Streptokinase: Preparation, Comparison with Streptococcal Proteinase, and Behavior as a Trypsin Substrate (32664)

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Streptokinase and streptococcal proteinase are both extracts of cultures of streptococci. The latter, from Group-A streptococci, is activated by a reducing agent after conversion of the zymogen to an enzyme (1,2). Streptokinase, from Group-C or Group-A bacteria, is not directly proteolytic although it reacts with plasminogen to yield a fibrinolytic product (3,4). This paper describes experiments with these two proteins, including the effects of streptococcal proteinase on clot lysis and attempts to elicit proteolytic activity from streptokinase by treating it with trypsin and mercaptoethanol. It also reports a method of purification of streptokinase.

Experimental and Results. Preparation of streptokinase. Prior to chromatography on diethylaminoethyl (DEAE) cellulose, streptokinase was fractionated in either of two ways, viz., by hydroxylapatite chromatography or by $(NH_4)_2SO_4$ precipitation.

Hydroxylapatite (Hypatite C, Clarkson Chemical, Williamsport, Pennsylvania) was equilibrated with 0.02 *M* phosphate buffer, pH 6.8, and packed into a 32 × 2-cm column. Crude streptokinase, purchased from the Lederle Company as Varidase, was extensively dialyzed against 0.02 *M* phosphate buffer, put on the column, and eluted in a step-wise manner at a flow rate of approximately 25 ml/hour. Phosphate buffers (pH 6.8) of the following concentrations were used for step-wise elution: 0.02 *M*, 0.06 *M*, and 0.5 *M*. The peak eluted by 0.06 *M* phosphate contained 15% of the initial absorbance (280 mμ) and 56% of the starting activity, a 3- to 5-fold increase in specific activity. Total recovery from the column was 80–85% of the absorbance and 90–95% of the activity.

For $(NH_4)_2SO_4$ precipitation, crude streptokinase which had been dialyzed against distilled water was treated with an equal volume of saturated $(NH_4)_2SO_4$. After centrifugation the precipitate was taken up in distilled water. The precipitate contained 35–50% of the initial optical density and 95–99% of the activity.

DEAE-cellulose chromatography. Whatman C-2 microgranular DEAE cellulose pretreated with 1.0 *N* NaOH, washed extensively with H_2O, and equilibrated with 0.09 *M* NaCl, was packed under pressure into a 30 × 2-cm column. Streptokinase from either the hydroxylapatite column or the $(NH_4)_2SO_4$ precipitation was dialyzed against 0.09 *M* NaCl and pumped onto the DEAE cellulose. The material was eluted by a gradient achieved through the use of four chambers of a "Varigrad" (5) containing 250 ml of 0.09 *M*, 0.15 *M*, 0.09 *M*, and 0.30 *M* NaCl, respectively (Fig. 1). The major peak of activity was concentrated by ultrafiltration (Diaflo membrane, Amicon Corp., Cambridge, Massachusetts) to 1–10 mg/ml. Its specific activity was increased 2- to 3-fold over the calcium phosphate streptokinase and 3- to 5-fold over the $(NH_4)_2SO_4$ material. The final preparation contained 3–5% of the original protein and 22–40% of the initial activity, a 6- to 10-fold increase in specific activity over crude streptokinase.

Characteristics of streptokinase. Several preparations were subjected to gel filtration on a 75 × 1-cm column of Sephadex G-100 equilibrated with Tris NaCl buffer (pH 7.10, 0.020 *M* Tris, 0.167 *M* NaCl). At each succeeding stage of fractionation, the gel-filtration elution pattern revealed less extraneous protein (Fig. 2). Although some purification (2–3-fold) could be achieved by gel filtration, the relatively small amounts of material which could be handled limited its use as a preparative procedure. Streptokinase prepared by DEAE-cellulose chromatography had an elution volume from G-100 between those of albumin and α-chymotrypsin, corresponding to a molecular weight of approximately 58,000.

