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Staphylococcal Hyaluronate Lyase: Purification and Characterization Studies

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Staphylococcal hyaluronate lyase (hyaluronidase) derived from a pathogenic strain of staphylococcus was purified by means of salt fractionation with ammonium sulfate and gel filtration through Sephadex G-100. Most of the enzyme activity from concentrated culture supernatant fluids of staphylococci was obtained in a fraction precipitated by 90 to 100% saturation with ammonium sulfate. A small amount of enzyme was also precipitated by 80 to 90% saturation with the salt. The hyaluronidase-rich fractions did not contain other staphylococcal enzymes, such as coagulase, protease, lipase, and staphylokinase. These enzymes were present in the original concentrates. Molecular sieving chromatography of the partially purified enzyme by filtration through Sephadex G-100 resulted in a further increase in specific enzyme activity. However, more than one active peak was obtained after gel filtration, thus suggesting that there may be more than one molecular form of the enzyme. Immunodiffusion in agar gel of the chromatographically purified enzyme fraction, with immune serum from rabbits injected with concentrated staphylococcal culture supernatant fluids, indicated that there was one major antigen. A similar antigen, giving reactions of identity with the purified material, was present in the original culture supernatant fluid.

Hyaluronate lyase (hyaluronidase) is secreted by many strains of coagulase-positive staphylococci, as well as by other microorganisms (C. Abramson, Ph.D. Thesis, Temple University, 1965; 7, 9–11, 17, 19, 23, 27–31). Production of this enzyme is characteristic of many coagulase-secreting staphylococci which are considered pathogenic. A relation between these two enzymes and virulence of the organism has been suggested (Abramson, Ph.D. Thesis; 9–11, 17, 29, 30). However, there have been only a few studies concerning purification and characterization of staphylococcal hyaluronidase. In contrast, there have been a number of reports concerning the biological and physical characteristics of this and many other enzymes secreted by staphylococci (4, 6, 10, 13, 18, 19, 26).

The medical and biological significance of hyaluronidase in infections caused by staphylococci has been suggested by several authors (10). However, before specific studies can be initiated concerning the importance of this enzyme in a host-parasite relationship, it is apparent that relatively purified preparations from pathogenic staphylococci should be available. The object of the present study was to purify and characterize staphylococcal hyaluronidase in order to more clearly define some of its physicochemical and immunochemical properties.

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MATERIALS AND METHODS

Cultural procedures. Staphylococcus aureus (AEMC 1801), previously described (1, 2; C. Abramson and H. Friedman, Federation Proc., p. 244, 1965), was used as the starting material for these studies. This strain secreted relatively large amounts of hyaluronidase, α-, β-, and Δ-hemolysins, free and bound coagulase, egg yolk lipase, staphylokinase (fibrinolysin), phosphatase, protease, and deoxyribonuclease. The organism was maintained on blood-agar slants [Tryptose Blood Agar base (Difco) and 10% sheep blood] and was subcultured approximately every 2 weeks. Cultures for large-scale production and purification of the enzyme were prepared by inoculating 15 ml of Brain Heart Infusion (Difco) with an inoculum of about 10^9 saline-washed microorganisms. After overnight incubation at 37 C, the 15-ml seed cultures were washed three times with sterile saline and inoculated into 2 liters of dialysate medium contained in sterile Erlenmeyer flasks, as described below. The culture was incubated in a water bath at 37 C, without shaking or aeration, for 24 hr and tested for purity by subculture and Gram stain.

Medium. Dialysate medium was prepared as previously described (1) by dialyzing 0.85% saline, in cellulose dialysis tubing, against 20 liters of Brain Heart Infusion for 72 hr at 5 C. The resulting dialysate was enriched with 5% dextrose (*p*H adjusted to 7.2 with NaOH) and sterilized by steam at 121 C for 15 min.

Purification procedures. Crude concentrate was prepared as previously described (1) by 100% crystalline ammonium sulfate saturation according to the methods of Green and Hughes (15). Dialyzed sulfate-free supernatant fluid was lyophilized and stored at −20 C.

