for 18 hours at 37°C. and pH 6.5. Samples from each were taken periodically and tested for their coagulating effect on milk with and without the addition of sodium thioglycollate.

All samples taken from the tubes containing chymotrypsin coagulated the milk substrate regardless of the presence of thioglycollate; the coagulation time was approximately 16 minutes for those tubes containing 0.01 mg. chymotrypsin per cc., and it was more than 150 minutes for those containing the lower concentrations of the enzyme. For each tube, the coagulation

TABLE V

Effect of pH on Stability of Proteinase and Precursor

	Culture filtrate incubated at pH indicated	pH				
		3.52	4.3	6.5	8.48	9.46
	Length of treatment	Coagulation time (min.) after neutralizing and adding milk-thioglycollate mixture				
	hrs.					
Trypsin-activated proteinase	0	33	22.5	15	18	25
	1	180	34	16	19	39
	4	840	45	15	26	72
	18	No clot	65	15	37	80
Precursor*	0	33	22.5	15	18	25
	1	90	35	20	30	30
	4	270	23	30	23	75
	18	90	35	15	20	100

* Activated with trypsin after incubating at pH indicated before testing with milk-thioglycollate mixture.

time remained fairly constant and showed no tendency to decrease throughout the 18 hour period during which it was incubated. Under these conditions, coagulation of the milk was, therefore, attributable to the direct action of the chymotrypsin; no effect of streptococcal proteinase was demonstrable. Samples taken from the culture filtrates which had been incubated with trypsin coagulated milk in 15 minutes but only in the presence of thioglycollate; samples taken from the filtrate alone did not coagulate milk except in the presence of thioglycollate and then only after several hours' incubation with the substrate. The filtrate, therefore, contained precursor which could be converted into the proteinase by trypsin.

From these results it was concluded that, under the conditions of this experiment, precursor was converted into proteinase by trypsin but not by chymotrypsin. In this respect, therefore, the precursor is similar to trypsinogen and chymotrypsinogen which are transformed into active enzymes by trypsin but not by chymotrypsin.

Autocatalytic Conversion of the Enzyme Precursor to Proteinase

1. Autocatalytic Conversion in Undialyzed Pfanstiehl Broth Cultures.—It will be seen from Table III that filtrates of cultures grown in undialyzed Pfanstiehl peptone broth were strongly proteolytic and showed little increase in activity after incubating with trypsin. Presumably, in these cultures, which had been grown for 24 hours at 37°C., the proteinase was present almost entirely in the active form. It seemed possible, however, that the enzyme had been derived from its precursor, possibly at an earlier stage in the growth of the culture. A Pfanstiehl broth culture was, therefore, sampled at various stages of growth and tested for the presence of both the proteinase and its precursor.

TABLE VI

Conditions Governing the Autocatalytic Conversion of Precursor to Proteinase in Cultures Grown in Undialyzed Pfanstiehl Broth

Cultural conditions		Untreated filtrate						Trypsin-treated filtrate					
		Final dilutions						Final dilutions					
Period of incubation	Degree of oxygenation	1:2	1:4	1:8	1:16	1:32	1:64	1:2	1:4	1:8	1:16	1:32	1:64
		Coagulation time after adding milk-thioglycollate mixture											
hrs.													
14	Deep layer (tube)	±	−	−	−	−	−	+++	+++	++	+	+	±
	Shallow layer (flask)	±	±	−	−	−	−	+++	+++	++	+	+	±
18	Deep layer (tube)	+++	++	+	+	±	−	+++	+++	++	+	+	±
	Shallow layer (flask)	+	+	±	−	−	−	+++	+++	++	+	+	±
24	Deep layer (tube)	+++	+++	++	+	+	±	+++	+++	++	+	+	±
	Shallow layer (flask)	+	±	±	−	−	−	+++	+++	++	++	+	±

For interpretation of symbols − to +++, see footnote to Table II.

