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Purification and Characterization of *Pseudomonas aeruginosa* Exotoxin

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A trypsin-sensitive, heat-labile exotoxin of *Pseudomonas aeruginosa* strain P-A-103 has been purified by a procedure that includes membrane ultrafiltration, hydroxylapatite chromatography, ion-exchange cellulose chromatography, and gel filtration chromatography. The procedure resulted in the recovery of 48% of the exotoxin with a 40-fold increase in specific activity (micrograms of protein per median lethal dose [LD_{50}]). The mean lethal dose of the purified toxin administered intravenously into mice weighing 20 g was approximately 6 μg of protein. The toxin contained virtually no nucleic acid, detectable pigment, or lipopolysaccharide. When subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, the toxin separated into at least six protein components which appeared to have similar molecular weights. The estimated molecular weight of the toxin is 54,000, and its isoelectric point is 5.0

Pseudomonas aeruginosa has received considerable attention in recent years as a pathogen for a large number of patients debilitated or immunologically compromised by primary disease, trauma or antineoplastic therapy (3, 5, 8). Studies of the pathogenesis of *P. aeruginosa* infections have revealed that the organism produces in addition to endotoxin, a variety of extracellular substances which may contribute to the virulence of the organism (6, 7, 9, 10, 12-14, 16). The least-characterized, yet most likely major, virulence factor is the trypsin-sensitive, heat-labile exotoxin originally described by Liu (13).

This toxin has been shown to be lethal for mice (13) and to be capable of eliciting hypotensive shock in monkeys (O. R. Pavlovskis and C. M. Herman, personal communication) and in dogs (4) after intravenous administration. Previous investigations in this laboratory have shown that the toxin inhibits uridine and amino acid uptake by tissue culture cells (18) and inhibits the respiration of mitochondria derived from mouse liver cells (17). These experiments were done with relatively crude and ill-defined toxin preparations.

This investigation was prompted by the importance of studying the toxin in the absence of contaminating materials such as pigment, endotoxin, and nucleic acid. This paper describes the successful separation of the toxin from these other components and presents some of its biochemical properties.

MATERIALS AND METHODS

Microorganisms. A nonproteolytic strain (P-A-103) of *P. aeruginosa* (12) was used throughout this investigation. Stock cultures were transferred to Trypticase soy agar (BBL) slants and incubated overnight at 37 C. The resultant heavy growth was suspended in Trypticase soy broth dialyzate (TSBD) containing 15% (vol/vol) glycerol and stored in small volumes at −70 C. For each experiment, a fresh vial was thawed and used to inoculate two slants of Trypticase soy agar. After overnight incubation at 37 C, the growth was suspended in 1 ml of sterile saline and used to inoculate a TSBD culture (100 ml). After 6 h of growth at 37 C with shaking, the TSBD culture was used as the inoculum for the cultures needed in an experiment.

Toxin extraction procedure. Twenty 32-ounce (approximately 0.95 liter) prescription bottles, containing TSBD and 1% agar, were inoculated each with 2.5 ml of the TSBD broth culture and incubated at 37 C for 18 to 24 h. After incubation, the cultures were frozen and thawed and the expressed liquids were collected and centrifuged (16,000 × *g*, 30 min). The supernatant fluid was passed through a prewashed 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) and stored at −20 C until used.

Toxin assay. The mouse lethality bioassay was used to measure toxicity. Filter-sterilized samples (0.2 ml), undiluted and serially diluted in saline, were injected intravenously into female Swiss white mice of the NIH-NMRI inbred strain, weighing 20 ± 2 g (four mice per dilution). Mortality was recorded for 5 days, and the median lethal dose (LD_{50}) of each preparation was expressed as micrograms of protein per 20 g of body weight.

Protein measurement. Protein concentrations

were determined by the method of Lowry et al. (15) using chemically defined LAB-TROL (Dade) as standard.