Sequential ammonium sulfate fractionation. Solid ammonium sulfate was added to sequential samples of crude concentrate dissolved in distilled water at 25 C (1, 15, 33), as follows (g/100 ml): 33.1 (0 to 50% saturation); 13.7 (50 to 70% saturation); 7.2 (70 to 80%); 7.7 (80 to 90%); 7.9 (90 to 100%); and equilibrated for 45 min at 25 C with the aid of a magnetic stirrer. Each fraction was centrifuged at 32,000 × *g* for 15 min at 5 to 10 C; supernatant fluids were decanted, brought to 25 C, and treated with the next concentration of ammonium sulfate necessary to obtain the desired salt concentration (15). All precipitates were dissolved in a minimal amount of distilled water, dialyzed against three to four changes of 20 volumes of cold, distilled water at 5 C, and tested for the absence of sulfate ion with 10^{-3} M $BaCl_2$. Fractions negative for sulfate ion were rapidly frozen and stored at −20 C.

Gel filtration column chromatography. Sephadex G-100 (Pharmacia Fine Chemical Co., Uppsala, Sweden) was prepared in a column (10 × 1 cm) according to the method of Porath and Flodin (25) and eluted with 0.05 M NaPB buffer containing 0.5 M NaCl (1).

Protein determination. Protein contents of the culture supernatant fluids and the fractions were determined by the Folin Ciocalteu phenol color reaction as modified by Lowry et al. (20) using Bovine Serum Albumin, 5 × crystallized (Pentex Inc., Kankakee, Ill.), as a standard.

Enzyme determinations. The mucin clot prevention (MCP) method was used for assay of hyaluronidase activity with culture supernatant fluids and purified fractions (17). This procedure depends on the fact that nondepolymerized hyaluronic acid precipitates in the presence of acetic acid and protein to form a "mucin" clot. Serial twofold dilutions of test material, prepared in 0.5-ml volumes with phosphate-buffered saline, were treated with 0.2 ml of substrate consisting of 3% crude potassium hyaluronic acid, prepared from human umbilical cords, in distilled water containing 10% (v/v) sterile horse serum. Final concentration of substrate was 0.4 mg/ml. The enzyme-substrate mixture was incubated for 20 min at 37 C and the reaction was stopped by immersing the tubes in an ice bath for 5 min. Addition of 0.2 ml of 2 N acetic acid to each tube resulted in development of a clot, except where enzyme was present. One unit of enzyme activity was expressed as the highest dilution of test material which prevented mucin clot formation after 30 min of incubation at 37 C.

The potency of hyaluronidase preparations was also expressed in turbidity reducing units (TRU). One unit was arbitrarily defined as the amount of enzyme which, in 30 min at 37 C, reduced the turbidity obtained with 0.2 mg of hyaluronic acid to that obtained with 0.1 mg of hyaluronic acid (33). The assay depends upon the fact that purified hyaluronic acid forms a colloidal suspension with acidified protein reagent. The resulting turbidity is related to the concentration of hyaluronic acid. When hyaluronic acid has been depolymerized by hyaluronidase, the turbidity is reduced. Therefore, turbidity is a function of the substrate concentration and can be related to enzyme activity.

Enzyme preparations were diluted in buffer in a series of concentrations dependent upon specific activity. The hyaluronic acid substrate was dissolved in sufficient buffer to give a concentration of 0.4 mg/ml. The buffer was 0.1 M sodium phosphate in 0.15 M sodium chloride, *p*H 7.2, modified from *p*H 5.3 as suggested by Tolksdorf (33). Albumin powder Fraction Five (Armour and Co., Alliance, Ohio) was dissolved in 250 ml of 0.5 M sodium acetate buffer, *p*H 4.2. The *p*H was adjusted to 3.0 with HCl and the solution was heated to 95 C for 30 min and cooled. The volume was adjusted to 1 liter with sodium acetate buffer, *p*H 4.2 (Acidified Albumin Reagent).

