Purification and Characterization of a Staphyloolytic Enzyme from *Pseudomonas aeruginosa*

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A strain of *Pseudomonas aeruginosa* has been shown to produce an enzyme that lyse 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 phosphoric acid cellulose and Bio-Gel P2. The staphyloolytic 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 staphyloolytic enzyme in 1 m sucrose or human serum.

In recent years, many enzymes have been reported which lyse viable cells of *Staphylococcus aureus*. These staphyloolytic 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 staphyloolytic 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 staphyloolytic 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

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2 Predoctoral Fellow of the Iowa State Research Foundation.
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 staphyloolytic enzyme. Preliminary experiments revealed that the highest yields of the staphyloolytic 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 staphyloolytic 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 staphyloolytic 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 NaHPO\(_4\), 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 staphyloolytic enzyme (△). Strain X was grown in a shake flask culture at 37 C in TSB. Growth (OD) was determined at 540 m\( \mu \). The staphyloolytic activity was determined in 0.01 M phosphate buffer (pH 8.5).](http://jb.asm.org/Downloaded from http://jb.asm.org)
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 en-
zeyme solution recovered from the P cellulose 
column was reduced about fourfold with dry 
Aquacide 1 (Calbiochem) at 0 C. Approximately 
50% of the staphyloytic activity recovered from 
P cellulose was lost in this step. To remove the 
extra 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 staphyloytic enzyme. 
Within the pH range of 3.0 to 11.0, maximal ac-
itivity of the enzyme was observed at pH 8.5 (Fig. 
3). Figure 4 presents data indicating that maximal 
activity of the staphyloytic enzyme was obtained 
in 0.01 M sodium phosphate. The same results 
were obtained with K2HPO4 and ammonium ace-
tate buffers of various molarities (all pH 8.5). 
With organic buffers such as triethanolamine and 
is(hydroxymethyl)aminomethane (Tris), the 
highest staphyloytic activity was obtained at 
0.1 M; at molarities higher or lower than 0.1 M, 
the staphyloytic 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 com-
pletely 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 Na2HPO4, although the activity

![Figure 2: Elution of the staphyloytic 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 Na2HPO4, adjusted to pH 10.0 with 1.0 M NaOH. Flow rate was 60 ml/hr, and each tube contained 10 ml.](http://jb.asm.org/)

<table>
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<th>Table 1. Purification of the staphyloytic enzyme from Pseudomonas aeruginosa strain Xs</th>
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<td>Procedure</td>
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*a One unit of staphyloytic 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: MgCl₂, MnCl₂, mercaptoethanol (all 10⁻⁴ M), 0.01% bovine serum albumin (BSA), and 0.1 M Na₂HPO₄. 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, NaN₃PO₄, and MnCl₂ all enhanced the stability of the enzyme to significant degrees, whereas MgCl₂ afforded only a slight stabilizing effect. CaCl₂ caused a slight inactivation, and mercaptoethanol resulted in complete inactivation of the staphylolytic enzyme.

The effects of various substances on staphylo-
lytic activity were tested by their addition to the assay buffer containing substrate. MgCl₂ (10⁻⁴ M) and 1% human serum had no effect on the staphylytic activity of the enzyme; 10⁻³ M ethylenediaminetetraacetic acid (EDTA) caused 15% inhibition; MnCl₂, BaCl₂, FeSO₄, CaCl₂ (all 10⁻⁴ M), and 10⁻² M mercaptoethanol caused from 25 to 50% inhibition. The enzyme was completely inhibited by 10⁻³ M EDTA, 10⁻⁴ M HgCl₂, and 10⁻⁴ M CuSO₄.

**Spheroplast formation.** Suspensions of viable cells of *S. aureus* strain 655 were prepared in phosphate buffer, phosphate buffer containing 1 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 × 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 staphylytic 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-
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 staphylocytic enzyme. Zyskind et al. (14) demonstrated that broth cultures of laboratory strains of *P. fluorescens* and *P. aeruginosa* possessed staphylocytic activity, although a broth culture of *P. saccharophila* did not. Thus, the production of a staphylocytic 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 *Pseudomonadaeae* with respect to the guanine plus cytosine content of their deoxyribonucleic acid. Therefore, the isolation of staphylocytic 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 *Pseudomonadaeae*.

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

The significance of staphylocytic enzymes to the study of *S. aureus* is already becoming apparent. Staphylocytic 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 staphylocytic enzyme permits detailed genetic and physiological studies of *S. aureus*. The staphylocytic 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).

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

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