Cultures were grown in 15 cc. amounts of Pfanstiehl peptone broth in a test tube (6 inches × ½ inch) and in an Erlenmeyer flask of 100 cc. capacity; in the latter instance, the broth formed a thin layer over the base of the flask so that the streptococci grew under markedly aerobic conditions. After 14, 18, and 24 hours' incubation at 37°C., samples from both cultures were removed and filtered. Part of each filtrate was treated with trypsin as in previous experiments, and the treated and untreated portions were then compared as regards their proteinase content by titrating against the milk-thioglycollate mixture.

It will be seen from Table VI that, in both tube and flask cultures, after 14 hours' incubation the proteinase was present almost entirely in the form of precursor which, at this stage of growth, had apparently reached its maximum concentration. Thereafter, active proteinase increased in both cultures, rapidly in the tube culture, slowly in the flask. It would seem reasonable to suppose that, under the partially anaerobic conditions prevailing in the depths

of the tube culture, the enzyme precursor was converted into the proteinase by traces of enzyme present in the earlier stages of growth but requiring reducing conditions for full activity. In the flask culture, this would occur much less rapidly since the small amount of proteinase present would remain relatively inactive under the markedly aerobic conditions prevailing therein.

As an alternative hypothesis, it might be supposed that, in broth cultures after 14 hours' incubation, some substance, either originally present in the broth or elaborated during growth of the microorganisms, inhibited the action of the enzyme but was destroyed by the added trypsin. An experiment was therefore designed to investigate this possibility.

Streptococci were grown for 24 hours in Pfanstiehl peptone broth; the culture filtrate (A) contained a good yield of proteinase, active under reducing conditions. A second culture,

TABLE VII

Demonstration That Lack of Activity of Young Cultures Is Not Due to Presence of Inhibitor

Diluents	pH	Dilutions of filtrate A (active proteinase)				
		1:2	1:4	1:8	1:16	1:32
		Coagulation time (min.) after adding milk-thioglycollate mixture				
*Culture filtrate B (inactive)	6.5	<3	4	8	19	38
" " " "	8.0	<3	5	10	21	42
Uninoculated broth	8.0	<3	3	8	19	42
Phosphate buffer	7.0	<3	5	10	31	67

* Culture filtrate B alone coagulated milk-thioglycollate mixture in 3 to 4 hours; after treatment with trypsin it coagulated milk-thioglycollate mixture in 5 minutes.

in the same medium, was grown for 14 hours and then filtered; this filtrate (B) contained only a negligible amount of the enzyme but a good yield of precursor. Two series of dilutions of filtrate A were made using as a diluent filtrate B adjusted either to pH 6.5 or to pH 8.0. These series of dilutions, therefore, contained falling concentrations of proteinase present in A and rising concentrations of hypothetical inhibitor present in B. Two control series of dilutions of filtrate A were made in uninoculated broth (pH 8.0) and phosphate buffer (pH 7.0) respectively. All four series of dilutions were incubated for 1 hour at 37°C. and then tested against milk-thioglycollate mixture. The coagulation times are shown in Table VII.

It can be seen from the results shown in Table VII that the coagulating activity of the proteinase in filtrate A was unaffected by the medium in which the enzyme preparation was diluted. The results of this experiment, therefore, gave no indication of a proteinase inhibitor in either the precursor-containing filtrate B or the uninoculated Pfanstiehl peptone broth.

2. Autocatalytic Conversion in Dialysate Broth Cultures.—In cultures grown in peptone dialysate broth the proteinase remains mostly in the form of its

precursor even after prolonged incubation (Table III). Such cultures, so far as proteinase production is concerned, resemble cultures grown under strongly aerobic conditions in undialyzed broth. It seems possible that in dialysate broth reducing conditions are inadequate for the activation of the small amount of proteinase present in the cultures, and therefore the autocatalytic reaction is not initiated. In confirmation of this hypothesis, it was found that by growing cultures in dialyzed culture media under anaerobic conditions, the proteinase appeared entirely in the active form. Such anaerobic conditions were achieved either by the addition to the culture media of neutral sodium thioglycollate (final concentration 0.05 M) or by growing the cultures under a seal of sterile paraffin.

