

Purification and Characterization of a Staphylolytic Enzyme from *Pseudomonas aeruginosa*¹

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A strain of *Pseudomonas aeruginosa* has been shown to produce an enzyme that lyses viable cells of *Staphylococcus aureus*. The maximal yield of the enzyme was obtained from shake flask cultures of *P. aeruginosa* which were grown for 18 to 22 hr at 37 C in Trypticase Soy Broth. A 333-fold purification of the enzyme was obtained by acetone precipitation of the culture liquor, followed by column chromatography on phosphonic acid cellulose and Bio-Gel P2. The staphylolytic enzyme exhibited maximal activity at 37 C in 0.01 M sodium phosphate (pH 8.5) and was stable at 37 C in the pH range of 7.5 to 9.5. The inhibition and stabilization of the enzyme by various organic and inorganic materials was investigated. Spheroplasts of *S. aureus* were formed by treating viable cells with the staphylolytic enzyme in 1 M sucrose or human serum.

In recent years, many enzymes have been reported which lyse viable cells of *Staphylococcus aureus*. These staphylolytic enzymes have been isolated from *Streptomyces albus* G (3-5, 11) and from specific species of *Chalaropsis* (6, 13), *Flavobacterium* (7), *Staphylococcus* (1, 11, 12), and *Cytophaga* (J. C. Ensign and R. S. Wolfe, Bacteriol. Proc., p. 33, 1964). In 1965, Zyskind et al. (14) reported the production of a staphylolytic enzyme by a species of *Pseudomonas*. This enzyme caused the rapid lysis of viable cells of *S. aureus*, *S. roseus*, *Gaffkya tetragena*, and *Sarcina lutea*. This report is concerned with studies of the production, purification, and characterization of this enzyme.

MATERIALS AND METHODS

Bacterial strains. The strain of *Pseudomonas* reported by Zyskind et al. (14), and hereafter referred to as strain X, was used in this study. Strain 655 of *S. aureus* (9) was used as substrate for the staphylolytic enzyme and in spheroplast studies. Both microorganisms were maintained at 4 C on Brain Heart Infusion Agar slants.

Production of the crude enzyme. The inoculum was prepared by streaking the entire surface of a Trypticase Soy Agar (BBL) plate with an active culture of strain X. After incubation at 37 C for 14 hr, the cells were harvested from the plate and inoculated into 1 liter of Trypticase Soy Broth (TSB, BBL) contained

in a 2,800-ml Fernback flask. The flask was shaken at 37 C on a New Brunswick model VL platform shaker at a setting of six. At various intervals during the fermentation, samples were removed from the flask, centrifuged at room temperature, and assayed for staphylolytic activity. After 18 to 22 hr, the cells were removed by centrifugation at 10,000 × g, and the resulting culture liquor was cooled rapidly to 0 C.

Enzyme assays and protein determination. The activity of the staphylolytic enzyme was determined at 37 C in phosphate buffer. (Unless otherwise specified, phosphate buffer refers to 0.01 M Na₂HPO₄, pH 8.5.) The substrate for the assay was prepared daily by rehydrating a vial of lyophilized cells of *S. aureus* strain 655 in phosphate buffer. Sufficient rehydrated cells were added to 9.0 ml of phosphate buffer to obtain an optical density (OD) reading of 0.50 at a wavelength of 535 mμ with a Bausch & Lomb Spectronic-20 colorimeter. A 1-ml amount of the staphylolytic enzyme, diluted when necessary to obtain OD reductions of 0.15 to 0.25 in 5 min, was then added to the assay tube. At 1 min after the addition of the enzyme, the OD was recorded at 1-min intervals. One unit of activity was defined as the amount of staphylolytic enzyme required to reduce the OD of a suspension of cells of strain 655 by 0.01 OD unit per min.

Protein was determined by the method of Lowry et al. (8) with bovine serum albumin (BSA, Fraction V, Nutritional Biochemicals Corp., Cleveland, Ohio) as standard.

Protease activity was determined by a modification of the procedure of Ghuysen et al. (4). A mixture of 0.5 ml of 1% casein (Calbiochem, Los Angeles, Calif.) in phosphate buffer and 0.1 ml of an appropriate dilution of enzyme was incubated at 37 C. After 15 min, 4.5 ml of 5% trichloroacetic acid was added, and the mixture was incubated at 45 C for 15 min, cooled,

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and centrifuged at $3,000 \times g$ for 15 min. The absorption of the supernatant fluid was then determined at $280 m\mu$ with a Beckman model DB spectrophotometer.