Limulus assay. The limulus assay for endotoxin was performed by R. D. Meyer and A. R. Kraft by the method of Levin and Bang (11). Lysates or amoebocytes from the horseshoe crab, *Limulus polyphemus*, were obtained from either the Sigma Chemical Co. (St. Louis) or were prepared from bleedings of horseshoe crabs by the method of Levin and Bang as modified by Yin et al. (22). Pyrogen-free glassware was prepared by washing in detergent and rinsing with 0.1 N NaOH and distilled water. Glassware was then autoclaved and heated for 18 h at 180 C in a hot-air oven. Reaction mixtures consisted of 0.1 ml test or control and 0.1 ml of lysate. Negative controls consisting of sterile distilled water were run with each measurement. Each assay was calibrated using lipopolysaccharide (LPS) from *Escherichia coli* 0111-B4 (Difco, Detroit) and *P. aeruginosa* (described below) in known concentrations as standards. Only solid gelation after 24 h incubation at 37 C was considered positive.

Membrane ultrafiltration. Macromolecular ultrafiltration was performed at 4 C under positive nitrogen pressure using Amicon ultrafiltration cells and membranes (Amicon Corp., Lexington, Mass.) The membranes used were XM-300, XM-100A, and PM-10, which have molecular weight cut-off points of 300,000, 100,000, and 10,000, respectively.

Chromatographic beds. Hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) was suspended in 0.005 M NaH_2PO_4-0.10 M NaCl, pH 7.0, and packed under positive pressure. The column was equilibrated by elution with 25 bed volumes of the phosphate buffer, and was charged with approximately 3 mg of protein per ml of bed volume.

Anion exchange chromatography was performed using diethylaminoethyl (DEAE) cellulose. The cellulose powder was decolorized by washing in 0.5 N NaOH. It was then acidified with 0.5 N HCl and washed free of acid with distilled water. The cellulose was washed again in 0.5 N NaOH, filtered, and washed free of alkali with distilled water. The adsorbent was poured into a column (14 by 1.5 cm) and equilibrated with 500 ml of 0.01 M tris(hydroxylmethyl)aminomethane (Tris), 0.10 M NaCl, pH 8.0.

Sephadex G-150 was allowed to swell for 2 days in 0.01 M NaH_2PO_4, 0.85% NaCl, pH 7.5. The swollen Sephadex was degassed by heating in a boiling water bath for 4 h and was poured into a column measuring 70 by 1.6 cm. The packed column was equilibrated with 1 liter of the phosphate buffer. Blue dextran 2000 (Pharmacia Fine Chemicals) was used to determine the void volume (V_o) and NaCl was used to measure the salt volume (V_s) of the column. Protein was measured at 280 nm using a Buchler Fracto-Scan ultraviolet monitor (Buchler Instruments, Fort Lee, N.J.), and 3.5-ml fractions were collected at a flow rate of approximately 20 ml/h.

Isoelectric focusing. An LKB 8101 (110 ml) column (LKB Instruments, Rockville, Md.) was used to focus toxin in a sucrose density gradient (0 to 50%) containing 1% (vol/vol) Ampholine (pH 3 to 10 range, LKB Instruments). Toxin (2.0 mg) was dialyzed against 1% glycine and then added to the solution containing no sucrose prior to the preparation of the gradient. The column was cooled to 4 C by a Neslab RTE-8 (Neslab Instruments, Portsmouth, N. H.) circulating-water bath. The final potential was 400 V, and 26 h was allowed for equilibrium to be reached. Fractions (4 ml) were collected, and the pH of each fraction was determined while maintaining the temperatures of each sample at 4 C. Absorbance at 280 nm was used to measure protein in each sample. The 280-nm absorbance was corrected for background absorbance contributed by the ampholytes and sucrose.

Polyacrylamide gel electrophoresis. Toxin preparations were assayed for purity by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis by the method of Schnaitman (19). Gels were stained with Coomassie blue by the method of Weber and Osborn (20).

Molecular weight determination. The molecular weight of toxin was estimated by the method of Andrews (1, 2) using Sephadex G-150 gel filtration chromatography (described above). The K_{av} value of toxin was compared with the K_{av} values of proteins of known molecular weights: bovine albumin (Pentex, Miles Laboratories) ovalbumin, chymotrypsinogen A, and ribonuclease A (Pharmacia Fine Chemicals).

Extraction and purification of endotoxin. Strain PA-103 LPS was extracted from heat-killed organisms by the hot phenol-water method described by Westphal and Jann (21). The final product represented a mixture of ribonucleic acid (RNA) and LPS. To remove the RNA, the mixture was centrifuged at 80,000 $\times$ *g* for 8 h. The supernatant fluid was decanted and the pellet was suspended and centrifuged for 3 h at 105,000 $\times$ *g*. The final pellet was used as the purified LPS preparation and represented approximately 1% (wt/wt) of the starting dry weight of cells.