3. Autocatalytic Conversion in Filtrates of Dialysate Broth Cultures.—It follows from the results of the foregoing experiments with dialysate broth cultures, that precursor present in dialysate broth culture *filtrates* should be autocatalytically converted to proteinase provided there is present, to initiate the reaction, a small amount of active enzyme and suitable reducing conditions. An experiment illustrating this reaction is described below.

To two tubes, each containing 9 cc. of dialysate broth culture filtrate (precursor preparation), was added either 1 cc. of 0.5 molar neutral sodium thioglycollate (Tube A) or 1 cc. distilled water (tube B). Another two tubes each contained 9 cc. of dialysate broth culture filtrate previously treated with trypsin in order to convert precursor to proteinase; to each of these tubes was added either 1 cc. of sodium thioglycollate (tube C) or 1 cc. distilled water (tube D). Samples were taken from each of the four tubes and tested against milk for proteinase activity before and at hourly intervals during incubation of the filtrate mixtures at 37°C. It should be noted that in this experiment the enzyme preparations were tested for activity against milk and not, as in previous experiments, against milk-thioglycollate mixture.

The results of this experiment, shown in Table VIII, clearly demonstrate the fundamental difference between the activation of proteinase by sodium thioglycollate and the conversion of precursor to proteinase, which takes place in the presence of this reducing agent: in the absence of thioglycollate, the proteinase, produced by the action of trypsin on the precursor, had no effect on the milk substrate (tube D); when thioglycollate was added, however, activation of the proteinase was immediate (tube C). By contrast, the precursor was converted into proteinase only after prolonged incubation in the presence of thioglycollate (tube A).

By comparing in Table VIII the coagulation times of tube A (precursor preparation) with tube C (in which precursor had been previously converted to proteinase by trypsin), it can be seen that, in the presence of thioglycollate, about half of the available precursor in A was converted to active proteinase during 8 hours' incubation at 37°C., for, at the end of that period the coagulation time for tube A was 18 minutes while that for tube C was 8 minutes. Between the 3rd and 8th hours of incubation, the rate at which this reaction proceeded strongly suggests an autocatalytic process in that an approximately

twofold increase in the concentration of proteinase, indicated by a halving of the coagulation time, occurred with each successive time interval. Presumably, this reaction was initiated by the small amounts of proteinase originally present in the filtrate but requiring adequate reducing conditions for full activity. The existence of this small amount of proteinase in the original culture filtrate was indicated by its capacity to clot milk only after incubation with that substrate for 10 hours under reducing conditions (tube B).

Effect of Casein on the Autocatalytic Conversion of Precursor to Proteinase.—At the conclusion of the preceding experiment, the four enzyme preparations A, B, C, and D were titrated for their content of proteinase. As had been

TABLE VIII

Conditions Governing the Autocatalytic Conversion of Precursor to Proteinase in Filtrates of Dialysate Broth Cultures

Treatment of filtrate	Duration of treatment of filtrate (hrs.) before testing for activity									
	0	1	2	3	4	5	6	7	8	18
	Coagulation time (min.) after adding milk*									
(A) Filtrate + thioglycollate.....	570 (approx.)	570 (approx.)	390	300	195	100	50	35	18	20
(B) " + water.....	—	—	—	—	—	—	—	—	—	—‡
(C) Trypsin-activated filtrate + thioglycollate.....	15	12	12	12	10	8	9	9	8	10
(D) Trypsin-activated filtrate + water.....	—	—	—	—	—	—	—	—	—	—§

— indicates no coagulation in 18 hours.

* The enzyme preparations were tested for activity against milk and not against milk-thioglycollate mixture.

‡ In presence of thioglycollate, coagulated milk in 600 minutes.