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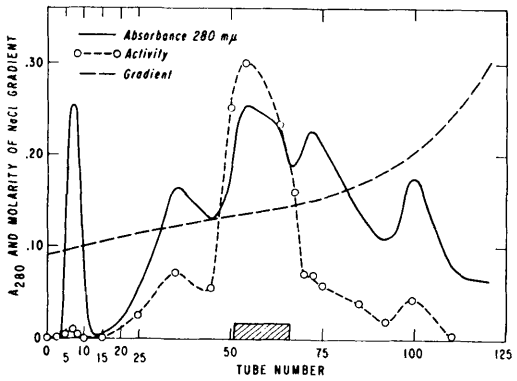

FIG. 1. DEAE cellulose chromatography of streptokinase after prior treatment with hydroxylapatite or $(NH_4)_2SO_4$. Flow rate was 50 ml/hour. Each tube contained approximately 9 ml of eluate. Streptokinase activity is shown as arbitrary units per tube. Hatched region indicates material subjected to gel filtration (Fig. 2, bottom).

Disc electrophoresis of this streptokinase preparation was done by the method of Davis (6); one major band and 1–2 faint bands were seen.

Streptokinase assay. Streptokinase was assayed by one or more of three methods. In all assays, the human plasminogen preparations used were prepared by Dr. J. S. Finlayson by a modification of the ether fractionation method of Derechin (7,8). The specific activities of these materials ranged from 15 to 50 Remmert and Cohen (9) casein units (cu) per milligram protein N; a crude preparation (4 cu/mg protein N) was used only in the clot-lysis assay for streptokinase. Approximately 1 cu of plasminogen was used per test sample. All streptokinase samples were assayed in several dilutions over a 1000-fold range.

The clot-lysis assay was a modification of Christensen's method (10) using plasminogen-poor human fibrinogen (11) and bovine thrombin (Parke-Davis, Lot No. 8854). The casein assay was a modification of Derechin's method (7) using Hammarsten casein (Mann Research Laboratories) which had been treated to diminish its β-casein content (12). Four ml of test solution were added to 9 ml of prewarmed 1% casein and incubated in a 40°C water bath. At predetermined intervals, 4-ml aliquots were mixed with 2 ml of 1.8 *M* perchloric acid, allowed to stand at room temperature for 10 min, filtered (Whatman No. 2 paper), and read at 276 mμ in a Beckman spectrophotometer.

The lysine methyl ester assay was based on that of Sherry *et al.* (13) as modified by T'sao and Kline[1] in which 0.25 ml of 2.5% lysine methyl ester (Mann Research Laboratories) in pH 6.0 phosphate buffer plus 0.75 ml of the streptokinase-plasminogen test solution were added to 0.5 ml of 0.75 *M* perchloric acid. To a 0.5-ml aliquot of this mixture,

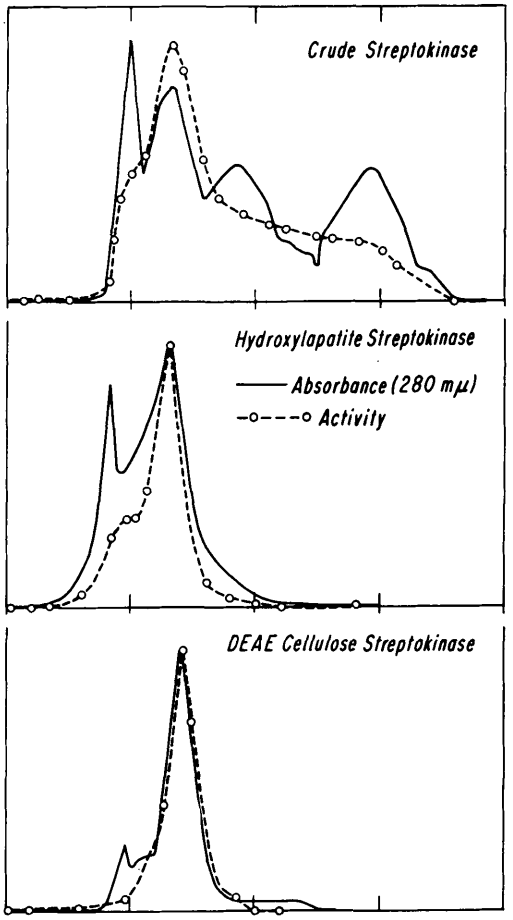

FIG. 2. Sephadex G-100 elution patterns of crude streptokinase (top); hydroxylapatite-treated streptokinase (middle); and streptokinase from DEAE cellulose (bottom). Flow rate was 25 ml/hour. Graph depicts absorbance at 280 mμ and streptokinase activity in arbitrary units (ordinate) versus cumulative eluate volume (abscissa).

[1] Personal communication.

TABLE I. Fibrin-Clot Lysis by Streptococcal Proteinase Enzyme Preincubated with Various Substances.