RESULTS AND DISCUSSION

Purification. The toxin purification procedure outlined below consists of four major steps: membrane ultrafiltration, hydroxylapatite adsorption chromatography, anion exchange chromatography on DEAE-cellulose, and Sephadex G-150 gel filtration chromatography.

After preliminary experiments had shown that an XM-100A ultrafiltration membrane retained about 40% of the toxin, an XM-300 membrane was used and found to retain less than 10% of the starting toxin activity. Consequently, an XM-300 ultrafiltration membrane was used routinely to remove components having molecular weights of at least 300,000 daltons from the toxin. For the purpose of concentrating the XM-300 membrane filtrate, a PM-10 ultrafiltration membrane was used with negligible loss of toxin through the membrane. The PM-10 retentate, representing a 30-fold reduction in

volume, was made 80% saturated with ammonium sulfate, and the resulting precipitate was collected by centrifugation, dissolved, and dialyzed overnight at 4 C against a solution of 5.0 mM NaH_2PO_4-0.10 M NaCl, pH 7.0.

The concentrated material was then subjected to adsorption chromatography on hydroxylapatite (Fig. 1) with a stepwise increase in sodium phosphate concentrations of 0.01 M, 0.06 M, and 0.36 M (pH 7.0) in 0.10 M NaCl. Three absorbancy peaks were obtained, designated A, B and C, respectively. When individual fractions were assayed for toxicity, only fractions eluted with 0.06 M sodium phosphate (peak B) contained toxin. When assayed for LPS, peak A contained 100 μg, peak B contained 1 μg, and peak C contained 10 μg of limulus-positive material per ml. It also was observed that peak A contained the major portion of pigment, but smaller amounts were visible in peaks B and C. The 280 nm/260 nm absorption ratio for peaks A, B, and C were 0.95, 1.10, and 0.60, respectively, indicating that peak C contained a comparatively greater concentration of 260 nm absorbing material (i.e., nucleic acid) than either peaks A or B. Toxin which eluted as peak B was concentrated by ammonium sulfate precipitation and equilibrated against 0.01 M Tris-hydrochloride-(pH 8.0)-0.10 M NaCl on a Bio-Gel P-6 column.

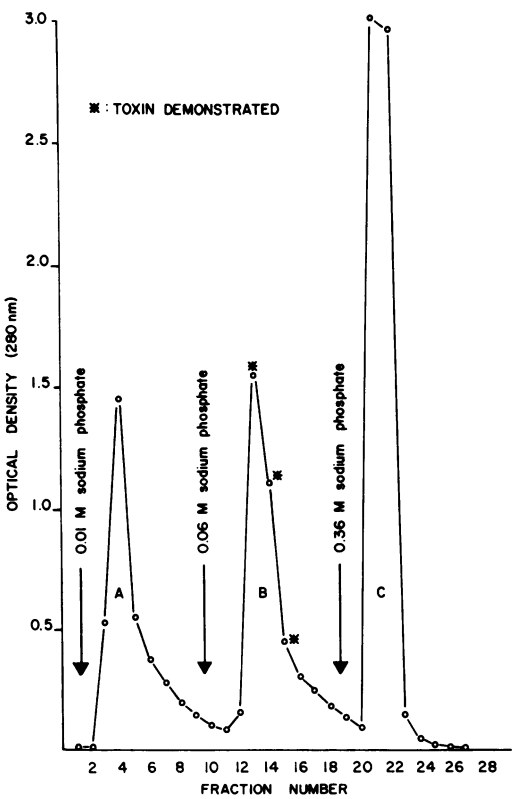

FIG. 1. *Hydroxylapatite (HTP) chromatography of P. aeruginosa exotoxin preparation. Approximately 40 mg of protein was applied to a 12-ml bed volume of HTP in 0.005 M NaH_2PO_4-0.10 M NaCl (pH 7.0). Elution was achieved under positive pressure with a stepwise increase in sodium phosphate concentrations as indicated. Fractions (4 ml) were collected and assayed for toxin by the mouse lethality bioassay.*