§ In presence of thioglycollate, coagulated milk in 20 minutes.

anticipated, it was found that the precursor in preparations A, C, and D had been converted into proteinase, whereas in preparation B this transformation had not taken place. Furthermore, the precursor in preparation B did not undergo autocatalytic conversion to proteinase during the titration procedure. It might have been expected that the transformation of precursor to proteinase would take place when serial dilutions of the precursor preparation were incubated with milk-thioglycollate mixture. This suppression of the autocatalytic process was not due to dilution of the filtrate since a separate experiment showed that, in the absence of milk, the reaction proceeded in two- and fourfold dilutions as rapidly as in the undiluted filtrate. It seemed possible, therefore, that the milk inhibited the autocatalytic process and an experiment was therefore designed to show whether casein would suppress or retard the reaction.

To each of two tubes were added 4 cc. dialysate broth culture filtrate and 0.5 cc. of 0.5 molar neutral sodium thioglycollate solution; to the first tube was then added 0.5 cc. of a 5 per cent solution of casein (tube 1) and to the second 0.5 cc. of water (tube 2). The same culture filtrate was used in this as in the preceding experiment. Both tubes were incubated at 37°C. and at intervals samples were taken and tested for their coagulating effect on an equal volume of milk-thioglycollate mixture. One control tube contained 4 cc. of filtrate and 1 cc. of water (tube 3). Two other control tubes each contained 4.5 cc. of filtrate in which the precursor had been previously converted autocatalytically to proteinase by incubation for 18 hours in the presence of thioglycollate; to one of these was then added 0.5 cc. of the casein solution (tube 4) and to the other 0.5 cc. of water (tube 5). The control mixtures were incubated with the others and at the beginning and conclusion of the experiment their coagulating effect on milk was tested by the same method.

From the results shown in Table IX it appears that casein retards the autocatalytic conversion of precursor to proteinase which takes place under

TABLE IX

Effect of Casein on Autocatalytic Conversion of Precursor to Proteinase in Filtrates of Dialysate-Broth Cultures

Treatment of filtrate	Duration of treatment of filtrate (hrs.) before testing for activity						
	0	6	12	17	19	21	23
	Coagulation time (min.) after adding milk-thioglycollate mixture						
1. Filtrate (precursor) + thioglycollate + casein	420	225	210	52	35	20	14
2. " " + " + water	420	15	7	7	7	7	7
3. Filtrate (precursor) + water	420						420
4. Autocatalytically activated filtrate (proteinase) + casein	7						7
5. " " " " + water	7						7

reducing conditions: in tube 2, the reaction was completed within 12 hours, whereas in tube 1 (containing casein), it was still incomplete at the end of 23 hours' incubation. From a comparison of the milk-clotting activity of the preparations in tubes 4 and 5, it can be seen that casein had no effect on the activity of proteinase in such concentrations as were present in those tubes; that is, the inhibitory effect is on the conversion of the precursor to the active enzyme rather than on the further proteolytic activity of the proteinase as indicated by its ability to clot milk.

A similar retarding of the autocatalytic reaction was observed when streptococci were cultured in Pfanstiehl peptone broth containing 0.4 per cent of casein: complete transformation of the enzyme precursor to proteinase was not achieved after 36 hours' incubation, whereas, in the absence of casein, it was complete within 24 hours.

It is of some interest that autocatalytic conversion of the precursor is suppressed in the presence of casein. This may be the result of competition

between the precursor and the comparatively large excess of casein, an alternative substrate, for the small amount of active proteinase available. Observations which may bear a somewhat similar interpretation have been made by Chopra (5). Working with proteinases of thermophilic bacteria, he found that their proteolytic action on gelatin was depressed by the addition of peptone which, however, was not itself attacked by the enzymes.

DISCUSSION

The experiments which have been described throw some light on the conditions under which streptococcal proteinase and its precursor are formed in broth cultures. They also give some idea of the conditions governing the conversion of the precursor to the active enzyme and indicate their relative stability under varied environmental conditions. It is obviously desirable to confirm and extend these observations with purified preparations of the enzyme and work has now been undertaken with this end in view.