Standards were prepared by adding 0.1 to 0.6 ml of substrate into a series of colorimeter tubes and adding buffer to 1.0 ml. A blank contained 1.0 ml of buffer. The tubes were heated in a boiling-water bath for 5 min, cooled in an ice bath for 5 min, and 4.0 ml of acidified albumin reagent was added. The tubes were gently mixed; after 10 min, turbidity was determined with a Klett-Summerson photoelectric colorimeter, test tube model, using a 540-nm blue filter. Milligrams of hyaluronic acid substrate were plotted against photometer readings, resulting in a straight-line standard curve.

For the assay, 0.5 ml of substrate was added to a series of colorimeter tubes in an ice bath. At timed intervals, 0.5 ml of enzyme dilution was added. The tubes were incubated for 30 min at 37 C, placed into a boiling-water bath for 5 min to stop the reaction, cooled in an ice bath for 5 min, and 4.0 ml of acidified albumin reagent was added. The turbidities were determined as above after 10 min of incubation.

Coagulase. By using the tube and plate methods, enzyme activity was determined semiqualitatively by incubating serial dilutions of test material with rabbit plasma at 37 C. Formation of a visible clot in a tube or opacity in an agar plate was taken as evidence of coagulase activity (2).

Staphylokinase (fibrinolysin). A qualitative agar plate method was used in which presence of enzyme activity was estimated by determining the ability of the test material to form a clear area in agar plates containing 20% human plasma in 1% agar (2).

Hemolysins. Lysis of rabbit, sheep, or human erythrocytes, washed and suspended in phosphate-buffered saline, was taken as evidence for the presence of specific hemolysins (2).

Lipase. Egg yolk lipase activity was determined

qualitatively by demonstration of an opacity in agar plates containing egg yolk broth (2).

Phosphatase. Phosphatase activity was determined qualitatively by using a modification of the Barber and Kuper method (4) in which phenolphthalein diphosphate sodium salt was used as an indicator in agar plates. After incubation of the substrate for 3 hr with the fractions, treatment of the plates with 0.5 N ammonium hydroxide resulted in a purple color in the absence of phosphatase activity (2).

Protease. The clearing of hemoglobin in agar plates was used as a qualitative test for presence of protease activity (2).

Deoxyribonuclease. Qualitative assay for deoxyribonuclease in agar substrate plates containing 1% deoxyribonucleic acid was recorded as a clear zone following addition of 1 N hydrochloric acid (2).

Immunological procedures. New Zealand white rabbits, approximately 2.5 to 3 kg each, were initially inoculated subcutaneously with crude staphylococcal culture supernatant preparations, rich in hyaluronidase activity. The antigenic preparation was emulsified in a 1:1 ratio with complete Freunds adjuvant (Difco) to contain 10 mg/ml. Additional injections administered at bimonthly intervals were given intravenously. Crude staphylococcal hyaluronidase (180 mg per rabbit) was injected over a 2-year period. Each 1-mg amount contained 2,000 MCP units of enzyme activity. Blood samples were collected prior to each immunization and sera were tested by Ouchterlony analysis (24).

Ouchterlony agar gel-double diffusion analysis was performed in flat-bottomed petri dishes containing 10 ml of 1% Noble agar (Difco) in phosphate-buffered saline (*p*H 7.2) as previously described (1).

RESULTS

Ammonium sulfate fractionation. Clarified culture supernatant fluids of staphylococci concentrated by ammonium sulfate precipitation were used as the starting material for sequential fractionation. Lyophilized concentrates of the supernatant fluids were dissolved in 100 to 200 ml of distilled water at a concentration of 50 or 100 mg/ml. Precipitates recovered by centrifugation were taken up in a minimal amount of distilled water, dialyzed against distilled water, lyophilized, and designated as fraction I-P (50%), II-P (70%), III-P (80%), IV-P (90%), and V-P (100%). Hyaluronidase activity was detected in the precipitates obtained by 80 to 90% and 90 to 100% salt saturation. The highest specific activity for hyaluronidase was associated with fraction V-P (100%), obtained by 90 to 100% saturation.