Amount of streptococcal proteinase enzyme (μg)	Minutes required for clot lysis (preincubations at 37°C[a])			
			Fibrinogen	
	Plasmin 0.4 cu, 5 min	Plasminogen 0.1 cu, 5 min	1 min	15 min
None	23.5	144	176	177
.002	—	142		
.004	25.5	—		
.040	23.0	—		
0.20	—	120		
18.0	—	87		
35.0	19.0	—	145	Did not clot
70.0	17.0	—		

[a] Varying amounts of unreduced streptococcal proteinase enzyme were preincubated with plasmin or plasminogen or fibrinogen before being assayed in the clot-lysis system at 37°C.

the following were added in succession: 0.05 ml 2% $KMnO_4$, 0.05 ml 10% Na_2SO_3, and 4.0 ml chromotropic acid solution (10 ml 2% chromotropic acid, 60 ml concentrated sulphuric acid, 30 ml distilled water). This combination was placed in boiling water for 15 min, cooled, and read at 580 mμ.

Streptococcal proteinase. Streptococcal proteinase was kindly supplied by Dr. Teh-Yung Liu in both the zymogen and enzyme form in 0.04 *M* NaCl with 10^{-4} *M* EDTA. The enzyme had been prepared from the zymogen through the use of trypsin; neither had been activated by a reducing agent. These were stored in the frozen state. They were analyzed in a manner similar to that described in the casein assay above except that mercaptoethanol (final concentration 0.05 *M*) was added to the test solution (12,14). It was found that the streptococcal proteinase enzyme had an activity equivalent to 2.99 Kunitz (15) trypsin units/mg protein; the zymogen, 0.44 units/mg protein.

Several dilutions of nonreduced streptococcal proteinase enzyme and zymogen were incorporated into clots formed by plasminogen-poor human fibrinogen and bovine thrombin. Clot lysis occurred after 3–4 hours of incubation at 37°C; the same lysis time was observed for controls which contained no proteinase. This experiment was repeated with streptococcal proteinase which had been preincubated for 5 min at 37°C with either 0.4 cu of glycerol activated plasmin or 0.1 cu of plasminogen. In each case, plasmin or plasminogen plus streptococcal proteinase enzyme lysed fibrin more rapidly than plasmin or plasminogen alone; the less streptococcal proteinase added, the closer the lysis times to the plasmin or plasminogen controls (Table I).

To determine if the streptococcal proteinase could directly affect fibrinogen, 1.4 mg of the nonreduced enzyme was incubated with 2 ml of 1% fibrinogen at 37°C. Aliquots were removed after 1 and 15 min, added to buffer, and clotted. As a control, fibrinogen and the enzyme were incubated separately and added sequentially to buffer before clotting. The samples and controls were allowed to lyse at 37°C. The enzyme–fibrinogen combination preincubated for 1 min lysed 15% more rapidly than the control. After a 15-min preincubation, this combination would not clot at all (Table I).

Finally, to ascertain whether plasmin, like trypsin, could convert the zymogen to the enzyme, 720 mg of zymogen was incubated with one cu of plasmin at 37°C for 4 hours. Mercaptoethanol was added to a final concentration of 0.05 *M* and the mixture allowed to stand 30 min at room temperature before being assayed on casein. Controls of zymogen and mercaptoethanol, plasmin and zymo-

gen, and plasmin alone were run. No significant conversion to active streptococcal proteinase could be detected.

Trypsin–streptokinase interaction. It was hoped that, in a manner analogous to streptococcal proteinase, streptokinase might undergo conversion to an active proteolytic enzyme. Therefore, streptokinase was treated with trypsin (trichloroacetic acid precipitated, Worthington) and mercaptoethanol as described for the proteinase, and assayed on casein. Mercaptoethanol was without effect, but the streptokinase–trypsin mixtures were more caseinolytic than trypsin alone. In addition, these mixtures were not readily inactivated by diisopropylfluorophosphate (DFP). Under no circumstances did streptokinase alone show proteolytic activity.

When analyzed by disc electrophoresis, a streptokinase-trypsin mixture (molar ratio 1/3.5) exhibited no single major band, thus giving no evidence of complex formation. However, the nature of the electrophoretic pattern suggested that proteolysis had occurred.