Preparation of chromatography columns. A 25-g amount of phosphonic acid (P) cellulose (Cellex P, Bio-Rad Laboratories, Richmond, Calif.), washed in distilled water, was settled to form a column, 4 by 20 cm. The column was equilibrated with 0.01 M phosphate buffer (pH 7.0) before use.

A 90-g amount of Bio-Gel P-2 (Bio-Rad) was settled to form a 3.5 by 33 cm column, which was then equilibrated to pH 8.5 with phosphate buffer.

RESULTS

Identification of strain X. Strain X was a gram-negative rod which produced a green, water-soluble pigment, was oxidative, possessed a polar flagellum, grew readily at 42 C, and produced the odor of trimethylamine. The results of these tests agree with the description of *P. aeruginosa* given in *Bergey's Manual of Determinative Bacteriology*.

Production of the staphylolytic enzyme. Preliminary experiments revealed that the highest yields of the staphylolytic enzyme were obtained from shake flask cultures of strain X grown at 37 C. Among a variety of media examined, TSB supported the highest yields of the enzyme. No enzyme was produced in a synthetic medium composed of amino acids, vitamins, and basal salts, although an OD value of 1.3 was obtained after 24 hr. Although luxurious growth occurred in all media examined, the production of the enzyme varied considerably.

As shown by the relationship of the growth of

strain X to the production of the staphylolytic enzyme (Fig. 1), significant amounts of the enzyme were not detected in the culture liquor until the onset of the stationary phase of growth (OD = 2.0). Maximal concentrations of the enzyme (70 to 80 units per ml) were characteristically obtained between 18 and 22 hr after inoculation.

Precipitation of the staphylolytic enzyme from the culture liquor. Acetone was added slowly to the culture liquor with continuous stirring at 0 C. The precipitate obtained after the addition of 2.3 volumes of acetone was allowed to settle for 1 hr at 0 C, after which the supernatant fluid was decanted and discarded. The heavy, brown precipitate was dissolved in 100 ml of cold phosphate buffer, and insoluble material was removed by centrifugation at $10,000 \times g$ for 30 min at 0 C. The supernatant fluid contained 73% of the original activity, with a 4.4-fold increase in specific activity.

Column chromatography on P cellulose. All operations were conducted at 4 C. A 25-ml sample of the enzyme obtained after the acetone precipitation and centrifugation was applied to a column of P cellulose. The column was then washed with 200 ml of 0.01 M phosphate buffer (pH 7.0) and was eluted with a linear gradient of 0.02 M phosphate buffer (pH 8.5) to 0.1 M Na_2HPO_4 adjusted to pH 10.0 with 1.0 M NaOH. The flow rate was 60 ml/hr, and 10-ml fractions were collected. Most of the proteolytic activity was eluted as an early front, although trace amounts were detected throughout the entire elution (Fig. 2). The con-

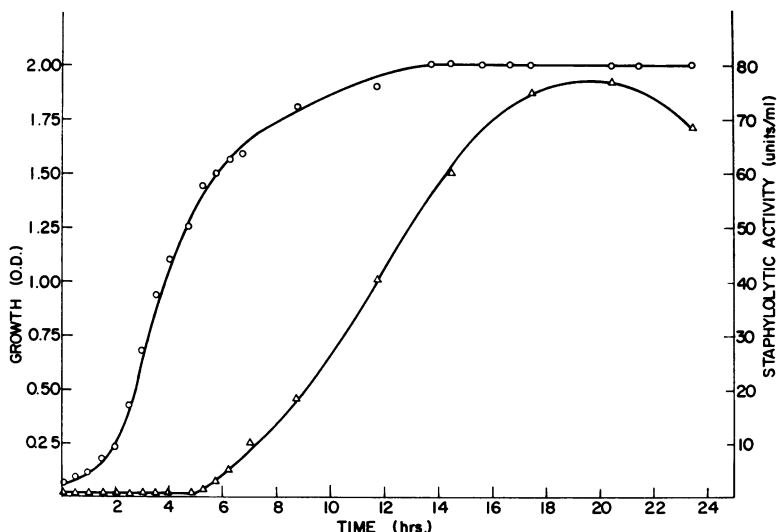


FIG. 1. Relationship of the growth of *Pseudomonas aeruginosa* strain X (○) to the production of the staphylolytic enzyme (△). Strain X was grown in a shake flask culture at 37 C in TSB. Growth (OD) was determined at $540 m\mu$. The staphylolytic activity was determined in 0.01 M phosphate buffer (pH 8.5).

tents of tubes 125 to 140 were combined, and were found to contain 41% of the original staphylo-lytic activity, with a 500-fold increase in specific activity over the original culture liquor.