The five salt fractions were assayed qualitatively for various enzyme activities. There was no evidence of coagulase, deoxyribonuclease, α- β-, or Δ-hemolysins, lipase, phosphatase, protease, or staphylokinase activity in fraction V-P (100%). Some hyaluronidase of low specific activity was detected in fraction IV-P (90%). Hyaluronidase was not detected in any of the other fractions (Table 1).

Gel filtration. Samples of the partially purified hyaluronidase-rich fraction V-P were subjected to further purification by gel filtration. Figure 1 indicates a typical profile showing one major and three minor peaks of hyaluronidase activity. These purification procedures, summarized in Table 2, showed a 4,000-fold increase in specific activity after filtration, as compared to the activity of the initial crude culture supernatant concentrate. Gel diffusion analysis of the enzyme-rich fraction obtained after gel filtration indicated a single detectable antigen in well 4, merging in a line of "identity" with the antigen band in the initial V-P fraction, which exhibited two precipitin bands (Fig. 2).

Physicochemical properties. Because small quantities of relatively purified hyaluronidase-rich fractions were obtained after gel filtration, most of the physicochemical studies were performed with fraction V-P (100%), as follows. (i) Enzyme activity remained relatively stable during 1 hr of incubation at 37 C, with only a slight decrease thereafter (Fig. 3). There was an initial decrease

TABLE 1. *Distribution of detectable staphylococcal extracellular enzyme activity in various fractions obtained from culture supernatant fluids by sequential ammonium sulfate fractionation*

Ammonium sulfate fraction	Enzyme activity present in fraction									
	Lipase	Hemolysin			Coagulase	Staphylokinase	Protease	Deoxyribonuclease	Phosphatase	Hyaluronidase
		α	β	Δ						
Initial concn	+	+	+	+	+	+	+	+	+	+
I-P (0–50%)	+	+		+	+					
II-P (50–70%)			+			+	+			
III-P (70–80%)								+		
IV-P (80–90%)									+	+
V-P (90–100%)										+

in enzyme activity at 50 C, without further decrease after an incubation period of 40 min. Marked thermolability was observed at 56 C. There was a variable decrease in activity during the first 10 min at 56 C and then almost complete loss of activity during the next 20 min of heating (Fig. 3). (ii) Samples of the V-P ammonium sulfate fraction containing partially purified hyaluronidase were dissolved in distilled water (10 mg/ml) and passed through 5 cycles of freezing and thawing with dry ice in alcohol and warm water at 37 C. After each cycle, samples were removed and a sample was assayed for hyaluronidase activity. The MCP titers remained unchanged throughout the 5 cycles.

Effect of pH on enzyme stability. The stability of enzyme activity in the partially purified V-P fraction, at a concentration of 5 mg/ml, was determined in acid and alkaline solutions and buffers ranging from *p*H 1 to *p*H 14. Concentration of buffers was 0.1 M, except where indicated. Total activity was lost after 18 hr of incubation at 37 C, at *p*H 1 and 14. There was marked loss of activity after similar incubation at *p*H 13. There was only a slight to moderate loss of activity at other *p*H values (Fig. 4).

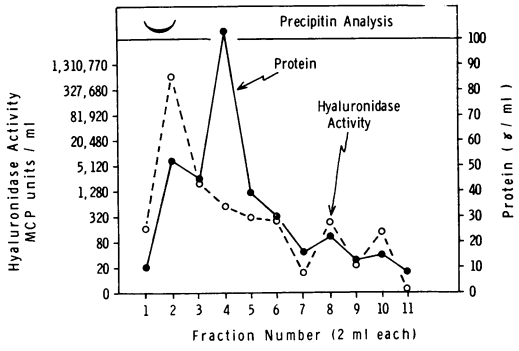

FIG. 1. *Gel filtration of partially purified salt-fractionated staphylococcal hyaluronidase on Sephadex G-100 in 0.05 M sodium phosphate buffer with 0.05 M sodium chloride, pH 7.2. Column was 10 × 1 cm. Precipitin band was detected in major peak by gel diffusion with rabbit antistaphylococcal serum.*

Stability of partially purified hyaluronidase. The hyaluronidase activity of the partially purified fraction V-P remained unchanged after storage for 1 year at −20 C. Incubation at room temperature for 15 hr in 0.15 M phosphate-buffered saline, *p*H 7.2, resulted in no detectable loss of activity. Incubation in 0.3% Noble agar, prepared with phosphate-buffered saline, at room temperature for 15 hr resulted in a fourfold increase in titer.