From the evidence presented in this report it is clear that active streptococcal proteinase is derived from an inactive precursor of the enzyme. This precursor is found in culture filtrates and can be converted into the active proteinase by low concentrations of trypsin; the conditions under which this reaction proceeds most rapidly are those which are optimal for tryptic activity. Under the conditions normally prevalent in broth cultures of streptococci, the conversion of precursor to proteinase is effected autocatalytically. This reaction is initiated by the small amounts of streptococcal proteinase usually found present with the precursor but requiring adequate reducing conditions for full activity. For this reason the precursor, which is elaborated mainly during the period of maximal bacterial proliferation, remains in this form until the later stages of growth when those reducing conditions essential for the autocatalytic reaction are established.

It follows that the autocatalytic conversion of precursor to proteinase may be suppressed by factors which establish in cultures conditions unfavorable for activity of the enzyme. Thus, when the enzyme is maintained in its inactive, oxidized form, the conversion of precursor to proteinase is inhibited. It appears likely that this accounts for the great preponderance of precursor over proteinase which occurs when cultures are grown in peptone dialysate broth. In such media, the enzyme remains mainly in the precursor form; but experiments have shown that this is converted into active proteinase if reducing conditions are established either by the addition of sodium thioglycollate or by growing the culture anaerobically. It seems probable that the non-dialyzable components of peptone, excluded from dialysate broth, are responsible for a similar fall in the redox potential of cultures in undialyzed peptone broth.

In a previous report (1) it was shown that, by cultivating the microorganisms at 22°C. instead of at 37°C., the activity of streptococcal proteinase in broth cultures could be suppressed. Experiments have shown that, under such conditions, the proteinase remains almost entirely in precursor form irrespective of the peptone incorporated in the medium. Here, the low temperature of incubation retards the autocatalytic reaction and the necessary reducing conditions, if established naturally, would not be maintained over a sufficiently long period to allow the reaction to proceed to completion.

The problem remains as to whether the precursor is a distinct and separable form of the proteinase or whether it represents a combination of the active enzyme and some inhibitory substance present in the broth cultures. For the former hypothesis, analogies are to be found in the precursors of trypsin and chymotrypsin, both of which are converted into active enzymes by low concentrations of trypsin but not by chymotrypsin. The same holds good for the precursor of streptococcal proteinase: it is converted to the proteinase by trypsin but not by chymotrypsin. There is also some evidence that the precursor of streptococcal proteinase is more resistant to acid than is the active enzyme; this would suggest that they are, indeed, separable entities. Against the alternative hypothesis, that of an inhibitor coexisting with the proteinase in broth cultures, no such inhibitor is demonstrable either in uninoculated Pfanstiehl peptone broth or in cultures which have been examined at a stage in growth where the proteinase is almost entirely in the precursor form. Furthermore, attempts at separating the hypothetical inhibitor from the proteinase have so far proved unsuccessful.

In summarizing the available data, it can be said that the evidence for a distinct and separable precursor of streptococcal proteinase is, at present, based largely on analogy and upon lack of evidence for a proteinase inhibitor in culture filtrates. Final proof awaits the isolation of both the proteinase and its precursor in a purified form.

SUMMARY

1. Streptococcal proteinase is derived from an inactive precursor found in culture filtrates of proteinase-producing streptococci.

2. The precursor can be converted into the proteinase by low concentrations of trypsin but not by chymotrypsin.

3. In cultures grown in suitable media the conversion of precursor to proteinase is effected autocatalytically. This reaction occurs under reducing conditions and is initiated by active proteinase present in low concentrations with the precursor.

4. The autocatalytic reaction is suppressed or retarded by conditions which decrease the activity of the proteinase, *e.g.* by growing cultures at 22°C.

instead of at 37°C. or by growing them under markedly aerobic conditions. It is also retarded in the presence of casein.

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