Removal of excess salts. The volume of the enzyme solution recovered from the P cellulose column was reduced about fourfold with dry Aquacide 1 (Calbiochem) at 0 C. Approximately 50% of the staphylo-lytic activity recovered from P cellulose was lost in this step. To remove the excess buffer salts, the enzyme was passed through the P-2 gel filtration column at 0 C. The resulting enzyme solution was then lyophilized to dryness and stored at -10 C in sealed vials. The results of the purification procedure are summarized in Table 1.

Properties of the purified staphylo-lytic enzyme. Within the pH range of 3.0 to 11.0, maximal activity of the enzyme was observed at pH 8.5 (Fig. 3). Figure 4 presents data indicating that maximal activity of the staphylo-lytic enzyme was obtained in 0.01 M sodium phosphate. The same results

were obtained with K_2HPO_4 and ammonium acetate buffers of various molarities (all pH 8.5). With organic buffers such as triethanolamine and tris(hydroxymethyl)aminomethane (Tris), the highest staphylo-lytic activity was obtained at 0.1 M; at molarities higher or lower than 0.1 M, the staphylo-lytic activity was reduced.

The effects of temperature, pH, molarity of buffer, and various additives on the stability of the enzyme were also examined. When stored for 24 hr at 4 C in 0.01 M phosphate buffers of various pH values, the enzyme was stable between pH values of 7.5 and 9.5, but was inactivated outside this range. Incubation of the enzyme in phosphate buffer at various temperatures revealed almost no loss in activity at 37 C in 2 hr; however, after 20 hr of incubation at 37 C, only 64% of the activity remained. At 65 C, the enzyme was completely inactivated in 15 min. Storage of enzyme samples in phosphate buffers of various molar concentrations at 4 C for 24 hr indicated maximal stability in 0.1 M Na_2HPO_4 , although the activity

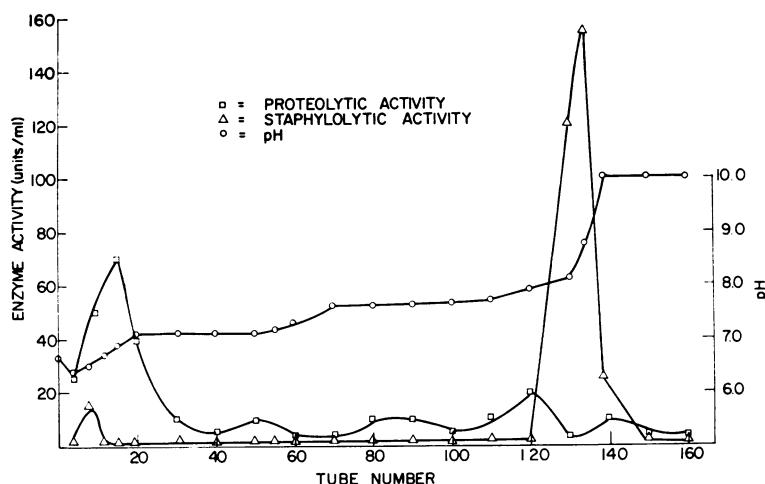


FIG. 2. Elution of the staphylo-lytic enzyme from P cellulose. The column was washed with 200 ml of 0.01 M phosphate buffer (pH 7.0) and was eluted with a linear gradient of 0.02 M phosphate buffer (pH 8.5) to 0.1 M Na_2HPO_4 , adjusted to pH 10.0 with 1.0 M NaOH. Flow rate was 60 ml/hr, and each tube contained 10 ml.

TABLE 1. Purification of the staphylo-lytic enzyme from *Pseudomonas aeruginosa* strain X^a

Procedure	Vol	Concn	Total amt	Protein	Specific activity	Yield	Purification
	ml	units/ml	units	mg/ml	units/mg	%	
Initial extract.....	5,400	65	351,000	6.2	10	100	1
Acetone precipitate.....	138	1,850	255,300	42	44	73	4.4
P cellulose.....	967	150	145,050	0.03	5,000	41	500
P-2.....	737	100	73,700	0.03	3,333	21	333

^a One unit of staphylo-lytic activity is the amount of enzyme required to reduce the OD of a suspension of cells of *Staphylococcus aureus* 655 by 0.01 unit per min. Specific activity is expressed as units per milligram of protein.

of the enzyme was completely inhibited under these conditions (Fig. 4).