Effect of dialysis and ethylenediaminetetraacetic acid. Exhaustive dialysis of fractions IV-P and V-P against demineralized distilled water or 10^{-3} M ethylenediaminetetraacetic acid resulted in variable decreases of activity. Fraction V-P had a

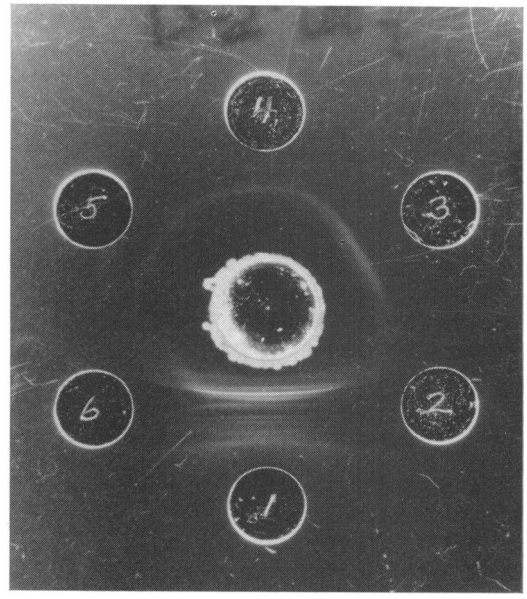

FIG. 2. *Agar-gel diffusion analysis of crude staphylococcal concentrate (well 1) and purified fractions rich in hyaluronidase activity in other wells. Well 4 contains Sephadex-purified fraction and well 5 contains agar-electrophoresis-purified fraction, reported elsewhere (1). Well 3 contains ammonium sulfate-partially purified fraction V-P. A 10-mg/ml antigen sample in each peripheral well. Center well contains rabbit antistaphylococcal serum.*

TABLE 2. *Summary of hyaluronidase activity after purification procedures*

Prepn	Vol	Total protein	Total enzyme units[a]	Specific activity	Recovery
	ml	*mg*	*TRU/ml*	*units/mg*	%
Initial crude conc	200	1.7×10^4	1.9×10^4	1.1	100
Fraction V-P (90–100% Ppt)	9.1	4.4	1.0×10^4	2,272	52
Sephadex G-100; fraction 6	2.0	0.1	438	4,380	2.3

[a] One enzyme unit equivalent to the amount of enzyme which reduces, in 30 min at 37 C, the turbidity of 0.2 mg of hyaluronic acid to that of 0.1 mg.

30-fold loss in activity, whereas fraction IV-P had a 4- to 10-fold loss. Attempts at reactivation by addition of dialysate growth medium, Brain Heart Infusion, divalent cations (10^{-3} M Ca^{++}, Mg^{++}, Mn^{++}), or mercaptoethanol were unsuccessful.

pH optimum. The pH optimum of enzyme activity in fraction V-P was determined over the range of *p*H 4 to *p*H 11 (Fig 5). Two peaks, one at *p*H 6 and a second at *p*H 8.6, were observed, suggesting a diphasic optimal activity range. There was a rapid fall in activity below *p*H 6 and above *p*H 9.

DISCUSSION

Microbial hyaluronidases have been studied primarily from the point of view of their relation to virulence and pathogenicity (10, 19). Many reports have been concerned with streptococcal hyaluronidase, while clostridial, pneumococcal, and staphylococcal hyaluronidases have been studied less extensively (10). None of these enzymes has been purified sufficiently to permit physicochemical examination.