Toxin was further purified by ion-exchange chromatography on DEAE-cellulose (Fig. 2). Three elution buffer systems were used to achieve a stepwise increase in ionicity: 0.10 M, 0.23 M, and 1.0 M NaCl, respectively, in 0.01 M Tris-hydrochloride (pH 8.0). Fractions representing the peak material eluted at each step were pooled and assayed for toxin. Toxin activity was only detected in the material eluted with 0.23 M NaCl. The concentration of LPS in this toxin preparation was 1 ng/ml. The majority of the remaining pigment adsorbed strongly to the cellulose and eluted only with 1.0 M NaCl.

Additional toxin purification was achieved by gel-filtration chromatography. Toxin purified through the step of DEAE-cellulose chromatography was concentrated by ammonium sulfate precipitation and chromatographed on Sephadex G-150 (Fig. 3). Toxin eluted from the column as the frontal portion of the first and major absorbancy peak. Fractions containing toxin activity were pooled and found to contain no detectable LPS by the limulus assay.

A summary of the results obtained by monitoring the purification of toxin at each of the steps described above is presented in Table 1. The yields were calculated by arbitrarily designating the material recovered after the step of membrane ultrafiltration and concentration as 100%. This designation was used because the loss of toxin ($<10\%$) prior to ultrafiltration was not measured with accuracy. The total recovery of toxin after gel filtration chromatography was 48%. The degree of purification was determined by comparing the amount of protein required for the LD_{50} at each step of purification with that of the starting material. On this basis, a 40-fold purification of the toxin was achieved.

Since, for the purpose of our studies, endotoxin was the most important contaminant, the degree of LPS contamination was monitored at each step in purification. After Sephadex G-150 chromatography, the toxin contained no LPS demonstrable by limulus assay or less than 10^{-9} g/ml.

Properties of purified toxin. A visible and

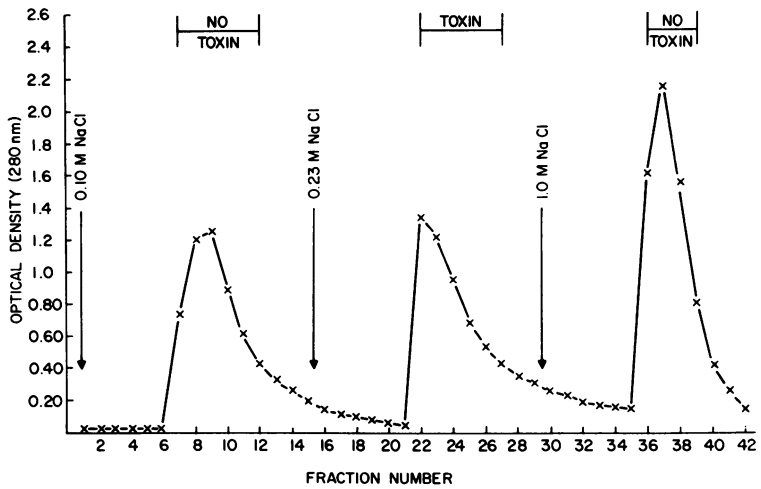

FIG. 2. *DEAE-cellulose chromatography of P. aeruginosa exotoxin preparation. Toxin eluted from hydroxylapatite was placed on a cellulose column (14 by 1.5 cm) equilibrated with 0.10 M NaCl-0.01 M Tris-hydrochloride (pH 8.0). Elution was achieved by a stepwise increase in ionicity as indicated. Fractions (4 ml) representing the peak 280 nm absorbancy material eluted at each step were pooled and assayed for toxin.*