Phosphate buffers were prepared containing the following materials: $MgCl_2$, $MnCl_2$, mercaptoethanol (all 10^{-4} M), 0.01% bovine serum albumin (BSA), and 0.1 M Na_2HPO_4 . An enzyme sample was added to each buffer and incubated for 22 hr at 22 C. The enzyme was then diluted 1:10 in phosphate buffer and assayed for staphylolytic activity. BSA, Na_2HPO_4 , and $MnCl_2$ all enhanced the stability of the enzyme to significant degrees, whereas $MgCl_2$ afforded only a slight stabilizing effect. $CaCl_2$ caused a slight inactivation, and mercaptoethanol resulted in complete inactivation of the staphylolytic enzyme.

The effects of various substances on staphylolytic activity were tested by their addition to the assay buffer containing substrate. $MgCl_2$ (10^{-4} M) and 1% human serum had no effect on the staphylolytic activity of the enzyme; 10^{-3} M ethylenediaminetetraacetic acid (EDTA) caused 15% inhibition; $MnCl_2$, $BaCl_2$, $FeSO_4$, $CaCl_2$ (all 10^{-4} M), and 10^{-3} M mercaptoethanol caused from 25 to 50% inhibition. The enzyme was completely inhibited by 10^{-2} M EDTA, 10^{-4} M $HgCl_2$, and 10^{-4} M $CuSO_4$.

Spheroplast formation. Suspensions of viable cells of *S. aureus* strain 655 were prepared in phosphate buffer, phosphate buffer containing 1

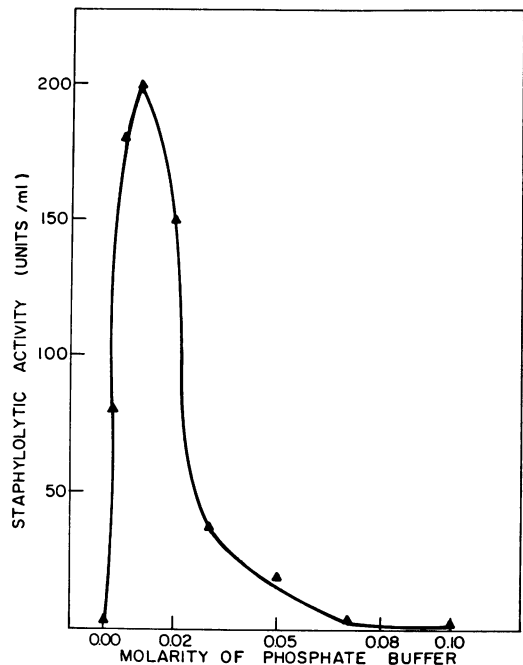


FIG. 4. Relationship of molarity to the staphylolytic activity of enzyme. All buffers were pH 8.5.

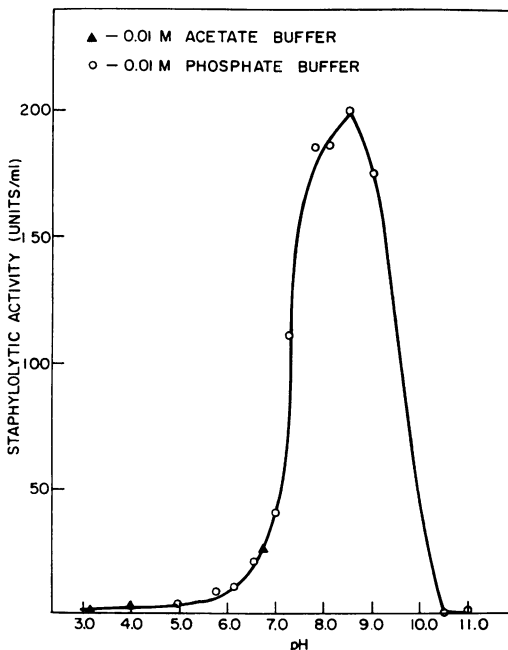


FIG. 3. Relationship of pH to the staphylolytic activity of the enzyme.

M sucrose, and in indiluted human serum. Purified enzyme was added to each suspension, and the OD was recorded at 1-min intervals. The results (Fig. 5) obtained in 1 M sucrose and in human serum were similar; after an initial reduction in turbidity, the OD stabilized and remained stable for at least 0.5 hr at room temperature. However, if sodium lauryl sulfate was added to each preparation after 8 min, the remaining cells in sucrose and human serum were rapidly lysed. Furthermore, if cells exposed to the enzyme in sucrose or human serum were collected by centrifugation ($10,000 \times g$ for 30 min at 22 C) and resuspended in phosphate buffer, a rapid reduction in turbidity and a marked increase in viscosity occurred.