Several methods for partial purification of this enzyme from a variety of sources have been reported. In this regard, a method for partial purification of bovine testicular hyaluronidase using ammonium sulfate and cold ethyl alcohol precipitation (8) was improved upon by ion-exchange chromatography on diethylaminoethyl Sephadex A-50 of a dialyzed ammonium sulfate

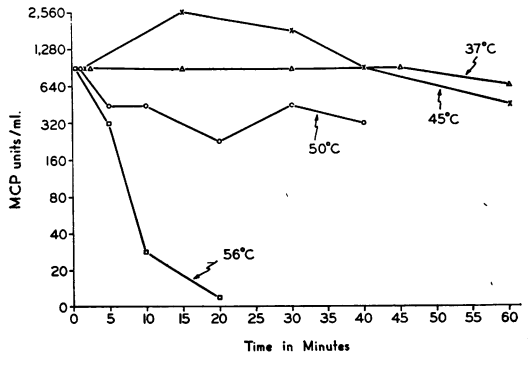

FIG. 3. *Influence of temperature on crude staphylococcal hyaluronidase. Volumes (2.0 ml) containing 10 mg of enzyme-rich fractions per ml, incubated at various temperatures for time period indicated. Residual enzyme activity determined with 0.1-ml samples by MCP method.*

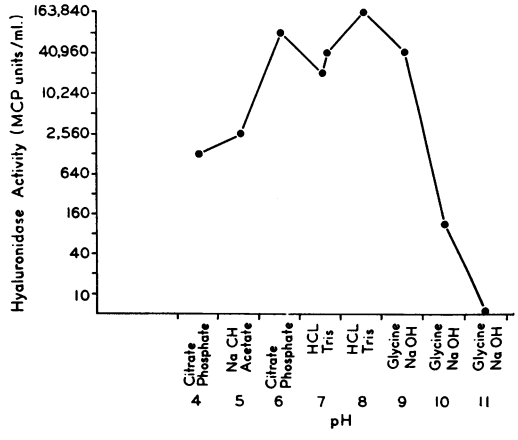

FIG. 5. *Effect of pH on enzymatic activity of partially purified staphylococcal hyaluronidase (10 mg/ml). Various pH values obtained with buffers listed.*

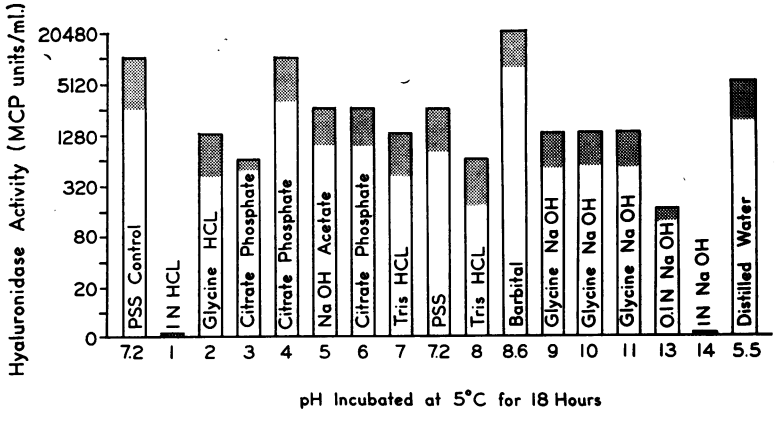

FIG. 4. *Effect of pH on activity of partially purified staphylococcal hyaluronidase. Samples (2.0 ml) containing 10.0 mg of enzyme per ml maintained at 5 C for 18 hr at pH indicated, with buffer or diluent listed. Samples were neutralized with dilute acid or alkali and brought to a constant volume with pH 7.2 phosphate-buffered saline. Enzyme activity was determined by the MCP method, with 0.1-ml samples.*

precipitate (32). Brunish and Mozersky (5) reported a 132-fold increase in specific activity of hyaluronidase from *Escherichia freundii* when extracts were chromatographed on IRC-50 cation exchange resin. Baker et al. (3) purified *Clostridium perfringens* hyaluronidase 1,000-fold by using ammonium sulfate fractionation followed by three ethyl alcohol fractionations at *p*H 9.0, 6.8, and 7.5, respectively. Streptococcal hyaluronidase has been purified by ammonium sulfate fractionation followed by treatment with cholesterol (22).