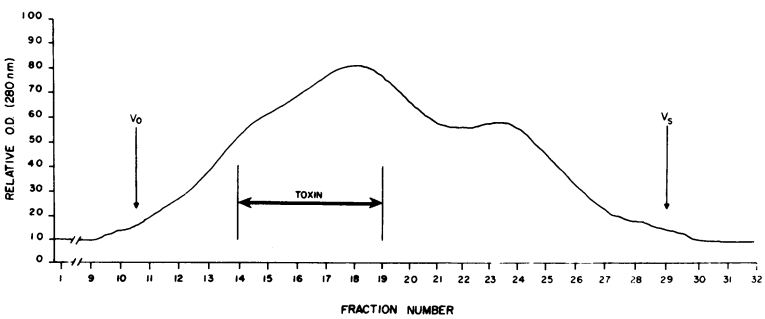

FIG. 3. *Sephadex G-150 gel filtration of P. aeruginosa exotoxin. Toxin purified through the step of DEAE-cellulose chromatography was chromatographed on a Sephadex G-150 column (70 by 1.6 cm) in 0.01 M NaH_2PO_4-0.85% NaCl (pH 7.5). The flow rate was 20 ml/h, and fractions of 3.5 ml each were collected. The void volume (V_0) and salt volume (V_s) were determined with blue dextran 2000 and NaCl, respectively.*

TABLE 1. *Purification of P. aeruginosa exotoxin*

Step in purification	Total protein (mg)	LPS[a] (μg/ml)	Protein needed for mouse lethality (μg)	Median lethal dose		Fold purification
				Total	Recovery (%)	
Starting material			240			1
Ultrafiltration[b]	266.0	100	110	2,418	100	2.2
Hydroxylapatite	76.6	1	36	2,128	88	6.7
DEAE-cellulose	11.7	0.001	9	1,300	54	26.7
Sephadex G-150	7.0	0[c]	6	1,167	48	40.0

[a] Lipopolysaccharide (LPS) was measured by the limulus assay. Total volumes ranged from 20 to 35 ml.
[b] Ultrafiltration was performed using an Amicon XM-300 membrane.
[c] Negative reaction with undiluted material, which indicates that the concentration of LPS was $< 10^{-3}$ μg/ml.

ultraviolet light spectrum of toxin showed a single symmetrical absorbancy peak at 277 nm indicating the presence of protein and the absence of any detectable pigment. The 280 nm/260 nm absorbancy ratio was 1.5 to 1.6, indicating the virtual absence of nucleic acid.

When a purified preparation of toxin containing 17 LD_{50} units was heated at 56 C, approximately 80% loss of biological activity as measured by mouse lethality occurred after 5 min and complete loss of activity occurred after 10 min. The toxin was also inactivated by exposure to trypsin (50 μg/ml for 60 min at 25 C) but was unaffected by similar treatments with ribonuclease or deoxyribonuclease.

When subjected to SDS-polyacrylamide gel electrophoresis, the toxin preparation appeared to contain at least six protein components. However, the proteins migrated during electrophoresis at similar rates and therefore appeared to fall within a close range of molecular weights. A comparison of the Sephadex G-150 K_{av} value of toxin with the K_{av} value of proteins having known molecular weights indicates that the toxin has a molecular weight of approximately 54,000 (Fig. 4).

Isoelectric focusing of toxin elicited a single band of precipitation corresponding to a single zone of 280 nm absorption between pH 4.5 and 5.0 (Fig. 5). Toxin activity was located in one fraction, at pH 5.0, and not in any of the other fractions. However, only about 25 to 30% of the total activity was recovered in this fraction. The poor recovery of toxin, after isoelectric focusing, suggests the toxin activity was partially destroyed at low pH. This is in agreement with the finding of Liu (13).

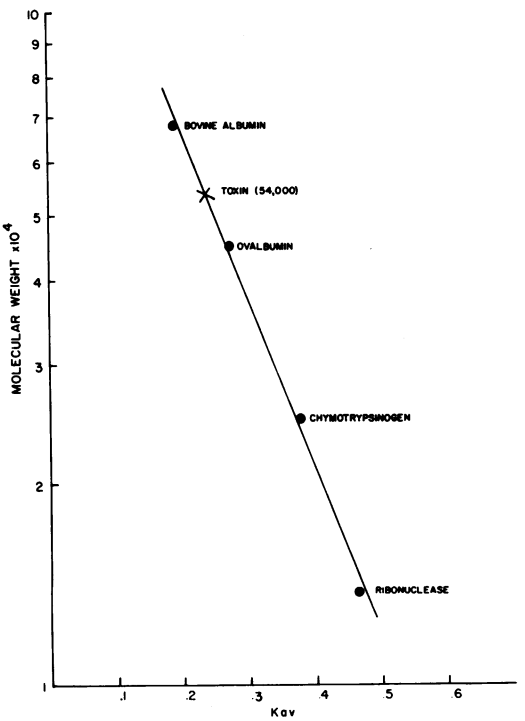

FIG. 4. *Molecular weight determination of P. aeruginosa exotoxin. The molecular weight of toxin was estimated by gel filtration using the Sephadex G-150 column described in Fig. 3.*