To investigate this apparent proteolysis, streptokinase–trypsin mixtures of 1/1 and 10/1 molar ratios were incubated for 5 min at room temperature. The reaction was then interrupted with DFP (final concentration 6×10^{-4} *M*) in propanol whereupon the mixtures were put on a 1 × 80-cm column of Sephadex G-75 and eluted with the same Tris NaCl buffer described previously. The flow rate was about 30 ml/hour. The elution volumes of streptokinase and trypsin with DFP were each determined; the former appeared in the breakthrough region, the latter considerably later.

None of the protein of the streptokinase–trypsin mixtures, as measured by absorbance at 280 mμ, appeared in the breakthrough peak, indicating that no intact streptokinase remained. Only those fractions in the trypsin region showed caseinolytic activity. Since this material could also be inhibited by DFP, it was evidently trypsin. Thus, there appeared to be neither a complex nor a new enzyme formed. It appeared possible that streptokinase could be a substrate for trypsin. This was tested first by adding an excess of streptokinase to trypsin (150/1 molar ratio), incubating at 37°C and precipitating aliquots with perchloric acid (final concentration 0.6 *M*) over a period of 4 hours. After precipitation the material was allowed to stand 10 min at room temperature before being filtered and read at 276 mμ. The progressive increase of perchloric acid soluble material indicated that trypsin was digesting streptokinase (Fig. 3).

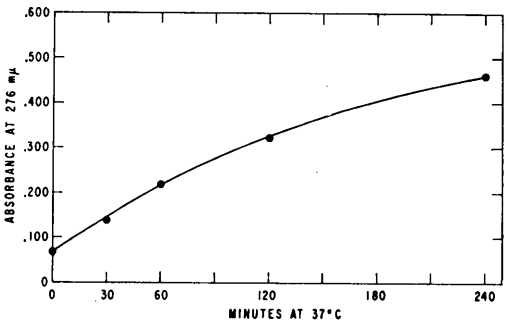

FIG. 3. Digestion of streptokinase by trypsin. Aliquots of the streptokinase–trypsin (150/1) mixture were withdrawn at intervals during incubation at 37°C, treated with perchloric acid, and the absorbance (276 mμ) of the perchloric acid-soluble material determined.

Trypsin alone was incubated at 37°C and its rate of autolysis at pH 7.8 determined by periodic assays on casein. Molar ratios of streptokinase–trypsin of 1/10, 1/1, 10/1, and 100/1 were then treated in the same manner. The retardation of loss of trypsin caseinolytic activity by streptokinase was observed, being most evident in the 100/1 sample (Fig. 4); the higher the streptokinase/trypsin ratio, the

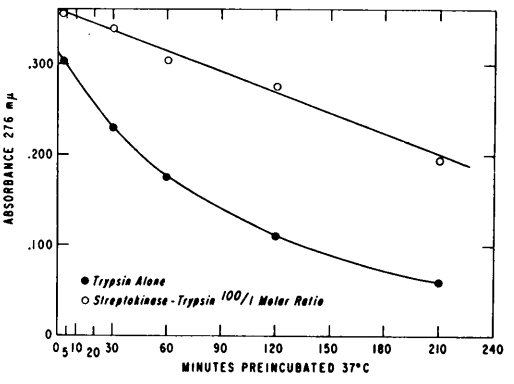

FIG. 4. Protection against autolysis. Aliquots of trypsin alone and streptokinase–trypsin (100/1) were assayed on casein during incubation at 37°C.

TABLE II. Effect of Streptokinase on the Inhibition of Trypsin by DFP.

Materials assayed	Caseinolytic activity	
	No preincubation	Preincubated 1 hour
Streptokinase-trypsin 1/1[a]	.341[b]	.180[b]
Trypsin	.300	.164
Streptokinase-trypsin 1/1[a] + DFP[c]	.117	.037
Trypsin + DFP[c]	.050	.040

[a] Molar ratio.

[b] Increase in absorbance (276 mμ) of perchloric acid soluble fraction during 20-min assay.

[c] DFP was used as a 0.1 M solution; 0.01-ml aliquots were added to 4-ml samples.

longer the duration of protection against autolysis. Neither bovine albumin at a molar ratio of 150/1 nor human gamma globulin at 10/1 exhibited significant protection of trypsin.

To test the effect of streptokinase on DFP inhibition of trypsin a 1/1 molar mixture of streptokinase and trypsin was incubated at 37°C and 0.01 ml of 0.1 *M* DFP added to 4-ml aliquots of this mixture taken at 0 and 60 minutes. These aliquots remained at room temperature for 10 min and were then assayed on casein. Controls of trypsin alone, trypsin with DFP, and the streptokinase–trypsin mixture without DFP were also assayed. Streptokinase afforded trypsin measurable protection against DFP inhibition; streptokinase incubated with trypsin for 60 min no longer gave this protection (Table II).