Staphylolytic activity of various *P. aeruginosa* isolates. Twenty clinical isolates of *P. aeruginosa* were examined for their ability to produce a staphylolytic enzyme. The supernatant fluids from cultures of all strains exhibited relatively high staphylolytic activity when cultivated in shake flasks of TSB.

Lysis of *P. aeruginosa* with the staphylolytic enzyme. The effect of the purified enzyme (100 units) on cells from 12-hr (young) and 24-hr (old) cultures of strain X was examined. The cells from an old culture lysed quite slowly, the OD of the suspension being reduced by one-third in 1 hr. Both viable and heat-killed young cells were com-

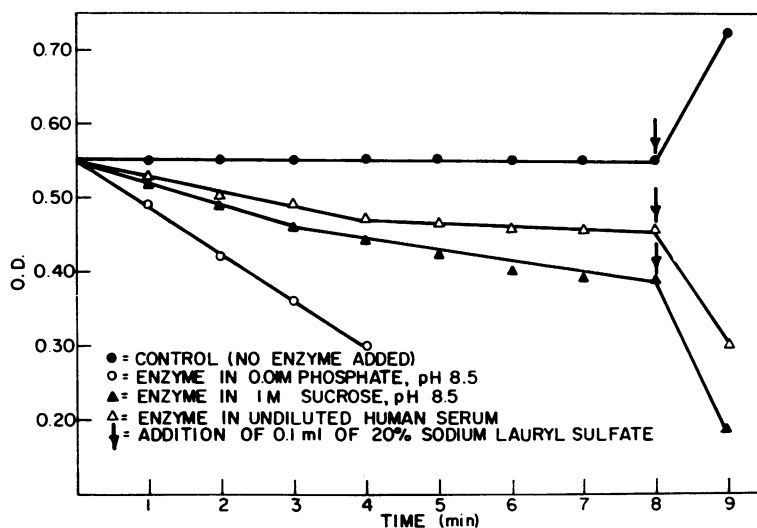


FIG. 5. Formation of spheroplasts. Staphylolytic enzyme (170 units) was added to viable cells of *Staphylococcus aureus* strain 655 in 1 M sucrose and undiluted human serum. Enzyme (17 units) was added to cells in 0.01 M phosphate buffer for comparison. The control contained cells in phosphate buffer with no enzyme.

pletely resistant to the action of the enzyme. However, acetone-dried young cells were susceptible to lysis by the enzyme at a rate similar to that observed with the untreated old cells.

DISCUSSION

All clinical isolates of *P. aeruginosa* tested during the course of this study produced a staphylolytic enzyme. Zyskind et al. (14) demonstrated that broth cultures of laboratory strains of *P. fluorescens* and *P. aeruginosa* possessed staphylolytic activity, although a broth culture of *P. saccharophila* did not. Thus, the production of a staphylolytic enzyme appears to be a common property of this genus. De Ley (2), in a recent review of *Pseudomonas* and related genera, discussed the heterogeneity of the *Flavobacterium* and *Cytophaga*, and the close proximity of certain members of these genera to the *Pseudomonadaceae* with respect to the guanine plus cytosine content of their deoxyribonucleic acid. Therefore, the isolation of staphylolytic enzymes from unidentified species of *Flavobacterium* (7) and *Cytophaga* (J. C. Ensign and R. S. Wolfe, *Bacteriol. Proc.*, p. 33, 1964) may provide further evidence for the relatedness of certain members of these genera to the *Pseudomonadaceae*.

The staphylolytic enzyme appears to function as an autolytic enzyme in *P. aeruginosa* strain X. This conclusion is supported by the observation that when unusually high levels of the enzyme (80 to 100 units per ml) are obtained in broth cultures, autolysis of the cells is apparent. Corre-

lated with this observation is the greater sensitivity of old cells of strain X to lysis by the enzyme.

The significance of staphylolytic enzymes to the study of *S. aureus* is already becoming apparent. Staphylolytic enzymes have been used extensively in the study of the structure and synthesis of the cell wall of *S. aureus* (cf. 6). Furthermore, the isolation of macromolecular components from within the cell after treatment with a staphylolytic enzyme permits detailed genetic and physiological studies of *S. aureus*. The staphylolytic enzyme from strain X is now being used in this laboratory for the routine isolation of highly polymerized deoxyribonucleic acid from *S. aureus* (Zyskind, Guerin, and Pattee, unpublished data).

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