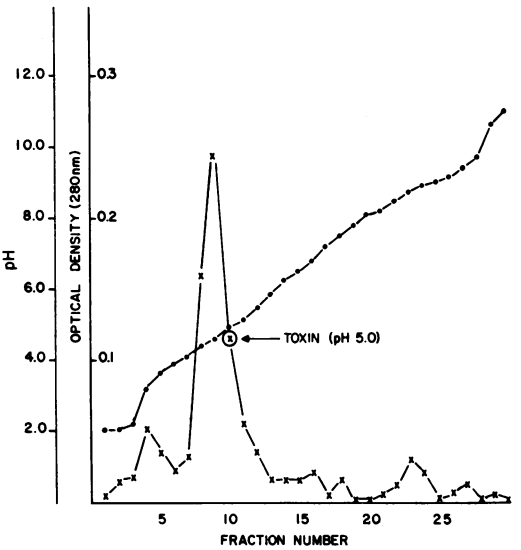

FIG. 5. *Isoelectric focusing of P. aeruginosa exotoxin. An LKB 8101 (110 ml) column was used to focus 2.0 mg of purified toxin in a sucrose density gradient (0 to 50%) containing 1% Ampholine (pH 3 to 10 range). The column was cooled to 4 C, the final potential was 400 V, and 26 h was allowed for equilibrium to be reached. Fractions (4 ml) were collected and the pH (●) and 280 nm absorbancy (×) of each fraction was determined.*

Mouse lethality has been used to measure the toxicity of both crude and purified exotoxin preparations. Crude preparations, however, contained large quantities of LPS whereas the purified exotoxin contained no detectable LPS. It appeared important, therefore, to determine the independent effects of endotoxin (LPS) and exotoxin. Mice given intravenous injections of as much as 2.8 mg (dry weight) of purified LPS from the same strain of *P. aeruginosa* used for the purification of exotoxin displayed the characteristic ruffled fur, crusted eyes, and reduction in activity typical of endotoxin poisoning within 24 h postinjection, but they fully recovered 2 to 3 days later. Mice injected with as little as 4 to 6 μg of purified exotoxin protein displayed no signs of morbidity within 24 h and did not develop any major signs of distress until a few hours before death, which occurred about 72 h postinjection. Mice injected with 2 LD_{50} units of crude material (480 μg of protein) displayed the signs of endotoxin poisoning within 24 h and died within 72 h postinjection. Thus, it appeared that there was a sufficient

quantity of endotoxin (LPS) in the crude material to elicit severe signs of morbidity shortly after administration, but because the endotoxin was approximately a thousandfold (wt/wt) less lethal for mice than exotoxin, as shown with purified preparations, the mouse lethality of the crude preparations reflected exotoxin activity.

O. R. Pavlovskis and F. B. Gordon (18) and O. R. Pavlovskis (17) described a number of cytotoxic effects of crude exotoxin preparations. It was not clear from their studies, however, whether the effects shown were elicited by one or several toxic components of the crude material. Since the purification of exotoxin, described in this paper, was achieved with good recovery (about 50%) by methods which separate components on the basis of their molecular weight and ionic charge, it is reasonable to assume that the exotoxin in a single component or a family of closely related proteins. Preliminary observations by O. R. Pavlovskis (personal communication) indicate that the purified exotoxin elicits cytotoxic effects comparable to those previously described.

Further purification and characterization of the biological properties of the exotoxin is now in progress.

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ADDENDUM IN PROOF

Since the submission of this paper, Liu and associates (P. V. Liu, S. Yoshii, and H. Hsieh. 1973. Exotoxins of *Pseudomonas aeruginosa*. II. Concentration, purification, and characterization of exotoxin A. J. Infect. Dis. **128**:514–519) reported on a purification procedure different from mine but resulting in a preparation which is quite similar. The specific activity of the toxin reported by Liu et al. is comparable to that of more recent toxin preparations obtained in this laboratory.

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