Discussion. Several investigators have reported methods of preparation of streptokinase (4,16,17,18). The final product obtained in the present work appears to be similar in purity and activity to these preparations.

After Elliott discovered streptococcal proteinase (1), he, Liu, and co-workers ascertained that neither the zymogen nor the enzyme was fully active unless treated by a reducing agent such as mercaptoethanol (14). Moreover, the enzyme was shown to render fibrinogen unclottable in a recalcification assay (1). This fibrinogenolytic quality of streptococcal proteinase enzyme was confirmed in the current study (Table I). No such proteolysis was seen when the clot was already formed. Thus either no adequate reducing agents were present or fibrin was far less amenable to proteolysis than was fibrinogen.

Preincubating the streptococcal proteinase enzyme with plasmin created a more potent fibrinolytic agent than plasmin alone; similarly preincubating the proteinase with plasminogen yielded more fibrinolytic activity than was present in the plasminogen preparation alone (Table I). If it is assumed that plasmin was already maximally activated, the suggestion that plasmin activated the streptococcal proteinase enzyme may be tenable. It is premature to postulate whether or not the streptococcal proteinase enzyme activated plasminogen or vice versa, since the small amount of plasmin present in the plasminogen preparation probably entered into the reaction. It is interesting that, unlike trypsin, plasmin did not significantly convert the zymogen to streptococcal proteinase enzyme.

Amino acid analyses of streptococcal proteinase and streptokinase show notable differences, i.e., cystine was absent in the latter (4,14). Furthermore, treatment with trypsin and a reducing agent did not produce a proteolytic enzyme from streptokinase. It becomes obvious that streptococcal proteinase and streptokinase are quite dissimilar proteins.

Although streptokinase could not be converted by trypsin to a proteolytic enzyme, it did serve as a trypsin substrate and, in so doing, protected trypsin from autolysis and DFP inhibition. By analogy, it may be possible that streptokinase is a substrate for plasmin, as suggested by the recent reports of De Renzo *et al.* (19) and by preliminary data in this laboratory.

Summary. Streptokinase was prepared by ion-exchange chromatography on DEAE cellulose after prior treatment with $(NH_4)_2SO_4$ or hydroxylapatite. The final product had 22–40% of the starting activity and 3–5% of the initial absorbance (280 mμ). Streptokinase and streptococcal proteinase were found to be quite dissimilar proteins, and streptokinase was shown to be a substrate for trypsin which protected the latter against autolysis and DFP inhibition.

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Further Purification of a Human Placental Inhibitor to Hemagglutination by H-1 Virus* (32665)

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A previous communication (1) described the preliminary purification of a substance, observed by Toolan (2) in all human placental fluids, that inhibits hemagglutination by H-1 and HB viruses, but not hemagglutination by the closely related RV or H-3 viruses; this material is not present either in maternal or fetal sera. It was learned that the inhibitor is an immunologically homogeneous glycoprotein that produces a single band on paper electrophoresis. Although a number of glycoproteins have been found that inhibit hemagglutination by influenza virus (1), the only two which have been successfully purified to date are urinary mucoprotein (3) and a group of inhibitory glycoproteins found in the submaxillary glands of ruminants (4). In both instances gentle chemical procedures were employed to obtain the final fractions. More drastic methods appear to break down the glycoprotein molecule. Since one of the steps previously reported in the method used for purification of the H-1 hemagglutination inhibitor involved precipitation with sulfosalicylic acid, it seemed possible to us that some denaturation with breakage of the native molecule into smaller units might have occurred during this procedure. Further work was done, therefore, to develop a simple and gentle technique for isolating the placental inhibitor in a pure form. The present paper describes such a method, together with several physical and chemical properties of the inhibitor.

Materials and Methods. Fluid was obtained from fresh unwashed placentas by removing the fetal membranes and allowing liquids of the placental mass to leak out into the containing vessel. Most of the placentas were delivery-room specimens handled with asceptic precautions. A few were obtained under sterile conditions at caesarian section. Approximately 20–75 ml of fluid were collected from each full-term placenta and spun for 15 min at 2,000 rpm to separate the red blood cells. Samples (0.3 ml) of the supernatant fluids

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