Rogers purified staphylococcal hyaluronidase 100-fold by ammonium sulfate precipitation, ferric hydroxide precipitation, and elution with sodium carbonate (28). By using two different assay methods, a double *p*H optimum was determined (5.5 and 6.6). He attributed this to the presence of two hyaluronidases. Attempts to separate the two activities by adsorption-elution with calcium phosphate and aluminum oxide columns were unsuccessful.

Davison et al. (7) attempted purification of staphylococcal hyaluronidase by 4% sodium flavianate precipitation but observed a threefold loss in specific activity. Isoelectric precipitation was more successful, resulting in what appeared to be evidence for three hyaluronidases. These were precipitated at *p*H 3, 4, and 5 with 250-, 15-, and 6-fold purifications, respectively.

In the present study, ammonium sulfate sequential fractionation was used as an initial method for partial purification of staphylococcal hyaluronidase after concentration of clarified culture supernatant fluids with 100% ammonium sulfate. Hyaluronidase activity was consistently recovered in two fractions, one with low activity [fraction IV-P (90%)] and the other one with extremely high specific activity [fraction V-P (100%)]. There was a 2,000-fold increase in specific activity of the most active fraction in comparison with the initial crude concentrates. Two major antigens were detected in the fraction with high activity, whereas the crude concentrate had many antigens. In other studies, agar-gel electrophoresis was utilized as an additional method for further purification, but there was only a slight increase in specific activity over that of the 90 to 100% ammonium sulfate fraction (Abramson, Ph.D. Thesis). The fraction obtained by electrophoresis was free of other detectable enzymes tested and revealed a single precipitin band when tested by gel diffusion with specific antiserum. It appeared that a minor antigen contained in the ammonium sulfate-purified fraction may have been eliminated in the fraction obtained by gel electrophoresis, suggesting a further purification in regard to contaminating antigens (Abramson, Ph.D. Thesis).

A report by Hallander indicated that staphylococcal exoenzymes could be readily fractionated by gel filtration (16). Similarly, Zolli and San Clemente (34) achieved a 4,000-fold purification of staphylococcal coagulase by using Sephadex G-200.

In this study, specific activity of hyaluronidase was found to increase nearly twofold over that obtained by ammonium sulfate fractionation when using gel filtration and elution with high-salt buffer. The resulting enzyme-rich fraction was free of coagulase, deoxyribonuclease, α-, β-, and Δ-hemolysins, lipase, phosphatase, protease, and staphylokinase. In addition to the major hyaluronidase peak, there were also several minor peaks with hyaluronidase activity following gel filtration. These results, which are reported in greater detail elsewhere (1), plus the results of Rogers (28) and Davison (7), suggest that there may be multiple molecular forms of staphylococcal hyaluronidase.

In the present study, it was found that enzyme activity in the 90 to 100% ammonium sulfate fraction appeared to adsorb to Sephadex G-100 and could not be eluted with phosphate buffers of low molarity (0.02 M). When the molarity of the phosphate buffer was increased by addition of 0.5 M sodium chloride, hyaluronidase activity was quantitatively recovered in the eluates (Abramson, Ph.D. Thesis). Similar adsorption has been found to occur with basic proteins such as lysozyme and ribonuclease and is reportedly due to the presence of negatively charged carboxyl groups in the dextran (12, 14, 21). Basic proteins of low molecular weight apparently are adsorbed to Sephadex and can be quantitatively "desorbed" by elution with a salt gradient. Hyaluronidase appears to have properties characteristic of a basic protein, as suggested by experiments indicating an isoelectric point of *p*H 9.7 (Abramson and Friedman, Federation Proc., p. 244, 1965), and its properties on Sephadex.

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